INSTITUTE FOR FERMENTATION

OSAKA

ANNUAL REPORT

1961-1962

1963

財団法人 発酵研究所

Annual Report

No. 1

(1961-1962)

1963

INSTITUTE FOR FERMENTATION

4–54, *Juso-Nishinocho, Higashiyodogawa-ku,*

Osaka, Japan

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FORWORD

The Institute for Fermentation was established at Osaka in November, 1944, as one of the research institutes for microbiology owing to the state policy to be pursued then. Following the termination of the World War its functions have been exercised under the financial protection of Takeda Chemical Industries, Ltd. Up to the year 1960, the activities of the Institute covered various fields for researches into applied and fundamental microbiology, including the production of antibiotics, ergot alkaloids and nucleotides, the microbial transformation of organic substances, and the physiological and taxonomical studies of microorganisms. The results of its researches were published in domestic and foreign scientific journals while these activities have hitherto been reported in the Annual Reports of the Takeda Research Laboratories as no periodical of its own was then available for that purpose.

Originally the Institute was established for forming valuable contributions to the development of fundamental microbiology essential for industry, for which purpose, a type culture collection was attached to the Institute. Under the development of things after the War researches within the Institute had been more in the nature of practical applications than in that of fundamental studies. According to an increase both in the number of research staff and in the amount of equipment, an astronomical budget was required for administrative purposes.

In the summer of 1960 when a new department of applied microbiology was established in the Takeda Research Laboratories, the Institute for Fermentation was so reorganized as to carry on, as its main objective, studies in the basic field of industrial microbiology, this making it necessary for the Institute to publish its own annual report. It would be a great pleasure for the Institute to seek the advice of acknowledged authorities in all countries on this field of study.

Chōbei Takeda

Chairman of the Board of Trustees

ADMINISTRATIVE REPORT

REPORT OF THE DIRECTOR

The reorganization of the Institute was completed in April of the year 1961. The membership of the direction committee was then changed as given on the next page. Changes were also effected in the director and research members, the present staff being composed of twenty members including executive assistants. Plans are now on foot for considerable extensions of laboratories.

The organization of the Institute consists of three sections majoring in branches of the microbiology —mycology, bacteriology and microbial genetics—, the type culture collection attached to the Institute being maintained under the guidance of these sections. For the reported years, researches were carried on into taxonomic studies on micro-fungi and yeasts, nutritional aspects of lactic acid bacteria, and also into genetic studies on temperate phages.

It is a pleasure for the Institute to have the first publication of the annual report. One review and four communications given on the following pages are originals, all written in elucidation of laboratory subjects and for publishing research results thereof.

November, 1963.

Takezi HASEGAWA

DIRECTION COMMITTEE *alphabetical*

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Yuji	SASAKI	Professor, Faculty of Agriculture, Hokkaido University
Kikiti	SATO	ex-Director of the Institute for Fermentation
Sueo	TATSUOKA	Director of the Takeda Research Laboratories

Heartfelt condolences have to be offered on the death of Prof. Izue YAMAZAKI, Trustee of the Institute for Fermentation, who passed away on July 25, 1962.

RESEARCH PROGRAM FOR 1961-1962

1. Studies on *Rhodotorula* yeasts
   Persons in charge: Takezi HASEGAWA, Dr. Ag., Isao BANNO, B. Ag.
2. Lactic acid bacteria and their application
   Person in charge: Reijiro KODAMA, Dr. Ag.
3. Taxonomic studies on micro-fungi
   Person in charge: Keisuke TUBAKI, Dr. Sc.
4. Genetic studies on temperate phage
   Person in charge: Teiji IIJIMA, Dr. Sc.

JOINT RESEARCHES SUPPORTED BY A GRANT-IN-AID FROM THE MINISTRY OF EDUCATION

1. Studies on the genus *Pediococcus* (1960 - 1962)
   Chief: Kakuo KITAHARA, Professor of the University of Tokyo
   Personnel: Reijiro KODAMA
2. Studies on the taxonomic basis of microorganisms (1960 - 1962)
   Chief: Toshinobu ASAI, Professor of the University of Tokyo
   Personnel: Keisuke TUBAKI
3. Studies on the antarctic organisms (1961 - 1962)
   Chief: Denzaburo MIYADI, Professor of Kyoto University
   Personnel: Keisuke TUBAKI
4. Fundamental studies on the application of lactic acid bacteria (1962 -)
   Chief: Kakuo KITAHARA, Professor of the University of Tokyo
   Personnel: Reijiro KODAMA

ACTIVITIES OF THE CULTURE COLLECTION SECTION

From January of 1961 till December of 1962, 1042 strains of fungi and bacteria were collected from nature and from other research organizations. As a result, 2895 cultures of molds, 1428 of yeasts and 787 of bacteria were being maintained in the collection at the end of 1962. To domestic and foreign organizations, 5810 cultures were distributed during the same period.

LIST OF CULTURES (Third edition, 167pp) which includes about three thousand names of available cultures was issued by the Institute in March, 1962.

PRESENTATION OF PAPERS AT SCIENTIFIC MEETINGS

Author(s)	*Title*	*Scientific Meeting*
R. Kodama and M. Nishio	Studies on lactic acid bacteria from various materials in the process of *saké*-brewing (II)	General Meeting of the Agricultural Chemical Society of Japan in Fukuoka (April, 1961)
K. Tubaki	Fungi isolated from the Antarctic materials (I)	Regular Meeting of the Mycological Society of Japan in Tokyo (June, 1961)
R. Kodama	The nutrition of the genus *Pediococcus*	Bioassay Conference in Tokyo (September, 1961)
K. Tubaki	Studies on a slime-forming fungus in polluted water	General Meeting of the Botanical Society of Japan in Tokyo (October, 1961)
R. Kodama and M. Nishio	Studies on lactic acid bacteria from various materials in the process of *saké*-brewing (III)	General Meeting of the Agricultural Chemical Society of Japan in Sendai (April, 1962)
K. Tubaki	Fungi isolated from the antarctic materials (II)	Regular Meeting of the Mycological Society of Japan in Tokyo (June, 1962)
T. Harada† T. Fukui† J. Nikuni† I. Banno and T. Hasegawa	The composition of extracellular polysaccharides of *Rhodotorula*	Regular Meeting of Kansai subdivision of the Agricultural Chemical Society of Japan in Tottori (June, 1962)
T. Hasegawa and I. Banno	Taxonomic significance of the vitamin requirement of *Rhodotorula* species.	VIII International Congress of Microbiology in Montreal (August, 1962)
K. Tubaki	On the Phialophora-stage of Ascomycetes	General Meeting of the Botanical Society of Japan in Nagoya (October, 1962)

† The Institute of Scientific and Industrial Research, Osaka University.

REPORTS FROM THE LABORATORIES

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No. 1, p. 11~24, 1963

THE NUTRITION OF *LACTOBACILLUS FRUCTOSUS* AND ITS APPLICATION TO MICROBIOLOGICAL DETERMINATION OF NICOTINAMIDE AND FRUCTOSE

Reijiro KODAMA

Three strains (No. 353, No. 825, and No. 2112) of lactic acid bacteria isolated from flowers failed to grow in a modified Thompson's synthetic medium (Kodama, 1956a). These strains required at least two substances for growth, both of which are contained in tomato juice at a relatively high level. One of these substances was not adsorbed on charcoal, the other was, and they were tentatively named Factor A and B, respectively. The active principle of Factor A was identified as fructose (Kodama, 1956b), and that of Factor B as nicotinamide (Kodama, 1956a).

The morphological, fermentative and other characteristics of these strains were distinguishable from those of any other hitherto fore described lactobacilli. The author, therefore, did not hesitate to describe these strains as a new species and designate them as *Lactobacillus fructosus* (Kodama, 1956d).

This paper deals with the nutritional requirements of *Lactobacillus fructosus* 353, one of these strains, and microbiological methods for the determination of nicotinamide and fructose by means of this organism.

I. Vitamin requirements.

(A) Nicotinamide.

Lactobacillus fructosus requires nicotinamide as one of its essential growth factors. In general, when bacteria require nicotinic acid or nicotinamide for growth, they can utilize both of the two. However, it is well known that nicotinamide is somewhat more active than nicotinic acid for *Staphylococcus aureus* (Knight, 1937, 1945) and dysentery bacilli (Knight, 1945; Dorfman *et al.*, 1941; Saunders *et al.*, 1941), and, further, that some members of the *Pasteurella* group (Berkman *et al.*, 1940; Koser *et al.*, 1941) respond to nicotinamide, but not to nicotinic acid. On the contrary, *Leuconostoc mesenteroides* ATCC 9135 (Shive, 1950; Johnson, 1945) which is usually used for the microbiological determination of the above vitamin, has been proved to respond to nicotinic acid, but not to nicotinamide at low concentrations.

Among nicotinic acid and its analogues tested, only nicotinamide was found to be specifically active for the growth of *Lactobacillus fructosus*, while nicotinic acid, isonicotinic acid, picolinic acid, trigonelline, ethylnicotinate, quinolinic acid, and coramine were all inactive. Nicotinuric acid, however, had 0.02% of the activity of nicotinamide.

On the other hand, nicotinamide and Coenzyme-I had the same activity on a molar basis.

(B) Other vitamins.

In addition to nicotinamide, *Lactobacillus fructosus* requires thiamine, riboflavin, calcium pantothenate essentially, and B_6-group vitamins, folic acid and biotin stimulatorily for the growth. Vitamin B_{12} and p-aminobenzoic acid were not required. These results are summarized in Table 1 (Kodama, 1956c).

Table 1. Vitamin Requirements of *Lactobacillus fructosus*

Vitamin	Requirement	Concentrations at which the growth response continues to increase	Concentrations at which maximum growth is achieved
		γ per ml	γ per ml
Thiamine·Hcl	E*	0~0.01	0.02 ~0.1
Riboflavin	E	0~0.02	0.02 ~0.1
Calcium pantothenate	E	0~0.02	0.02 ~0.05
Nicotinamide	E	0~0.05	0.01 ~0.2
Biotin	S*		0.0005~0.001
Pyridoxal·Hcl	S		0.002 ~0.05
Folic acid	S		0.002 ~0.005
p-Aminobenzoic acid	N*		
B_{12}	N		

* E, Essentially required; S, Stimulatorily required; N, Not required

The activity of cocarboxylase was equal to that of an equimolar amount of thiamine, while 4-methyl-5-(β-hydroxyethyl) thiazole was only 0.2% as active as thiamine. The activity of thiamine monophosphate was approximately 50 - 70% of that of thiamine.

The nutritional pattern of this organism comformed to type-V among six types of vitamin requirement which the present author observed with heterofermentative lactobacilli (Kodama and Nishio, 1961).

II. Purine, pyrimidine, and amino acid requirements.

Purine, arginine, aspartic acid, glutamic acid, isoleucine, histidine and valine were required essentially, and uracil, leucine, lysine, methionine, serine, threonine, tryptophan, tyrosine and cysteine were stimulatorily for the growth of this organism. Alanine, glycine, phenylalanine and proline were not required. Asparagine replaced aspartic acid completely whereas aspartic acid replaced asparagine only partially.

III. Requirement for fructose.

Lactobacillus fructosus utilized only fructose successfully among all the other sugars tested, except glucose, which was metabolized only in a small quantity, when each of these sugars was used as a sole carbon source (Table 2). That is, fructose was an

Table 2. Utilization of Carbohydrates by *Lactobacillus fructosus* (Incubated at 36° for 3 Days)

	Growth	Acid produced (ml of 0.1 N NaOH per 5 ml of media)		Growth	Acid produced (ml of 0.1 N NaOH per 5 ml of meida)
Starch	—*	0	Arabinose	—	0
Dextrin	—	0	Xylose	—	0
Inulin	—	0	Ribose	—	0
Raffinose	—	0	Salicin	—	0
Maltose	∓* or —	0	α-Methylglucoside	—	0
Sucrose	∓ or —	0	Dulcitol	—	0
Lactose	—	0	Adonitol	—	0
Trehalose	—	0	Mannitol	—	0
Glucose	+*	0.62	Glycerol	—	0
Fructose	++*	2.04	Sodium acetate	—	0
Galactose	∓	0.10	Calcium lactate	—	0
Mannose	—	0	Sodium gluconate	—	0
Rhamnose	—	0			

* —, No growth; ∓, Negligible growth; +, Slight growth; ++, Good growth

essential nutrient required for the prompt and heavy growth of this organism in stationary culture.

The physiological significance of the fructose requirements is mentioned below.

(A) Fermentation products.

Lactobacillus fructosus is heterofermentative and is to be included in the "inactive group" of Pederson (1938). When this organism was compared with *Lactobacillus fermenti* 36 as to their fermentation products, it was found that both were similar in that their principal product from fructose was mannitol, but the former organism was different from the latter in glucose comsumption and in ethanol production (Table 3).

Table 3. Fermentation Products from Glucose and Fructose

		Sugar (mg per 100 ml of media)		Product (mg per 100 ml of media)			
		Initially added	Consumed	Ethanol	Acetic acid	Lactic acid	Mannitol
Lactobacileus fructosus 353	Glucose	4628	790	0	296	327	
	Fructose	4322	4322	0	705	758	2836
Lactobacillus fermenti 36	Glucose	4628	4628	800	190	2282	
	Fructose	4322	4322	40	557	877	2620

Since heterofermentative lactic acid bacteria so far described generally produced lactic acid, ethanol and carbon dioxide when glucose was employed as the sole carbon source, the fact that *Lactobacillus fructosus* did not produce ethanol from glucose, appeared to be closely related to its fructose requirement.

(B) Growth stimulating effect of glucose and of gluconate in the presence of fructose (Kodama, 1957a).

While fructose was essentially required for prompt and heavy growth of *Lactobacillus fructosus*, glucose stimulated the growth of this organism in the presence of fructose particularly in the early stage. Such an effect, however, could never be obtained when fructose or glucose was used independently, even in larger amounts (Fig. 1). As seen

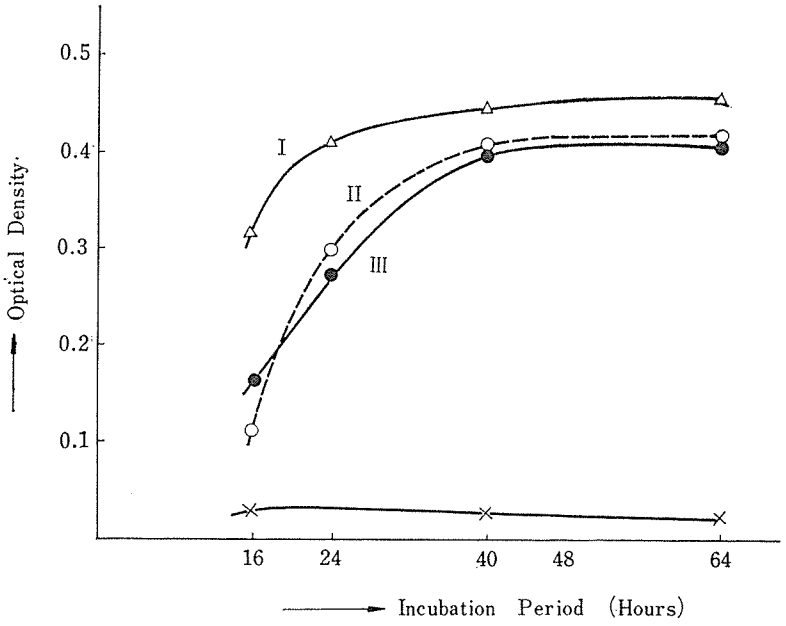

Fig. 1. The stimulating effect of glucose on the growth of *Lactobacillus fructosus* in the presence of fructose.
Curve I: Fructose 20 mg/ml + Glucose 20 mg/ml
Curve II: Fructose 40 mg/ml
Curve III: Fructose 20 mg/ml
Curve IV: Glucose 20 mg/ml

from the figure, combination of the two sugars not only reduced the hours to reach "inflection point" on the growth curve, but also somewhat improved the amount of growth. The effect was observed even with the addition of 0.2 mg of glucose per ml of the medium containing 20 mg of fructose, and the maximum was reached with 2 mg of glucose.

Growth promoting effects by a combination of two kinds of sugars were already described with *Lactobacillus bulgaricus* (lactose plus glucose) by Snell *et al.* (1948) and with *Lactobacillus fermenti* (glucose plus maltose, fructose plus maltose, or glucose plus xylose) by Fang and Butts (1951), Snell and Lewis (1953) and MaciasR (1957). But with *Lactobacillus fructosus*, miscellaneous sugars other than glucose, such as arabinose, xylose, galactose, sucrose, maltose, lactose, and starch, did not have such a growth promoting activity as glucose. However, gluconate, which was not utilized when used as a sole carbon source, also showed a similar effect on the growth of this organism only in the presence of fructose (Table 4).

Table 4. The Stimulating Effect of Sodium Gluconate on the Growth of *Lactobacillus fructosus* in the Presence of Fructose

Carbon-source (mg per ml of the medium)	Optical density	
	Incubation period (Hours)	
	16	40
Fructose 20	0.042	0.347
Sodium gluconate 10	NG*	NG
〃 1	NG	NG
Fructose 20+Glucose 10	0.238	0.475
〃 +Sodium gluconate 10	0.158	0.492
〃 + 〃 4	0.141	0.465
〃 + 〃 2	0.133	0.450
〃 + 〃 1	0.122	0.450
〃 + 〃 0.5	0.111	0.407

* No growth

On the other hand, glucose-6-phosphate, fructose-6-phosphate, or fructose-1, 6-diphosphate could replace glucose as a growth promoting agent but not fructose as an essential requirement.

(C) Influence of autoclaving on the utilization of fructose (Kodama, 1957b).

According to the conditions of the inocula tested, a clear disparity was observed between the growth of the same organism when fructose used as a sole carbon source was autoclaved with the medium at 10 pounds for 10 minutes, and the growth when fructose was added aseptically (Fig. 2).

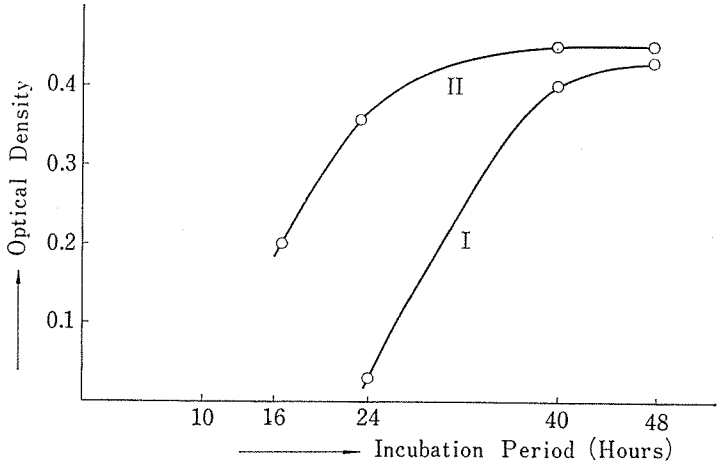

Fig. 2. The growth of *Lactobacillus fructosus* when fructose was autoclaved with the medium or added aseptically.
Curve I: The growth curve of the organism when fructose was added aseptically after autoclaving in distilled water at 10 pounds for 10 minutes.
Curve II: The growth curve of the organism when fructose was autoclaved with the medium at 10 pounds for 10 minutes.

Up to the present time, the reasons why the growth of lactic acid bacteria was promoted by autoclaving of the medium are considered to be due to the formation of

1) Hydrogen acceptors such as acetaldehyde, pyruvate etc.

2) Reducing agents, the requirements for which is satisfied by ascorbic acid, cysteine etc.

or 3) Substances such as glucosylglycine, which is presumed to be an intermediate in the heat activation reaction. (Snell *et al*; 1948; Smiley *et al*; 1943; Orla-Jensen, 1933; Rabinowitz and Snell, 1947; Rogers *et al*; 1953)

In *Lactobacillus fructosus*, the delayed growth which occurred when fructose was not autoclaved with the medium, was closely related to the age of the slant culture used for the inocula (Table 5). As shown in Table 5, when young inocula were used, successful growth of the organism was obtained on the synthetic medium to which fructose had been added aseptically as a sole carbon source, whereas no growth or delayed growth occurred when old inocula were used (Table 6). Even when old inocula were used, however, the addition of glucose in addition to fructose, or fructose autoclaved with the medium permitted rapid growth. That is, the same organism required both glucose and fructose for the prompt and heavy growth according to the condition of the inocula. In addition the time of growth initiation depended on the amount of glucose supplied (Table 6).

On the other hand, gluconate also showed a growth stimulating effect similar to that of glucose in the presence of fructose, but, unlike glucose, only when fructose could be utilized by this organism (Table 7).

