THE SPECIFIC EFFECT OF ZINC AND OTHER HEAVY METALS ON GROWTH AND FUMARIC-ACID PRODUCTION BY RHIZOPUS[1]

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Raulin (1869) first demonstrated that *Aspergillus niger* requires, besides the usual elements, traces of certain other elements, notably zinc, iron, silicon and possibly manganese. Although Raulin's claims have in some instances been refuted (Coupin, 1913; Lepierre, 1913; Wehmer, 1893), the investigations of Javillier (1907) and Bertrand (1911), and, more recently, of Steinberg (1919), Bortels (1927), and Roberg (1928) have confirmed and extended Raulin's original observations, especially with regard to the requirements for zinc and iron. It was found that if either of these two elements were removed completely from the medium, no growth would occur. Since the physiology of the fungus was modified in a unique fashion (Javillier 1912) by the presence of zinc, Javillier (1913) attributed to this element the rôle of a catalyst. Steinberg (1934) characterized zinc and iron as nutrients, as opposed to the stimulant concept of Pfeffer (1895). A detailed review of the literature on the heavy metal nutrition of fungi will be presented elsewhere (Foster 1939).

Few reports are found in the literature dealing with the relation of heavy metals to the growth of species of *Rhizopus*, most investigators previously having used *A. niger*. McHargue and Calfee (1931) found that the presence of zinc, especially when combined with manganese and copper, resulted in increases in *Rhizopus* growth. More recently zinc was found to play a vital rôle in the physiology of lactic-acid-producing strains of *Rhizopus* (Lockwood *et al.*, 1936; Waksman and Foster, 1938). Relatively

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poor growth occurred and large quantities of lactic acid accumulated in the medium unless zinc was added, when growth became greatly accelerated accompanied by sharp reduction in lactic acid yield. The zinc effect was interpreted (Waksman and Foster, 1938) as a catalyzation of the more complete destruction of the glucose thus rendering available more energy and carbon for the synthesis of cell substance.

EXPERIMENTAL

No special effort was made to free the medium completely of traces of heavy metals. The results, therefore, reflect the effect of the added zinc. Two hundred ml. portions of the basal medium containing Merck's reagent grade anhydrous glucose, 2.5 per cent; $(NH_4)_2SO_4$, 0.2 per cent; $MgSO_4 \cdot 7H_2O$, 0.05 per cent; K_2HPO_4, 0.05 per cent, were distributed into 500 ml. Erlenmeyer flasks. A strain of *Rhizopus nigricans*, previously found (Foster and Waksman, 1938) to produce fumaric acid from glucose vigorously and abundantly, was used in these investigations.

Zinc or other elements[2] were added to flasks in quadruplicate, which then were sterilized for 1 hour on 2 consecutive days at 100°C., and inoculated with spores from a zinc-free culture of *Rhizopus nigricans*. After incubation at 28°C. for 3 days, 5 gram portions of sterile $CaCO_3$ were added and the cultures shaken several times daily to neutralize the acid formed, care being taken not to injure the pellicle. After 7 and 11 days growth, the liquid was poured off aseptically and the pellicles washed twice with sterile distilled water which then was added to the original liquid. The pellicles were saved for later use. Glucose was determined by the Bertrand method, NH_3—N by distillation with MgO, calcium by $KMnO_4$ titration, after double precipitation as the oxalate; fumaric acid was determined gravimetrically as mercurous fumarate according to Ölander's (1929) modification of Hahn and Haarman's method. Since the results after 7 days are exactly comparable to the older set, only the latter are presented in table 1. All data are averages of duplicates.

Of the 5 elements tested, zinc exerted the greatest effect upon

[2] $ZnSO_4 \cdot 7H_2O$; $Fe_2(SO_4)_3$; Na_2MoO_4; $MnSO_4 \cdot 4H_2O$; $CuSO_4 \cdot 5H_2O$.

growth and acid production. Zinc ion, in a concentration of 1.2 parts per million, profoundly modified energy utilization by this organism, growth being decidedly stimulated. The organism grew vigorously and formed an abundant aerial mycelium over a

TABLE 1

Effect of trace elements upon growth and fumaric-acid production by Rhizopus nigricans

Milligrams per 200 ml. of culture, containing 5,088 mgm. glucose and 82.8 mgm. NH_3—N

