

## INCUBATION AT 44° C. AS A TEST FOR FAECAL *COLI*

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(With 3 Figures in the Text)

### FOREWORD

THE research recorded by Dr Clegg and Mr Sherwood was undertaken as part and parcel of a series of investigations originating in certain happenings, at first disturbing and apparently inexplicable, which occurred in connexion with shellfish purification. Its presentation, therefore, would normally form an important integral part in a pending general report on the more comprehensive research referred to. In view, however, of the fact that the subject-matter deals with a problem which is much to the fore at the present time, not only in respect of shellfish, but in a much wider field, it has been considered desirable to give it separate and prior publication.

Briefly, the stimulus to the main investigation was provided by occasional anomalous results observed by us in the bacteriological analyses of mussels which had undergone the process of purification in operation at Conway and elsewhere. It appeared, at such times, that the mussels had not only undergone no purification, but were much worse than before treatment, judging by the tests of samples incubated at 37° C. It gradually emerged that these fantastic results were due to bacteria of the *Aerogenes* group (and, in some cases, almost exclusively to organisms of the *Cloacae* type), and that their presence in such overwhelming numbers was due to multiplication in the shellfish (Dodgson 1936, 1937, 1938).

The upshot was that, unless the purification installations in question were to be considered as potential "insanitary areas", bacteriological tests must be confined to the determination of the *Escherichia coli* content of the shellfish.

At the suggestion of Prof. G. S. Wilson experiments with incubation at 44° C. were undertaken. It became increasingly evident that an important factor was the strict regulation of the temperature of incubation. After numerous trials, the device described in the paper was suggested and constructed by Dr Clegg, the results of its use proving most satisfactory. The burden of the present communication is the essential importance of such close regulation.

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It would appear to be logical to adopt incubation at 44° C. for all shellfish, whether purified or not. The question of a "standard of purity", on such a basis, is one that presents some difficulty, but, in the light of data already accumulated, it should not prove insuperable.

R. W. D.

#### INTRODUCTION

*Escherichia coli* as the sole index of faecal pollution was first employed by Eijkman (1904); its use has now been advocated by Dodgson (1938) and Perry (1935) for assessing the degree of pollution of shellfish. Members of the intermediate-*aerogenes-cloacae* (I.A.C.) group, which are considered by water authorities to be an index of remote faecal pollution only, have been frequently suspected of multiplying in shellfish and shellfish waters, and have been found in enormous numbers in unpolluted oysters and mussels and in the barnacles attached thereto (Dodgson, 1936, 1937, 1938; Perry, 1935).

Assuming—and there is a mass of evidence upon which to base this assumption—that *Esch. coli* is a reliable indicator for assessing the degree of faecal pollution of water or shellfish for human consumption, we need hardly emphasize the value of any test for the differentiation of *Esch. coli* which could be used alone without severe and justifiable criticism. At present, however, for classifying members of the coliform group in water investigations, the Ministry of Health (1937) prescribes five differential tests and suggests four confirmatory tests. A combination of two or more tests is likewise specified by the American Public Health Association (1933) for this purpose, no single test being yet accepted as adequate.

Perhaps the most important attempt to devise a single test for differentiating typical faecal *coli* was Eijkman's use of the fermentation of dextrose with the production of acid and gas at 46° C. Of late, favourable reports have appeared about the use of a modification of this method (Perry, 1935). The fact that more than thirty years after its evolution, this method is still receiving serious attention points to its potential value.

It appeared from a review of the literature that the most serious criticism of the Eijkman test was that of the temperature of incubation. Eijkman's original test was carried out at 46° C., as is Perry's modification of it; the Metropolitan Water Board (1937) favours a temperature of 42° C., while Wilson (1935), in connexion with his extensive milk investigation, adopted 44° C. In this laboratory a long investigation, arising out of the apparent multiplication in shellfish of lactose fermenters other than faecal types, has included trials of modifications of Eijkman's test for differentiating faecal *coli*. In this Harding (unpublished data) found a 95% negative correlation between incubation at 44° C. and the citrate test, among lactose fermenters isolated from polluted shellfish: the plate counts, however, after incubation at 44° C. appeared to be unduly low in comparison with parallel plates at 37° C. Wilson had already carried out a test on similar lines with cultures obtained from

milk and his report was very favourable inasmuch as only 1 strain out of 496 tested gave a citrate-positive result and also a positive Eijkman test. He states: "It seems likely that those workers who have reported on the test favourably have had incubators permitting a fairly constant temperature of about 44° C. in their Eijkman tubes, while those who have reported on it unfavourably have been working with incubators in which the temperature of the medium was too high, too low, or inconstant."