Orla-Jensen (1933) found that most of the carbohydrates were partly converted into others during sterilization, and thus 9% of the glucose and not less than 20% of the fructose was converted; the glucose mostly to fructose, the fructose mostly to glucose.

Table 5. The Effect of Age of Inoculum on the Growth of *Lactobacillus fructosus*

	Age of slant culture for incoulum (Days)	Optical density: Incubation period (Hours) 16	24	40	64
Fructose autoclaved with the medium at 10 pounds for 10 minutes, was used as a sole carbon-source.	2	0.305	0.395	0.445	0.455
	7	0.240	0.375	0.445	0.470
	10	NG*	NG	0.335	0.455
	15	NG	NG	0.340	0.440
	21	NG	NG	0.368	0.450
Fructose added aseptically, was used as a sole carbon-source.	2	0.050	0.383	0.477	0.530
	7	NG	0.027	0.405	0.480
	10	NG	NG	NG	NG
	15	NG	NG	NG	NG
	21	NG	NG	NG	NG

* No growth

Table 6. The Effect of Amount of Glucose on the Growth of *Lactobacillus fructosus*

Carbon-source (mg per ml of the medium)		Optical density					
		Incubation period (Hours)					
		16	19	23	38	46	62
Fructose 20		NG*	NG	NG	NG	NG	NG
〃 +Glucose 10	Both fructose and glucose were added aseptically.	0.065	0.212	0.307	0.444	0.480	
〃 + 〃 4		0.025	0.160	0.254	0.415	0.440	
〃 + 〃 2		0.009	0.117	0.240	0.422	0.447	
〃 + 〃 1		NG	0.002	0.087	0.408	0.440	
〃 + 〃 0.4		NG	NG	0.005	0.370	0.410	0.442
Fructose 20	Fructose was autoclaved with the medium.	NG	NG	0.005	0.326	0.368	0.407

* No growth

Table 7. The Difference Between the Stimulating Effect of Glucose and That of Sodium Gluconate on the Growth of *Lactobacillus fructosus*

Carbon-source added aseptically (mg per ml of the medium)	Optical density				
	Age of slant culture for inoculum				
	10 Days			18 Days	
	Incubation period (Hours)				
	24	40	64	20	48
Fructose 20	NG*	0.070	0.375	NG	NG
〃 +Sodium gluconate 0.5	NG	0.250	0.408	NG	NG
〃 + 〃 1	NG	0.297	0.445	NG	NG
〃 + 〃 2	NG	0.313	0.473	NG	NG
〃 + 〃 4	NG	0.353	0.490	NG	NG
〃 +Glucose 10	0.232	0.483	0.515	0.100	0.490

* No growth

The above-mentioned effect of autoclaving of the media containing fructose on the growth of *Lactobacillus fructosus* seems to probably be due to the glucose converted from the fructose. There has not been any lactic acid bacteria so far recorded which showed such a specific requirement for carbon source.

(D) Metabolic products in the coexistence of fructose and glucose or gluconate (Kodama, 1957c).

The above facts appear to indicate that fructose and glucose or gluconate are utilized together. Through the results obtained from the analysis of metabolic products in the coexistence of these carbon sources, the following facts were made clear.

1) As seen from Table 8, glucose which was utilized only slightly by *Lactobacillus fructosus* when given as a sole carbon source, could be readily utilized in the presence

Table 8. The Consumption of Sugars and Their Fermentation Products in Media that Contain Increasing Amounts of Fructose and a Definite Amount of Glucose Simultaneously.

Sugar									Fermentation product*								
Initially added			Consumed*						Ethanol		Acetic acid		Lactic acid		Mannitol		
			Total		Glucose (Total minus Fructose)		Fructose										
Total (Glucose plus Fructose)	Glucose	Fructose	mg per 100 ml of media	Per cent on total sugar initially added	mg per 100 ml of media	Per cent on glucose initially added	mg per 100 ml of media	Per cent on furctose initially added	mg per 100 ml of media	Per cent on total sugar consumed	mg per 100 ml of media	Per cent on total sugar consumed	mg per 100 ml of media	Per cent on total sugar consumed	mg per 100 ml of media	Per cent on total sugar consumed	Per cent on fructose consumed
mg per 100 ml of media																	
1793	1793	0	123	6.9	123	6.9	0	0	0	0	39.8	32.3	65.6	53.3	0	0	0
2175	1793	382	681.5	31.3	299.5	16.7	382	100	0	0	139.9	20.5	171.7	25.0	356.5	52.3	93.3
2619	1793	826	1413.5	53.9	587.5	32.8	826	100	0	0	237.0	16.7	285.0	20.1	757.5	53.6	91.7
3613	1793	1820	2950.2	81.6	1130.2	63.0	1820	100	0	0	409.1	13.8	486.0	16.3	1528.7	51.8	84.0
5556	1793	3763	5556	100	1793	100	3763	100	0	0	751.2	13.5	938.9	16.8	3037.8	54.7	80.7

* The analysis was conducted after 9 day's incubation of the organism tested.

of fructose. The amount of glucose utilized increased proportionally to the amount of fructose present, resulting in the production of mannitol to amounts corresponding to the greater part or almost all of the fructose, particularly in the linear ascending part on the response curve of fructose shown in Fig. 4. In other words, a proportional relation was observed between the amounts of the growth and that of mannitol produced. The ratios of the lactic acid as well as acetic acid produced to the total sugar initially added approached that of the following equation proposed by Pederson (1929) with increase in the amount of fructose.

$$3C_6H_{12}O_6 \rightarrow 2C_6H_{14}O_6 + C_3H_6O_3 + C_2H_4O_2 + CO_2$$

Fructose Mannitol Lactic acid Acetic acid

2) Gluconate, which was not utilized at all by the same organism when given as the sole carbon source, also could be utilized in the presence of fructose, resulting in the production of larger amounts of lactic acid and acetic acid than those in the medium that contained fructose alone, and the amount of mannitol produced exceeded the theoretical value, 67.3%, obtained from the above-mentioned equation.

The formation of mannitol by the resting cells of this organism was inhibited by monoiodoacetate.

(E) Physiological significance of the fructose requirement (Kodama, 1960).

From these results, it is inferred that the fructose requirement for the prompt and heavy growth of *Lactobacillus fructosus* is attributable to the character of this sugar as a profitable hydrogen acceptor in the process of glycolysis. The fact that ethanol is not produced from glucose by this organism unlike usual heterofermentative lactic acid bacteria, appears to give support to this inference.

Nevertheless, glucose should be considered as a more essential carbon source than fructose, which performs an important role in the growth of this organism only as a hydrogen acceptor. This consideration is supported by the fact that glucose stimulates growth in the presence of fructose, and that both glucose and fructose are essentially required for the successful growth when old inocula are used. As fructose is regarded as "carbon source plus hydrogen acceptor", this sugar can also be partially utilized as a carbon source only for young inocula. Therefore, it is concluded that when a single carbon source is used, only fructose permits satisfactory growth of the same organism.

Neither methylene blue, riboflavin, triphenyltetrazolium chloride, nor acetaldehyde can serve as a substitute for fructose as the hydrogen acceptor so fas as the growth is concerned. Pyruvate, however, can partially replace fructose, that is, "glucose plus pyruvate" permits growth.

Thus, it is concluded that *Lactobacillus fructosus* is characterized by requiring an exogenous hydrogen acceptor for satisfactory growth, and moreover, by its preference for fructose as one of the most effective exogenous hydrogen acceptors.

IV. An improved method for the microbiological determination of nicotinamide (Kodama, 1957d).

Up to the present, either *Shigella paradysenteriae* (Isbell *et al*; 1941) or *Leuconostoc mesenteroides* ATCC 9135 (Johnson, 1945) has been used for the microbiological determination of nicotinamide contained in natural materials. *Shigella paradysenteriae* responds not only to nicotinamide, but also to nicotinic acid and a number of other related compounds (Table 9), so that the values obtained can only be taken as what Isbell *et al.* (1941) called "equivalents to nicotinamide." On the other hand, samples have to be subjected to hydrolysis prior to assay with sulfuric or hydrochloric acid in order to convert nicotinamide to nicotinic acid, because *Leuconostoc mesenteroides* responds to nicotinic acid, but not to nicotinamide at low concentrations.

Table 9. Specificity of the Nicotinic Acid Group
(Activity*, per cent)

	Leuconostoc mesenteroides ATCC 9135 (Shive, 1950)	Dysentery bacilli (Shivc, 1950)	*Lactobacillus fructosus* 353
Nicotinic acid	100	10～30	0
Ethylnicotinate		10～30	0
Picolinic acld		0	0
Nicotinuric acid	0.03	3	0.02
Quinolinic acid		0.3	0
Nicotinamide	0.03～ 0.1	100	100
Trigonelline		0	0
Isonicotinic acid		0	0
Coramine	0.2	0.1	0
Coenzyme-I	50～70	50	100

* Activities calculated in terms of per cent of most active form of the vitamin.

As already described, *Lactobacillus fructosus* requires nicotinamide as an essential growth factor. From this point of view, this organism may be suitable for assay purposes.

(A) Synthetic medium, standard curve, recovery, and reproducibility.

A medium prepared by removing only nicotinamide from the synthetic medium shown in Table 10, was used as the basal medium for the assay.

The response to the nicotinamide added is shown in Fig. 3. A standard curve similar to Fig. 3 must be obtained with each set of assays. The nicotinamide content of each unknown tube was then read from the standard curve. From these values, the nicotinamide content of the sample is calculated. As in other microbiological assays, it is necessary to take the average of the values obtained at several levels on the curve within the assay limits.

The values obtained here are reproducible within $\pm 6\%$. Recoveries of the

Table 10. Composition of the Synthetic Medium
(Quantities given are needed for the preparation of 10 ml of final strength medium.)

Adenine sulfate	200γ	Ascorbic acid	2 mg
Guanine hydrochloride	200	Tween 80	10
Xanthine	200	L-Tryptophan	1
Uracil	200	L-Cysteine hydrochloride	1
Thiamine hydrochloride	5	L-Asparagine	2
Riboflavin	10	Glucose	100
Calcium pantothenate	5	Fructose	200
Nicotinamide	10	Bacto Vitamin Free Casamino Acids	50
Biotin	0.05	Salts A*	0.1 ml
Folic acid	1	Salts B*	0.1 ml
Pyridoxal hydrochloride	5	Adjusted to pH 6.4	

* Prepared according to Snell and Wright (Snell and Wright, 1941)

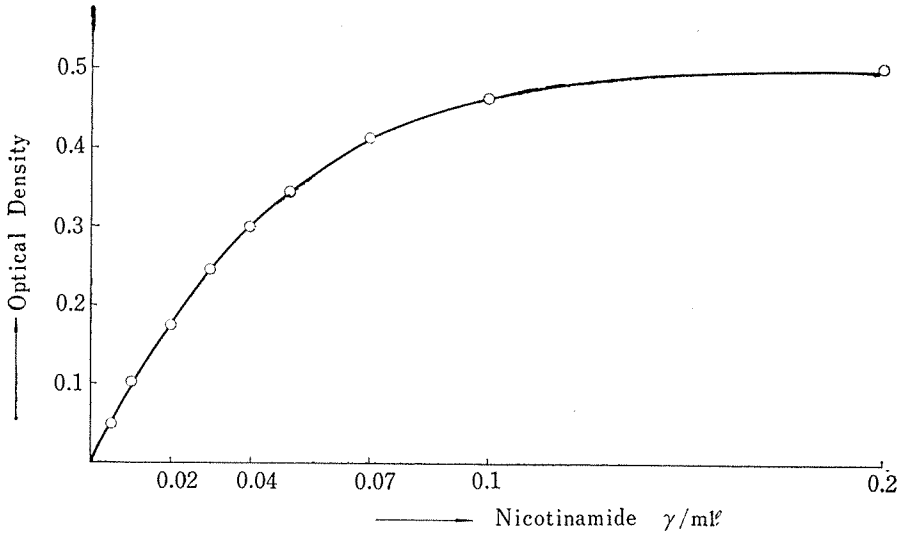

Fig. 3. Response of *Lactobacillus fructosus* to nicotinamide added.

nicotinamide added to malt extract, liver extract or yeast extract can be made within ±6%.

(B) Comparison between the values obtained by the present method and by other methods.

Comparable results obtained from selected samples, when assayed for nicotinamide by the procedure employing *Lactobacillus fructosus*, and according to the procedure of Krehl *et al.* (1946) employing *Leuconostoc mesenteroides*, are given in Table 11. Both gave similar results, but the samples tested showed somewhat higher values with *Lactobacillus fructosus* than with *Leuconostoc mesenteroides*.

It should not be concluded that nicotinamide, when measured with *Lactobacillus fructosus*, is all composed of "free amide," since this method does not distinguish between

Table 11. Comparative Values of Nicotinic Acid and Nicotinamide Obtained with *Lactobacillus arabinosus*, *Leuconostoc mesenteroides*, and *Lactobacillus fructosus*

	Organism used				
	Lactobacillus arabinosus	*Leuconostoc mesenteroides*			*Lactobacillus fructosus*
	Total nicotinic acid	Nicotinic acid		Nicotinamide (Total minus Free)	Nicotinamide
		Free	Total		
	γ per ml	γ per ml	γ per ml	γ per ml	γ per ml
Spinach extract	0.7	0.27	0.58	0.31	0.42
Liver extract	40.0	7.2	40.6	33.4	34.3
Yeast extract	61.8	33.0	55.0	22.0	25.3
Tomato juice	9.0	1.5	8.7	7.2	7.3

the free amide and that bound as cozymase. However, this method has several advantages, as shown in the following:

1) The response of *Lactobacillus fructosus* to nicotinamide is only one-tenth of that of *Leuconostoc mesenteroides* to nicotinic acid,

2) The values obtained are independent of the effects owing to the acid hydrolysis of samples,

and 3) The process is simple, since nicotinamide is directly determined.

V. Microbiological determination of fructose (Kodama, 1960).

As mentioned before, when a single sugar is used as carbon source, *Lactobacillus fructosus* utilizes only fructose successfully and glucose slightly in stationary cultivation, but it does not utilize other carbon sources at all. However, glucose or gluconate can be utilized effectively in the presence of fructose. This fact appears to indicate the possibility that carbon sources other than glucose or gluconate also may be utilized in the presence of fructose. Therefore, the growth stimulating effects of various carbon sources (starch, dextrin, inulin, glucose, galactose, mannose, raffinose, trehalose, rhamnose, maltose, sucrose, lactose, arabinose, xylose, ribose, adonitol, dulcitol, mannitol, α-methylglucoside, glycerol, salicin, lactate, and gluconate) were tested in the presence of each level of fructose corresponding to the linear ascending part on the response curve of fructose in Fig. 4. As a result of the tests, it was found that other carbon sources other than glucose or gluconate had no such effect. This fact indicates that these sugars, except glucose and gluconate, are not utilized even in the presence of fructose.

Eventually, *Lactobacillus fructosus* responds quantitatively to fructose even in the presence of glucose, gluconate or all the other carbohydrates tested. Pyruvate can partially replace fructose in the presence of glucose, but both fructose-6-phosphate and fructose-1,6-diphosphate cannot.

From these facts, the microbiological determination of fructose can be carried out

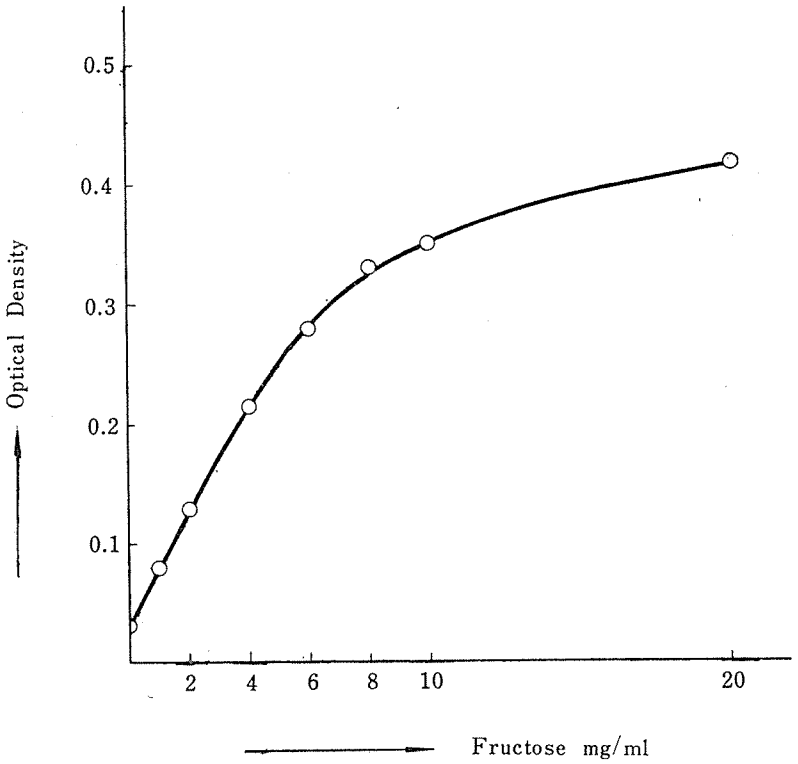

Fig. 4. Response of *Lactobacillus fructosus* to fructose added in the presence of glucose.

with some natural products, e.g. honey, in which fructose is contained in much larger amounts than pyruvate.

A medium prepared by removing only fructose from the synthetic medium shown in Table 10, was used as the basal medium. The response curve of the fructose added is shown in Fig. 4. Goto (1956) obtained satisfactory results in the microbiological determination of fructose by the present method.

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Ann. Rep. Inst. Fermentation, Osaka
No. 1, p. 25~54, 1963

TAXONOMIC STUDY OF HYPHOMYCETES

Keisuke TUBAKI

Introduction

Fungi Imperfecti (Deuteromycetes) are a taxonomic group which can not be classified as Ascomycetes, Basidiomycetes or Phycomycetes because their method of sexual reproduction is unknown, hence this group consists of a "waste basket" assemblage of fungi. Therefore, the fungi whose sexual organs have not been found are artificially brought into this group. The method of sexual reproduction is the most important criterion for the systematic classification of fungi, but for those fungi which lack sexual organs, there has been no other way to delimit taxa except on the basis of their morphological characteristics such as conidia or other asexual structures. Among others, Hyphomycetes include Moniliales and Mycelia Sterilia and constitute an important part of fungi imperfecti. Hyphomycetes are extensively distributed in the natural world such as air, animals and plants, not to mention soil. They have been found to play a major role in the decomposition of daily necessities, especially in food spoilage. Formerly they were recognized as plant- and animal-pathogens, however, the importance of Hyphomycetes is now distinct because of the increase of industrial value and also from the ecological point of view.

The final object of taxonomy is to make clear phylogenetic relationships among taxa. From this point of view, there have been many contradictions in the method of classification of Hyphomycetes so far employed, such as Saccardo's System (1886) which is being used even at present. His classification was truly artificial and stress was given to the host relationships, color and size of asexual organs such as features of conidiophores, but no consideration was given to a natural system.

Hence, in some cases, two kinds of Hyphomycetes belonging to the same genus were not related systematically, but rather were connected more closely to fungi belonging to other genera. This leads to the unreasonable situation where naturally related species of fungi imperfecti are not always found in the same or neighbouring genera.

The first contradiction encountered in the classification of Hyphomycetes by Saccardo's system is that different observers do not agree in distinguishing the coloration between Moniliaceae and Dematiaceae. Moreover, the system includes many criteria which depend upon changeable and unstable characteristics. With expansion of the utility of Hyphomycetes, their properties on artificial media pose a great problem, and it is understandable that the description of Saccardo which was based too much on characteristics on plant host is incompatible with modern taxonomy. It is often dif-

ficult to determine a fungus from culture since its original description was made only from material occurring on natural substrates, because the external appearance of a fungus may be variable depending upon the kinds of substrates (hosts) as well as such enviromental conditions, as humidity, temperature, and light. The color and feature of fungi observed by the naked eye are also subject to biochemical change. Taxonomically, comparison must be always conducted on comparatively stable features, which prove uniform even if the organisms are put under quite different conditions. This requires a more reasonable classification of the Hyphomycetes.

