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Numerical Taxonomy of the Genus *Nocardia*

By M. TSUKAMURA

The National Sanatorium, Chubu Chest Hospital, Obu, Chita-gun, Aichi-ken 474, Japan

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SUMMARY

By a numerical classification, strains received as *Nocardia asteroides* were divided into two clusters; these two groups were named as *N. asteroides* (Eppinger) Blanchard and *N. farcinica* Trevisan, respectively, recognizing the name of *N. farcinica* for the group which included strain ATCC 3318, received as *N. asteroides*.

The genus *Nocardia* was divided into 7 groups; (1) *N. asteroides* (in a new sense); (2) *N. brasiliensis*; (3) *N. caviae*; (4) *N. farcinica*; (5) a group provisionally named *N. rubra*; (6) *N. pelletieri*; (7) *N. madurae*.

The species provisionally named *N. rubra* was made up of strains which, when received from other workers, bore 8 species names: *N. rubra*, *N. erythropolis*, *N. globerula*, *N. convoluta*, *N. minima*, *N. lutea*, *N. rubropertincta* and *N. polychromogenes*.

Relationships between seven species of *Nocardia* are shown as a dendrogram, and descriptions of characteristics (here called Hypothetical Mean Organism) of the species are presented.

A method called numerical identification is presented by which identification can be made, without subjective judgement, by comparison of the characters of a test strain with the Hypothetical Mean Organism after permissible limits of S-values for the Hypothetical Mean Organism have been established.

INTRODUCTION

After the publication of *Bergey's Manual of Determinative Bacteriology* (1957), the taxonomy of the genus *Nocardia* was advanced by Gordon & Mihm (1957, 1959, 1962*a*, *b*) and Gordon (1966*a*, *b*), who characterized the species by biochemical tests. In other groups of organisms numerical taxonomy (Sneath, 1957*a*, *b*, 1964; Sneath & Cowan, 1958; Sokal & Sneath, 1963) has made a contribution to the elucidation of taxonomic problems, and since no numerical classification has been made on the genus *Nocardia* (see Appendix), it was decided to apply this method to the genus.

METHODS

One hundred and twelve strains received as nocardiae were used for the study. Thirty-four strains were received in 1967 from Dr Ruth E. Gordon, Institute of Microbiology, Rutgers University, New Brunswick, New Jersey, U.S.A., under the following species names: *N. asteroides* (10 strains); *N. brasiliensis* (7 strains); *N. caviae* (7 strains); *N. pelletieri* (5 strains); and *N. madurae* (5 strains). For these strains we retained the strain names. The other 78 strains were received in 1965 and 1967 from Dr I. Uesaka, Tuberculosis Research Institute, Kyoto University, Kyoto, Japan;

among these, 66 had been supplied to Dr Uesaka by Dr N. M. McClung, University of South Florida, Tampa, Florida, U.S.A., and these 66 strains were designated by the letter M. Eight strains had been supplied to Dr Uesaka by Dr McClung through Dr Arai, Chiba University, Chiba, Japan. These also were designated with the letter M. Four strains had been supplied to Dr Uesaka by Dr Gordon through Dr Arai; these were designated by the letter C. During investigations in our laboratory the strains were given a laboratory number so that their identities were concealed.

All strains were subcultured on glucose agar and kept at $-20°$. The composition of the glucose agar was as follows: glucose, 10 g.; sodium glutamate, 4·0 g.; KH_2PO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; agar, 20·0 g.; distilled water, 1,000 ml.; adjusted to pH 7·0 by addition of 10 % (w/v) KOH.

One hundred characters were tested (see below); the methods employed were described previously (Tsukamura, 1967*a*); incubation was at 28°, unless specially noted.

Among the 100 characters 97 were set up originally for differentiating between the members of the genus *Mycobacterium*. Only 3 other characters were added; these were: (1) partial acid-fastness; (2) permanent mycelium; and (3) temporary mycelium.

