THE DETECTION OF ACETYL-METHYL-CARBINOL IN BACTERIAL CULTURES[1]

A COMPARATIVE STUDY OF THE METHODS OF O'MEARA AND OF BARRITT

By C. G. BATTY-SMITH, M.A., M.D.

From the Public Health Laboratory, Wakefield, Yorkshire

CONTENTS

Historical survey

The early work on the chemistry of the Voges-Proskauer reaction is too well known to need consideration here. Several workers tried to hasten and strengthen the reaction by the addition of various oxidizing agents, but with little success. O'Meara (1931) made the first real advance when he investigated anew the chemistry of the reaction and found the accepted views incorrect in several respects. As a result he added creatine to the culture, used a much stronger alkaline solution than had hitherto been customary, and so achieved a marked improvement in the rapidity of development and intensity of the colour. He recommended the use of 24 hr. cultures.

Levine, Epstein & Vaughn (1934) obtained a larger number of positive results after 48 hr. incubation at 37° C. than after 5 days. They used a modified reagent for the O'Meara test consisting of 0·3 % creatine in 40 % potassium hydroxide solution and found that 100 % of positive results were obtained after the test had stood for 4 hr., whereas 99·4 % of positive results were given by the standard test only after 12 hr. standing.

Dorner & Hellinger (1935) compared the standard Voges-Proskauer, O'Meara and other methods but found no great difference in the results. They also tested various peptones on the market from the point of view of making media for this reaction, but the brand of peptone proved to be of little consequence. They found that clearer and more positive results were obtained by O'Meara's method if ferric chloride were added.

[1] This paper formed part of a thesis submitted to the University of Cambridge for the degree of M.D.

https://doi.org/10.1017/S0022172400059817 Published online by Cambridge University Press

Wilson, Twigg, Wright, Hendry, Cowell & Maier (1935) recommended performance of the test upon 2-day cultures because they found that many cultures which were positive after 24 hr. incubation became negative by the third or fourth day. They used O'Meara's method and found that it never missed a positive result. Compared with the old or standard method on sixty-five strains of aerogenes-cloacae organisms, it obtained ten positive results which were negative by the old method. They concluded that O'Meara's method was much the more delicate of the two.

Barritt (1936) recorded experiments with the addition of α-naphthol resulting in a great increase in sensitiveness and intensification of the colour change but without any loss of specificity. With this test it was found that certain organisms showing no reaction with the ordinary Voges-Proskauer test or with the O'Meara modification did in fact produce acetyl-methyl-carbinol. Barritt claimed that this test could detect diacetyl in a dilution of one part in one million, and that if creatine was added an even greater delicacy was secured, the substance being detected at one in five millions. It is interesting to note that O'Meara claimed for his modification that it was sensitive to a concentration of one part in 50,000.

Barritt gave warning that absolute ethyl alcohol must be used as a solvent for the α-naphthol because methylated spirits gave a positive reaction.

He advised that 3-day cultures should be used and mentioned Difco Bacto peptone as being particularly suitable for making the glucose-phosphate medium for the test.

The results obtained by using Barritt's method with the intermediate types of the coli-aerogenes group showed that a great many were α-naphthol-positive though negative by O'Meara's method. This finding brings these organisms into close relationship with *Bact. aerogenes* and suggests, Barritt thinks, that many, if not all, of those strains which are M.R.+, V.-P.+ should no longer be classified as irregular but as intermediates which produce just sufficient acetyl-methyl-carbinol to be detectable by ordinary methods.

He concluded that it was clear that the ordinary Voges-Proskauer test had no real value as negative evidence of acetyl-methyl-carbinol production.

Harold (1936) made comparative tests of O'Meara's modification and of Barritt's method on 150 'citrate +, M.R.+'-strains from polluted waters, the 'old' or 'standard' method being compared at the same time.

Both the creatine and α-naphthol tests revealed more positives than the old method (α-naphthol, 62 positives; creatine, 57) The colour given by Barritt's method was very distinct, and very few faint reactions were obtained as compared with the other two tests The results obtained with typical *Bact. coli* and aerogenes strains showed complete agreement by all three methods. Among the intermediates, on the other hand, marked discrepancies were found; the Barritt method giving more V.-P. positives

Cultures giving the reactions, M.R. +, citrate +, V.-P. + and indole + or − were carefully retested by repeated platings, and although some were converted into pure types, no mixtures were encountered. The majority continued to give anomalous results, and it is clear that with the Barritt test more of these V.-P. + organisms would be detected.