ELEMENT	GLUCOSE CONSUMED	FUMARIC ACID FOUND	CONVERSION	NH_3—N CONSUMED	TOTAL CALCIUM IN SOLUTION	CALCIUM DUE TO ORGANIC ACIDS	PER CENT OF CALCIUM DUE TO FUMARIC ACID
mgm. per liter			*per cent*				
0	4,702	2,349	50.0	31.0	760.2	715.9	113.2*
5Zn	5,088	452	8.9	82.1	337.8	220.4	70.8
15Zn	5,088	439	8.6	81.0	332.5	216.7	70.0
5Fe	4,593	2,034	44.3	36.6	726.0	673.7	104.1
15Fe	4,227	1,878	44.4	25.4	652.8	616.5	104.4
5Mo	4,833	2,189	45.3	34.5	777.0	727.7	103.7
15Mo	4,873	2,114	43.4	36.6	770.3	718.0	101.5
5Mn	4,873	2,188	44.9	35.5	778.4	727.6	103.7
15Mn	4,812	2,009	41.8	36.0	763.8	712.6	97.2
2Cu	4,798	2,119	44.2	36.6	756.0	703.7	103.8
5Cu	4,862	1,643	33.8	47.4	653.3	585.6	96.2
5Zn + 5Fe	4,958	598	12.1	81.5	360.8	244.3	84.5
5Zn + 15Fe	4,863	597	12.2	82.2	352.5	235.0	77.4
5Zn + 5Fe + 5Mo	4,840	561	11.6	73.0	351.0	246.6	71.6
5Zn + 5Fe + 5Mo + 5Mn	4,968	377	7.6	82.2	289.5	172.0	75.6
5Zn + 5Fe + 5Mo + 5Mn + 5Cu	4,250	629	14.8	73.0	372.1	267.7	82.3

* Values greater than 100 per cent are due to fact that the acid was incompletely neutralized by the $CaCO_3$; later work showed this to be close to 100 per cent.

thick pellicle as contrasted to slow growth, very thin pellicle formation and lack of profuse aerial mycelium when zinc was deficient. The zinc-containing cultures made about two and a half times as much growth as the controls, as measured by nitrogen consumption.[3] The slight increase in glucose consumption

[3] The nitrogen content of *Rhizopus* cell substance was found to be approximately 5 per cent.

in the former cultures could in no way be compared to stimulation of growth. Zinc evoked even a more pronounced effect in the cultures which had additional supplementary trace elements (Fe, Mn, Mo, Cu). These elements alone had no significant effect, but the simultaneous presence of zinc brought about the characteristic response. One may thus speak of a "specific effect" of zinc on the growth of *Rhizopus*. Out of a large number of other so-called trace elements tested, none produced this effect.

The data on acid production (table 1) suggest the nature of this specific effect. In every case, a pronounced diminution in the actual amount of fumaric acid produced occurred in the zinc cultures. Despite the slightly greater utilization of glucose, the yields of acid which ordinarily approximated 44 per cent were reduced from $\frac{1}{3}$ to $\frac{1}{8}$ of this value by a trace of zinc. In the absence of this element, the organism transformed a large portion of the glucose into fumaric acid, whereas in its presence only a small fraction of the sugar consumed was left in the form of this acid. Zinc enabled the organism to utilize more completely its energy source instead of leaving a large part of it tied up in the form of fumaric acid. One may thus ascribe to zinc the rôle of a catalyst in the metabolism of *Rhizopus*. Mere traces seem to catalyze a more complete destruction of the glucose molecule and consequently its more efficient utilization as a source of energy and of carbon for cell synthesis. Zinc is not a mere accelerator of the rate of utilization of the original glucose as proven by comparing glucose-carbon disappearing with the carbon synthesized into cell substance.

$ZnSO_4$	GLUCOSE CARBON CONSUMED	CARBON SYNTHESIZED INTO CELL SUBSTANCE[4]	$\frac{\text{CELL SUBSTANCE C}}{\text{GLUCOSE C}}$
mgm. per liter	*mgm.*	*mgm.*	*per cent*
0	1,881	279	14.8
5	2,035	739	36.3
15	2,035	729	35.8

[4] This was calculated from the C:N ratio of the *Rhizopus* pellicle, which was taken as 9:1. Carbon content of *Rhizopus* was found by analysis to be approximately 45 per cent.

The efficiency of carbon utilization for cell synthesis was thus increased by 2 to 3 times under these conditions. Further evidence of the specific physiological effect of zinc upon the metabolism of *Rhizopus* was brought out by comparing the relation of fumaric acid to total acidity. When zinc was not added, fumaric acid accounted for practically 100 per cent of the total acidity measured as calcium in solution; in its presence this was only 70 to 80 per cent. These results have been repeatedly confirmed. In other words, zinc induced the formation of a very significant amount of other, as yet unidentified, acidic constituents.