Morphological characters were observed on smears from organisms growing on Ogawa egg medium or glucose agar, and on slide cultures of glucose agar after 1, 2, 3, and 7 days of incubation, by the Ziehl–Neelsen method. The term 'permanent' mycelium was used for well-developed mycelium showing fragmentation to rods or coccoids, mycelia being observed throughout the incubation period; 'temporary' mycelium for mycelia visible at an early stage of growth which soon became rods and coccoids. The term 'complete' acid-fastness was applied to organisms as acid-fast as growing mycobacteria.

Amidase tests were done according to the method of Bönicke (1962), the time of incubation being modified to 16 hr at 37°. To avoid contamination of the organisms with components of medium in the amidase tests, only glucose agar-grown cultures were used. Egg media seemed to be unsuitable for nocardiae, producing growth adherent to egg media.

All tests were done three times and the results obtained in at least two replications were used for preparation of charts of characters. The results were expressed by symbols (+, −), and the S-value was calculated as follows:

$$\text{S-value } (\%) = (n_s/n_s+n_d) \times 100\ \%,$$

where n_s is the number of characters in which two strains showed similar code symbols (++ or −−) and n_d is the number of characters in which two strains showed different symbols (+−). The method used for calculation of S-values was described previously (Tsukamura, 1966). Clustering was made by single linkage (Sokal & Sneath, 1963).

Among the characters used 25 were ineffective for differentiation between the members of the genus *Nocardia*, giving either all positive or all negative results for the whole set of organisms. Nevertheless, these were retained in the test series. On the other hand, several characters reported as useful for differentiation between the members of the genus *Nocardia* (Gordon & Mihm, 1957, 1962*a*) were not included in this study because the same test series was applied to both mycobacteria and nocardias.

Hypothetical Mean Organism (HMO) is the term used to express a mean charac-

terization of a species, which is a slight modification of the 'Calculated Median Organism' of Liston, Wiebe & Colwell (1963).

For preparation of HMOs, the mean number of positive characters (mean of the characters showing a positive reaction in a strain) is calculated for every species or group. The number of positive characters in an HMO is equal to the mean number of positive characters per strain. The characters to be used for preparation of an HMO are chosen, first taking a character appearing at the highest frequency and then taking a character appearing at the next (lower) frequency, and the choice of such positive characters continues until the number of characters obtained equals the mean number of positive characters.

The number of characters used for the preparation of HMOs is not always equal to the mean number. If, after selection of one less than the mean number of characters, three characters showing the same frequency are found, these three are added, obtaining two characters more than the mean number. The HMOs thus prepared are similar to the Calculated Median Organisms of Liston *et al.* (1963). The average S-value between the HMO and the Calculated Median Organism in various mycobacterial species was 98·5 % (Tsukamura & Mizuno, 1968). Details of the method of preparation of the HMO and a few advantages in the use of the HMO (for example, it is possible to calculate the degree of deviation in the characters of an HMO) were described previously (Tsukamura & Mizuno, 1968).

RESULTS

Numerical classification of Nocardia (strains provided by Dr Gordon)

Numerical classification of 34 strains of *Nocardia* received from Dr Gordon as reference strains showed clearly 6 clusters: (1, 2) *N. asteroides* (two clusters); (3) *N. brasiliensis*; (4) *N. caviae*; (5) *N. pelletieri*; (6) *N. madurae* (Fig. 1). The clusters of *N. asteroides* were labelled group A (from ATCC 9970 to R-553) and group B (from R-764 to ATCC 3318), respectively. Strain R-1351 received as *N. caviae* was not in the cluster of *N. caviae* and, therefore, was omitted from the group of *N. caviae*.

A dendrogram showing relationships between the species was prepared from the S-value table shown in Fig. 1 and is shown in Fig. 2.