Literature Review on Classification and Discussion

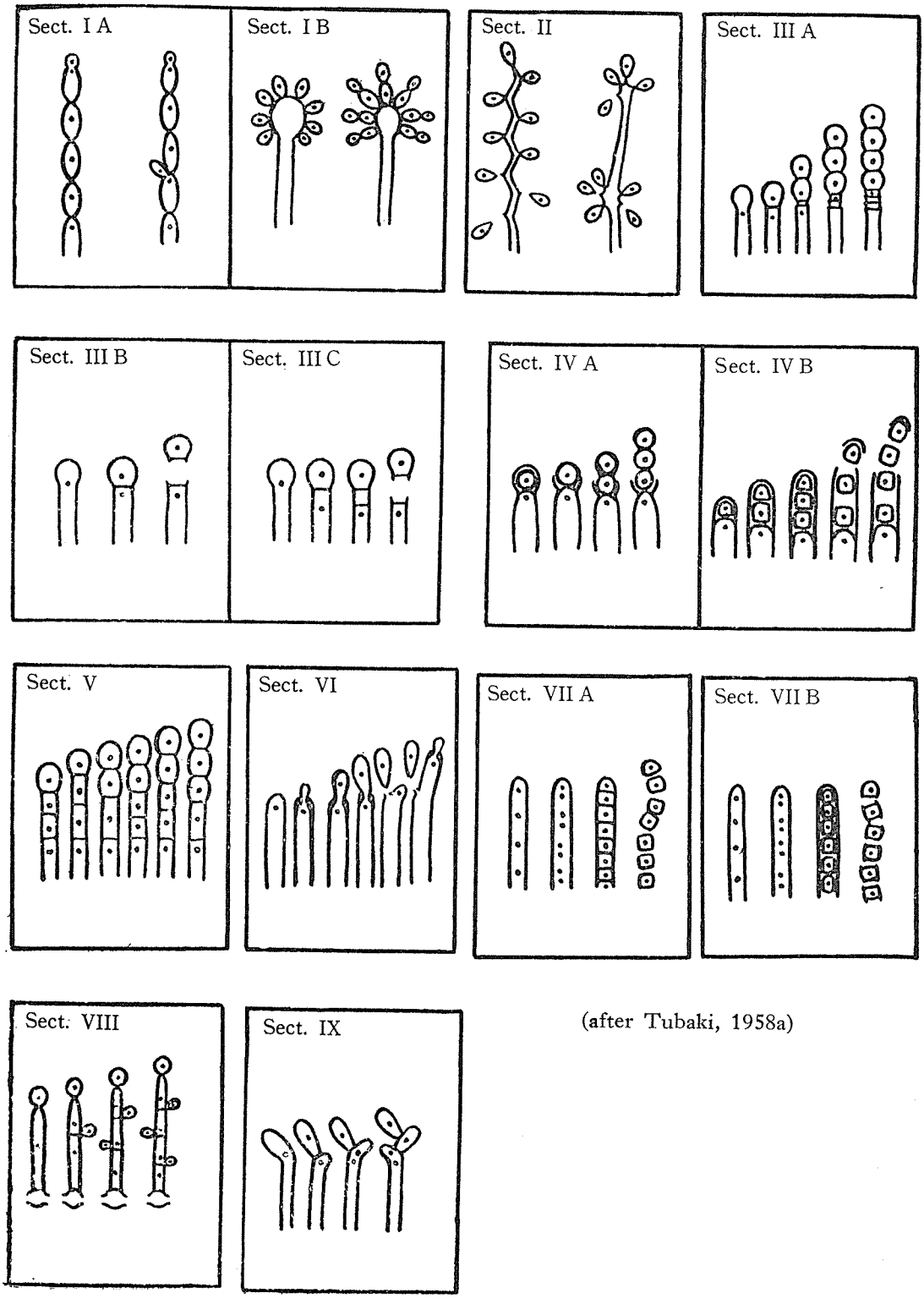
As mentioned before, Hyphomycetes lack a preferential standard as seen in the perfect fungi, so their classification has been based on Saccardo's System up to the present, because there are no standard taxonomic systems such as Bergey's manual for bacteria and Lodder & Kreger-van Rij for yeasts. The section of fungi in the Kryptogamen-Flora by Rabenhorst (1907, 1910) and the manual of Lindau (1922) are also similar reference materials. The "Genera of Fungi" by Clements & Shear (1931) is a revision and amplification of Saccardo's system and is used most extensively at present.

As early as 1888, Costantin proposed a classification of Hyphomycetes giving primary importance to the mode of insertion of the spore on conidiophore, but unfortunately it has never been accepted. Of all studies, that by Vuillemin (1910) was richest perhaps in originality. He proposed a classification based upon spore-forms, not upon the single term "conidium". He recognized two basic terms, *thallospores* and *conidia vera*. This classification is summarised by Langeron and Vanbreuseghem (1952). Vuillemin's definition of spore-types has been reviewed and amended by Mason (1933). The standard advocated by Mason (1937), i.e. classification of conidia into *dry spores* and *slimy spores* from the ecology of conidia, is significant from the view point of the distribution of fungi in the natural world. Wakefield and Bisby (1941) used Mason's major groupings and classified Hyphomycetes into Xerosporae (dry spores) and Gloiosporae (slimy spores) in their listing of British Hyphomycetes, but as the work does not contain all fungi, it cannot be used for ordinal classification but is rather useful from the ecological point of view. G. Smith (1954) also adopted Wakefield's idea which was based on Mason's one. Ingold (1942) suggested a third biological spore type after working with aquatic Hyphomycetes. Moreau (1953) adopted the system of Langeron and Vanbreuseghem and arranged the imperfect fungi in their style, thus extending Vuillemin's concepts. He arranged not only the Hyphomycetes but the Melanconiales and Sphaeropsidales using Vuillemin's conception of spore types. Hoehnel (1924) proposed a system of Fungi Imperfecti, but was not followed by other workers. Grigoraki (1936) discussed on the aleuriospore-producing Hyphomycetes and divided them into four groups. The method of Barnett (1960) is based on the system of Saccardo. In the excellent work of Hughes (1953), which seems to have

been derived from Vuillemin's ideas, Hyphomycetes were classified into eight large groups. He also discussed on classification from a pure taxonomic view point. In 1958, Hughes also investigated type specimens of imperfect fungi and put the genera and species of Hyphomycetes in order, thus making a great contribution to their future classification. Using the type-species method in classifying Hyphomycetes, Mason (1933, 1937, 1941) elucidated the generic characters after examining the type specimens and inspired many critical subsequent studies on the same organisms. These considerations were extended in the subsequent papers of Hughes (1958) as already mentioned. In 1956, Goos discussed the classification of the Fungi Imperfecti and stated that the true value of Hughes' classification will not be known until it is applied to species other than those included in his system, but certainly some of the ideas presented are worthy of serious consideration. He also listed and briefly defined the terms which have been used in designating spore types. Tubaki (1958b) added to Hughes' grouping a ninth section and divided some sections into sub-sections. In the meantime, he attempted to discover a pattern of relationships between perfect and imperfect stages. To my knowledge, the most recent attempt at classification of Hyphomycetes has been that of Subramanian (1962). In his brief discussion, he recognized six morphological categories of spores, based on their method of formation, and also proposed six families for the bulk of Hyphomycetes. In the same year, 1962, Donk dealt with the generic names based on the imperfect states of Hymenomycetes, exclusive of the Sporobolomycetaceae, and he treated many hyphomycetous fungi from view point of type concept.

Aside from the fungi having primitive characters, such fungi as Hyphomycetes which have fairly complicated characters ought to have stable properties reflecting the natural system. The older method of classifying Hyphomycetes observed only a cross section of the propagation and natural selection of fungi. Even if a mutation arises, the resulting strain ought to show characters reflecting the properties of the parent strain. If a system based on such a fundamental character is presented, it will be very useful.

When Hyphomycetes are classified with the foregoing in mind, the most inherent and stable character is the method of conidial development, as mentioned before. Besides this, pigmentation, enzymatic activity, hereditary and serological characters, and pathogenicity can be considered as aids in classifying the fungi. Pigmentation may be a valuable character in classification, but more detailed chemical data are needed. The value of pigmentation will increase when considered not as the final product, but at each metabolic stage. The study of hereditary characters is also very important, and the variation of fungi must be reflected in their classification. When artificial mutations, as now produced for practical uses, are better known, they will become a powerful tool for classification. Serological characters are also important as in the classification of bacteria and yeasts. The presence or absence and degree of pathogenicity mainly concern classification beyond "species" from the theoretical point of view,



(after Tubaki, 1958a)

Fig. 1

but from the practical view point they also must be clarified. Chemical nature of the cell wall and the protein structure of fungi will also become extremely powerful tools for the classification. The same applies to other biological properties.

The method of conidial development is a character which is always adopted in the classification of fungi as far as they form conidia, and the method is very stable and fixed for each species or often for each genus. Fortunately the majority of Hyphomycetes can be subjected to pure culture under similar conditions, but on artificial media they do not necessarily grow in the same way as under natural conditions. Hence, selection of standard condition for comparison is very difficult. In 1958, Subramanian described that during the recent past outstanding progress in taxonomy has been made both on "classical" lines as well as on "experimental" lines and the experimental knowledge should be used to modify and strengthen the "classical" taxonomy.

Formerly, 1958, I attempted to classify the Hyphomycetes by the above described method. That is, employing the standard used by Hughes, I have classified many Hyphomycetes of coprophilous, fungicolous, leaf & stem groups, etc. into nine great sections and fourteen small sub-sections (fig. 1). The color of mycelium and conidia, the shape of conidia, and existence or nonexistence of septa, which were so far employed in older classifications, were regarded as of secondary importance. In that work, the sections were not named and no key was presented. In each section and sub-section, one species was chosen to show typical conidial development. It was not clear whether the natural relationships of these fungi are reflected in the above classification. Then, many ascomycetous fungi in their imperfect stages were tried according to the above method, and a solid trend was found. Namely, according to Saccardo's system, these ascomycetous fungi are classified into innumberable form-genera from the view point of imperfect fungi, but according to my system, they showed a fairly definite trend. Especially, Dothideales, Erysiphales, Hypocreales, Helotiales and Pezizales show a strong definite trend in the method of their sporulation as already described.

The previous report was written using microscopic features for grouping and arranged so as to show phylogenetic relationships, but the sections were not named. In the present study, the writer has extended the above study and the sections, including sub-sections, are reconsidered and named. An arrangement of Hyphomycetes into six divisions, as described later, is primarily based on the above concept on conidial ontogeny and also on behaviour of conidium-growing point which is described in the following discussion. No key of the genera to each division is presented in this paper. It is under preparation and will appear in the next paper concerning the taxonomic problems of Hyphomycetes after a precise examination of more fungi.

Considerations on the Conidium-growing Point

In addition to the above described features, the following structure also seems to be a important tool for classification. Namely, the behaviour of the conidium-growing point in relation to the conidia and conidiophores subsequent to their development;

in other words, "acropetal", "basipetal" and others. This character of the conidial development is also considered as reflected of the behabiour of the active, multiplying nucleus in the conidium-growing mother cell.

In the acropetal production of conidia, they are produced successively in the direction to the apex, i.e. the apical member is the youngest one (also called basifugal). In this case, the conidium-growing point ascends into the newest apical conidium from which a new one may develop as well. The daughter nucleus migrates into the new conidium and the same process is repeated. On the contrary, in basipetal conidial production which is in the direction of the base, the apical part is the oldest. In this case, the conidium-growing point does not move from the upper part of the conidiophore. Nuclear behaviour involves mitosis in the conidiophore and one daughter nucleus migrates through the tube into a new conidium in which it rests until that conidium begins to germinate. The other nucleus remains in the conidiophore and the process is repeated.

Therefore, the distinctive behaviour of the conidium-growing point also appears to be basic in attempting classification in Hyphomycetes as well as distinguishing conidial types.

It is surprising, at present, to find the following statement by Mason (1937): "when the first spore remains attached to its hyphae and produces by acropetal growth a long chains of other conidia from its apical end, the obvious presumption is that organic connection has not been severed at the base ……. The first formed spores, which are at the base of the chain, first act as part of thallus, in that they support in the air, and carry food to the later formed spores." Exactly, in acropetal succession of conidia, each one is connected by minute septal pores to each other and the apical conidium may be supplied with nutrients which come up from the first formed basal conidium. On the contrary, in the basipetal succession of conidia, such a phenomenon does not occur; only a newly formed basal conidium may be supplied with nutrients. To my knowledge, acropetal vers. basipetal succession are not mutually changeable in conidial ontogeny, which characteristic is considered to be a stable and reliable one in classification. From the above point of view, these two categories in the successive formation of conidia are adopted for the classification of Hyphomycetes. Therefore, the grouping of Hyphomycetes being proposed here is primarily based on the conidial development method and behaviour of the conidium-growing point.

Considerations of Terminology

Before discussing the classification of Hyphomycetes based on the categories which have been described, it is necessary to reconsider the terminology used in classifying Hyphomycetes.

The term "conidium" has been used for those spores produced without nuclear fusion followed by meiosis unenclosed in a sporangium. Unless otherwise provided, the term conidium (pl. -ia) is used in this general sense. The term "chlamydospore"

is used for a spore formed by rounding up of a mycelial unit, coupled with a thickening of the cytoplasm and wall generally sphaerical, thick walled, larger than the hypha which bears it, terminal or intercalary, single or in chains.

To clarify the meaning of terms used in designating conidial types, they are listed and briefly defined into the morphological categories with reference to the papers of Mason (1933, 1937), Hughes (1953), Goos (1956), Subramanian (1962), etc. With regards to the new terms, the reasons for their proposal will be described in the explanatory remarks of each division.

Blastospore: produced as a blown-out end from a parent cell, or directly from a fertile hypha; originally they develop in acropetal succession.

botryose solitary blastospore: blastospore occur in botryose clusters.

botryose blastospore: blastospore in chain.

Radulaspore: formed side by side upon small denticulated sterigma (spicule projection) which cover the surface of the conidiophore-tip, or which may be produced upon intercalary swelling; not in chains. Radulaspores are divided into two types as follows:

termino-radulaspore: terminates the growth of the conidiophore and is formed side by side upon small denticulated sterigma (spicule projection), usually solitary. Conidiophore increases in length as new ones develop.

pleuro-radulaspore: formed side by side upon small denticulated sterigma (spicule projection) which cover the surface of the terminal or intercalary swellins of the conidiophore, solitary or in short chains. Originally not terminate the growth of the conidiophore.

Aleuriospore: formed as a blown-out end of a hyphal tip or as a lateral protrusion, which is cut off by a septum; usually with flattened base encircled by a minute frill, single or in chains.

meristem aleuriospore: aleuriospore formed due to the meristematic growth of the conidiophore.

Phialospore: abstricted from the mouth of a phialide endogenously in basipetal succession; forming chain or grouped into a false head.

Porospore: formed through minute terminal or lateral pore in the wall of conidiophore.

Classification

Though more data appears necessary as to the conidia-forming mechanism, the above categories were used for major grouping of Hyphomycetes. They are:

Division	Blastosporae
〃	Radulasporae
〃	Aleuriosporae
〃	Phialosporae
〃	Porosporae
〃	Arthrosporae

Blastosporae
Hyphomycetae producentes blastosporas
Genus Typicum: *Cladosporium* Link 1815
Hyphomycetes producing blastospores.
Type Genus: *Cladosporium* Link 1815

Radulasporae
Hyphomycetae producentes radulasporas
Genus Typicum: *Beauveria* Vuillemin 1912
Hyphomycetes producing radulaspores.
Type Genus: *Beauveria* Vuillemin 1912

Aleuriosporae
Hyphomycetae producentes phialosporas
Genus Typicum: *Scopulariopsis* Bainier 1907
Hyphomycetes producing aleuriospores
Type Genus: *Scopulariopsis* Bainier 1907

Phialosporae
Hyphomycetae producentes phialosporas
Genus Typicum: *Catenularia* Grove 1886
Hyphomycetes producing phialospores
Type Genus: *Catenularia* Grove 1886

Porosporae
Hyphomycetae producentes porosporas
Genus Typicum: *Helminthosporium* Link 1822
Hyphomycetes producing porospores.
Type Genus: *Helminthosporium* Link 1822

Arthrosporae
Hyphomycetae producentes arthrosporas
Genus Typicum: *Geotrichum* Link
Hyphomycetes producing arthrospores.
Type Genus: *Geotrichum* Link

In the classification of Hyphomycetes, it is desirable to attempt to classify all the known genera on the basis of the above described criteria. However, it is fairly difficult to treat all of these bibliographic genera. If the peculiar type of conidial production is examined in each genus and is considered as of permanent importance in classification, these too much splintered genera may be adjusted in number. In fact, there are still too many genera and too widely divergent points of view current in present mycology.

At present, therefore, nearly 130 genera were selected after studying typical cultures or materials, or referring to authoritative literature. The arrangement is not entirely completed and many more developmental studies are now needed. Often, well-known genera are omitted because of uncertainties in their conidial structures. A key will be presented in the near future. The generic names included are restricted

to those of common members and their cultural features were considered of great importance to their arrangement at present. In this respect, a revision of Hughes (1958) was introduced in many cases. Generic names which were considered as synonyms are listed under the appropriate headings. Generic synonyms proposed by Hughes (1958) were often omitted except for a few cases. The writer did not examine all of the type specimens or cultures derived from them, so no indication by "=" or "≡", meaning facultative or obligate synonyms was expressed.

For arranging the genera in each division, ecological characters were not stressed; special cases such as aquatic were treated elswhere (Tubaki, 1958a, 1960a).

In general, the drawings were borrowed from previous papers of the present author as is indicated in the description. The appearances of fungi under different magnifications are shown. Regarding many species which were included here and not reported previously from Japan, the sources of the collections were given. The sourse of other fungi were already reported by the present author.

Key to the divisions:

1. conidium-growing point accompanied by a newly formed apical conidium; of the blastospore-type developed acropetally or borne simultaneously on a swollen base. ..... Blastosporae
   conidium-growing point on the upper part of conidiophore, not acropetal 2
2. conidiophore increases in length as conidia develop 3
   conidiophore does not increases in length 4
3. conidia of the radulaspore-type ..... Radulasporae
   conidia of the aleuriospore-type, conidiophore with annellation ..... Aleuriosporae
   conidia of the porospore-type ..... Porosporae
4. conidia of the phialospore-type ..... Phialosporae
   conidia of the arthrospore-type ..... Arthrosporae
   conidia solitary or catenulate and of the aleuriospore-type .. Aleuriosporae

Blastosporae

In this group those fungi which produce blastospores are included. Previously (1958b) the writer included these fungi in Sect. I and divided them into two subsections, IA and IB. However, in the present study, these Hyphomycetes are united into the division Blastosporae. This division is also treated by Petersen (1963) and corresponds to the Ciferri's family Aureobasidiaceae and also to Subramanian's family Torulaceae Corda emend. Subramanian (1962).

The term "blastospore" is less clear in its meaning and has been used loosely. Under certain cultural conditions, differentiation between blastospore, radulaspore and phialospore is not satisfactory as pointed out by Cooke (1962). In the present discussion, distinct morphological categories of the blastospore is recognized as pre-

viously described. In addition to the above concept, I believe the meaning of the term blastospore could well be limited to those conidia developing acropetally in chains by further budding or to those in rows arising as buds. Accordingly each conidium in a chain, except for the terminal one, acts not only as blastospore but as blastospore-mother cell. The chain may frequently branches due to the formation of more than one bud from a single cell. Sometimes the lateral branches of conidiophores are modified entirely into a number of conidia or into solitary conidia and in these instances the conidia are borne simultaneously on conspicuous denticles. The solitary conidium or short, simple or branched chains of conidia may be aggregated on well differentiated swollen cells. Hughes (1953) proposed the term "botryose solitary blastospore" for those blastospores occur in botryose clusters and "botryose blastospore" for conidia in chains.

Important genera of the division Blastosporae.

conidia are blastospores in acropetal succession

Cladosporium Link 1915

Hormodendrum Bon. emend Harz 1871

Hyalodendron Diddens 1934

Septonema Corda 1837

Bispora Corda 1837

Speiropsis Tubaki 1958

Strumella Fr. 1825

Tricellula v. Beverwijk emend Haskins 1958

Volucrispora Haskins 1958

conidia are botryose solitary blastospores

Botryosporium Corda 1833

Phymatotrichum Bon. 1851

Cephaliophora Thaxter 1903

Gonatobotrys Corda 1839

Oedocephalum Preuss 1851

conidia are botryose blastospores

Gonatorrhodiella Thaxter 1891

Radulasporae

This group includes those fungi which produce radulaspores, which were assigned to Sect. II in a previous paper (1958b). The term radulaspore was introduced by Mason (1933). Here, we must note a term "terminus spore", proposed by Mason (1933). He defined terminus spore as one which terminates the growth of the sterigma and "radula spore" as one which does not necessarily terminate the growing point of a hypha. However, it is not clear if these radula-spores are different from terminus-spores, as well be seen in the genera *Arthrobotrys*, *Aureobasidium*, etc. Accordingly,

it is desirable to divide the term radula-spore into the two terms, namely *termino-radulaspores* and *pleuro-radulaspores*, as defined before. Termino-radulaspores are in marked contrast to the pleuro-radulaspores as they terminate the growth of the conidiophore.

Kendrick (1962) proposed the term " sympodula" for the sporogenous cells of the present group and the conidia were specifically called sympodioconidia.

