LVI.—*The Chemical Actions of some Micro-organisms.*

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THE present research was commenced with the intention of isolating, if possible, the nitrifying organism contained in soil. In the autumn of 1886, I spent, through Dr. Klein's kindness, some time in the laboratory of the Brown Institution, learning, under his instruction, the methods usually employed for the separation of different species of bacteria. On returning to the Rothamsted laboratory, I commenced the study of five organisms—two isolated from visible growths in some of my nitrifying solutions; two obtained from soil at the Brown Institution; and one, the well-known *Bacillus subtilis*, obtained from hay.

A short time afterwards I became acquainted with the paper by W. Heraeus (*Zeit. f. Hygiene*, 1886, 193). In this communication he names seven well-known organisms:—*M. prodigiosus*, *Staphylococcus citreus*, Finkler's bacterium, the spirillum of cheese, the bacteria of anthrax and typhoid fever, and the "Wurzelförmige Bakterien," as capable of nitrifying urine, and concludes that the power of producing nitrification is by no means unusual among bacteria. In April, 1887, Dr. Klein kindly supplied me with pure cultures of 12 bacteria, including several of those specifically named by Heraeus. The study of these organisms afforded results of considerable interest, and in consequence others were from time to time supplied by Dr. Klein. The investigation thus deviated from its original intention, and became, to a considerable extent, a study of some of the chemical properties of a variety of organisms, many of them well known as pathogenic. The action of these organisms has been tested in four particulars:—1. The hydrolysis of urea. 2. Action on milk. 3. Capacity for reducing nitrates. 4. Power of producing nitrification. It is proposed, after publishing the results at present obtained, to return to the investigation originally intended, and recommence the study of the micro-organisms in soil.

THE MICRO-ORGANISMS STUDIED.

The following were received, in the state of pure cultures, from Dr. Klein:—

1. *Bacillus* of swine fever.
2. " of typhoid fever.
3. " of infantile diarrhoea.

4. *Bacillus* of anthrax.
5. „ of septicæmia (mouse).
6. „ „ (guinea-pig).
7. „ *subtilis* (jequirity extract).
8. „ „ (scurf of scarlatina).
9. „ *fluorescens*.
10. „ *fluorescens liquescens*.
11. „ *intestinalis*.*
12. *Spirillum*, Koch's cholera asiatica.
13. „ Finkler's cholera nostra.
14. „ Deneke's cheese.
15. „ Lingard's noma.
16. *Micrococcus aureus*.
17. *Staphylococcus luteus*.
18. „ *candidus*.
19. „ *candidus liquescens*.
20. *Streptococcus scarlatinae*.

From Dr. W. R. Smith was received :—

21. *Micrococcus ureæ*.

The following five were isolated under Dr. Klein's superintendence :—

22. *Bacillus subtilis* (hay).
23. „ *floccus*.
24. „ *toruliformis*.
25. „ *sulphureus*.
26. „ *tardecrescens*.

There was also separated from specimens of *M. prodigiosus*—

27. *Micrococcus gelatinosus*.

A considerable number of the organisms here enumerated are well known, and need no description. In some other cases a reference to the original description will suffice.

1. *Bacillus* of swine fever (Klein). See *Reports of Med. Offic. Loc. Gov. Board*, 1877-8, 169; 1886-7, 446, Pl. XXV.

3. *Bacillus* of infantile diarrhæa (Klein). See same *Reports*, 1886-7, 447, Pl. XXVII. It will be more fully described in the following *Report*.

5. *Bacillus* of septicæmia, mouse (Klein). *Ibid.*, 447, Pl. XXVI.

6. *Bacillus* of septicæmia, guinea-pig (Lingard). *Ibid.*, 1885-6, 178, Plate XIX.

* When this paper was communicated to the Chemical Society the organism now called *B. intestinalis* was spoken of provisionally as *Bacterium termo*.

9 and 10. *Bacillus fluorescens*. These two fluorescent bacilli will be described by Dr. Klein in *Med. Offic. Report*, 1887-8.

11. *Bacillus intestini*. Obtained by Dr. Klein from the large intestine in a case of diarrhœa in a rabbit. Grown in broth it appears as a short bacillus with rounded ends. The longer forms are $1.0-1.5 \mu$ in length, and about 0.4μ in thickness.

In a plate culture, it appears the first day as small translucent dots, which afterwards develop in the depth to opalescent spheres, and on the surface produce a shining expanse of pearl-like lustre.

In a stabbed culture, the growth at first is chiefly in the depth. The track of the needle is bounded by rows of opaque spheres. There is no liquefaction.

In broth at 22° , or 33° , a dense white turbidity is produced, with no film.

15. *Spirillum of noma* (Lingard). This non-liquefying spirillum is described in the *Practitioner*, **38**, 121, Figs. 32-35.

18. *Staphylococcus candidus* (Klein). This non-liquefying organism was obtained from condensed milk. *Med. Offic. Report*, 1886-7, 386, No. 3.

19. *Staphylococcus candidus liquescens* (Klein). Obtained from the blood of a scarlatina patient. *Ibid.*, 370, Pl. VII.

20. *Streptococcus scarlatinæ* (Klein). *Ibid.*, 1886-7, 374, Pls. I-XII. The organism used in the experiments described in this paper was obtained from the blood of a scarlatina patient.

21. *Micrococcus ureæ* (Smith). *Quart. Jour. Micro. Science*, 1887, 371.

23. *Bacillus floccus*.—Obtained from garden soil at the Brown Institution. A straight bacillus, $3.0-7.5 \mu$ in length, and 1.0μ in thickness; motile, in broth forms long interlacing threads.

In gelatin plate cultures at 22° , it forms the first day faint colonies which, under a low magnifying power, resemble balls of cobweb; from these fine interlacing threads, resembling the mycelium of a fungus, spread in every direction. Liquefaction commences the first day, and on the second extends over most of the surface.

In stabbed gelatin cultures at 22° , it forms a liquefied channel in the course of the needle the first day. From this channel numerous fine branches, resembling knotted threads, pass into the gelatin. The second day liquefaction has extended over the surface; the channel has disappeared, and the gelatin is filled with finger-like branches, starting from the old tube. The fifth day liquefaction is completed. A soft, thick film forms on the surface; below this are two layers of growth with clear spaces between. These two layers finally become one, and sink to the bottom. The liquefied gelatin becomes brown near the surface.

On agar agar at 22°, a grey, finely crinkled film quickly spreads over the whole surface.

In diluted urine, or in very weak broth at 22°, it forms dense white flocks resembling those produced by anthrax, the rest of the liquid remaining clear.

In normal broth at 33°, it forms, after some days, a thick soft film, which falls as ribbons.

24. *Bacillus toruliformis*.—Obtained from garden soil at the Brown Institution. Maximum length 5.3 μ , a few 6.4 μ ; thickness 1.0—1.6 μ . The ends thickened, and much rounded. Many short, thick forms joined together. The general arrangement very crooked and irregular.

In gelatin plate cultures, it forms the first day white dots, having a granular margin. The second day there are large white colonies, many $\frac{1}{16}$ or $\frac{1}{8}$ inch in diameter. Under a low magnifying power they appear of granular structure. Liquefaction has commenced.

In stabbed gelatin, the first day's growth is chiefly confined to the needle channel, and consists of moderately opaque dots. The second day liquefaction commences. It occurs principally at the surface. A funnel of liquefied gelatin is formed with a short conical pipe, and white organism in the pipe, the upper liquid being clear without film. The gelatin at the bottom of the tube remains long unliquefied.

On agar agar at 22°, it grows rapidly, forming a pasty, moderately opaque, white film.

In broth at 33°, it grows luxuriantly, producing great turbidity, an abundant fine deposit, but no distinct film.

25. *Bacillus sulphureus*.—This was isolated from a surface growth which had appeared on some solutions which had nitrified. In a deposit from dilute broth it had a length of 1—1.5 μ , and a thickness of 0.3 μ . Taken from the surface of the broth it appeared as a network of bacilli, the sheaths faintly stained, with well-stained oval dots within. The bacillus is motile.