Since a type strain of *Nocardia asteroides* seems to be non-existent, it was desired to consider which group contained, as nearly as possible, the characters attributed to *N. asteroides*. All strains showed the characters described by Gordon & Mihm (1957, 1959, 1962*a*), but three characters used by them were variable. Not all of the 7 strains of group A formed acid from rhamnose, or grew at 45°, and 5 of 7 showed reduction of nitrate to nitrite. On the other hand, all 3 strains of group B formed acid from rhamnose, grew at 45°, and failed to reduce nitrate. Gordon & Mihm (1957, 1959, 1962*a*) described these characters as follows: (a) 32 % of the strains tested formed acid from rhamnose; (*b*) 42 % of the strains grew at 45°; and (*c*) 88 % of the strains reduced nitrate to nitrite (Gordon & Mihm, 1962*a*). These characters resemble our group A rather than group B; accordingly, group A was named *N. asteroides*, but group B required another name. Group B included strain ATCC 3318, named *N. farcinica* Trevisan (Gordon & Mihm, 1957, 1962*a*), which name seemed to be suitable for group B.

Differentiation between groups A and B could be made not only by these characters

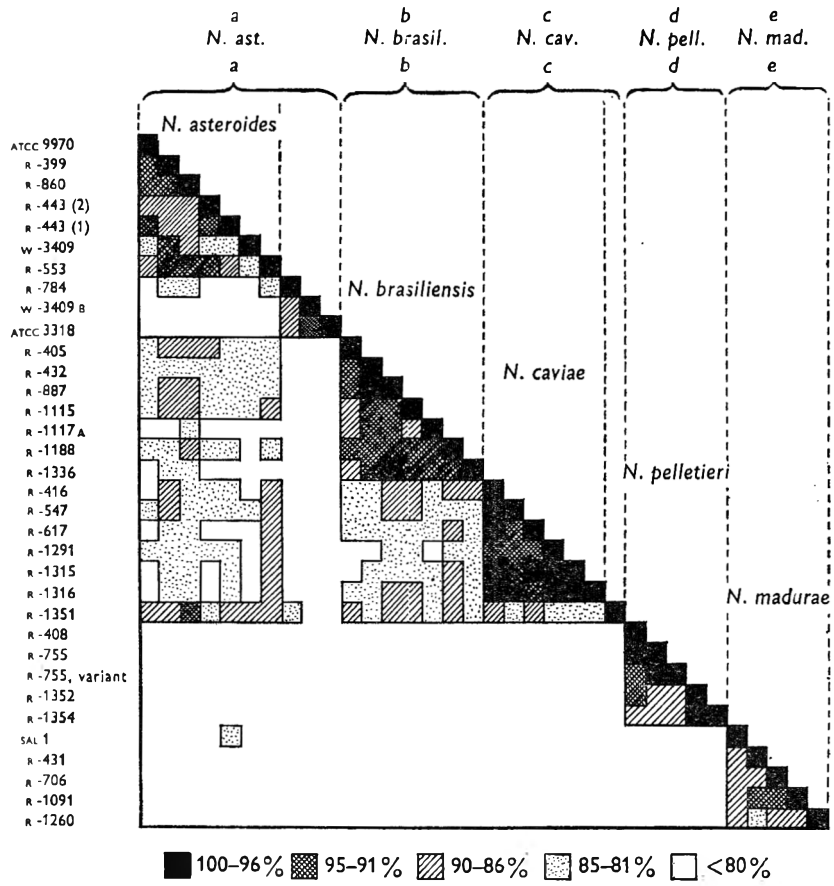

Fig. 1. Diagrammatic representation of the S-value table prepared by shading the square of a S-value between the strains.

The species names above are the names received.

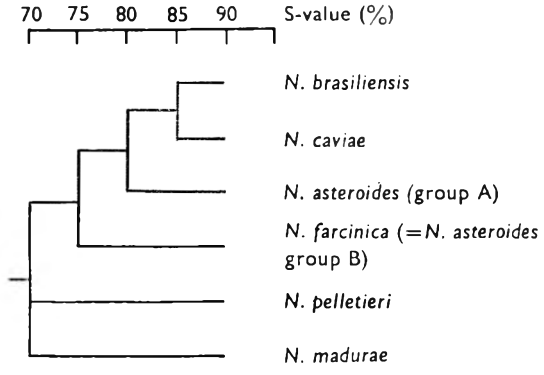

Fig. 2. Dendrogram prepared from the S-value table shown in Fig. 1.

but more distinctly by many other characters (see below). It is now intended to demonstrate differences between these two species in tests of more strains and to make acceptable the proposal for the division of the species *N. asteroides* into two separate species.

