CONDITIONS Modifying the EFFECT of DELETERIOUS AGENTS upon the FUNGI.

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For some time it has been known that traces of metals in solution can be removed from such solutions by the introduction of insoluble substances. Nägeli found in experiments with Spirogyra that distilled water,as well as tap water,often proved injurious. By careful experimental study of all the factors which might be involved,he was able to trace the injury to soluble copper,which in the first case came from the distilling apparatus,and in the second,from the brass faucet. By varying the quantity of the solution into which a definite number of Spirogyra filaments were placed,it was determined that the amount of solution was an important factor.

Filaments placed in 1000 c. c. of harmful distilled water died,while the same number placed in 100 c. c. were able to survive. It may be concluded from the above that there is an inverse relation between the deleterious agent and the bulk of the organism acted upon. With a few organisms the toxic agent,even when in great dilution,may manifest itself in time. In this connection,it may be said also,that the surface of the containing

vessel offers a place of attachment for poisonous molecules or ions. Nägeli found by the introduction of insoluble substances such as granite,starch grains,and cotton fibres that the toxicity of the solution could be reduced,and rendered harmless to Spirogyra. He also attempted to determine the effect of the presence of insoluble substances on nutrient solutions,but found that they exerted none. Nägeli experimented with many of the heavy metals,platinum, gold, mercury, silver and copper, though chiefly with copper. The copper used as alloy in a gold crown was sufficiently soluble that its presence could be distinctly detected in water into which such coins had been dropped.

Following Nägeli,little was done with this problem until the summer of 1902 when True and Geis made preliminary experiments to determine the effect of insoluble substances on toxic solutions. In their work,a solution of $CuSO_4$ of sufficient concentration to permit growth for about twenty-four hours was required. Such a solution was experimentally determined,and to it insoluble substances were added. Lupinus albus seedlings were placed into these solutions,where they were left twenty-four hours. The results were so favorable,that in the summer of 1903,C. S.

Oglevee continued the work.

In the more extended scope of the work, $CuSO_4$, $AgNO_3$, $HgCl_2$, NaOH, Thymol, Resorcinol and Phenol were used. A maximum concentration permitting growth was determined, and from this dilutions were made. These solutions were poured into beakers of 300 c. c. capacity, a duplicate being made for each. As in the previous experiment, Lupinus albus seedlings were used as test objects. The cultures stood twenty-four hours, sometimes longer, at the end of which time, the average growth made was taken as the growth for each concentration. In all cases cited from True and Oglevee, the molecular weight of the toxic agent is dissolved in the number of liters of distilled water mentioned. The following data may be taken as typical of their results ;-

35,000 L. +	Mol. wt. $CuSO_4$	4 m. m.
"	" " " + glass	10 m. m.
"	" " " + filter paper	18 m. m.
"	" " " + starch grains	12 m. m.
"	" " " + paraffin	8 m. m.
Distilled H_2O		12 m. m.

The growth of the control for twenty-four hours was 12 m. m. Distilled water plus the toxic agent gave 4 m. m.,

or 8 m. m. less than the growth of the control. The presence of glass gave an increase of 6 m. m. , filter paper, an increase of 50 % above the control. All of these insoluble substances acted as agents of dilution, filter paper being so effective in reducing the toxic action that solutions containing this substance in conjunction with the toxic agent were sufficiently diluted to cause stimulation, or increased growth above that given by the control culture.

Mercuric Chloride and silver nitrate gave the following results:-

2000 L.	+	$HgCl_2$	10
"		" +glass	16.9
Distilled H_2O			12

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20,000 L.	+	$AgNO_3$	13 m. m.
" "		" + sand	16 m. m.
35,000 L.		"	14 m. m.
" "		" +glass	14 m. m.
" "		" + filter paper	19 m. m.
Distilled H_2O			14

In the case of the addition of glass to the $HgCl_2$ solution, an increase of 6.9 m. m. was obtained, an acceleration of 4.9 m. m. above the control, 12 m. m. Silver

nitrate also gave some interesting results as may be seen from the above table.

