The Germicidal Action of Metals and its Relation to the Production of Peroxide of Hydrogen.

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That sundry metals possess not merely a distinct inhibitory action upon the growth of moulds, bacteria, and other micro-organisms, but even possess germicidal properties has been known for a long period, and there have been numerous observations demonstrating this action. The earliest observation known to us is that by Raulin in the seventies, upon the remarkable inhibition produced by traces of silver upon the growth of the *Aspergillus niger*. More recent work has in general followed the lines of the experiment employed by Miller, in his observations upon the bactericidal properties of gold and various alloys employed in dentistry; namely, plate cultures have been made of various micro-organisms, and into the inoculated culture media, while still fluid, small pieces or plates of the metals have been dropped, the presence or absence of a clear surrounding zone indicating arrest of growth of inoculated bacteria. As a result of these observations, it has been shown that certain metals possess inhibitory powers. More than this, according to Behring, reinoculation of the clear zone after the removal of the metal may lead to continued negative results. This would indicate that not merely is there inhibition, but an alteration of the gelatin, and suggests strongly the presence there of substances having a distinct germicidal effect. A full study of this nature was made by Bolton. Other observations (Ficker, Von der Dois, Clarke and Gage) have been along the lines of either employing metal vessels to hold the inoculated fluids, or placing strips of the metal in glass vessels holding water containing known cultures of bacteria. In these experiments also, metals like copper, zinc, and silver have been noted to possess distinct bactericidal effects. But in the above observations there has been no adequate attempt to determine the means whereby the metals exert their bactericidal action. Leedham-Green, however, made the observation that iron was inert or active according as to whether the conditions of the experiment prevented or permitted free oxidation of the metal. It was through oxidation that inhibitory action was brought about. The general opinion has been that there is a diffusion of the metal into the water or

other medium, and that in some direct way the metal arrests the growth or actually brings about the death of the micro-organisms.

Certain observations made by Prof. Barnes, and a recent paper by Barnes and Shearer, have opened up the possibility of gaining a more direct knowledge of the mechanism by which these metals act, and the present series of observations owes its origin to Prof. Barnes. Barnes and Shearer working with aluminium showed experimentally that aluminium foil in water containing dissolved oxygen generated peroxide of hydrogen according to the following hypothetical equation:—

$$2Al + 6H_2O + 6O = Al_2(OH)_6 + 3H_2O_2.$$

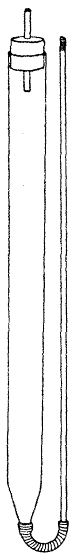
Concerning the production of the peroxide they write as follows:—"We placed pure aluminium sheet in ordinary pure distilled water open to the air and thoroughly charged, and after standing for a few hours we applied the well-known potassium iodide test for peroxide with marked success. Having obtained evidence of the production of the peroxide, we found that the yield could be increased by using considerable quantities of aluminium foil in small pieces, and leaving it for two or three days in water, through which air bubbled." They further showed that the production of peroxide of hydrogen depended upon the presence of dissolved oxygen, and subsequently that zinc acted in a manner similar to aluminium, and that with copper there was no production of peroxide of hydrogen.

The germicidal action of hydrogen peroxide is well known. It became, however, a matter of interest to observe in the first place in connection with those metals which in water gave origin to hydrogen peroxide, how far their bactericidal properties bore relationship to the presence of the peroxide, and, in connection with the metals causing no development of peroxide, whether any cause could be determined for their activity. The problems to be solved demanded the development of a series of experiments; in the first place, upon the maximal production of hydrogen peroxide through the agency of the pure metals, and later the effects of the metal in water containing dissolved oxygen, and lastly in oxygen-free water.

After some preliminary investigation during which many experiments were performed, the technique to be described was adopted, and, although some of the results here made use of were obtained prior to the improved method of investigation generally adopted, subsequent experiments showed them to be sufficiently accurate as compared with the newer method, the results varying slightly in degree, but not in kind. The metals used in the experiments were pure and were supplied by Prof. Barnes. The water used was the Montreal tap water, which is a soft alkaline water derived from the Ottawa River, containing variable, but in general small numbers of natural micro-organisms.