Wilson himself refers to a variation of  $\pm 1.0^\circ$  C. in his incubators.

In the testing of pure cultures of organisms and of samples of minced mussel and other shellfish at 44° C. in this laboratory, considerable difficulty has been encountered in controlling the variation of the temperature of the incubators to less than 1° C.; this applied to water-jacketed air-incubators and water-baths, the heating of both of which was controlled by capsules. With the adoption of a specially constructed mercury-toluene thermo-regulator (see later) the variation in temperature was reduced to  $\pm 0.1^\circ$  C. (i.e. one-tenth of that allowed by Wilson) as recorded by a maximum and minimum thermometer kept in the bath throughout the experiments. Consequently it seemed desirable to investigate, with the aid of the new equipment, precisely which temperature between 41 and 46° C. was the most suitable for the growth of *Esch. coli* and the elimination of the I.A.C. group as judged by the citrate test, in view of the possibility that a variation of 1° C. might make all the difference between the fermentation of the carbohydrate with the production of gas, and no evolution of gas; an effect which we were soon able to demonstrate.

Thus this research was undertaken, as a self-contained portion of a larger investigation which has provided a favourable starting point, to discover the most effective modification of Eijkman's test, in the hope that it would be sufficient in itself as a method for identifying faecal *coli* especially in the testing of shellfish.

#### DESCRIPTION OF THERMO-REGULATOR

It was not originally intended to include a description of the thermo-regulator in this paper as Novy's mercury-toluene thermo-regulators have been on the market for a number of years, and our home-made apparatus is only a form of the original modified to fit a water-bath with a lid. However, as we have had several enquiries concerning it and requests suggesting that it should be included in this paper, a brief description together with a diagram is given.

In Fig. 1 the glass bulb and part of the connecting tube are filled with toluene and the remaining portion (the **U** part) of the tube with mercury. The amount of mercury in the reservoir is adjusted by means of a screw clip so that when the desired temperature in the bath is reached the opening of the capillary is almost covered with mercury. This cuts off the main supply of gas to the burner and permits only a very meagre supply through the capillary tubing in the by-pass. When the temperature of the water in the bath cools a fraction of a degree the toluene in the bulb contracts, the level of the mercury in *A* falls,

exposing the mouth of the capillary, and the supply of gas to the burner is increased.

The apparatus which we have in use gives a maximum variation in temperature of not more than  $\pm 0.1^{\circ}$  C.; in fact, it is frequently less than this.

The sensitivity of any such regulator is dependent on its construction in which care should be exercised. The following points may be of some assistance:

(a) The bulb containing the toluene should be of such size and shape as to permit the expansion of the toluene to raise and lower the mercury column with only a very slight change in temperature.

(b) There should be a constriction in the tube *A* just below the capillary gas inlet to augment the rise and fall of the mercury column.

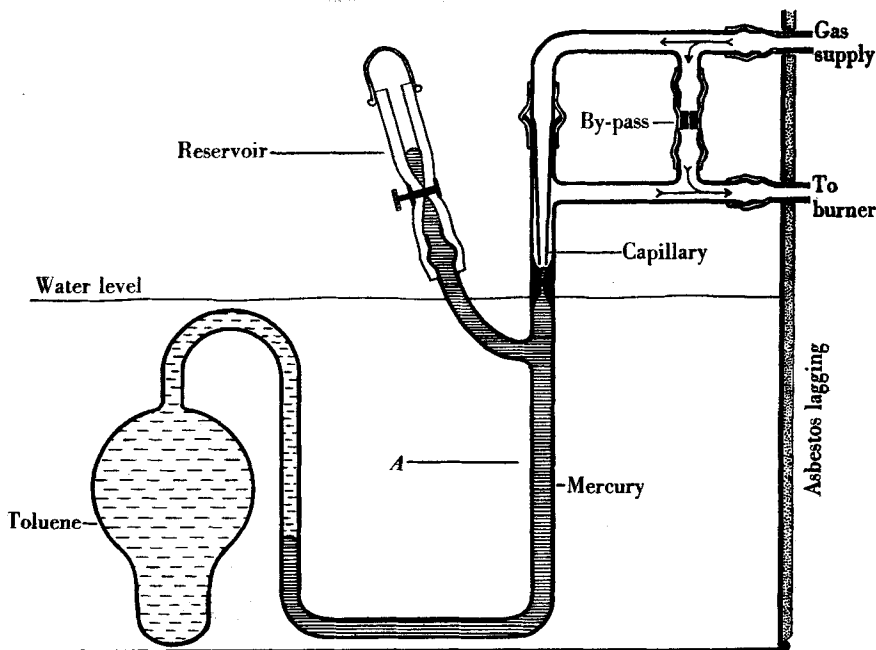


Fig. 1. Diagram of thermo-regulator in the corner of a water bath.