To show the relative effect of a more concentrated solution,as compared with a highly ionized one,results obtained with Thymol are given;-

2800 L.	+	Thymol	0.5
"		" + sand	7.0
Distilled H_2O			10.

In this special case,the results were marked. In general,however,True and Oglevee found dilute solutions of stronger toxic agents to be more effective than more concentrated solutions of weaker poisons.

One other experiment is deserving of special mention. A solution of $CuSO_4$ m/35,000 was taken,and to equal volumes of the same,different amounts of sand were added. The solution gave a growth of 2 m. m. The solution + 40 grams of sand gave 8 m. m. The addition of 80 grms. gave 10 m. m.; of 120 grms.,18 m. m. ; 160 grms.,14 m. m. ; while 200 grms. gave practically 12 m. m. The addition of 40 and of 80 grms.of sand diluted the solution sufficiently that 8 m. m. and 10 m. m. were the resulting growths as compared with the greatly depressed growth of 2 m. m. 120 grms.still

further reduced the toxic agent so that that remaining in its solution was sufficient to cause acceleration,as is shown by 18 m. m. growth. 160 grms + 200 grms. caused such a great dilution that the accelerating effect was no longer manifested,growth gradually approaching that of the control.

Dandeno has also prosecuted studies along a similar line using inorganic and organic salts. His experiments dealt particularly with Mass action. Seedlings of Lupine,corn and pea were used in amounts of toxic solution varying from 1 c. c. to 25 c. c. Results showed that the smaller the amount of solution used,the greater the concentration which could be resisted, e.g. a seedling may grow several millimeters in 1 c. c. of a solution,and be killed by 2 and 1/2 c. c. of the same solution. By the introduction of an amount of sand nearly equal to the volume of the solution,the toxicity was in some cases reduced thirty-two times.

Since in the work already done along this line, phanerogams have been used,it was thought that something interesting as well as profitable might result from a repetition of the above using fungus forms. Experiments which employ seedlings must of necessity run only a short time. It is also difficult to determine at just what point

death occurs.

In order to insure uniformity,it was not possible to use Erlenmeyer flasks,as on the addition of insoluble solutions,the level was changed and the surface area reduced. Bostom prescription bottles of 125 c. c. capacity were used instead,as the parallel sides gave the same surface whatever the variation in volume of the solution. The solubility of the glass,which must have been slight,entered as a common factor. After each experiment, the bottles were thoroughly cleaned,and sterilized from one half to one hour at from 120°C to 140°C.

Sea sand of two grades,crushed window glass of three grades,absorbing porous plates,and ash free filter paper were used as insoluble substances. The sea sand and filter paper were boiled in several changes of distilled water so that all soluble parts might be removed. The sand was not treated with acid for the reason that it has been shown to be practically impossible to remove all traces even by very careful washing. The glass,however,was treated with acid as it came from the laboratory.

Beet decoction and beef bouillon served as media. In the preparation of beet decoction,450 grms.of

sugar beet root were allowed to each liter of tap water. This was boiled for two hours,filtered, and cleared by heating with white of egg. In bouillon,1 lb. (453+grms.) of lean beef was allowed to each liter of tap water. All fat was trimmed from the beef,after which it was finely ground in a chopper. Late in the afternoon,one liter of water was added for 453 grms.of chopped beef. This was allowed to stand all night in a cool place. The following morning,the liquor was drained off,and cleared by heating under pressure. To each liter,10 grms. of peptone and 5 grms.of salt were added. This decoction was now thoroughly sterilized. NaOH was used in neutralizing.

Toxic agents used were $CuSO_4$ and H_2SO_4 (Merck. 1 - 84). The solutions were made as gram equivalent solutions, n/4 or n/8 being the usual concentration,and dilutions made from these. Twenty-five c. c. of the nutrient solution plus the toxic agent were used in each bottle.