The medium used was plain nutrient agar, and the plates were incubated at 37° C. In the later experiments a medium containing iron, esculin and taurocholate of sodium, such as has recently been described by Prof. Harrison, was used. *B. coli* were added to the water drawn from the tap, so that the number of organisms very greatly exceeded that found usually in contaminated water.

A tube 40 cm. long by 3·5 cm. in diameter was drawn out to a nipple at one end. Over this was fitted a piece of rubber tubing with a section of glass tubing fitted on to the rubber at its distal end (see figure). The rubber was of such length that it was possible to attach the smaller tube to the side of the large one. The small tube extended above the level of the large one, and was, when sterile, plugged with cotton wool. The whole was then relatively sterilised by passing water at 70° C. through it for one hour. The metal having been previously cleaned and thoroughly washed in running water was dropped into the tube by the unsterile hand; 155 c.c. of the inoculated tap water was then added, and the apparatus attached to a Sprengel pump, and air bubbled through the water for a period of one hour. At the end of that time the rubber at the base of the large tube was pinched with the fingers, the attachment to the Sprengel pump disconnected, and a quantity of water removed from the larger tube by means of a sterile pipette, plated on agar, 1/20 c.c. in each case, and incubated at 37° C. for 24 hours, when the plates were examined and compared with plates to which a similar quantity of the untreated inoculated water had been previously added. In the tables these are referred to as Plate 2 and Plate 1 respectively. For determining the amount of peroxide of hydrogen present Bach's test was employed, as well as the potassium iodide test.



Control Experiment.—Using the above technique, inoculated water, containing *B. coli*, was bubbled for a period of one hour without the addition of any metal. There was slight, if any, reduction in the number of organisms in the water. The simple bubbling of air for one hour through water containing *B. coli*, then, does not cause any noticeable reduction in the number of organisms present in the water.

Effects of Metal without "Bubbling."—The following experiments were performed to see what effects metallic aluminium, zinc, and copper had upon bacteria when placed in contact with water at rest containing *B. coli* in the apparatus described. The results are shown below. It will be observed that zinc exerts some effect upon *B. coli* in one hour. It is noteworthy that aluminium and copper exerted none :—

Metal.	Water.	Time.	1st plate, 1/20 c.c.	2nd plate, 1/20 c.c.	Hydrogen peroxide.
	c.c.	hour.			
Aluminium ... 50 grammes	150	1	Innumerable*	Innumerable	No reaction.
Zinc .... 50 „	150	1	„	Slight reduction	„
Copper .... 50 „	150	1	„	Innumerable	„

* At least 7000 to 8000 colonies per 0·05 c.c.

The water used in these experiments was obtained from the tap and held a fair amount of oxygen in solution. It is seen from this experiment that there is a bactericidal action (in the case of zinc) without demonstrable production of peroxide of hydrogen. In studying this it seemed possible that the oxygen in solution in the water played, nevertheless, some part in the destruction of the organisms, presumably by interaction with the metal.

Effects of Oxygen-free Water.—To investigate this the following experiments were performed. Tap water was again used, but previous to placing it in contact with the metals it was boiled for half an hour and then cooled :—

Metal.	Water.	Time.	1st plate, 1/20 c.c.	2nd plate, 1/20 c.c.	Hydrogen peroxide.
	c.c.	hour.			
Aluminium ... 50 grammes	180	1	Innumerable	Innumerable	None.
Zinc .... 50 „	180	1	„	„	„
Copper .... 50 „	180	1	„	„	„

Here we see that there is practically no reduction with any of the metals.

Effects of Bubbling of Air on the Solubility of the Metal.—In the next experiment water was added to the three metals and air was drawn through the apparatus for a period of one hour, and subsequently the water was tested quantitatively for metal in solution.

