THE APPLICATION OF THE PRINCIPLE OF EIJKMAN'S FERMENTATION TEST FOR DETERMINING THE *COLI* TITRE OF WATER[1]

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(With 2 Figures in the Text)

At present the *coli* titre of water is almost universally determined by fermentation tests in sugar-containing media; the fermentation method of Eijkman (glucose-peptone solution at 46°[2]) and that of Bulir (neutral-red mannite bouillon at 46°) are current in many European countries, while in America fermentation in lactose bouillon at 37° is the standard test. Fermentation tests at 37° in lactose-containing media with the addition of bile and bactericidal dyes are also widely used (Great Britain, America).

The absence of a single universally accepted method of determining the *coli* titre of water by fermentation is explained by a difference of opinion regarding not only the most favourable culture medium, but also the most suitable temperature of cultivation.

The founder of the fermentation method at a temperature of 46°, Eijkman (1904), based his investigations on the following considerations. *Bact. coli communis*, originating from the intestinal tract of man and warm-blooded animals, can multiply and produce gas in glucose at 46° whereas the great majority of saprophytic water microbes and other microbes having no sanitary significance, such as coliform bacteria from the intestinal canal of cold-blooded aquatic animals, either do not develop under these conditions or are greatly suppressed.

Eijkman's first follower, Bulir (1907), in estimating the temperature factor (46°) agreed with his predecessor on the chief points.

The correctness of Eijkman's and Bulir's view, that the capacity to ferment at 46° differentiates the typical *Bact. coli communis* of man and warm-blooded animals from varieties of coliform bacteria from the intestines of cold-blooded animals and insects, was confirmed by numerous authors (Christian, Hilgermann, Mallanah, Thomann, Mordberg, Leiter, Magalhães, Huss, Salus and Hirn, Schröder, Jungeblut, Minkevich and his collaborators, and others).

[1] Reported on March 22, 1935, at the 242nd sitting of the Leningrad Microbiological Society.

[2] All temperatures in ° C.

An objection raised against the fermentation test at 46° suggested by Eijkman and Bulir is that the small numbers of intestinal *coli* contained in the maximum dilution of water under examination may die at 46° and thus remain undiscovered in cases where they undoubtedly exist (Neumann, Nowack, Venema, Sachse, Barth and others).

This question was sharply raised recently by Brown and Skinner (1930) and Skinner and Brown (1934). These authors cultivated in parallel maximum dilutions of emulsions of faeces from sick and healthy men in Eijkman's and Bulir's culture media and in American standard lactose bouillon, by placing the cultures in the first two media in a water-bath at 46° and in a bath containing water at room temperature which was gradually heated for 5 hours until it reached 46°. Cultures in lactose bouillon were placed in a thermostat at 37·5°, and the results of the tests were recorded after 24 and 48 hours. It was thus established that in many cases when cultures were grown at 46°, especially if the test-tubes were placed directly in a water-bath at that temperature, *Bact. coli communis* gave no growth, whereas fermentation tests on lactose bouillon at 37·5° in similar test-tubes gave a positive result. Comparing the data obtained from cultures at 46° and 37·5°, Skinner and Brown plotted curves demonstrating a numerical balance in favour of the fermentation test on lactose bouillon at 37·5°. Skinner and Brown explain the discrepancy between their conclusions and those of the advocates of the fermentation test at 46° by pointing out that in the majority of cases the validity of the fermentation test at 46° was determined on materials containing large numbers of *Bact. coli* (faeces, pure cultures), and, in some cases, without a sufficiently strict recording of the real temperature in the thermostat.

This latest work of the American authors could not but induce us to examine critically once more the question of the validity of the fermentation test at 46°.

The first step taken in our investigations was to verify how far the capacity to produce gas on Bulir's medium in test-tubes at 46° is characteristic of *Bact. coli communis* of intestinal origin, using massive doses of culture in loops from colonies in conjunction with a simultaneous negative test on Simmons' or Koser's citrate medium, which we had found a valuable test in previous work.

For this purpose in November-December 1934, the faeces of sixty-five healthy persons were investigated by us, being cultivated in the usual way on plates containing Endo's medium. Red metallically glistening colonies, characteristic of *Bact. coli*, were transferred by loops, ten from each plate, into test-tubes containing 5 c.c. of Bulir's medium, and then placed in a large thermostat for 24 hours at 46°.[1]

[1] According to a carefully verified thermometer on the same level with the test-tubes. We purposely gave up the use of a water-bath, as Eijkman's fermentation test in practice is made in a thermostat. We found that the conditions of heating test-tubes containing culture medium in the water of the bath and in the air space of the thermostat are not identical.

