

## INORGANIC NUTRITION OF MYROTHECIUM VERRUCARIA

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(WITH 4 FIGURES)

The composition of inorganic salt solutions used for the cultivation of most fungi has generally been obtained empirically, it being early recognized that while variations in the formulae would affect growth, the effects would probably be of minor significance for the intended purpose of the experiment. The present paper reports experiments concerning growth of *Myrothecium verrucaria* on several substrates in solutions of systematically varied composition. This imperfect fungus was unknown in experimental microbiology until recently. It has high cellulolytic activity, rapid growth and simple nutritional requirements; the organism sporulates readily, the spores occurring in moist masses. These characteristics have led to its wide use in experimental microbiology and as a test organism in studies of microbiological degradation. Taxonomic studies of the organism have been made by White and Downing, 1947. Studies of its cellulolytic activity have been carried out by Greathouse, 1942, 1950; Saunders *et al.*, 1948; Siu and Sinden, 1951; Reese *et al.*, 1952; Blum and Stahl, 1952; and Whitaker, 1953. It has been used to develop an accelerated test for the determination of susceptibility of various materials to microbiological degradation (Mandels and Siu, 1950) and for the rapid screening of compounds for fungitoxicity (Mandels and Darby, 1953). The respiratory metabolism of its mycelium has been studied by Darby and Goddard, 1950. Several papers have appeared characterizing the carbohydrate metabolism and surface location of certain enzymes in its spores (Mandels, 1953a, 1954). Additional studies with its spores include a general treatment of respiration and germination (Mandels and Norton, 1948), and descriptions of an atypical surface-located ascorbic acid oxidase (Mandels, 1953b). Shirk and Byrne (1951) have used its spores in a respirometric assay of a large series of nitrophenols. The production of antibiotics by this and related species has been noted by Brian *et al.* (1948). Nearly all of the 70 papers in which studies on this organism have been reported have appeared within the last decade. The references cited in this brief review are not

complete. The reader is referred to the papers listed for additional references.

## METHODS

Stock cultures of *M. verrucaria* (PQD 460 = USDA 1334.2) were carried on potato dextrose agar or on filter paper on salt agar. Spore suspensions for inocula were prepared by flooding the surface of cultures with distilled water and shaking gently. The suspensions were not washed. Unless stated otherwise, the inorganic nutrient solution hereafter referred to as basic salts having a pH of 6.4 contained  $\text{NH}_4\text{NO}_3$ —3.0 g (37.5 mM);  $\text{KH}_2\text{PO}_4$ —2.68 g (19.7 mM);  $\text{K}_2\text{HPO}_4$ —2.09 g (12 mM);  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ —2.22 g (9 mM); 0.1 g Difco yeast extract; dist.  $\text{H}_2\text{O}$ —1 liter. Two percent sucrose or ground cellulose (40 mesh, 12 oz gray cotton duck) was used as a carbon source. CP grade chemicals were used. At least three replicates were used for each treatment.

TABLE I

EFFECT OF SHAKING AND RATIO OF FLUID VOLUME TO FLASK SIZE ON GROWTH  
(Basic salts, 2% sucrose, 0.01% yeast extract)

Condition	Vol. of sol'n	Flask size	Dry weight of mycelium per ml		
			2 days mg	5 days mg	28 days mg
Still	25 ml	125 ml	1.1	4.0	2.5
Shaken	25 ml	125 ml	3.4	5.8	3.0
Shaken	25 ml	250 ml	5.6	5.1	2.4
Shaken	50 ml	125 ml	2.3	4.8	2.9
Shaken	50 ml	250 ml	3.7	5.7	3.1

Erlenmeyer flasks received 25 ml/125 ml or 50 ml/250 ml of medium containing ca.  $10^6$ – $10^7$  spores/ml and were incubated at 30° C on a reciprocal shaker having 90 3" strokes per minute. These conditions were selected as a result of preliminary work shown in TABLE I.

