A RESEARCH INTO THE NATURE AND ACTION OF THE ENZYMES PRODUCED BY THE BACTERIA.

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THE researches of Nencki, Brieger, Drechsel, and others have greatly advanced our knowledge respecting the composition of proteid bodies. How great the advance has been will be seen by anyone comparing their researches with the earlier investigations of Liebig, Hlasiwetz, and Schutzenberger. The data obtained are, however, not yet sufficient to enable one to formulate a satisfactory hypothesis with regard to the constitution of proteids. The varied nature of the bodies that arise from the disintegration of proteids indicates how complex their chemical structure must be. The proteid molecule is itself a "complex" of groups of atoms. These groups yield different decomposition products, as *e.g.* Leucin, Indol, Skatol, Methylmercaptan, &c. Chemical research has not yet exhausted the number of these groups or of their derivatives. The chemical structure of the proteids of the living cell is still more complex. Of their properties we know something, of their constitution nothing or next to nothing. These proteids have, however, certain marked characteristics which differentiate them from the metabolic products of the cell itself. The Enzymes are the most familiar example of this group of proteids. In these bodies the atoms of the proteid molecule have undergone a change in position. This may be expressed by saying that they have passed from a stable to an unstable condition. If we consider proteids like the peptones we find that they are stable bodies. Heat, light, &c., do not change their properties; their atoms seem to be in a state of rest. It is different with the enzymes. By the action of heat, light, acids, &c., they lose in part or wholly their characteristic features, and pass from an unstable to a stable modification. They have therefore been termed by the chemist "groups in motion" on account of their close resemblance in

molecular constitution to unstable organic compounds of an aldehyde nature. The enzymes thus share the susceptibility of the protoplasm that produces them to the action of external agents.

As already stated, the most familiar examples of these unstable albumens are the enzymes. Recent bacteriological research has, however, made us acquainted with a group of bodies to which the name of Toxalbumens has been given. Though only a few have as yet been isolated, their number is probably large. The toxalbumens, as regards their properties, have much in common with the enzymes. Just as heat, light, acids, &c. weaken or destroy the ferment action of the enzymes, so do the same agents weaken or destroy the toxic action of the toxalbumens. The toxalbumens are therefore unstable bodies. Like the enzymes they are soluble bodies, and can be precipitated out of their solutions by alcohol. The continued action of alcohol weakens or destroys their poisonous properties—*e.g.*, the toxalbumens of diphtheria and tetanus. The enzymes are innocuous in the digestive tract; they are toxic when injected subcutaneously. The toxalbumens behave in the same way.

The toxalbumens and the enzymes have therefore many points in common, and they are probably closely related in constitution.

It is now generally accepted as proved that the unicellular organisms produce enzymes. It seemed probable to the writer that the study of the formation of these enzymes would also throw light on the mode of production of the toxalbumens. Further, that the methods found best adapted for the demonstration of the action of the enzymes would be the most suitable to use for the study of the toxalbumens. An answer might also be found to an important question: Are the toxalbumens true proteids formed within the bacterial cell itself? I do not refer here to the albuminates. This research is confined to the genuine albumens elaborated by the living cell; which stand closest to it in constitution, and cannot be heated above 50° C. for any length of time without losing wholly or in part their characteristic properties. I now proceed to give the results of my experiments. I am greatly indebted to Professor

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I.

Upon the production of a proteolytic Enzyme by the Bacteria.

The first series of experiments was made with a group of Comma organisms, viz. :—

1. Koch's Comma bacillus of Cholera asiatica.
2. Deneke's cheese spirillum.
3. Vibrio Metschnikoff.
4. Finkler and Prior's spirillum.

These micro-organisms are convenient to work with, as a comparatively low heat kills them. It is thus possible to sterilise cultures of these organisms at a temperature below 100° C. without completely destroying the ferment action of the fluid. Four series of experiments were made.

A. With gelatine cultures.
B. With simple meat-broth cultures.
C. With peptone meat-broth cultures.
D. With extracts of the bacteria.

A. *Experiments with Gelatine Cultures.*

Pure cultures of the above-mentioned bacteria were made in 10% gelatine. These were kept in the incubator at 20° C. for 7–10 days. The tubes were then sterilised in the water-bath at 60°–65° C. for ½ to ¾ of an hour. A series of tubes containing 10 c.c. of sterile 10% and 7% gelatine respectively were liquefied, and a few crystals of menthol added. To each of these tubes were added 2 c.c. or 1 c.c. of the sterilised gelatine cultures of the bacteria. They were then placed in the incubator at 39° C. along with control tubes of 10%, 7%, and 5% gelatine. The inoculated and control tubes were taken out of the incubator at intervals of 24 hours. The gelatine was then cooled down, and kept in a cool place to see if any restiffening took place, or if it remained liquid. Sterile gelatine was also inoculated from the tubes in order to make sure that no living bacteria were present.