(c) The by-pass should permit only the minimum amount of gas to keep the flame alight.

(d) The capillary should be placed in a central position so as to come in contact with the top of the meniscus of the column of mercury.

(e) When filling the apparatus (this is rather a laborious procedure, as only a small amount of toluene can be admitted at once) sulphur-free toluene should be used. Even so, a slight reaction will take place between the toluene and the mercury. To obviate this the mercury and toluene should first be placed together in a bottle and shaken for some hours to allow this reaction to go to completion. The toluene and mercury can then both be filtered and used to fill the thermo-regulator.

These regulators may need cleaning and refilling every three or four months according to the purity of the gas supply. It will be found that in time a black sulphide deposit is formed on top of the mercury. If the pressure of gas is good it is possible to employ a scrubbing device to remove these sulphur compounds from the gas.

We have also in use an electrically heated and controlled water-bath (the component parts of which were purchased from a well-known manufacturer of chemical apparatus). This bath gives a slightly smaller variation in temperature than the gas-heated ones, but it is much more expensive to construct and costs more to run.

#### EXPERIMENTS

##### *First series of cultures*

In the first instance 196 colonies were isolated from samples of polluted mussels, portions of which had been plated out on lactose, bile salt, peptone, neutral red agar at 37° C., the technique described by Dodgson (1928) being followed. These colonies after being "pricked out" and cultured on nutrient agar slopes were inoculated into Koser's citrate medium at 37° C., and into MacConkey's broth at 37, 41, 42, 43, 44, 45 and 46° C., in an accurately controlled water-bath in which the variations from the desired temperature did not exceed  $\pm 0.1^\circ$  C. as recorded by a maximum and minimum thermometer. MacConkey's broth, containing bile salt and lactose, has been in use in this laboratory for over 20 years and has proved very satisfactory in inhibiting moulds and spore-forming bacteria (Dodgson, 1928). MacConkey's medium has been reported by Wilson (1935) to be more favourable for gas production by *Esch. coli* at 44° C., than his modification of Eijkman's medium, and Perry (1935) has recently changed over to it from his own modification of Eijkman's medium. The use of this medium was therefore decided upon in preference to Eijkman's in which the fermentable carbohydrate is dextrose.

It should perhaps be mentioned that the MacConkey tubes inoculated from these "prick-out" colonies were not pre-heated before inoculation. Criticism on this subject has been raised and is probably quite justified when water-jacketed air incubators are used, but when the tubes are incubated in a water-bath this precaution is unnecessary. This was demonstrated by placing twenty-five tubes, each containing 10 c.c. of liquid at 14.8° C., in the 44° C. water-bath. The temperature of the contents of the tubes was read at intervals of 1 min., and it was found that the desired temperature of 44° C. was reached after 9 min. incubation.

When preparing plates from polluted mussels for incubation at 37° C., high dilutions of the juice from the minced mussels were used, so that it was possible to prick out every colony from a plate or from a given area. Thus the ratio of the faecal to non-faecal types appearing on the plates would not be upset by the unconscious and perhaps natural tendency of the experimenter to select the largest colonies from a plate. It was realized that this precaution would lead to the

inclusion of a large number of non-lactose fermenters, especially as it was decided (for the purpose of collecting other data) to incubate the plates for 48 hr. instead of 24 hr. as is usually done when making "prick-out" cultures. It would have been misleading to have selected only red colonies from the plates because at 48 hr. certain vigorously growing lactose fermenting colonies, which give a definite acid reaction with the neutral red in 24 hr., become decolorized.

The results of inoculating the 196 cultures into MacConkey broth and incubating for 48 hr. at the various temperatures are shown in Table I and Fig. 2.