In contrast to zinc, iron alone usually tended to depress growth but the fumaric-acid-yielding capacity of the organism was not reduced thereby (see also table 5). When combined with a trace of zinc, the effect of iron was not only completely obliterated, but the specific effect of zinc was even accentuated. The 3 elements (Mo, Mn, Cu) alone brought about no particularly specific response. Here again, regardless of what other elements were present in the medium, zinc favored the formation of another acid (or acids) amounting to 25 to 30 per cent of the total. Further study brought out that adjustment of the Fe:Zn ratio can reduce the specific effect of zinc and that the characteristic response of the organism to iron is manifested, i.e., a partial neutralization of the two elements takes place. It must be emphasized, therefore, that the associative or antagonistic nature of these two elements depends upon their ratio.

The marked response which zinc induces in *Rhizopus* suggests that the need for this element may be such that no growth would occur if it were completely removed from the medium. Fifty grams of $CaCO_3$ were added to two liters of the above medium which was then autoclaved for 15 minutes at 15 pounds pressure, according to Steinberg's (1919) purification technique and filtered hot. All glassware with which the medium came in contact had previously been thoroughly rinsed with distilled water purified in the same way. One milligram of the heavy metal salts was added to 100 ml. of medium in 250 ml. Erlenmeyer flasks, the media sterilized 1 hour at 100°C., and inoculated with a minimum of spores from a heavy-metal-deficient culture. The same flasks had been used for previous experiments of a similar nature, each flask receiving identical metal treatment, so that contamination from the glass was at a minimum. After incubation at 28° for 4 days, the fungus pellicles were washed, dried at 80°C., over night and at 100°C. for 2 hours and weighed.

The scant few milligrams growth in the controls (table 2) is probably due to metals leaching from the glass as well as from the inoculum. The results clearly show that the combination of zinc and iron is indispensable for the growth of *Rhizopus*. Although there is some indication that manganese and copper may

TABLE 2

Growth of Rhizopus nigricans in purified basal nutrient solution as influenced by heavy metals

HEAVY METAL TREATMENT*	DUPLICATE DRY WEIGHT OF FUNGUS	SPORULATION
	mgm.	
Basal medium	11, 7	Sterile
Zn, Mn	25, 21	Sterile
Zn, Fe	264, 246	Very slight
Fe, Mn	10, 10	Sterile
Fe, Zn, Cu	318, 319	Abundant
Fe, Zn, Mn	325, 323	Abundant
Fe, Mn, Cu	12, 8	Sterile
Fe, Zn, Mn, Cu	355, 368	Abundant

* All metals except Cu were added as 10 mgm. of the following salts per liter of nutrient solution: $ZnSO_4 \cdot 7H_2O$, $Fe_2(SO_4)_3$, $MnSO_4 \cdot 4H_2O$; 2 mgm. per liter $CuSO_4 \cdot 5H_2O$.

also be essential, it is obvious that the amounts of these elements required by the organism are extremely low as compared with the demand for zinc and iron. The absence of either of the latter elements from the otherwise complete medium resulted in practically no growth, while the two elements combined always gave abundant growth.

The pellicles from the 7-day cultures of the first experiment were used to determine the influence of glucose concentration upon fumaric-acid production. Sterile glucose solutions (150 ml.) of varying concentrations were added to the flasks containing the pellicles together with 10 grams $CaCO_3$. Incubation was no growth occurred, but the pellicles continued to convert the glucose into fumaric acid.

The results (table 3) show clearly that the rate of glucose consumption is influenced decidedly by its initial concentration. The maximum fumaric-acid production occurred when the initial concentration of glucose was from 5 to 15 per cent. Higher concentrations caused progressively smaller transformation of the sugar to acid. It is clear that efficiency of conversion is inversely proportional to glucose concentration. No consideration was given, in this experiment, to the differences in the size of the pellicles and to the possible effects of the various trace elements in the formation of the pellicles upon their activities. Many of the pellicles produced so much fumaric acid that abundant crystallization of calcium fumarate occurred in the cultures.