The helicosporous group, Helicosporae Saccardo (1886), seems to be adequately characterized a present. In this group, helicoid conidia arise in succession on short lateral sporogenous cells of conidiophores. Though the sporulating mechansim of these fungi is homologous throughout the group, these members are distributed in the families Moniliaceae, Dematiaceae or Tuberculariaceae by the older system. For example, in the hyaline conidia-bearing fungus, *Helicodendron*, conidiophores and aerial hyphae are both dark colored in culture. Consequently, color of conidia is not a reliable criterion for delimiting the major groups of Hyphomycetes.

As already described (1958b), I speculated that *Calcarisporium* and *Verticicladium* could be derived from Ophiostomataceae. *Hyalodendron*, also known as the imperfect stage of *Ceratocystis*, fits closely to the so-called *Cladosporium*-type of some *Ceratocystis*. Further studies are necessary to further clarify these relationships.

Acrostaphylus, typified by *A. hypoxyli* Arnaud, includes *Nodulisporium* and differs from *Calcarisporium* only in its fuscous color (Tubaki, 1958b). The former is considered as the conidial stage of *Daldinia*. *Calcarisporium* also is close to *Hansfordia* and precise comparative study is necessary to differentiate them.

Verticicladiella, differing from *Verticicladium* in the absence of a slimy head, was redescribed by Kendrick (1962) and several *Leptographium*-like species were placed in this genus, e.g. *V. penicillata* (Grosm.) Kendrick = *Leptographium penicillatum* Grosm. In these species, conidia are of the radulaspore-type, not of the aleuriospore-type, thus differing from those of true *Leptographium* which shows annellation. Kendrick's work is of interest, since a conidial stage of the *Verticicladium*-type was found in *Ceratocystis minor* as already mentioned (1958b) and *V. penicillata* K. was formerly linked with its perfect stage, *Ceratocystis penicillata* (Grosm.) Moreau. Therefore, some of the imperfect stages of the Ophiostomataceae are in this group.

Sympodiella acicola Kendrick (1958) produces cylindrical conidia acropleurogenously on the terminal position of the conidiophore and can be included in this group.

Brachydesmiella biseptata Arn. was redescribed by Hughes (1961) including a latin diagnosis and can be included in this group.

The generic name *Streptothrix* Corda was commonly used for the fungi which produce radulaspores on helically twisted conidiophores, but was recently replaced by *Conoplea* Pers. (Hughes, 1960).

Aureobasidium is a genus which was studied precisely on the structure of radulaspore production. The genus *Aureobasidium* was formerly known as *Dematium*, etc. The typical species, *Aureobasidium pullulans* (De Bary) Arnaud was known as *Dematium*

pullulans De Bary, the so-called "black yeast". Following the taxonomic work by Arx (1957) and Ciferri (1956), Cooke (1959, 1962) published a life-historical and taxonomical work on this species. The present author treated it in Sect. IB, however, because it is now clear that this fungus produces radulaspores (1958). This fungus is included in the Radulasporae in the present study. (Pl. 1C)

Regarding the genus *Heterosporium*, De Vries (1952,) Hughes (1958) and Barron and Busch (1961) suggested that it has no taxonomic status and *Scolecobasidium* is accepted as proper for the disposition for these and similiar forms. In these species, conidia are of the radulaspore-type and are produced in clusters or in acropetal series from the ends of tubular extensions of the conidiophores.

Beltrania, based on *B. rhombica* Penzig, produces biconic conidia on denticulate conidiophores or on separating-cells, so these conidia are originally of the radulaspore -type.

Tritirachium differs from *Beauveria* in the absence of a bulbous base to the verticillate branches. Conidiophores of *Beauveria* are clustered on the mycelium on the entomogenous host, but in culture they are not. The establishment of *Beauveria virella* Mangenot (1952) in which conidiophores are *Tritirachium*-like and not clustered on the mycelium is correct. At present, therefore, *Tritirachium* is treated under the genus *Beauveria*. (Pl. 2I)

Recently (1962), Meredith reported on the spore discharge of *Cordana musae* and *Zygosporium oscheoides*, and the conidial development of the former species was described so that it can be included in this group.

Important genera of the division Radulasporae

conidia are terminus-radulaspores

Acrothecium Corda 1839

Beauveria Vuillemin 1912

Tritirachium Limber 1940

Brachydesmiella Arnaud 1954

Brachysporium Sacc. 1881

Cercospora Fres. 1863

Cercosporella Sacc. 1825

Chaetopsis Grev. 1825

Chloridium Link 1809

Dactylaria Sacc. 1880

Dactylella Grove 1884

Dicranidion Harkness 1885

Diplocladiella Arnaud 1953

Diplorhinotrichum Harkness 1902

Fusicladium Bon. 1851

Graphium Corda 1837 (radulaspore-type)

Ramularia Unger 1833

Acrotheca Fuckel 1860
Ovularia Sacc. 1880
Scolecotrichum Abbott emend Barnett et Busch 1962
Heterosporium Klotz. 1877
Sporotrichum Link 1809
Sympodiella Kendrick 1958
Verticicladium Preuss 1851
Verticicladiella Kendrick 1962
Virgaria Ness 1816

conidia are pleuro-radulaspores
Arthrobotrys Corda 1859
Aureobasidium Viala et Boyer 1981
Beltrania Penzig 1882
Botrytis Pers. 1801
Calcarisporium Preuss 1851
Cordana Preuss 1851
Hansfordia Hughes 1951
Acrostaphylus Arnaud 1953
Papularia Fr. 1825
Pseudobotrytis Krzem. et Badura 1954

Aleuriosporae

In this group are included those fungi which produce aleuriospores. The term aleuriospore was coined by Vuillemin (1911) and is not clear in its meaning and the differentiation is different depending on such authors as Mason (1933), Langeron and Vanbreuseghem (1952), Ingold (1942), Vuillemin (1911), Subramanian (1962), Hughes (1953), Goos (1956), etc. The writer agrees with the concept of Goos (1956), which distinguishes between aleuriospores and chlamydospores. This division is also treated by Petersen (1962, 1963) and may correspond to the family Bactridiaceae of Subramanian (1962).

In the previous paper (1958b), I included aleuriospore-forming fungi in Sect. III and divided them into three sub-sections: A, producing typical annellophores; B, producing solitary aleuriospores; C, with a separating cell between conidiophore and aleuriospore, which breaks down to liberate the aleuriospore. At present, the writer divided Aleuriosporae into two sub-groups: those forming true aleuriospores and those forming *Trichothecium*-type aleuriospores, *meristem aleuriospores*. In the former case, aleuriospores are in a chain or solitary and annellation can be found in those fungi which produce aleuriospores in succession. Conversely, those fungi which produce successively true aleuriospores have annellations on their conidiophores in most cases and these conidia have a somewhat flattened bases with the same width as the apex of the conidiophores.

In the latter sub-group, those which form *Trichothecium*-type conidia, is peculiar in the method of conidial development and is treated in Section IX (1958b). *Trichothecium*-type is typical of this group. The primary conidium is produced at the apex of the conidiophore and is delimited by a septum. Immediately below this septum, the conidiophore swells to one side, forming the secondary spore-primordium, which elongates. A septum is then formed in the conidiophore cutting off the secondary conidium, which now has the primary conidium attached to it. A swelling is formed to one side below the secondary conidium where a septum then cuts off the tertiary spore-primordium, and so on. Usually these conidia have nipple-like projections with an encircling frill at the points of attachment. Because the matured conidia were cut off by septa and the dimension of the point of the attachment is equal to that of tips of the conidiophores, they can be looked on as aleuriospores as already mentioned by Vuillemin (1910). They are produced basipetally due to the meristematic growth of the conidiophore. The conidiophores are meristematic in the upper parts and do not increase in length, thus differing from the *Scopulariopsis*-type. So, these conidia are called *meristerm-aleuriospores*. The term meristem arthrospore was proposed by Hughes (1953) and included those conidia which develop true chain of conidia basipetaly from poorly differentiated conidiophores which posesses a generative or meristematic region towards the apex. The conidiophore merges imperceptibly with the chain of conidium-initials which exhibit a gradual maturation toward the distal end of the chain. *Sirodesmium*, a typical fungus, was previously described in Sect. V. The conidiophore does not increase in length, and conidium-initials are characteristically not distinct because they develop from the meristematic part of the conidiophore. Therefore, conidial production is basipetal and it seems better to treat these conidia under the type of meristem-aleuriospores, not an arthrospores. Although many kinds of fungi are included in this division, phylogenetical lines are different depending on the groups. For example, those producing meristem-aleuriospore are derived from Hypocreales, e.g. *Trichothecium*-stage of *Hypomyces* (Tubaki, 1960b) and *Dactylium dendroides* which is a conidial stage of *Hypomyces rosellus*. On the contrary, those of the *Scopulariopsis*-type with annellation can be found in the conidial stages of *Microascus* (Udagawa, 1962).

Bibliographically (Hughes, 1953, etc), typical annellations were recognized in the following genera: *Annellophora*, *Arthrobotryum*, *Deightoniella*, *Ceratosporella*, *Farlowiella*, *Leptographium*, *Masoniella*, *Phaeoscopulariopsis*, *Pollaccina*, *Scopulariopsis*, *Podoconis*, *Spilocaea*, *Sporidesmium*, *Mastigosporium*, *Monosporella*, *Stigmia*, *Stysanus*, *Trichurus*, *Triposporium*. Actually, the writer has seen isolates of the following species which have annellations: *Arthrobotryum* sp., *Deightoniella torulosa*, *Gliomastix convoluta*, *Leptographium lundbergii*, *Masoniella grisea*, *Phaeoscopulariopsis* sp., *Scopularipsis brevicaulis*, *Sporidesmium niligirense*, *Stysanus stemonitis*, *Trichurus terrophilus* and *Acremoniella atra*, as can be seen in Plate 1a. Mason's opinion (1933) that conidia of *Acremoniella atra* are terminal chlamydospores is correct, but in the present study, it

appears that the fungus forms not only a terminal aleuriospore but successive aleuriospores with annellations. In *Trichurus terrophilus*, annellations are evident. but *Trichurus* differs from *Stysanus* only in the presence of a sterile spine among conidiophores even in cluture.

Among those fungi which produce solitary aleuriospore (no annellation occurs in this case), two kinds of aleuriospore-production can be found, e.g. in the Dermatophytes. For example, *Microsporum* produces terminal aleuriospores, namely "macroconidia", which have separating cells between conidia and conidiophores. In my previous paper (1958b), fungi which have such separating cells were assigned to Sect. IIIC. When such conidia (aleuriospores) with separating cells have separated, the separating cell is a fairly conspicuous little collar at the ends of the conidiophores and the bases of conidia. (Pl. 2H)

Judging from the original figure of *Monocillium humicola* (Barron, 1961), the genus *Monocillium* is very similar to *Masoniella*. Systematically, it seems to be closely related to *Masoniella* and also to *Scopulariopsis*. *Phaeoscopulariopsis* differs from *Scopulariopsis* only in its darker colored conidial apparatus and is treated as an earlier name for *Masoniella* (Hughes, 1953). However, dark colored fungi of the *Phaeoscopulariopsis* are included in *Scopulariopsis* in the present study, and the genus name *Masoniella* is applied to those fungi which produce successive aleuriospores from rather narrow, unbranched annellophores. There is no reason that the name *Scopulariopsis* should be restricted to those members which produce hyaline or light colored aleuriospores.

Important genera of the division Aleuriosporae

conidia are aleuriospores in chains; with annellation

Acremoniella Sacc. 1886
Annellophora Hughes 1951
Arthrobotryum Ces. 1854
Bactrodesmium Cooke 1883
Deightoniella Hughes 1952
Dictyosporium Corda 1836
Speira Corda 1837
Gliomastix Gueguen 1905
Leptographium Lagerb. et Melin 1927
Monocillium Saksena 1954
Scopulariopsis Bainier 1907
Masoniella Smith 1952
Phaeoscopulariopsis Ota 1928
Spilocaea Fr. 1825
Sporidesmium Link 1809
Stysanus Corda 1837
Trichocladium Harz 1871
Trichurus Clement et Shear 1896

conidia are solitary aleuriospores; without annellation

Allescheriella Henn. 1897

Coccospora Wallr. 1833

Amallospora Peznig 1897

Bactridium Kuntze 1817

Camposporium Harkness 1884

Candelabrum v. Beverwijk 1951

Chrysosporium Corda 1833

Chrysosporium-state of the Dermatophytes

Clasterosporium Schw. 1832

Humicola Traaen 1914

Microsporum Gruby 1843

Keratinomyces Vanbreuseghem 1952

Mycogone Link 1809

Nigrospora Zimm. 1902

Piricauda Bubak emend. Moore 1956

Sepedonium Link 1809

Stephanoma Wallr. 1833

Triposporium Corda 1837

conidia are meristem-aleuriospores

Coniosporium Link 1809

Sirodesmium de Not 1849

Cylindrophora Bon. 1881

Dactylium Nees 1817

Trichothecium Link 1824

Phialosporae

This group includes those fungi which produce phialospores. The conidium-bearing structure should be called a phialide. The phialides are unicellular, terminally or intercalarly placed on aerial hyphae. They are cylindrical, ovoid, or flask-shaped, with a narrower-distal neck, commonly have a terminal belly collarette; the length of phialide does not increase except in a few cases.

As already discussed in a previous paper (1958b), there are all gradations in the type of phialospore-production, such the degree of the collarette-development and as to whether the conidia are endogenous or not. The present author formerly included the phialospore-forming fungi in Sect. IV which was divided into two sub-sections as IVA and IVB: the former in which conidial production is truly endogenous. In both cases, the phialospores are morphologically similar. However the phialospores are more deeply produced in the latter. In the former, the collars are often small and inconspicuous and are not distinctly flared outwards, however, it is possible to recognize them

under the high-power of the microscope or with phase-contrast microscopy. This kind of conidial development is sub-endogenous. So, an endogenous lateral production must be recognized. A number of fungi can be included in this group.

This division corresponds to the family Tuberculariaceae of Subramanian (1962).

In addition, the following two forms must be discussed: biphialide and polyphialide. The term polyphialide was proposed by Hughes (1951) to include phialides which produce more than one open end from each of which a basipetal succession of phialospores is produced. The term biphialide was shown by van Beyma (1940) to include phialides which have double tops, surrounded by a small single collar, from which conidia can be produced at the same time.

The following fungi were found to have polyphialides: *Catenularia fuliginea* Saito (Plate IG), *Catenularia heimii* Mangenot (Pl. IIA), *Catenularia*-state of *Chaetosphaeria innumera* Tul., *Cat. myriocarpa* (Fr.) Booth and *Chloridium chlamydosporis* (v. Beyma) Hughes(=*Bisporomyces chlamydosporis*) (Mangenot, 1952; Tubaki, 1963). Therefore, forms developing polyphialides and biphialides are related to each other.

Catenularia fuliginea Saito was erroneously used as a synonym for *Torula sacchari* Corda or *Sporendonema epizoum* (Corda) Ciferri et Redaelli. However, conidia of this osmophilic fungus are not of the arthrospore-type, but of the phialospore-type and are produced through distinct collarettes. In addition, this fungus developes polyphialides, so it must be included in the genus *Catenularia* as originally described by Saito (1904). (Pl. 1G)

Camposporium is placed near *Acrothecium*, but differs from it in the presence of bi- or polyphialides.

Members of *Cylindrocladium* are well known for their cylindrical, several-celled conidia which are packed together. It can be commonly found in the southern Japan. As discussed previously, the present author considered the following genera as congeneric with *Cylindrocladium: Gliocladiopsis* Saksena and *Moeszia* Bubak.

As for the genus *Memnoniella*, the conclusions of Zach (1946), Bisby & Ellis (1949) and Barron (1962) were discussed and it was reduced to synonymy with *Stachybotrys*.

The line separating *Acrophialophora* from *Paecilomyces* is not distinct.

Isaria, a unique entomogenous fungus forming a light-colored synnemata, usually developes powdery thin colonies on most media and the microscopical features are similar to those of *Paecilomyces*. Accordingly, the former genus is placed in the same position as *Paecilomyces* along with those of the synnema-forming group in the present study.

The line separating *Margarinomyces* from *Phialophora* also is not distinct, as was already mentioned (1958b), and the author concluded that the former genus is congeneric with the latter in the present study. Even in *Margarinomyces*, the collarette of the phialides is distinct like that of *Phialophora* (Pl. 2D). This is especially striking in a culture of *Margarinomyces bubaki* derived from the type culture. This was already discussed by Cain (1952) and Cooke who derived *Margarinomyces* from *Phialophora*

by reduction of the collar and *Aureobasidium* from *Margarinomyces* by a loss of the phialide (1962).

In addition, we must discuss the separation of *Phialophora* from *Catenularia*. The generic name *Catenularia* is usually accepted for organisms which produce a short chain of colored conidia from conidiophore with flaring collars. However, after examining a culture of *Catenularia heimii* Mangenot, it appeared that these conidia often may be grouped in a head under high humidity in addition to catenulation in a chain. So, it is of doubtful validity to separate these genera from the view point of the artificial classification of Hyphomycetes. Then, from the rule of priority, the generic name *Catenularia* must be chosen to typify the given species when both genera are treated as congeneric. Mangenot (1952) discussed the endogenous sporulation of this fungus. From the viewpoint of phylogenetical lines, both genera can be derived from different ancestors: namely, some *Phialophora* from Discomycetes, especially from the genus *Mollisia* (Mangenot, 1958, 1961) and some *Catenularia* from Sphaeriales, especially from the genus *Chaetosphaeria* (Booth, 1958). As described by many authors, phialospore-production coincides morphologically with spermatium-formation of Ascomycetes. For example, *Phialophora*-like spermatium-formation can be found bibliographically in the following ascomycetous fungi: *Guignardia*, *Martinia*, *Monilinia*, *Nectria*, *Lambertella*, *Rutstroemia*, *Sclerotinia*, *Septotinia*, etc. *Phialophora*-like structures are also found in the spermatial structures of *Coryne sarcoides*. In any event, it will be assumed that many species of *Phialophora* can be derived from Discomycetes, especially from Helotiaceae.

As noted elswhere, some *Leptographium*-like hyphomycetes produce radulaspores, not aleuriospores like the true *Leptographium* which were named *Verticicladiella* Hughes. Another *Leptographium*-like fungi are also maintained as distinct from that genus on the bases of spore ontogeny and were named under the generic name *Phialocephala* Kendrick (1961a, 1963) which was placed fairly near *Thysanophora* Kendrick (1961b).

Recently, Hughes (1963) published a monographic study of the genus *Menispora* and described its phialospore-production in detail.

There are peculiar fungi which produce phialospores endogenously and they were previously included into Sect. IVB: *Chalara*, *Chalaropsis*, *Hughsiella*, *Sporoschima* and *Thielaviopsis*. *Chalaropsis* is similar to *Chalara* except for production of chlamydospores. *Chalara quercina* is the imperfect stage of *Ceratocystis fagacearum*, and *Thielaviopsis paradoxa* is of *Ceratocystis paradoxa*. This phylogenetical fact indicates that the presence of chlamydospores seems not to be a reasonable character of differentiating these genera. However, for practical purpose *Chalara* and *Thielaviopsis* were separated in the present study, and *Chalaropsis* was treated as congeneric with the latter genus.

Important genera of the division Phialosporae

Phialospores found endogenously within the phialides

Chalara Rabenh. 1844
Hughsiella Batista et Vital 1956
Thielaviopsis Went 1893
Chalaropsis Peyron. 1916

phialospores formed sub-endogenously through the collarettes
Aspergillus Mich. emend Thom et Church 1926
Cacumisporium Preuss 1851
Catenularia Grove 1886
Cephalosporium Corda 1842
Cylindrocladium Morgan 1892
Gliocladiopsis Saksena 1954
Moeszia Bubak 1914
Diplosporium Link 1824
Fusarium Link 1809
Gliocladium Corda 1840
Gonytrichum Nees et Nees 1818
Graphium Corda 1837
Menispora Pers. 1822
Metarrhizium Sorok. 1879
Mycogone Link 1809 (*Cephalosporium*-, *Verticillium*-stage)
Paecilomyces Link emend Brown et Smith 1957
Acrophialophora Edward 1960
Isaria Pers. 1801
Penicillium Link emend Thom 1930
Phialocephala Kendrick 1961
Phialophora Medlar emend Binford, Hess et Emmons 1944
Margarinomyces Laxa emend v. Beyma 1943
Sepedonium Link 1809 (*Cephalosporium*-, *Verticillium*-stage)
Stachybotrys Corda 1837
Memnoniella Hoehn. 1923
Thysanophora Kendrick 1961
Trichoderma Pers. 1801
Verticillium Nees 1817
Stachylidium Link 1809

Porosporae

Fungi which produce porospores are included in this group. The pore is delimited from the conidiophores abruptly and the base of the liberated porospores is rounded or truncate, but the wall is continuous around the base except for the basal pore. These porospores are produced in verticils apically and laterally, often in chains. The term

porospore was proposed by Hughes (1953). Typically, they are recognized in *Helminthosporium*, *Curvularia*, *Alternaria*, *Stemphylium*. They were included in Sect. VI (Tubaki, 1958b).

