**Microbiology.** — *On the production of acetylmethylcarbinol by Bacterium coli.* By A. J. KLUYVER and E. L. MOLT.

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## 1. *Introduction.*

As is well-known, the so-called VOGES–PROSKAUER-test plays a rather important rôle in the differentiation of *Bacterium coli* from *Aerobacter aerogenes*. We owe to HARDEN [1]) the insight that a positive result of this test, as obtained in the ordinary media in which glucose has been fermented by the last mentioned species, is due to the presence of small amounts of acetylmethylcarbinol in these media. On addition of alkali to the media this compound is readily oxidised to diacetyl which then combines with some nitrogenous constituent of the commercial peptone (probably arginine), the complex thus formed imparting to the solution a pink colour.

A negative outcome of this test has since many years been a primary requisite for the characterization of a bacterium as a typical faecal strain of *Bacterium coli*.

Under these conditions the contention that also quite typical faecal strains of this species are able to produce small amounts of acetylmethylcarbinol in sugar fermentation bears a rather alarming character.

Nevertheless statements to the said effect have been made from time to time. In 1926 the senior author together with DONKER [2]) have on more or less theoretical grounds expressed their conviction that under certain conditions acetylmethylcarbinol will also appear among the products of the fermentation of sugar by *B. coli*. A positive result of a preliminary experiment made in a medium buffered at a relatively high pH was reported at the same time.

When, however, some years later the American bacteriologist RUCHHOFT inquired into this matter, it was not at once found possible to reproduce this result, and accordingly it was accepted that some error might have crept into the earlier experiment [3]).

In 1937 a short publication by REYNOLDS and WERKMAN [4]) has appeared

[1]) A. HARDEN, Proc. Royal Soc. Ser. B., **77**, 424 (1906).

[2]) A. J. KLUYVER und H. J. L. DONKER, Chemie der Zelle und Gewebe, **13**, 134 (1926).

[3]) Cf. C. C. RUCHHOFT, J. G. KALLAS, BEN CHINN and E. W. COULTER, Journ. of Bact., **22**, 125 (1931).

[4]) H. REYNOLDS and C. H. WERKMAN, Archiv. f. Mikrobiol., **8**, 149 (1937).



in which these authors communicate that they have been able to induce *B. coli* to form quite appreciable amounts of acetylmethylcarbinol in a thoroughly aerated mineral glucose-medium made weakly alkaline by addition of some sodium bicarbonate.

The experiments described below were undertaken with the primary aim of corroborating these results. Secondly, however, it was felt necessary to reinvestigate whether also under the usual conditions of the VOGES–PROSKAUER-test *B. coli* might possibly produce slight traces of acetylmethylcarbinol. Such a reinvestigation was especially desirable with a view to the recent studies of BARRITT [1]) who succeeded in greatly increasing the sensitivity of the VOGES–PROSKAUER-reaction by the addition of $\alpha$-naphtol to the medium before the alkalinisation.

In his first paper BARRITT proved that acetylmethylcarbinol is produced by many organisms usually considered unable to do so. Amongst these organisms were several so-called intermediate types in the *coli-aerogenes* group which gave a negative VOGES–PROSKAUER reaction (in O'MEARA's creatine modification). To the contrary, two characteristic faecal strains of *B. coli* were found to give negative reactions even on applying the so sensitive $\alpha$-naphtol-test.

Taking into account the above mentioned results of REYNOLDS and WERKMAN the latter result asked for confirmation. The consideration that in bacterial taxonomy so much importance is attached to the property of producing acetylmethylcarbinol — in BERGEY's well-known system the generic differentiation between *Escherichia* and *Aerobacter* is mainly based on this character — it seemed worth-while to collect additional data regarding this point.

## 2. *Production of acetylmethylcarbinol in a medium buffered with sodium bicarbonate.*

In this first series of experiments we used a quite typical faecal strain of *B. coli* (V.P. —, methylred +, citrate —, uric acid —, indole +) from the collection of the laboratory. This strain — E.V. 6. 4. 1. — has been used previously for the quantitative investigation of the products of sugar fermentation [2]). The medium contained 1 % peptone, 2 % glucose, 2 % $NaHCO_3$, 0.1 % $K_2HPO_4$ and 0.5 % NaCl. For the preparation we followed the prescription given by REYNOLDS and WERKMAN [3]), in so far that the medium was sterilized in two separate parts: a solution of the bicarbonate and the phosphate, and a solution of the other ingredients. Before mixing these together in the desired proportion the loss of carbon

[1]) M. M. BARRITT, Journ. of Path. and Bact., **43**, 441 (1936); ibid., **44**, 679 (1937).

