ON THE LIQUEFACTION OF GELATIN BY THE BACILLUS CLOACAE.

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In the examination of a drinking water the chemist has a great advantage over the bacteriologist in that he can begin and complete his analysis in one day. The bacteriologist, on the other hand, is retarded in his work by several causes, one of which is that certain organisms liquefy gelatin only very slowly. The *Bacillus cloacae,* for instance, may not show any change in this medium for 30—40 days or even more. It is obvious that a delay of this duration may render a report valueless for practical purposes. Besides, the differentiation of *B. cloacae* from *B. lactis aerogenes* hinges upon the former's power of movement and of liquefying gelatin. It seemed, therefore, important to endeavour to devise some means by which it could be determined in a few days whether an organism belonged to the class of liquefiers. Various devices were tried, such as frequent sub-cultivation on various media prior to inoculation on to gelatin, the use of low percentages of gelatin, incubating at 37° C., or 42° C., and combinations of these, but all without success. It was, however, observed that old gelatin cultures which showed a stratum of liquefied medium about one quarter of an inch deep, became completely liquefied during a sojourn of 48 hours at 37° C., though they solidified on cooling if they had been kept only 24 hours at this temperature.

While I was talking over this point with Dr George Dean he suggested that it might be a question of the quantity of the organism or of the products of its growth used for inoculation which determined the time of commencement and completion of liquefaction, and for this suggestion I desire to thank him heartily as it has enabled me to make a step forward towards the solution of this problem.

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In a previous number of this *Journal* (1905, Vol. v. p. 372), reference was made to certain bacilli of the *B. cloacae* group which took from six weeks to three months to liquefy $\frac{1}{4}$—$\frac{1}{2}$-inch of a gelatin tube. These partially liquefied tubes were placed at 37° C. for 48 hours, and the now completely liquid cultures were used to make fresh gelatin stab cultures (a loopful being used for each stab) which were kept at room temperature. At the end of 30 days there was no apparent liquefaction but in 40 days the liquefaction had extended about $\frac{1}{4}$-inch down the tube. A stay of 48 hours at 37° C. rendered the liquefaction complete.

Experiment I.

In this experiment three of these liquid tubes were used to inoculate two series of fresh gelatin tubes; Series I, consisting of three tubes, each of which received 1·0 c.c. of the liquid culture; and Series II, also of three tubes, each of which received 0·5 c.c. of the corresponding culture. Both series were incubated at 37° C., together with control tubes of uninoculated medium. Each day they were removed from the thermostat and placed in cold running water for 2—4 hours, during which period they were examined from time to time. If the gelatin set the tubes were at once returned to the incubator, but if at the end of four hours it was still liquid the tubes were left on the laboratory bench until the close of the experiment. This procedure brought out an interesting point in connection with the liquefaction of gelatin. It was noticed that some tubes which remained liquid after having been in cold water at 14—15° C. for four hours, became solid by the next morning when allowed to remain in a rack on the laboratory bench all night; the temperature of the room at the time of observation being about 19° C. This behaviour suggests the existence of a phase of delayed setting as a preliminary to complete liquefaction. In the results of all the following experiments this point has been taken into consideration and no tube has been considered to be liquefied unless it remained permanently liquid.

Results of Experiment I.

Day:—	1	2	3	4	5	6	7
Series I:							
1 c.c. in each tube	S	L	—	—	—	—	—
Series II:							
0·5 c.c. in each tube	S	S	S	S	semi-S	more liquid	L

S = solid on cooling. L = liquid on cooling.

Thus we see that 1 c.c. of an old liquefied gelatin culture of a *B. cloacae* liquefied 8—9 c.c. of gelatin in 48 hours at 37° C., while 0·5 c.c. took seven days to produce the same effect; and consequently that we cannot entirely leave out of consideration the part played by quantity in the production of this reaction.

Experiment II. To ascertain whether agar cultures would act like old gelatin cultures.