Requirements for trace elements are apparently satisfied by impurities in the reagents used and in the yeast extract. Addition of these metals collectively at the concentrations indicated below was slightly inhibitory to growth on sucrose. Dry weights at 2, 5 and 7 days were 40, 99, 109 without, and 25, 63, 98 ml/25 ml with trace elements. Trace elements were added as the following salts (mg/l):  $\text{Fe}_2(\text{SO}_4)_3$  hydrate, 54;  $(\text{NH}_4)_3\text{P}(\text{Mo}_3\text{O}_{10})_4$ , 24;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 50;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 2.5;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 5.5.

Agar cultures (1.5% Difco agar) contained ca. 40 ml/10 cm petri dish. Linear growth was determined as the mean of two diameters

each of three replicate plates and is expressed as radial increase in mm/day.

Dry weights were obtained by collecting and washing the mycelium on filter paper discs and drying overnight at 80° C. An average tare weight was automatically subtracted by use of a counter-weight with a Roller-Smith torsion balance. This was justified by the following analyses:<sup>1</sup> (a) 16 mm diameter discs, sharkskin filter paper (cut by a special hand punch),  $\bar{X}$  = 8.58 mg,  $s$  = 0.185 mg,  $n$  = 116; (b) 40 mm discs (Schleicher and Schuell No. 589-1H),  $\bar{X}$  = 76.83 mg,  $s$  = 2.482 mg,  $n$  = 294. Thus in 95% of the cases ( $\pm 2s$ ) the maximum error introduced was 0.37 and 4.96 mg for (a) and (b) respectively. For net weights of 10 and 100 mg the paper error is less than 5%. Since most weights exceeded these values, the error was correspondingly less.

## RESULTS

## (1) Effect of modification of nutrient salt solution on growth in solution

To determine the effect of wide variations of the inorganic salt concentrations on growth with sucrose as carbon source, the basic salt solution was varied by 5-fold steps. Each of the 3 salts was used at three levels, making 27 solutions in all, plus a no-salts control. Solution F-2 (TABLE II) approximates the basic salts solution. The phosphate was changed to contain equal molarities of  $\text{KH}_2\text{PO}_4$ - $\text{K}_2\text{HPO}_4$ , pH 6.8. Dry weights and final pH at 2, 4 and 7 days are shown in TABLE II. Best results were obtained with solution B-3 containing  $\text{NH}_4\text{NO}_3$  = 7.5 mM,  $\text{K}_x\text{H}_x\text{PO}_4$  = 160 mM,  $\text{MgSO}_4$  = 1.8 mM.

In a further experiment the relative concentration of the salts of the B-3 solution was maintained, but the total salt concentration was varied from  $5 \times$  to  $1/500$  (TABLE III). Most rapid initial growth occurred in the  $5 \times$  solution, although greatest total growth in 5-7 days was obtained with the  $2 \times$  solution. Growth falls off with decreasing total salts until at the lower levels it approaches that of the no-salts control. At this point the effects of the carry-over of unknown quantities of salts with the unwashed inoculum and that contained in the yeast extract become complicating. Differences in results between similar solutions in TABLES II and III are ascribed to normal variation.

$$\bar{X} = \text{arithmetic mean, } \frac{\text{sum of individual weights}}{\text{number}}$$

$$s = \text{standard deviation} = \sqrt{\frac{\sum x^2}{n}}$$

where  $x$  = deviation of the individual values from the mean  
 $n$  = number of determinations.

TABLE II

EFFECT OF VARIOUS CONCENTRATIONS OF SALTS ON MYCELIAL WEIGHT AND pH  
(2% sucrose, 0.01% yeast extract)