In the next experiment, a study was made of the effect of zinc, especially in combination with other elements, upon the specific activity of the pellicles produced. Pellicles were grown for 11 days, washed twice with sterile water, drained, 150 ml. sterile 5 per cent glucose solution and 5 grams sterile $CaCO_3$ added, and the flasks incubated 6 days at 28°C. with frequent shaking. The results presented in table 4 point to the fact that the history of the pellicle may alter the fumaric acid metabolism of *Rhizopus*. Pellicles grown with zinc alone and in combination with other elements were unfavorable to fumaric acid accumulation, whereas for glucose consumption their vigor was considerably reduced. A combination of Zn, Fe, Mo, and Cu in the original medium was most effective in repressing the production of acid by the fungus pellicles, both as regards percent conversion and total acids formed. In addition, considerable amounts of acids other than fumaric, as measured by the calcium in solution, were found among the products formed by the zinc pellicles. Further, the fact that fumaric acid accounted for only about 90 per cent of these acids, even in the case of the zinc-free pellicles shows that the pellicles metabolized the sugar differently from the growing cultures of the organism, the latter giving practically 100 per cent fumaric acid. Bernhauer (1927) reported similar behavior for citric-acid formation by mature pellicles of *Aspergillus niger*

TABLE 3

Influence of initial glucose concentration upon glucose consumption and fumaric-acid production by Rhizopus

150 ml. glucose solution used

INITIAL GLUCOSE CONCENTRATION	GLUCOSE CONSUMED	FUMARIC ACID FOUND	CONVERSION
per cent	*mgm.*	*mgm.*	*per cent*
2.5	3,323	1,559	46.9
5.0*	9,000	3,534	39.3
10.0	10,575	3,563	33.7
15.0	12,215	3,681	30.1
20.0	10,060	2,986	29.7
25.0	10,440	1,954	18.7
30.0	5,125	631	12.3
40.0	5,125	399	7.8

* These 2 flasks received 200 ml. of 5.0 per cent glucose solution.

TABLE 4

Influence of pellicle nutrition upon its effectiveness in producing fumaric acid

ELEMENTS IN ORIGINAL MEDIUM	GLUCOSE CONSUMED	FUMARIC ACID FOUND	CONVERSION	TOTAL CALCIUM IN SOLUTION	CALCIUM AS FUMARIC ACID
	mgm.	*mgm.*	*per cent*	*mgm.*	*per cent*
0	6,901	1,840	26.7	638.0	99.5
5Zn	3,235	1,010	31.2	392.0	88.5
15Zn	3,180	971	30.6	379.0	88.4
5Fe	6,820	2,879	42.2	1,048.0	94.9
15Fe	6,805	2,793	41.0	1,032.0	90.3
5Mo	7,157	2,681	37.5	938.0	98.6
15Mo	5,910	2,524	42.7	900.0	96.7
5Mn	6,820	2,395	35.1	841.0	98.2
15Mn	7,057	2,503	35.5	898.0	96.8
2Cu	7,057	2,409	34.1	853.0	97.4
5Cu	5,130	2,375	46.3	844.0	97.0
5Zn + 5Fe	1,987	319	16.1	131.0	83.9
5Zn + 15Fe	1,762	274	15.5	120.0	78.8
5Zn + 5Fe + 5Mo	2,125	319	14.1	136.0	80.9
5Zn + 5Fe + 5Mo + 5Mn	3,100	140	4.5	190.0	25.4
5Zn + 5Fe + 5Mo + 5Mn + 5Cu		344		190.0	81.3
10V	6,510	2,425	37.3	900.0	92.9

at 28°C., for 9 days, with frequent shaking. No nitrogen or nutrient minerals were present in these pellicle cultures. Thus, cultivated in the presence of zinc and acting on sugar replacements.

A study was now made of the effect of zinc upon fumaric acid production, as influenced by the age of the fungus. Growth of the organism was interrupted at 3 different stages: (*a*) after 5 days, when the culture in the flasks with the regular medium had just formed a complete pad upon the surface of the medium and before the profuse aerial mycelium and sporulation occurred; (*b*) after 7 days, when a well-formed thick pad was produced, accompanied by heavy aerial mycelium and abundant sporulation, especially in the zinc cultures; (*c*) after 10 days, well past the maximum growth stage of the fungus growth. The experiment was set up in a manner similar to the previous, duplicate flasks of each treatment being withdrawn at each of the 3 periods of growth. The various pellicles were freed from the medium, washed, replaced as usual with 10 per cent glucose and $CaCO_3$ and incubated for 5 days. Besides zinc and iron alone, a combination of Fe, Mo and Cu was used, in order to demonstrate conclusively that it was zinc and not the other elements which resulted in the characteristic responses obtained in the previous experiment. Analyses of the culture liquid during the growth of the fungus are presented in table 5. The per cent conversion in the zinc cultures dropped from 22.6 to 12.8 per cent, with advancing age, thus tending to confirm definitely the assumption that the function of zinc becomes increasingly important in repressing fumaric acid production with increased age of the organism.