Metal.	Water.	Metal in solution.
	c.c.	
Aluminium......... 50 grammes	150	Trace—hydrate thrown down.
Zinc.................. 50 „	150	„ „ „
Copper .... 50 „	150	Less than 0·0015 part per 100,000.

It can hardly be possible that this small quantity of metal in solution can be responsible for any germicidal action exhibited in the experiments to

follow. The work of Clarke and Gage would also point to the fact that it was not so. To investigate the point, however, the following experiment was performed:—

Aluminium, zinc, and copper were bubbled for a period of one hour in tap water, and the water was then withdrawn into a sterile bottle and allowed to stand overnight. These three portions of water were then inoculated with *B. coli;* plates were made immediately, and again in one hour. The results are given below:—

Metal.	Water.	Time.	1st plate, 1/20 c.c.	2nd plate, 1/20 c.c.
	c.c.	hour.		
Aluminium... 50 grammes ...	150	1	Innumerable	Innumerable.
Zinc............ 50 " ...	150	1	"	"
Copper ...... 50 " ...	150	1	"	Slight reduction.

These results conform to the previous experiments, and were to be expected. Others have shown that small quantities of copper may at first inhibit the growth of *B. coli,* but that subsequently the results are very similar to those obtained when *B. coli* are added to sterile water in glass vessels and allowed to stand. The results with aluminium and zinc show that the amounts of these metals in solution exert no bactericidal action; that the small quantity of peroxide of hydrogen still present exerts no effective action.

Production of Peroxide of Hydrogen.—Quantitative tests of the amount of *available oxygen* in the water after air had been bubbled through for one hour gave the following figures:—

With zinc—
(1).............. 0·318 per cent. by volume, equivalent to 0·00096 per cent. hydrogen peroxide by weight.
(2).............. 0·299 per cent. by volume, equivalent to 0·00090 per cent. hydrogen peroxide by weight.

With aluminium—
(1).............. 0·252 per cent. by volume, equivalent to 0·00076 per cent. hydrogen pexoxide by weight.
(2).............. 0·195 per cent. by volume, equivalent to 0·00059 per cent. hydrogen peroxide by weight.

With copper......... None.

I do not need to emphasise the point that these amounts of peroxide per cent. can have little germicidal effect. Obviously the results to be noted below

are not due to the mere presence of peroxide, but are due to some action with which is associated the production of the peroxide.

Germicidal Effect of Aluminium.—Aluminium strip cut into small squares and cleaned was then experimented with. The metal, in various proportions by weight, was bubbled with water containing *B. coli.* The results are shown below :—

Quantity of metal.	Water.	Time.	1st plate, 1/20 c.c.	2nd plate, 1/20 c.c.	Peroxide.
	c.c.	hour.			
Aluminium, 125 grammes	150	1	Innumerable	1 colony	Present.
" 100 "	150	1	"	5 colonies	"
" 75 "	150	1	"	1 in 48 hrs.	"
" 50 "	150	1	"	1	"
" 25 "	150	1	"	Many	Slight trace.
" 25 "	150	1	"	"	"

The first two experiments in the series were performed prior to a change in technique, and it was subsequently shown that 50 grammes of aluminium caused, depending upon the cleanliness of the metal, a good production of peroxide of hydrogen, and would generally give a plate free from colonies. A fair amount of peroxide of hydrogen is formed and a hydrate is thrown down. The hydrate has no opportunity of sedimenting, the bubbles of air keeping it constantly in motion and thoroughly mixed with the water. In taking the test water, therefore, the hydrate is thoroughly mixed with the water, thus excluding the possibility of live organisms being entangled in the precipitate and carried down with it, and thus not appearing on the plates. Quantitative tests for aluminium in solution showed only traces of the metal present.