Numerical classification of Nocardia (strains provided by Dr Uesaka and Dr McClung)

Results of the numerical classification of 78 strains received from Dr Uesaka, which were mainly from the collection of Dr McClung, are shown in Fig. 3. Two distinct clusters were present, but half of the strains remained unclustered. One of the clusters consisted of 34 strains (from C-407 to M-175); the other of 7 strains (from M-1 to

Fig. 3. Diagrammatic representation of the S-value table prepared by shading the square of a S-value between the strains.

Table 1. '*Hypothetical Mean Organisms*' (*HMOs*) *of the species of the genus Nocardia* (*comparison with Table 2*)

	N. asteroides (group A)	*N. rubra* (Kyoto-II)	*N. farcinica* (Kyoto-I)	*N. brasiliensis*	*N. caviae*	*N. pelletieri*	*N. madurae*
1. Gram reaction	+	+	+	+	+	+	+
2. Acid-fastness, complete	−	−	−	−	−	−	−
3. Acid-fastness, partial	+	+	+	+	+	+	+
4. Mycelium, permanent	−	−	−	−	−	+	+
5. Mycelium, temporary	+	+	+	+	+	+	+
6. Rod shape (with or without coccoids)	+	+	+	+	+	+	+
7. Cord formation (compact grouping)	+	+	+	+	+	+	+
8. Colonial morphology, rough	+	+	+	+	+	−	−
9. Colonial pigmentation	−	+	−	−	−	+	−
10. Photochromogenicity	−	−	−	−	−	−	−
11. Growth rate, rapid	+	+	+	+	+	−	−
12. Catalase	+	+	+	+	+	+	+
13. Nitrate reduction	+	−	−	−	+	−	−
14. Three-day arylsulphatase	−	−	−	−	−	−	−
15. Two-week arylsulphatase	−	−	−	−	−	−	−
16. Salicylate degradation	−	−	−	−	−	−	−
17. PAS degradation	−	−	−	−	−	.	.
18. Growth on 0·2 % PAS medium	+	+	+	+	+	.	.
19. Growth on NH_2OH medium (62·5 μg./ml.)	+	+	+	+	+	.	.
20. Growth on NH_2OH medium (125 μg./ml.)	+	+	+	+	+	.	.
21. Growth on NH_2OH medium (250 μg./ml.)	−	+	+	−	−	.	.
22. Growth on NH_2OH medium (500 μg./ml.)	−	+	−	−	−	.	.
23. Tolerance to 0·1 % picric acid	+	+	+	+	+	+	+
24. Tolerance to 0·2 % picric acid	+	+	+	+	+	+	+
25. Growth at 28°	+	+	+	+	+	+	+
26. Growth at 37°	+	+	+	+	+	+	+
27. Growth at 45°	−	−	+	−	+	+	+
28. Growth at 52°	−	−	−	−	−	−	−
29. Acetamidase	−	+	+	−	−	−	−
30. Benzamidase	−	−	+	−	−	−	−
31. Urease	+	+	+	+	+	−	−
32. Isonicotinamidase	−	−	−	−	−	−	−
33. Nicotinamidase	+	−	+	−	+	+	−
34. Pyrazinamidase	−	−	+	−	+	+	−
35. Salicylamidase	−	−	−	−	−	−	−
36. Allantoinase	−	−	−	−	+	−	−
37. Succinamidase	−	−	−	−	−	−	−
38. Malonamidase	−	−	−	−	−	−	−
39. Acetate (as C source)	+	+	+	+	+	+	+
40. Citrate (as C source)	+	+	−	+	−	−	−
41. Succinate (as C source)	+	+	+	+	+	−	+
42. Malate (as C source)	+	+	+	+	+	−	+
43. Pyruvate (as C source)	+	+	+	+	+	+	+
44. Benzoate (as C source)	−	−	−	−	−	−	−
45. Malonate (as C source)	−	−	−	−	−	−	−
46. Fumarate (as C source)	+	+	+	+	+	−	+
47. Acid from glucose	+	+	+	+	+	+	+
48. Acid from mannose	−	−	−	−	−	−	−
49. Acid from galactose	−	−	−	+	−	−	−
50. Acid from arabinose	−	−	−	−	−	−	+