Important genera of the division Porosporae

Alternaria Nees 1817
Curvularia Boedijn 1933
Helminthosporium Link (as *Helmisporium* Link after Hughes, 1958)
Stemphylium Wallr. 1833

Arthrosporae

Fungi which produce arthrospores are included in this group. They are characterized by the production of conidia by the septation and breaking up of simple or branched hyphae. Previously (1958), this group was included in Sect. VII and was divided into two sub-sections: VIIA and VIIB, depending on whether the arthrospores were produced exogenously or endogenously. In the exogenous method, transverse septa are laid down in hyphae and the outer wall. The protoplasm of the hyphae may break down by the fragmentation of transverse septa at nearly the same time and conidia set free. In the second method, conidia develop endogenously within the outer wall of the hyphae and a papilla or a minute pore is sometimes clearly visible at each end of the conidium. When these endogenous conidia have been liberated, minute frills may remain at both ends which are remnants of the outer wall of the hyphae.

This division corresponds to Ciferri's family Geotrichaceae (1958b) and also to Subramanian's family Geotrichaceae (1962).

Geotrichum candidum Link emend Charmichael (1957) is a typical species in this group and known as typical fungi in polluted water (Tubaki, 1962). Members of the genus *Geotrichum* are considered as those lacking completely the enzymatic system which controlls the production of blastospores according to Nickerson's enzymatic theory (1948).

Endogenous production of arthrospores can be found in *Coremiella ulmariae* (MacWeeney) Mason, *Sporendonema casei* Desm. (Pl. 2J) and *Sporendonema purpurascens* (Bon.) Mason et Hughes (Pl. 2K). In *Sporendonema*, the arthrospores are formed endogenously within the conidiophore, and either extruded or liberated finally by the decay of conidiophore wall. In his study on *Sporendonema*, Ciferri (1958a) treated *Catenularia fuliginea* Saito as synonymous with *Sp. epizoum* (Corda) Ciferri et Redaelli. However, *C. fuliginea* produces phialospores through distinct collarette and also has polyphialides. Accordingly, this species must be reduced to the genus *Catenularia* as already mentioned. In 1960, Caretta reexamined and amended the genus *Coprotrichum*, separating it from *Geotrichum* and *Sporendonema*. *Oidiodendron* resembles *Coremiella* in the production of their thick-walled spores within the parental hyphal walls, but was separated from the latter morphologically and culturally by Barron (1962).

Important genera of the division Arthrosporae.

conidia are endogenous arthrospores

Coremiella Bubak et Krieger 1912

Oidiodendron Robak 1932

Sporendonema Desm. emend Oud. 1885

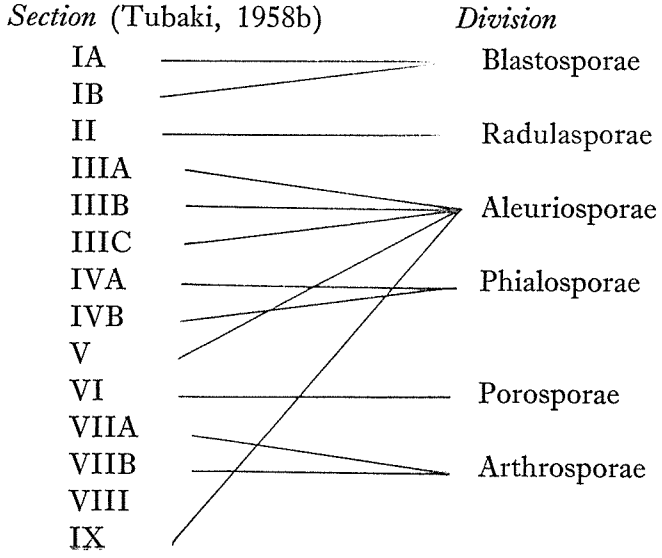
Coprotrichum Bon. 1851

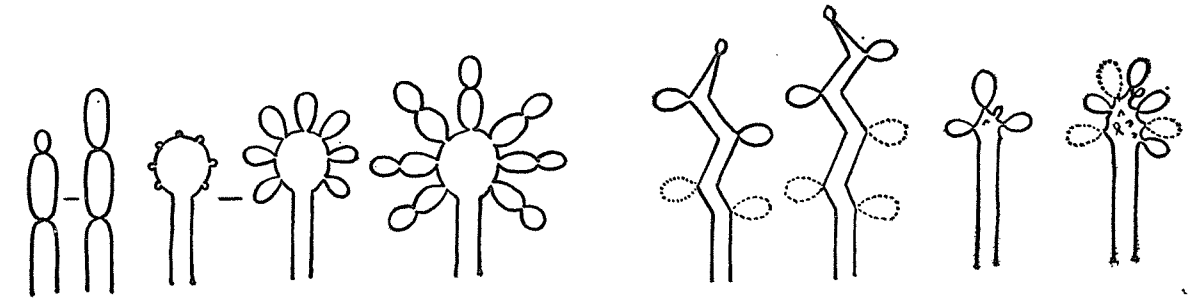
conidia are exogenous arthrospores

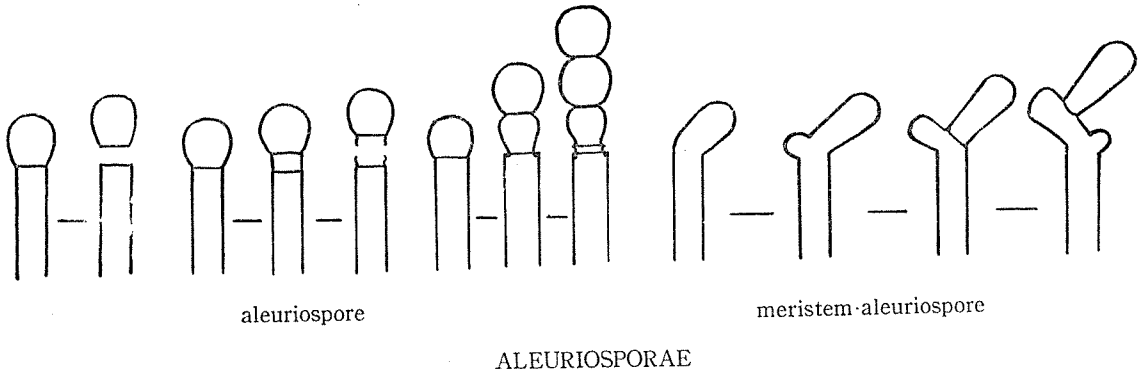
Geotrichum Link 1809

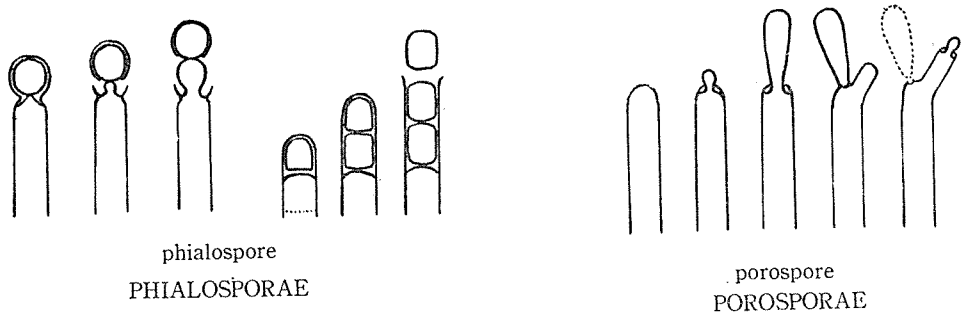
Discussion and Conclusion

Natural features of Hyphomycetes have been considered to be displayed only when they are growing under natural enviromental condition and numerous fungi have been thus described, under the assumption that these natural features are displayed on the host plant. As results, too many new taxa has been proposed and too much stress have been given to the host-relationships and color and size of asexual reproductive structures. The only way to observe the characteristics of fungi either in culture or on field material is to study the precise method of conidial development. From the viewpoint of conidium-ontogeny, the author earlier proposed a classification of Hyphomycetes (1958b) (fig. 1). Since the characteristics of the conidial development of Hyphomycetes have become better known, the author has continued to study this feature of various fungi, and, at present, proposes to assign them to six taxonomically named divisions, i.e. Blastosporae, Radulasporae, Aleuriosporae, Phialosporae, Porosporae, and Arthrosporae. Six terms for conidia: blastospore, radulaspore, aleuriospore, phialospore, porospore and arthrospore were defined. New terms, *termino-radulaspore*, *pleuro-radulaspore* and *meristem-aleuriospore* were proposed; respectively representing the *Beauveria*-stage, the *Aureobasidium*-stage and the *Trichothecium*-stage. A comparative table between the previous and the present concept is as follows:









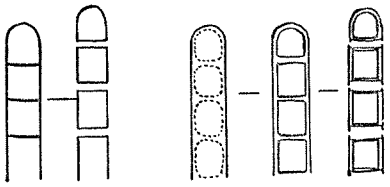


Fig. 2 Schematic figures of Divisions

In the present study, emphasis has been given to the conidium-ontogeny as in the previous work (1958b), and in addition, the behaviour of the conidium-growing point was also considered as an important character for classification. The behaviour of the conidium-growing point was divided into two categories, i.e. acropetal vers. basipetal. In the first case, the conidium-growing point along with the uppermost newly formed conidium, and conidiophore does not increase in length, and the terminal conidium also resumes the function of a conidium-bearing mother cell. In the latter case, the conidium-growing point terminates the conidiophore. These can be further differentiated by the nuclear behaviour, namely in acropetal development the active multiplying nucleus is in the newest conidium whereas in basipetal development remains at the top of the conidiophore. A key to genera in each division is being constructed and will appear in the next paper.

A study of more genera by the precise examinations of each conidial ontogeny is necessary for a clear understanding of the relationships of Hyphomycetes.

Thanks are due to Mr. I. Asano, T. Ito and Y. Furukawa for their helpful assistance.

Part of this work was supported by a grant-in-aid from the Science Research Fund of the Ministry of Education.

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IFO-6795, CBS; *Mollisia discolor* (Mont.) Phill., *Moll. ligni* (Desm.) Karst. and *Moll. heterosporum* Le Gal apud Le Gal et Mangenot, from Univ. Nancy (Mangenot); *Piricauda nodosa* (Preuss) Moore, Herb. IFO-10361, on *Machilus thunbergii*, Oosumi Penn., Japan, 20 July, 1962; *Scolecotrichum graminis* Fuckel, IFO-6684, Nat. Hug. Lab. Japan (Kurata), B-12; *Sporendonema casei* Desm., IFO-7656, Dept. Agr. Hokkaido Univ. (Sasaki, 1907); *Sporendonema purpurascens* (Bon.) Mason et Hughes, IFO-7659, IMI-45638; *Stysanus stemonitis* (Pers.) Corda, IFO-7677, CBS; *Thielaviopsis paradoxa* (de Seynes) v. Hohn., IFO-6190, Nat. Inst. Agr. Sci. Tokyo; *Trichurus terrophilus* Swift et Povah, IFO-7660, IMI-46251.

Explanation of the Plates

Pl. 1. A, *Acremoniella atra*, showing annellations; B, aleuriospores of *Allescheriella crocea*; C, radulaspore-formation of *Aureobasidium pullulans*; D, blastospore-formation of *Botryosporium pulchrum;* E-F, sporophore and porospore of *Corynespora gigantea;* G, polyphialide of *Catenularia fuliginea;* H, biphialides of *Chloridium chlamydosporis;* I, biphialide of *Cistella* sp.; J, porospore-formation of *Curvularia lunata;* K, endogenous phialospores of *Chalaropsis thielavioides*. Pl. 2. A, polyphialide of *Catenularia heimii;* B-C, development of meristem-aleuriospores of *Dactylium dendroides;* D, phialide with collarettes of *Phialophora fastigiata;* E, blastospores of *Oedocephalum coprophilum;* F, bladder-like projection of *Helicomyces* sp., after liberation of the helicoid radulaspores; G, bulbous conidiophores of *Deightoniella torulosa;* H, separating cell of *Microsporum canis;* I, zig-zag formed sporophores of *Tritirachium purpureum* after liberation of the radulaspores; J-K, endogenous arthrospore-formation of *Sporendonema casei* and *Sp. purpurascens*: L, annellation in *Stysanus stemonitis;* M, endogenous phialospores of *Thielaviopsis paradoxa*.

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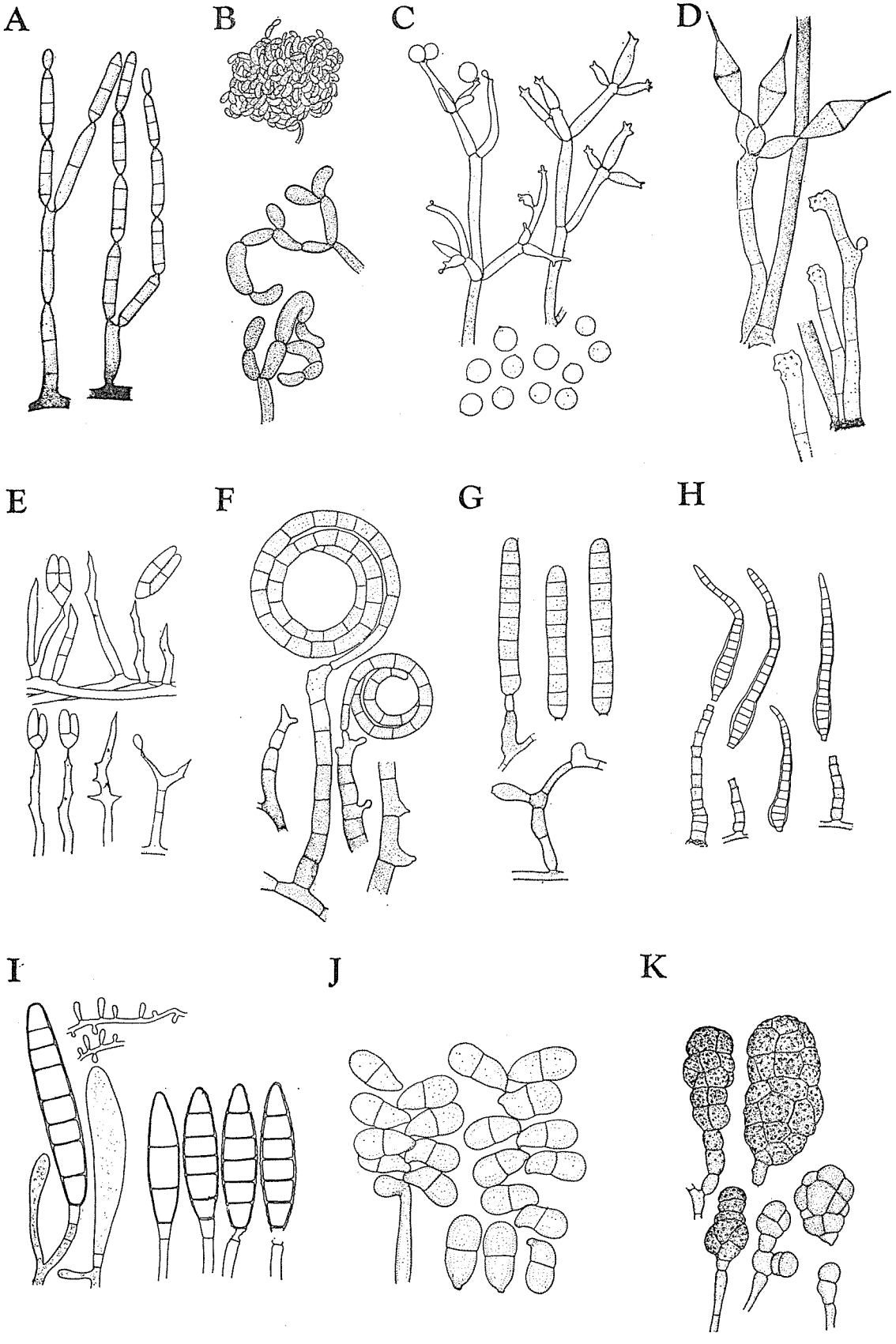
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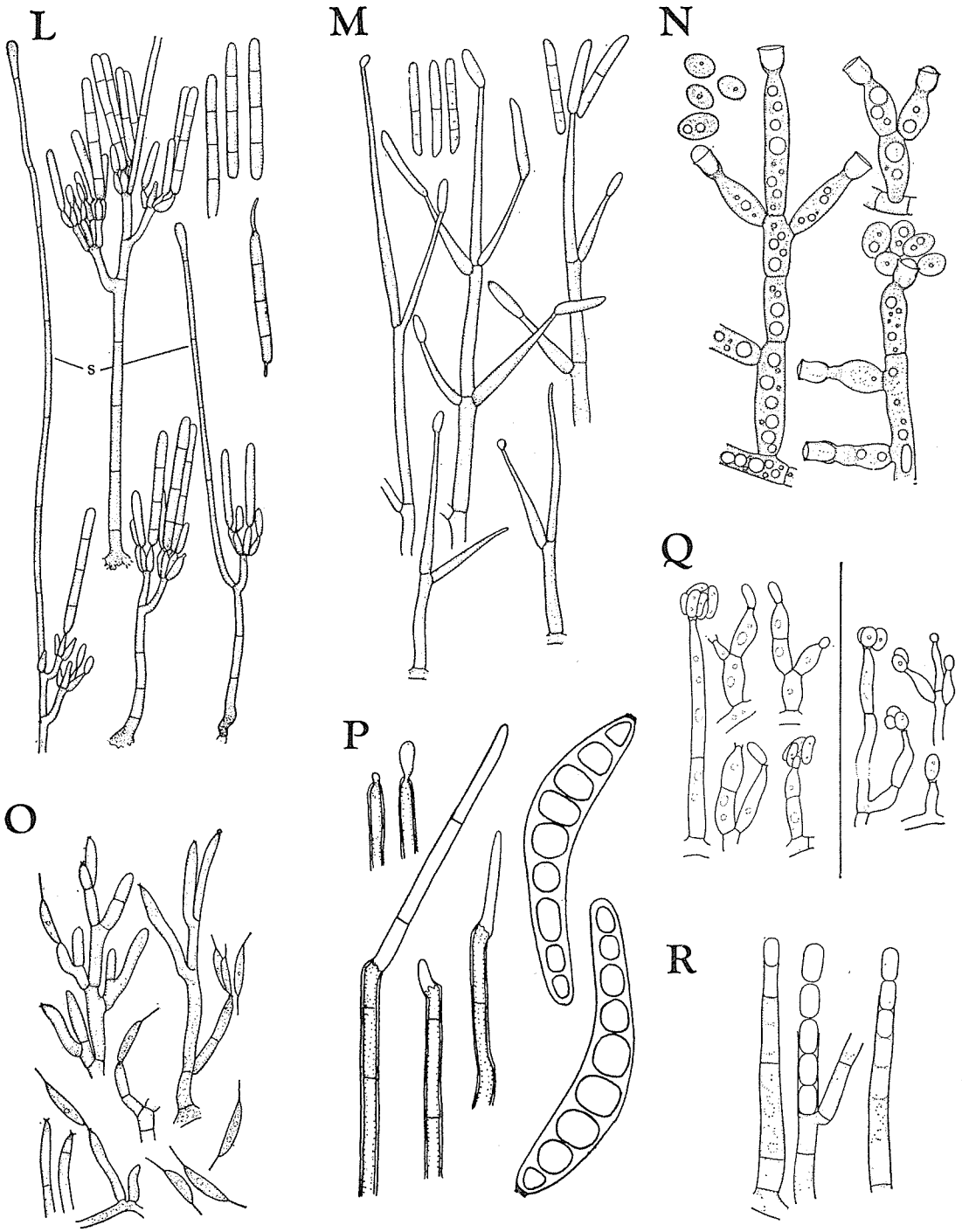

Fig. 3 Representation of conidial developments
A-B. Blastosporae C-F. Radulasporae G-K. Aleuriosporae L-O, Q, R, Phialosporae P. Porosporae
A. *Septonema chaetospira* B. *Strumella griseola* C. *Hansfordia grisella* D. *Beltraina rhombica* E. *Dicranidion fragile* F. *Helicosporium panacheum* G. *Camposporium antennatum* H. *Sporidesmium niligirense* I. *Microsporum gyspseum* J. *Trichothecium roseum* K. *Sirodesmium olivaceum* L. *Cylindrocladium ilicicola* M. *Moeszia cylindroides* N. *Phialophora* O. *Menispora minuta* P. *Helminthosporium oryzae* Q. *Margarinomyces bubaki* and *M. atrovirens* R. *Thielaviopsis paradoxa* [after, Tubaki (1958b)]