Germicidal Effects of Zinc.—A series of experiments similar to those performed with aluminium were carried out with granulated zinc. The

Metal.	Water.	Time.	1st plate, 1/20 c.c.	2nd plate, 1/20 c.c.	Peroxide.
	c.c.	hour.		hours.	
Zinc...... 125 grammes	150	1	Innumerable	24 none 48 a few	Strong.
" ...... 100 "	150	1	"	24 none 48 one	"
" ...... 75 "	150	1	"	24 none 48 three	Fair.
" ...... 50 "	150	1	"	24 none 48 two	"
" ...... 25 "	150	1	"	24 six 48 twenty-four	Slight.
" ...... 10 "	150	1	"	24 thirty	"

peroxide reaction was stronger than with aluminium, and the metal exhibited a more pronounced germicidal action.

With 50 grammes of zinc the plate was usually free from colonies, although occasionally a few colonies would develop. It was noticeable again that if the surface of the metal was not clean the yield of peroxide was slight and the reduction of the organisms in the water not so marked.

Germicidal Effects of Copper.—Pure sheet copper was then substituted for zinc. The following experiment sets forth the germicidal action of copper in this method of experimentation. It will be seen that copper exhibits quite a marked bactericidal effect, and that *without the production of peroxide of hydrogen.* Small quantities of copper could be detected in the water in about 0·0015 part per 100,000.

Metal.	Water.	Time.	Plate before, 1/20 c.c.	Plate after, 1/20 c.c.	Peroxide of hydrogen.
	c.c.	hour.			
Copper...... 125 grammes	150	1	Innumerable	None	None.
,, ... 125 ,,	150	1	,,	,,	,,
,, ... 125 ,,	150	1	,,	,,	,,
,, ... 100 ,,	150	1	,,	,,	,,
,, ... 75 ,,	150	1	,,	,,	,,
,, ... 50 ,,	150	1	,,	30	,,
,, ... 50 ,,	150	1	,,	33	,,
,, ... 25 ,,	150	1	,,	Numerous	,,
,, ... 10 ,,	150	1	,,	,,	,,

By reference to this table, it will be seen that 50 grammes of copper is not as strong a germicide as a similar quantity of zinc. Nevertheless, under the conditions of the experiment, it exhibits a strong germicidal action. This, as already noted, is: (1) in the absence of production of peroxide of hydrogen, and (2) under the conditions in which the amount of copper passing into solution is inadequate to explain the effects. We further have determined (3) that copper in tap water, with a free supply of oxygen, exhibits a stronger germicidal action than does ordinary tap or boiled water, and also (4) that when water is treated in the same manner, and for the same length of time, without copper being present, and is inoculated with *B. coli* in similar proportions, there is little, if any, reduction of the number of *B. coli* in the water.

Little attempt has been made in the past to associate the bactericidal influence of metals with oxidation or other effects produced by those metals, We learn that Novy and Hendry have carried out certain studies upon the production of "organic peroxides" by metals, and the effect of the same upon bacteria, but although some of their results were communicated verbally

to a meeting of the Association of American Bacteriologists there has been no publication, nor am I acquainted with their findings. The only observation known to me is that by Leedham-Green already noted, upon the negative effects of unoxidised iron and the arrest of growth of bacteria when the iron is allowed to undergo oxidation. The general conception has been that the metal undergoes solution, and in this condition is directly active upon the bacteria.

As bearing upon the mode of action of metals, the results of the present investigations may be briefly summed up.

(i) Air drawn for one hour through water containing abundant colon bacilli exercises no perceptible inhibitory action upon their subsequent growth.

(ii) Relatively large amounts of pure zinc with large surface area placed in water, contaminated with abundant colon bacilli, and allowed to act for one hour, bring about a recognisable but not extreme destruction of the bacteria. Aluminium and copper, under similar circumstances, have no perceptible effect.

(iii) When the same experiment is repeated, with the one difference that the oxygen has been driven out of the water by previous boiling, none of these metals has any decided influence upon the subsequent growth of the bacteria.

It is thus obvious that such bactericidal activity of zinc as manifests itself is associated with the coincident presence of oxygen.