Table 1 (*cont.*)

	N. asteroides (group A)	*N. rubra* (Kyoto-II)	*N. farcinica* (Kyoto-I)	*N. brasiliensis*	*N. caviae*	*N. pelletieri*	*N. madurae*
51. Acid from xylose	−	−	−	−	−	−	+
52. Acid from rhamnose	−	−	+	−	−	−	+
53. Acid from trehalose	−	−	−	+	−	−	+
54. Acid from raffinose	−	−	−	−	−	−	−
55. Acid from inositol	−	−	−	+	+	−	−
56. Acid from mannitol	−	+	−	−	+	−	+
57. Acid from sorbitol	−	+	−	−	−	−	−
58. Glycerol (as C source)	+	+	+	+	+	+	+
59. Glucose (as C source)	+	+	+	+	+	+	+
60. Mannose (as C source)	−	+	+	−	+	−	−
61. Galactose (as C source)	−	−	−	+	−	−	−
62. Arabinose (as C source)	−	−	−	−	−	−	+
63. Xylose (as C source)	−	−	−	−	−	−	+
64. Rhamnose (as C source)	−	−	+	−	−	−	+
65. Trehalose (as C source)	−	+	−	+	−	+	+
66. Raffinose (as C source)	−	−	−	−	−	−	−
67. Inositol (as C source)	−	−	−	+	+	−	+
68. Mannitol (as C source)	−	+	−	+	+	−	+
69. Sorbitol (as C source)	−	+	−	−	+	−	+
70. Fructose (as C source)	+	+	+	+	+	−	+
71. Sucrose (as C source)	−	+	−	−	−	−	−
72. Ethanol (as C source)	−	+	+	−	−	−	+
73. Propanol (as C source)	+	+	+	+	−	−	+
74. Propylene glycol (as C source)	−	+	+	−	−	−	−
75. 1,3-Butylene glycol (as C source)	−	−	+	−	−	−	−
76. 1,4-Butylene glycol (as C source)	−	−	−	−	−	−	−
77. 2,3-Butylene glycol (as C source)	−	−	+	−	−	−	−
78. L-Glutamate (as N and C sources)	+	+	+	+	+	+	+
79. L-Serine (as N and C sources)	−	−	−	+	+	−	−
80. Glucosamine (as N and C sources)	+	+	+	+	+	−	+
81. Acetamide (as N and C sources)	−	+	+	−	−	−	−
82. Benzamide (as N and C sources)	−	−	−	−	−	−	−
83. Monoethanolamine (as N and C sources)	−	+	+	−	+	−	−
84. Trimethylene diamine (as N and C sources)	−	−	−	−	−	−	−
85. L-Glutamate (as N source)	+	+	+	+	+	+	+
86. L-Serine (as N source)	+	+	−	+	+	−	+
87. L-Methionine (as N source)	+	+	−	+	+	−	+
88. Acetamide (as N source)	+	+	+	+	+	−	+
89. Benzamide (as N source)	−	−	−	−	−	−	−
90. Urea (as N source)	+	+	+	+	+	−	+
91. Pyrazinamide (as N source)	+	+	+	+	+	−	+
92. Isonicotinamide (as N source)	+	+	−	−	−	−	+
93. Nicotinamide (as N source)	+	+	+	+	+	−	+
94. Succinamide (as N source)	+	+	+	+	+	−	+
95. Nitrate (as N source)	+	+	+	+	+	−	+
96. Nitrite (as N source)	−	−	−	−	+	−	−
97. Nicotinic acid	−	−	−	−	−	−	−
98. Growth on TCH medium (10 μg./ml.)	+	+	−	+	+	.	.
99. Growth on salicylate medium (0·05 %)	+	+	−	+	+	.	.
100. Growth on salicylate medium (0·1 %)	+	+	+	+	+	.	.