Not more than a hundred test-tubes were placed in a thermostat simultaneously, and the temperature, which fell, after loading the thermostat, to 44–44·5°, was brought quickly to 46° by additional heating. In this way 650 colonies were investigated, of which number 609 produced gas at 46°. Control growths were made on Bulir's medium at 37° of the forty-one which did not produce gas at 46°, with the result that twenty-five of them were unable to produce gas even at this temperature. These must be excluded from the calculation, leaving only sixteen colonies of the remaining 625, *i.e.* 2·6 per cent., able to ferment at 37° but not at 46°.

These results fully confirm the data previously obtained by us, which show that the number of *Bact. coli communis* strains from the faeces of man and warm-blooded animals unable to ferment at 46° did not exceed 4·5 per cent. Altogether, 1349 strains from the faeces of man, pigs, horses, cows and birds have been examined by us. Of these only forty-nine (3·6 per cent.) failed to produce gas in Bulir's medium at 46°.

These data, in conjunction with the investigations of many authors, showing that coliform bacilli from the intestines of cold-blooded animals and insects are incapable of fermentation at 46°, confirm the statement that the fermentation test at 46° under conditions of massive inoculation in conjunction with the citrate test is a very valuable auxiliary method for the purpose of identifying *Bact. coli communis*.

The problem of the survival of all faecal *coli* at 46°, which was the chief object of Skinner's and Brown's investigations, was not hitherto specially studied by us. One of us (Minkevich, 1932) described one experiment on the survival, in maximum dilutions, of two strains of *coli* in Bulir's medium at 46° and 37°. In both cases the results of this experiment showed a reasonable agreement of the fermentation titre at 46° and at 37°; and, at the time, did not give us any cause to doubt the sensitiveness of the fermentation test at a temperature of 46°.

However, we now undertook a more thorough investigation of this subject. As test material for studying the comparative survival of *coli* at 37° and 46° we used sewage-polluted Neva water, taken within the boundaries of the city of Leningrad near the Bridge of Freedom, and made a series of experiments in Bulir's medium at 37° and 46°.

The water under examination was diluted with sterile water to the limit of its fermentation titre in tenfold ratios and was introduced in 1 c.c. quantities with a separate sterile pipette for each dilution into test-tubes containing 5 c.c. of Bulir's medium, each water dilution being introduced with the same pipette into corresponding test-tubes of each row. Immediately on the introduction of the culture, one row was placed in a thermostat at 37°, the other row in a thermostat at 46°; the temperature of the thermostat, which fell after loading by 1–2°, was immediately restored to 46° by additional heating. Thirteen parallel experiments were thus made. The results were recorded at the end of 24 hours. A summary is given in Table I.

Table I. *Number of water samples in which fermentation took place*

Temp. ° C.	Water dilutions						
	Undiluted	1/10	1/100	1/1000	1/10,000	1/100,000	1/1,000,000
37	13	13	13	12	10	6	2
46	8	3	0	0	0	0	0

The data obtained by us, as recorded in Table I, show plainly that a temperature of 46°, applied at once, inhibits the development of *Bact. coli communis* not only in maximum but also in low dilutions of water. The *coli* titre of the water at 37° varied within the limits of 0·01–0·0001 c.c. These data are in complete agreement with that part of Skinner's and Brown's experiments in which they determined the survival and fermentation capacity of *coli* in maximum dilutions, placing the cultures at once in a water-bath at exactly 46°.

This finding, however, is contrary to the ordinarily satisfactory use of Eijkman's and Bulir's fermentation test at 46° in the examination of water in different countries; and we, personally, after using Bulir's method for about 10 years, could not record any noticeable discrepancy between the *coli* content determined by this method and that given by the milk coagulation test at 37° proposed by us (*Laboratory Practice*, No. 9, 1934, Moscow).

Analysing the causes of the discrepancy, we consider that it is correctly explained by Skinner and Brown. The latter affirm that the satisfactory results obtained in practice at 46° depend on the fact that in reality those who use the fermentation test at 46° never deal with an actual temperature of 46° from the very beginning of the experiment. In the thermostat adjusted at 46° this temperature inevitably falls by several degrees after loading and reaches 46° only in the course of several hours, so that during the first hours in the thermostat the development of *coli* takes place at a temperature below 46°.

This consideration is in general confirmed by Skinner and Brown in that part of their experiment in which, by gradually raising the temperature of the water-bath during 5 hours until it reaches 46°, they obtained better results, and also by four experiments made by us, when the cultures were kept for the first 4 hours at 37° and then transferred to a thermostat at an exact temperature of 46°, warming to correct the fall due to loading.