[2]) M. A. SCHEFFER, De suikervergisting door bacteriën der coligroep. Diss. Delft 1928.

[3]) H. REYNOLDS and C. H. WERKMAN, Journ. of Bact., **33**, 603 (1937).

dioxide in the first solution was restored by passing sterile carbon dioxide through the solution till phenolphtaleine was decolourized.

Portions of 100 ccm of the sterile medium were brought in Erlenmeyer flasks of 200 ccm and then inoculated with a young culture of the bacterium.

After 4 days incubation at 30° C 75 ccm of the medium were brought into a distillation flask, to which were also added 2.5 gr $FeSO_4$ [1]), 20 ccm 40 % $FeCl_3$ solution and some drops of oleic acid to prevent foaming during the distillation. After heating the mixture slowly in order to oxidise all acetylmethylcarbinol to diacetyl, the latter compound was distilled off. Owing to the great volatility of the diacetyl the small quantities present were found to accumulate completely in the first 10 ccm of the distillate.

1 ccm of this distillate was subjected to the test for diacetyl according to O'MEARA's prescription [2]). Hereto some creatine crystals and afterwards 1 ccm 40 % KOH were added. If diacetyl is present a red colour results.

Another 1 ccm of the distillate was subjected to the reaction after BARRITT (l.c.) in its most sensitive modification. Hereto some solid creatine was again added, and further 0.6 ccm of a 5 % solution of $\alpha$-naphtol in alcohol and 0.2 ccm 40 % KOH. Depending on the amounts of diacetyl present the solution shows a pink to deep crimson colour. With dilute diacetyl solutions of known concentration a colour standard can be obtained which allows of an approximate estimation of the quantity present in the distillate.

Since colour reactions always contain an element of uncertainty we thought it desirable to confirm all positive results in the following way. The remainder of the distillate (8 ccm) was subjected to a second distillation in a small flask (contents ± 15 ccm) with joint dephlegmating device. To the first 2 ccm of the distillate obtained in this way 1 ccm 10 % $NiCl_2$, 1 ccm 20 % $NH_2OH$ . HCl and 2.5 ccm 20 % sodium acetate were added. After boiling and consecutive cooling the characteristic red needles of nickel dimethylglyoximate will appear, if diacetyl is indeed present in the distillate [3]).

The production of acetylmethylcarbinol was studied both in cultures which were strongly aerated by passing finely divided air bubbles through the medium, and in cultures which were not treated in this way.

The results of several tests performed according to the scheme outlined above were quite consistent, as may be judged from the examples collected in Table I.

From these results it is clear that the particular strain of *Bacterium coli* ferments glucose under the conditions of alkaline buffering with production

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1) Cf. L. E. C. KNIPHORST und C. J. KRUISHEER, Zeitschr. f. Unters. d. Lebensm., **73**, 1 (1937).

2) R. A. Q. O'MEARA, Journ. of Path. and Bact., **34**, 401 (1931).

3) Cf. C. B. VAN NIEL, Biochem. Zschr., **187**, 472 (1927).



of small quantities of acetylmethylcarbinol. It should, however, be observed that these quantities are so small that they escape detection, if the sensitive reaction of BARRITT is directly applied to the fermented medium.

Moreover it is evident that under the conditions employed artificial

TABLE I.