NRRL 61531). As shown later, strains of the large group were identical with group B, that is, *N. farcinica*, and another group was shown to be a species distinguishable from the other species. In the following, the course of classification and identification will be described in detail.

Table 2. *Properties of 7 groups of Nocardia (mean no. of positive characters, intragroup-S-value for each group, and mean S-value to the HMO in each group)*

Group	No. of strains	Mean no. of positive characters	Intragroup-S-value (%)	Mean S-value to the HMO (%)
N. asteroides (group A of *N. asteroides*)	7	44·3 ± 4·03 (n = 7)	89·1 ± 3·38 (n = 21)	93·6 ± 2·58 (n = 7)
N. rubra (group Kyoto-II)	7	56·6 ± 3·05 (n = 7)	91·9 ± 2·74 (n = 21)	95·3 ± 1·98 (n = 7)
N. farcinica (group Kyoto-I)	30	52·7 ± 4·26 (n = 30)	91·0 ± 3·87 (n = 435)	94·6 ± 3·17 (n = 30)
N. brasiliensis	7	48·3 ± 3·15 (n = 7)	92·8 ± 2·25 (n = 21)	95·9 ± 2·55 (n = 7)
N. caviae	6	52·5 ± 2·43 (n = 6)	96·0 ± 1·46 (n = 15)	97·5 ± 1·38 (n = 6)
N. pelletieri	5	20·8 ± 1·48* (n = 5)	92·2 ± 3·84 (n = 10)	96·6 ± 1·34 (n = 5)
N. madurae	5	46·8 ± 3·11* (n = 5)	89·5 ± 2·22 (n = 10)	92·4 ± 2·07 (n = 5)

* Data on egg media were excluded. Accordingly, a total of 91 characters was tested. In other groups, a total of 100 characters was tested.

Table 3. *S-value table (%) of the 'Hypothetical Mean Organisms' (HMOs) of 7 groups of Nocardia*

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
(1) *N. asteroides* (group A)	—	—	—	—	—	—	—
(2) *N. brasiliensis*	88	—	—	—	—	—	—
(3) *N. caviae*	84	84	—	—	—	—	—
(4) *N. rubra* (Kyoto-II)	81	79	77	—	—	—	—
(5) *N. farcinica* (Kyoto-I)	80	74	78	79	—	—	—
(6) *N. madurae*	76	78	79	78	70	—	—
(7) *N. pelletieri*	74	67	67	64	69	67	—

Intragroup-mean S-values to the HMO are as follows: (1) 93·6 ± 2·58 % (n = 7); (2) 95·9 ± 2·55 % (n = 7); (3) 97·5 ± 1·38 % (n = 6); (4) 95·3 ± 1·98 % (n = 7); (5) 94·6 ± 3·17 % (n = 30); (6) 92·4 ± 2·07 % (n = 5); (7) 96·6 ± 1·34 % (n = 5). (Refer to the right column in Table 2.)

Figure 3 was prepared by a rearrangement of the S-value table of 78 strains. When a diagrammatic representation of the S-value table of the strains was first prepared, two clusters were recognized, one large cluster consisting of 30 strains (from C-407 to M-200) and another small cluster consisting of 7 strains (from M-1 to NRRL 61531). These clusters were named provisionally Kyoto-I and Kyoto-II, respectively. In order to compare these two groups with the 6 groups obtained by a numerical classification of Dr Gordon's strains, HMOs were prepared for each of the following groups (Table 1): (1) Kyoto-I; (2) Kyoto-II; (3) *Nocardia asteroides* (group A); (4) *N. brasili-*

ensis; (5) *N. caviae*; (6) *N. pelletieri*; (7) *N. madurae* (Table 1). The strains used for preparation of the HMOs were as follows: (1) Kyoto-I (30 strains, C-407 (received as *N. madurae*) to M-200 (received as *N. brasiliensis*) in Fig. 3); (2) Kyoto-II (7 strains,