In the above experiments, 4-day cultures incubated at 37° C. in glucose-phosphate-peptone-water were used, the tests being carried out exactly according to the instructions given in the *Ministry of Health Report* No. 71 (1934), and the Barritt test exactly according to Barritt's directions.

Iyer & Raghavachari (1939), in a paper in which they praised highly the sensitiveness and specificity of Barritt's method, advocated the use of a single tube of glucose-phosphate medium for each culture on which to carry out, first the methyl-red test and then the Voges-Proskauer test, instead of using two separate tubes. They stated that controlled tests had shown that this might be done without the results being vitiated in any way. They claimed that this procedure conduced to economy, simplified the technique, and also ensured identical conditions as regards initial inoculum and final strength of the culture used for the two tests.

In this connexion Barritt's warning against false positive reactions produced by methylated spirit should be remembered and the methyl-red solution should be made with absolute alcohol.

Vaughn, Mitchell & Levine (1939) made some interesting observations upon the influence of incubation temperature on acetyl-methyl-carbinol production and also upon the periods needed for the full colour development when using Barritt's and O'Meara's methods.

They adopted the modification of Levine *et al.* (1934) which has been previously quoted and found the percentage of positive V.-P. reactions was greater by all methods if the cultures were incubated at 30° C.

The higher percentage of positives was given by Barritt's method, 23·1% of the cultures being positive at 30° C. incubation and 20·4% at 37° C.

These workers noticed that (1) the citrate results were not affected by the temperature or duration of incubation, (2) the number of typical *Bact. coli* obtained was the same at both temperatures, (3) the tendency of the higher temperature was to yield a larger number of M.R. +, V.-P. − reactions, and they recommended incubation for 24–48 hr. at 30° C. on the grounds that if the higher temperature was employed, some aerobacter strains might be incorrectly classed as intermediate or irregular.

Using known V.-P. positive strains, they tested the speed of development of the colour reaction and found that Barritt's method detected 99·5% of the positives if allowed to stand for 1 hr. Their modification of O'Meara's method gave 94·5% after 4 hr. while the standard method (10% potassium hydroxide) gave only 83·8% after 24 hr.

Experiments

Technique

This work was originally undertaken as a subsidiary investigation during an enquiry into the value of incubation at 44° C. in the bacteriological examination of water. So many coliform strains were to be isolated and typed in the course of this enquiry that it seemed a good opportunity to determine the relative sensitiveness and specificity of O'Meara's and of Barritt's method for the detection of acetyl-methyl-carbinol.

It is unnecessary to describe in detail the methods by which the organisms were isolated. In short, MacConkey agar plates were made from positive MacConkey broth tubes, some of which had been incubated at 44° C. and others at 37° C., colonies were picked and subjected to the usual differential tests as described in the *Ministry of Health Report* (1939).

Voges-Proskauer reaction. Barritt (1936) admits that slightly stronger reactions are given by his method if a knife-point of creatine is added, and this procedure was adopted throughout. The present writer tried the effect of adding ferric chloride in addition and found that the depth of colour of the positive reactions was slightly enhanced. A further slight increase in speed of development and in intensity of colour was found to be produced by 5 min. shaking after addition of all the reagents.

Barritt's method was therefore performed in the following way throughout the present work:

To 5 c.c. of a 2-day culture in glucose-phosphate medium, a knife-point (about 25 mg.) of creatine and 2 drops of 2% ferric chloride solution were added and the mixture lightly shaken. 3 c.c. of a 5% solution of α-naphthol in absolute alcohol were then added followed by 1 c.c. of 40% potassium hydroxide solution. The mixture was then vigorously shaken for 5 min. in a 'Kahn' shaking machine.

Since O'Meara's method is generally considered to be thoroughly reliable it was decided to perform it in parallel with the above modification of Barritt's method in order to test the specificity of the latter.

The tests were strictly comparable, 10 c.c. of a single culture being divided into two portions of 5 c.c. each for the tests.

O'Meara's method was performed as follows:

To 5 c.c. of a 2-day culture, a knife-point (about 25 mg.) of creatine was added, followed by 5 c.c. of a 40% solution of potassium hydroxide, and the mixture shaken for 5 min. in a mechanical shaker.