A Roux bottle of nutrient agar was inoculated with *B. cloacae* and another similar bottle with *B. lactis aerogenes*. Both bottles were placed at 37° C. After six days' incubation the growths were swept off the agar and each emulsified in 10 c.c. of sterile NaCl (0·85 %) solution. A gelatin tube was inoculated with 0·5 c.c. of the emulsion of *B. cloacae* and another tube with the like amount of the emulsion of *B. lactis aerogenes*. Both tubes were incubated at 37° C., and each day they were treated with cold water as in the previous experiment.

Result :

	Days :—5th	6th	9th	10th	15th
B. cloacae, 0·5 c.c.	not quite firm at R.T. 21° C.	semi-solid at 20° C. R.T.	semi-solid at 16° C. C.W.T.	liquid at C.W.T. 16·5° C.	—
B. lactis aerogenes, 0·5 c.c.	solid at R.T.	solid at R.T.	solid at R.T.	solid at R.T.	no sign of liquefaction

R.T. = room temperature. C.W.T. = cold water temperature.

The surface of the medium in a Roux bottle[1] equals that of 15—20 tubes containing sloped agar, therefore 0·5 c.c. of the emulsion, the amount of culture used for inoculation, would equal about one six-day agar tube. This amount liquefied 8—9 c.c. of ordinary nutrient gelatin in 10 days and was, therefore, not so powerful as the 0·5 c.c. of the old gelatin culture used in Exp. I.

Experiment III. To ascertain the effect of differences in the quantity and thickness of the emulsion.

A Roux bottle of nutrient agar was inoculated with *B. cloacae* and incubated at 37° C. for six days, when the growth was swept off, emulsified in sterile NaCl (0·85 %) solution (the whole emulsion measured about 10 c.c.) and centrifugalised. Gelatin tubes were inoculated with 1 c.c., 0·5 c.c., and 0·25 c.c. respectively of the slightly turbid supernatant fluid, and then the remainder was thoroughly stirred up to form again a thick emulsion, of which 1 c.c., 0·5 c.c., and 0·25 c.c. were put into gelatin tubes. Both sets of tubes were incubated at 37° C., and cooled each day as in previous experiments.

[1] The agar in these Roux bottles is solidified while the bottle lies flat on its side. The agar surface thus obtained equals in area that of 15—20 ordinary slant agar tubes.

Result:

Material used for inoculation	Amount	Day:—3	5	7	11
Thick Emulsion	1 c.c.	solid on cooling	liquid at 14·5° C.	-	—
,,	0·5 c.c.	,,	,,	—	—
,,	0·25 c.c.	,,	solid	liquid at 14·5° C.	—
Turbid Supernatant fluid	1·0 c.c.	,,	solid	,,	—
,,	0·5 c.c.	,,	solid	,,	—
,,	0·25 c.c.	,,	solid	solid	liquid at 14·5° C.

This shows a difference in the time of liquefaction due to differences in the amount of material inoculated. The fact that the supernatant fluid liquefied the gelatin so soon suggests that the NaCl solution dissolved something which assisted in the solution of the gelatin.

Experiment IV. To ascertain the effect of young agar cultures in large quantity and also of a temperature of 37° C. *compared with the temperature of the room.*

A series of slant agar tubes were inoculated all over the surface with the *B. cloacae* and a similar series with *B. lactis aerogenes*. After 24 hours' incubation at 37° C., the growths were scraped off and gelatin tubes inoculated and incubated as recorded in the following Table (p. 27), which also gives the results.

On the 42nd day tube H was put into the 37° C. incubator. After 24 hours: solid on cooling; after 48 hours: liquid on cooling.

From these observations we must conclude that large inoculations by themselves are not of very great use but that they must be assisted by a temperature of 37° C. to obtain the best results.

Experiment V. To ascertain the effect of still larger inoculations of young agar cultures.

Slant agar tubes were inoculated with *B. cloacae*, the inoculating material being spread over the entire surface of the agar. After 24 hours' growth at 37° C., the growth was scraped off and used for inoculating tubes containing 8—9 c.c. nutrient gelatin which were then incubated at 37° C., and cooled down each day as before.