Each culture was set up with a duplicate. In series where Penicillum was used,the cultures stood two weeks ; in the case of Aspergillus,however, one week was a sufficient length of time. Cultures of Aspergillus were kept at 28° C. in order that conditions might be the same. After the solutions were poured out,and the insoluble substance added,the bottles were sterilized at 120° C. and

when cool,inoculated from pure cultures. After standing the required length of time,the growths were filtered off on papers of known weight,washed,and afterwards dried for several hours at from 100° C to 110° C. From the oven,they were placed in a dissicator until weighed. In all cases the average dry weight of two cultures was taken as the growth for the culture.

In many instances,there was quite a difference in the two weights. If it were possible to get approximately the same number of spores into each bottle,it seems reasonable that this difference might be lessened.

Preliminary experiments were made using $CuSO_4$ as the toxic agent,and Penicillum as the test object. Two amounts of sand and three grades of glass were used. Results were not very satisfactory owing to precipitation,and to impurity of the sand used.

In the experiment given,an equal amount of insoluble substance was added to each culture.

I. $Cu\ SO_4$ - Penicillium in Beet decoction two weeks,-

n/256 Solution	Large glass	.1614 grms.
	Medium	.1580
	Small	.1760
	None	.1692
Control in decoction alone		.1190

From this table it is seen that the presence of Cu SO_4 in the nutrient media had a stimulating effect when .1692 grms. is compared with .1190 grms. or the growth in the control. The addition of fine glass acted as an agent of dilution reducing the toxicity of the solution so much that it acted even more effectively as a stimulating agent. Large pieces of glass had little effect, while pieces of medium size for some reason seemed, from the result, to have increased the toxic effect of the solution.

Penicillium grew slowly, and as an usual rule, did not form a continuous stroma over the surface. Because of its more thrifty habits and more rapid growth, Aspergillus niger was used in the subsequent experiments. H_2SO_4 was also substituted for $CuSO_4$ as it gave less precipitate, thus insuring greater accuracy in results.

In one experiment it was thought desirable to test widely differing absorbing surfaces. Porous plates and window glass of the same thickness were obtained, and pieces two inches square cut from each. These were weighed to ascertain the amounts of each it should be necessary to add in order that equal surfaces would be offered.

The following table gives the results obtained:-

II,- Aspergillus niger in Beet decoctions,-

Insoluble subst.	H_2SO_4					Control
	n/8	n/16	n/32	n/64	n/128	
Pottery(22.25grms)	.0444	.0785	.0956	.1081	.1008	
Glass(12.47 grms)	.0080	.1142	.1295	.1707	.1465	
None	.0014	.1439	.1371	.1523	.1493	
None	—	—	—	—	—	.1588

The growths obtained in solutions containing pottery,when considered by themselves show a gradual increase which reaches a maximum at n/64 . With a slight irregularity in the decoction+the toxic agent,the same thing is true of the other two. It is at n/8 with pottery and glass,and at n/64 with glass that growth exceeded that produced in solutions in which no insoluble substances were present. These then,in this experiment,mark the places at which insoluble substances were most effective. Pottery showed an injurious effect,as will be more fully treated later,yet even under such unfavorable conditions,growth showed a gradual increase and a decline wholly comparable with that in decoctions to which H_2SO_4 had been added.

III,- Aspergillus niger in Beet decoction,-

Insoluble subst.	H_2SO_4					Control
	n/8	n/16	n/32	n/64	n/128	
Pottery(22.25 grms)	.0800	.1139	.1307	.1439	.1548	
Glass(12.47 grms)	.0665	.1732	.1939	.1802	.1836	
None	.1028	.1506	.1539	.1859	.1932	
None	—	—	—	—	—	.1558

Results obtained in the repetition of the previous experiment are more satisfactory, though they do not agree in all particulars. Here again, growth in the cultures containing pottery did not in any instance equal that in decoction $+H_2SO_4$. There was a gradual increase from n/8 to n/128. Cultures containing glass showed greatest growth at n/32. n/128 in decoction H_2SO_4 gave .1932 grms. dry weight, n/32 in the solution to which glass was added gave .1939 grms. To produce the same growth, the solutions must have been of practically the same concentration, a condition, in this case, produced by the glass. Concentrations of n/64 and n/128 gave, with slight irregularity, the characteristic decline which approached growth in the control.