The earlier experiments showed that the formation of another unidentified acid is always associated with zinc. Further proof that this is correct is to be found in the fact that, with progressive age, when the effect of zinc became more marked, there was a progressive increase from 7.7 to 33.7 per cent in the quantity of the other acid found. Malic and succinic acids have been identified in culture solutions of this organism.

On comparing glucose and nitrogen consumption during the growth of the organism as influenced by iron, it is seen that this element repressed the latter. These cultures possessed a higher sugar-fermenting capacity per unit of cell substance than the iron-free cultures. Both iron-containing and iron-free cultures, especially during the later periods of growth, had a fermentative capacity far greater than the zinc cultures. This is a highly important fact pertinent to the fundamental physiology of the organism, for the efficiency of its energy utilization thus becomes inversely proportional to its fermentative capacity. In other words, the zinc cultures consumed a small amount of glucose per unit of growth but utilized it very efficiently, whereas the zinc-free cultures consumed a large amount of glucose but very inefficiently.

Throughout the course of these investigations, the maximum conversion yields of fumaric acid were mostly from 40 to 46 per cent, yet, sporadically, yields were obtained as high as 58 per cent. In order to obtain a total increase from 36.5 to 45.1 per cent in the 5 and 7 day iron cultures, the rate of increase during the 2 day interval necessarily must have been considerably higher than 45.1 per cent. This increment can be calculated to be 54.5 per cent. Thus, this strain of *Rhizopus* can convert glucose actually at a much higher rate than that apparent from the data. A comparison of the rates of acid formation is presented in figure 1.

Alcohol is produced in *Rhizopus nigricans* cultures during the early period of growth and decreases in the very old stages with a coincident increase in fumaric acid (Butkewitsch and Federoff, 1930); this may explain the higher conversion rates. In order to demonstrate that a high rate of conversion could be obtained from alcohol, *Rhizopus* pellicles were allowed to act on ethyl alcohol. As much as 70 per cent of the alcohol consumed was found to have become converted to fumaric acid, thus establishing beyond doubt the ability of the organism to form fumaric acid from ethyl alcohol and the probability that the latter is the intermediary product in the formation of this acid.

The negative value for fumaric acid production obtained in the zinc cultures, during the 7 to 10 day period, may possibly be due to actual consumption of the acid by the fungus, as suggested by Butkewitsch and Federoff (1930) or to its transformation into malic acid (Bernhauer and Thole, 1936). To test this, the

TABLE 5

Effect of trace elements upon growth and acid production by Rhizopus at different periods of growth

TREATMENT, MGM. PER LITER	0			10Zn			10Fe			10Fe + 5Mo + 5Mn		
Age of culture, days	5	7	10	5	7	10	5	7	10	5	7	10
Glucose consumed, mgm.	1,368	3,167	4,756	2,375	4,236	4,938	1,394	2,675	4,438	1,308	2,691	4,284
Fumaric acid found, mgm.	463	1,473	1,898	536	719	629	503	1,201	2,054	420	1,258	2,210
Conversion, per cent	33.8	46.5	39.9	22.6	17.0	12.8	36.1	44.9	46.3	32.1	46.8	51.6
Nitrogen consumed, mgm.	18.0	21.5	42.5	28.8	64.4	74.7	17.1	18.9	30.7	13.8	18.6	29.3
Total calcium in solution, mgm.	184.0	506.5	657.0	241.6	397.2	440.0	192.3	408.1	712.9	184.8	421.0	749.6
Calcium due to organic acids, mgm.	158.3	475.7	596.2	200.5	305.1	333.4	167.8	381.1	668.8	165.1	414.4	707.9
Calcium as fumaric acid,* per cent	104.9	107.0	110.0	92.3	81.8	66.3	103.7	108.6	105.4	97.0	105.0	108.0

* The values above 100 per cent are due to incomplete neutralization of the acid by $CaCO_3$; later experiments showed them to be close to 100 per cent.