Table I. *The results of different temperatures of incubation for 48 hr. on 196 cultures inoculated into MacConkey's broth*

Temperature of incubation, ° C.	No. of cultures showing full acid and gas*				Non-lactose fermenters or limited gas	
	Citrate-positive		Citrate-negative		Nos.	%
	Nos.	%	Nos.	%		
37	116	59.2	29	14.8	51	26.0
41	18	9.2	17	8.7	161	82.1
42	7	3.6	17	8.7	172	87.7
43	3	1.5	17	8.7	176	89.8
44	1	0.5	17	8.7	178	90.8
45	0	0	14	7.1	182	92.9
46	0	0	8	4.1	188	95.9

\* "Full acid and gas" refers to an amount of gas which more than fills the concavity of a Durham's tube (see below).

Data collected in this laboratory over a number of years showed that out of 1615 colonies isolated at 24 hr. from neutral-red agar plates, 1439 or 89.1% produced acid and gas when inoculated into MacConkey's broth at 37° C. In Table I the percentage of lactose fermenters from the colonies isolated is 74. This is at variance with the figure stated above, but when the fact is taken into consideration that this second percentage is from *all* the colonies which developed on neutral red agar plates up to 48 hr. and not from red colonies alone which developed in 24 hr., it will be seen that the two results are not comparable. These results, however, are not in agreement with that of Bigger (1934), who found only seven lactose fermenters out of 145 colonies isolated, and on that score condemned out of hand shellfish analysis on lactose—bile salt—peptone—neutral-red agar ("MacConkey's agar").

The two important facts arising out of Table I are: (a) the reduction of the citrate-positive strains from 59.2% at 37° C. to 0.5% at 44° C. and (b) the constant number of citrate negative strains between 41 and 44° C., and the quick fall at higher temperatures, tending to indicate that temperatures above 44° C. are too high (Fig. 2).

Perry & Hajana (1935) found by their modified Eijkman method that out of 223 colonies of lactose fermenting organisms, isolated from unpurified oysters obtained from four localities differing topographically and geographically, only 11.2% were *Esch. coli*. In this series of our results, out of 145 strains of lactose fermenters 17 or 11.7% were citrate-negatives which fer-

mented lactose at 44° C. Such close agreement is perhaps only a coincidence and is not stressed.

It will be noted that there is a total reduction of 6·1% in the citrate-negative strains between 37 and 44° C. If this percentage is calculated on the basis of the citrate negatives alone it becomes 41·6%: this seems to be alarmingly high. However, it must be remembered, that these cultures were also inhibited at 41° C.: the faecal significance of citrate-negatives which are

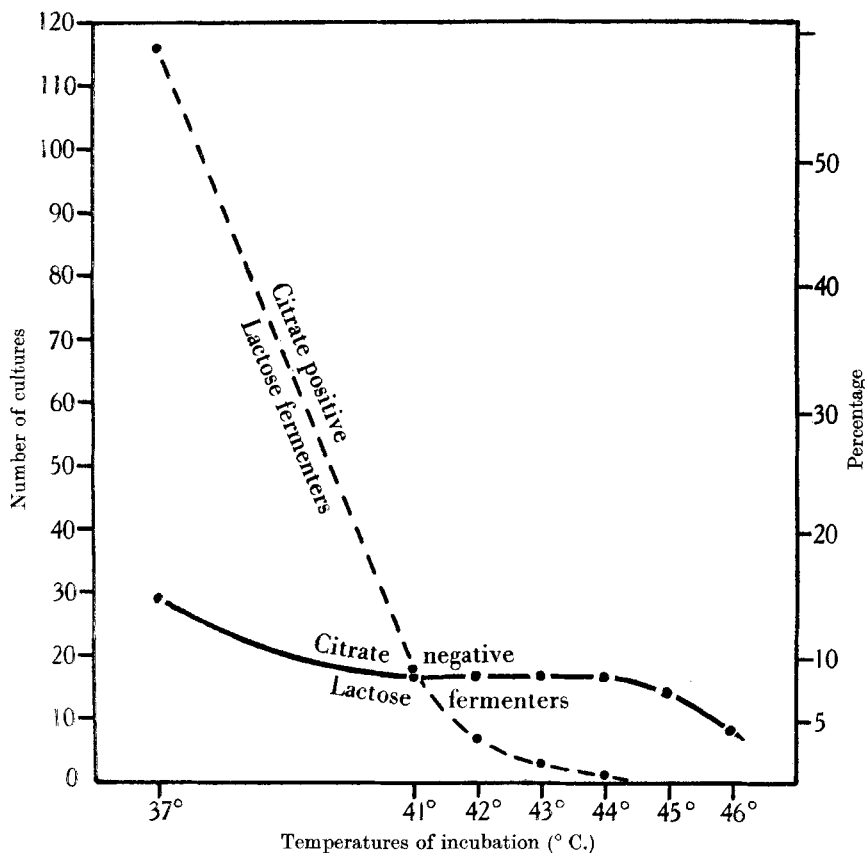


Fig. 2. Showing effect of different temperatures of incubation on lactose fermenters isolated from shellfish (series 1).