In a plate culture, no growth appeared for several days. The colonies were at first milky points, becoming afterwards translucent spherical masses. The colour of the colonies is at first yellowish-white. By exposure to light the colonies become opaque, and of a bright sulphur colour. There is no liquefaction.

In a stabbed culture, there is scarcely any growth in the depth of the gelatin. Growth soon appears upon an inoculated surface.

In broth at 22°, it produces turbidity. After some days small clots are deposited, some strung together in ropes, together with some finer threads. Festoons of these ropes hang from the surface, but

there is no film. The deposit in broth turns yellow on exposure to light.

The bacillus does not grow at 35°.

26. *Bacillus tardecrescens*.—This organism was isolated from floating gelatinous masses occurring in some solutions of ammonium carbonate which had nitrified. Grown in dilute broth it appears as a small oval bacillus, 1.0 μ in length, and about 0.5 μ in thickness. Grown on gelatin it is somewhat smaller, and appears more like an oval coccus. It is stained by gentian-violet very slowly.

The bacillus is, under all circumstances, slow in growth. In a plate culture, the colonies became visible after a week or more; they grow into small translucent droplets, of a somewhat smoky tint.

In a stabbed culture, the growth was chiefly in the depths, it consisted of strings of spherical colonies. There was no liquefaction.

In broth, growth is slow, and only produces a very moderate turbidity.

27. *Micrococcus gelatinosus*.—Two specimens of *M. prodigiosus*, from different sources, produced a mixture of red and white growths on the surface of agar agar. The red I did not succeed in obtaining pure; the white was easily isolated by cultivation on agar agar at 35°, when the white only was developed. The white organism resembles the red in microscopical characters, but grows much better than the red at high temperatures, and preserves its vitality for a longer period.

Grown in broth, it appears as an oval coccus, or short bacillus, 0.5—1.3 μ in length, and 0.3—1.0 μ in thickness.

In plate cultures of gelatin, it appears the first day as milk-white dots, which the next day are increased to $\frac{1}{8}$ or $\frac{1}{4}$ inch diameter, and liquefied.

In stabbed gelatin, liquefaction in the channel commences the first day, and has extended over the surface by the second day. A funnel is then formed with a wide pipe. There is no film.

On agar agar it grows rapidly at 22°, less rapidly at 35°, forming a thick, pasty, whitish mass, moderately opaque, covering the whole surface.

In broth, it produces an abundant turbidity, and a considerable amorphous deposit, but no film.

GENERAL METHOD.

The methods of culture on gelatin, and agar agar, were those originated by Koch, and now generally employed. The nutritive gelatin, and agar agar, were of the composition employed by Dr. Klein, the former containing 10 per cent. of gelatin. The prepara-

tion of the various liquid mediums will be described under the head of each investigation.

The microscope was one kindly placed at my disposal by the Chemical Society for the purpose of this investigation. The powers used were Zeiss' D, and Leitz's $\frac{1}{20}$ oil immersion lens. The stained preparations were made with gentian-violet.

All inoculations of solutions were made by means of Klein's capillary glass pipette or hollow needle, the cotton-wool stopper not being removed from the tube or bottle containing the sterilised liquid. By means of the same pipette, the acidity or alkalinity of a solution could be at any time ascertained without removing the stopper. In examining a liquid for nitrites or nitrates, a much larger pipette was employed, the capillary tube of which was passed between the stopper and the glass; sterilised wool was held against the pipette at the point where it entered the bottle, so that only filtered air might enter when suction commenced. The large pipettes were kept in boiling water till the moment when required. By proceeding in this way, the cultures were commenced and brought to a close without once removing the cotton-wool stoppers.

As a check on the results, a culture was generally made on gelatin or agar agar at the end of each experiment, or series of experiments, made in a fluid medium, the object being to prove that the action had proceeded without the entrance of any foreign organism. This check was at first employed in every case, but after confidence in the mode of work had been attained, it was made use of only to check striking or irregular results, or to ascertain the growth of an organism in milk. The method was especially necessary for the latter purpose, as successful inoculation with an organism which produced no striking change in the milk could in no other way be ascertained.

THE HYDROLYSIS OF UREA.

The hydrolysis of urea, and its conversion into ammonium carbonate, which takes place in putrefying urine, is now universally acknowledged to be due to the action of micro-organisms. Pasteur has described a micrococcus which acts in this manner. More recently (1885) Leube and Graser (*Virchow's Archiv*, **100**, 555) have separated the organisms occurring in samples of ammoniacal urine, and have found four which were capable of converting urea into ammonium carbonate. The most active of these was a short bacillus, which they name *Bacterium ureæ*. The next in activity, and the most abundant, was a micrococcus. There were besides two bacilli, which exerted a much feebler action. The authors also name a fifth

organism, "Lungen Sarcin," as capable of converting urea into ammonia. Heraeus (*Zeit. f. Hygiene*, 1886, 215, 221) isolated four bacilli which effected an ammoniacal fermentation in urine; three of these liquefied gelatin, and were therefore distinct from the organisms of Leube and Graser. During last year, Dr. W. R. Smith (*Quart. Jour. Microscopical Science*, 1887, 371) has separated about 20 organisms from ammoniacal urine, and found among them only one which was capable of producing ammonia from urea. The active organism separated by Dr. Smith was a micrococcus; it was apparently not identical with the micrococcus of Leube and Graser, as it slowly liquefied gelatin, whilst their micrococcus did not. It appears therefore that at least nine organisms are known which are capable of hydrolysing urea.

Cultivations in diluted urine have been attempted with all the organisms studied during the present investigation. Nos. 13, 14, 16, 17, and 26 have not occasioned a distinct turbidity in the solutions; Nos. 19 and 20 have given only a slight turbidity; the remainder have afforded plain evidence of growth. The urine solutions were of two kinds; the first was a 1 per cent. solution, containing gypsum, used for the experiments on nitrification. The cultures in this series of solutions were tested with Nessler's reagent after being kept for two or three weeks at 32°; the presence of gypsum in this case preventing volatilisation of the ammonia. The tint given by Nessler's reagent in the various cultures was compared with that produced by the same test in similar solutions unseeded with organisms. The organisms Nos. 1, 2, 4, 5, 11, 13, 15, 17, 18, 19, 20, 22, 23, 24, 25, and 26 were tried in this manner. In one case only (No. 18) was any distinct increase of ammonia perceived over that present in the unseeded solution.

The second urine solution contained 25 per cent. of urine, and was prepared especially to determine the action of the organisms on urea. The test-tubes containing the seeded urine were kept at the temperature of 22° for 7—10 days, the alkalinity of each cultivation was then determined by means of standard acid and alkali, and the result compared with that given by unseeded urine placed under the same conditions. The method did not admit of extreme accuracy, as the indicators employed failed to determine the point of neutrality with great exactness. All of the organisms under investigation, with the exception of Nos. 7, 8, 16, and 19, were tried by this method; in the case of 13, 14, and 17, however, no quantitative result was attempted, as there was no evidence of any growth in the solution. With the exception of two organisms, to be presently mentioned, none proved capable of distinctly affecting the alkalinity of the urine.

In the case of the *Micrococcus ureæ* (Smith), a very distinct effect

was produced on the urine. In two experiments, made with different solutions, the urine acquired a distinct ammoniacal odour. In one case the alkalinity at the end of seven days was increased to an extent equivalent to 0·0066 gram of ammonia per 10 c.c. of solution, while in the second experiment the increase of alkalinity in eight days was equal to 0·0043 gram of ammonia.

A very distinct increase in alkalinity also occurred in the case of *B. fluorescens*. In the first experiment, the increase of alkalinity in eight days was equivalent to 0·0039 gram of ammonia in 10 c.c. of the solution. In the second experiment, the increase in seven days was equal to 0·0037 gram. It seems probable from this result that the *B. fluorescens* is identical with the bacillus giving a green fluorescence obtained by Heraeus from soil, as this organism was found by him to convert urea into ammonia.

