AERATION OF FLUID CULTURE MEDIA
SUPPLEMENTARY OBSERVATIONS
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After a long stay in Europe caused by the international situation, I returned to Blukwa Laboratory in May 1941, and was able to set up once more the aeration apparatus described in an earlier paper.¹ A highly virulent sample of B. pestis of local origin was aerated in order to confirm the effect of this procedure on the proliferation of the organisms, and on their loss of virulence.² By analogy with such terms as anaerobiosis, hypo-oxybiosis, oxybiosis or aerobiosis, it will be convenient to designate the state induced by this method of aeration as "hyperoxybiosis,"³ which would represent a more advanced stage in the series.

The strain of plague bacilli employed bore the number 343. It was obtained from an infection which occurred in July 1941 amongst guinea-pigs at Blukwa. The virulence of this organism was tested by the method of Sokhey and Maurice.⁴ A subculture grown in broth at 26° C. for forty-eight hours was diluted 1 in 10 million, and 0·5 c.c. of this dilution was inoculated into three guinea-pigs (9856, 9857 and 9858). Two of these animals died on the third day and the other on the fifth. Numerous tests were carried out to confirm the virulence of this strain. The strain was then preserved on blood-gelatine in a refrigerator, and it still retains its full virulence.

The strain 343 was then submitted to aeration for weekly periods, being subcultured regularly on ordinary gelatine and blood-gelatine. After five weeks of aeration, a subculture was made in peptonised broth (Sokhey and Maurice). When this culture contained about 200 million living organisms per cubic centimetre, three guinea-pigs (1209, 1210 and 1211) were respectively inoculated with the fluid, 1 c.c. subcutaneously, 2 c.c. subcutaneously, and 2 c.c. intraperitoneally. The animal which received the intraperitoneal injection died unexpectedly on the following day. The other animals succumbed on the second and third days. After this the stock culture was again aerated for a further period of seven days, and a broth culture was prepared. Two cubic centimetres of this broth was injected subcutaneously into a guinea-pig (1381), and it died from plague five days later.

I could only suggest one possible explanation for these unexpected results. The methods employed in these experiments were strictly similar to those already described,¹ except that calcium carbonate (la chaux vive du marbre) was used instead of concentrated potash to remove carbon dioxide from the air before bubbling it through the
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culture. The calcium was now replaced by a 30 per cent. solution of caustic potash and the aeration of stock 343 was continued.

On the forty-ninth day, that is after seven days of exposure to air treated with potash, I inoculated 2 c.c. of the aerated broth culture into a guinea-pig (1514). This animal died six days later. At the site of the injection was a large hæmorrhagic lump surrounded by an area of gelatinous consistency. The liver and spleen were large and roughened, and scrapings from these organs showed some plague bacilli, but a gelatine slope culture made from the heart blood remained negative.

On the fifty-eighth day, after sixteen days' exposure to air treated with potash, 2 c.c. of broth culture was injected into a guinea-pig (1630). This animal survived. A new experiment was then tried: 400 million living organisms were injected into a guinea-pig (2079) according to the method of Sokhey and Maurice. Between the fifth and the tenth days the temperature rose to 40° C.-40.5° C. and the animal died from hyperpyrexia on the eleventh day. The site of inoculation appeared normal, no organisms could be found in the body or on culture, but the liver and spleen were enlarged and showed many pale necrotic areas.

A further period of aeration seemed desirable. On the sixty-fifth day, after twenty-three days' exposure to potash, the strain was again tested. As in the last experiment, 400 million plague bacilli were injected into a guinea-pig (2285). No febrile reaction followed and the local swelling disappeared in a few days.

Referring to the earlier experiments with the strains 102 and 158 of plague bacilli which had been made non-virulent after thirty and thirty-six days of aeration, it seemed that an exposure of forty-two days at 37° C. in the absence of potash had no more effect on attenuation of virulence than an aeration of seven to thirteen days in the presence of potash. It would appear, therefore, that when the air was washed through potash it acquired some new property which caused a rapid attenuation of virulence. Potassium is a radioactive substance, whose radiobiologic properties are well known since the work of Zwardemaker on the frog's heart. Is it not possible that the treated air owes its attenuating property to the beta emanations of the potassium? It will be recalled that Pasteur attenuated the virus of rabies by suspending the spinal cord of the rabbit over caustic potash, and that this change is attributed to desiccation. If the drying is carried out rapidly, no attenuation results. May it not be that the change is really due to the electronic bombardment from the potassium?

According to certain workers, some bacteria, especially B. coli, Staph. aureus and B. anthracis (spores), lose their vitality and pathogenic activity under the influence of the beta radiation of radium. This has also been shown to occur in the case of the viruses of encephalitis, herpes and hydrophobia and of the bacteriophages. Potassium, like radium, emits beta radiation though in much less
amount. The coefficient of absorption of the potassium emanation is 22 to 38 cm. of aluminium, while that of the radium emanation is 5500 cm. In other words, potassium is 200 times less active than radium. Thus it would appear likely that the attenuation of virulence, which occurred after the method of aeration was changed, was due to emanation from the potassium and not simply to oxygenation as I had thought at first. This supposition would seem to merit further investigation.

