A BACTERIOLOGICAL INVESTIGATION OF ARTIFICIAL MINERAL WATERS.

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THE practical aim of the following investigation was to determine how far the use of artificial aërated waters was a safeguard against the transmission of water-carried diseases. Under long continued hygienic teaching, the public has become fully alive to the dangers of contaminated water, and there is no doubt that a very large proportion of the mineral water drunk is consumed because, in addition to its pleasantness, or the supposed therapeutic value of the contained alkaline salts, it is thought to offer practical immunity from the danger of specific infection. That this is the case, and that manufacturers recognise it, is obvious from the advertisements of the various makers in which they insist either on the purity of the source of supply, or on the precautions taken during manufacture to secure the biological purity of the aërated water.

The danger arising from metallic contamination, especially lead, owing to the increased solvent action of water saturated with carbon dioxide on the pipes, pressure cylinders, syphon heads, etc., has been recognised for some years. This source of danger has now, however, been practically eliminated in the better class of mineral waters by the use of block tin pipes, thickly tinned cylinders, and syphon heads consisting of almost pure tin, or protected by porcelain linings or electro-plating.

The dangers arising from the possibility of contamination with the micro-organisms of specific diseases have more recently attracted attention, and it is to the diminution of risks from this source that the manufacturers have lately directed their efforts. It is with this side of the question, the biological purity of the water, that this inquiry is concerned, and it was undertaken to discover how far the opinion as to the greater safety of aërated waters was justified, and how far the methods of manufacture intended to increase the organic purity of the waters were efficient.

The points investigated were—

1. The source of the water and the methods employed in its purification.
2. The number and nature of the micro-organisms present in mineral waters derived from various sources.
3. The action of CO_2 on the microbes present in artificial waters.
4. The action of CO_2 under varying pressures on certain pathogenic organisms.

The mineral waters examined were either simple aerated or alkaline waters, obtained, by the courtesy of various leading London makers, direct from the factories, and were selected to show what was the influence of variation in source of supply, or subsequent treatment on the bacteriology of the finished waters.

I. THE SOURCES AND METHODS OF PURIFICATION.

It was found that the waters employed in London might be grouped into four chief varieties, according to the original sources of the water or the methods of purification employed before aëration. These groups are—

- A. River or deep-well water as supplied by one of the London water companies. The water was used direct from the mains or after charcoal filtration and subjection to a softening process.
- B. Artesian-well water from private wells situated in different parts of the London district. This water was generally filtered through either animal or block charcoal.
- C. Distilled water. This was water derived from one of the water companies, distilled under pressure, the pressure varying from 20 to 35 lbs. per square inch. Occasionally the water was distilled at 100°C . The distilled water after condensation, which was effected in tin-lined condensers, was filtered through either animal or wood charcoal, in order to remove the peculiar mawkish flavour of distilled water. The charcoal apparently acts by oxidation of a volatile organic compound which, as well as the want of aëration, is the cause of the distinctive taste.
- D. Boiled water. The water supplied by a water company after filtration through spongy iron filters was treated with sodium carbonate (a softening process) and potassium permanganate, and then boiled by steam coils, in covered tanks. The openings to the tanks were protected by cotton-wool plugs in inverted U-tubes. The water was then syphoned off and filtered through pounded glass into another covered and protected tank, from which it passed to the aerating machines.

The waters employed, then, are very varied, and include river, deep-well, artesian-well, distilled and boiled water; and as these varieties are known to be biologically very different, it might be expected that the

finished aerated waters prepared from these varied sources would present strongly marked differences.

II. BACTERIOLOGICAL EXAMINATION OF ARTIFICIAL AERATED WATERS.

The waters examined were simple aerated, soda, potash, or lithia waters. They were contained in syphons or corked bottles. The examination in the case of the syphons was conducted as follows: The syphon nozzle was rinsed by discharging some of the water, then

TABLE A.

Examination of Aerated Waters within 24 Hours of Aëration.

Source of Water.	Hours after Aëration before Examination.	Description of Water.	Bottle or Syphon.	Day of Numeration.	Organisms per c.c.			Ammonia per 100,000 pts.	
					Bacteria.	Moulds.	Total.	Free.	Albuminoid.
1. Water, Class B; ¹ block carbon filter.	5	Simple aerated.	Syphon.	3rd.	477	3	480	·001	·006
2. Do.	3½	Do.	Do.	3rd.	431	2	433	...	...
3. Do.	4½	Do.	Do.	6th.	2874	45	2919	·006	·01383
4. Water, Class B; animal charcoal filtration.	18	Soda-water.	Syphon.	8th.	1048	...	1048	Trace.	·00184
5. Do.	18	Do.	Do.	3rd.	594	51	645	...	..
6. Water, Class D.	5	Simple aerated.	Syphon.	5th.	649	...	649	...	...
7. Do.	18 (ice)	Do.	Corked bottle.	8th.	1170	44	1214	...	...
8. Water, Class C; animal charcoal filtration.	18	Simple aerated.	Corked bottle.	6th	1007	82	1089	...	...
9. Do.	Do.	Do.	Do.	6th	350	4	354	...	...
10. Do.	Do.	Soda-water.	Do.	4th	1066	469	1535	...	...
11. Do.	Do.	Do.	Do.	4th	353	226	579	...	...

¹ The classes given are those into which the varieties of water used are grouped in the text.

swabbed out with alcohol, then rinsed once more with the water, the outside heated by passing through the flame, and a portion of the water then drawn into a sterile plugged test tube. From this tube the water was withdrawn in a sterile bulb pipette.

In the case of the corked bottles, after carefully washing the neck with mercury perchloride, and subsequently with alcohol, the cork was loosened, the gas allowed in great part to escape, and then the cork rapidly removed and replaced by a sterile wool plug. Through this

plug the water was withdrawn into sterile bulb pipettes. With water so withdrawn nutrient gelatine was inoculated in the usual way, and spread out in Petri's capsules. In nearly all cases the amount of water employed was estimated by weighing, but in a few cases graduated pipettes were used, the latter only where the amount taken was comparatively large.

The use of pipettes to measure the water was found not to be sufficiently accurate where small quantities of water were employed, owing to the formation of gas bubbles in the pipette. Even when the waters had

TABLE B.

Examination of Aërated Waters kept 48 Hours to 7 Days after Aëration.