Vaughn *et al.* (1939) found that approximately the maximum yield of positives by these two methods was given after they had been standing for definite periods, these periods being: for Barritt's method 1 hr., and for O'Meara's method 4 hr. They considered that false positives might be given by Barritt's method if a longer period was used, and this is in accordance with Barritt's original remarks. The above periods were adopted in each case.

It will be remembered that Iyer & Raghavachari (1939) found it satisfactory to perform both the methyl-red and Voges-Proskauer tests on a single culture. In view of this, it seemed to the writer that it would be a pity to waste the methyl-red cultures, when, by using them as these workers suggest, an additional Voges-Proskauer result at 3 days could be obtained. This was in fact carried out by O'Meara's method, and several positives by Barritt's method were confirmed which otherwise would not have been supported.

Analysis and discussion of experimental findings

Table 1 shows that Barritt's method failed to detect only 4 % of the total positive cultures and that, if O'Meara's method is allowed to be reliable, only 4 % of the positive reactions by Barritt's method could in any sense be described as 'false', for the remainder were all corroborated in some degree by O'Meara's method. It was noticed that two cultures which were positive by Barritt's method showed a trace by O'Meara's method, the trace being a colour so faint that it could be detected only when the result was compared with a negative culture; without this comparison a negative result must have been recorded.

On repetition of the tests, of the five cultures which were negative by Barritt's method and positive by O'Meara's method, four were confirmed as

Table 1

	No. of cultures
Positive by Barritt's method; trace by O'Meara's method	2
Positive by both methods	106
Trace by both methods	4
Positive by Barritt's method; negative by O'Meara's method	5
Trace by Barritt's method; negative by O'Meara's method	2
Trace by Barritt's method; positive by O'Meara's method	1
Negative by Barritt's method; positive by O'Meara's method	5
Total cultures positive by one or both methods	125
Total cultures negative by both methods	477
Total cultures tested	602

(The results by O'Meara's method at 2 days and at 3 days are here considered as one.)

Table 2. *Comparative results after different incubation periods*

	No. of cultures	Percentage of total positive cultures
Positive by O'Meara's method after 2 days (9 trace only)	103	82·4
Positive by O'Meara's method after 3 days (4 trace only)	114	91·2
Positive by Barritt's method after 2 days (7 trace only)	120	96

negative by both methods, while in one a trace was detected by O'Meara and the Barritt test was a weak positive. In spite of the negative confirmations the original results (O'Meara positive at 3 days in each case) were probably not due to clerical errors, for all five organisms (save one which produced indole) were of the same type. Evidently since the highly sensitive Barritt method failed to detect acetyl-methyl-carbinol on the second day when the

less sensitive O'Meara method was able to detect it only on the third day, this organism must have produced the substance during the third 24 hr. after inoculation.

As to the five cultures which were positive by Barritt's method and negative by O'Meara's method, in two the positive result was confirmed by O'Meara's method, in one both tests were negative, in one the results remained unchanged and the fifth culture was no longer available.

These ten cultures in which a definite discrepancy occurred between the results of the two methods were all organisms of doubtful character and probably not coliforms. In other words, as far as definite coliform types were concerned there was no discrepancy between the results of Barritt's test and of O'Meara's test *when left to stand for the necessary time to develop all its positive reactions.*

It will be seen from Table 2 that when O'Meara's method was performed on 3-day cultures 8·8 % more positives were obtained than if the cultures were tested after 2 days; also that Barritt's method at 2 days detected 96 % of the positive cultures as compared with 91·2 % shown by O'Meara's method at 3 days. The inferences are that, on the average, positive reactions are more likely to be obtained from 3-day cultures, and that, if Barritt's method were used at this period, nearly 100 % of positives would be detected.

The opinion was expressed by Vaughn *et al.* (1939) that 4 hr. at least were necessary in order that the O'Meara method should develop its full number of positives. This view was amply substantiated. Although the results after 5 min. (the time recommended in the *Ministry of Health's Report*, 1939) were not recorded for comparison with those obtained after 4 hr., it was frequently observed that definite positives would have been lost had the readings been taken after 5 min. This is perhaps a factor which had led some workers to criticize Barritt's test on the grounds of lack of specificity, and if they have based their views upon a comparison with the old method, no doubt there was an even greater gulf fixed between the two sets of results.