(iv) A much more intense bactericidal action is produced when air is permitted to bubble for one hour through water holding the colon bacilli in suspension, in the presence of aluminium, zinc and copper. Using a sufficiency of the pure metal it is possible to render the water completely sterile with all three metals, and that, when it contains abundant bacteria.

(v) In the case of zinc and aluminium, the sterilisation process is accompanied by the production of easily recognisable amounts of peroxide of hydrogen, and formation of hydrates of the two metals.

(vi) While this is the case, the peroxide itself cannot be regarded as the sterilising agent, and this because :—

(*a*) The amount of peroxide developed in one hour by bubbling air through the like quantity of sterile water in the presence of zinc or aluminium, while easily recognisable, is nevertheless very small in amount; a similar dilution of the peroxide added to a suspension of colon bacilli has no perceptible effect.

(*b*) Bactericidal action of the same or greater intensity is exerted by pure copper under like conditions of experiment, and *this with no associated recognisable production of hydrogen peroxide.*

(vii) It is thus evident (*a*) that oxygen must be present in order that these three metals, zinc, aluminium, and copper, may manifest bactericidal properties (in Montreal tap water), and (*b*) that in the process of interaction between the oxygen and the first two of these metals hydrogen peroxide becomes developed, whereas it is not detected in the case of the third.

Are we justified in drawing any conclusions as to the essential nature of the process of bacterial destruction that occurs in the presence of these—and possibly of other—metals? I am inclined to think that our general conception of the mode of production, and, it may be added, of the mode of disinfectant and sterilising action of hydrogen peroxide supplies the clue. We may lay down that the molecule of oxygen coming into contact with any of these three metals becomes dissociated with the liberation of free ions, of which, in the case of zinc and aluminium, some combine with the molecule of water to form the peroxide, and that it is these free ions, or in more old-fashioned language, "nascent oxygen," that is the essential agent in the bactericidal process. It is in this way that we explain the bactericidal activity of peroxide of hydrogen (for although I have laid down that in these particular experiments the amount of hydrogen peroxide developed was inadequate to explain the marked destruction of the bacteria, it must be remembered that this compound has notable disinfectant powers). We believe that the molecule of the peroxide coming into contact with the bacteria becomes dissociated, and that the liberated ion of oxygen is the destructive agent. It is quite possible that the hydrogen peroxide formed is thus a subsidiary agent in the bactericidal process where zinc and aluminium are present. It seems, however, simpler to presume that the dissociation of the oxygen molecule in the presence of water is the feature common to all three metals, and that in the case of the zinc and aluminium experiments the free ions of oxygen act upon the bacteria prior to, or, more accurately, in preference to, becoming associated with the molecules of water to form the peroxide, a certain excess, however, undergoing the latter combination. With regard to copper it must be postulated that the greater affinity of the free ions to the copper molecules than to those of water leads to non-formation of the peroxide or to rapid dissociation of such molecules as may become formed.

Why copper and zinc have diverse actions in the matter of the production of hydrogen peroxide I do not as a bacteriologist pretend to explain; I can but call attention to the facts.

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NOTE.—Subsequent to the above experiments, further analyses have been made, using boiled *distilled* water in the place of tap water. It was found that zinc in contact with distilled water, without the bubbling process, has a decided germicidal action. Copper has a variable effect, never as strong as that exhibited in the foregoing experiments. Aluminium usually corresponds in effect to that exhibited by plain boiled distilled water, which causes, in an hour, slight reduction in the number of organisms.

Away from the metal—that is, in water which has been in contact with the metal for one hour and then placed in a clean vessel—zinc water (distilled) still causes a reduction in the number of organisms. It will be seen that this is in line with the observations of Behring noted in our introductory paragraph. It is difficult to ascribe this to anything but the direct effect of the metal in solution, and yet, only the minutest traces of the metal are present, quantities which in tap water could hardly be responsible for the effects produced, and indeed would not produce them.

As bearing upon these somewhat divergent results it may be recalled, as a matter of familiar knowledge, that the action of distilled and of acid waters upon copper and other metals differs from that of the (usually) alkaline tap waters.

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