The experiments gave the following results :—

Series 1.—2 c.c. of the sterilised gelatine cultures of the bacteria

(7–10 days growth) added to 10 c.c. of 10% gelatine and 7% gelatine.

Day Examined.	10% Gelatine.				7% Gelatine.			
	1	2	3	4	1	2	3	4
1. Cholera, . . .		+	+	+		+	+ +	
2. Deneke, . . .		+	+	+		±	+ +	
3. Metschnikoff, . .		+	+	+	+	+ +		
4. Finkler and Prior, . .		+	+	+ +	+ +			

Series 2.—1 c.c. of the sterilised gelatine culture of the bacteria (7–10 days growth) added to 10 c.c. of 10% and 7% gelatine.

Day Examined.	10% Gelatine.				7% Gelatine.			
	1	2	3	4	1	2	3	4
1. Cholera, . . .			+	+			+	+ +
2. Deneke, . . .			+	+			+	+ +
3. Metschnikoff, . .		+	+	+			+ +	
4. Finkler and Prior, . .		+	+	+ +	+	+ +		

\+ = partial liquefaction of the gelatine.
\+ + = complete liquefaction of the gelatine.

Series 3.—2 c.c. of the sterilised gelatine cultures of the bacteria (4–6 weeks old) added to 10 c.c. of 10% and 7% gelatine.

It will not be necessary to tabulate the results of these experiments. Only the sterilised gelatine cultures of the Metschnikoff and Finkler and Prior organisms produced any distinct liquefaction of the fresh gelatine. The liquefaction also was after four days partial and not complete.

Series 4.—2–5 c.c. of the sterilised gelatine cultures of the bacteria (7–10 days growth) were added to *Fibrin*.

After four days there was no apparent disintegration of the fibrin by the Cholera and Deneke cultures. The Metschnikoff and Finkler and Prior cultures produced a partial disintegration.

These experiments lead one to the following conclusions:—
The bacteria which liquefy gelatine do so by means of a ferment-

like body or enzyme which is capable of exercising its liquefying power on gelatine apart from the cells that produced it. The *sterilised* cultures of the bacteria liquefied fresh gelatine. This fact had already been proved by the researches of Sternberg, Bitter, Lauder Brunton and the writer, and more recently by Fermi. The above experiments confirm therefore the results obtained by these observers. I may, however, mention here that *boiled* gelatine cultures of the bacteria did not liquefy fresh gelatine. The liquefaction was not, therefore, due to the action of any acid. The boiling of the cultures destroyed not only the bacteria, but also the ferment produced by them.

With regard to the liquefying power of the sterilised cultures of the bacteria on gelatine, I found that the most active were the Metschnikoff and Finkler and Prior cultures. The latter, in their turn, had a more energetic action than the Metschnikoff cultures, and were the only ones able to liquefy completely 10% gelatine.

A complete liquefaction of 7% gelatine was produced by sterile cultures of all four organisms, but here again the Metschnikoff and Finkler and Prior cultures had a more energetic action than the Deneke and Cholera. As regards liquefying power on gelatine they stood as follows:—1, Finkler and Prior; 2, Metschnikoff; 3, Cholera; 4, Deneke.

The *old* cultures of the organisms either did not liquefy gelatine, or their action was very feeble. After 4–6 weeks the Cholera and Deneke cultures did not liquefy gelatine. The Metschnikoff and Finkler and Prior cultures showed a very faint action. In these old cultures the metabolic products of the bacteria had probably weakened or destroyed the ferment produced by the cells. Finally, the action on fibrin was very feeble.

The experiments made to determine the production of enzymes by the bacteria have in most cases been carried out with gelatine cultures of the micro-organisms. The method, however, does not give completely satisfactory results. The action on fresh gelatine is often only partial or very slow, whilst on fibrin it sinks to a minimum. The gelatine cultures seem to contain very little of the proteolytic enzyme. It is probable that in a highly nutrient medium like peptone gelatine the bacteria secrete

very little of the enzyme. There is so much easily assimilated nutriment present that the bacteria are able to live without any "struggle for existence" on their part. In such a rich soil the amount of food is far in excess of the requirements of the bacteria, and there is less necessity for an active secretion of their ferment. Further, in a solid medium less of the enzyme passes out of the cells by simple solution than in a fluid medium.

The ferment, too, is mixed with the various metabolic products of the gelatine, which tend to weaken its action. The old gelatine cultures of the bacteria had little or no liquefying power on fresh gelatine. The above factors would explain the relatively small effect of the sterile liquefied gelatine cultures on 10% gelatine, and the feeble action on fibrin. It is probably mainly conditions of nutrition that determine the amount of the ferment secreted by the bacteria.

B. *Experiments with Simple Meat-Broth Cultures.*

The usual meat infusion was used, *i.e.*, the broth obtained by adding to 1 lb. of beef one litre of water.