The property of effecting the hydrolysis of urea is apparently but rarely met with among micro-organisms; in the present case, out of 24 organisms tried, only two could certainly be shown to possess it. I am doubtful, however, if either of these organisms is the cause of strong ammoniacal fermentation. At all events, in two comparisons of the result produced by the *M. ureæ* with that yielded, under the same circumstances, by the mixed organisms of arable soil, the latter proved far more effective. The soil was from heavy, unmanured, arable land. The fragment used was about the size of a large pin's head; it was taken half an inch below the surface. The two comparisons were made in different solutions. The amounts of ammonia found per 10 c.c. of diluted urine were as follows:—

	Soil.	<i>M. ureæ</i> .
I.	0·0252 gram	0·0066 gram.
II.	0·0116 „	0·0043 „

It would appear probable, therefore, that the soil contained a more powerfully hydrating organism than the micrococcus of Dr. Smith; it possibly contained the active bacillus of Leube and Graser.

ACTION ON MILK.

Milk is an excellent medium for studying the chemical action of micro-organisms, being a liquid susceptible of many changes. In all the experiments I have made, skim-milk has been employed; the inconvenience of a surface layer of cream was thus avoided. The first trials were made with skim-milk kindly supplied by the Aylesbury Dairy Company. This milk had been separated in the centrifugal machine, it was thus perfectly sweet, and as nearly as possible free from fat. The milk had, however, been sterilised by heat before it reached me, and had of course again to be heated after it had been

transferred to test-tubes. Owing probably to this double heating of the milk, it required a longer time to curdle than the milk subsequently employed. The milk afterwards used in the experiments was taken directly from the cow into a sterilised stoppered bottle; the portion collected was never the first or the last milk from the udder. The bottle of milk was stood in a cool place for 36 hours. The milk below the cream layer was then drawn off by a syphon, and 10 c.c. measured into each test-tube. The test-tubes, closed of course with sterilised cotton-wool, were then heated at 100° for 20 minutes in a steamer. Only three imperfectly sterilised tubes were observed during the whole investigation.

The precipitation of the casein, which determines the curdling of milk, may be brought about either by an acid, or by a soluble ferment, as rennet; the latter is capable of solidifying milk even when it has been made alkaline with sodium carbonate.

The proportion of acid required to curdle milk is smaller the higher is the temperature; thus milk that is only slightly sour may be completely curdled if heated to boiling. Attempts were made to ascertain what was the smallest proportion of lactic acid that would suffice to curdle milk. Very dilute lactic acid was slowly dropped into 10 c.c. of sterilised skim-milk; milk acidified with different proportions of acid was then heated, and the effect observed. With 0.15 gram of lactic acid per 100 c.c. of milk, a tolerably complete solidification of the milk was obtained when the test-tube was plunged into boiling water. A considerably smaller proportion of lactic acid sufficed to produce a partial curdling. With 0.32 gram of lactic acid per 100 c.c. of milk, the whole became nearly solid in two hours at 33° . The proportion of lactic acid required to curdle at lower temperatures could not be ascertained by this method, as it was found impossible to mix the necessary proportion of acid with the milk without at once curdling it, the acid being unavoidably present in great excess at the spot on which the drop of acid fell. It is difficult to determine the quantity of acid in milk with much accuracy, the reaction with litmus-paper being far from sharp. Neutral litmus-paper was used as the indicator in all the experiments.

To Hueppe (*Mittheil. a. d. k. Reichsgesundheitsamt*, 2, 309) we are indebted for the fullest study of the organisms affecting milk. He names five as capable of souring milk. One, a bacterium or bacillus, which curdles with the evolution of carbonic acid gas; two cocci obtained from the mouth; *M. prodigiosus*; and the coccus of osteomyelitis.*

* Marpmann (*Arch. Pharm.*, 1886, 243) states that he has obtained five organisms producing lactic acid from the milk supplied to Göttingen; his descriptions do not seem to be published yet.

Among the organisms which I worked with, five have distinctly acidified milk; most of these organisms are certainly different species from those enumerated by Hueppe. The acidification by these organisms has been in very different degrees. The three first named act apparently simply by the production of acid; in the case of the fourth and fifth, the action is more complicated.

Staphylococcus candidus.—At 22° this organism distinctly acidified the milk. The culture was continued for 43 days without visible change in the milk, save the production of a slight sediment.

At 32°, after eight days, the acidity of the milk was equal to 0·13 gram of lactic acid per 100 c.c. The milk was not curdled, and did not solidify when placed in boiling water.

At 33°, with another batch of milk, the acidity after 17 days was 0·15 per cent.

Bacillus intestini.—Grown in milk at 23°, it produced in 11 days an acidity equal to 0·33 per cent. of lactic acid; in 21 days an acidity of 0·33 per cent.; in 31 days an acidity of 0·33 per cent. The milk did not curdle at 23°; when the cultures were placed in boiling water they immediately solidified.

At 34° the milk became solid in two days; the acidity was then equal to about 0·35 per cent. of lactic acid.

Bacillus of Infantile Diarrhœa.—A culture at 22° has been quite fluid after 43 days, but examined at 83 days was found to be solid. The following amounts of acidity were found in experiments made at different times:—At 10 days, 0·33 per cent.; at 11 days, 0·34 per cent.; at 7 days, 0·31 per cent.; at 14 days, 0·35 per cent.; at 21 days, 0·38 per cent.; at 31 days, 0·44 per cent. of lactic acid.

At 33° the milk became solid in two days; the acidity was then 0·32 per cent.

At 36° the milk curdled in one day, and gas was distinctly produced.

No peptone was found in old cultures either of the curdled or uncurdled milk.

Micrococcus ureæ.—At 22° the milk begins to thicken in 12 days, and some days later becomes solid; the curd is, however, always soft. The acidities determined were—at 10 days, 0·17 per cent.; at 7 days, 0·16 per cent.; at 14 days, 0·24 per cent.; at 21 days, 0·22 per cent. The last-named culture was tested for peptone, but only a slight reaction was obtained.

At 33° a soft curdling takes place in 5 days. The amounts of acidity in different experiments were—at 7 days, 0·19 per cent.; at 8 days, 0·24 per cent.

Micrococcus gelatinosus.—At 10° the milk becomes solid in about 15 days. The curd afterwards very slowly shrinks, or partly dissolves. Peptone was distinctly present in the whey of an old culture.

At 23° the milk solidifies in 2 days; the amount of acid at that time is about 0·15 per cent. The curd very slowly becomes smaller; the whey then contains peptone very distinctly.

At 32° the milk curdles in 1 day; the acidity is then about 0·14 per cent.

On reviewing the results given by these five organisms, some points of interest appear. The first three organisms apparently do little more than produce lactic acid. The amount of acid produced by the second and third is considerably larger than that yielded by the first, but with each organism the proportion of acid which can be formed seems to be nearly a fixed quantity; at least after a certain acidity is reached further increase takes place with extreme slowness, so that apparently a culture of six weeks will not enable *Staph. candidus* to curdle milk at all, nor will it enable *Bacillus intestini* and the *Bacillus of infantile diarrhœa* to curdle milk at 22°. We may probably assume that when a certain proportion of lactic acid has been produced the further growth of the organism is checked, and with this the progress of chemical action. That the curdling which does take place is determined solely by the lactic acid produced appears very probable, for the amount of acidity in cultures freshly curdled at 33° of *B. intestini* and the *Bacillus of infantile diarrhœa* is practically the same as the amount found requisite to produce curdling in the previous experiments with lactic acid.

When we turn to the last two organisms, *M. ureæ* and *M. gelatinosus*, the circumstances are very different. *M. gelatinosus* is far more active in curdling milk than either *Bacillus intestini* or the *Bacillus of infantile diarrhœa*, for these fail entirely to curdle at 22°, while *M. gelatinosus* not only curdles in 2 days at 22°, but also after 15 days at 10°, and yet *M. gelatinosus* produces far less acidity than these two organisms. They fail to curdle milk at 22° with an acidity of 0·38 per cent.; *M. gelatinosus* succeeds in curdling milk at this temperature with an acidity of only 0·15 per cent. ! It is clear, then, that in the case of *M. gelatinosus* some curdling agent other than lactic acid takes a considerable share in the reaction. The same may be said of the far less perfect curdling effected by *M. ureæ*, as here too the amount of acidity produced is by itself insufficient to effect the curdling which takes place. Further light is thrown on this subject by the results afforded by the next three organisms.