	Time days	Concentration		Dry weight per culture (mg/25 ml)			Final pH		
		NH <sub>4</sub> NO <sub>3</sub> mM	MgSO <sub>4</sub> mM	Concentration K <sub>2</sub> H <sub>2</sub> PO <sub>4</sub> (mM)					
				6.4	32.0	160	6.4	32	160
		1	2	3	1	2	3		
A	2	7.5	0.36	27	25	34	5.8	6.4	6.6
	4			55	52	85	6.6	6.7	6.9
	7			59	60	85	7.5	6.6	6.6
B	2	7.5	1.8	30	23	45	6.0	6.5	6.8
	4			72	64	84	6.1	7.1	7.3
	7			94	95	142	6.6	6.8	6.6
C	2	7.5	9.0	36	25	39	6.0	6.4	6.7
	4			65	64	98	6.2	6.9	7.0
	7			83	78	127	6.2	6.7	6.8
D	2	37.5	0.36	28	22	43	5.8	6.4	6.6
	4			53	51	54	7.0	7.1	7.1
	7			56	57	74	7.0	7.1	6.7
E	2	37.5	1.8	28	23	44	5.8	6.0	6.5
	4			60	75	95	6.1	6.3	7.1
	7			70	78	105	6.5	6.5	6.8
F	2	37.5	9.0	28	38	35	6.4	6.2	6.6
	4			84	68	94	6.4	6.7	6.9
	7			64	46	120	6.8	6.9	6.5
G	2	187.5	0.36	23	25	32	5.9	5.9	6.9
	4			41	45	47	6.9	6.1	7.1
	7			46	41	57	6.5	6.9	6.7
H	2	187.5	1.8	38	23	34	5.6	5.9	6.4
	4			60	69	74	6.2	7.1	7.0
	7			58	68	101	6.8	6.7	6.6
I	2	187.5	9.0	22	26	24	5.7	6.0	6.5
	4			33	48	66	6.0	6.7	6.8
	7			52	71	89	6.4	6.5	6.7
No salts (control)									
				2			5.6		
				4			5.5		
				7			4.7		

Further data on the total salt effect can be seen by comparing A1, E2 and I3; A2 and E3; B1 and F2; B2 and F3; D1 and H2; D2 and H3; and E1 and I2 in TABLE II. In almost every case better growth occurs at the higher concentrations.

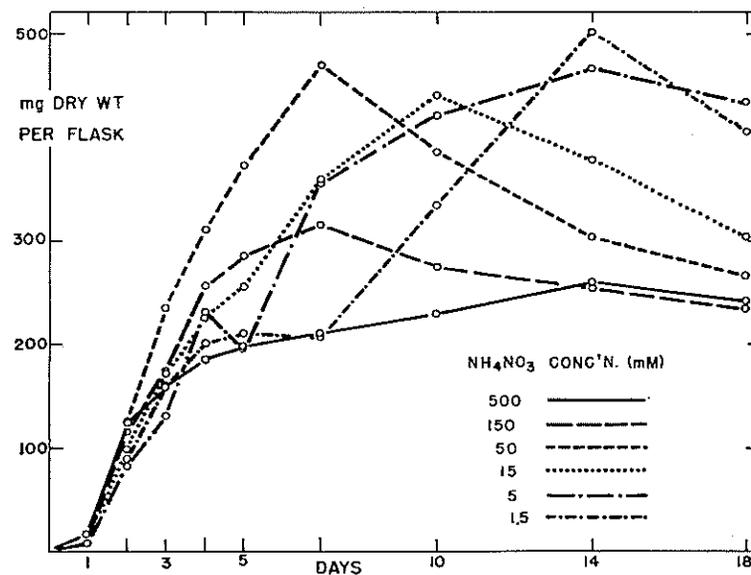
TABLE III

EFFECT OF TOTAL SALT CONCENTRATION ON DRY WEIGHT  
(2% sucrose, 0.01% yeast extract)

Expt.	Concentration of salts (mM)			Dry weight per culture (mg/25 ml)			
	NH <sub>4</sub> NO <sub>3</sub>	K <sub>2</sub> H <sub>2</sub> PO <sub>4</sub>	MgSO <sub>4</sub>	Rel. conc'n*	2d	5d	7d
1	37.5	800	9.0	5	158	142	160
1	15.0	320	3.6	2	130	172	158
2	15.0	320	3.6	2	36	161	203
2	7.5	160	1.8	1	28	112	160
2	1.5	32	0.36	1/5	21	76	108
2	0.15	3.2	0.036	1/50	32	54	86
2	0.015	0.32	0.0036	1/500	30	73	35

\* In comparison with solution B-3 in TABLE I.