Source of Water.	Days after Aëration before Examination.	Description of Water.	Bottle or Syphon.	Day of Numeration.	Organisms per c.c.			Ammonia per 100,000 pts.	
					Bacteria.	Moulds.	Total.	Free.	Albuminoid.
12. Water, Class C ; wood charcoal filtration.	2	Non-aërated.	Corked bottle.	2nd.	777	...	777	·0276	·004
13. Do.	2	Soda-water.	Do.	3rd.	280	18	298	...	...
14. Do.	5	Simple aërated.	Do.	7th.	750	2	752	·004	·004
15. Water, Class A ; unfiltered, softened.	4	Soda-water.	Corked bottles.	2nd.	2615	...	2615	·0032	·0138
16. Do.	5	Lithia-water.	Do.	3rd.	410	...	410	...	...
17. Water, Class C ; animal charcoal filtration.	6	Simple aërated.	Do.	8th.	38	13	51	·0044	·003
18. Do.	6	Soda-water.	Do.	8th.	218	340	558	...	...
19. Do.	6	Potash-water.	Do.	8th.	215	18	233	·001	·004
20. Origin unknown.	?	Soda-water.	Bottle, ball cork.	2nd.	2006	...	2006	·006	·001

been uncorked for some time, and all evolution of gas had apparently ceased, suction of the water into the pipettes was followed by the development of bubbles in greater or less number. The bottles or syphons were not specially shaken to diffuse the organisms, as ordinary handling, together with the extremely vigorous agitation following the escape of the gas, was quite enough to effect this object. The same

remarks apply to the syphons, the washing out of the nozzle and tubes leading to general agitation by escaping CO_2 .

The results of the examination are arranged in Tables A, B, and C. Table A shows the results of analysis—bacteriological and, in some cases, chemical—of aerated waters made within 24 hours of manufacture. Table B shows similar results from analyses undertaken between 2 and 7 days after aëration, and Table C of the analysis of waters more than 3 weeks old.

TABLE C.

Examination of Aerated Waters 3 Weeks or more after Aëration.

Source of Water.	Weeks after Aëration before Examination.	Description of Water.	Bottle or Syphon.	Day of Numeration.	Organisms per c.c.			Amounts per 100,000 pts.	
					Bacteria.	Moulds.	Total.	Free.	Albuminoid.
21. Water, Class A. charcoal filtration, softening.	3	Soda-water.	Corked bottle.	9	25	9	34	·00184	·0092
22. Water, Class D ;	3	Simple aerated.	Syphon.	8	2	1	3	·001	·001
23. Do.	4	Potash-water.	Do.	8	4	38	42	Trace.	Trace.
24. Do.	4	Soda-water.	Do.	8	265	3	268	·004	·004
25. Distilled water, no filtration.	4	Lithia-water.	Do.	8	2	1	3	·003	Trace..
26. Water, Class D.	4	Simple aerated.	Corked bottle.	7	0 (?)	36	36	} ·006	·002
27. Distilled unfiltered.	4	Lithia.	Do.	10	16	8	24		
28. Water, Class A ; unfiltered, softened.	6	Lithia-water.	Corked bottle.	10	33	1	34	...	...
29. Water, Class D.	10	Simple aerated.	Corked bottle.	10	49	3	52	...	...
30. Water, Class A ; animal charcoal filtration.	20	Lithia.	Corked bottle.	10	2000	0	2000	...	...

The number of micro-organisms present in the different samples is in most cases considerable, but varies between somewhat wide limits. The largest number observed was 2919, the smallest 354 (24 hours old) to 3 (in water 3 weeks old). In no case did my results, even where the enumeration could be postponed to the sixth or eighth day, show the presence of so large a number of organisms as was found by Hochstetter

in German mineral waters; the number in 8 bottles examined by this observer varying from 10,000 to 75,000.

A comparison of the results of analysis of simple aerated waters and those containing alkaline salts, especially sodium carbonate, would seem to show, where comparison is possible, that the latter contain the greater number of organisms. Sohnke has shown that solutions of sodium chloride and sodium bicarbonate, made in sterile vessels with sterile water and unfiltered, contain considerable numbers of moulds and bacteria. Numbers which indicate this increased richness of the alkaline waters are—

Simple aerated water,	{	1. 1089 p. c.c.	Soda water, same source	{	1. 1535 p. c.c.
Plates counted 6th day,	{	2. 354 „	and date of manufacture,	{	2. 579 „
			Plates counted 8th day.		
Simple aerated,	{		Water same source	{	Soda, 558 „
Plates counted 8th day,	{	1. 51 „	Plates counted on	{	Potash, 233 „
			8th day,		

Variations of individual samples are, however, so great that it is difficult to draw any decided conclusion on this point.

The colonies which developed were for the most part composed of bacilli. Micrococci were present in much smaller numbers, and yeasts (*torula alba* and *rosea*) occurred very rarely, and generally in aerated waters, made by manufacturers who were also bottlers of beer, etc. The moulds varied greatly in numbers, and seemed on the whole to be more numerous in corked bottles than in syphons. The most common mould was *P. glaucum*, but *aspergilli* and *mucors* also occurred.

The number of varieties of bacteria present was rarely large, and in most cases was restricted to three or four, even in those waters containing a large total number.

The constant and most conspicuous were—(1) a rapidly growing bacillus which liquefies the gelatine and produces a brilliant green fluorescence—probably *B. fluorescens liquefaciens* (Flügge), and a slowly developing, non-liquefactive bacillus producing a yellow pigment—*B. luteus* (Flügge). There appeared to be several varieties of the fluorescing organism, indistinguishable under the microscope, but showing differences in rate of liquefaction, amount of pigment produced, and form of colonies. There was also in certain waters a non-liquefactive brilliantly fluorescing bacillus, possibly *B. fluorescens putidus* (Flügge). The *B. fluorescens liquefaciens* was met with in every water, and when present in any considerable number prevented the counting of the colonies after the second or third day. In looking at the total organisms per c.c. given in the Table, it is therefore necessary to look also at the date of numeration, as the maximum appearance of colonies occurred on the third or fourth days, so that while some of the totals represent the actual numbers of organisms present, with the usual reservations necessary in regard to all bacteriological analyses, others merely represent a minimum. The actual proportion of liquefying to non-liquefactive

organisms present, even in those waters containing a considerable number, was never large.

