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10. Supported by grants from the National Science Foundation and the National Foundation. I acknowledge the assistance of M. Grabowski, M. Milan, S. Lehrer, and S. Kaplan, and the valuable suggestions of Dr. J. S. Clegg.

11 September 1963

Rhizomorph Production by Armillaria mellea Induced by Ethanol and Related Compounds

Abstract. Armillaria mellea *produced abundant rhizomorphs when grown on a chemically defined medium containing ethanol at concentrations as low as 50 parts per million. In the absence of ethanol no rhizomorphs were formed. Rhizomorph production was also stimulated by 1-propanol, isopropanol, and 1-butanol as well as by acetaldehyde (to a lesser extent). Potassium acetate had very slight stimulating effect, and methanol was completely ineffective.*

Armillaria mellea (Vahl) Quél. is a widely prevalent and important plant pathogen in California. This fungus is unique in its production of highly organized rhizomorphs whose development and morphology have been recently investigated by Townsend (*1*) and Snider (*2*). These structures function in the penetration of host roots by this pathogen. Investigations of the nutritional requirements for rhizomorph production and growth have been published (*2, 3*). In these studies it was necessary to include in the medium a complex substrate such as peptone or yeast extract in order to obtain vigorous development of rhizomorphs. During an investigation to determine the nutritional factors responsible for rhizomorph formation and growth it was found that ethanol would markedly stimulate rhizomorph development in a chemically defined synthetic medium.

The production of ethanol by fungi is well known (*4*). The utilization of ethanol as a sole carbon source for growth is less common but has been reported (*5, 6*). Miller and Halpen (*6*) reported that ethanol at certain concentrations supported abundant sporulation of yeast. However, the action of ethanol in stimulating growth or influencing the type of growth has received little attention. Springer and Gernet (*7*) reported that addition of 0.5 to 2 percent ethanol would stimulate the synthesis of citric acid by *Aspergillus niger,* and Cochrane (*8*) found that low concentrations of ethanol would stimulate germination of the macroconidia of *Fusarium solani.*

The basal medium in this investigation was composed of 5 g of glucose, 0.75 g of $MgSO_4 \cdot 7H_2O$, 1.75 g of KH_2PO_4, 2 g of L-asparagine, 1 mg of thiamine, and 20 g of agar in 1000 ml of distilled water. The *p*H of the medium was adjusted to 5.8. Early in the study the requirement of *Armillaria mellea* for thiamine and the poor growth on inorganic forms of nitrogen, reported by Garrett (*3*), were confirmed.

The fungus was grown in glass 4-oz (118 ml) prescription bottles containing 20 ml of medium. The bottles were laid flat to form a layer of agar measuring 90 by 45 by 5.0 mm. Inoculum consisted of 5-mm disks of water-agar containing mycelium of the fungus. The water agar was seeded with a small piece of rhizomorph and was ready for use in 2 to 3 weeks. Inoculum over 5 weeks of age was not used. After the test bottles had been seeded they were incubated in the dark at 25°C. The total length (in centimeters) of rhizomorphs formed was determined with a Keuffel and Esser map-reading device (*1, 2*). This method provided a satisfactory relative measurement of rhizomorph growth. Only qualitative evaluations of mycelial growth were made.

On the basal medium *A. mellea* showed good mycelial growth but did not produce rhizomorphs. When redistilled ethanol was added to give a final concentration of 150 or 1500 parts per million by weight (3.2 and 32 mmole/liter), mycelial growth was stimulated and rhizomorphs were produced (see cover). The first evidence of rhizomorph development was apparent after 5 to 6 days, and there was extensive development at 14 days. The stimulating effect of ethanol was detected at a concentration of 25 ppm. Optimum concentration appeared to be at 500 ppm, and very little increase in rhizomorph development occurred at 1000 and 2000 ppm ethanol. Distorted and stunted rhizomorphs resulted when the alcohol concentration was 4 percent. No inhibition occurred at 1 percent ethanol. The stimulatory effect of ethanol was observed with all of five isolates tested. The isolates all originated from single spores and varied in their ability to produce rhizomorphs.

When related compounds containing two carbon atoms were tested, acetate was only slightly stimulatory and acetaldehyde, while toxic at 0.01*M*, induced rhizomorph formation at lower concentrations (Table 1). Of other alcohols tested, methanol had no stimulatory activity whereas 1-propanol and 1-butanol markedly enhanced rhizomorph development (Table 1). Isopropanol was similar to 1-propanol in stimulatory activity. Compounds that stimulated rhizomorph development also stimulated mycelial growth. In all cases the *p*H of the medium after growth was 5.8 to 6.0.