The results of our experiments are recorded in Table II.

Table II. *Number of water samples in which fermentation took place*

	Water dilutions							
	Undiluted	1/10	1/100	1/1000	1/10,000	1/100,000	1/1,000,000	1/10,000,000
Cults. at 37° for 4 hr., then at 46° up to 24 hr.:	4	4	4	2	1	0	0	0
Cults. at 37°:	4	4	4	3	3	2	0	0

As can be seen from Table II, a preliminary growth of short duration at 37° followed by removal to a thermostat at 46° reduces the difference between fermentation titres determined at 37° and 46° (compare with Table I).

The next series of experiments was made by us as follows: The cultures were placed in a small thermostat fixed at 46° in which, after loading, the temperature fell to 42° and reached 46° again only after 5–6 hours, as in this case no additional heating was applied. The result of these tests are given in Table III.

Table III. *Number of water samples in which fermentation took place*

	Water dilutions							
	Undiluted	1/10	1/100	1/1000	1/10,000	1/100,000	1/1,000,000	1/10,000,000
At 46° under conditions of ordinary heating of thermostat when completely loaded:	4	4	4	2	1	0	0	0
At 37°	4	4	4	3	3	2	0	0

We believe that the summary of experiments presented in Table IV illustrates with sufficient clearness the conditions necessary for obtaining good results at 46° and explains the practical success of this test as well as the contradictory data.

In practice, in the majority of cases, the primary reproduction of *coli* occurs below 46°, and at the moment this temperature is attained, already enriched growths, capable of easily supporting the temperature of 46°, are subjected to its action as is the case in experiments with massive doses of culture.

Hence the experiments of Skinner and Brown in placing cultures at once in a water-bath of 46° with additional heating of the thermostat after loading it, from the viewpoint of ordinary practice are to a certain degree artificial and can be correct only when a thermostat of great capacity, adjusted at 46°, is loaded with a small number of test-tubes. In view of the fact, however, that the primary fall of temperature in the thermostat after loading, the degree of the fall and the length of time elapsing before the temperature again reaches 46°, are factors which cannot be standardised, we set before ourselves the task of discovering the temperature which would not, under any conditions, prevent the development of even single cells of *Bact. coli communis* in fermentation tests in Bulir's medium.

With this purpose in view we made a number of experiments, analogous to those mentioned above, in which the action of a temperature of 45°, 44° and 43° on cultures of maximum dilutions of water were investigated. The thermostat, after its loading, was immediately warmed up to the experimental temperature.

These experiments proved that the highest temperature harmless for *coli* in maximum dilutions is 43·5°. At 46°, 45°, and even 44°, maximum dilutions of Neva water and suspensions of *coli* cultures are not always able to ferment in Bulir's medium, being inhibited more by the higher temperatures. At 43–43·5° delay was not observed in any of our numerous experiments. Even the maximum dilutions of Neva water and suspensions of *Bact. coli communis* manifested with great constancy a full capacity to produce gas in Bulir's medium at 43–43·5°.

Table IV shows the results of parallel investigations of twenty water samples made at 43–43·5° and at 37°.

Table IV. *Number of water samples in which fermentation took place*

Temp. ° C.	Water dilutions							
	Undiluted	1/10	1/100	1/1000	1/10,000	1/100,000	1/1,000,000	1/10,000,000
43–43·5	20	20	20	8	4	0	0	0
37	20	20	20	12	4	3	0	0

On examining Table IV one can see that only in water dilutions of 1/1000 and 1/100,000 do fermentation tests at 37° show a difference of 4 in one dilution and of 3 in the other. A further examination proved, however, that in all these instances the fermentation was caused not by typical *Bact. coli communis*, but by other varieties of the *coli-aerogenes* group which developed luxuriantly on Simmons' medium and which did not produce any gas in Bulir's medium at 46° with massive inoculations. The maximum positive dilutions at 43–43·5° produced in culture typical *Bact. coli communis*.

These experiments show that a temperature of 43–43·5° retains in full or nearly in full the advantage of 46°, *i.e.* it eliminates the majority of the members of the *coli-aerogenes* group which do not indicate faecal pollution..

Having convinced ourselves of the harmlessness of the temperature of 43–43·5° for maximum dilutions of *Bact. coli communis* and at the same time of its destructive action in maximum dilutions of organisms of the *coli-aerogenes* group which are not significant of faecal pollution, we set before ourselves the further task of investigating the influence of this temperature on the gas-producing capacity and the reproduction of *Bact. coli communis* in Bulir's medium in comparative tests at 43–43·5°, and at 37°.