Bacillus fluorescens liquescens.—At 23° milk seeded with this organism thickens in 3 days, and becomes solid in 5 days. The milk is neutral at the time of curdling. The small quantity of whey at the surface exhibits a slight bluish-green colour. In a culture a month old the curd remained undissolved; it had become distinctly acid. Tests for peptone in cultures a fortnight old showed none, or a trace only.

Spirillum of Asiatic Cholera (Koch).—At 22° the milk exhibited a loose curdling in 4 days; it was then neutral. Milk made distinctly alkaline with sodium carbonate became solid in 3 days. After 25 days the milk was still solid; it had then become distinctly acid.

At 33° milk curdled firmly in 4 days; in 11 days it was slightly acid. Alkaline milk became solid in 1 day, and was then still alkaline. The curd in the last-named experiment shrank a good deal. The whey, tested after 15 days, was distinctly acid, and gave a strong reaction of peptone. Peptone was also found in smaller quantity in cultures in the ordinary milk 11 days old.*

Spirillum of Cheese (Deneke).—This organism acts but slowly on milk. At 22° a bright yellow ring forms at the surface of the milk in 4–6 days. A tough crust is then developed, giving the milk the appearance of solidification. Later, at somewhere about 11 days, the milk gelatinises. It is still neutral, and has a butyric odour. Still later the opaque jelly partially redissolves; the whey becomes slightly acid, and contains a distinct amount of peptone. In alkaline milk the gelatinisation begins in about 12 days, and gradually increases in solidity.

At 33° a similar course of change takes place, but apparently more slowly. The milk after 21 days is found to be alkaline, and little or no peptone is present.

In the case of the *B. fluorescens liquescens* and the *Spirillum of Asiatic cholera* we have clear instances of the complete curdling of the casein without the formation of any acid; the action is in fact similar to that produced by the ferment contained in rennet. It has been frequently assumed that when an organism does work exactly similar to that performed by a ferment, that it does so by the production of a ferment. There is at least one undoubted instance of the production of a ferment by a micro-organism, namely, the formation of invertin by yeast. According to S. Lea (*J. Phys.*, 6, 136) the bacteria which convert urea into ammonia also act by the production of a ferment. Microphytes would thus appear to effect certain hydrolytic actions by the same agency as similar actions are accomplished in the cell sap of plants of more complex organisation. Facts and analogy thus seem to support the hypothesis that ferments are produced by bacteria; and until more certain light is thrown on the subject we may well assume with Hueppe and others, that the rennet-like curdling of milk is due to the production of a ferment.

With the information gained by the study of the last group of

* Since writing the above, Dr. Klein has informed me that the faculty of curdling milk has not been hitherto recognised as belonging to Koch's spirillum. The experiments have therefore been repeated, but with the same results. The gelatin cultures, prepared from the curdled milk as a check, gave normal growths.

organisms, we can now understand how *M. gelatinosus* and *M. ureæ* are able to curdle milk with the production of only a part of the acidity necessary to effect this purpose. They act in fact as ferment producers, as well as acidifiers.

The cheese spirillum possesses only a feeble gelatinising power; it appears to occupy an intermediate position between the group of rennet-like organisms, and the group of peptonising organisms which we have next to consider.

Hueppe names four bacilli and two cocci as capable, from his own observation, of gelatinising the casein, and then redissolving it with conversion into peptone. According to him this is by no means an uncommon property of bacteria; he ascribes it to the presence of two ferments, a rennet ferment and a trypsin ferment.

Peptone has been looked for in the present experiments by the application of the usual test of sodium hydrate and copper sulphate; and also by means of phosphotungstic acid, a reagent considerably more delicate. The milk to be tested was in every case placed in a small dialyser of parchment-paper; after 24 hours, the diffusate was examined for peptone. According to Vines and Green (*Phil. Trans.*, 178, 43), dialysis is needed to avoid a confusion between peptone and hemialbumose, both of which give the same reaction with chemical tests, whilst only the first will pass through a membrane.

In the case of a large number of organisms small quantities of peptone have been found in the milk cultures, especially when they became old. The peptone is usually most abundant in cultures made at a low temperature (23°); at a higher temperature (33°), less peptone and more ammonia is found. Milk cultures have, for the same reason, more tendency to become alkaline at the higher temperature.

The following five organisms do not produce a solidification of the milk which corresponds to typical curdling, but the casein is more or less gelatinised, and then redissolved.

Bacillus subtilis (hay).—Milk cultures of this organism at 22° begin in two or three days to show a translucent space immediately below the surface. Each day this space increases, and the milk is resolved into a nearly clear liquid above and a soft jelly below, the latter steadily diminishing by re-solution. The clear liquid afterwards becomes turbid from bacterial growth. The reaction of the milk is neutral during the early stages of the reaction, but becomes strongly acid later; the odour is then pungent, and acids of the fatty series are clearly present. As soon as the separation into fluid and jelly is clearly established, peptone can be distinctly found, and it becomes abundant when the re-solution of the casein has made some progress.

At 33° the action commences earlier, but the re-solution of the casein seems to proceed more slowly than at a lower temperature.

The bacilli from scarlatina scurf, and from jequirity extract, peptonised as vigorously as the bacillus from hay.

Bacillus anthracis.—The experiments were made at 22°. The action goes through the same course as that described in the case of *B. subtilis*, the production of acids in the latter stage of the reaction did not apparently occur.

B. floccus.—The peptonising action is apparently as vigorous as with the two preceding organisms. No production of acid was observed.

Spirillum of Cholera nostra (Finkler).—The culture was made both at 23°, and at 37°. At the higher temperature the entire gelatinisation of the milk preceded the formation of a clear solution. Peptonisation was more vigorous at the lower temperature. This organism did not apparently act quite as rapidly as the three previously named.

Bacillus toruliformis.—The experiment was made at 22°. The action was considerably slower than in any of the preceding cases. The milk did not gelatinise for rather more than a week, and after 26 days one half of the jelly was still undissolved. The culture remained neutral.

The remaining organisms produced little effect on milk, although nearly all grew freely in it, as was proved by making gelatin cultures from the milk many days after it had been seeded.

Streptococcus scarlatinæ is stated by Klein to curdle milk at 37° in three days, or a little later. I obtained no curdling with this organism.

At 22° the milk became distinctly acid, but at the end of 43 days was quite fluid.

At 32° the acidity after eight days was equal to 0·07 gram of lactic acid per 100 c.c.; the milk was quite fluid, and did not curdle when placed in boiling water. A culture at 37°, continued for several weeks, was equally unsuccessful in producing curdling.

Fresh cultures on gelatin were used for inoculation in all these experiments.

On reporting these results to Dr. Klein, he informed me that the cultures of *Str. scarlatinæ* in his possession had also lost the power of curdling milk. Since then he has obtained a fresh supply of the organism, and finds that, as before, it curdles milk completely at 37°, rendering it very acid. This loss of the power of producing lactic acid during a long series of cultures on gelatin is a fact of great interest. The organism I experimented with had been grown on gelatin for a year and four months before the experiments in milk were made.

The two *Bacilli of septicæmia* make the milk distinctly alkaline, a change which is visible to the eye, as the milk loses much of its

opacity. The bacillus from the mouse acted more energetically than that from the guinea-pig. A trace of peptone was found in both cases in cultures three weeks old. The *Bacillus of swine fever*, *B. fluorescens*, and *B. tardecrescens* also slowly produce an alkalinity in milk.

The *Typhoid bacillus*, and *Staphylococcus luteus*, slowly render the milk slightly acid, without producing further change. *B. sulphureus* grew without producing any appreciable change. The *Spirillum of noma* did not grow easily in milk. I obtained a culture in alkaline milk only. It produced no apparent change. My stock of *Staphylococcus candidus liquescens*, and *Micrococcus aureus* was unfortunately dead before the milk experiments commenced.