Barritt's method possesses two advantages: speed and definiteness. Of the first, Barritt (1936) himself and Vaughn *et al.* (1939) have given 1 hr. as the period necessary for the development of the full number of positive reactions, but the writer encountered few additional positive reactions after 15 min., and there is no doubt that the extensive shaking adopted hastened the development of the colour.

Of the second, equivocal reactions were commendably rare with this method. Barritt, in his original paper, mentions that negative results occasionally show a faint trace of pink, and this was found to be the case though the number of such results was small. The colour referred to is a coppery-pink and is so characteristic that the danger of confusion is slight after even a short experience of taking readings; indeed, on three occasions only during the examination of 602 tests was the writer in any difficulty for a decision.

In the matter of so-called 'false positives' it has been shown here that 4 % only of positive results by Barritt's method went unsupported, and that in spite of the test having been used in a slightly modified form which gave stronger reactions and therefore might have been expected to cause still more false positives.

That the 4 % referred to are false is, however, unlikely for two reasons: first, that still more positives would almost certainly have been revealed if the test had been performed also upon 4- and 5-day cultures; and secondly, that Vaughn *et al.* (1939) observed that about 3 % more positive reactions could be obtained if the cultures were incubated at 30° C. instead of at 37° C.

Although some workers consider that the test should not be performed upon cultures less than 4 days old, it is probable that the loss of positive reactions resulting from the use of 3-day cultures is very small. There is an advantage in testing on the third day in that one culture may be used both for the methyl-red and Voges-Proskauer tests, which saves time and material when many tests have to be done.

In the writer's view the 'old' or 'standard' method for the Voges-Proskauer reaction should now be discarded and should cease to be described in official publications. Barritt's test should be more widely used, for it gives results which are practically parallel with those given by O'Meara's method without the necessity of waiting 4 hr. for them.

'MIXED' STRAINS

It is pertinent to refer here to the question of strains which give positive results by both the methyl-red and Voges-Proskauer tests.

Such strains have been noticed by many workers. Ruchhoft, Kallas, Chinn & Coulter (1931), Skinner & Brudnoy (1932) and Wilson *et al.* (1935) regarded them as mixtures, but Harold (1936) and Barritt (1936), both of whom encountered them while experimenting with the sensitive Barritt method, admitted their real existence. Doubtless the supposed negative correlation between the methyl-red test and Voges-Proskauer reaction owes its existence largely to the use of the old method, but it is impossible not to agree with Barritt that its negative results have no value as evidence of the absence of acetyl-methyl-carbinol.

Wilson attempted the separation of a series of these so-called 'mixed' strains and found that they reacted typically on reinvestigation. This result was difficult of explanation, and he came to no definite conclusion, but suggested that they might merely have reacted atypically on first examination or have resulted from the different rates of growth of mixed organisms in culture.

Quite recently Stuart, Mickle & Borman (1940) have thrown new light upon this subject. They found many strains which were positive 'to some degree' in both the methyl-red and Voges-Proskauer tests if the cultures were incubated at 37° C., but which were methyl-red negative and strongly Voges-Proskauer positive after incubation at 20° C.

In the present study a fair number of strains were encountered which gave a doubtful or positive methyl-red result with a positive Voges-Proskauer reaction. On repetition of the methyl-red test, some of these remained doubtful and a few became negative. In the case of those which continued doubtful, the methyl-red result was taken to be negative and most of the strains then corresponded to *Bact. aerogenes* type I.

However, twenty-seven strains were persistently methyl-red-positive and Voges-Proskauer-positive, so it was decided to plate some of them out from the stored citrate cultures as suggested by Ruchhoft *et al.* (1931). It was hoped thus to discover whether they were mixed strains, and a few of the cultures giving a persistently doubtful methyl-red result were treated in the same way.

There were twelve cultures on an organism which gave quite typical convex, mucoid colonies and which was M.R.+, V.-P.+ and citrate +, and eight of the twelve strains fermented cellobiose. Another which grew in similar colonies was M.R.+, V.-P.+, but failed to grow in citrate. Late and non-lactose-fermenting varieties of each of these types were encountered.