To the infusion ½ per cent. of sodic chloride was added, and the fluid was rendered faintly alkaline with carbonate of soda.

Flasks containing 100 c.c. of this broth were inoculated from cultures of the four common organisms, and placed in the incubator at 39° C. (In the case of Deneke's spirillum, the optimum temperature was 30° C.) On the third or fourth day the broth cultures were transferred to test-tubes, and sterilised at 60°–65° C. in the water-bath, *i.e.*, they were treated in exactly the same way as the gelatine cultures.

I will here detail only the experiments with 10% gelatine and with fibrin, as they will amply illustrate the results obtained.

Series 1.—2 c.c. of the sterile meat-broth cultures of the bacteria, added to 10 c.c. of 10% gelatine + menthol.

Day Examined.	1	2	3
1. Cholera, ...		+	+ +
2. Deneke, ...		+	+ +
3. Metschnikoff, ...		+	+ +
4. Finkler and Prior, . . .	+	+ +	

\+ = partial liquefaction of gelatine.
\+ + = complete liquefaction of gelatine.

Series 2.—5 c.c. of the sterile meat-broth cultures of the bacteria, added to fibrin + menthol.

Results:—

1. *Cholera*, Complete disintegration of the fibrin on the 5th day.
2. *Deneke*, „ „ „ 5th day.
3. *Metschnikoff*, „ „ „ 4th day.
4. *Finkler and Prior*, „ „ „ 3rd day.

In control tubes containing 5 c.c. of fresh broth + fibrin and menthol, the fibrin remained unchanged.

Fresh gelatine was also inoculated from the tubes containing the sterilised meat-broth cultures + fibrin or gelatine. In neither case did any bacteria grow in the control tubes.

Series 3.—The meat-broth cultures of the bacteria were also concentrated to one-half of their volume in Brieger's vacuum distilling apparatus. Of this concentrated fluid 5 c.c. was added after sterilisation at 60°–65° C. to fibrin + menthol.

Results:—The Cholera and Deneke cultures, treated in this fashion, digested the fibrin on the fourth day. The Metschnikoff and Finkler and Prior cultures digested the fibrin on the third day.

The digestive action on fibrin was therefore a little stronger in the concentrated than in the unconcentrated meat-broth cultures.

Series 4.—A series of experiments made with *Eggalbumen*.

The results of these experiments may be mentioned here, as they serve to bring out one interesting point.

5 c.c. of the sterilised and unconcentrated meat-broth cultures of the bacteria were added to coagulated eggalbumen + thymol. The tubes were placed in the incubator at 39° C.

The Cholera and Metschnikoff cultures disintegrated the eggalbumen on the *second* or *third* day.

The Deneke and Finkler and Prior cultures only disintegrated the eggalbumen on the *fourth* or *fifth* day. The ferment action of the Cholera and Metschnikoff cultures was therefore more energetic on eggalbumen than on fibrin, and exceeded that of the Deneke and Finkler and Prior cultures. On the other hand, the Finkler and Prior cultures had a more energetic action on fibrin than any of the others. Thus the proteolytic action of the enzymes produced by the bacteria varies on different forms of albumens. The proteolytic action may be weak on fibrin, and yet strong on eggalbumen. The reverse also holds true. The ferment action of the bacteria varies, therefore, with the nature of the soil in which they grow.

C. *Experiments with Peptone Meat-Broth Cultures.*

2% of peptone and 0·5% of sodic chloride were added to the meat infusion, and the fluid made faintly alkaline with carbonate of soda.

Flasks containing 100 c.c. of this culture fluid were inoculated from pure cultures of the bacteria, and placed in the incubator at

39° C. On the third or fourth day the peptone broth cultures were transferred to test-tubes, and sterilised at 60°–65° C. in a water-bath. The ferment action of these sterilised cultures was tested on gelatine and on fibrin.

Series 1.—2 c.c. of the sterilised peptone meat-broth cultures of the bacteria, added to 10 c.c. of 10% gelatine + menthol.

Results:—

Day Examined.	1	2	3	4
1. Cholera, ...		+	+	++
2. Deneke, ...		+	+	+
3. Metschnikoff, . . .		+	+	++
4. Finkler and Prior, . .		+	+	++

\+ = partial liquefaction of gelatine.
\+ + = complete liquefaction of gelatine.

Series 2.—5 c.c. of the sterilised peptone meat-broth cultures of the bacteria added to fibrin + menthol.

Results:—

1. Cholera, } *No* complete disintegration of fibrin on the *fifth* day.
2. Deneke, }
3. Metschnikoff, } Complete disintegration of fibrin on the *fifth*
4. Finkler and Prior, } day.