TABLE 6

*Effect of trace elements during the growth of Rhizopus upon fumaric acid production by pellicles of different ages**

TREATMENT, MGM. PER LITER	0			10Zn			10Fe			10Fe + 5Mo + 5Mn		
Age of culture, days	5	7	10	5	7	10	5	7	10	5	7	10
Glucose consumed, mgm.	3,433	7,057	7,783	7,907	12,877	12,291	3,160	5,724	7,626	2,317	5,032	5,924
Fumaric acid found, mgm.	1,676	2,877	2,732	3,646	4,380†	3,988†	1,285	2,643	3,104	997	2,389	2,613
Conversion, per cent	48.8	40.8	35.1	46.1	34.9	32.4	40.7	46.2	40.7	43.3	47.5	44.1
Total calcium in solution, mgm.	522.6	994.5	973.0	1,301.5	1,587.3	1,411.1	505.5	946.6	1,123.3	376.6	873.4	955.3
Calcium as fumaric acid, per cent	92.5	99.7	97.0	97.0	95.3	97.7	87.0	96.4	96.3	91.7	94.4	94.8

* Incubation of pellicles was 5 days in all cases.

† Abundant crystallization of Ca fumarate occurred on the 5th day.

organism was grown on 1 per cent fumaric acid as the only source of energy. One-half of the solution was neutralized with NH_4OH and the other boiled with $CaCO_3$ and filtered. Each portion was divided into 2 lots, only one receiving $ZnSO_4 \cdot 7H_2O$, at the rate of 10 mgm. per liter. Definite growth was obtained in all cases, but it was slow and scant so that it appears that fumaric acid could serve only as a very poor source of energy; no difference was apparent due to zinc. These results suggest the possibility that the reduced yields of fumaric acid in the zinc cultures are not

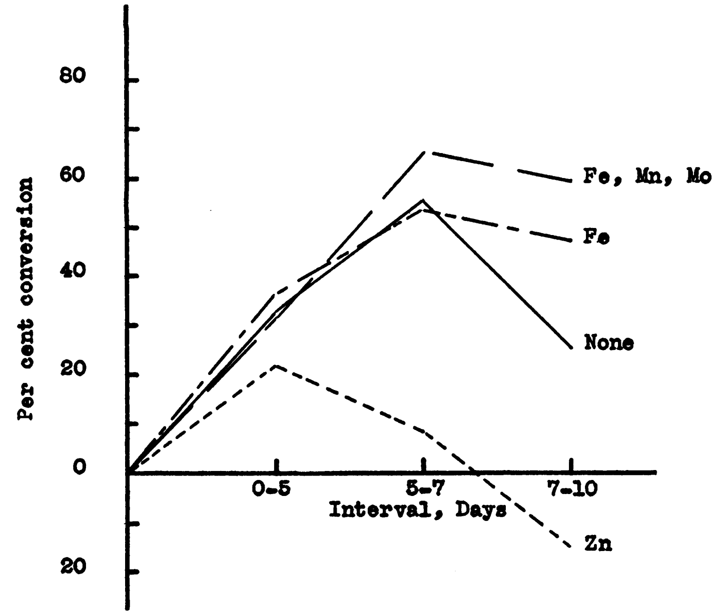

FIG. 1. RATE OF CONVERSION OF GLUCOSE INTO FUMARIC ACID DURING DIFFERENT STAGES OF GROWTH

due to the actual consumption of the preformed fumaric acid but of its precursor and also to its transformation into another acid.

The results on the action of the pellicles from the experiment reported in table 5 are presented in table 6. The zinc pellicles behaved in two respects differently from those of the previous experiment. These pellicles, far heavier than those previously used, consumed glucose at a much higher rate, without, however, any appreciable increase in the quantity of acids other than fumaric. An explanation of these contrasting results is lacking at present, but it may be associated with the size of the pellicle and glucose concentration. Whereas maximum growth of the organism, as measured by glucose consumption and by nitrogen uptake, was obtained in 10 days, the greatest pellicle activity was found in the case of the 7 day old pellicles. There is, therefore, a period of physiological youth for the fungus which occurred between the 5th and 7th days of growth. This is clearly brought out in figure 1.

Antagonistic actions between iron and zinc on *Aspergillus* was indicated by Roberg (1928, 1931) and Bortels (1927); iron re-

TABLE 7

Influence of zinc, iron and glucose concentration upon growth and fumaric-acid formation by Rhizopus