Occasionally a water was found in which the cocci bore a much larger proportion to the bacilli than was usual. This was the case with water No. 4, Table A, in which *M. aurantiacus* (Schröter) occurred in considerable numbers.

Other organisms met with were *B. flavus liquefaciens* (Macé), *Sarcina flava*, *B. subtilis*, and a large non-liquefactive motile bacillus.

It would appear, then, that the differences in the source of the water supply, or the purifying treatment it undergoes, can scarcely be traced in the finished waters. Hochstetter found in his examination of Berlin mineral waters that individual samples made from the same bulk of water vary greatly. Reinl, too, noticed the great variation in individual samples of bottled natural mineral waters. My results show the same thing. It is certain that the greater or less biological purity of the original supply is masked by contaminations which occur during manufacture. These secondary sources of contamination comprise—(1) the storage tanks, (2) the filters employed, (3) the bottles, and (4) the corks. The latter two sources of contamination should be the chief cause of the variation of individual samples. The certainty of originally sterile water becoming once more full of organisms in its passage through storage tanks, filters, etc., or by bottling in non-sterile bottles, is so obvious that discussion of the matter would be useless were it not important to discover whether at any of these points pathogenic germs, as distinguished from ordinary saprophytes, might gain entrance. As shown above, the bacteriological examination gives no information as to the original purity of the source of supply. It is useless and would be foolish to demand that makers should send out sterile water, if it can be shown that the precautions taken effectually prevent the access of pathogenic germs. In discussing, then, the sources of contamination, attention is here directed solely to the possible entry of disease-producing organisms. First, with regard to the original supply. The waters of Group A are the ordinary drinking waters supplied to the metropolis. Opinions differ as to the absolute freedom of these waters from at least the risk of contamination with disease germs. It must be remembered that in most of the cases of typhoid, which are attributed to the use of impure drinking water, the water impurities can be shown to have gained access owing to local conditions of improper storage, communication with drains, etc., and not to have existed in the water as supplied from the mains. The second group, the artesian-well waters, are admittedly very pure and safe waters, always provided contamination with surface water is efficiently prevented. In London the water of artesian-wells rises only part way to the surface, and has to be raised by pumping. This often means that the upper part of the well is not the simple impermeable iron pipe which constitutes the lower part. The waters of Groups C and D are practically microbe free as they leave the stills or

boiling tanks, and certainly could not contain the germs of any of the ordinary water-borne diseases. The water, however, is not used as it comes direct from the stills, but is first filtered through animal or vegetable charcoal to remove the peculiar distilled water taste. Here the biological purity is at once destroyed, but as the charcoal appears to be very carefully selected, freshly burnt, frequently changed, and very thoroughly washed, *in situ*, with the purified water before use, the risk of dangerous contamination must be infinitesimal. The boiled water was filtered through glass to remove the deposited lime, etc. These filters were cleansed by washing with sulphurous acid, potassium permanganate, and final washing with boiled water.

For the storage of the waters slate tanks are usually employed, and these, in the factories visited, were well situated, carefully protected, frequently emptied in the ordinary course of manufacture, and kept scrupulously clean. In considering the last sources of contamination, the bottles and corks, distinction must be made between syphons and corked bottles. Syphons are cleansed by partially filling with aerated water, rinsing them with this, and then discharging the water so used. They are, therefore, washed with the same quality of water which is afterwards used for drinking, and no fresh source of contamination is introduced. The bottles are cleansed by soaking in hot water, brushing out with hand or revolving brushes, and finally inverting over a jet of water at high pressure which thoroughly rinses them. The bottles are subsequently carefully inspected before use. The thoroughness with which the cleansing is done and the amount of inspection varies considerably in different factories, and during the summer months, when there is a difficulty in keeping up with the demand, the washing is made as short as possible and perhaps occasionally omitted.

The spray with which the final rinsing is done is usually obtained from the mains of the company in whose district the factory lies, this supply being the most convenient on account of the available pressure. As a result, the water which has been boiled, distilled, or otherwise purified, is finally bottled with an admixture of ordinary tap water, thus reintroducing, though in a diluted form, the dangers surrounding the use of untreated water. The use of main water for rinsing, by the manufacturer whose aerated water is made from the same supply, is logical enough, but those makers who work on the assumption that purification processes are necessary to secure the safety of the waters should avoid thus stultifying their efforts. An examination of bottles has shown, as was to be expected, that they are far from sterile. They are used almost directly as they come from the spray, that is while still very wet, or after standing inverted for a day or two to drain. It was found by experiment, however, that in the great majority of inverted bottles, 48 hours after rinsing, there was a considerable quantity of unevaporated water, which gave rise to copious growths in nutrient bouillon.

Corks must also add to the number of organisms present in the

bottled waters, though the risk of the entrance of pathogenic germs by this route is probably very small. Some makers, however, pay attention to this matter, and wash the corks in alkaline solutions, in solutions of sulphurous acid, and finally in water. Others enclose the corks in wire cages and immerse them in boiling water, while others merely dust them by rolling, and soften them with hot water.

In a few instances it has been possible to examine the water on its way to the aërating cylinders, and the same water after aëration and softening, *i.e.* the water before and after the final source of contamination in the bottles and syphons.

TABLE D.

Examination of Water before and after Aëration.

Origin of Water.	Time of Examination.	Day on which Plates were counted.	Number of Organisms per c.c.			
			Bacteria.	Moulds.	Total.	
1. Water of Class B; filtered through block charcoal, drawn in sterile bulb pipette.	3 hours after drawing.	4th.	2162	10	2172	Before aëration.
2. The same water after aëration, in syphon.	3 hours after aëration.	3rd.	431	2	433	After aëration.
3. Water of Class D; drawn in sterile india-rubber-stopped tubes.	4 hours after drawing.	7th.	1750	10	1760	} Before aëration.
4. A second sample of same water as 3.	4 hours.	7th.	1820	12	1832	
5. Same water after aëration, in syphon.	4 hours after aëration.	5th.	649	0	649	After aëration.
6. Water of Class D; drawn in Pfuhr's tubes.	4 hours after drawing.	10th.	905	0	905	} Before aëration.
7. A second sample of same water as 6.	18 hours in ice.	9th.	593	0	593	
8. Same water after aëration, in corked bottle.	4 hours after aëration.	8th.	1170	44	1214	After aëration.