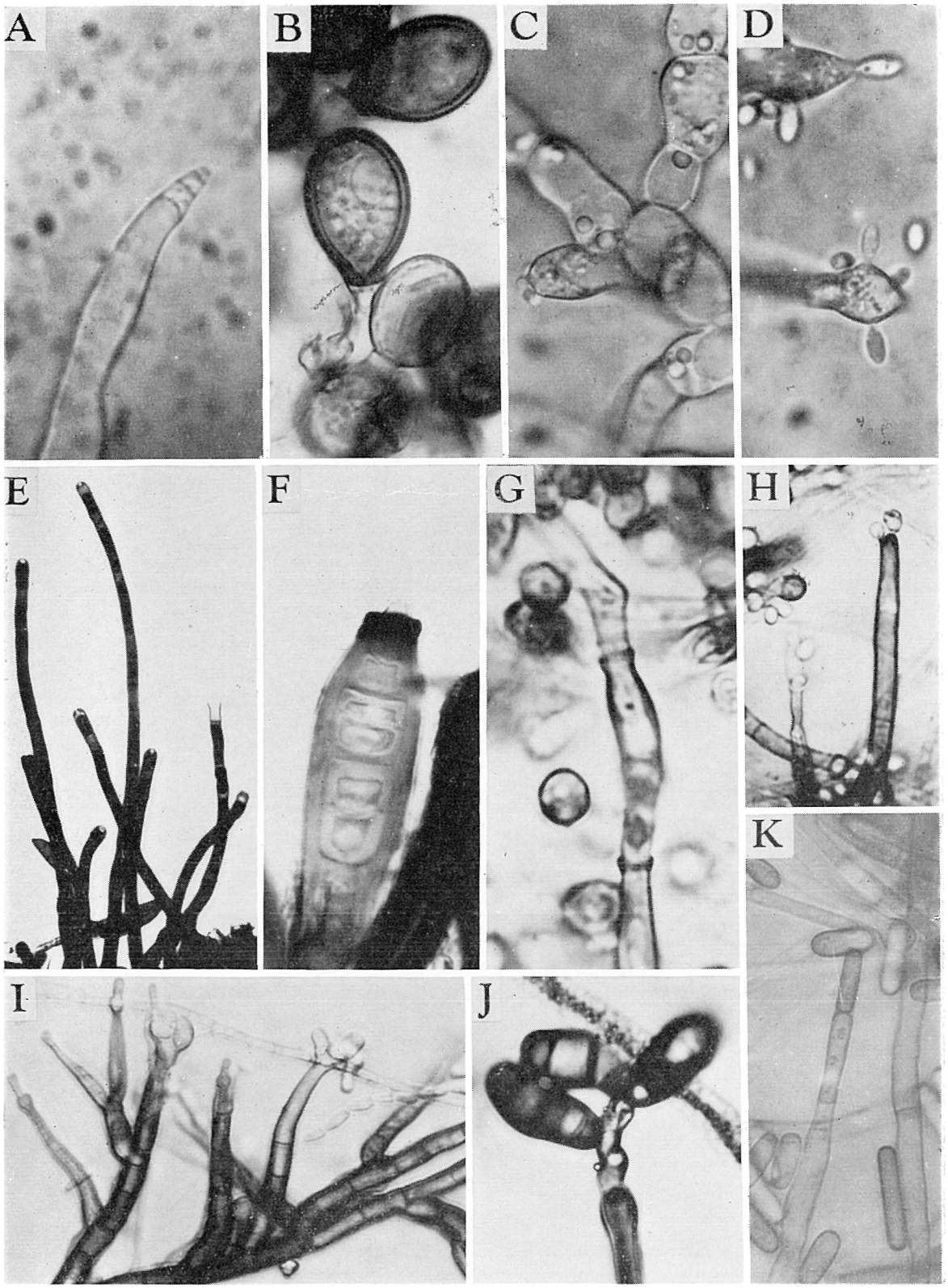

Plate 1

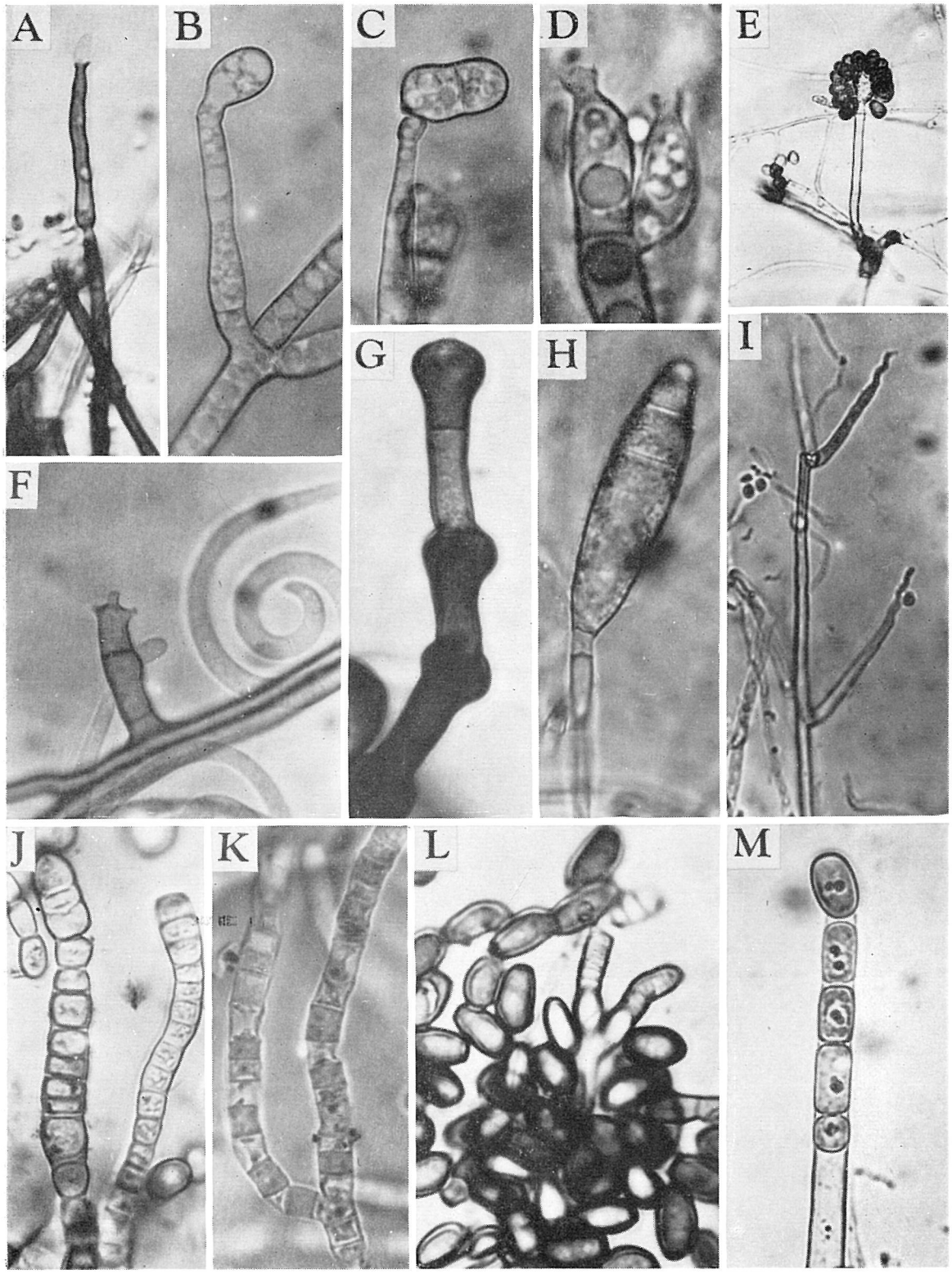

Plate 2

Ann. Rep. Inst. Fermentation, Osaka
No. 1, p. 55~60, 1963

ISOLATION OF TEMPERATE PHAGES FROM NATURAL SOURCES

Teiji IIJIMA

Lysogeny, the hereditary potentiality of bacteria to produce bacteriophage without external infection with phage particles, is a wide-spread characteristic of a large number of bacterial species (Lwoff 1953, Bertani 1958). A lysogenic bacterium transmits this property from generation to generation. In *Escherichia coli* several temperate phages were isolated from lysogenic strains and their specific locations on the host chromosome of *E. coli* K 12 were determined by the technique of genetic recombination. The relationship between the locus of a temperate phage on the host chromosome and the transducing genetic marker that was transfered with the same phage was also investigated (Lederberg and Lederberg 1953, Morse *et al.* 1956, Jacob and Wollman 1957, Matsushiro 1961).

By way of surveying temperate phages whose multiplication in a host cell depends on the presence (or absence) of *F* factor (or other episomes such as colicinogenic factor and multiple resistance factor), several temperate phages, whose activities do *not* depend on the presence of the episomes mentioned above, were isolated from natural sources.

This report describes the method of isolation and some of the properties of these phages.

Experimental

The phages were isolated from *E. coli* which had been brought to the Research Institute for Microbial Diseases, Osaka University for clinical examination along with other enteric bacterial specimens.

E. coli was picked from these specimens and plated on nutrient agar plates and incubated for 18 hours at 37°C. The colonies which developed on these plates were replica-plated onto second plates on which an appropriate indicator strain (usually a nonlysogenic derivative from K 12, W 3110, was used) had been previously seeded. The replica plates were exposed to ultra violet light for 30 to 40 seconds at a distance of 60 cm to induce the vegetative multiplication of prophage. After incubation overnight at 37°C, colonies which showed clear inhibition zones on the replica plates, were isolated from the master plates.

By this procedure, about 1600 colonies were examined and 96 suspected lysogenic strains were isolated. These strains were designated as BE 1 to BE 96. The BE strains thus isolated were further tested for their ability to liberate phage particles after treatment with an inducing agent. Overnight cultures of BE strains were rejuvenated by shaking in fresh medium for two hours at 37°C. The cells were harvest-

ed by centrifugation and then resuspended in a synthetic medium (Davis and Mingioli 1950). The suspension was exposed to UV light for 30 seconds at a distance of 60 cm. The irradiated suspension was then supplemented with an equal volume of two-fold strength nutrient broth and cultured on a rotary shaker at 37°C. During incubation, the optical density of the cell suspension at 600 mμ increased during the first 2 hours and then decreased rapidly owing to lysis of the treated cells. Cell suspensions of certain strains did not show a decrease of optical density. The supernatant of the culture was collected by centrifugation at 3000 rpm, and the remaining cells were sterilized by treatment with chloroform. The activity of the supernatant was tested by cross-streaking against an indicator strain (W 3110) or by spotting the supernatant on a lawn of the indicator strain layered on a nutrient plates with soft agar. The activity of the supernatant was also examined by plaque count. An aliquot of appropriate dilution of the supernatant was mixed with 1 ml of the indicator strain in 3 ml of melted soft agar and plated on nutrient agar plates.

Plaque morphology was checked after 18 hours incubation at 37°C. The ability to lysogenize to W 3110 was tested by the following method: Cells were picked from the center of a plaque, when it was turbid, and aliquots of appropriate dilutions of the cells were spread over nutrient agar plates. The colonies which developed on these plates were replica-plated on a plate seeded with an indicator, W 3110, and irradiated with UV for 30 seconds. Colonies which showed a lytic zone on the indicator were replated on a nutrient plate and their ability to liberate phage particles was retested after treatment with the inducing agent. If all colonies were defined as lysogenic, they were stored as lysogenic strains of W 3110 for the phage. Among 96 strains tested by this method, 71 liberated a temperate phage or phages and 10 of these phages established a stable lysogenic strain for W 3110 respectively.

Results

Plaque morphology of some of these phages is shown in Plate I, II and Table 1. A strain designated as BE 35 liberated three types of phages, which were different from each other with respect to their plaque morphology. Two of them, ϕ 35S and ϕ 35L, were able to lysogenize to W 3110, but the remaining one ϕ 35G-21, was very virulent and unable to lysogenize to W 3110 under the conditions described above. Furthermore, lysate of ϕ 35G-21 was mixed with a large number of W 3110 cells and incubated for 18 hours at 37°C. After this procedure the remaining cells were either lysogenic or resistant cells, but all of them proved to be resistant after testing. One of the resistant strains was designated as W 3110/GR-1. Host range mutants of ϕ 35G-21 were easily obtained. The plaque forming ability of mutant *h* (ϕ 35G *h*) on the original W 3110 and W 3110/GR-1 is shown in Table 2.

Phages refered to as ϕ 170 and ϕ 17c were isolated from the lysate of W 3110 (ϕ 170) after UV induction. Phage ϕ 170 forms turbid plaques while ϕ 17c forms clear plaques on the same indicator strains. Both ϕ 170 and ϕ 17c cannot attack W 3110 (ϕ

Table 1. Plaque morphology of the isolated temperate phages

Phage	original	lysogenicity to W 3110	Diameter	Description
ϕ 170	BE 17	+	1.3-2.0 *mm*	turbid
ϕ 17c	BE 17	+	1.3-2.0	clear
ϕ 35S	BE 35	+	1.1	turbid
ϕ 35L	BE 35	+	1.5	clear
ϕ 35G-21	BE 35	*	1.5	clear
ϕ 38	BE 38	+	0.5	very turbid
ϕ 60	BE 60	+	1.5-1.8	turbid
ϕ 68	BE 68	+	0.5	turbid
ϕ 731	BE 73	+	0.8	turbid

* very virulent, no lysogenic strain was obtained.

Table 2. Plaque froming ability of a host range mutant of ϕ 35G

Phage	indicator	
	W 3110	W 3110/GR-1
ϕ 35G-21	4×10^9/ml	1×10^3/ml
ϕ 35G *h*	6×10^9	7×10^7
ϕ 35L	5×10^7	1×10^2

35S) and ϕ 35S also cannot attack W 3110 (ϕ 170) and W 3110 (ϕ 17c), that is, ϕ 170 and ϕ 17c are co-immune to ϕ 35S. However, ϕ 170 and ϕ 17c differ from ϕ 35S in plaque morphology and their action spectrum to some strains. For example, both ϕ 170 and ϕ 17c can attack and lysogenize to an Hfr strain, F 385, but ϕ 35S has no activity on this strain. It seems that ϕ 17c is a clear mutant of ϕ 170, and ϕ 35S is a closely related strain to ϕ 170.

The location of a temperate phage on the host chromosome was determined by genetic recombination. It was known that Hfr H injects its chromosome in the order of *O-T-L-Lac-Gal*, and markers distal to *Gal* were injected with a low frequency. An Hfr strain, F 385, was isolated from Hfr H by UV irradiation. This strain injects its chromosome in the order *O-Xyl-T-L-Lac-Gal.*, and the markers distal of *Gal* also were injected at a high frequency. A lysogenic strain of F 385 was isolated by mixing a phage with F 385 and was crossed with W4573, prototroph $Lac^-Gal^-Mal^-Xyl^-Mtl^-S^r$. The mating mixture was plated on various selected media after 2 hours incubation. The recombinants were analysed for their unselected marker, and the location of the prophage was determined. This method was not applicable to ϕ 38 and ϕ 60, because these phages do not attack Hfr H. This method was also not applicable to ϕ 35L, because the fertility of F 385 (ϕ 35L)$\times$W 4573 was much lower than the corresponding cross of F 385$\times$W 4573. This lowered fertility was due to zygotic induction, because the lytic zone was found around the recombinant colonies when F 385 (ϕ 35L) was replica-plated on the lawn of the recipient W 4573.

The locations of prophage ϕ 170 and ϕ 731 were determined by this method. Prophage ϕ 170 is located in the neighbourhood of *Gal* locus and prophage ϕ 731 is located more distally from ϕ 170.

Phage ϕ 170 transduced *Gal* marker from W3110 to W 4573 at a frequency of 10^{-6} or 10^{-7}, but other markers such as *Lac*, *Xyl* and *Mal*, have never been transduced.

The precise locus of other phages and transducing ability are now being studied.

Summary

Many strains of *Escherichia coli* were isolated from natural sources and were tested their lysogenicity. Some of temperate phages from these strains were lysogenized to non-lysogenic strain of *E. coli* K 12. Some of the properties of these phages; plaque morphology, location on the chromosome of K 12 were described as well as an ability of transduction.

Acknowledgement

A part of this work was done in the Department of Genetics, Medical School, Osaka University, in collaboration with Dr. M. Eguchi in Kyoto University of Industrial Arts and Textile Fibers.

The Author wish to thank to Prof. H. Kikkawa in Osaka University for his active interest, and I am also indebted to Dr. K. Kotera in the Research Institute for Microbial Diseases for his many help to collect of *E. coli* strains and Mr. T. Sakane in the Institute for Fermentation for his technical assistance during this work.

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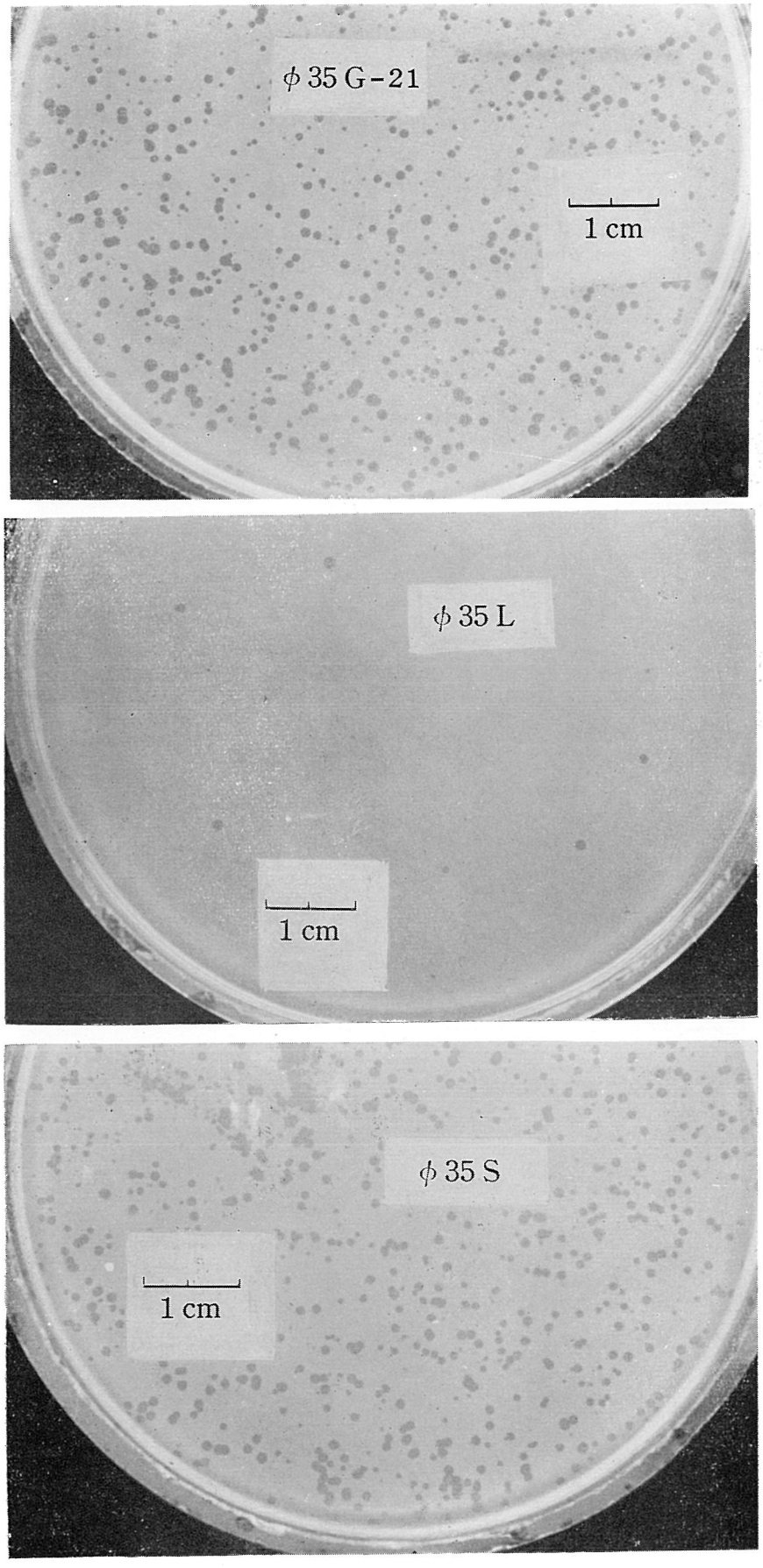
Plate I