Varying the total nitrogen concentration over a wide range results in strikingly different types of growth curves (FIG. 1). At the same level of sucrose (0.1M) the NH<sub>4</sub>NO<sub>3</sub> concentration was varied from 1.5 to 500 mM, the other salts being the same as in the basic solution. The initial rate of growth—i.e. for the first two days—is a direct func-

FIG. 1. Effect of nitrogen concentration on growth. (Mg. dry weight per 50 ml. basic salts + 0.1 M sucrose (3.4%) + NH<sub>4</sub>NO<sub>3</sub> as indicated, 30°.)

tion of the nitrogen concentration. On the other hand it is surprising to note that the maximum amount of mycelium produced tends to be an inverse function of the amount of nitrogen. More unusual are the double peaked curves for the two lowest levels of nitrogen—one early, followed by a short period of autolysis, then a resurgence of growth to a second high peak. This suggests a shift in metabolism, perhaps due to utilization of excreted organic nitrogen compounds. Only minor pH

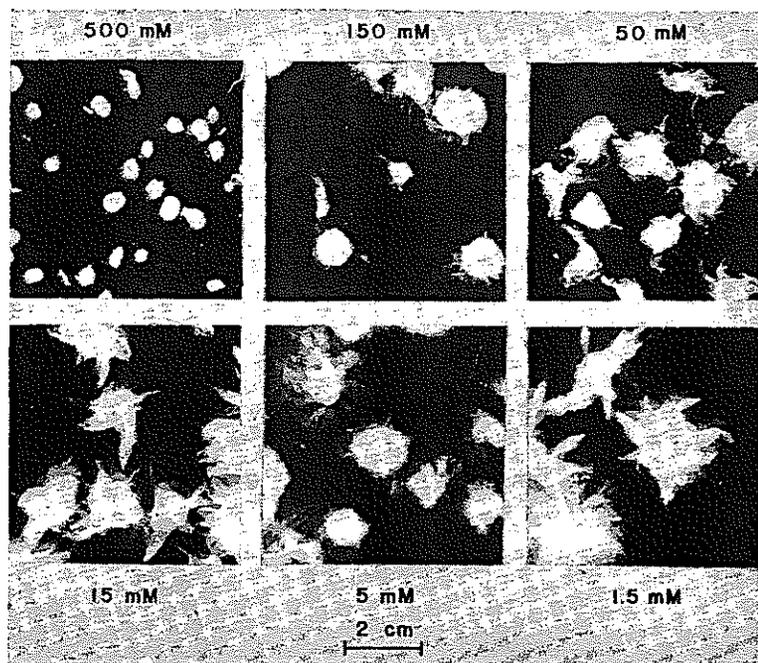


FIG. 2. Types of pellets formed with different concentrations of  $\text{NH}_4\text{NO}_3$ . (32 mM  $\text{K}_2\text{P}_2\text{O}_7$  + 9 mM  $\text{MgSO}_4$  + 0.1 M sucrose (3.4%),  $\text{NH}_4\text{NO}_3$ , as indicated, 30°, 18 days.)

changes occur during growth, the extremes being about 6.0 and 7.5, and these do not coincide with the breaks in the growth curves.

Differences in the character of growth in shaker flasks at nitrogen concentrations are very pronounced (FIG. 2). At high levels the pellets are small, compact and smooth. As the nitrogen is decreased the pellets become larger, more diffuse, with a more hairy type of growth. Also,

the pale yellow pigment which is characteristically produced with most media is much reduced at the lower nitrogen levels.

Observations of the size and shape of pellets in other experiments were not recorded, although more or less comparable variations have been observed.

TABLE IV  
EFFECT OF SALT CONCENTRATION ON CELLULOSE BREAKDOWN

I. Loss in dry weight of cellulose-mycelium mixture (500 mg ground cellulose/25 ml, 0.01% yeast extract added, 13 days growth)					
	$\text{NH}_4\text{NO}_3$ (mM)	$\text{MgSO}_4$ (mM)	1	2	3
			$\text{K}_2\text{H}_2\text{P}_2\text{O}_7$ (mM)		
			3.2	32	320
A	7.5	0	—	—	—
B	7.5	0.9	17	13	19
C	7.5	9.0	38	12	10
D	75	0.9	17	24	27
E	75	9.0	17	47	21
F	750	0.9	41	46	23
G	750	9.0	47	37	31

No salts control = 10% loss in dry weight

II. Loss in breaking strength of cotton duck (1" X 3" strips on salt agar, 0.01% yeast extract added, 13 days growth)					
	$\text{NH}_4\text{NO}_3$ (mM)	$\text{MgSO}_4$ (mM)	1	2	3
			$\text{K}_2\text{H}_2\text{P}_2\text{O}_7$ (mM)		
			3.2	32	320
A	7.5	0	—	—	—
B	7.5	0.9	56	—	92
C	7.5	9.0	94	54	42
D	75	0.9	83	82	43
E	75	9.0	78	100	74
F	750	0.9	93	98	68
G	750	9.0	98	97	92

No salts control = 38% loss in breaking strength.