GLUCOSE CONCENTRATION	GLUCOSE CONSUMED			FUMARIC ACID PRODUCED			CONVERSION			NITROGEN CONSUMED		
	Fe	Fe+Zn	Zn	Fe	Fe+Zn	Zn	Fe	Fe+Zn	Zn	Fe	Fe+Zn	Zn
per cent	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*per cent*	*per cent*	*per cent*	*mgm.*	*mgm.*	*mgm.*
2.5	3,414	4,138	4,276	1,567	101	None	45.9	2.5	0.0	24.0	82.9	82.8
5.0	5,309	7,793	8,293	2,035	1,810	1,355	38.3	23.2	16.4	36.8	82.9	83.1
10.0	5,075	12,973	12,969	1,620	2,838	2,326	31.9	21.9	17.9	40.4	81.7	81.2
15.0	5,550	9,850	11,100	834	1,016	893	15.0	10.3	8.0	27.8	74.0	67.5
20.0	7,275	11,850	11,050	501	541	816	6.9	4.6	7.4	26.0	65.3	61.0
30.0	8,100	9,838	9,638	190	None	67	2.3	0.0	0.7		42.0	33.5

duced the toxic effect of high and even optimal (Gollmick 1936) concentrations of zinc on sporulation. Since glucose concentration is important in controlling fumaric acid production, it is evident that these three factors govern the relation between growth and fumaric acid formation by *Rhizopus*. An experiment was therefore set up to elucidate these interrelationships. Three series of flasks received the usual basal medium with the following concentrations of glucose: 2.5, 5, 10, 15, 20 and 30 per cent. One series received iron, the second iron and zinc, the third zinc alone as 10 mgm. per liter of salt. All treatments were carried out in duplicate. Seven day cultures were analyzed (table 7).

By increasing the concentration of glucose, there was an increase in glucose consumption only in the iron-containing cultures; this cannot be directly associated with increments in growth, for maximum growth, as measured by nitrogen assimilation, was reached with 10 per cent glucose and then dropped off. Above that concentration, a large part of the glucose was changed to alcohol, reference being previously made to the high fermenting capacity of the iron cultures. Glucose was repressive to acid formation in direct proportion to its concentration. From a yield of 45.9 per cent with the lowest concentration of glucose, the production of fumaric acid was reduced to a mere trace with the highest concentration. The optimum concentration of glucose for the activities of *Rhizopus*, in the presence of an iron salt, therefore depends upon the physiological unit of measurement.

The 10 per cent sugar cultures with zinc alone gave the maximum response in glucose consumption, in growth, in yield of fumaric acid and in per cent conversion. Above and below this concentration, there was a falling off, and, as found previously, no fumaric acid was formed in the 2.5 per cent glucose cultures. The high concentrations of glucose, here as well, blocked almost completely the formation of fumaric acid.

Attention has been called earlier to the fact that there exists a critical carbohydrate-nitrogen ratio, below which fumaric acid is not formed in Zn cultures. This experiment gives evidence of the existence of two carbohydrate-nitrogen ratios; no acid is formed below one and above the other. The $\frac{\text{carbon}}{\text{nitrogen}}$ ratios are calculated to be 25:1 and 300:1, respectively; only within these limits was fumaric acid produced under the experimental conditions.

The combination of iron and zinc was similar to the zinc series, with respect to the maximum activity at the 10 per cent glucose level. The evidence that the antagonistic effects of these two elements partially neutralize one another is quite definite. For example, the favorable influence of iron on fumaric acid formation was clearly manifested with the 2.5 per cent glucose concentration, for the acid was definitely formed under these conditions and not in the corresponding zinc cultures. Similarly, with the 5, 10, and 15 per cent glucose concentrations, the net result was plainly that of neutralization; the values were increased and reduced as compared with the zinc and iron cultures, respectively.

There is also a distinct associative effect. In the 20 per cent glucose concentration, the presence of both Fe and Zn gave a definite lowering in acid yield and with 30 per cent they completely repressed the formation of the acid while it was still formed in the zinc cultures. The net effect, therefore, of iron and zinc upon fumaric acid production by *Rhizopus* may be either antagonistic or associative, depending upon the sugar concentration; up to 20 per cent glucose, the effect was antagonistic; above this concentration, it was associative.

DISCUSSION

Zinc is indispensable for *Rhizopus* and, on this basis, may be called a "nutrient" according to Steinberg (1934). However, iron and manganese are also essential, but their effect is not comparable to that of zinc under the conditions used. Thus, the term "nutrient" does not serve to indicate the rôle of the particular element in the nutrition of an organism. An element may be vital to the organism in one of two ways—structurally or functionally. Structurally it acts as a building block in the formation of cellular material—it comprises the unit of which cell substance is composed. In this category one usually places carbon, nitrogen, hydrogen, oxygen, sulfur and phosphorus. On the other hand, a substance becomes functional when it is indispensable to some specific process or transformation carried out by the cell substance. Enzymes and catalysts belong here. In some cases, an element may serve in both capacities, as claimed by Buromsky (1936) for potassium and magnesium.