*Bacterium coli* in medium of the following composition: 1% peptone, 2% glucose, 2% $NaHCO_3$, 0.1% $K_2HPO_4$ and 0.5% NaCl.

| Nr. of experiment | Artificial aeration | $\alpha$-naphtol-reaction in medium directly | 1st distillate | | 2nd distillate Ni-dimethyl-glyoxim.-reaction |
|---|---|---|---|---|---|
| | | | creatine + KOH | creatine + $\alpha$-naphtol + KOH | |
| 1 | Yes | n.t. *) | n.t. | n.t. | positive |
| | No | n.t. | n.t. | n.t. | positive |
| 2 | Yes | negative | weakly positive | strongly positive 0.05 mgr/ccm | positive |
| | No | negative | weakly positive | strongly positive 0.05 mgr/ccm | positive |
| 3 | Yes | negative | weakly positive | strongly positive 0.025 mgr/ccm | positive |
| | No | negative | weakly positive | strongly positive 0.05 mgr/ccm | positive |

*) n.t. = not tested.

aeration is not a requisite for the production of the carbinol. This result which at first sight conflicts with that of REYNOLDS and WERKMAN may be due to the circumstance that in our experiments the non-aerated cultures have also had free access to the air, whilst the American investigators refer to their controls as representing "anaerobic fermentations".

It seemed desirable to extend these observations to other typical strains of *Bacterium coli*. It should be stressed that all strains used answered the usual requirements, *i.e.*, V.P. —, methylred +, citrate —, uric acid —, indole +. In total 8 strains have been tested; five of these were obtained from the laboratory collection, whilst the others were freshly isolated strains.

The results of this part of the investigation are collected in Table II.

Table II leaves no doubt that the production of traces of acetylmethylcarbinol in the medium buffered with bicarbonate is a quite general property of *Bacterium coli*. It is worthy of notice, however, that the amount

was always so small that even BARRITT's sensitive method — which is able to detect diacetyl in a dilution of 1 : 1.000.000 — yielded negative results when applied directly on the medium.

It should be added that several blank experiments were made on the medium before inoculation; in all these cases the reactions were negative.

TABLE II.

Various strains of *Bacterium coli* in the same medium as mentioned in Table I.

| Indication of strain | Number of days incubated | α-naphtol-reaction in medium directly | 1st distillate creatine + α-naphtol + KOH | 2nd distillate Ni-dimethyl-glyoxim.-reaction |
|---|---|---|---|---|
| E.V. 6.4.1 | 4 | negative | distinctly positive | positive |
| E.V. 6.4.2 | 4 | „ | „ „ | „ |
| E.V. 6.4.3 | 5 | „ | „ „ | „ |
| E.V. 6.4.4 | 5 | „ | „ „ | „ |
| E.V. 6.4.5 | 5 | „ | „ „ | „ |
| Nr. 1 | 5 | „ | „ „ | „ |
| Nr. 2 | 6 | „ | „ „ | „ |
| Nr. 3 | 7 | „ | „ „ | „ |

### 3. *Production of acetylmethylcarbinol in media as usually applied in the* VOGES–PROSKAUER*-reaction.*

The foregoing results made it desirable to investigate whether the production of acetylmethylcarbinol by *Bacterium coli* was indeed bound to the special conditions adhered to until now, or that with the very sensitive methods applied such a production could also be observed under the conditions usually prevailing in the VOGES–PROSKAUER-test.

As a rule this is made in a medium containing 1 % peptone, 0.5 % NaCl, and variable concentrations of glucose. We decided, therefore, to investigate media with glucose concentrations of 0.5, 1 and 2 %.

In one experiment the influence of artificial aeration was examined once more.

In all these experiments the same strain, *i.e.*, E.V. 6.4.1, was used. An incubation time of 4 days was maintained throughout.

The results obtained are collected in Table III.

Table III shows clearly that also in the media as usually applied in the VOGES–PROSKAUER-test *Bacterium coli* produces well-detectable amounts of acetylmethylcarbinol. However, the quantity formed is so slight



that even the sensitive reaction after BARRITT, when applied directly on the fermented medium, yields negative results.

TABLE III.

*Bacterium coli* in media as usually applied in the VOGES-PROSKAUER-test.

| Nr. of experiment | % glucose | Artificial aeration | α-naphtol reaction in medium directly | 1st distillate | | 2nd distillate Ni-dimethyl-glyoxim.-reaction |
|---|---|---|---|---|---|---|
| | | | | creatine + KOH | creatine + α-naphtol + KOH | |
| I | 0.5 | no | negative | positive | positive 0.025 mg/ccm | positive |
| II | 1 | no | „ | „ | positive 0.025 mg/ccm | „ |
| III | 1 | no | „ | „ | positive 0.05 mg/ccm | „ |
| IV | 2 | no | „ | „ | positive 0.05 mg/ccm | „ |
| V | 2 | no | „ | „ | positive 0.05 mg/ccm | „ |
| VI | 2 | yes | weakly positive | „ | strongly positive 0.2 mg/ccm | „ |

Only in the artificially aerated medium a faint, although unmistakably positive reaction was obtained.