cut out at 41° C., we would venture, is rather dubious. Again, were we not to cut out this 41·6% of citrate-negatives, we should have to include a significant number of citrate-positives.

It would have been interesting to have performed methyl red and Voges-Proskauer tests on all these strains that were cut out at 44° C., both citrate-positive and citrate-negative, to see if the different temperatures segregated the different types of the *coli-aerogenes* group into definite zones; but it was deemed more important to repeat this experiment on a larger scale to provide a wider basis for conclusions, rather than to attempt to draw conclusions from

the somewhat meagre evidence already available; and to obtain such data as is mentioned above from a further experiment.

In recording the data from the 196 cultures, the tubes, wherever necessary, were incubated for 48 hr. It was seldom found that tubes which were negative at 24 hr. were positive at 48 hr., but occasionally a tube showing a limited production of gas at 24 hr. showed "full" gas after 48 hr. incubation.

#### *Gas formation*

In this work the problem arose as to what amount of gas in a Durham's tube should constitute a positive result. On this point depends, to a limited degree, Tables I and III and Figs. 2 and 3. The criterion we have adopted in recording tubes as positive is that the concavity of a Durham's tube 1 cm.<sup>1</sup> in diameter should be full of gas, i.e. approximately 10% of the tube. Perry (1935), who uses a temperature of 46° C. for his modified Eijkman test, permits either visible gas (one or more small bubbles in the Durham's tube) or effervescent gas (that which may be seen on shaking the tube) to denote a positive result. Our view is, that if a certain culture will give only a limited production of gas (i.e. filling less than 10% of the Durham's tube) at a certain temperature, but yet will give a vigorous production of gas at a temperature of one or more degrees lower, and an even smaller amount of gas or no gas at all at a temperature one degree higher, then the temperature at which the limited production of gas takes place should be regarded as too near the thermal death-point to be taken as a stable temperature for that culture to produce gas. For example: a number of the cultures which gave acid and gas at 44° C. (i.e. 10% gas or more) gave a considerably smaller volume of gas at 45°, and still less or none at 46°. One 44° C. positive strain yielded no gas even at 45° C. The reactions of ten cultures (from a later experiment) shown in Table II bear out this point.

Table II. *Reactions of ten cultures at different temperatures in MacConkey's broth*

Culture no.	Citrate utilization	Reaction in MacConkey's broth			
		43° C.	44° C.	45° C.	46° C.
162	—	AG	AG	A <sup>P</sup>	A <sup>Ph</sup>
167	—	AG	AG	A <sup>P</sup>	A
155	—	AG	AG	A <sup>P</sup>	A
168	—	AG	AG	A <sup>P</sup>	—
18	—	AG	AG	A <sup>P</sup>	—
31	—	AG	AG	A	A
28	—	AG	AG	—	—
107F	+	AG	A <sup>P</sup>	—	—
15F	+	AG <sup>C</sup>	A	—	—
119F	+	A <sup>P</sup>	A	—	—

AG, acid and gas (more than concavity); AG<sup>C</sup>, acid and gas (concavity full); A<sup>P</sup>, acid and "pisiform" amount of gas (less than concavity); A<sup>Ph</sup>, acid and "pin-head" amount of gas; A, acid only; —, no change.

<sup>1</sup> This is an unusually large size for a Durham's tube, but in our work it has proved more satisfactory than the smaller size.



The three citrate-positive cultures in the above table are not representative: most of the citrate-positive cultures were cut out at lower temperatures. They are included to illustrate our argument. They also point out the absolute necessity for careful regulation of temperature in the incubator.

When stipulating that a certain amount of gas shall constitute a positive tube, we must legislate for "border-line" cases, in which it would seem that confirmatory tests, such as are in general routine use, would have to be made. Most of the cultures which ferment lactose at 44° C. do so *vigorously*. The very small percentage which give slightly less than a concavity of gas should be tested further.