These results may also serve as an illustration of the general effects of toxic agents. When the toxic solution is too concentrated, suppression of growth results. As the concentration is reduced, growth gradually approaches that of the control. Further dilution weakens the solution to such an extent that the toxic agent becomes an agent of growth acceleration. As dilution is continued, the toxic agent becomes so much weakened that it loses its effect and growth approaches that of the control. The above experiment shows that reduction of the toxic agent may be brought

about in two ways.-either by dilution with the medium,or by the introduction of insoluble substances.

To determine the cause of the results obtained by using pottery as an agent of dilution,a third series was set up. In two sets of bottles,the usual weight of pottery was placed,and the solutions poured over it. In set two of the above,however,the solutions were transferred to other bottles containing no pottery. This was to determine whether or not there were soluble substances in the pottery. The results were as follows:-

IV,- - Aspergillus Niger in Bouillon,-

Insoluble subst.	H_2SO_4		
	n/8	n/16	n/32
Pottery(12.47grms)	.0072	.0594	.0433
Poured from Pottery	.0071	.0650	.0467
None	.0660	.0791	.0715

Growth in the presence of pottery,and in the solutions poured from pottery were practically the same, hence it was just to conclude that the solutions were the same. The same depressed growth again resulted,so it may be concluded without a doubt that this depression was due to some injurious soluble substance which came from the pottery.

In the next experiment,the absorptive power of fine and of coarse sand was compared with that of fine and of

coarse glass.

V. - Aspergilus Niger in Beet decoction,-

Insol.Subst.	H_2SO_4.					Control.
(10 grms) .	n/4	n/8	n/16	n/32	n/64	n/128	
Fine sand	.1507	.2144	.2133	.2204	.1900	.2073	
Coarse sand	None	.2120	.2031	.2143	.1985	.1692	
Fine glass	"	.1374	.1466	.1602	.1507	.1582	
Coarse glass	"	.1328	.1427	.1394	.1553	.1601	
None	"	.1337	.1533	.1530	.1443	.1583	
None	—	—	—	—	—	—	.1569

VI:- Aspergillus niger in Beet Decoction,-

Insol.Subst.	H_2SO_4.				Control.
(10 grms)	n/4	n/8	n/16	n/32	
Fine sand	.1430	.1871	.2032	.2141	
Coarse sand	.0434	.1768	.2137	.2344	
Fine glass	None	.0520	.1575	.1613	
Coarse glass	"	.0437	.1644	.1663	
None	"	.0069	.1199	.1800	
None	—	—	—	—	.1569

In series V. both fine and coarse sand showed themselves to be good absorptive surfaces, the fine sand being the more effective. This property may well be attrib-

uted to the greater surface area offered. The presence of the fine sand in the n/4 solution gave a growth nearly the same as that of the control. The maximum point of accelerated growth was reached at n/32 , and was followed by a marked decline in n/64 and n/128. With fine glass also,maximum growth was at n/32, while cultures containing coarse glass seemed to reach their maximum at n/128 . The effect of fine and of coarse sand,in series VI. was especially marked in the n/4 solution. As a whole,this series was not as uniform as series V. It showed the common tendency of a gradual increase in growth which,judging from the previous series would have reached a maximum near the n/32 concentration.

VII.-Aspergillus Niger in Bouillon,-

Insol.Subst.	H_2SO_4.					Control.
	n/4	n/8	n/16	n/32	n/64	n/128	
20 grms.sand	.1541	.1733	.1649	.0998	.0998	.0820	
10 " "	.1505	.1797	.1325	.1061	.0906	.0694	
5 " "	.1048	.1756	.1014	.0963	.1131	.0704	
None	None	.1049	.1006	.0965	.0769	.0685	
None	—	—	—	—	—	—	.0794

VIII.- Aspergillus niger in Beet Decoction,-

Insol. Subst.	H_2SO_4.				Control.
	n/4	n/8	n/16	n/32	
20 grms. sand	.2303	.2064	.2497	.2646	
10 " "	.1094	.2327	.2288	.2782	
5 " "	None	.1420	.2328	.2397	
None	"	.0324	.0811	.2207	
None	—	—	—	—	.1853