It is of great interest when one property of an organism can be correlated with another. I venture to think that these experiments with milk enable us to do this. The whole of the organisms which fail to gelatinise milk are organisms that do not liquefy gelatin. The three organisms first mentioned in this section, which apparently simply attack the milk-sugar and produce lactic acid, are also non-liquefying. On the other hand, the whole of the organisms which act on milk as ferments liquefy gelatin. This conclusion is quite in accordance with the view already taken by some investigators, that the liquefaction of gelatin is itself an action produced by a ferment. We may venture therefore to predict that every liquefying organism will be found capable of gelatinising the casein of milk. This precipitation of the casein may take place with or without the formation of lactic acid, and with or without a subsequent re-resolution of the casein as peptone.

Some experiments were made to see how the mixed organisms of soil would attack milk; the soil employed was a heavy loam from an arable field long unmanured.

At 10°, milk seeded with a small fragment of soil became gelatinised in nine days; the milk was then slightly acid. In 11 days, gas began to appear. Half the volume of the milk was then an opaque jelly. The odour was very bad, a circumstance that had not occurred with any culture made with pure organisms. Peptone was distinctly present.

At 22°, the milk curdled in 2—3 days, and became slightly acid. In 4 days the evolution of gas commenced, and continued active for some days. The curd slowly redissolved, and the solution contained peptone very distinctly. The reaction of the milk was for some time only slightly acid, but at the end of 5 weeks the acidity had become very large. The odour passed through various unpleasant stages, which it is difficult to describe.

Soil thus showed itself possessed of organisms acting like rennet

and trypsin ferments. It differed in action from the pure organisms tried, first by the production of much gas, and secondly by the formation of putrefactive products having a powerful odour.

THE REDUCTION OF NITRATES.

The reduction of nitrates to nitrogen in sewage, and in waters containing sewage, seems to have been first noticed by Angus Smith (*Mem. Lit. and Phil. Soc. Manchester*, 1867 [3], **4**, 56). He subsequently made many experiments on the subject, which are described in his Reports to the Local Government Board on the Pollution of Rivers, 1882 and 1884.

Schloesing in 1868 (*Compt. rend.*, **66**, 237) showed that during the fermentation of tobacco juice, or of putrefying urine, or during the lactic fermentation of sugar, any nitrate that was originally present disappeared, nitrous oxide, nitric oxide, and nitrogen gas being produced.

Schloesing further showed in 1873 (*Compt. rend.*, **77**, 353) that a vigorous reduction of nitrates to nitrogen gas occurs in moist vegetable soil, when a change of atmosphere is prevented. Muntz has shown (*Ann. Chim. Phys.*, 1887, **11**, 125) that under the same circumstances chlorates are reduced to chlorides, bromates to bromides, and iodates to iodides.

Some experiments were made by myself in the Rothamsted laboratory in 1880 (*Jour. Roy. Agri. Soc.*, 1881, 332) on the reduction of nitrates in soil. 7 lbs. of arable soil were placed in a percolator, forming a column 8 inches deep. The soil was saturated with water, and sodium nitrate, in quantity equal to a large agricultural dressing, placed on the surface. After a week, water was applied daily for nine days, in quantity sufficient to keep the surface covered, and the drainage was collected and analysed. At the end of this time nitrates and nitrites ceased to appear in the drainage water. Only 21 per cent. of the nitrates applied were recovered as nitrates and nitrites in the drainage. Large transverse fissures were formed in the soil by the production of gas.

The power of soil to reduce nitrates to nitrites, and finally to destroy the latter, nitrogen being probably evolved, was further shown by later experiments not hitherto published.

To 125 c.c. of a sterilised 40 per cent. solution of urine, contained in a bottle closed by a cotton-wool stopper, about 0.2 gram of arable soil was added, and the bottle placed in a cupboard having a temperature of about 10°. In five days, nitrites were distinctly present, resulting from the reduction of the nitrates naturally occurring in the urine. In 12 days neither nitrites nor nitrates could be found, the solution giving no reaction with diphenylamine.

To 100 c.c. of a 20 per cent. solution of urine, containing 1 gram of nitre per litre, about 0.5 gram of arable soil was added, the surface of the solution was covered with a thin layer of paraffin oil, and the bottle then placed in an incubator at 20°. In two days, a slight evolution of gas commenced, and nitrites were distinctly present. In nine days evolution of gas ceased. Nitrites have permanently remained in this solution.

A similar experiment was made at the same time, the only difference being that 5 grams of nitre, and 5 grams of glucose per litre were present in the solution. Gas was evolved as before, but in much larger quantity. In 11 days all nitrites and nitrates had disappeared.

In a duplicate experiment to the preceding, but conducted at a temperature of 35°, gas was evolved in one day, and ceased in four days, by which time the nitrites first formed had disappeared, and no nitrates could be found.

The oxidisable organic matter contained in the soil and urine is seen by the first two experiments to be capable of reducing only a small amount of nitrate; the addition of glucose in the last two experiments determined the reduction of a much greater quantity of nitrate, and the final disappearance of nitrite from the solution. That the reduction of nitrates is due to a reaction between the nitrates and the organic matter present has been recognised from the first by every investigator.

Meusel, in 1875. (*Jour. pharm.* [4], 22, 430), was the first to prove that the reduction of nitrates to nitrites in natural waters is brought about by the agency of living organisms, which he pronounced to be bacteria.

Dehérain and Maquenne, in 1882 (*Compt. rend.*, 95, 732), were the first to establish the same agency in the case of the reduction of nitrates in soil. It has, in fact, been abundantly shown that if sewage or soil is sterilised by the action of heat or antiseptics, no reduction of nitrates will take place.

The conditions necessary for the reduction of nitrates include a nourishing medium and temperature suitable for the growth of micro-organisms; also the presence of organic matter capable of oxidation. The exclusion of air is favourable to reduction, but not essential to it; thorough aëration is, however, fatal to the process. When the organism is capable of growing at a high temperature, such a temperature is most favourable to reduction. The organic matter suitable for effecting reduction is very varied. According to different observers, albuminoids, sugar, propyl alcohol, ethyl alcohol, fats, glycerol, glycol, acetates and tartrates, are all active in this respect. The amount of reduction, if other conditions are equal, depends entirely on the quantity of oxidisable organic matter present. Thus Gayon and Dupetit found that in sewage, seeded with putrid urine,

not more than 0·1—0·2 gram of nitre per litre was reduced; while in chicken broth, seeded from the same source, 50 grams of nitre per litre could be reduced.

The products of the reduction of nitrates are very varied. The reduction may be to nitrites, to nitric oxide, to nitrous oxide, or to nitrogen. The formation of ammonia has been sometimes observed, but the evidence generally points to its origin in the decomposition of nitrogenous organic matter rather than from the reduction of nitrates. The formation of nitric oxide has been frequently noticed during the fermentation of sugar-beet molasses, when the solution is not kept sufficiently acid (*Compt. rend.*, **66**, 171, 237). The production of nitrous oxide has been observed by Dehérain and Maquenne during the reduction of nitrates by soil (*Compt. rend.*, **95**, 691, 854).

The differences in the products of reduction are determined:—
1. By the conditions of the experiment; 2. By the specific nature of the acting organism. Till within the last few years all experiments have been made with mixtures of various organisms, such as naturally occur in the air of the laboratory, in sewage, or in soil. Working with such natural mixtures one is easily led to conclude that the character of the reaction depends entirely on the composition of the solution, or on the other conditions of the experiment. Thus Munro (*Trans.*, 1886, 667) found that river-water readily oxidised ammonia to nitric acid, but after the addition of a tartrate reduction set in, and all nitrates disappeared from the solution. From such facts, some have concluded that not only the extent of reduction, but the whole difference between oxidation and reduction is simply determined by the composition of the solution. These ideas become, however, greatly modified when we become acquainted with the specific properties of the different organisms which together produce the actions in question, it then becomes evident that in many cases one stage of the work accomplished is performed by one organism, while a second stage is effected by another.