*Second series of cultures*

In a repetition of the first experiment seven samples of polluted oysters and mussels from six different localities were examined. It was hoped thus to ascertain if the correlation of the citrate test and the fermentation of lactose at 44° C. was a phenomenon general to most localities, and if local peculiarities in the bacterial flora of any one place could be readily demonstrated. The seven samples were obtained from the following places:

Sample	I: Duddon (mussels).
,,	II: Conway (mussels).
,,	III: Conway (mussels).
,,	IV: Brightlingsea (oysters).
,,	V: Lympstone (mussels).
,,	VI: Lytham (mussels).
,,	VII: Aberdovey (mussels).

From each sample a pool of ten shellfish was prepared for plating, and 48 hr. colonies were pricked out on to agar slopes from each sample, making a total of 336 cultures for the whole test. The same technique as practised before, of pricking out all the colonies from plates or portions of plates, was used. Of these 336 colonies ten failed to grow on agar slopes. The results of inoculating the remaining 326 strains into Koser's citrate medium at 37° C., and MacConkey's broth at the different temperatures are given in Table III and Fig. 3.

Table III. *The results of different temperatures of incubation on 326 cultures inoculated into MacConkey's broth*

Temperature of incubation, ° C.	No. of strains showing full acid and gas*				Non-lactose fermenters or limited gas	
	Citrate-positive		Citrate-negative			
	Nos.	%	Nos.	%	Nos.	%
37	53	16.3	83	25.4	190	58.3
41	18	5.5	70	21.5	238	73.0
42	11	3.4	69	21.2	246	75.4
43	6	1.8	69	21.2	251	77.0
44	4	1.2	69	21.2	253	77.6
45	1	0.3	47	14.4	278	85.3
46	0	0	8	2.5	318	97.5

\* See Table I, note, relating to gas.

In this second experiment it will be seen at a glance that, although the conditions differ from the first experiment as regards the proportions of lactose fermenters to non-fermenters and the number of citrate-positives to citrate-negatives, the fundamentals remain the same. The numbers of citrate-negative-lactose-fermenting cultures which remained constant in the first experiment between the temperatures of 41 and 44° C., here show a drop of 1 in 70. Above 44° C. the curve (Fig. 3) tails off much as before. The number of citrate-positive cultures which fermented lactose at 44° C. is very slightly higher, being 1.2% of the total instead of 0.5% as previously found.

The number of non-lactose fermenters at 37° C. is not comparable with any data previously obtained in this laboratory, being 58.3% instead of *ca.* 10%. As mentioned before all colonies were taken from 48 hr. plates. It is known that colonies which appear *after* 24 hr. incubation are mostly non-lactose fermenters, whilst the large proportion of those which appear within 24 hr.

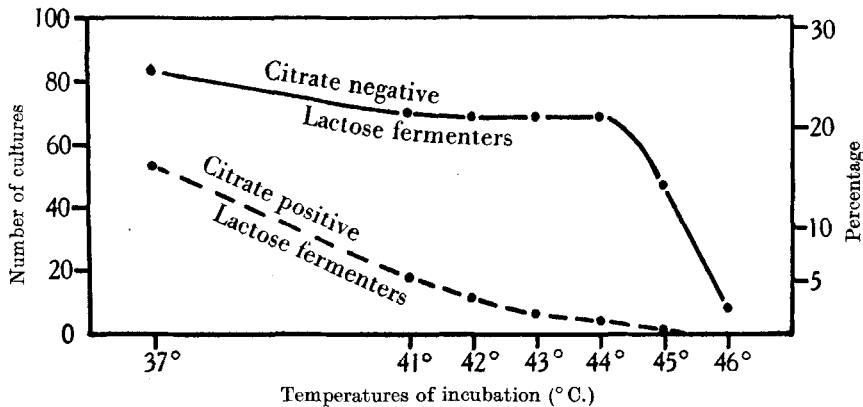


Fig. 3. Showing effect of different temperatures of incubation on lactose fermenters isolated from shellfish (series 2).

will ferment lactose. Thus the result here obtained should not be regarded as suggestive of the ineffectiveness of MacConkey plates.

A change of bacterial flora may have contributed to this difference. Such a possibility is suggested by Dodgson (1937) who states: "That waves of bacterial flora may occur is suggested by the fact that for a period of years the residual red colonies in lactose plates were almost invariably found to be staphylococci, whilst during the last two years (1935-7) such cocci have been conspicuous by their absence."