The experiments detailed under headings B. and C. lead one to the following conclusions:—

The simple meat-broth cultures of the bacteria yield a more active proteolytic enzyme than the gelatine cultures. There is more of the enzyme present in the simple meat-broth than in the gelatine. Further, the simple meat-broth cultures have a more energetic action on fibrin than the richer and more nutrient broth which had been rendered so by the addition of 2% of peptone. The amount of the soluble ferment albumen secreted by the cells is determined by the nutritive requirements of the micro-organisms. Less is secreted in a rich soil than in a poor soil, where a greater effort has to be made for nutrition. The luxuriance of the bacterial growth is no criterion as to the amount of the enzyme present in the culture fluid. The growth of the bacteria was more abundant in the peptone

broth than in the simple meat-broth, yet the latter had a more energetic ferment action.

When a micro-organism is placed under conditions inimical to its growth, more of the soluble cell proteids pass over into the surrounding medium than is the case where the conditions are favourable. This fact would have great pathological interest if we can prove that these unstable soluble proteids of the bacterial cells possess toxic properties. I will detail later on some animal experiments with reference to this point.

The above experiments have shown that the best medium for the productions of these soluble cell proteids is simple meat-broth, and that with it the best results are to be obtained.

There is evidently only a fractional amount of the enzymes present in the gelatine cultures of the bacteria. A much larger amount is present in the meat-broth cultures, though the amount is also probably fractional. The enzymes being soluble bodies, would it not be possible to extract them directly from the bacterial cells? If successful, this would undoubtedly be the best method. We would obtain the bacteria as free as possible from their metabolic products; and the extracts would contain the soluble cell proteids in a purer form.

The following experiments were made with the view of elucidating this point.

D. *Experiments with Extracts of the Bacteria.*

A small quantity of nutritive Agar-Agar was placed in Erlenmeyer flasks of 200–250 c.c. capacity. The Agar-Agar, after sterilisation, was allowed to stiffen on one side of the flask. In this way a large surface was obtained for inoculation with the bacteria. The Agar was inoculated from pure cultures of the Cholera; Deneke; Metschnikoff and Finkler and Prior organisms. The bacteria, by means of a platinum wire, were well distributed over the whole surface of the Agar. The flasks were then placed in the incubator at 39° C., with the exception of the Deneke cultures, which grew best at 30° C.

In this way a large surface growth of the bacteria was obtained in two to three days. The growth was then scraped off with a blunt, sterilised platinum spatula, and transferred to small Erlenmeyer flasks or to test-tubes.

The following methods of extraction were tested.

(1) *Extraction with Chloroform Water.*

Chloroform water was added to the bacteria, and the tubes or flasks placed in the incubator at 39° C. for 3–4 days. They were

then kept at 30° C. for three days longer. At the end of that time they were taken for the experiments. It was found that the bacteria settled at the bottom of the tube or flask, and the supernatant fluid could thus be removed with a pipette from the deposit.

5 c.c. of the watery extract were added to fibrin or eggalbumen. I found that the digestive action on these proteids was very slight, and was not to be compared with the ferment action of the meat-broth cultures of the same bacteria. It was evident that simple extraction with water alone would not suffice. These experiments were therefore abandoned.

(2) *Extraction with pure undiluted Glycerine.*

These experiments also did not give any satisfactory results.

(3) *Extraction with Glycerine + Water.*

It was found that this method gave the best results, and that the best concentration to use was a 40% solution of glycerine in water. The bacteria were cultivated on agar in the manner already described. On the third or fourth day the growth was scraped off with a platinum spatula, and placed in small Erlenmeyer flasks or in tubes. The 40% glycerine was then added. The bacteria were extracted for four days at 39° C., and for three days at 30° C., altogether one week.

The same bacteria were used as on the previous experiments. I found that the comma organisms were particularly well adapted for extraction with glycerine. For example, the solvent action of the glycerine on Koch's comma bacillus is so great that the organisms disappear almost entirely. The comma micro-organisms would therefore probably yield the best results. If one succeeded at all, it would be with these bacteria. Experiments made with them would furnish data for further researches with other bacteria. For these reasons the first series of experiments was made with the spirilli.

When the remains of the bacteria had settled at the bottom of the tube or flask, the supernatant fluid was removed with a pipette. An advantage of this method is that it obviates the necessity of sterilising the fluid by heating. The glycerine itself has an inimical action on the bacteria, and if one adds a small amount of thymol or menthol the extracts remain absolutely sterile.

The glycerine extracts prepared as above were tested on gelatine, fibrin, and eggalbumen.

I. *Experiments with Gelatine.*

1*st Series.*—1–2 c.c. of the glycerine extracts of the bacteria were added to 10 c.c. of 7% gelatine in test-tubes, so that the extract formed a layer on the surface of the gelatine. A mark was made on the outside of the tubes at the line of junction between the gelatine and the extract. In this way one could observe if any liquefaction of the gelatine took place from above downwards. I am

indebted to Dr Fermi for this method. The tubes were kept in the incubator at 20° C.