Within the last few years considerable progress has been made towards ascertaining the reducing faculty of individual species of bacteria. In 1882 Gayon and Dupetit (*Compt. rend.*, **95**, 1365) published quantitative results showing the amount of nitrate reduced in the same time by seven distinct organisms. The most active was a small, mobile bacillus, producing few spores, anaerobic. This organism, cultivated in chicken broth at 35°, with exclusion of air, reduced 9·6 grams of nitre per litre to nitrite in one day. Several other organisms only reduced at the rate of about 0·5 gram per day. In 1886, the same authors published a splendid research upon the reduction of nitrates by bacteria (*Ann. de la science agronomique*, 1885, **1**, 226).

A good abstract of this paper will be found in this Journal (Abstr., 1886, 823). The authors found that the reduction of nitrates to nitrites was a very usual property of the bacteria examined; they met with only one species (they notice 11) which could be cultivated in broth containing nitre without occasioning reduction. They state that a large class of bacteria reduce only to nitrites, no nitrogen gas being produced. They experimented with, but did not succeed in isolating in a pure state, the organism producing nitric oxide. They isolated two bacilli from sewage, which they named *Bacterium denitrificans* α and β ; these reduced nitrates to nitrogen gas, nitrites being formed under most circumstances as a stage in the reaction. The action of the α -organism was under favourable circumstances very energetic, the liquid in which it is cultivated becoming covered with foam, and evolving in one day its own volume of nitrogen, the temperature at the same time considerably rising. The same organism when grown in an artificial solution, containing nitre and asparagine, produced a considerable amount of nitrous oxide; when the asparagine was omitted, no nitrous oxide was formed.

Heraeus (*Zeit. f. Hygiene*, 1886, 215) grew 10 bacteria, isolated from water and soil, in solutions containing a nitrate and sugar. Six grew well in this medium, and of these, two bacilli reduced the nitrate to nitrite and ammonia.

In the autumn of 1887, I communicated to the British Association (*Report*, 1887, 653) a preliminary account of the results obtained with 20 organisms, part of those forming the subject of the present paper.

In March of the present year, a paper was read before our Society by Dr. Percy Frankland (*Trans.*, 1888, 373), in which the author describes the results he has obtained, respecting the reduction of nitrates to nitrites, with 32 species of bacteria, separated by himself from the atmosphere or from natural waters. The main series of experiments were made in a weak solution, containing as organic matter 0.25 gram of peptone, and 0.3 gram of sugar per litre, at the temperature of 30°. Of the organisms tried, 15 or 16 were found incapable of reducing nitrates to nitrites; it is to be remarked, however, that seven of these organisms produced either no visible growth, or a very slight turbidity. It would seem possible, therefore, that under conditions more favourable to growth the proportion of active organisms might be increased.

In the experiments with pure organisms which I have now to describe, the power of each organism to reduce nitrates has been tested in nearly every case in two solutions:—1. Beef broth containing 5 grams of potassium nitrate per litre; 2. A 20 per cent. urine solution, containing 1 gram of nitre per litre. In some cases

peptone has been added to the broth, and glucose to the urine solution; but these were for additional trials, outside the main series.

For the broth, 2 lbs. of lean beef were taken, cut small, treated with cold water, slowly heated with stirring, and finally boiled for half an hour. The filtered broth was made slightly alkaline to neutral litmus-paper by the addition of sodium carbonate, brought to boiling, and again filtered. The perfectly clear filtrate then received the necessary amount of nitre, and was diluted to 1200 c.c.

The urine solution was in some cases clarified by making it distinctly alkaline with potassium or sodium carbonate, boiling, and filtering; the filtrate was then neutralised with phosphoric acid. Urine thus prepared remains perfectly clear when heated for sterilisation, and also when it becomes alkaline, a fact of considerable advantage if the growth of an organism is to be determined by turbidity, or by the formation of a deposit in the solution. Urine so clarified is not, however, so nutritive as urine which has not been filtered after boiling.

The nitrated broth, or urine, was placed in small wide-mouthed bottles, previously baked at 140° ; each bottle received 100 c.c., which nearly half filled it. The mouths were closed with plugs of sterilised cotton-wool, a paper cap tied on, and the contents of the bottles sterilised by heating for several hours at a temperature near 100° .

The trials were, as a rule, made at two temperatures; the lower one varied at different times from $20-23^{\circ}$, the higher from $32-35^{\circ}$.

The formation of nitrite was ascertained by two reagents: 1. The zinc iodide and starch solution of Trommsdorf; 2. Metaphenylenediamine. The first reagent is so extremely delicate that one is apt to conclude from its indications that a large amount of nitrate has been reduced, when in fact the amount of reduction has been extremely small; the extent of reduction has therefore always been judged from the results of the second test. No strictly quantitative determinations of the nitrous acid formed have been made.

The reducing power of each organism must be concluded mainly from its behaviour in the experiments with broth. The reduction of nitrate in the urine solutions was never large, the proportion of nitrous nitrogen very seldom exceeding 1 per million of the solution. The results with urine also varied a good deal at different times, according probably to the varying nutritive character of the solution.

With freely growing organisms the reduction of nitrate generally takes place speedily (if it occurs at all) both in broth and urine solutions, and most speedily at the higher temperature, if the organism will bear it. To avoid, however, repeated testing of the solutions, the cultures in broth at 32° to 35° were seldom examined

till at least three days old, and the cultures at 20—23° not before five days. The cultures of slowly growing organisms, and many of the cultures in urine, were allowed a longer time before examination.

Looking first at the bacilli, which form the largest class examined, I am disposed to place the following three first, as possessing the greatest power of reducing nitrates to nitrites.

Bacillus floccus.

„ *fluorescens non-liquescentis.*

„ of swine fever.

In the case of these organisms, the culture in broth gave an exceedingly strong reaction with metaphenylenediamine, a large precipitation of the colouring matter taking place immediately.

Next in order stand the following eight bacilli, the cultures of which in nitrated broth gave a very strong reaction with metaphenylenediamine, but with no immediate precipitate of colouring matter.

Bacillus intestini.

„ of typhoid fever.

„ of infantile diarrhoea.

„ of septicæmia (mouse).

„ „ (guinea-pig).

„ *anthracis.*

Spirillum of Asiatic cholera.

„ of cheese.

Far removed from these in reducing power stands—

Bacillus subtilis.

The hay bacillus does not reduce nitrates in urine, even when glucose is added. In broth at 22°, there is frequently no reduction for a week or more, but generally a slight reduction afterwards occurs. In broth at 35°, a small reduction can be perceived after a few days. Reduction does not apparently occur till the broth becomes alkaline. The addition of peptone favours the reduction. The largest amount of nitrite noticed has probably not exceeded 5 parts of nitrous nitrogen per million of solution.

B. subtilis from the scurf of a scarlatina patient gave the same amount of reduction as the bacillus prepared from hay. The bacillus from jequirity extract gave a somewhat greater reduction. Frankland speaks of *B. subtilis* as giving no reduction; this is probably owing to the small amount of reduction it occasions when a feebly nutritive solution is employed.

The following six bacilli have given no reduction of nitrates to nitrites :—

Bacillus fluorescens liquescens.

„ *toruliformis.*

„ *sulphureus.*

„ *tardecrescens.*

Spirillum of cholera nostra.

„ *of noma.*

B. fluorescens liquescens, and *B. sulphureus*, have not been tested at a temperature above 23°, as they refused to grow in the hot incubator.

B. toruliformis, Finkler's spirillum, and the spirillum of noma, have each been grown with an attempt at complete exclusion of air, the surface of the culture-liquid being covered, after seeding, with a layer of paraffin oil; this addition did not at all prevent the growth of the organisms. The use of paraffin did not in any case determine a reduction of the nitrate. It was thought at first that the layer of oil would effectually protect the liquid from contact with oxygen, but a subsequent experiment, in which a solution of cuprous oxide in ammonia was substituted for the culture-fluid, showed that oxygen did in fact pass beneath the layer of oil. I hope at some time to try the effect of an absolute exclusion of oxygen.