Metalnikov and Yakimach, using radon, described the powerful action of radium emanation on a strain of sarcinae. Small quantities of emanation stimulate multiplication, and profound morphological changes may occur. They note also the high dissociating power of the irradiation, and the production of new races of the bacteria which preserve their tendency to further mutation after subculture.

Bonet-Maury and Olivier studied the action of radon on the tubercle bacillus, and reported that “the action of radon on the bacteria allows us to obtain Koch’s bacilli living but not virulent, and ‘de réaliser les meilleures conditions pour espérer obtenir l’immunité antituberculeuse.’”

Further work is necessary to prove my thesis, and with the limited means at my disposal I have carried out further experiments, which are described below.

A. Action on B. dysenteriae and B. typhosus of Air bubbled through Potash

The organisms employed for this study were a typhoid bacillus of local origin and a dysentery bacillus obtained from the laboratory of Kilo-Moto (Dr Janssens). Unfortunately, the guinea-pig, the animal most readily available in Blukwa, is not readily infected with these organisms. Sanarelli recommends the following method for enhancing virulence. A twenty-four hours’ broth culture of typhoid bacilli is inactivated and 5 c.c. is injected into the cellular tissue of the guinea-pig, and at the same time there is introduced into the peritoneal cavity 10 c.c. of an old sterilised broth culture of B. coli. I employed a satisfactory strain of B. coli recently isolated from a dysenteric stool; a seventeen-hours’ broth culture of this served my purpose. This strain, unsterilised and given intraperitoneally, was very pathogenic both for mouse and guinea-pig. Mouse 2299 died within twenty-four hours after receiving 0.5 c.c. Guinea-pig 2302, given 1 c.c., died next day with large numbers of bacilli in its organs. On the other hand, mouse 2300 survived after receiving 0.5 c.c. hypodermically. When treated with 10 drops of formol to 50 c.c. of broth culture, this strain no longer killed a guinea-pig but only caused a slight rise of temperature to 39.3° C. Guinea-pig 2676 was inoculated subcutaneously with 2 c.c. of an eighteen-hour broth culture of B. typhosus, and at the same time 2 c.c. of the formolised B. coli
was given intraperitoneally. Two days later it died in hypothermia, and a pure culture was obtained from the heart blood. A second guinea-pig (2677) was inoculated with a young culture of *B. dysenteriae* and the same amount of *B. coli*, and died on the third day with a positive blood culture. Intraperitoneal inoculation of a young culture of either the typhoid or the dysenteric organism, without the addition of formolised *B. coli*, caused only a slight rise of temperature.

Having established these standards, it was now possible to compare with them the virulence and toxicity of these strains after exposure to air treated with potash for varying lengths of time. As a result of a temporary shortage of fresh guinea-pigs, it was necessary to make use of certain animals which had already been used for standardising a vaccine (E.V.).

### TABLE I

| Guinea-pig. | New or Re-employed | Subcutaneous Inoculation of 2 c.c. *B. typhosus* | Intraperitoneal Inoculation of 2 c.c. B. typhosus | Result |
|-------------|---------------------|-----------------------------------------------|-----------------------------------------------|--------|
| 2676        | R                   | 18-day broth. Untreated                        | Formolised *B. coli*                          | Died 2nd day. Blood culture positive |
| 2645        | R                   | 17-day ,, Aerated 9 days                       |                                               | Survived. 39-5° C. for 2nd-5th days |
| 2658        | R                   | 18-day ,, ,, 16                                 |                                               | Survived. 40° C. for 2nd-5th days |
| 2561        | N                   | 27-day ,, ,, 23                                |                                               | Died 7th day. Blood-culture positive |
| 2529        | N                   | 16-day ,, ,, 30                                |                                               | Survived. 39-5° C. for 2nd-5th days |

| Guinea-pig. | New or Re-employed | Subcutaneous Inoculation of 2 c.c. *B. dysenteriae* | Intraperitoneal Inoculation of 2 c.c. *B. dysenteriae* | Result |
|-------------|---------------------|-----------------------------------------------------|-----------------------------------------------------|--------|
| 2677        | R                   | 18-day broth. Untreated                             | Formolised *B. coli*                                | Died 3rd day. Blood culture positive |
| 2646        | R                   | 17-day ,, Aerated 7 days                            |                                                    | Survived. 39-5° C.-40° C. for 1st-5th days |
| 2657        | R                   | 4-day ,, ,, 14                                     |                                                    | Survived. 39° C.-40° C. for 1st-6th days |
| 2560        | N                   | 17-day ,, ,, 21                                    |                                                    | Died 10th day. Blood culture negative |
| 2530        | N                   | 16-day ,, ,, 28                                    |                                                    | Died 12th day. Blood culture negative |

While the experiments were vitiated by the employment of guinea-pigs which had been used before, they do show a definite attenuation of the two strains from the first week of aeration of the cultures with potash-treated air. This attenuation is less complete for dysentery (death of 2530), but further treatment of the culture might have produced a different result.