A comparison of the results of series made in beet decoction and in bouillon showed that growth in the former was always greater than in the latter. There was always a shifting of the maximum growth towards the stronger solutions when bouillon was used. This was very evident in the two preceeding tables, bouillon giving a maximum growth at n/8 , beet decoction at n/32. The presence of twenty grams and of ten grams of sand in n/4 of series VII. gave a growth nearly twice that obtained in the control. In series VIII. growth in the n/4 solution to which twenty grams of sand were added, was greater than that in the decoction + H_2SO_4 at n/32.

IX,- Aspergillus Niger in Bouillon.-

Insol.Subst.	H_2SO_4						Control.
	n/4	n/8	n/16	n/32	n/64	n/128	
2 grms.filter paper	.0084	.0976	.0841	.0876	.0665	.0652	
1 grm.filter paper	.0179	.1018	.0885	.0696	.0716	.0627	
2 grms sand	.0364	.1538	.1267	.0821	.0764	x	
1 grm.sand	.0124	.1189	.1142	.0844	.0706	x	
None		.0946	.1012	.0721	.0679	.0691	
None							.0656

x = Contaminated.

X.- Aspergillus Niger in Beet Decoction,-

Insol.Subst.	H_2SO_4.				Control.
	n/4	n/8	n/16	n/32	
2 grms filter paper	None	.0239	.2048	.2030	
1 grm filter paper	"	.0654	.1999	.2213	
2grms.sand	"	.1166	.2380	.2692	
1 grm sand	"	.1605	.1734	.2468	
None	"	.0506	.1872	.2316	
None					.2308

The results of series IX. were interesting

for several reasons. Maximum growth was obtained at n/8 where insoluble substances were present, and at n/16 in their absence. In the higher concentrations, one gram of filter paper proved more effective than two grams. The converse was true with regard to the sand. A repetition of series IX, the results of which are recorded under table X. strengthen statements made with regard to series IX. In two cases out of three, one gram of filter paper was more effective than two grams. In two cases out of three, again two grams of sand proved an effective dilution agent. Weight for weight comparisons of the two was not possible owing to peculiar properties exhibited by the two decoctions.

In conclusion, it may be said that insoluble substances act as agents of dilution or of absorption removing in some way toxic molecules or ions. This effect is most noticeable in solutions of high concentration, usually n/8, and in solutions giving maximum growth, — n/32 or n/64.

A toxic solution gives greatest growth at n/128. On the addition of a sufficient amount of an effective insoluble substance, greatest growth is obtained at n/32 or n/64. Such results lead to the conclusion that the immediate effect of the presence of insoluble substances is to move

the region of growth from one of lower to one of higher concentration.

Of the insoluble substances used, sand proved the most effective diluting agent ; glass was somewhat effective when of medium fineness, but large pieces showed little or no effect. Powdered glass is undesirable since in such a finely divided state it is known to be soluble to a considerable extent. Although filter paper was effective in some instances, its peculiar effect will be a problem for further investigation. Pottery has been previously ruled out as harmful.

Beet decoction is the better medium. Bouillon is not so favorable on account of the facts which are to be found in some of the tables given.

The results of these experiments confirm conclusions drawn by Nägeli, Dandeno and True and Oglevee, namely, that the presence of insoluble substances in toxic solutions reduces the toxicity of such solutions. True and Oglevee found filter paper to be more effective than sand. Present evidence is to the contrary. Fungus forms as test objects are undoubtedly more desirable than the seedlings used by the above experimenters, as the growth in each culture is an accurate and delicate response to the conditions

under which it was produced.

It is a fact worth noting that two such nutrient media as beet decoction and beef bouillon have such different effects upon the same fungus form. When the toxic agent is added, it also is found to be affected differently in the two media as is indicated by reference to the tables. In future work, just such variations may be expected unless the same medium is used throughout.

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This thesis is never to leave this room.
Neither is it to be checked out overnight.