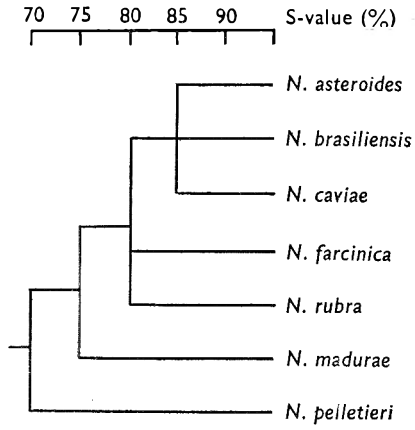

Fig. 4. Dendogram prepared from the S-value table of HMOs of the species of *Nocardia*.

Table 4. *Comparison between Nocardia asteroides (group A) and N. brasiliensis*

	N. asteroides (group A) (%)	*N. brasiliensis* (%)
Mean S-value to own HMO	93·6 ± 2·58 (n = 7)	95·7 ± 2·55 (n = 7)
Mean S-value to other HMO	83·7 ± 2·01 (n = 7)	86·4 ± 2·51 (n = 7)

(Mean) ± (standard deviation) %. (n = number of strains.)

Table 5. *Intra- and inter-groups mean S-values, Nocardia asteroides and N. brasiliensis*

	(1) (%)	(2) (%)
(1) *N. asteroides* (group A) (7 strains)	89·1 ± 3·38 (n = 21)	—
(2) *N. brasiliensis* (7 strains)	82·9 ± 3·37 (n = 49)	92·8 ± 2·25 (n = 21)

Intra-group mean S-values are significantly larger than inter-groups mean S-value (P less than 5 %).

M-1 (received as *N. rubra*) to NRRL 61531 (received as *N. polychromogenes*) in Fig. 3); (3) *N. asteroides* (7 strains, ATCC 9970 to R-553 in Fig. 1); (4) *N. brasiliensis* (7 strains, R-405 to R-1336 in Fig. 1); (5) *N. caviae* (6 strains, R-416 to R-1316 in Fig. 1); (6) *N. pelletieri* (5 strains in Fig. 1); (7) *N. madurae* (5 strains in Fig. 1). Group B, *N. farcinica*, was omitted from the above groups, as it consisted of only 3 strains.

Table 6. *Intra- and inter-groups mean S-values, Nocardia brasiliensis and N. caviae*

	(1) (%)	(2) (%)
(1) *N. brasiliensis* (7 strains)	92·8 ± 2·25 (n = 21)	—
(2) *N. caviae* (6 strains)	84·6 ± 2·62 (n = 42)	96·0 ± 1·46 (n = 15)

Intra-group S-values are significantly larger than inter-groups mean S-value (P less than 5 %).

Table 7. *Intra- and inter-groups mean S-values, Kyoto-I and Kyoto-II groups*

	(1) (%)	(2) (%)
(1) Kyoto-I (30 strains)	91·0 ± 3·87 (n = 435)	—
(2) Kyoto-II (7 strains)	77·9 ± 3·87 (n = 210)	91·9 ± 2·74 (n = 21)

Intra-group mean S-values are significantly larger than inter-groups mean S-value (P less than 5 %).

Table 8. *Identification of strains ATCC 3318, W-3409B and R-784 (group B of Nocardia asteroides)*

Hypothetical Mean Organism (HMO)	S-value to HMO (%) ATCC 3318	W-3409B	R-784	Permissible low limit of S-value for species* (%)
N. farcinica (Kyoto-I)	93	93	89	88·3
N. rubra (Kyoto-II)	78	75	78	91·3
N. asteroides (group A)	77	74	83	88·4
N. brasiliensis	74	68	77	90·8
N. caviae	71	70	79	94·7
N. pelletieri	70	76	74	93·9
N. madurae	73	67	69	88·3

* The permissible low limit of the S-value to the HMO for members of a species is at (mean) − 2 × (standard deviation) %.