The greater resistance of the animals which had been previously in contact with plague or the E.V. strain should be specially noted. This recalls the phenomenon described by Girard,” where he describes a reduction of the general mortality in Madagascar amongst natives vaccinated with living E.V. plague (4.8 per 1000 among 46,879 vaccinated against 9.7 in 60,000 unvaccinated).
B. Effect of Air washed in a Radioactive Solution of a Uranium Salt

Not having at my disposal either radium or other radioactive element, I used a 50 per cent. solution of uranium nitrate in distilled water to replace the caustic potash in the wash-bottle of the aeration apparatus. For test I employed the original *B. pestis* (343) of standard virulence, preserved on blood-gelatine in the refrigerator.

It should be noted that the cultures exposed to uranium air were always much less abundant than those exposed to air treated with potash or calcium, and were even more sparse than those in ordinary broth. Moreover, the culture of the thirty-sixth day was grumous and gave no subculture. Further experiments with this strain were therefore impossible.

A series of guinea-pigs were inoculated subcutaneously with 2 c.c. of the plague culture after varying lengths of exposure to uranium air. The results are shown in Table II.

**TABLE II**

| Guinea-pig | Sample of | Died from Plague | Observations          |
|------------|-----------|------------------|-----------------------|
| 2841       | 7th day   | 4th day          | Poor culture          |
| 2934       | 15th "    | 4th "            | " "                   |
| 2965       | 22nd "    | 4th "            | " "                   |
| 2977       | 29th "    | 4th " Survived  | Cloudy "culture. Subcultures failed |
| 3039       | 36th "    |                  |                       |

The strain survived till the twenty-ninth day, but no reduction of virulence was evident as all the animals died of typical plague on the fourth day. The last guinea-pig (3039) did not die, because the culture itself at the time of inoculation had lost its virulence and could not be subcultured.

The absence of attenuation in this experiment is probably to be explained by the fact that the uranium emanations were of the alpha variety.

C. Effect of Hydrogen washed in Caustic Potash on the Tetanus Bacillus

I next tried the effect of an inert gas (hydrogen) washed in caustic potash on an anaerobe (*B. tetani*). The strain employed came from the Pasteur Institute of Morocco and was labelled "*Bacille tétanique. Souche Alger."

It was kindly sent by Dr Martin, to whom I tender my best thanks. A Kipp's apparatus was used to provide the hydrogen. Special care was taken to see that the zinc and sulphuric acid were sufficiently pure to avoid the production of sulphurated or arseniuretted.
hydrogen in quantity sufficient to damage the culture. The hydrogen was washed in a 50 per cent. solution of caustic potash, and then was bubbled through an ordinary 1 per cent. glucose broth culture of tetanus bacillus in the incubator at 37°C.

From the first passage this strain grew very abundantly in the hydrogenated broth, giving an opacity comparable to that obtained with aerobic organisms when exposed to air treated with potash. When aeration was discontinued an abundant powdery deposit settled below a clear fluid. Hydrogenisation was carried out at weekly intervals. For inoculation of the guinea-pig the deposit was shaken up and 2 c.c. of the turbid suspension was injected under the skin of the inner surface of the thigh.

**TABLE III**

*Effect of Potassium-treated Hydrogen on B. tetani*

| Guinea-pig | Sample of | Result                        |
|------------|-----------|-------------------------------|
| 2673       | 9th day   | Died in a few hours           |
| 2683       | 16th "    |                               |
| 2935       | 23rd "    | Died in about 36 hours        |
| 2966       | 30th "    | Died between 36 and 48 hours  |
| 2976       | 37th "    | Died in about 90 hours *      |
| 3038       | 44th "    | Survived †                    |

* Guinea-pig 2976 after 24 hours showed a tightness of the skin; at 48 hours the stiffened leg was useless; by the third day the contractions had spread to the flank on the inoculated side, with deviation of the spinal column.
† Guinea-pig 3038 showed nothing pathological.

Subcultures of the organism remained active and typical, but they had quite lost their toxicity.

**SUMMARY AND CONCLUSIONS**

Investigations supplementary to those previously published on "The Aeration of Cultures by Bubbling Air" and on "The Bacillus of Yersin in an Aerated Medium" seem to justify the following remarks:

1. The bubbling of gas through a culture medium significantly increases growth by setting up a condition of "hyperoxybiosis" when air or oxygen is used, and an analogous condition of "anaerobiosis" when an indifferent gas such as hydrogen is used for anaerobes. This is abundantly evident from the observations of Topley and Wilson,17 Findlay and MacCallum,18 and more recently Rahn and Richardson,19 also from my own experiments.

2. When the gas is washed with caustic potash before being bubbled through the culture, it exerts a manifest attenuating effect on the toxic and pathogenic activities of both aerobic and anaerobic organisms.

This property seems to be related to the radioactivity of potassium (beta emanation). Proof of such probability is afforded by observations of numerous workers on the attenuating action of radium emanation (beta radiation like that of potassium) and by my observation that
calcium (non-radioactive) and uranium (mainly alpha radiation) do not produce this attenuation.

The technique described has enabled me to obtain non-virulent strains of B. pestis, greatly attenuated strains of B. dysenteriae and B. typhosus, and a non-toxic strain of B. tetani.

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