The Table shows that the number of organisms present in the water used is considerable, that this number is slightly diminished or unaltered (taking into consideration the intervals between examination and numeration) in the case of the syphons, but that in the case of the corked bottle specimen there is a decided increase of bacteria in the aërated water, together with the appearance of moulds which were originally absent. The bottles in this case were cleansed and rinsed out with ordinary unfiltered main water.

A glance at the results of chemical analysis shows that in nearly all cases the organic purity of these waters is very considerable. The

chemical and bacteriological results do not show any close connection. This might be anticipated from the nature and sources of the biological contamination.

III. THE ACTION OF CO_2 ON THE ORGANISMS FOUND IN MINERAL WATERS.

The results of the analysis of waters made between 2 and 7 days after aëration show no very marked differences from the results obtained with 24-hours-old waters; the organisms were similar in kind and the numbers present not much less. Towards the end of the week, however, there seems to be a diminution in the rate of growth which rendered it possible to keep the plates longer.

With waters kept for 3 weeks and longer, however, the case is very different. There is a quite unmistakable diminution in the total number of bacteria present, as shown in Table D. The proportional number of moulds also generally increases, though there are great individual differences in samples. The number of liquefying organisms, both actual and relative, becomes less, and, as a result, the plates can be kept and counted between the eighth and tenth days. Besides the diminution in number there is also a very decided change in the organisms, which renders the rate of development of the colonies much more slow. A culture of one of these old waters frequently shows no colonies for the first three days, a few on the fourth, and then an increase on the fifth or sixth day. In old kept waters I have rarely found more than a few moulds, an occasional colony of *B. fluorescens liquefaciens*, and a variable and sometimes large number of colonies of *B. luteus*. This latter seems to be one of the most resistant of organisms to the action of CO_2 .

This injurious action of CO_2 on the organisms contained in water has been pointed out by Leone and Sohnke. The latter insisted on the comparative freedom from organisms of waters kept 1 to 9 months, and found three bottles examined when 3 to 4 years old to be absolutely sterile. Hochstetter came to the conclusion, on the contrary, that this diminution of micro-organisms in aërated water did not occur, and suggested that the results of Leone and Sohnke are due to their having destroyed the culture plates too soon, in ignorance of the retardation in development, due to the action of the CO_2 . This explanation will not apply to my results, which point strongly to an actual reduction in the number of microbes present. Hochstetter's results were obtained by examination, at successive dates, of different samples made (presumably?) from the same bulk of water. The great opportunities afforded in manufacture for variations in the biological purity of individual samples have already been pointed out, and it seems probable that Hochstetter's conclusions are due to these variations, the individual differences being more than sufficient to mask the progressive decrease. Thus in a distilled aërated water in corked bottles, examined when

freshly made, the organisms varied from 73 to 2400, and in a similar water kept 161 days the variations were between 770 and 66,000.

The following Table, E, shows the results of an examination of the same sample at different intervals. In the case of the bottled waters the samples were drawn by means of sterilised screw taps inserted through the corks. The results show a decided diminution of organisms under the prolonged action of CO_2 .

TABLE E.

Showing Result of the Examination of the same Samples at Different Intervals after Aération.

Origin of Water.	Day on which Colonies were Counted.	Number of Organisms per c.c.			
		Moulds.	Bacteria.	Total.	
1. Water of Class B; in syphon.	6th.	2874	45	2919	4 hours after aération.
2. Same sample.	6th.	374	...	374	7 days after aération.
3. Same sample.	9th.	410	...	410	15 days after aération.
4. Water of Class D; in syphon.	5th.	649	...	649	4 hours after aération.
5. Same sample.	10th.	259	...	259	35 days after aération.
6. Water, Class D; in corked bottle.	8th.	1170	44	1214	18 hours after aération.
7. Same sample.	6th.	12	356	368	26 days after aération.
8. Water from same bulk as 6, but not same sample.	8th.	59	22	81	26 days after aération.

It must always be borne in mind that the apparent diminution will depend on the variety of organisms present. Bacteria differ remarkably in their resistance to the action of CO_2 . In a water containing chiefly one of the resistant forms the diminution would be ill-marked. The water containing the greatest variety of organisms would probably show the greatest change. Two of these exceptional waters were met with in the course of this investigation. In one, an artesian-well water (soda) in syphon, the organisms were found to have increased rather than diminished, and the *B. fluorescens liquefaciens* was present in considerable numbers. The second was a charcoal-filtered ordinary tap water, which after five months contained about 2000 organisms per c.c. (Table C, No. 30). The bacteria consisted of three varieties — (1) a motile, round-ended, thick bacillus, forming spores in cells of altered shape, and growing as a non-liquefactive thick opaque white colony spreading with irregular festooned edges on the surface of the gelatine; (2) a very small fine bacillus, like the *B. murisepticus*, forming circular white moist

non-liquefactive colonies ; and (3) an organism very similar, but forming yellow non-liquefactive growths. The last was present in the greatest numbers, and No. 1 was most scanty.

The observations on these waters were repeated several times, but identical results were obtained and the differences could not be due to experimental errors.

The diminution in numbers which appears to occur is due to the action of the CO_2 . Leone has shown that it is not due to the displacement of the oxygen present, as removal of this gas by hydrogen or the air-pump is not so injurious as its removal by CO_2 . It is also not due to the normal dying out of microbes, such as was noted by Meade Bolton in ordinary water, under the influence of time. This is shown by an examination of the aerated water, drawn and kept under sterile conditions after the carbonic acid has been allowed to escape. Such examination shows that after the loss of CO_2 there is a rapid multiplication of organisms.

TABLE F.
Effect of Keeping Water after Loss of CO_2 .

Before loss of CO_2 .			After loss of CO_2 .		
Source of Water.	Date of Counting Colonies.	Organisms per c.c.	No. of Days Water kept after loss of CO_2 .	Date of Counting Colonies.	Number of Organisms.
1. Water, Class B ; 4 hrs. after aëration.	6th day.	2919	...	...	...
2. Same water, 7 days aëration.	6th do.	374	7	1st day.	Colonies innumerable in '33 c.c.
3. Water, Class B ; soda, 24 hrs. after aëration.	3rd day.	645	28	1st day.	Colonies innumerable in '22 c.c.
4. Water, Class A ; soda, 4 days after aëration.	2nd day.	2615	36	1st day.	Colonies innumerable in '14 c.c.
5. Water, Class A ; soda, 20 days after aëration.	9th day.	34	28	1st day.	Innumerable in '5 c.c.