(2) *Effect of modification of nutrient salts solution on cellulose breakdown*

Modification of the salts solution appears to have somewhat different effects when cellulose is the substrate (TABLE IV). Optimum growth, as measured by loss in total dry weight of the cellulose-mycelium mixture, is noted in solutions E-2, F-1,2 and G-1,2. Compared to growth on sucrose, the requirements for phosphate are considerably less, while high nitrogen is more beneficial.

The breaking strength data in this particular experiment are of limited value as measures of relative rates of cellulose breakdown because the prolonged incubation could have permitted complete loss in strength in solutions where breakdown was actually relatively slow. In general the results are comparable to those noted above. The high activity in the absence of added  $MgSO_4$ , which has been noted previously (Siu and

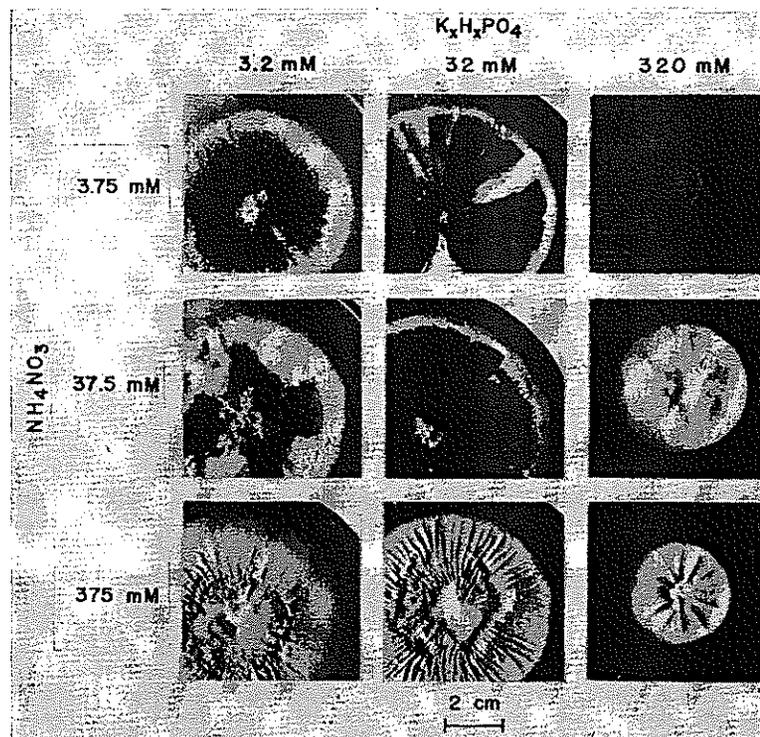


FIG. 3. Effect of inorganic composition of the medium on growth on agar + 2% sucrose, 0.01% yeast extract, and 9 mM  $MgSO_4$ . 15 days. Initial pH 6.8.

Sinden, 1951), indicates a low requirement which is met by impurities in the cellulose.

### (3) Effect of modification of nutrient salts solution on growth on agar

The effects of modification of the basic salts solution on growth on agar containing sucrose are quite different from those in liquid culture (FIG.