### *Discussion of results.*

In the first place the foregoing experiments corroborate the findings of REYNOLDS and WERKMAN that also true faecal strains of *Bacterium coli* are able to produce slight quantities of acetylmethylcarbinol in the fermentation of glucose. In addition the investigations made show clearly that the said production is not bound to the special conditions prevailing in the experiments of these authors, but also occurs under the conditions usually applied in the VOGES–PROSKAUER-test.

Since the amounts of carbinol produced in all these cases are so small that the VOGES–PROSKAUER-reaction — even in BARRITT's recently proposed highly sensitive modification — fails to show the presence of the carbinol, the results obtained need not necessarily conflict with the routine method for the differentiation of *B. coli* and *A. aerogenes*.

Nevertheless, it cannot be denied that the theoretical basis on which the significance of the VOGES–PROSKAUER-reaction rests is considerably weakened. The property "do not produce acetylmethylcarbinol", as a generic character of *Escherichia* in BERGEY's system, can no longer be maintained.

On the other hand one should not loose sight of the fact that there remains a marked difference in the types of sugar fermentation as brought about by *B. coli* and by *A. aerogenes* respectively. Under ordinary conditions the former species produces only traces of acetylmethylcarbinol and probably also traces of 2—3 butylene glycol (REYNOLDS and WERKMAN), whilst the latter species produces easily detectable amounts of the carbinol and 2—3 butylene glycol in an amount of over 30 % of the weight of the sugar fermented (SCHEFFER).

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**Anatomy.** — *On insignificance of cranial vault-height in phylogenetic brain growth.* By EUG. DUBOIS.

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Two months ago Dr. EDWARD JACOBSON sent me (as one of his last gifts) a small squirrel, preserved in alcohol, which animal died in the zoological garden of Bandoeng (Java) and formerly lived in an Australian zoological garden. The weight of this adult male animal, at death, was 62 gr. [1]), and I measured the length of head and body to be about 14 cm. Dr. JUNGE, conservator at the Rijksmuseum van Natuurlijke Historie, diagnosed the species, from the external characters, as *Sciurus palmarum* L. This diagnosis of the genus was upheld by subsequent preparation of the skeleton. Especially the baculum substantiated that the species, undoubtedly, is *Sciurus* (*Funambulus*) *palmarum* [2]).

Having prepared the skull and comparing it with a fine and typical skull of *Sciurus vulgaris*, also an adult male, I observed that, contrary to expectation in such a comparison of a small species with a large one belonging to an identical genus, the brain case of the small animal was not relatively high-vaulted but indeed strikingly low-vaulted, and such even in comparison with the gigantic subgenus *Ratufa*. *Obviously the smaller species only attained a lower degree of cranial vault-height.* However, the cranial capacity of this palm squirrel skull appearing to be 2.3 cc, we find a cephalization factor (in terms of volumes) of 0.238, whereas for our *Sciurus vulgaris*, the body weight of which was 335 gr. and the cranial capacity is 6.2 cc, we find the cephalization factor 0.245, about the common value for the typical or arboreal Squirrels.

The body weight, 62 gr., of this palm squirrel increased a little, in consequence of the long living in confinement. The skull is perhaps not quite normal. It was fortunate, therefore, that I had an opportunity to examine a much finer and apparently normal skull of the same species, again from an adult male, a specimen from the Leiden Natural History museum. It has an equal cranial capacity, of 2.3 cc, but the length of this skull is considerably greater and to all appearance normal; the dimensions are equal to those given for the skull of this species by W. T. BLANFORD [3]). Comparing this skull with that of our *Sciurus vulgaris*, we find it to be much more oblong. The cranial low-vaultedness in *Sciurus palmarum* may

[1]) Erroneously, as demonstrable, noted 112 gr.

[2]) R. I. POCOCK, The Classification of the Sciuridae. Proceedings of the Zoological Society of London. 1923, pp. 215—218.

[3]) Fauna of British India. Mammalia, p. 383.