For the purpose of solving the first part of this question (gas production) we constructed a simple closed fermentation apparatus (Fig. 1, schematic) which enabled us to make exact quantitative determinations of gas production during the growth of *Bact. coli communis*.[1]

The apparatus consists of an ordinary glass flask of 250–300 c.c. capacity which is half-filled with tap water and into which is plunged a test-tube graduated to 15–20 c.c. completely filled with water and turned upside down. This part of the apparatus is not sterilised, and in the test is placed in the thermostat beforehand to heat it to the temperature of the thermostat.

The second half of the apparatus consists of a bent glass tube on one end of which is firmly fixed an indiarubber stopper to which an ordinary test-tube of corresponding diameter is fitted. This part of the apparatus is sterilised, the glass tube with the indiarubber stopper being boiled in water, whereas the test-tube filled with a definite quantity of Bulir's medium is autoclaved at 120° for 15 min.

Into test-tubes containing 10 c.c. of Bulir's medium a definite quantity of

[1] Ordinary fermentation tubes with one closed elbow are unfit for precise quantitative records of gas production in view of a free escape of gas through the open end of the apparatus.

a greatly diluted emulsion of *Bact. coli communis* or of the water under test is introduced, and immediately the test-tubes are firmly closed with the indiarubber stopper fixed to the bent end of the glass tube. The stopper is then carefully coated with wax or paraffin along the margins of the test-tube and tube.

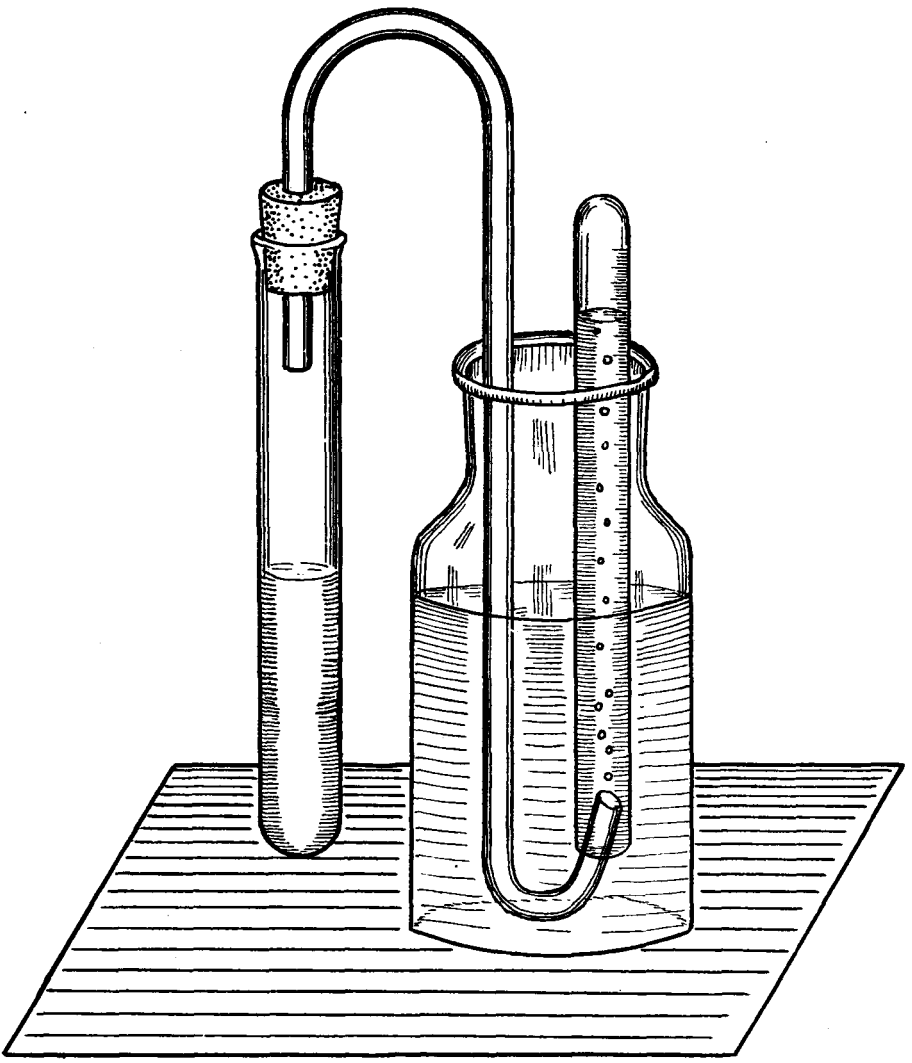

Fig. 1. Apparatus for determining the quantity of gas formed in cultures of *Bact. coli* on liquid sugar containing medium.