Results:—The amount of liquefaction noted on the fourth day was as follows:—

1. Cholera, . . . $\frac{1}{4}$ inch of gelatine liquefied.
2. Deneke, . . . $\frac{1}{4}$ inch ,, ,,
3. Metschnikoff, . . $\frac{1}{2}$ inch ,, ,,
4. Finkler and Prior, . $\frac{3}{4}$ inch ,, ,,

2nd Series.—1 c.c. of the glycerine extracts of the bacteria was added to 10 c.c. of 7% gelatine. The tubes were kept at 30° C. and 39° C. On the third day the glycerine extracts of the bacteria had completely liquefied the gelatine. In the case of Finkler and Prior the gelatine was liquefied on the second day. The results were the same when the tubes were kept at a temperature of 39° C. or 30° C.

II. *Experiments with Fibrin.*

3–5 c.c. of the glycerine extracts of the bacteria were added to fibrin + menthol. The tubes were kept in the incubator at 39° C.

Results:—

1. *Cholera*, } Complete disintegration of the fibrin on the third to
2. *Deneke*, } fourth day.
3. *Metschnikoff*, ,, ,, on the second to third day.
4. *Finkler and Prior*, ,, ,, on the second day.

III. *Experiments with Eggalbumen.*

3–5 c.c. of the glycerine extract of the bacteria added to eggalbumen + menthol.

Results:—

1. *Cholera*, Complete disintegration of eggalbumen on second to third day.
2. *Deneke*, ,, ,, on fourth to fifth day.
3. *Metschnikoff*, ,, ,, on second to third day.
4. *Finkler and Prior*, ,, ,, on fourth to fifth day.

Control inoculations were in all cases made on fresh gelatine, to make sure that the fluids had remained sterile.

These experiments demonstrate that the glycerine extracts of the bacteria contain a proteolytic enzyme which liquefies gelatine and digests fibrin and eggalbumen.

Glycerine extracts which had stood in the laboratory for two months had little or no effect on gelatine, fibrin, or eggalbumen. The proteolytic ferment produced by these bacteria is evidently of a very unstable nature, and when separated from the cells that produce it becomes readily converted into an inactive modification.

Whilst the fresh glycerine extracts had a distinct ferment action, there were variations in the action due to the nature of the soil. Thus the Cholera and Metschnikoff extracts had a more energetic action on eggalbumen than the extracts of the Finkler and Prior spirillum. On the other hand, the Finkler and Prior extract had a more energetic action on fibrin than either the Cholera or Metschnikoff extract.

To sum up shortly the results of these experiments:—

1. The fresh glycerine extracts made from young agar cultures of the Cholera, Deneke, Metschnikoff and Finkler and Prior spirilli contain an enzyme which, like trypsin, is capable of digesting proteids.

2. The enzyme, when extracted from the cells that produce it, does not retain its ferment action for any length of time. At the end of two months the glycerine extracts had no action on proteids.

3. The ferment action of the glycerine extracts varies according to the nature of the soil, as the experiments with fibrin and eggalbumen prove.

The filtering of the glycerine extracts through biscuit porcelain cells has presented difficulties which I have not yet satisfactorily overcome.

In a second paper the results will be given of experiments made with the glycerine extracts of anthrax and other pathogenic bacteria.

II.

Upon the presence of a diastatic Enzyme in the Glycerine extracts of the Bacteria.

It was next of interest to ascertain if the glycerine extracts of the bacteria contained a *diastatic enzyme.*

The action of pure cultures of the bacteria on starch was first tested.

A starch paste was made by boiling 1·5 – 2% of starch in water. This paste was poured whilst still hot into test-tubes, and sterilised in the usual way. On cooling, some starch flocculi settled at the bottom of the tubes: these flocculi enable one to gauge in a rough fashion the action of the bacteria on the starch. The first change undergone by the starch is its conversion into a soluble form. If one shakes a tube containing sterile starch paste, the flocculi do *not* disappear: on the other hand, if one shakes a tube in which a micro-organism is diastasing the starch, the flocculi disappear and pass

completely into solution. Further, the paste loses its milky appearance, it becomes watery and transparent. The control tube, on the other hand, remains viscid, milky, and opaque. In this fashion one can note with what rapidity the starch is being diastased.

The starch paste was inoculated from pure cultures of the Cholera, Deneke, Metschnikoff, and Finkler spirilli. It is an advantage to add at the same time a few drops of meat-broth. The tubes were kept at 39° C. or 30° C.

Results:—

1. *Cholera*, Starch flocculi disappear on second day.
2. *Deneke*, „ „ on third day.
3. *Metschnikoff*, „ „ on third day.
4. *Finkler and Prior*, Starch flocculi still present on fifth day.

One sees from these preliminary experiments that the first three microbes had an action on the starch, and that the fourth produced no apparent change. The starch cultures were next tested chemically. If one wishes to compare the results obtained with the different bacteria, other tests must be employed besides Fehling's. It is then important to ascertain not only if sugar is present, but if the starch has been wholly or partially diastased by the bacterium.