Proceeding next to arrange the micrococci examined according to their reducing effect, and classifying them as far as possible on the same principles as the bacilli, we place in the first rank—

Micrococcus ureæ.

„ *gelatinosus.*

Staphylococcus candidus.

„ *luteus.*

These are apparently practically equal in efficacy to the three bacilli previously named as possessing the highest reducing power.

In the second rank stands one coccus—

Staphylococcus candidus liquescens.

Far removed from these comes—

Streptococcus scarlatinae.

This must, for practical purposes, be classed as without a reducing power for nitrates, but in fact a trace of nitrous acid is shown by the delicate iodide test when it is cultivated for some time in nitrated broth. *Strept. scarlatinae* has been grown covered with a layer of paraffin oil without developing further reducing powers.

One other micrococcus,

Micrococcus aureus,

has been grown in nitrated broth, with and without a layer of paraffin on the surface, without any production of nitrite.

Among the organisms examined by Frankland it was the bacilli only which exhibited reducing properties ; among those now reported on, the micrococci have proved to be in many cases as powerful as the bacilli.

The table (p. 750) summarises the whole of the experiments on reduction. The letters R, r, r, tr., 0, indicate the extent of reduction observed. The letters n. gr. signify "no growth."

As already mentioned, the results in urine varied a good deal at different times, the whole period of experiment being more than a year. In some cases single experiments showed a distinct amount of nitrite by the iodide test, though none is mentioned in the table, a majority of experiments, made at other times, showing no nitrous acid. In several cases in which a large reduction occurred in broth at a low temperature, the experiment was not repeated at a high one. It may be safely concluded that in each of these cases the reduction would have been equally great at the high temperature, there being in none of these instances any difficulty of growth at a high temperature. Whenever an organism showed no, or very little, reduction, a trial was always made at both temperatures.

In several cases of small or no reduction, the experiment was repeated with an addition to the broth of 5 grams of peptone per litre ; in no instance was the amount of nitrite increased by this addition, the broth being in itself sufficiently rich in organic matter. The addition of glucose to the urine was chiefly tried with organisms which afterwards proved to be destitute of reducing power ; in one case in which the organism was able to reduce, its action was distinctly increased by the glucose.

The whole number of organisms reported on is 25 ; of these 7 were entirely without reducing power, 1 only produced a mere trace of nitrite, and 1 only a very small quantity. The remaining 16 reduced nitrates in broth with considerable vigour.

Of those which failed to reduce, one was of feeble growth, producing only a very limited turbidity in the broth, but others in the same class were among the most vigorously growing organisms examined. The power of reducing nitrates is thus in no way determined by rapidity and vigour of growth.

As far as I am able to judge, the reduction which has occurred has been simply from nitrates to nitrites, but this point can only be decided by strictly quantitative experiments. No production of gas has been observed in any of the broth cultures, although the amount of nitrate present was quite sufficient to occasion visible gas if a reduction to nitrogen had taken place. *B. floccus* was grown in broth under a layer of paraffin, with the especial object of observing any bubbles of gas that might be formed, but without any such result ;

Experiments on the Reduction of Nitrates to Nitrites by various Micro-organisms.

No.		In broth.				In diluted urine.			
		20—23°.		32—37°.		20—23°.		32—37°.	
		Number of trials.	Reduction.	Number of trials.	Reduction.	Number of trials.	Reduction.	Number of trials.	Reduction.
23	<i>B. floccus</i>	1	R	2	R	3	r	2	r
9	" <i>fluorescens non-liquescens</i>	1	R	1	R	1	tr.	1	tr.
1	" of swine fever	1	R	—	—	2	tr.	2	r
11	" <i>intestinalis</i>	1	R	—	—	2	0	2	tr.
2	" of typhoid fever	1	R	—	—	3	0	3	0
3	" of infantile diarrhoea	1	R	—	—	4	0	5	tr.
12	Sp. of Asiatic cholera	1	R	1	R	1	—	1	r
5	B. of septicaemia (mouse)	1	R	—	—	2	tr.	2	r
6	" " (guinea-pig)	1	R	—	—	2	tr.	2	r
4	" of anthrax	3	R	2	R	7	0	6	0
14	Sp. Deneke's	2	R	2	R	3	0	2	n. gr.
22	<i>B. subtilis</i>	6	tr.	2	r	8	0	6	0
10	" <i>fluorescens liquescens</i>	4	0	3	0	6	0	3	0
24	" <i>toruliformis</i>	1	0	2	0	—	—	3	0
25	" <i>sulphureus</i>	1	0	1	n. gr.	2	0	2	n. gr.
26	" <i>tardescens</i>	1	0	1	0	—	—	—	—
13	Sp., Finkler's	1	0	4	0	—	—	3	0
15	" of noma	1	0	2	0	—	—	—	—
21	<i>M. urea</i>	1	R	—	—	—	—	3	0
27	" <i>gelatinosus</i>	1	R	—	—	2	0	3	0
17	<i>Staph. luteus</i>	2	R	1	R	3	0	1	tr.
18	" <i>candidus</i>	1	R	2	R	1	0	1	n. gr.
19	" " <i>liquescens</i>	1	R	—	—	3	0	2	0
20	<i>Streptococcus scarlatina</i>	2	0	1	R	—	—	3	0
16	<i>M. aureus</i>	1	0	5	0	—	—	1	0
				3	0	1	n. gr.	1	0

this bacillus, which was obtained from soil, is thus not one of the denitrifying bacteria of Gayon and Dupetit.

Another means of judging whether reduction has proceeded further than the stage of nitrite is by studying the alkalinity of the solution. If the reduction of potassium nitrate yields gaseous oxides of nitrogen, nitrogen, or ammonia, the solution must become alkaline from the formation of potassium carbonate. With the exception of the broth cultures of *Staph. candidus liquescens*, I have observations of the alkalinity of the broth cultures of each organism at the time when trial was made for nitrites; at this time, 3—10 days from starting the cultures, the reaction of the broth was slightly alkaline in cultivations of *M. ureæ* and *B. fluorescens liquescens*, and decidedly alkaline in the case of *B. fluorescens non-liquescens*; in all other cases, the broth was neutral, or, in a very few instances, feebly acid. With the exception, possibly, of the culture of *B. fluor. non-liq.*, the reduction to nitrites would, therefore, appear to have occurred without the production of nitrogen, oxides of nitrogen, or ammonia. After a considerable time, most of the broth cultures became distinctly alkaline; but this in itself is no proof of the destruction of nitrites, for many organisms render simple broth alkaline after growing in it for some time, the production of alkali being due to the destruction of the organic salts which the juice of meat contains.

As far then as this imperfect evidence goes, none of the organisms examined (with the possible exception of *B. fluor. non-liq.*) possessed the power of reducing nitrates to nitrogen, or to its gaseous oxides. That soil does actually contain organisms which are capable, under certain circumstances, of so reducing nitrates, has been confirmed by my own experiments already described (pp. 742–3).

Nitrites are not normally present in well aerated fertile soils, or at least only to that minute extent in which they can be shown by delicate tests to occur everywhere. The drainage waters collected at Rothamsted only rarely contain sufficient nitrite to give a distinct reaction with the delicate iodide test. Misapprehension has sometimes occurred on this point, as some analysts are in the habit of reporting the quantity of nitrogen present as “nitrates and nitrites,” without apparently ascertaining in each case the presence of nitrites in the water.

NITRIFICATION.

A good many investigations have been published during the last two years, having for their object the discovery of nitrifying organisms, or the farther elucidation of the process of nitrification: little success seems, however, to have attended these endeavours.

Celli and Zuco (*Gazz. chim. Italiana*, **17**, 99) found that a solution of ammonium chloride, containing $\frac{1}{1000}$ its weight of mercuric chloride, passed and repassed two or three times a day through a column of sterilised sand and calcium carbonate, gave, after a few days, a distinct reaction with diphenylamine. The amount of nitric or nitrous acid present was increased when spongy platinum was substituted for the sand. They conclude that the nitrification of ammonia in soil may take place without the intervention of an organism, although organisms may assist in nitrification.