IV. THE VITALITY OF PATHOGENIC ORGANISMS IN AERATED WATERS.

The present investigation had for its end a practical object, viz. to determine what safeguards the use of aerated waters offered against infectious-water-carried diseases. The organisms selected for experiment were *B. typhosus*, *Spirillum cholerae* (Koch), *Spirillum cholerae nostras* (Finkler and Prior), *B. coli communis*, *Staphylococcus pyogenes aureus*, and

B. fluorescens liquefaciens. The first three were chosen as being the specific organisms of diseases admittedly spread by contaminated water; the *B. coli communis* because of the close relation which this organism bears to the *B. typhosus*, whether considered as a variety of the latter according to the Lyons school, or as aiding the growth and generalisation of *B. typhosus* in the infected body, as shown by Sanarelli and Chantemesse and Widal. The *Staphylococcus pyogenes aureus* was selected as typical of the group of micrococci, and as presenting the experimental advantage of being easily recognised, while the *B. fluorescens* (isolated from a sample of water) served to furnish a basis of comparison between pathogenic and the normally occurring non-pathogenic water organisms. The injurious action of CO_2 on various organisms has been noted by many observers, but their experiments have usually reference to the action of gaseous CO_2 on organisms in pure culture, suspended in fluid or disseminated throughout solid culture media. Buchner pointed out that the *Spirillum cholerae*, exposed to an atmosphere of CO_2 , showed no signs of growth even in good culture media, while *Emmerich's bacillus* and the *B. typhosus* would still grow. C. Fränkel has very fully examined the action of gaseous CO_2 on organisms diffused through thin layers of culture media, from which oxygen had been thoroughly removed. He found that the growth of the *Spirillum cholerae* and of the *Finkler's spirillum* was stopped absolutely, and that though the organisms were not all killed, yet their number was diminished and the rate of development slowed. The same observations were made as to a *B. fluorescens* isolated from water. *Staphylococcus pyogenes aureus* grew slowly at 37°C . (not at the ordinary temperature), and was injured by its exposure to the gas. *B. typhosus*, on the contrary, grew as in air. Fränkel showed that the organisms were not attenuated if not killed. Frankland made similar observations on the spirilla of cholera and cholera nostras, but found that an exposure of eight days to CO_2 prevented subsequent development in air. Leone, Sohnke, and Reinl have examined the action of CO_2 on organisms in water, but the most complete examination of the duration of vitality of pathogenic germs in aerated waters, under ordinary conditions, is by Hochstetter. He inoculated a number of corked aerated water bottles with suspensions of various germs in water, by means of a specially constructed syringe, and examined the bottles at different intervals. His results accord very closely with those obtained in my experiments.

Scala and Sanfelice examined the action of that amount of CO_2 which normally occurs dissolved in water, at 15°C . and atmospheric pressure, on various pathogenic organisms (*Spirillum cholerae*, *B. anthracis*, *Staphylococcus aureus* and *albus*, *B. typhosus*, and the bacillus of rabbit septicæmia), and concluded that it was without effect. If the amount of CO_2 was increased, as by passing a stream of the gas through the water, then a harmful effect was exerted on *Spirillum cholerae* and *B. anthracis*. Apparently the action of the gas under

pressure¹ was examined on *B. subtilis* and *Proteus vulgaris* only, with the result that the gas proved harmful to the vitality of *B. subtilis*, but left *Proteus* unaffected. The spores of *B. anthracis* and *B. subtilis* did not develop if exposed to a constant stream of CO₂.

My own experiments were made to determine, if possible, the duration of the vitality of the organisms—

1. In simple unsterilised aerated water.

2. In sterilised water which had been made alkaline, and was subsequently aerated. For this purpose a soda-water made from artesian-well water, having an alkalinity equal to 0.636 (Na₂ CO₃) per 100 c.c., was used.

The attempt was also made to gauge the pressure of the CO₂, and by experimenting at different pressures to determine the effect of the pressure factor on the duration of vitality. A number of small soda-water bottles, containing about 150 c.c., were fitted with india-rubber corks, through which passed a short glass or metal tube, terminating just within the bottles. To the outer ends of the tubes was attached strong india-rubber tubing, wound round with linen for support, and securely wired on. By this tubing the bottle could be attached to a metal cross piece with four arms. To one arm the bottle was attached, the second passed to the source of aerated water, the third communicated with a pressure gauge, and the fourth was fitted with an india-rubber tube and screw pinch cork, and was used to blow off the air contained in the apparatus. The air was swept out by a stream of CO₂, and in all cases the pressure was allowed to fall 5 or 10 lbs., two or more times, so that the air was thoroughly removed. The bottles plugged with cotton wool were sterilised by heating for one hour at 100° C. The corks and attached tubes, inserted in test tubes, were, together with the cross piece and its connection, sterilised in the autoclave, the open ends of the tubes being plugged with wool. The bottles were infected with the specific germ, the wool-plugs of the bottles replaced by the sterilised corks, which were then wired in, and the bottles attached and wired on to the arm of the cross piece intended for them. Aerated water was then driven into the bottles, avoiding splashing as much as possible; the air driven out as described above, and then more water driven in, until the bottle was nearly full and the desired pressure reached. The screw clamp was then screwed down, the charged bottle detached, and a sterilised nozzle or tap inserted into the tube attached to the bottle. To examine the charged water, the bottle was inverted, the clamp unscrewed so as to allow the water to slowly drop from the nozzle, and after some drops had escaped two tubes of suitable nutrient medium were inoculated with about six drops each. Each examination employed about eighteen drops—six wasted, and six used in each of two tubes. The loss of pressure was therefore slight. The taps and nozzle were heated before each examination.

¹ An abstract of this paper in the *Centralbl. f. Bakteriologie* was alone consulted, as the original paper was not available.