The second part of the apparatus thus prepared is placed in the thermostat in which the first part of the apparatus has previously been placed and the loose end of the bent glass tube is immersed in the water filling the flask. In about 30 min., after the excess of air expanded by heating has been forced

out of the pipe, the bent end of the glass tube immersed in water is quickly placed under the test-tube for the collection of the gas and the whole apparatus is left in the thermostat for the desired period of time.

By recording the quantity of gas accumulating in the test-tube at intervals the rate of gas production may be followed.

By this method series of experiments were made on various strains of *Bact. coli communis* and Neva water. The cultures were diluted to the limit and 1 c.c. of this maximum dilution was introduced with one and the same pipette into ten test-tubes forming part of our apparatus, each containing 10 c.c. of Bulir's medium.

Five sets of apparatus were placed in a thermostat at 43–43·5° and five at 37°. Fourteen series of experiments in all were made on *Bact. coli communis*, ten museum strains being examined (six from men, two from bulls and two from pigs) and four freshly isolated from human faeces. At the end of 24 and of 48 hours the quantity of gas produced and the number of bacteria contained per 1 c.c. of medium were determined.[1]

Table V illustrates the results of these experiments: the figures of the quantities of gas produced and of the number of organisms per c.c. at 43·5° given in the record columns are numerators and at 37° are denominators, the calculations having been made to only one decimal place.

Table V

No.	Name of strain	Time of recording hours	Quantity of gas in c.c. in apparatus No. 1	2	3	4	5	No. of microbes in millions per c.c.	Average ratio of gas vol. at 43° and at 37°	Average ratio of number of microbes per c.c. at 43° and at 37°
1	*Bact. coli commune*, No. 12	24	8·2 / 4·5	9·0 / 5·7	9·0 / 4·4	9·0 / 6·0	9·8 / 5·6	400 / 676	1·7 : 1	1 : 1·6
		48	8·2 / 9·0	9·0 / 9·7	9·2 / 8·8	9·2 / 10·5	9·8 / 9·6	48 / 628	1 : 1	1 : 1·3
2	*Bact. coli commune*, No. 1	24	9·0 / 5·0	8·5 / 5·0	9·0 / 5·3	7·3 / 5·6	8·5 / 4·5	160 / 400	1·6 : 1	1 : 2·5
		48	9·0 / 8·8	9·0 / 7·8	9·2 / 8·3	7·5 / 10·0	9·0 / 9·5	0 / 380	1 : 1	—
3	*Bact. coli commune*, No. 16	24	8·7 / 5·0	7·5 / 5·8	8·4 / 5·7	8·6 / 7·0	8·5 / 7·4	612 / 1020	1·3 : 1	1 : 1·8
		48	8·7 / 9·0	8·0 / 8·7	8·4 / 7·8	8·6 / 9·4	8·5 / 9·6	0 / 972	1 : 1	—
4	*Bact. coli commune*, Kem 71	24	7·2 / 6·0	6·6 / 6·5	7·6 / 6·0	7·5 / 6·0	7·7 / 7·3	462 / —	1·1 : 1	—
		48	7·3 / 6·9	6·7 / 6·5	7·6 / 7·0	7·5 / 6·6	7·7 / 7·9	15 / 1500	1 : 1	1 : 100

The data in Table V give the following ratios with great regularity. Whereas at 43–43·5° the maximum quantity of gas is produced within the

[1] The volumes of gas determined at 43°, for the purpose of comparison with the amount produced at 37°, were reduced to the level of the latter temperature by deducting 6/273rds of it from the volume determined at 43° (Gay-Lussac law).

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first 24 hours and hardly increases at all during the next 24 hours, at 37° there is a distinct delay in gas production which becomes equal to that produced at 43–43·5° only towards the end of the second 24 hours. At the same time one's attention is drawn to the fact, which seems paradoxical, that in contradistinction to intense gas production at 43–43·5° the figures of reproduction recorded in 24–48 hours are in inverse proportion, *i.e.* at 43–43·5° they are definitely less than the figures of reproduction at 37°, this being particularly noticeable after 48 hours.

The next series of experiments, which consisted of four parallel ranges, was made on Neva water, the results of two of these are given in Table VI (designations as in Table V).