Frank (*Forschungen auf dem Gebiete der Agrikulturphysik*, **10**, 56) expresses the opinion that nitrification is in greatest part an inorganic process. In his hands, soil which had been ignited was still capable of nitrifying ammonium chloride. Frank's experiments have been since repeated by Plath (*Landw. Jahrbücher*, **16**, 891), who comes to the opposite conclusion, that soil when sterilised has no power of oxidising ammonia.

We surely need not controvert at length these conclusions of Celli and Frank, contrary as they are to the accumulated evidence of many well-ascertained facts. I may perhaps refer to one of my own early experiments (*Trans.*, 1878, 44), which seems to give a distinct answer to the question at issue. Similar soil was placed in three tubes, through which air was daily drawn, the air, in one case, containing a little chloroform vapour, in another the vapour of carbon bisulphide. Where air alone was aspirated, the nitric nitrogen in the soil increased from 8 to 50 parts per million; where the air contained the vapour of chloroform, or carbon bisulphide, no appreciable increase of nitric nitrogen took place.

Although, however, we may strongly hold that the nitrifying power of soil is due to the action of a living organism, we have still to explain the appearance of small quantities of nitric or nitrous acid in the experiments above mentioned. It is clear that in all such experiments the presence of traces of nitrites in the atmosphere must always be taken into account. I have already shown (*Trans.*, 1881, 229) that distilled water cannot be freely exposed to air for any length of time without containing traces of nitrous acid, and the presence of this acid is soon manifested if the exposure takes place in a room in which coal-gas is burnt. When, therefore, an experimenter on nitrification makes use of the diphenylamine test, which gives a blue coloration with 1 part of nitric or nitrous nitrogen in 10 millions of water, or the far more delicate iodide and starch test, which will indicate 1 part of nitrous nitrogen in 100 millions of water, he will not improbably obtain a distinct reaction sooner or later, without a living organism having had any share in the operation, or indeed without any oxidation of ammonia having occurred in his solutions.

We turn next to the investigations with living organisms. Celli and Zuco isolated various organisms from the subsoil waters of Rome; these organisms were then grown in a solution containing an ammonium salt. In every case the solution after a time gave a small reaction with diphenylamine. The amount of nitrous or nitric acid did not increase during several months, even when a current of oxygen was passed through the solution. When the cultures were passed and repassed through a column of sand, the reaction with diphenylamine was distinctly increased. All the organisms tried seem to have behaved in a similar manner.

Adametz (*Forsch. Agr.-Phys.*, 1886, 381) isolated 22 organisms from soil. Four of these were grown in an ammoniacal solution. At the end of five weeks an extremely small quantity of nitric acid was present.

Heraeus (*Zeit. f. Hygiene*, 1886, 193) has conducted an investigation on nitrification in Koch's laboratory; about 29 organisms were examined. He obtained a powerful nitrification when a large quantity of soil was placed in a solution of ammonium carbonate. The bacterial skin which formed on the surface of the solution in this experiment was also very active in setting up nitrification. In 6 days the solution seeded with this skin oxidised 1.6 c.c. of indigo, and in 10 days 5.5 c.c. From this bacterial skin he isolated a bacillus, and a streptococcus; these, together with a bacillus and yeast isolated from fermenting urine, were grown in an ammoniacal solution similar to that already employed. All the solutions gave a distinct reaction for nitrous acid with the iodide and starch test, but the amount was too small to determine with indigo. About 13 organisms of well-known kinds were also grown in a 20 per cent. urine solution. Seven of these organisms produced in one day's growth in the incubator sufficient nitrous acid to give a strong reaction with the iodide and starch test. Heraeus concludes that these seven organisms, and the four previously mentioned, possess oxidising properties, and are capable of forming nitric acid.

Frank (*loc. cit.*) separated a number of organisms from soil, but failed on trial to obtain nitrification with any of them.

Leone (*Atti d. R. Accademia d. Lincei*, 1887, 37) concludes from his experiments that all micro-organisms are more or less capable, under favourable conditions, of producing nitric acid, and that the same organisms in the presence of organic matter are capable of reducing nitrates. Muntz (*Ann. Chim. Phys.*, 1887, **11**, 128) also cautiously observes, that organisms which appear identical with those which produce nitrification are capable of reducing nitrates when air is excluded.

Manly Miles (*Agricultural Science*, 1887, 102) writes as if he had

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pure cultures of the nitrifying organism under experiment, but he gives no description of the organism.

P. Frankland (Trans., 1888, 389) grew 32 organisms obtained from air and water in a nutritive solution containing an ammonium salt for 40 days; he obtained in no case more than a faint indication of nitrous acid.

It seems very clear that not one of the investigators who have experimented with isolated species of bacteria has obtained in his solutions more than a trace of nitrous or nitric acid; *no one has obtained an amount that could be determined quantitatively*. Another point which generally appears is that every organism tried gives nearly the same result. The failure to obtain the nitrifying organism in a separate form is most conspicuous in the case of Heraeus. He had in his hands a bacterial skin which possessed energetic nitrifying properties; but the two organisms which he separated from this skin gave, in a solution of the same composition as that previously nitrified, nothing more than the usual trace of nitrous acid.

The statement of Heraeus that seven of the organisms examined commenced the nitrification of a 20 per cent. urine solution in one day is apparently due to a mistake. My own experiments show that a urine solution of that strength cannot be nitrified by soil without the addition of gypsum: the commencement of nitrification in a strong solution is also extremely slow (Trans., 1884, 661). The nitrous acid which so speedily appeared in his solutions was doubtless due to the reduction by the organisms of the nitrates naturally present in the urine (Trans., 1884, 669).

Celli and Zuco, Leone, and others, apparently believe that the oxidising or reducing action of an organism is determined by the conditions in which it is placed, and that the same organism is capable of discharging both functions. The capacity for exercising the entirely opposite functions of nitrification, and the reduction of nitrates, is indeed possessed by soil and by river water, mediums which contain a multitude of different organisms, but it cannot as yet be said to be proved of any single organism growing in a pure culture. The investigations of P. Frankland, and myself, have shown that all bacteria do not reduce nitrates, even under specially favourable conditions, and one that is capable of unmistakably oxidising ammonia has not apparently been at present isolated.

My own results respecting the nitrifying power of isolated organisms admit of a very brief description. It has been already stated that two of the organisms, *B. sulphureus*, and *B. tardecrescens*, had been separated from visible growths occurring in solutions which had nitrified. Two other organisms, *B. floccus* and *B. toruliformis*, had been obtained from soil. The remaining organisms had not an origin

that would suggest their possession of the nitrifying properties belonging to soil. The four organisms which appeared most likely to include one possessing nitrifying properties were submitted to several trials; 20 other organisms were mostly tried but once. Four different solutions were employed, the composition being varied with the view of obtaining growth with a minimum amount of carbonaceous matter present. The examination of the solutions extended in every case over many weeks. When a fragment of arable soil was added to any of these solutions, nitrification distinctly commenced within a fortnight, and in another fortnight all the ammonia (equal to about 25 of nitrogen per million of solution) had disappeared. None of the pure organisms experimented with gave any such result. A distinct reaction with diphenylamine was in some cases obtained, but this did not appear to grow in amount, although in such cases the examination was specially prolonged. The amount of nitric or nitrous nitrogen in the solutions did not apparently in any case exceed 1 per million, and all of this could not be attributed to the action of the organism, as the unseeded solutions in the incubator also gave some reaction with diphenylamine. When we have discounted the trace of nitrites probably obtained from the atmosphere, there is clearly very little left that can be attributed to the action of the organism. The question whether any part of the nitrate or nitrite present was produced by the organism I am unable to decide; but it is quite clear that none of the organisms examined possessed any nitrifying power in any way comparable with that possessed by soil. An organism which nitrifies as soil nitrifies has yet to be isolated.