TABLE V  
GROWTH ON VARIOUS INORGANIC NITROGEN SOURCES  
(2% sucrose + 0.01% yeast extract)

	Dry weight per culture (mg/25 ml)				pH		
	Days	$NH_4Cl$	$KNO_3$	$NH_4NO_3$	$NH_4Cl$	$KNO_3$	$NH_4NO_3$
Expt. 1	2	28	25	36	2.8	6.2	6.2
	4	40	39	62	2.7	6.2	7.2
	7-8	53	96	94	2.6	7.0	7.2
Expt. 2	3	47	19	45			
	6	113	117	112			
	10	135	150	125	6.4	6.4	6.4

Expt. 1  $K_2H_2PO_4$  = 32 mM,  $MgSO_4$  = 9 mM.  
Expt. 2  $K_2H_2PO_4$  = 320 mM,  $MgSO_4$  = 1 mM.  
Total nitrogen in all flasks was 105 mM.

3). Since varying magnesium sulfate from 0.09-9 mM had no effect on the rate or character of growth, only the results at 0.9 mM are illustrated. High potassium phosphate markedly suppresses linear growth and sporulation, while at low concentrations the thickness of growth and amount of sporulation are slightly decreased. High concentrations of nitrogen suppress sporulation. Sectoring is occasionally encountered in this organism. In the plates shown in FIG. 2 frequent sectoring is noted where sporulation has occurred. The apparent absence of sectors where no sporulation occurred is of interest. Sparse growth at the normal linear rate occurs on sucrose-agar to which no salts have been added.

TABLE VI  
UTILIZATION OF ORGANIC NITROGEN

N source*	Dry weight per culture (mg/25 ml)		
	3d	6d	10d
Sodium monoglutamate	78	182	157
dl-Isolenine	35	177	150
dl-Aspartic acid	63	155	148
dl-Alanine	45	128	166
Glycine	30	101	150
1(+)-Arginine monohydrochloride	12	74	111
Asparagine	34	150	173
Urea	28	98	138
$NH_4NO_3$ (control)	45	112	125

Total N in all flasks = 20 mM,  $K_2H_2PO_4$  = 320 mM,  $MgSO_4$  = 10 mM, pH 6.4, 2% sucrose + 0.01% yeast extract.

\* Autoclaved separately.

## (4) Nitrogen nutrition

In shaker flasks with sucrose as substrate, no great differences in growth occur with nitrogen supplied as  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  or as both, except in poorly buffered media containing  $\text{NH}_4^+$  where the pH drops rapidly and growth is poor (TABLE V). On agar, with either cellulose or glucose as substrate, the character of growth varies considerably with these sources of nitrogen (FIG. 4). Effects of pH changes or their magnitude are not known here.

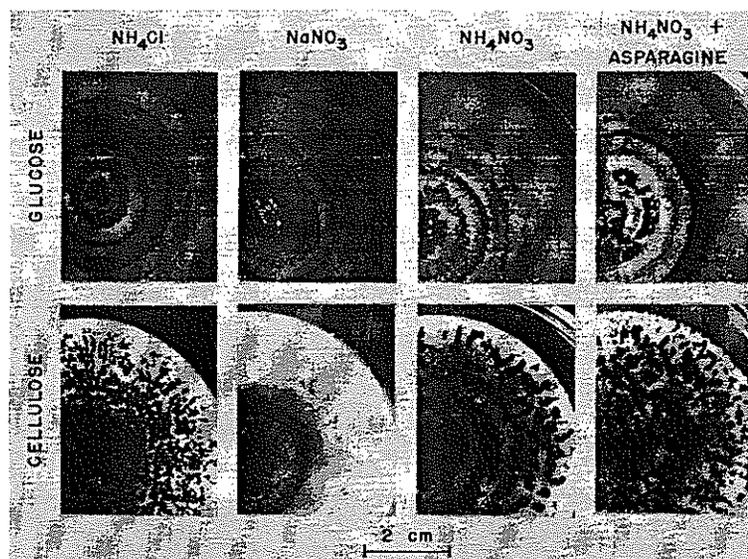


FIG. 4. Effect of inorganic nitrogen source on character of growth with glucose and cellulose as substrates. Basic salts plus 0.5% glucose or Whatman No. 2 filter paper. Nitrogen supplied as a)  $\text{NH}_4\text{Cl}$ , b)  $\text{NaNO}_3$ , c)  $\text{NH}_4\text{NO}_3$ , d)  $\text{NH}_4\text{NO}_3$  + 0.1% asparagine. 17 days old.