Result:

		Day:—2	3	4	5	6
Tube I	inoculated with the growth from 6 agar tubes	solid on cooling	solid on cooling	liquid	—	—
Tube II	inoculated with the growth from 3 agar tubes	,,	,,	not quite solid	semi-solid	liquid

Thus by using very large inoculations we can still further shorten the time required for liquefaction.

Table relating to Experiment IV.

	Amount of growth used for inoculation	Incubation temp.	Day :—6	8	9	11	26	37	42
A.	1 loopful *B. lactis aerogenes*	37° C.	solid at 20° C.	solid at 19·5° C.	solid at R.T.	solid at R.T.	solid	solid	solid
B.	3 tubes *B. lactis aerogenes*	,,	,,	solid at R.T. 19·5° C.	,,	,,	,,	,,	,,
C.	1 loopful *B. cloacae*	,,	,,	solid at R.T. 19·5° C.	,,	,,	liquid at 19° C. R.T.		
D.	3 tubes *B. cloacae*	,,	not quite firm	liq. at 19·5° semi-solid at 16·5° C.	liq. at C.W.T. 16·5° C.	—	—	—	—
E.	1 loopful *B. lactis aerogenes*	R.T.	solid	solid at R.T. 19·5° C.	solid at R.T.	solid	solid	solid	solid
F.	3 tubes *B. lactis aerogenes*	,,	,,	,,	,,	,,	,,	,,	,,
G.	1 loopful *B. cloacae*	,,	,,	,,	,,	,,	slight softening at one spot	3—4 drops of liquid gelatin	—
H.	3 tubes *B. cloacae*	,,	,,	,,	,,	small spot of softened gelatin	—	—	about $\frac{1}{4}$-inch of liquid gelatin

R.T. = room temperature. C.W.T. = cold water temperature.

Experiment VI. To ascertain the effect of lowering the percentage of gelatin.

A Roux bottle of nutrient agar (=15—20 agar tubes) was inoculated with *B. cloacae*, incubated at 37° C. for 24 hours, and then at room temperature for 4 days. The growth was then swept off and emulsified in salt solution (0·85 %). The emulsion measured from 14—15 c.c.

Two series of 12 tubes each were prepared, each tube containing 8 to 9 c.c. of nutrient gelatin. In Series I the percentage of gelatin in the medium was 5 % and in Series II 10 %. Into each tube was put 0·5 c.c. of the emulsion of *B. cloacae*. The tubes were sealed off in the flame to prevent evaporation, and incubated at 37° C. Beginning with Series I a tube was taken out of the incubator each day and cooled in water down to 13° C., the rest of the tubes being left at 37° C.

Of the tubes of Series I (5 % gelatin) the first to become liquid was that taken out of the incubator on the 8th day. As this one remained permanently liquid the remaining tubes of this series were cooled down and all were found to be liquefied. As all the tubes of Series I were completely liquefied a tube of Series II was also examined on this day and found to solidify on cooling.

The first tube of Series II (10 % gelatin) to show any sign of liquefaction was the tube removed from the incubator on the 12th day. This was not quite solid on cooling. The 14th day tube was liquefied and so were the rest of this series. The results are shown in the following Table.

	% gelatin	Day:—1	2	3	4	5	6	7	8	9	10	11	12	13	14
Series I	5	S	S	S	S	S	S	S	L	—	—	—	—	—	—
Series II	10	,,	,,	,,	,,	,,	,,	,,	S	S	S	S	S	S	L

S = solid. L = liquid.

Thus we see that the time of liquefaction can be shortened by using lower percentages of gelatin than the usual 10 %, a five-day agar growth liquefying 8—9 c.c. of 5 % gelatin in eight days, while it required 14 days to do the same in the case of 10 % gelatin.

Experiment VII. To ascertain the effect of large inoculations upon nutrient 5 % gelatin.

Inoculations were made with *B. cloacae* all over the surface of slant agar tubes which were incubated at 37° C. for 48 hours. The growth was then scraped off and used for inoculating tubes of nutrient 5 % gelatin. The incubation temperature was 37° C., and the tubes were examined each day as before.