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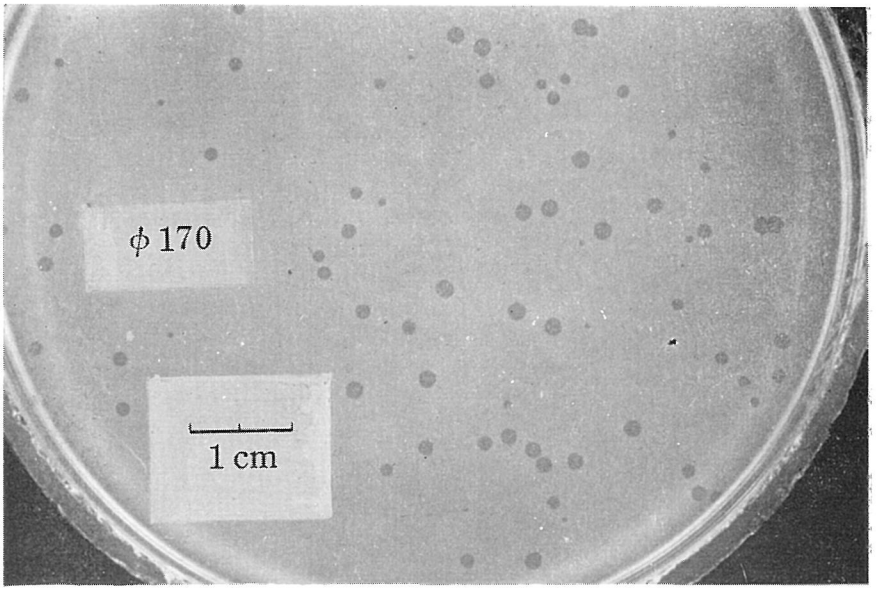

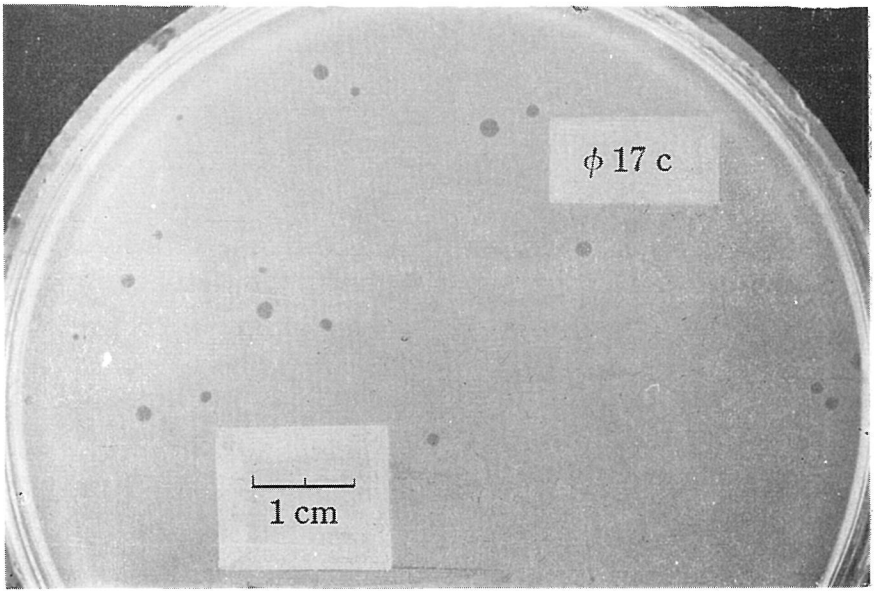

Ann. Rep. Inst. Fermentation, Osaka
No. 1, p. 61~66, 1963

INACTIVATION AND INDUCED MUTATION OF *RHODOTORULA GLUTINIS* BY IRRADIATION PART I WITH ULTRAVIOLET RAYS

Isao BANNO

There have been few reports of genetical and cytolgical investigation on the yeasts of the family Cryptococcaceae, in which no sexual phenomena are known. Their phylogenetic relationships to higher fungi and the affinities among taxa of the family are still in doubt.

Investigations of these yeasts from a genetical and cytological point of view would contribute to the elucidation of interrelationships among them. A genetical study on *Rhodotorula* yeast of the family, of which taxonomical investigations have been carried out in this laboratory (Hasegawa et al. 1960a, b. 1963), was attempted. The investigation was started by the collection of biochemical mutant of *Rhodotorula* yeasts. The following report concerns the inactivation of cells and the yield of mutants in *Rhodotorula glutinis* by ultraviolet irradiation.

Materials and Methods

Two strains of *Rhodotorula glutinis*, IFO 0559 (strain *gracilis*) and IFO, 0880 (strain *koishikawensis*), were used in all the mutation experiments. These strains were chosen because of their similarity in vigorous growth, the large amount of lipid contained in their cells and in cell morphology.

Their cells grow by normal budding, are oval to long oval in shape, and are non -clustered. They do not require vitamins, amino acids, or nucleic bases for growth. They grow satisfactorily in a simple synthetic medium composed of 0.3% $(NH_4)_2SO_4$, 5% glucose, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl, 0.01% $CaCl_2$ and trace mineral elements*. This synthetic medium was used as minimal medium. Besides, potato -yeast-glucose agar (PYG) composed of 20% potato decoction, 0.5% yeast extract, and 3% glucose** was used both as a complete medium and a maintaining medium.

The cells to be irradiated were harvested from 48 hrs culture at 25°C on PYG agar slant, washed twice and vibrated to produce a suspension of single cells in saline. The source of ultraviolet rays was a low-pressure mercury vapor lamp (Matsuda

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* H_3BO_3, $CuSO_4$, KI, $FeCl_3$, $MnSO_4$, Na_2MoO_4, and $ZnSO_4$

** Pelled sliced potato 200g was boiled for 30 min. and centrifuged after cooling. To the supernatant fluid yeast extract 5g and glucose 30g were added and the mixture was diluted to 1000 ml with tap water.

10 W sterilize-lamp). One tenth ml of a cell suspension was spread onto the surface of complete medium in a 9cm Petri-dish and irradiated by the lamp at a distance of 45cm. Serial dilutions of the cell suspension were spread so that 100 to 300 survival colonies could be recovered in a Petri dish after the exposure to various dosage. After incubation at 28°C for 4 to 6 days, colony counts were made and the percent of viable cells determined.

The colonies representing cells that had survived exposure to ultraviolet were then tested for nutritional requirements by replica-plating to agar media containing various nutrients in this sequence: (a) minimal medium, (b) minimal medium supplemented with amino acids, (c) minimal medium supplemented with vitamins, (d) minimal medium supplemented with adenine, guanine, uracil, cytosine and their ribosides and ribotides, (e) minimal medium containing all the above supplements and (f) complete medium.

Colonies failing to grow on medium (a), (b), (c) and/or (d) were transferred from

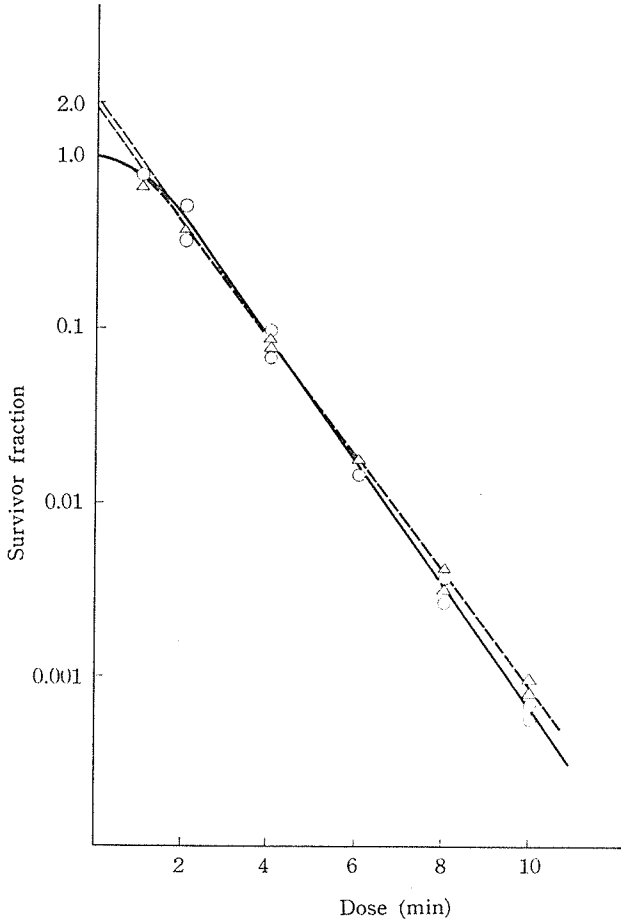

Fig. 1. UV irradiation survival curves of *Rh. glutinis* IFO, 0559.
○—○ irradiated immediately after preparation
△--△ irradiated after 120 hrs. starvation of nitrogen source

the master plates to complete agar slants and subsequently tested twice to confirm their deficiencies.

In order to determine which of the nutrients is required by each mutant, they were transfered to a series of agar plates containing minimal medium supplemented with different combinations of from 5 to 10 of the nutrients, and followed by a final screening in liquid minimal medium with all the supplements, omitting one each time.

Results

Figs 1 and 2 show survival of cells of IFO 0559 and IFO 0880 as an exponential function of the ultraviolet dose when the yield was measured on complete medium. The solid curves were made when the cells were exposed to ultraviolet rays immediately after preparation. Dotted ones represent results obtained with cells stored in saline supplemented with 1% glucose for 3 days at 10°C. During this storage the cells were nitrogen-

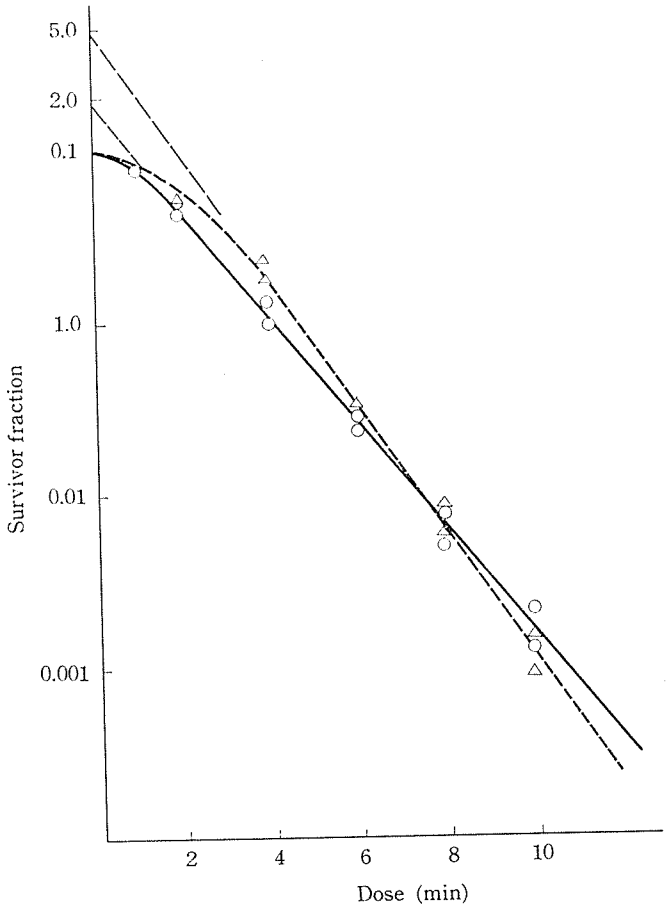

Fig. 2. UV irradiation survival curves of *Rh. glutinis* IFO, 0880.
see footnote of table I,

starved. Both the freshly harvested cells and the N-starved cells of IFO, 0559 and the freshly harvested cells of IFO, 0880 gave sigmoid survival curves, extrapolating back to approximately 2 at zero dose. While the N-starved cells of the latter strain gave a sigmoid curve extrapolating back to approximately 5. Appreciable difference in their sensitivity to ultraviolet irradiation, as judged by the final slopes of the survival curves, was not observed between the cells under the two physiological state.

The frequency of mutation was determined with cells subjected to doses required to inactivate approximately 99.9 per cent of them; The cells of IFO, 0559 and IFO, 0880 were irradiated for periods of 10 and 11 minutes respectively. The results are shown in tables 1 and 2. Exact survival percents are shown in the second column and the total numbers of colonies replica-plated appear in the third column. The number of mutants observed in the first replica-plating was included in the 4th column, and the number of mutants that were confirmed after being tested twice is shown in the last column. Figures in parentheses express the number of mutants per survivors on the percentage base.

Table 1. Frequency of nutritional mutants by UV irradiation in *Rh. glutinis* IFO, 0559

Exp. No.	Nitrogen starvation	Percent survivor	Colonies tested	Number of nutritional mutants	
				lst	3rd
1	no	0.14	1702	45 (2.6%)	1 (0.06)
2	no	0.09	3377	176 (5.2)	1 (0.03)
3	no	0.09	5708	216 (3.8)	2 (0.04)
4	120 hr.	0.07	1203	63 (6.3)	7 (0.57)

Table 2. Frequency of nutritional mutants by UV irradition in *Rh. glutinis* IFO, 0880

Exp. No.	Nitrogen starvation	Percent survivor	Colonies tested	Number of nutritional mutants	
				lst	3rd
1	no	0.07	2538	30 (1.8)	12 (0.08)
2	120 hr.	0.04	823	64 (7.8)	16 (1.7)

From these results it is clear that ultraviolet has very little effect on mutation of the two *Rhodotorula* strains. When the N-starved cells were irradiated by ultraviolet, the results as shown in the last line of tables 1 and 2 were obtained. The frequency of mutation is of a higher oder in the N-starved cells than in the freshly harvested cells. Nitrogen-starvation of cells enhanced the mutagenic effect of ultraviolet.

The distribution of the mutants from the strains IFO, 0559 and IFO, 0880 with respect to nutritional requirements is summarized in Tables 3 and 4 respectively.

Table 3. Distribution of UV induced mutants of *Rh. glutinis* IFO, 0559

Requirement	Number of mutants
Adenine	1
Methionine	2
Pantothenic acid	1
Adenine or niacine	1
Unknown	5

Table 4. Distribution of UV induced mutants of *Rh. glutinis* IFO, 0880

Requirement	Number of mutants
Adenine	4
Xanthine	1
Methionine	2
Aspartic acid	1
Thiamine	3
Niacine	2
Unknown	5

Discussion

The survival curves for the cells of the strain IFO, 0559 under the two physiological conditions were two hit. With the strain IFO, 0880 the cells freshly prepared showed a two hit curve whereas the N-starved cells showed a multihit curve, extraporating to approximately 5 at zero dose. The reason why the number of hits change according to the condition of cells of IFO, 0880 is obscure. If cell-inactivation by ultraviolet rays is well explained by the target theory, the form of survival curves must not change with the physiology of cells.

Iguchi (1959) obtained a one hit curve when cells of a strain of *Rhodotorula rubra* were treated with ultraviolet rays. From this, he regarded the strain as a haploid organism. The difference of the present result from his result may be attributed to the strains used to test.

Furthermore, it was reported that in ultraviolet irradiation the *saccharomyces* cells considered genetically to be haploid showed a exponential curve in some experiments and a sigmoid curve in others (Caldas et al. 1951, Delong et al. 1951, Sarachek et al. 1953, Pomper 1955) and also that shape of survival curves obtained with microbial cells exposed to ultraviolet rays varied with condition before or after irradiation (Pomper et al. 1953, Alper 1961). Consequently it seems impossible to conclude the ploidy of cell from the shape of ultraviolet survival curve. Therefore, a plodiy value cannot be ascribed to the strains IFO, 0559 and IFO, 0880 from the result of the present experiment.

As seen in the last column of tables 1 and 2 the frequency of stable mutants was unexpectedly rare. It is noticeable that in ultraviolet irradiation of the cells subjected to nitrogen starvation, mutant frequencies of both strains are of a order higher than in the freshly harvested cells. Through the starvation the genetic material in cells might become more sensitive to ultraviolet. Whether such an increase of mutation frequency by nitrogen-starvation in ultraviolet irradiation is found in other micro-organisms would be an interesting problem for investigators attempting to secure numerous and various mutants

None of the mutants obtained often reverted to wild type, so they are suitable for use in genetical experiments

Summary

The cells of two strains, IFO, 0559 and IFO, 0880 of *Rhodotorula glutinis* were exposed to ultraviolet rays. Their ultraviolet survival curves were examined and the survivors tested for nutritional requirements.

Ten and eighteen mutants were obtained from IFO, 0559 and IFO, 0880 respectively, and have been characterized with respect to their nutritional requirements. In addition these mutants were stable enough to be used in genetic experiments.

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Ann. Rep. Inst. Fermentation, Osaka
No. 1, p. 67~71, 1963

INACTIVATION AND INDUCED MUTATION OF *RHODOTORULA GLUTINIS* BY IRRADIATION PART 2 WITH X-RAYS

Isao BANNO

A mutation experiment with ultraviolet rays on the strains of *Rhodotorula glutinis* was reported in a preceding paper (Banno 1963a). The mutants with single marker from strains IFO, 0559 and 0880 were obtained in the experiment. The mutant with more than one maker is more useful in genetic investigation. Thus the present experiment was attempted to aquire by X-rays the mutants with another marker in addition to the ultraviolet-induced mutant-marker.

Materials and Methods

A pantothenate-less mutant (M-24) of *Rh. glutinis* IFO, 0559 and an adenine-less mutant (M-86) of *Rh. glutinis* IFO, 0880, obtained by ultraviolet were used in the present mutation experiments.

The cells to be exposed to X-rays were prepared by the same procedures as in the ultraviolet irradiation experiments (Banno 1963a). Four ml. of cells (10^6 /ml) suspended in sterilized distilled water was poured into a 5 cm Petri-dish and irradiated at a dosage of 60000r/hr with gentle agitation. X-ray generator with Cu target (Isodebye flex II) operated at 35 KV. 20 mA was used as a source of irradiation. Counts of viable cells after the irradiation and detection of mutant colonies were made in the same manner as described in the preceding paper.

Results and Discussion

The relation of the exponential fraction of surviving cells to doses of X-rays is shown in Fig. 1, when the yield was measured on complete agar medium. The form of the dose survival curves of both mutant strains followed a one hit pattern, indicating that according to the target theory their cells are haploid

These results are consistent with that reported by Magni (1951, 1953), who found a one hit survival curve for each of 19 different yeasts belonging to the genus *Rhodotorula* by X-ray irradiation and regarded them to be haploid.

A correlation between the form of survival curves in mutagenic treatment and the degree of ploidy of cells has been discussed by many investigators (Atwood and Norman 1949, Pomper and Atwood 1955, Alper 1961, Wolff 1961). Efforts to elucidate experimental observations on the basis of theoretical survival curves obtained by a mathematical method have not yet met with success (Lucke and Sarachek 1953, Magni 1956, Zelle and Ogg 1957). Pomper et al. (1954) warned against making, without

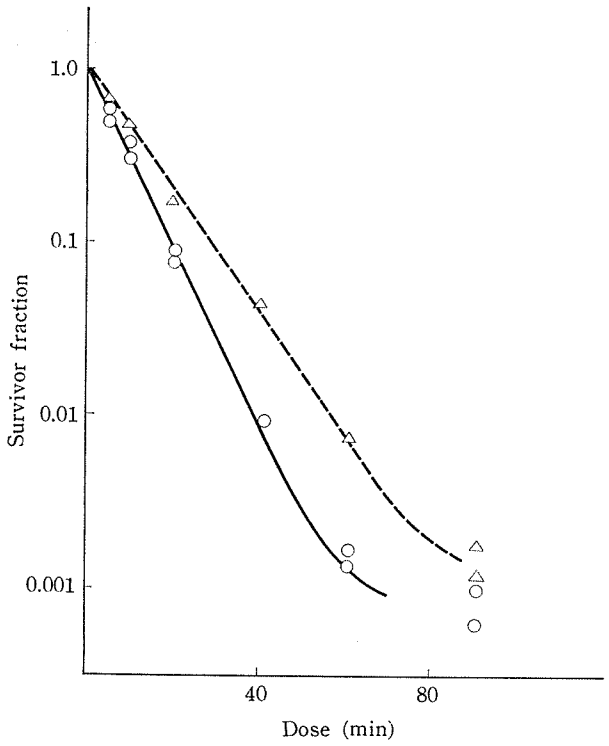

Fig. 1. X-irradiation survival curves of a mutant (M-24) of Rh. glutinis IFO, 0559 [○—○] and of a mutant (M-86) of Rh. glutinis IFO, 0880 [△---△].

genetic confirmation, a decision of ploidy only from survival curves. It was discussed in the preceding paper that in ultraviolet irradiation indeed the survival curves of cells varied according to various conditions and, in some cases, were incompatible with those expected from their ploidy confirmed on a genetic investigation.