When yeast extract (2 g/liter) or peptone (2 g/liter) were added to the basal medium, rhizomorphs were produced without the addition of alcohol. However, ethanol significantly increased rhizomorph production when it was added to medium containing either of these compounds. Rhizomorphs were produced on potato-dex-

Table 1. Effect of ethanol, related compounds containing two carbon atoms, and other alcohols, in different concentrations, on rhizomorph production by *Armillaria mellea.*

Conc. (mmole/liter)	Length (cm) at 14 days*					
	Ethanol	Acetaldehyde	Potassium acetate	Methanol	1-Propanol	1-Butanol
10.8	59.8 ± 2.8		17.5 ± 1.1	< 1.0	36.5 ± 1.3	79.3 ± 2.9
2.6	60.3 ± 4.3	21.3 ± 3.7	11.2 ± 6.0	< 1.0	54.5 ± 3.5	54.2 ± 4.7
1.08	28.0 ± 3.2	15.7 ± 3.5	2.5 ± 0.3		49.0 ± 4.4	43.7 ± 4.7
0.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0

* Each value is the mean of at least six replications; standard error is indicated.

trose agar, but development was markedly stimulated by the addition of ethanol. In addition, Raabe (*9*) has reported that aqueous extracts of wood from several plant species will stimulate rhizomorph production in *A. mellea*. Therefore it appears that many natural materials contain a substance or substances which are required by *A. mellea* for rhizomorph development. The fact that alcohols of low molecular weight can replace these complex substrates permits rhizomorphs to be produced on a chemically defined medium so that more precise information on the nutritional requirements of this fungus for rhizomorph production can be obtained.

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24 June 1963

Lysosomes in the Renal Papillae of Rats: Formation Induced by Potassium-Deficient Diet

Abstract. *Lysosomes appeared in the cytoplasm of cells of the renal papillae of rats fed on a potassium-deficient diet. The lysosomes were identified by their morphologic appearance when examined by electron microscopy, and by their acid phosphatase activity shown by both light and electron microscopic examination of Gomori-treated tissue.*

On the basis of biochemical studies of the products of cell fractionation, De Duve postulated that cells must contain organelles in which are localized acid hydrolases, such as acid phosphatase, cathepsin, and beta-glucuronidase (*1, 2*). He gave the name "lysosome" to these particles. Fractions of homogenized cells showing high activity of acid phosphatase, separated by centrifugation, were examined by electron microscopy and found to consist of bodies which were distinguishable from mitochondria, and which had a single limiting membrane (*3*). Since then, examination of sectioned tissue by electron microscopy has shown that bodies having the general morphology just outlined are present in many different kinds of cells (*4–6*). Nonspecific acid phosphatase has been demonstrated in bodies with the structure just described by applying the Gomori method for acid phosphatase to suitably prepared tissues and examining sections by electron microscopy (*5–7*).

Recently, while studying the kidneys of rats made potassium deficient, we recognized that formation and accumulation of lysosomes was taking place in the cells of the renal papilla, where they are not ordinarily found. This observation resulted from our investigation of the characteristic eosinophilic granules, which accumulate in the cytoplasm of renal papillary cells of rats within a week of feeding the animals a potassium-deficient diet (*8, 9*). In previous studies we have shown that the granules contain serum protein and, in addition, a sialic acid–containing mucopolysaccharide (*10, 11*). Since other investigators had already demonstrated histochemically that increased amounts of acid phosphatase accumulate in the cells of the papilla during potassium deficiency in the rat (*12*), it occurred to us that the granules might be lysosomes. This report outlines our evidence that the granules have the biochemical and morphological characteristics recently suggested for lysosomes (*7*).

Our observations were made on male Wistar rats which weighed from 175 to 200 g at the start of the experiment. They were fed a diet low in potassium, obtained from the Nutritional Biochemical Corporation of Cleveland. Control rats were fed the same diet supplemented with potassium salts (0.8 g KH_2PO_4 and 0.6 g KCl per 100 g diet). Each control rat was paired with an experimental animal and given the same daily amount by weight of the control diet as the experimental animal consumed of the low potassium diet. While eating this low potassium diet, rats do not gain weight, but do develop a potassium-deficient state which is characterized by low levels of potassium in the muscles and serum (*10*). Groups of both the experimental and control rats were anesthetized and killed at intervals of 1, 2, 3, 4, and 8 days, and 1 month after the initiation of the diet. Kidneys were removed from each rat through an abdominal incision before killing the animals, and the papilla quickly removed from each kidney.

Table 1. The activities of total, free, and bound acid phosphatase in papillary tissue of normal and potassium-deficient rats. The activities are expressed as micrograms of inorganic phosphorus liberated from β-glycerophospate by 100 mg of renal papillary tissue in 1 hour.

Total	Free	Bound
	Control rats	
65	—	—
82	—	—
66	66	0
77	77	0
119	92	27
107	73	34
100	90	10
75	74	1
*86 ± 7	78 ± 3.4	12 ± 6.6
	Potassium-deficient rats	
709	—	—
750	—	—
453	—	—
488	—	—
798	—	—
417	219	198
332	151	181
431	221	210
840	488	352
684	419	265
645	385	260
401	114	287
290	108	182
*557±52	263±52	242±21

* Mean ± S.E.M.

Papillae for chemical analysis were weighed, and the activity of total tissue acid phosphatase was assayed chemically by the method described by De Duve (*1*); in some papillae the activity of free and bound acid phosphatases was determined also (*1*). Those papillae which were to be examined by light microscopy were fixed in calcium-formol and stained for acid phosphatase by the method of Burstone (*13*). Some of these sections were washed in distilled water after the overnight incubation in Burstone substrate, and then stained by the Hale iron technique for acid mucopolysaccharide following the method of Mowry (*14*).

Tissue for electron microscopic examination was prepared in two ways: by fixation in osmium tetroxide, and by the method of Miller for acid phosphatase localization (*6*). All solutions for Miller's enzyme technique were made up with 0.35*M* sucrose, rather than the 0.22*M* sucrose suggested by