The method used in this research was based on the successive changes starch undergoes when being converted into sugar. These are as follows:—

1. *Original starch*,
2. *Soluble starch* (amylo dextrin) } + Iodine = blue.
3. *Dextrines*, Erythro-dextrin + Iodine = violet and red.
   Achroo-dextrin
   Malto-dextrin } + Iodine = 0.
4. *Maltose*, { Reduces Fehling's solution. Does not reduce Barfoed's reagent.
5. *Dextrose*, { Reduces both Fehling's solution and Barfoed's reagent.

Another test employed was found to be very useful,—viz., the addition of caustic soda to the starch paste. If no yellow coloration is produced, dextrose is not present; if the fluid becomes yellow, either dextrose, maltose, or milk sugar may be present. I found that the intensity of the colour produced by adding caustic soda was always proportional to the amount of reduction of Fehling's solution —*i.e.*, the deeper the tinge of yellow produced, the greater the reduction of Fehling, and *vice versa*.

Barfoed's reagent consists of 1 part of the neutral acetate of copper dissolved in 15 parts of water. To 200 c.c. are added 5 c.c. of a 38% solution of acetic acid.

Results:—Starch paste inoculated from pure cultures of the bacteria.

	Day of Examination.	Caustic Soda.	Iodine.	Fehling.	Barfoed.
1. Cholera.	1	faint yellow	blue	faint reduction	0
	2	deep yellow	violet	distinct reduction	,,
	3	orange yellow	red	copious pp.	,,
	4	,,	0	,,	,,
2. Deneke.	1	0	blue	0	,,
	2	faint yellow	,,	faint reduction	,,
	3	yellow	violet	distinct reduction	,,
	4	,,	red	,,	,,
3. Metschnikoff.	1	0	blue	0	,,
	2	yellow	,,	distinct reduction	,,
	3	,,	violet	copious pp.	,,
	4	orange yellow	red	,,	,,
4. Finkler and Prior.	1	0	blue	0	,,
	2	0	,,	,,	,,
	3	faint yellow	,,	,,	,,
	4	,,	,,	faint reduction (?)	,,

The tubes were kept at a temperature of 30° and 39° C.

From these experiments it will be seen that the Cholera, Deneke, and Metschnikoff organisms diastased the starch. The action of the Finkler and Prior spirillum was almost *nil*; on the fourth day there was only a faint reduction of Fehling's solution. The Cholera spirillum had the most active diastatic action; its action was more rapid and energetic than that of the Deneke or Metschnikoff organisms. As already stated, the action of the Finkler spirillum was almost *nil* on the starch. The Finkler spirillum which had the strongest action on gelatine, had the weakest action on starch. On the other hand, the Cholera spirillum, which had the weakest liquefying power on gelatine, displayed the strongest diastatic action on starch. The action on the starch was marked at the end of twenty-four hours, when Fehling's solution was reduced. The starch cultures of the Deneke and Metschnikoff organisms first reduced Fehling's solution after forty-eight hours. On the fourth day the starch

had disappeared altogether in the Cholera cultures, and no reaction was given with iodine. None of the other organisms completely diastased the starch. The experiments I have made lead me to the conclusion that the *diastatic* action of the Cholera spirillum greatly exceeds its proteolytic action. In other words, its ferment action is more marked on carbohydrates than on proteids. If we compare the ferment action of the Finkler with that of the Cholera spirillum, we may say that the characteristic enzyme in the former case is a proteolytic, in the latter a diastatic one.

The Cholera spirillum is thus specially adapted for growth in a carbohydrate soil. The presence of carbohydrates will rather favour than hinder its growth. If a carbohydrate food such as rice is present in the digestive tract, it will furnish a most favourable nidus for the Cholera organism. In such a soil it will rapidly multiply, and set up at the same time a most active fermentation.

The action of the glycerine extracts of the bacteria on starch paste was next tested.

The glycerine used had no reducing action on Fehling's solution, nor had it any action on the starch.

1 c.c. of the sterile glycerine extracts of the bacteria was added to the starch paste, with the following results :—

	Day of Examination.	Caustic Sod.	Iodine.	Fehling.	Barfoed.
1. Cholera.	1	0	Blue	0	0
	2	Faint Yellow	Blue	Reduction	,,
	3	Yellow	Violet	do.	,,
	4	do.	Red	do.	,,
2. Deneke.	1	0	Blue	0	,,
	2	Faint Yellow	do.	0	,,
	3	do.	do.	Reduction	,,
	4	Yellow	do.	do.	,,
3. Metschnikoff.	1	0	Blue	0	,,
	2	Faint Yellow	Violet	0	,,
	3	Yellow	do.	Reduction	,,
	4	do.	do.	do.	,,
4. Finkler and Prior.	1–4	0	0	0	,,

It will be seen that the glycerine extract of Finkler's spirillum had no action on the starch. On the other hand, the extracts of the Cholera, Deneke, and Metschnikoff spirilli diastased the starch. The Cholera extract was the most active. The starch

paste to which it was added reduced Fehling's solution on the second day. The negative results with Barfoed's reagent indicate that the sugar produced by the bacteria is probably of the nature of a maltose.