In the experiments with soda-water the apparatus was modified. The bottles were fitted with corks bored with two holes; through one of these passed a long tube reaching to the bottom of the bottle, and through the other was passed a short tube with a tap. The bottles were sterilised as in the first case, filled with sterile soda-water through the long tube, and infected through the short one. The bottle was then attached as before to the arm of the cross piece, and CO₂ passed in. The gas traversed a plug of sterile wool inserted in the tube leading to the gas supply. Air was drawn out by allowing the CO₂ to escape from the tap. In some of these experiments 20 lbs. pressure was used instead of 15, because it was found that the pressure dropped owing to the slowly progressive solution of the gas in the alkaline water.

The material used for inoculation was usually a young culture of the organism on nutrient agar, removed by a sterile wire and suspended in sterile distilled or sterile soda-water. The suspensions were rendered uniformly turbid, and about 1.5 to 2 c.c. were employed for each inoculation. Agar cultures were employed, in order that as little nutrient material as possible should be added to the water. The vitality of the organisms in the non-aërated waters used for infection of the bottles was also tested at intervals. This testing was done as a control on the aërated waters, but the conditions were not identical. The dilution was less, and the proportional amount of added nutrient medium, or organic matter derived from the organisms themselves, greater in these non-aërated than it was in the aërated waters; they are, however, comparable among themselves.

The pressures employed were one and two atmospheres. Ordinary bottled aërated water is at a pressure of 30–40 lbs. It was considered that 30 lbs. represented nearly enough the conditions of the commercial waters, without making the experiments unduly risky. The difference between one and two atmospheres should show the action of the pressure factor while solving the practical question as to whether the lowering of pressure in half-empty syphons would permit of a multiplication or a prolonged resistance of these pathogenic germs.

The results of the experiments are to be found in Tables I.–VI. :—

TABLE I.—*B. Fluorescens Liquefaciens*.

Age and Nature of Culture used.	Water used.	Pounds Pressure per sq. inch.	Hours or Days after Inoculation when Water was Examined.	Organism found to be still Alive.		First Experiment in which the Organism was found to be Dead.	
				Hours.	Days.	Hours.	Days.
1. Culture on gelatine at 22° C., 4 days old; organisms isolated from water.	Simple aërated, non-sterilised.	30	Days 2, 6, 9, 11, 13, 16.	..	16	..	..
2. " "	" "	30	Days 2, 6, 9, 11, 13, 16. ?	..	16?	..	..

TABLE II.—*B. Coli Communis*.

Age and Nature of Culture used.	Water used.	Pounds Pressure per sq. inch.	Hours or Days after Inoculation when Water was Examined.	Organism found to be still Alive.		First Experiment in which the Organism was found to be Dead.	
				Hours.	Days.	Hours.	Days.
1. Agar culture at 37° C., 3 days old, isolated from case of diarrhoea.	Simple aerated, non-sterile.	15	Days 2, 4, 6, 8, 9.	..	6	..	8
2. Similar culture, 5 days old.	"	30	Days 4, 6, 7, 9, 11.	..	7	..	9
3. Same culture as No. 1.	Sterile distilled, non-aerated.	0	Alive 50 days after inoculation.	..	..	..	..
4. Same culture as No. 2.	"	..	Alive 50 days after inoculation.	..	..	..	..

TABLE III.—*Staphylococcus Pyogenes Aureus*.

Age and Nature of Culture used.	Water used.	Pounds Pressure per sq. inch.	Hours or Days after Inoculation when Water was Examined.	Organism found to be still Alive.		First Experiment in which the Organism was found to be Dead.	
				Hours.	Days.	Hours.	Days.
1. Agar culture at 37° C., 18 hours old.	Simple aerated, non-sterile.	30	Days 2, 6.	..	2	..	6
2. Agar culture at 37° C., 18 hours old.	Simple aerated, non-sterile.	Saturated at ordinary pressure.	Days 1, 2, 3, 5, 6.	..	3	..	5
3. " "	" "		Days 1, 2, 3, 5, 6.	..	2	..	3
4. Agar culture, 37° C., 48 hrs. old.	Sterile soda-water.	20	Days 3, 4, 5, 7.	..	4	..	5
5. " "	" "	Not aerated.	Days 5, 7, 8, 9, 11, 12.	..	11	..	12

TABLE IV.—*B. Typhosus*.

Age and Nature of Culture used.	Water used.	Pounds Pressure per sq. inch.	Hours or Days after Inoculation when Water was Examined.	Organism found to be still Alive.		First Experiment in which the Organism was found to be Dead.	
				Hours.	Days.	Hours.	Days.
1. Culture on agar, 37° C., 24 hours old.	Simple aerated, non-sterile.	30	Days 2, 6, 9, 11. ?	..	11?	..	..
2. " "	" "	30	Days 2, 6, 9, 11, 13, 16.	..	11	..	13
3. Culture on agar, 37° C., 48 hours old.	Simple aerated, non-sterile.	15	Days 4, 6.	..	4	..	6
4. " "	" "	30	Days 4, 6, 7.	..	6	..	7
5. " "	Sterile distilled.	Not aerated.	Alive 50 days after inoculation.	..	..	..	..
6. Culture on agar, 37° C., 48 hours old.	Sterile soda-water.	20	Days 5.	..	..	..	5
7. " "	" "	30	Days 5.	..	..	..	5
8. " "	" "	Non-aerated.	Days 5, 7, 8, 9.	..	8	..	9

TABLE V.—*Spirillum Finkleri*.