Of the 136 lactose fermenters at 37° C., sixty-nine or 50.7% were citrate-negatives which fermented lactose at 44° C. This is considerably higher than the 11.7% previously found, but was caused in the main by an exceptionally high percentage of this type of organism from the sample of Brightlingsea unpurified oysters. However, even when the data for the Brightlingsea sample are not included, the percentage of this type is still 29% of the lactose fermenters. This is quite high. It was hoped that such conditions as this would

arise for it has given the 44° C. incubation a more thorough test than would have resulted from two experiments in which the conditions were similar.

The eighty-three citrate-negative cultures which fermented lactose at 37° C. (this figure includes those which fermented lactose at 44° C.) and the four citrate-positives which also fermented lactose at 44° C. were stained for Gram reaction and put through the indol, methyl red and Voges-Proskauer tests. The Voges-Proskauer test used was the modification suggested by O'Meara (1931). The medium used in this test will give the reaction in 24 hr. and, in a comparative study of a number of modifications of the Voges-Proskauer test in this laboratory, was found to be the most reliable.

Of these eighty-seven cultures the sixty-nine citrate-negatives which fermented lactose at 44° C. were found to be without exception Gram-negative non-spore-forming organisms which were methyl-red positive and Voges-Proskauer negative. All but three of these strains produced indol. The characteristics and reactions of the remaining eighteen cultures (four citrate-positive, 44° C. lactose-positive; and fourteen citrate-negative, 44° C. lactose-negative) are given in Table IV. These eighteen cultures were also Gram-negative non-spore-formers.

Table IV. *Characteristics of eighteen citrate-positive and citrate-negative cultures which did not correlate with the 44° C. test*

Culture no.	Highest temp. recorded for "full" AG in MacConkey's broth	Citrate	Methyl red	Voges-Proskauer	Indol	Gelatin	Classified as
246	37	-	-	-	-	-	Irregular VIII (Wilson)
25	37	-	+	-	-	-	
114	37	-	+	-	-	-	} <i>B. coli</i> type II (Ministry of Health)
127	37	-	+	-	-	-	
130	37	-	+	-	-	-	} Atypical <i>B. coli</i> (Bigger)
122	37	-	-	+	-	-	
124	41	-	-	+	-	-	} Intermediate type IX (Bigger)
284	37	-	-	+	-	-	
254	37	-	±	+	-	-	} Irregular V <i>aerogenes</i> -like I (Wilson)
255	37	-	±	+	-	-	
166	37	-	-	+	+f	-	} Intermediate type VII (Bigger)
249	37	-	-	+	+f	-	
136	37	-	±	+	+	+	} New types
244	37	-	±	+	+f	+	
108	45	+	-	+	-	-	
203	44	+	-	+	-	-	} Irregular <i>aerogenes</i> -like (Wilson)
123	44	+	+	-	+	-	
308	44	+	+	-	+	-	} Atypical <i>B. coli</i> (Bigger)

"f" denotes a faint reaction.

In Table IV the column headed "highest temperature recorded for 'full' acid and gas in MacConkey's broth" refers to the highest temperature of those used at which the production of acid and gas was noted and not the highest temperature at which the culture would give acid and gas; for no experiments on temperature were performed between 37 and 41° C.

Incubation for liquefaction of gelatin was carried on for 14 days only.

Of the four citrate-positive cultures which fermented MacConkey broth at 44° C., two (nos. 108 and 203) gave the reactions of *typical aerogenes*, but are called by Wilson (1935) *Irregular aerogenes-like* because of growth at 44° C. The other two (nos. 123 and 308) gave reactions similar to those of Bigger's *atypical coli*, though it is not known whether Bigger's *atypical coli* would give a positive 44° C. test. This type is not included in the classifications of Wilson or the Ministry of Health.

Of the fourteen citrate-negative cultures which failed to ferment MacConkey's broth at 44° C., four (nos. 25, 114, 127 and 130) are called by the Ministry of Health *Bact. coli type II*, and by Bigger *atypical coli*. Nos. 122, 124 and 284 correspond to Bigger's *Intermediate type IX* and to Wilson's *Irregular V aerogenes-like I*, but are not classified by the Ministry of Health. Nos. 254 and 255 conform to Bigger's *Intermediate type VII*, but not to anything set down by Wilson or the Ministry of Health. Again, no. 246 is given by Wilson as *Irregular VIII*, but not recorded by Bigger or the Ministry of Health. The remainder constitute three new combinations, the first nos. 136 and 244,<sup>1</sup> the second no. 166 and the third no. 249.