No. of tube	Amount inoculated	Day:—1	2	3	4
1.	Growth from 6 agar tubes	Both solid at R.T. 13° C.	Both not quite solid after 2 hours at R.T. 13° C.	Both liquid at R.T. 13° C. very viscid after 3 hrs. at C.W.T. 9° C.	Both liquid at the temp. of cold water 10° C.
2.	,, ,, 3 ,, ,,				

R.T. = room temperature. C.W.T. = cold water temperature.

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If we compare these results with those obtained with 10 % gelatin we find there is a slight advantage in using the less solid medium. It must be borne in mind that in all these experiments it is the time of complete liquefaction which has been noted. If one had been content only to cool the inoculated tubes down to a temperature at which control uninoculated tubes became solid the power of liquefaction could have been made evident at an earlier period than is shown in the tables.

The experiments detailed above have shown that by means of massive inoculations into 5 % gelatin and by incubating at 37° C., the power possessed by *B. cloacae* of liquefying gelatin can be demonstrated in a much shorter time than by the usual method.

The solution of the gelatin is no doubt due to a ferment such as has been proved by Bitter, Fermi, Hueppe, and others[1] to be a product of the metabolism of many organisms. Only two experiments in connection with this point will be given here, all questions as to its mode of action, etc., being left for discussion on a future occasion.

Experiment VIII. To ascertain whether cultures of B. cloacae killed by chloroform liquefy gelatin.

A Roux bottle of nutrient agar was inoculated with *B. cloacae* and incubated at 37° C. for 48 hours. The growth was then washed off with 10 c.c. of sterile salt solution and emulsified. A few drops of chloroform were now added, the mouth of the bottle was plugged tightly with wool and covered with a lead capsule and the bottle then placed at 37° C. for 48 hours. The whole emulsion measured 18 c.c. Tubes containing about 8 c.c. of gelatin were inoculated thus:

No. 1	with 5 c.c.	of emulsion				
,, 2	,, 4	,,	+1 c.c.	of sterile	tap	water
,, 3	,, 3	,,	+2	,,	,,	
,, 4	,, 2	,,	+3	,,	,,	
,, 5	,, 1	,,	+4	,,	,,	
,, 6	,, 1	,,				
,, 7	,, 1·5	,,				

The sterile tap water was added to make the volume of fluid added equal in the case of the first five tubes. The tubes were placed in the incubator at 37° C., and were examined and cooled in water each day as in previous experiments.

After 24 hours at 37° C., all the tubes soon became solid when allowed to remain at the temperature of the room.

On the second day tubes 6 and 7 became solid after about 15 minutes at room temperature but in the remainder the setting was very much delayed and was not absolutely complete in 4 hours.

[1] Cited by Kolle and Wassermann, *Handbuch der pathogenen Micro-organismen*, Bd. 1. 1903, p. 106.

On the third day 6 and 7 solid at room temperature; the others liquid. After 1 hour in cold water at 10° C.: No. 2 almost set. After 2 hours at 10° C.: No. 4 set, Nos. 1 and 5 almost quite solid, No. 3 liquid, except for about ¾-inch at the bottom of the tube where the gelatin was too solid to pour out. On this day a loopful of the liquid gelatin was taken out of tubes 1—5 and used to inoculate slant agar tubes which, after 48 hours' incubation at 37° C., showed no growth. Thus it may be accepted that tubes 1—5 did not contain any living organisms.

On the fifth day No. 6 solid at room temperature, 11·5° C., Nos. 1—5 and 7 liquid after 1½ hours in a rack at room temperature. After 3 hours at 9° C. cold water temperature: 7 a viscid liquid, and the others liquid. Left at room temperature overnight; they all remained liquid. These results are tabulated below.

Results of Experiment:

No. of tube	Material inoculated	1st day	2nd day	3rd day	5th day
1.	5 c.c. of emulsion of *B. cloacae*	solid R.T.	solid R.T.	liquid R.T. solid 10° C.	liquid at 9° C.
2.	4 ,, ,, +1 c.c. sterile water	,,	,,	,,	,,
3.	3 ,, ,, +2 ,, ,,	,,	,,	,,	,,
4.	2 ,, ,, +3 ,, ,,	,,	,,	,,	,,
5.	1 ,, ,, +4 ,, ,,	,,	,,	,,	,,
6.	1 ,, ,,	,,	,,	solid R.T.	solid R.T.[1] 11·5° C.
7.	1·5 ,, ,,	,,	,,	,,	liquid at 9° C.