The specific effect that zinc produces on *Rhizopus* is very similar to the functional or catalytic type of nutrient. The greater efficiency of energy utilization by the organism in the presence of this element results in the conversion of a large part of the consumed glucose into cell substance, and only a small portion converted to organic acids. The reduction in the economic coefficient $\left(\frac{\text{wt. sugar consumed}}{\text{wt. fungus growth}}\right)$ in the presence of zinc is similar to that of *Aspergillus niger* (Buromsky, 1913; Butkewitsch and Orlow, 1922; Ono, 1900). With 2.5 per cent glucose in the medium, *Rhizopus nigricans* consumed all the sugar but produced no fumaric acid, which is similar to the results of Butkewitsch and Orlow (1922) with oxalic acid in *A. niger* cultures.

If zinc were a structural element it would cause merely an increase in the *rate* of utilization of the glucose but no change in the *manner* of utilization of the sugar molecule, i.e., acid production per unit of glucose consumed would be the same. This clearly is not the case here. The formation of considerable amounts of other acidic constituents under the influence of zinc is further indication of the striking modification induced by this element. This specific effect of zinc appears to be sufficient justification for characterizing it as a biological catalyzer, according to the conception of Javillier. If it could be replaced by other heavy metals it would lose its characterization as a true physiological catalyst and would be considered simply as a mere excitant of protoplasmic phenomena. These results, however, are not in agreement with the findings of Bernhauer and Thole (1936), who included zinc in the culture medium and obtained increased yields of fumaric acid by *Rhizopus nigricans*.

Zinc appears to be closely associated with the breakdown of the carbohydrate. This view is supported by the increased efficiency of carbon utilization in the presence of zinc; also by the fact that, in some instances, pellicle cultures, in the absence of nitrogen and minerals and with no apparent increment in growth, gave a reduced proportion of fumaric acid to total acids similar to that of the cultures during the growth period. Further indication of this is shown by the fact that the most marked effect of zinc occurred after the fungus reached maturity when presumably nitrogen metabolism would not be very active while carbohydrate breakdown was still pronounced. Butkewitsch (1922) and Wassiljew (1927, 1930) likewise concluded that zinc is concerned with carbohydrate metabolism.

Wassiljew (1935) further demonstrated that the effect of zinc was not the same for biochemically different strains of *Aspergillus niger* and he went as far as to suggest a characterization of *A. niger* strains based on their reactivity to zinc.

The results of the studies of carbohydrate concentration indicate that this is an important feature in mold metabolism, especially when quantitative relations between sugar used and end products obtained are under consideration. This is especially true of those fungi which are susceptible to the influence of the heavy metals, but unfortunately this fact has seldom been considered. Unless special purification methods have been employed, it is not unlikely that the combination of these factors could influence the metabolism of the organism so as to give divergent and irreproducible results.

The accumulated evidence suggests that the requirement by filamentous fungi of traces of heavy metals, such as zinc, iron, manganese, copper and possibly others, is a general phenomenon in their nutrition. For the most part this has been proven by observing weight yields and sporulation of the fungi under highly controlled conditions. However, it must be emphasized that only by resorting to physiological studies can the true function of trace elements in the metabolism of fungi be elucidated.

SUMMARY

Zinc was found to be indispensable for the growth of *Rhizopus nigricans*. The presence of 1.2 p.p.m. of zinc ion in an otherwise complete nutrient medium markedly altered the physiology of the fungus. The organism utilized the available energy 3 times more efficiently when zinc was added to the medium. In the absence of added zinc, *Rhizopus* did not completely oxidize the glucose but converted a large part of it into fumaric acid, and made a relatively slow and poor growth. In the presence of zinc, growth was rapid and abundant, a much smaller part of the energy of the glucose being left in the form of fumaric acid. The function of zinc is interpreted as catalyzing a more complete destruction of the glucose molecule with a consequent greater efficiency of energy utilization by the fungus.

The effect of iron in the metabolism of *Rhizopus* was practically the opposite of that of zinc.

Glucose concentration was found to be an important factor governing the influence of zinc and iron. Maximum response to zinc in glucose consumption, growth, absolute yield of fumaric acid and per cent conversion was obtained with 10 per cent glucose medium. In zinc cultures a critical carbon:nitrogen ratio was found to exist. Below a C:N ratio of 25:1 and above 300:1 no fumaric acid was found. Up to 20 per cent glucose concentration, the net effect of iron and zinc was antagonistic; above this concentration, it was associative. Evidence is presented indicating that zinc is closely associated with carbohydrate dissimilation.

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