In X-ray irradiation, however, the shape of survival curve for fungi, especially for yeasts, depends on their ploidy. The more significant experimental data given by different investigators have consistently shown that the X-ray survival curves for haploid yeast are exponential or one hit whereas the curves for diploid yeast are sigmoid or multihit (Wyckoff and Luyet 1931, Latarjet and Ephrussi 1949, Lucke and Sarachek 1953, Zirkle and Tobias 1953, Uretz 1955). So X-rays survival curves would be considered as an important clue in determining the ploidy of microbial cells. Here as an experimental hypothesis in further investigations the author considers that the cells of these yeasts are haploid.

When the cells were irradiated at various doses, the frequency of mutation as showed in tables 1 and 2 were obtained. The numbers of mutants determined on the first screening are shown in the third column and those mutants which were stable after two more successive transfers are included in the 4th column of these table. Mutant frequencies are shown in percentage in parentheses. It can be seen in table 1 that the frequency of mutants in M-24 increased as the survival percent decreased to 0.26. But the frequency decreased again when the survival percent was 0.1. The

Table 1. Frequency of nutritional mutants by X-ray irradiation in mutant-24 of *Rh. glutinis* IFO, 0559

Exp. No.	Percent survivor	Colonies tested	Number of nutritional mutant	
			lst	3rd
1	0.83	3873	62 (1.6%)	1 (0.03)
2	0.45	3146	81 (2.6)	6 (0.19)
3	0.44	1227	29 (2.4)	3 (0.41)
4	0.26	886	22 (2.4)	5 (0.56)
5	0.10	2000	18 (0.9)	1 (0.05)

Table 2. Frequency of nutritional mutants by X-ray irradiation in mutant-86 of *Rh. glutinis* IFO, 0880

Exp. No.	Percent survivor	Colonies tested	Number of nutritional mutant	
			1st	3rd
1	0.60	5834	51 (0.9)	13 (0.22)
2	0.21	279	25 (9.0)	2 (0.71)

fact that the frequency of mutants has a maximum at a certain dosage has been observed in some mutagenic treatments of organisms. (Hollaender et al. 1941 Kölmark 1953, Markert 1953). In M-86, also, the frequency of mutants at 0.21 percent survival was higher than that at 0.6 percent survival.

The great fraction of the presumptive mutants isolated on the first screening failed to show a requirement on the second or third screening. Similarly as with ultraviolet irradiation, the mutation frequency was low with X-ray irradiation (see table 1 and 2). It is considered that any mutagenic agent may hardly induce mutation on the cells of *Rhodotorula* yeasts.

The distribution of the mutants of M-24 and M-86 with respect to additional requirements is summarized in tables 3 and 4 respectively. It should be noted in

Table 3. Distribution of X-ray induced mutants in M-24 of *Rh. glutinis* IFO, 0559

Requirement	Number of mutants
Methionine +Pantothenic acid	2
Unknown	41

Table 4. Distribution of X-ray induced mutants in M-86 of *Rh. glutinis* IFO, 0880

Requirement	Number of mutants
Tryptophan+Adenine	1
Methionine+Adenine	2
Arginine+Adenine	2
Lysine+Adenine	1
Methionine	1
Arginine	1
Lysine	3
Unknown	2

Table 4 that of the 13 mutants aquired from M-86, 5 failed to show the original requirement for adenine. Since the reversion frequency of adenine-deficiency with the same dose of X-ray irradiation was less than 10^{-3} in all, the reversion frequency in the mutant fraction is too high.

The fact that such a high frequency of reversion of the original mutation to wild type was found in cells which mutated at another site on a mutagenic treatment casts a doubt on the idea that mutational events induced by a mutagen occur independently of each other.

All the double mutants aquired by ultraviolet and X-ray irradiation, except one which was deficient for adenine and arginine, were very stable and did not revert to wild type at a frequency higher than 10^{-7}. Therefore they were stable enough to be used in an investigation on cell interactions reported in a separate paper (Banno 1953b).

Summary

Two single mutants (M24 and M86) of two strains of *Rhodotorula glutinis* were exposed to X-rays and the survivors were tested for nutritional requirements. The form of their X-rays survival curves suggested that their cells are haploid. Two double mutants from M24 and eight double mutants from M86 were obtained and all have been characterized with respect to their nutritional requirements. These mutants were stable enough to be used in genetic experiments.

The author wishes to thank Mr. K. Mikata and Mrs. K. Ijigawa for their generous assistance.

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PUBLICATIONS

SUMMARIES OF RESEARCH PAPERS

Takezi HASEGAWA, Isao BANNO and Kozaburo MIKATA,

Studies on the Genus *Rhodotorula*. VII. Production of Starch-like Compound

J. Fermentation Technol. 39: 157-159 (1961)

Forty strains belonging to *Rhodotorula*, *Cryptococcus*, *Candida*, *Trichosporon*, etc. were examined for their ability to produce a strach-like compound. The tests produced the same results as those already reported by other authors*. Although the property of producing a starch-like compound is one of characteristics available for classifying yeasts, it is considered not to be one of the distincitve features of the genus *Cryptococcus* as some species of other genera also produce a starch-like compound. [In Japanese]

* Mager, J. and M. Aschner (1947) J. Bacteriol. 53: 283-295; Lodder, J and N.J.W. Kreger-van Rij (1952) The yeasts, a taxonomic study. North Holland publ., Amsterdom. 713 p; Lodder, J., W. Ch. Slooff and N.J.W. Kreger-van Rij (1958) p. 1-62 in A.H. Cook, The chemistry and biology of yeasts. Academic Press, New York. 763 p.

Takezi HASEGAWA and Isao BANNO,

Studies on the Genus *Rhodotorula*. VIII. The Taxonomic Significance of the Color due to Carotenoid Pigments

J. Fermentation Technol. 39: 159-163 (1961)

Twenty-two strains of *Rhodotorula* were examined as to the optical nature of their carotenoid pigments extracted with acetone and petroleum ether.

Cells cultivated in two kinds of media were employed for the extraction of the pigments. One was a synthetic medium reported by Wickerham (1952) and the other was a potato and yeast extract medium. Each strain was incubated in 50 ml of both media at 28°C on a rotary shaker for 4 days. When the cells harvested from the potato and yeast extract medium were used, the red and yellow strains could be clearly distinguished from each other by differences of the absorption maxima of their pigment extracted; the extracts from the cells of red strains were found to exhibit absorption maxima at 480 mμ and those of the yellow strains were at 450 mμ.

The pigment extracted from *Rh. glutinis* var. *aurantiaca*, a peculiarly colored variety, showed an absorption maximum at 470 mμ. [In Japanese]

Takezi HASEGAWA and Isao BANNO,

Studies on the Genus *Rhodotorula*. IX. The Distribution of the Carotenogenic Species among the Asporogenous Yeasts

J. Fermentation Technol. 38: 173-176 (1961)

In order to investigate the distribution of the activity of carotenogenesis among the asporogenous yeasts, twenty-nine yellow or pale-colored strains were selected from among seven hundred and forty-three cultures belonging to seventy-two species of the following genera:

Cryptococcus (5 spp.), *Torulopsis* (22 spp.),
Pityrosporum (1 sp.), *Brettanomyces* (3 spp.),
Candida (30 spp.), *Kloeckera* (5 spp.),
Trigonopsis (1 sp.), *Trichosporon* (4 spp.),
(*Rhodotorula* was omitted).

The selected cultures were incubated at 28°C for 4 days or more in a shake culture, and the pigments were extracted with acetone and petroleum ether.

Among the above genera, only *Cryptococcus* was found to produce carotenoid pigments. As an exceptional species, none of the five strains of *Cr. neoformans* investigated, produced carotenoid pigments. Moreover, it was also found that the yellow pigmentation in these strains of *Cr. neoformans* was not influenced at all by diphenylamine even in a dose sufficient to inhibit the pigment production in other *Cryptococci*. These results led to the conclusion that the intracellular pigments of *Cr. neoformans* were not carotenoids. As a result, it was concluded that all carotenogenic strains of *Cryptococcus* should be placed in the genus *Rhodotorula* from the viewpoint of practical classification. [In Japanese]

Takezi HASEGAWA and Iaso BANNO,

Studies on the Genus *Rhodotorula*. X. The Vitamin Requirement of the Carotenogenic *Cryptococci*

J. Fermentation Technol. 39: 176-179 (1961)

The vitamin requirement of nine strains of the carotenogenic *Cryptococci* was investigated in order to elucidate their taxonomic relationships to the original *Rhodotorulas*. All of them required only thiamine except one autotrophic strain, *Torula flavescens*, and their modes of thiamine requirement coincided exactly with those of the yellow *Rhodotorulas*. [In Japanese]

Reijiro KODAMA and Masaaki NISHIO,

Studies on Lactic Acid Bacteria from Various Materials in the Process of *Sake*-Brewing.

J. Fermentation Assoc. Japan 19: 269-276 (1961)

Investigation was made on the taxonomical properties and the nutritional requirements of lactic acid bacteria isolated from various materials in the process of *saké*-brewing. The results are:

(1) Of fourteen homofermentative isolates, four strains were identified as *Lactobacillus saké* Kitahara, three strains as *Lactobacillus plantarum* (Orla-Jensen) Holland, and of fourteen heterofermentative isolates, six strains were identified as *Leuconostoc mesenteroides* (Cienkowski) van Tieghem, eight strains as *Lactobacillus batatas* Kitahara.

(2) The peptide-like substances, which were contained in yeast extract and other natural products, were practically essential for the growth of *L. saké* in the synthetic medium, but the strains of this species have also been able to grow slightly in the presence of L-glutamine.

(3) While all isolates required both nicotinic acid and calcium pantothenate essentially for the growth, the vitamin requirements of these lactic acid bacteria conformed to at least six patterns, which were classified according to the requirements of thiamine, riboflavin, folic acid and vitamin B_6-group.

(4) The isolates from unrefined *sake* which turned sour after preservation at 30°C for about one month, were all found to belong to *L. acidophilus*, and they, unlike *L. saké* could not grow at 3°C to 4°C, but could barely grow at 7°C to 8°C.

[In Japanese]

Keisuke TUBAKI,

Studies on a Slime-forming Fungus in Polluted Water.

Trans. Mycol. Soc. Japan 3:29-35 (1962).

Thirty water samples including thirteen slimy materials in the polluted water of the Ishikari River, Hokkaido, were examined under direct observation and under culture. Nearly 120 strains of fungi and yeasts were isolated and distributed into 27 species. Among them, 23 strains were identified as *Geotrichum candidum* Link ex Persoon emend. Carmichael, and they were divided into three groups: I-Ⅲ, with the texture of the growth. The group Ⅲ of *Geotrichum candidum* can make the plumose or tassel-like massive growth in the current media under the controlled laboratory condition, and this filamentous slimy growth is closely similar to those in nature.

Takeshi TAKAHASHI and Takezi HASEGAWA,

Studies on the Microbiological Transformations of Steroids II. The Steroid Oxidizing Property of *Rhizoctonia solani*

J. Agr. Chem. Soc. Japan 35: 1394-1399 (1961)

The steroid oxidizing property of *Rhizoctonia solani* Kühn was studied. It was observed that the organism oxidized 17α, 21-dihydroxypregn-4-ene-3, 20-dione (Reichstein's Compound S) (I) to form 6β, 17α, 21-trihydroxypregn-4-ene-3, 20-dione, 17α, 21-dihydroxypregna-1, 4-diene-3, 20-dione and a third product, compound No. 4, the structure of which was not determined. For the purpose of determining the structure of 6β, 17α, 21-trihydroxypregn-4-ene-3, 20-dione, the major product, it was converted to its mono- and di-acetate, 6β-hydroxyandrost-4-ene-3, 17-dione and androst-4-ene-3, 6, 17-trione. The production of these substances from (I) was demonstrated in 31 strains of the species.

In previous works*, it was found that *Corticium sasakii*, regarded as the same species as *Rhizoctonia solani* (=*Corticium vagum* Br. et Cav.) by European mycologists, produced 11β-, 11α- and 19-hydroxy-cortexolone from (I). After that, this reaction was reexamined with 920 strains of *Corticium sasakii* gathered in Japan, and it was confirmed that every strain of *C. sasakii* converted (I) into exactly the same products without exception. The reactions mentioned above may be considered as new taxonomic criteria of these two taxa. [In Japanese]

* Hasegawa, T. and T. Takahashi (1958) Bull. Agr. Chem. Soc. Japan 22: 212-217; Nishikawa M. and H. Hagiwara (1958) Chem. Pharm. Bull. 6: 226-228.

Takeshi TAKAHASHI and Takezi HASEGAWA,

Studies on the Microbiological Transformation of Steroids. III. The Steroid Oxidizing Property of *Pellicularia filamentosa* group.

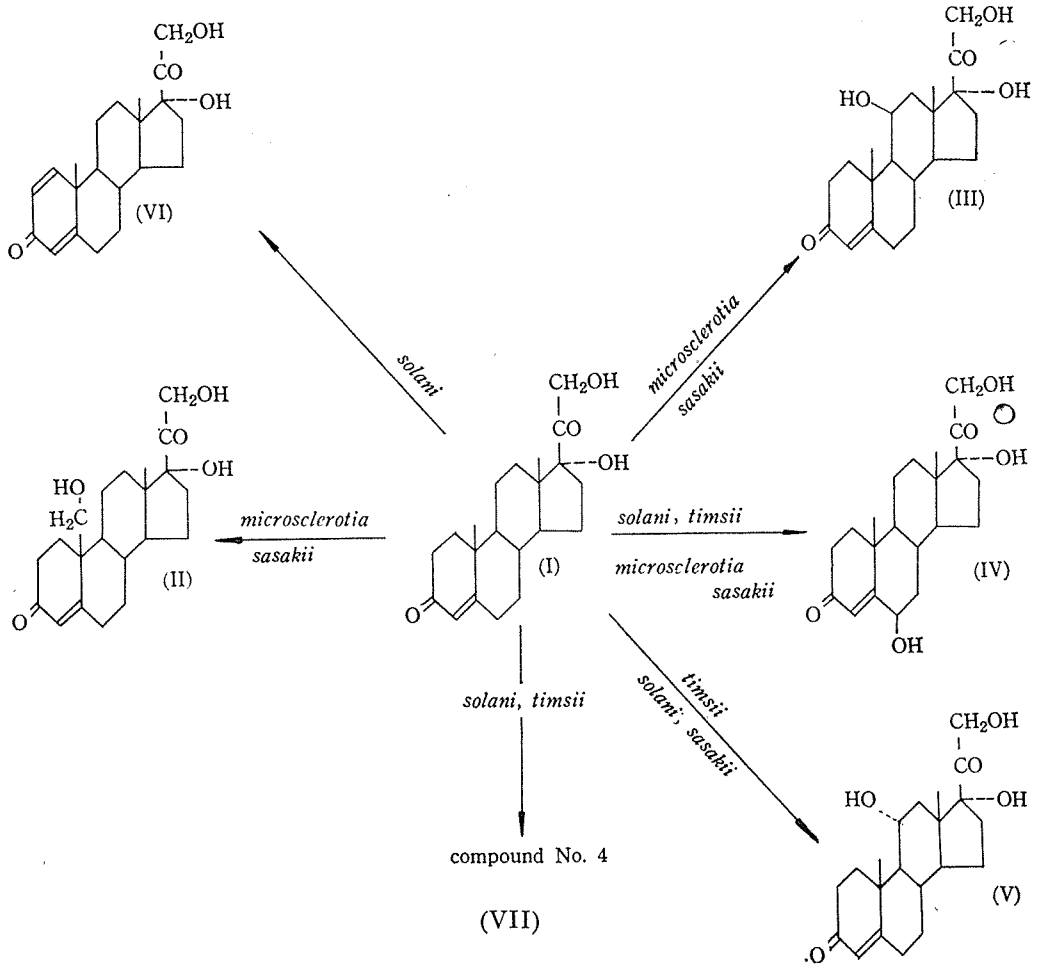
J. Agr. Chem. Soc. Japan 35: 1399-1404 (1961)

In previous works, the steroid oxidizing properties of *Corticium sasakii* and *Rhizoctonia solani* were studied. These microorganisms were taxonomically regarded as biotypes of *Pellicularia filamentosa* (Pat.) Rogers by Exner (1953); *Pellicularia filamentosa* f. sp. *sasakii* and *P. filamentosa* f. sp. *solani*. The others are *P. filamentosa* f. sp. *microsclerotia* (=*Corticium microsclerotia* (Matz) Weber) and *P. filamentosa* f.

sp. *timsii*. In the present paper, the steroid oxidizing properties of these two organisms were investigated. As a result, it was found that *P. filamentosa* f. sp. *microsclerotia* hydroxylated Reichstein's Compound S (I) to 19-hydroxycortexolone with a small amount of hydrocortisone, and that, by *P. filamentosa* f. sp. *timsii*, (I) was oxidized to *epi*-hydrocortisone in addition to small amounts of 6β-hydroxycortexolone and of an unidentified compound which was formed from (I) by *P. filamentosa* f. sp. *solani*. Then, the properties of the four taxa were compared. The results are shown in the table below.

As far as the steroid oxidizing property is concerned, *P. filamentosa* f. sp. *microsclerotia* seems to be related more closely to *P. filamentosa* f. sp. *sasakii*, while *P. filamentosa* f. sp. *timsii* to *P. filamentosa* f. sp. *solani*.

P. filamentosa f. sp.	II	III	IV	V	VI	VII
sasakii	+	++	±	+	—	—
microsclerotia	++	+	±	—	—	—
solani	—	—	++	±	+	+
timsii	—	—	+	++	—	+



Takeshi TAKAHASHI, Yuji UCHIBORI and Takezi HASEGAWA,

Studies on the Microbiological Transformations of Steroids. IV. 1-Dehydrogenation and 20-Hydrogenation by *Stereum fasciatum*

J. Agr. Chem. Soc. Japan 36: 67-72 (1962)

The transformation of steroids by *Stereum fasciatum* was studied. After incubation with a culture broth of the micro-organisms, Reichstein's Compound S (I) was dehydrogenated to Δ^1-cortexalone (II) and (II) in turn, was hydrogenated at the 20-carbonyl group to 17α, 20β, 21-trihydroxypregna -1,4-dien-3-one.

The dehydrogenation of (I) and the hydrogenation of (II) were brought about separately under the same conditions, and this is not a coupling reaction. The former reaction took place first, the latter following. 17α-methyltestosterone was also converted by the microorganism to Δ^1-methyltestosterone. [In Japanese]

Takezi HASEGAWA and Takeshi TAKAHASHI,

Studies on the Microbiological Transformation of Steroids. V. Oxidation of Steroids by *Polyporus orientalis*

Ann. Rep. Takeda Research Lab. (19): 164-171 (1960)

Polyporus orientalis brought about the hydroxylation of progesterone to 6β,11α-dihydroxyprogesterone. When incubated with Reichstein's Compound S, this microorganism converted it into 6β-hydroxycortexolone and *epi*--hydrocortisone in the ratio of 3 to 1. Similar reactions were found in some species of *Aspergillus** and *Rhizopus*** A brief consideration was made on patterns of transformation by these organisms. [In Japanese]

* Dulaney, E.L., E.O. Stapley and C. Hlavac (1955) Mycologia 47: 464-474

** Eppstein, S.H., P.D. Meister, C.H. Murray and D.H. Peterson (1956) Vitamins and Hormones 14: 359-432

Takeshi TAKAHASHI and Yuji UCHIBORI,

Studies on the Microbiological Transformation of Steroids. VI. Stereospecific Reduction of the 20-carbonyl Group by Fungi.

Agr. Biol. Chem. 26: 89-97 (1962)

Microbiological reductions of the 20-carbonyl group of steroids were investigated. *Candida pulcherrima* and *Sporotrichum gougeroti* converted the following substrates into the corresponding 20β-hydroxyderivatives (yields of the products are indicated in parentheses): cortexolone (60-70%) and 17α,21-dihydroxypregna-1,4-diene-3,20-dione (40-80%). A *strain of Rhodotorula glutinis* converted the following substrates into the corresponding 20α-hydroxyderivatives: cortexolone (65%), 17α,21-dihydroxypregna-1,4-diene-3,20-dione (80%), 11β,17α-dihydroxypregn-4-ene-3,20-dione (45%) and presumably 19-hydroxycortexolone (10%).

MISCELLANEOUS SCIENTIFIC PAPERS

1. Takezi HASEGAWA. 1962. Recent Problems on the Yeast Taxonomy. Trans. Mycol. Soc. Japan 3(15): 67-76 [In Japanese]
2. Keisuke TUBAKI. 1962. Fungi in Polluted Water. Seikatsu Eisei 6(4): 1-11 [In Japanese]

発酵研究所年報　第1号

昭和38年11月30日　印刷
昭和38年11月 5日　発行

（非売品）

編集兼
発行人　長谷川武治

発行所　財団法人 発酵研究所
大阪市東淀川区十三西之町4丁目54番地

印刷所　日本印刷出版株式会社
大阪市福島区亀甲町2丁目62番地