Table VI

No.	Water sample No. and quantity of culture	Time of recording hours	Quantity of gas in c.c. in apparatus No. 1	2	3	4	5	No. of microbes in millions per c.c.	Results of cultures on Endo's medium	Ratio of gas vol. at 43–43·5° and at 37°	Ratio of the number of microbes per c.c. at 43° and 37°
1	Test No. 1, 2 drops	24	3·7 / 1·0	3·5 / 2·7	3·9 / 3·5	3·6 / 3·3	3·0 / 2·9	18 / 2000	*Coli* + moderate / *Coli* + abundant	1·3 : 1	1 : 111·1
		48	3·7 / 3·0	3·6 / 5·5	3·9 / 5·0	3·6 / 5·1	3·0 / 5·5	0 / 1600	*Coli* – moderate / *Coli* + abundant	1 : 1·3	—
3	Test No. 3, 1 c.c.	24	2·5 / 2·0	3·8 / 2·0	4·2 / 2·5	4·5 / 2·9	3·5 / 1·6	—	*Coli* + moderate / *Coli* + abundant	1·6 : 1	—
		48	3·2 / 4·2	3·8 / 4·8	4·2 / 5·7	4·5 / 6·2	3·5 / 5·0	—	*Coli* – / *Coli* +	1 : 1·4	—

Table VI shows that the ratio in the course of reproduction and gas production of *Bact. coli communis* at 43–43·5° and at 37° in experiments in water cultures have in general the same regularity as in analogous tests on pure cultures. The fact, however, cannot be overlooked that the development and gas production of *Bact. coli communis* in mixed cultures from water are somewhat less than in pure cultures, the difference being proportionately greater at 43° than at 37°. It is of interest that in water cultures showing abundant gas production after 48 hours' incubation at 43° *coli* may not be found in subcultures on Endo's medium. This is in accordance with Neumann's phenomenon, described in 1906.

Since our work on the gas-producing capacity of *Bact. coli communis* at 43–43·5° showed that at this temperature the process is practically complete at the end of 24 hours, we followed in addition the fermentation of *Bact. coli communis* by determining the quantity of gas at different intervals during the first 24 hours of growth and after up to 72 hours.

The experiments were made on two strains of *Bact. coli communis* from man in maximum dilutions. The results are shown in Table VII. In column 4 designations as in Tables V and VI.

Table VII

No.	Names of strains	Time of first appearance of fermentation (frothing)	Time after which quantity of gas is recorded hours	Quantity of gas in c.c. in apparatus No. 1	2	3	4	5	Ratio of volumes at 43–43·5° and at 37°
1	*Bact. coli communis*, Mourmansk 29	At 43–43·5°, 8 hours	10	3·3 / 0	3·5 / 0	3·0 / 0	1·5 / 0	2 / 0	—
			16	7·4	7·5	6·5	3	5	—
				Unrecorded					
		At 37°, 12 hours	24	7·4 / 1·5	7·5 / 2·3	6·5 / 2·5	3·0 / 2·1	5·0 / 1·8	2·9 : 1
			48	7·4 / 5·6	7·5 / 6·5	6·5 / 5·4	3·0 / 5·5	5·0 / 5·8	1 : 1
			72	7·4 / 5·6	7·5 / 6·5	6·5 / 5·4	3·0 / 6·2	5·5 / 5·8	1 : 1
2	*Bact. coli communis*, O.M.M. 274	At 43–43·5°, 8 hours	10	3·7 / 0	2 / 0	3 / 0	2·5 / 0	2·2 / 0	—
			16	8	5·6	6·5	5·5	5·6	—
				Unrecorded					
		At 37°, 13 hours	24	8·0 / 2·5	5·6 / 2·5	6·5 / 3·5	5·5 / 2·5	5·6 / 3·1	2·2 : 1
			48	8·0 / 5·7	5·6 / 6·3	6·5 / 7·5	5·5 / 6·1	5·6 / 6·6	1 : 1
			72	8·0 / 5·7	5·6 / 6·3	6·5 / 7·5	5·5 / 6·5	5·6 / 7·4	1 : 1

Table VII shows that gas production at 43–43·5° is practically complete in 16 hours. Comparing this more intense activity of *Bact. coli communis* at 43–43·5° than at 37° during the first 16 hours of growth with the low figures of reproduction at 43–43·5° determined after 24 and 48 hours, it appears that the cycle of vital development of *Bact. coli communis* at 43–43·5° goes on very fast, and that at this temperature in 24 hours dying off commences which at 37° evidently occurs much later.