These experiments prove that the glycerine extracts of the bacteria which diastase starch contain an enzyme which, when separated from the cells that produce it, is capable of converting starch into sugar. Further, it was already shown that the glycerine extracts of the same bacteria contain also a proteolytic enzyme.

Experiments were also made with a view of determining the presence or absence of a *milk curdling ferment* in the glycerine extracts of the bacteria. These experiments are still being carried on, but the results are not yet of a sufficiently definite character.

It is of great importance to investigate the influence of the bacteria on the *coagulating power* of the blood. This question has also engaged the attention of the writer, and will be dealt with in a future paper. One interesting point may, however, be mentioned here.

A nutrient medium was prepared, consisting of blood serum, glycerine, and water. This mixture did not coagulate on boiling. It was inoculated from pure cultures of the Cholera and the Finkler spirillum. The organisms grew well in this soil. On the third day after inoculation there was a complete coagulation of the blood serum in the tubes inoculated with the Cholera spirillum. There was no coagulation in the tubes inoculated with Finkler's spirillum. This striking difference in the action of these two organisms on the blood serum opened up a fresh line of inquiry, and when sufficiently advanced its results will be given.

The action of the Glycerine extracts of the Bacteria on Loew's Reagent.

In the introduction to this paper mention was made of certain striking differences that exist between the proteids of the living cell, and other forms of albumens such as the Peptones. The latter are stable bodies. By the action of heat, light, &c. they do not change their properties. The Enzymes, on the other

hand, are unstable proteids. Heat, light, acids, and alkalies weaken and finally destroy their characteristic properties. In this respect they share the sensitiveness of the protoplasm itself to external agents. The toxalbumens produced by the bacteria have also an unstable constitution. They are readily transformed into an inactive modification, and lose their toxic properties. The chemical structure of the unstable proteids differ, therefore, from that of the stable. What that difference really is, remains a matter of conjecture. The distinction may consist in a different grouping of the atoms in their molecule. As regards their unstable chemical constitution, an analogy is to be found in certain organic chemical compounds. The aldehydes, for instance, are very unstable bodies. The aldehydes contain the aldehyde group $C\langle^{H}_{O}$. They easily undergo oxidation on simple exposure to the air,—*e.g.*,

$$\underset{\text{Acetaldehyde.}}{CH_3-C\langle^{H}_{O}} = \underset{\text{Acetic Acid.}}{CH_3COOH.}$$

Further, they easily reduce metallic oxides, especially the oxide of silver, in alkaline solution.

$$CH_3-C\langle^{O}_{H} + Ag_2O = CH_3.COOH + Ag_2.$$

Nencki also found that very dilute alkaline solutions of albumens absorbed oxygen slowly from the air. Loew's well-known researches prove that the living protoplasm of certain Algæ reduces a dilute alkaline solution of silver. In this respect the chemical structure of the albumens of the living cell may resemble that of those organic bodies which contain in their molecule the unstable and easily oxidised aldehyde group $C\langle^{H}_{O}$. The dead protoplasm does *not* reduce the oxide of silver.

The question suggested itself—Have the active ferments produced by the bacteria a similar action on the oxide of silver to that of the living protoplasm?

The glycerine extracts of the bacteria were accordingly tested with Loew's reagent. It was possible that Loew's reagent might be a valuable test for ascertaining the presence or absence of an active ferment in the glycerine extracts of a micro-organism.

The fresh glycerine extracts of the Cholera, Deneke, Metschnikoff, and Finkler spirilli were added to Loew's reagent along with a small quantity of menthol. The glycerine and the menthol did not reduce the silver solution. The fresh glycerine extracts of the Cholera spirillum and the vibrio Metschnikoff produced a distinct reduction of the oxide of silver. The extracts of the Deneke and Finkler spirilli also reduced the silver solution, though not so actively as was the case with the two former organisms. The tubes during the experiments were protected from the action of light.

The active reduction of the silver by the extracts of the Cholera spirillum and vibrio Metschnikoff might be due to the presence of a larger amount of the soluble cell proteids in the extracts than was the case in the extracts of the Deneke and the Finkler spirillum.

A large number of experiments will have to be made with other micro-organisms before one is in a position to estimate the value of this test. These experiments will decide if the reducing action on silver is a general one, or confined to certain bacteria.

Loew's Reagent is prepared as follows:—

No. I. 13 c.c. of a solution of Potash—1·333 spec. gr.
10 c.c. of Ammonia—0·96 spec. gr.
77 c.c. of Water.

No. II. 1 per cent. solution of Nitrate of Silver.