Age and Nature of Culture used.	Water used.	Pounds Pressure per sq. inch.	Hours or Days after Inoculation when Water was Examined.	Organism found to be still Alive.		First Experiment in which the Organism was found to be Dead.	
				Hours.	Days.	Hours.	Days.
1. Agar culture, 37° C., 48 hrs. old.	Simple aerated, non-sterile.	15	Hours 2, 3, 4, 5.	..	..	2	..
2. " "	" "	30	Hours 1, 2, 3, 4, 5.	..	..	1	..
3. " "	Distilled sterile.	Non-aerated.	Hours 4, 5, 6.	5	..	6	..
4. Agar culture, 37° C., 48 hrs. old.	Simple aerated, non-sterile.	15	Hours $\frac{1}{2}$, 1, 2.	$\frac{1}{2}$	..	1	..
5. " "	" "	30	Hours $\frac{1}{2}$, 1, 2.	$\frac{1}{2}$	..	1	..
6. " "	Distilled sterile.	Non-aerated.	Hours 3, 4, 5; days 1, 2.	..	1	..	2
7. Agar culture, 37° C., 4 days old.	Sterile soda-water.	20	Hours $\frac{1}{2}$, $\frac{1}{2}$, 55 mins.	55 mins.	..	..	..
8. " "	" "	Non-aerated.	Hours 4; days 1, 2.	4	..	..	1
9. Agar culture, 37° C., 48 hrs. old.	" "	30	Hours $\frac{1}{2}$, $\frac{1}{2}$, 1, $1\frac{1}{2}$.	..	..	$\frac{1}{2}$	..
10. " "	" "	Non-aerated	Hours 4; days 1, 2, 3, 4, 6.	..	4	..	6
11. Agar culture, ¹ 37° C., 48 hrs. old.	Sterile soda-water.	15	Hours $\frac{1}{2}$, $\frac{1}{2}$, $\frac{3}{4}$, 1, $1\frac{1}{2}$, 2, 3.	$\frac{3}{4}$	..	1	..
12. " "	" "	30	Hours $\frac{1}{2}$, $\frac{1}{2}$, $\frac{3}{4}$, 1, $1\frac{1}{2}$, 2, 3.	1	...	$1\frac{1}{2}$	..
13. " "	" "	Non-aerated.	Hours 4; days 1, 2, 3, 5, 6, 7, 8, 9, 10, 12.	..	10	..	12

TABLE VI.—*Spirillum Cholerae*.

Age and Nature of Culture used.	Water used.	Pounds Pressure per sq. inch.	Hours or Days after Inoculation when Water was Examined.	Organism found to be still Alive.		First Experiment in which the Organism was found to be Dead.	
				Hours.	Days.	Hours.	Days.
1. Culture on Agar, 37° C., 48 hrs. old.	Simple aerated, non-sterile.	15	Hours $\frac{1}{2}$, 1, 2, 3, 4.	..	..	$\frac{1}{2}$	..
2. " "	" "	30	Hours $\frac{1}{2}$, 1, 2, 3, 4.	$\frac{1}{2}$	..	1	..
3. " "	Sterile distilled.	Non-aerated.	Hours $1\frac{1}{2}$, $1\frac{1}{2}$, 3, 4, 5; days 1.	5	..	..	1
4. Culture on Agar, 37° C., 24 hrs. old.	Sterile soda-water.	15	Hours $\frac{1}{2}$, 1, $1\frac{1}{2}$, $2\frac{1}{2}$, $3\frac{1}{2}$, $4\frac{1}{2}$, $5\frac{1}{2}$, $6\frac{1}{2}$.	1	..	$1\frac{1}{2}$	..
5. " "	" "	30	Hours $\frac{1}{2}$, 1, 2, 3, 4, 5, 6, 7.	..	..	$\frac{1}{2}$	..
6. " "	" "	Non-aerated.	Hours 2, 3, 4, 5, 6, 7, 8, 9; days 1.	9	..	..	1
7. Agar culture, ² 37° C., 48 hrs. old.	Sterile soda-water.	15	Hours $\frac{1}{2}$, $\frac{1}{2}$, $\frac{3}{4}$, 1, $1\frac{1}{2}$, 2, $3\frac{1}{2}$.	$\frac{1}{2}$	..	$\frac{3}{4}$	..
8. " "	" "	30	Hours $\frac{1}{2}$, $\frac{1}{2}$, $\frac{3}{4}$, 1, $1\frac{1}{2}$, 2, $3\frac{1}{2}$.	$\frac{1}{2}$	..	$\frac{1}{2}$	..
9. " "	" "	Non-aerated.	Hours 5; days 1, 2, 3, 5, 6, 7, 8, 9, 10.	..	9	..	10

¹ This culture was made from a gelatine culture at temperature 18° C.² Culture made from a gelatine plate cultivation. Possibly the increased resistance in water was partly due to this.

The length of time during which the various organisms retain their vitality varied considerably. They could, however, be divided into well-marked groups, though in these groups individual differences appeared. That the results of experiment should vary, even considerably, was to have been expected from the great discrepancies in the length of vitality noted by previous investigators.

As was to be expected, the ordinary water organism, *B. fluorescens liquefaciens*, showed the greatest resistance. It was still living after sixteen days' exposure, when the experiments ceased; it was, however, met with in commercial waters living and uninjured after more than five weeks' exposure.

Of the pathogenic germs, the *B. typhosus* and the *B. coli communis* form a group able to retain their vitality after at least seven days' exposure, and not certainly destroyed until the lapse of a fortnight, in ordinary unsterile aerated water. The longest period during which *B. typhosus* was found alive was eleven days, and the shortest period in which it was found to be killed was six days. The earliest period at which the *B. coli communis* died was eight days. It was alive up to the seventh day.

In sterile distilled water the vitality of both bacilli persisted more than two months.

The use of alkaline as opposed to ordinary water seems to shorten the period of resistance of these organisms, in both aerated and non-aerated waters. *B. typhosus* dies before the fifth day in aerated alkaline water, and between the eighth and ninth in non-aerated soda-water. The organism is known to withstand large doses of acid, but seems affected by the alkali.

The *Staphylococcus pyogenes aureus* holds an intermediate position between the previous group and one formed by the two varieties of spirilla. It appears to live in simple aerated water, and also in soda-water from two to four days, dying before the fifth day. In sterilised non-aerated soda-water it was alive after eleven days.

The organisms of the fourth group show a very much smaller resistance to the action of the aerated water. The longest period during which either spirillum was found alive was one hour, and this was in a sterilised alkaline water. In ordinary aerated water the vitality was not found to persist beyond half an hour. The apparent increase in resistance in the alkaline water was to be expected, knowing, as we do, the importance of a decidedly alkaline medium for their culture. The action of the sodium salt was more marked in a non-aerated water.

The action of the pressure factor, in the effect produced by the gas on micro-organisms, does not appear to be very important. The results obtained vary capriciously, and the variations are apparently due to indirect causes, differences in cultures or methods of experiments rather than to variation in pressure.

The *Spirillum cholerae* was the only one in which the increase of pressure

seemed to cause diminished vitality with any constancy, though there are indications of the same relations in the case of *Spirillum finkleri*.