For the purpose of verifying this hypothesis we determined the figures of reproduction of *Bact. coli communis* in Bulir's medium at 43–43·5° and 37°. A typical strain of *Bact. coli communis* (No. 16) was maximally diluted and 1 c.c. of this dilution was introduced with the same pipette into two test-tubes containing 15 c.c. of Bulir's medium each, one tube being placed in a thermostat at 43·5°, the other at 37°. In the course of 30 hours' growth, at regular intervals of 2 hours, 0·1 c.c. of the contents was taken from each of the two test-tubes with sterile pipettes graduated to 0·01 c.c. and was exactly diluted to one-millionth of its original concentration. 1 c.c. of this millionth dilution from each test-tube was plated on gelatine in Petri dishes in duplicate to determine the number of colonies. The final count was made after 72 hours' incubation at 22°. The results are recorded in Table VIII and the corresponding curves are plotted in Fig. 2.

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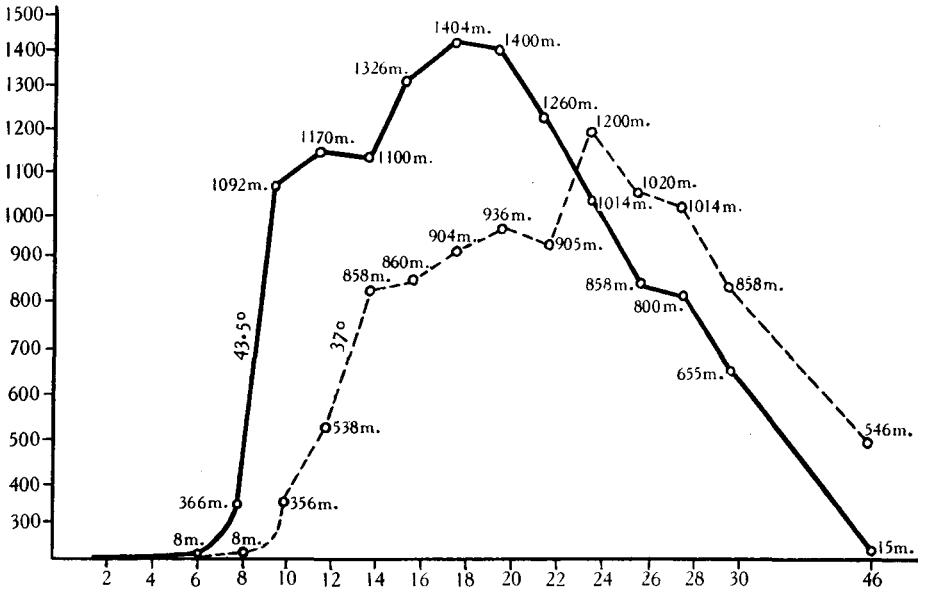

Fig. 2. Curves of *Bact. coli* reproduction in Bulir's medium at 37° and 43–43·5°. On the abscissa the hours are marked from the beginning of incubation; on the ordinate, the number of organisms in millions (m).

Table VIII

Time of introduction of culture and of parallel observations hours	At 43·5°		At 37°	
	No. of colonies in millions per c.c.	Changes in Bulir's medium	No. of colonies in millions per c.c.	Changes in Bulir's medium
2	0	None	0	None
4	1	None	0	None
6	8	None	0	None
8	366	Notable turbidity, froth appears on surface	8	None
10	1092	Great turbidity, gathers froth on surface	536	Slight turbidity
12	1170	Medium turbidity, froth keeps on; deposit noticeable	538	Notable turbidity; little froth on surface
14	1100	Turbidity and quantity of froth decreased, deposit increased	858	Great turbidity, much froth at surface and deposit
16	1326	—	860	—
18	1404	—	904	—
20	1400	—	936	—
22	1260	—	905	—
24	1014	—	1200	—
26	858	—	1020	—
28	800	—	1014	—
30	655	—	858	—
46	15	—	546	—

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In spite of the apparent paradox shown in Tables V and VI, Table VIII and the corresponding curves clearly show that the reproduction of *coli* as well as the gas production at 43·5° begins earlier, increases more intensively and reaches its maximum much sooner than at 37°. It may be pointed out that the absolute figures determined at a maximum stage of reproduction are also somewhat higher at 43·5° (1404 millions) than at 37° (1200 million). The difference, however, is within the experimental error of the method.

Having reached the maximum, reproduction is replaced by a phase of progressive dying, due to the accumulation of acids. This regressive process also occurs at a greater speed at 43·5°, and often, as is seen from a number of the preceding experiments, after 48 hours' growth at this temperature, the medium has spontaneously become sterile (Neumann's phenomenon). The reproduction of *coli* at 37° is subject in general to the same regularity, the whole process here lasting longer. Hence, it becomes quite clear why, when determining the figures of reproduction after 24–48 hours at 43·5°, as a rule lower figures are obtained than at 37°. In 24 hours we compare the culture at 37° at the summit of its reproduction with the culture at 43·5° already in a stage of dying, and in 48 hours we compare two regressing cultures of which the culture at 37° is in the middle stage of regression, whereas the culture at 43·5° is already in the final stage.