In all, eighteen suspected strains were plated but only four proved to be mixtures. Of these the only conclusive separation was that of one of the M.R.+, V.-P.+, citrate + strains; this was revealed as a mixture of intermediate type I with aerogenes type I. Two other cultures separated rather inconclusively, no doubt because insufficient colonies were picked.

The attempt to separate these strains was not very exhaustive, but in the majority of cases the colonies on each plate appeared identical. One strain which appeared particularly interesting was M.R.+, V.-P.+, citrate + and indole + and formed gas from cellobiose. A plate was made from the citrate tube and fifteen colonies were picked. Of these, fourteen gave the same reactions as the original strain but one was M.R.+, V.-P.−, citrate − and indole +. This raises a suspicion that if a still larger number of colonies had been picked a culture of aerogenes type I might have been obtained, thus accounting for the reactions of the original strain.

In view of the manner in which the methyl-red and Voges-Proskauer relationship can be reversed by different incubation temperatures, it is difficult to believe that M.R.+, V.-P.+ strains cannot exist. More work is still needed on this subject, but a very large number of suspected mixtures would have to be plated out and very large numbers of colonies picked from each plate. The use of modern methods for the detection of acetyl-methyl-carbinol may reveal more of these organisms, but whether they shall be classed as irregulars or, as Barritt (1936) and Harold (1936) suggest, with the intermediates, has yet to be decided. Perhaps, indeed, following the suggestion contained in the work of Stuart *et al.* (1940), they should not be classified at all except on the basis of results obtained with incubation of the cultures at 20° C.

SUMMARY

Upon each of 602 cultures, the Voges-Proskauer reaction was performed by O'Meara's method upon a 2-day and a 3-day culture, and by Barritt's method on a 2-day culture. Of 125 positive cultures, O'Meara at 2 days detected 82·4%; at 3 days 91·2%; and Barritt at 2 days, 96%. Only 4% of positives by Barritt's method were unsupported by O'Meara's method, and these strains were probably not coliforms. It is concluded that M.R. +, V.-P. + strains revealed by Barritt's test may have a real existence, for there is no valid reason for the view that this test gives false positives.

My thanks are due to Prof. P. L. Sutherland, Director of this laboratory, for permission to publish these results.

REFERENCES

BARRITT, M. M. (1936). The intensification of the Voges-Proskauer reaction by the addition of α-naphthol. *J. Path. Bact.* **42**, 441–54.

DORNER, W. & HELLINGER, E. (1935). Studies on the Voges-Proskauer test. *J. Bact.* **29**, 16–17.

HAROLD (1936). *Metropolitan Water Board, 31st Annual Report.*

IYER, P. V. S. & RAGHAVACHARI, T. N. S. (1939). Technique of methyl-red and Voges-Proskauer tests used in bacteriological analysis of water. *Ind. J. med. Res.* **26**, 885.

LEVINE, M., EPSTEIN, S. S. & VAUGHN, R. H. (1934). Differential reactions in the colon group of bacteria. *Amer. J. Publ. Hlth*, **24**, 505–10.

Ministry of Health Reports on Public Health and Medical Subjects, No. 71 (1934).

O'MEARA, R. A. Q. (1931). A simple delicate and rapid method of detecting the formation of acetyl-methyl carbinol by bacteria fermenting carbohydrate. *J. Path. Bact.* **34**, 401–6.

RUCHHOFT, C. C., KALLAS, J. G., CHINN, B. & COULTER, E. W. (1931). Coli-aerogenes differentiation in water analysis. *J. Bact.* **22**, 125–81.

SKINNER, C. E. & BRUDNOY, H. G. (1932). The utilization of citrates and the fermentation of cellobiose by strains of *Bacterium coli* isolated from human faeces. *J. Hyg., Camb.*, **32**, 529–34.

STUART, C. A., MICKLE, F. L. & BORMAN, E. K. (1940). Suggested grouping of slow lactose fermenting coliform organisms. *Amer. J. Publ. Hlth*, **30**, 499–508.

VAUGHN, R., MITCHELL, N. B. & LEVINE, M. (1939). *J. Amer. Wat. Wks Ass.* **31**, 993.

WILSON, G. S., TWIGG, R. S., WRIGHT, R. C., HENDRY, C. B., COWELL, M. P. & MAIER, I. (1935). *Spec. Rep. Ser. Med. Res. Coun., Lond.*, no. 206.

(*MS. received for publication* 10. x. 1941.—Ed.)