Growth with various sources of organic N are shown in TABLE VI. Sodium monoglutamate and isoleucine were superior to the other amino acids tested. Alanine, asparagine and aspartic acid were excellent, somewhat better than the several inorganic forms of nitrogen tried. Glycine was about as good as inorganic N; arginine was somewhat inferior to inorganic N. A mixture of amino acids as found in casein hydrolysate was excellent, as shown in other experiments (not reported).

## DISCUSSION

Growth occurs over wide modifications of the inorganic nutrient solution. Varying total salts by a factor of 1000, the ratios of the salts by factors of 25, or nitrogen by 300, affects the initial rate of growth on sucrose, for example, only by a factor of about 2 and total growth about 3.5 times. Variations from one experiment to another, using the same nutrient solution, are as great or greater than those induced experimentally. The type of growth is influenced greatly by modification of the inorganic nutrients. This is illustrated strikingly for growth on agar where sporulation is affected qualitatively and in solution using pellet morphology as a criterion.

The selection of a culture medium should, therefore, be governed by the end results desired. Media may thus be selected for ease of prepa-

TABLE VII  
RECOMMENDED CONCENTRATIONS OF SALTS FOR VARIOUS TYPES OF GROWTH

Criterion	Substrate	Composition (grams/liter) <sup>1</sup>			
		$\text{NH}_4\text{NO}_3$	$\text{KH}_2\text{PO}_4$	$\text{K}_2\text{HPO}_4$	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
Dry weight in solution	sucrose	1.2	21.8	27.9	0.9
Mycelium + spores on agar	sucrose	0.3	2.2	2.8	0.2
Mycelium on agar	sucrose	3.0	2.2	2.8	0.2
Dry weight loss	ground cloth	6.0	2.2	2.8	0.2
Tensile strength loss	cloth strips	6.0	2.2	2.8	0.2
Tensile strength loss <sup>2</sup>	cloth strips	0.3	0.7	0.9	0.05
Cx production <sup>3</sup>	ground cellulose	1.0	1.36	—	0.3
Spore production <sup>4</sup>	filter paper	3.0	2.7	2.1	2.2

<sup>1</sup> 0.01% yeast extract is usually added.

<sup>2</sup> Data of Siu and Sinden.

<sup>3</sup> Data of Reese (1950), Cx a cellulolytic enzyme, pH adjusted to 6.3 with N/1 NaOH.

<sup>4</sup> Mandels and Norton, 1948.

ration, maximum dry weight in solution, maximum growth on agar, cellulose breakdown, production of pigment, enzyme or other chemicals, spore production or suppression, pH control, etc.

A summary of approximate salt concentrations found to be most favorable for various types of growth and metabolic activity is presented in TABLE VII. While the conditions under which these results were obtained are not strictly comparable, the data represent an approach to the formulation of optimum solutions.

The favorable effect on dry weight of relatively high phosphate and low nitrogen and magnesium sulfate cannot be explained on its high

buffering capacity since the organism has never been observed to produce much acid, and it is tolerant of a wide pH range. On the basis of the data of Siu and Sinden (1951) its superiority might be expected to be due to the high phosphate rather than to potassium. Unpublished data support this idea.

The salt requirements for optimum cellulose breakdown vary with the criteria used and are widely different from those required for growth on sucrose. When based on loss in total dry weight of the cellulose-mycelium mixture the advantages of the high nitrogen solutions are evident, whereas high phosphate is not beneficial. When based on loss in tensile strength of cotton duck the addition of very low concentrations of salts is enough to give almost maximal breakdown. It is, in fact, possible to get very high breakdown without added magnesium sulfate, and the no salts control suffered 38% loss. Siu and Sinden (1951) found 0.6 mM potassium optimal for cellulose breakdown, although about 50% loss occurred without added potassium. Salts contained in the cloth represent an unknown factor.

#### SUMMARY

Optimum concentrations of inorganic salts for *M. verrucaria* depend upon whether the experimental requirements are for dry matter production, pellet size and shape, vegetative *vs.* reproductive growth on agar, cellulose breakdown, etc. Good growth occurs with either  $\text{NO}_3^-$  or  $\text{NH}_4^+$ , provided the pH is controlled, although better growth (with sucrose) occurs with organic nitrogen.

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