R.T. = room temperature.

It is thus evident that the liquefaction of gelatin is due to a ferment. It was expected that by using varying amounts of material for inoculation corresponding differences in the time required for liquefaction would be shown, but this has not been the case, and no explanation of the fact is at present forthcoming. It must be borne in mind that in measuring out an emulsion of this kind it is impossible to make certain of obtaining an equal amount of effective material in each cubic centimetre. But this will not account for the difference between tubes 5 and 6, which each received as nearly as possible the same amount of emulsion and contained the same quantity of material to be peptonised. Here the only explanation appears to lie in the different degrees of concentration of the medium in the two tubes. That this is not the entire explanation however is shown by the following experiment:

A Roux bottle of nutrient agar was inoculated with *B. cloacae* and incubated at 37° C. At the end of 48 hours the growth was scraped off and emulsified in 10 c.c. of distilled water. The whole emulsion measured 15 c.c. It was poured into a sterile flask, a few drops of chloroform added, the flask tightly corked and placed

[1] On the 8th day this tube remained liquid at R.T. = 15° C.

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Table relating to Experiment VIII.

Tube	Amount of material inoculated	Day:—1	2	3	4	5	7	8
1.	3 c.c. of emulsion + 3 c.c. sterile salt solution	Solid on cooling 10° C.	S 10° C. C.W.T.	L 10° C.	—	—	—	—
2.	2 ,, ,, + 4 ,, ,, ,,	,,	,,	,,	—	—	—	—
3.	1 ,, ,, + 5 ,, ,, ,,	,,	,,	,,	—	—	—	—
4.	0·5 ,, ,, + 5·5 ,, ,, ,,	,,	,,	,,	—	—	—	—
5.	0·25 ,, ,, + 5·75 ,, ,, ,,	,,	,,	S 10° C.	S 10° C.	L 10° C.	—	—
6.	3 ,, ,, only	,,	L 10° C.	—	—	—	—	—
7.	2 ,, ,, ,,	,,	,,	—	—	—	—	—
8.	1 ,, ,, ,,	,,	S 10° C.	L 10° C.	—	—	—	—
9.	0·5 ,, ,, ,,	,,	,,	S 10° C.	L 10° C.	—	—	—
10.	0·25 ,, ,, ,,	,,	,,	,,	S 10° C.	S 10° C.	S 9° C. C.W.T.	L 10° C.

C.W.T. = cold water temperature.

S = solid.
L = liquid.

at 37° C. After allowing autolysis to proceed for 48 hours the emulsion was used to inoculate tubes containing 6—6·5 c.c. of 10 % nutrient gelatin as in the preceding Table (p. 31).

With the idea of obviating the sedimentation and consequent unequal distribution of the organisms throughout the emulsion which takes place when measuring out emulsions with the usual large bore 10 c.c. pipette all the varying quantities of emulsion in this experiment were measured with the same 1 c.c. pipette. The tubes were incubated, etc., as before.

As soon as a tube became liquid, inoculations were made from it on to agar to ascertain whether the tubes were free from living organisms. Growth was obtained in the case of tubes 3 and 5, but as the organisms did not give the reactions of *B. cloacae* and did not liquefy gelatin their presence had no special influence on the result. The $\frac{\text{time}}{\text{quantity}}$ relation comes out fairly well in the series of tubes 6—10, but it cannot be said that dilution of the gelatin has had a very marked effect in tubes 1—5. We would not, therefore, be justified in saying that the rapidity of liquefaction of gelatin by this enzyme is proportional to the degree of concentration of the medium.

Summary.

The *Bacillus cloacae* liquefies gelatin very slowly, sometimes taking a month or more to do so. It is in consequence occasionally a source of delay and of great inconvenience to the bacteriologist. In this paper it has been shown that by using appropriate methods the time necessary for demonstrating the liquefaction of gelatin by this organism may be shortened to a week.