Immediately before use 1 c.c. of No. I. is mixed with 1 c.c. of No. II. and diluted to a litre.

The glycerine extracts of the bacteria do not, of course, contain the active ferments produced by the bacteria in a pure form. They are mixed with other soluble albumens of the cell protoplasm, of whose nature we as yet know little.

The glycerine extracts yielded precipitates with alcohol; ammonium sulphate; acetic acid and ferrocyanide of potassium. Also with acetic acid and sodic chloride. The glycerine extracts therefore contain a group of bodies of an albuminose nature. The

active ferments adhere to the alcoholic precipitates, though the alcohol weakens their action. The alcoholic precipitates produced a partial liquefaction of gelatine, and had also a feeble diastatic action on starch. The writer is still engaged upon the chemical examination of these precipitates.

Animal Experiments.

The action of the glycerine extracts on animals was next tested.

I will here detail experiments made with the extracts of the Cholera spirillum and the vibrio Metschnikoff.

Glycerine extracts not more than a fortnight old were used.

$\frac{1}{2}$ c.c. of the glycerine extract was diluted to 1 c.c. with water, and injected into a guinea-pig under the skin of the abdomen.

In both cases the result was a marked and rapid fall in temperature of the animals, as will be seen from the following Tables.

I.

Glycerine Extract of Cholera Spirillum.

The injection was made at 11.15 A.M.

Time		*Temperature*	
11.15	A.M.	98·4°	F.
11.30	„	97·9	
11.45	„	96·9	
12.15	P.M.	95·8	
12.30	„	94·8	
12.45	„	95	
2	„	95·6	
2.25	„	95·6	
3	„	95·6	
4	„	95·6	
5	„	95·8	
6	„	96	

On the next day the temperature of the animal was again normal. The animal did not die. The marked symptom was the great fall in temperature produced. One hour and a half after the injection the temperature had fallen to 95° F., and remained subnormal for seven to eight hours. After twenty-four hours it had risen again to 98·4° F. The glycerine extract of the Cholera spirillum produced therefore in guinea-pigs a characteristic symptom of cholera asiatica in man.

II.

Glycerine Extract of Vibrio Metschnikoff.

The injection was made at 11 A.M.

Time 11	A.M.	*Temperature* 98·4° F.
11.15	,,	97·2
11.30	,,	97·2
11.45	,,	96
12.15	P.M.	96·4
12.45	,,	97·2
1.15	,,	97
2.30	,,	98·4

Here also there was a marked and rapid fall in temperature. The minimum was 96° F., as compared with 95° F. in the Cholera experiments. The recovery also was more rapid, and three and a half hours after injection the temperature had risen to the normal.

The glycerine extracts of the Cholera and Metschnikoff spirilli therefore contain soluble cell proteids which have a toxic effect on animals. These toxalbumens, if not identical with the ferments produced by the bacteria, are closely related to them in constitution. They are in any case, like the ferments, *intra*-cellular products. They are, further, rapidly absorbed when injected into animals; producing an almost immediate toxic effect.

The results obtained with the Cholera and Metschnikoff spirillum have led me to extend these experiments to anthrax and other pathogenic bacteria. These investigations will be communicated in a further paper. The toxic effects of the glycerine extracts of the bacteria, and of the precipitates obtained from them, will then be fully gone into.

I will conclude this paper with a brief summary of the results of the above researches:—

1. The bacteria which liquefy gelatine do so by means of a soluble ferment or enzyme. The action of the enzyme on gelatine can be demonstrated apart from the cells that produced it.

2. The amount of this proteolytic enzyme secreted varies according to the nature of the soil. The amount present in gelatine cultures of the bacteria is relatively small. The amount secreted in simple meat-broth cultures is much larger.

3. It is probably conditions of nutrition which regulate the amount of enzyme secreted, as the simple meat-broth cultures had a more active ferment action than the gelatine or peptone meat-broth cultures.

4. Simple meat-broth is the best medium for the production of these soluble ferments.

5. The action of the ferments varies with the nature of the soil. The Cholera enzyme had a more energetic action on eggalbumen than on fibrin. The ferment produced by Finkler's spirillum had an opposite effect.

6. The glycerine extracts of the bacteria also contain the active ferments.

7. The glycerine extracts contain not only a proteolytic, but also a diastatic enzyme which converts starch into sugar. The soluble cell proteids can therefore be extracted from the cells by means of glycerine, and their action demonstrated.

8. The most active enzyme produced by the Cholera spirillum is a diastatic, and not a proteolytic.

9. The glycerine extracts of the spirilli reduce Loew's reagent, in this way acting like the protoplasm of the living cell.

10. The glycerine extracts which contain the active ferments of the bacteria, possess also toxic properties. The soluble cell proteids which possess these toxic properties are closely related in constitution to the ferments, and may be identical with them, or a modification of them. The account of these investigations will be continued in a second paper.