Thus only two types from the cultures described above (wherein the citrate and 44° C. tests cannot be correlated) appear to have any faecal significance. Only one of these types is eliminated by the 44° C. test. To the best of our knowledge the significance of these two types is still doubtful and the occurrence of such types is far from frequent; a fact which this experiment supports.

#### *General considerations*

As mentioned previously this work on incubation at 44° C. attempts to eliminate the need for confirmatory tests by providing a single test for the identification of *faecal coli*. The need for a single test, and the futility in the present state of our knowledge of multiplying tests, as far as practical shellfish pollution work is concerned, has already been stressed by Dodgson (1928, p. 381; 1938).

It is not suggested that this test would be of similar value in water examination, where the detection of members of the I.A.C. group is a matter of considerable importance, though it might well serve a useful purpose as a single confirmatory test.

On the other hand, in the routine examination of shellfish, because of the occasional multiplication of members of the I.A.C. group in the shellfish, and because of the presence of these organisms in coastal and estuarine waters which may be free from faecal pollution, it would be misleading to place any reliance on these organisms; it is therefore necessary to use a test which will differentiate *faecal coli*.

Criticism will probably be directed against incubation at 44° C., if put into use for the examination of shellfish or waters from shellfish beds, on the grounds

<sup>1</sup> On reading a recent paper by Parr (1938) we discovered that organisms giving the reactions of cultures 136 and 244 have been recorded by him.

that samples from known polluted areas incubated at this temperature may give very low counts when compared with the counts or "probable numbers" of coliform organisms at 37° C. This may be so, but we already have data, which it is hoped will be included in a later paper, tending to show that *Esch. coli* does appear to be a fairly reliable index of pollution. As the 44° C. test gives such close correlation with the citrate, indol, methyl red and Voges-Proskauer tests, it should not be hard to revise any standards based on previous bacteriological examination to correspond with the new and more reliable index.

Objections may be raised against this method on the grounds that it is not perfect and that it inhibits a few organisms *suspected* to be of faecal origin and permits others to grow which are not thought to be indicative of faecal pollution. However, in these experiments, the number of such strains was insignificant, there being, in the second experiment, only two *aerogenes* admitted and three *atypical coli* excluded out of a total of 326 cultures studied.

In conclusion it is suggested that all that is really necessary in estimating the pollution of shellfish is to utilize an organism which will give a warning of the possible presence of typhoid bacilli. *Esch. coli* will do this, whereas the I.A.C. group may or may not. Although there have been cases on record where from certain samples of faeces no *Esch. coli* have been isolated, we think it highly improbable that at any given time the effluent from a sewer would contain more than a minute percentage of stools of this type. On the other hand it is now generally accepted that wherever *Esch. coli* is, there will faecal pollution be also.

The present investigation is being extended to include another series of organisms isolated not only from shellfish but also from faeces, and it is hoped to present the results in a future paper.

#### SUMMARY

1. The use of *Esch. coli* alone as an index of faecal pollution for shellfish, and the correlation between the 44° C. MacConkey test and citrate tests are discussed.
2. The mercury-toluene thermo-regulator used in these experiments, which gives a maximum variation of  $\pm 0.1^\circ$  C., is discussed briefly and illustrated.
3. Experiments are described in which 522 colonies from polluted shellfish were isolated, inoculated into MacConkey's broth and incubated at temperatures of 37° C. and at successive 1° intervals from 41 to 46° C., in accurately controlled water-baths. An almost perfect negative correlation was found to exist between 44° C. incubation and the citrate test.
4. It appeared that temperatures above 44° C. are detrimental to the growth of *Esch. coli*.

5. Certain cultures of citrate-negative lactose fermenters at 37° C., which were inhibited at 44° C., were found on further investigation to be mostly intermediate types.

ACKNOWLEDGEMENT. Grateful acknowledgement is made to Dr R. W. Dodgson, Consultant for Shellfish Services (late Director), Ministry of Agriculture and Fisheries, for most helpful criticism and advice, and to whom the initiation of the research of incubation at 44° C. as a test for faecal pollution, in connexion with shellfish, is due.

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(MS. received for publication 5. iv. 39.—Ed.)