In the light of the data described in this paper it is stated that for the purpose of excluding the injurious influence of excessively high temperatures fermentation tests on the Eijkman principle must be made not at 46° but at 43–43·5°.

The data of Skinner and Brown and our investigations confirming part of them, do not lessen the value of the principle of fermentation tests at a raised temperature as proposed by Eijkman. The temperature of 43–43·5° recommended by us prevents the suppression of *Bact. coli communis* which occurs at 46° in Bulir's medium energetically stimulates its activity even when in very small numbers, and at the same time this temperature sharply represses the development of organisms of the *coli-aerogenes* group, which are not characteristic of faecal pollution but may occur in water.

Our proposal to make fermentation tests in Bulir's medium at 43° was practically anticipated by Eijkman. He regarded 46° as the maximum, and thought the test might actually be more sensitive at 42°.

Vincent and Henneckine (*Arch. méd. Belge*, **75**, 1922) recommended for the presumptive test nearly the same temperature (42°). The Hygienic Institute of the German University in Prague applies a fermentation test on glucose bouillon at 43° (see Singer (1929), *Zbl. f. Bakt.* I Abt., Ref., **95**, 445). According to a personal communication received by one of us (Minkevich) from C.-E. A. Winslow in 1935, when Eijkman's test is used in America, it is also preferably applied at 43°, not at 46°.

The accelerated multiplication and gas production of *coli* at 43·5° makes it unnecessary in nearly all cases to keep fermentation tests in the thermostat

more than 24 hours. This gives the best conditions for diminishing the number of false fermentation tests dependent on the presence of less thermo-resistant *coli-aerogenes* bacteria and gas-producing anaerobes, which appear later.

The comparison of fermentation tests in Bulir's medium at 43° with standard American tests on bouillon with lactose at 37° with a view to determining their comparative values is the object of a special investigation which is being made by us and the results of which we hope to publish in the near future.

Conclusions

1. A temperature of 46° is the maximum which will allow the development and gas production of *Bact. coli communis*. It does not hinder the manifestation of the fermentation capacities of cultures in Bulir's medium in massive inoculations, but in many cases represses their development when in small numbers.

2. It is proposed to make fermentation tests in Bulir's medium at 43–43·5° for determining the *coli* content of water.

This temperature does not prevent the development of even single cells of *Bact. coli communis* in maximum dilutions, stimulates sharply its development and gas production, and apart from exceptional cases limits the time of incubation to 24 hours.

3. The following scheme for determining the *coli* content of water can be recommended:

(*a*) Fermentation test in Bulir's medium at 43–43·5°; the incubation period being 18–24 hours.

(*b*) Subculture from the two last dilutions which produce gas on to a solid differential medium containing lactose (Endo, Levine) in Petri dishes. It is advisable, after making control reinoculations on the Endo's medium, to keep the cultures which have not caused fermentation for another 24 hours in the thermostat at 43° because sometimes *Bact. coli communis* does cause fermentation at this temperature later than 24 hours.

(*c*) Identification of colonies after their bacterioscopic examination according to the ordinary scheme. Instead of a long range, in large-scale work, the following two decisive tests can be recommended:

(i) A massive inoculation from colonies into test-tubes containing Bulir's medium for testing the fermentation capacity at 46°.

(ii) Slant culture in a test-tube on Simmons' medium. Only those colonies can be regarded as typical *Bact. coli communis* which 24 hours after massive inoculation into Bulir's medium produce gas at 46°, but which in Simmons'

medium at 37° after the same interval do not reveal any growth or change of colour of the medium.[1]

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[1] The combination of fermentation test at 45–46° with the Koser-Simmons' citrate test absolutely excludes the most common saprophytic coliform organisms in water: *Bact. aerogenes*, *Bact. cloacae*, *Bact. aquatilis communis* and coliform varieties from the intestines of cold-blooded animals. This is evident from Table IX, which gives the chief characteristics of different strains of these bacteria.

Table IX

Bacteria	Growth on citrate medium	Gas production in Bulir's medium at 45–46°
Bact. coli commune and its varieties	−	+
Bact. aerogenes	+	− (seldom +)
Coliform bacteria from the cold-blooded	+ (seldom −)	−
Bact. cloacae	+	−
Bact. aquatilis communis	−	−

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