The conclusion at which I have arrived, from the recorded experiments, and also from a number in which the experimental pressure was not kept up owing to failure of apparatus, etc., is that the pressure is unimportant, and that organisms die as quickly in water, thoroughly saturated with the gas, as they do in water in which the gas is under pressure. This conclusion is not opposed to the results of Scala and Sanfelice, as the amount of gas present in what I describe as thoroughly saturated water was not less than that occurring in water through which a stream of CO₂ is being passed, and indeed in most cases there was a residual pressure of $\frac{1}{2}$ to 1 lb. It does not appear, then, that the loss of CO₂ in partially used syphons would lead to the multiplication of any of the pathogenic organisms experimented with, and the results of the analysis of syphons at different dates show that the diminution in number of the ordinary non-pathogenic organisms proceeds notwithstanding the partial loss of pressure.

The conclusions which may be drawn from the foregoing observations may be summarised thus:—

1. That artificial aërated waters contain a considerable number of micro-organisms.

2. The number present in the finished waters gives no clue to the original purity of the source of supply, or to the efficacy of the methods of purification.

3. That there is a decided decrease in the number of micro-organisms present in the aërated waters when these are kept. The decrease is most noticeable after about three weeks. It is of general but not constant occurrence.

4. The decrease is due to the presence of carbon dioxide—loss of the gas being followed by increase in number of micro-organisms. The bacteria, therefore, are not in all cases killed, but have their development arrested.

5. Carbon dioxide in solution kills certain pathogenic germs. The period of resistance varies with the organism.

6. Pressure does not appear to be an important factor.

A bacteriological examination alone does not furnish complete data for answering the important question as to the safety of aërated waters as beverages. Supplementing bacteriological with ordinary hygienic inquiry, it appears that the water employed for aëration is drawn from trustworthy sources, and is carefully stored so as to show no risk of serious contamination. The processes adopted for purification are also satisfactory guarantees against dangers existing in the source of supply, though, in many cases, the initial biological purity of the water undergoes impairment in the process of manufacture. The advantage of using a purification process, such as distilling or boiling, is that risk attending accidental contamination of the source is eliminated. As a rule the

contamination during manufacture does not imply the possibility of the entrance of pathogenic germs, but there is certainly a risk, though it may be slight, in the use of improperly cleansed bottles.

Aëration undoubtedly increases the safety of water. It offers a practically absolute safeguard against cholera, since the *Spirillum cholerae* and *Spirillum Finkleri* are both so rapidly killed, that the water could hardly be used before these germs were dead.

The safeguards against typhoid are by no means so complete. Hochstetter quotes the case of an epidemic of typhoid occurring at Mainz in 1884, which was traced to the use of contaminated aërated water. Aërated waters are often drunk within a few days of manufacture. Safety would hardly be absolute unless the waters were kept for a fortnight after aëration before being consumed.

It is assumed here that the safety of the water is coincident with the death of the pathogenic organisms. It is possible, however, that practical safety may be assured at an earlier date, owing to the temporary attenuation of the organisms by the carbonic acid gas. Permanent attenuation is apparently not produced by the action of CO_2 . This was shown by Hochstetter to be true in the case of the spores of *B. anthracis* and *Aspergillus flavescens* exposed for 56 to 154 days in aërated water. Fränkel showed that the virulence of *M. tetragenus*, *B. anthracis*, and the organisms of chicken cholera and mouse septicaemia, was also unchanged by exposure to an atmosphere of carbon dioxide. On the other hand, that temporary attenuation may probably occur is indicated by the retardation in the rate of development of the organisms, and by the occasional, almost complete, loss of the power of liquefying gelatine produced by the action of the gas. It is probable that similar modifications of the pathogenic properties of the microbes may occur, though experiments are wanting on this point. Since, however, it has been shown that the association of micro-organisms may revive the pathogenic power of even highly-attenuated organisms (as in the case of *B. typhosus*), it would seem wiser, at present, not to trust to this loss of virulence, more particularly as the death of the pathogenic germs occurs within such a comparatively short time.

An aërated water made from a good source, and kept for more than fourteen days, would then appear to offer complete safety from the usual water-carried diseases.

APPENDIX.

Since the remarks (p. 475) on the use of a spray of untreated main-water for rinsing bottles were written, several manufacturers have modified their methods, and now use water which has been distilled or filtered through a Pasteur-Chamberland filter. In one case the artesian-well water used for aëration has always been employed to finally cleanse the bottles. To the methods used in purifying the original water must

now be added filtration through Pasteur-Chamberland or Berkefeld filters. An analysis of bottled aerated water prepared from main-water passed through a Berkefeld filter showed the presence of a comparatively small number of micro-organisms ; but, as specially sterilised bottles and corks had been used, the results cannot be compared with those given in the text; nor were the conditions those obtaining in commercial waters.

Though aerated water is often drunk, as stated, very shortly after manufacture, yet it is the endeavour of most makers to keep the water for a week or more before selling.

REFERENCES.

BUCHNER, ...	<i>Archiv f. Hygiene</i> , 1885, Bd. iii., p. 410.
LEONE, ...	<i>Do.</i> <i>do.</i> , 1886, Bd. iv., p. 175.
SOHNKE, ...	<i>Zeitschr. f. Mineralwasser-fabrikation</i> , 1886.
RIENL, ...	<i>Wiener. med. Wochens.</i> , 1888, Nos. 22 and 23.
HOCHSTETTER, ...	<i>Arbeiten. aus dem Kaiserlich. Gesund.</i> , 1887, Bd. ii., p. 1.
FRANKLAND, ...	<i>Zeitschr. f. Hygiene</i> , 1889, Bd. vi., p. 13.
C. FRÄNKEL, ...	<i>Do.</i> <i>do.</i> , 1889, Bd. v., p. 332.
MEADE BOLTON, ...	<i>Do.</i> <i>do.</i> , 1886, p. 76.
SANARELLI, ...	<i>Annales de l'Institut Pasteur</i> , 1892, tome vi., p. 71.
CHANTEMESSE AND WIDAL, ...	<i>Do.</i> <i>do.</i> , 1892, tome vi., p. 71.
SCALA AND SANFELICE, ...	<i>Bull. d. r. Accad. medica di Roma</i> , anno 16. <i>Centralbl. f. Bakteriologie</i> , 1891, Bd. ix., p. 110.