Table 9. *Strains identified, provisionally, as Nocardia rubra (Kruse) Chalmers & Christopherson*

Strain*	History
N. rubra M-1	N. M. McClung; CBS, Holland (= R-562)
N. erythropolis M-8	N. M. McClung; Waksman (3407)
N. globerula M-75	N. M. McClung; NRRL no. B1306 (= ATCC 9356 = R-417)
N. convoluta M-79	N. M. McClung; ATCC 4275
N. minima M-103	N. M. McClung; ATCC 8674
N. lutea M-192	N. M. McClung; D. Schneidau (365); R. E. Gordon (R-680) E. N. Azarowiez (147); Inst. Pasteur, Paris
N. polychromogenes NRRL 61531	N. M. McClung; NRRL 61531
N. polychromogenes M-6	N. M. McClung; *N. polychromogenes* (Vallée) Jensen; CBS; (= R-565)
N. rubropertincta M-191†	N. M. McClung; J. D. Schneidau

* Species name is the name when received.
† Suggested to be a member of this species (see Table 10).

Table 10. *Identification by comparison with Hypothetical Mean Organisms (HMOs) of strains not forming a cluster in preliminary sorting*

Species name and strain name as received	S-value (%) to the HMO of: *N. asteroides* (group A)	*N. rubra* (Kyoto-II)	*N. farcinica* (Kyoto-I)	*N. brasiliensis*	*N. caviae*	*N. pelletieri*	*N. madurae*	Identification*
Permissible low limit (%)†	88·4	91·3	88·3	90·8	94·7	93·9	88·3	
N. coeliaca M-122	69	84	71	75	71	50	72	.
N. madurae C-743	84	83	92	80	76	68	70	F
N. blackwellii M-81	83	84	95	77	77	65	76	F
N. asteroides M-130	83	80	97	77	77	69	74	F
N. asteroides M-163	80	77	84	72	74	72	68	F‡
N. corralina M-78	86	75	74	82	76	80	67	A‡
N. brasiliensis C-851	85	82	73	95	83	63	78	B
N. asteroides M-94	91	82	81	85	81	76	76	A
N. asteroides M-124	92	81	80	84	82	72	78	A
N. asteroides M-128	90	83	80	84	80	74	78	A
N. asteroides M-132	93	80	85	87	87	77	72	A
N. brasiliensis M-145	84	76	73	89	79	76	77	B‡
N. asteroides M-155	91	80	79	87	81	76	76	A
N. asteroides M-161	90	75	78	84	84	77	75	A
N. transvalensis M-193	79	77	73	81	81	60	71	.
N. brasiliensis M-199	83	78	75	91	85	68	72	B
N. brasiliensis M-204	86	79	76	96	82	68	77	B
N. asteroides M-206	91	82	83	85	81	68	75	A
N. brasiliensis M-168	87	78	75	93	85	67	76	B
Nocardia sp. M-54	80	77	80	82	92	75	75	C‡
N. asteroides M-73	83	80	81	83	93	71	76	C‡
N. asteroides M-180	75	70	75	77	89	77	68	C‡
N. tenuis convoluta M-203	78	80	75	83	81	55	72	.
N. restrictus M-9	72	61	64	66	62	83	57	.
N. asteroides M-10	94	79	84	82	82	72	75	A
N. asteroides M-93	88	79	78	80	76	79	72	A
N. asteroides M-129	97	80	81	89	85	77	75	A
N. asteroides M-156	94	75	76	82	82	71	69	A
N. bostroemi M-159	84	71	78	76	80	85	67	.
N. asteroides M-166	85	76	79	83	91	77	75	C‡
N. intracellularis M-170	80	67	74	72	72	83	64	.
N. pretoriana M-194	90	77	74	86	80	75	75	A
N. rubropertincta M-191	82	89	74	80	76	64	75	R‡
N. polychromogenes M-6	82	93	76	80	76	61	75	R
N. madurae M-172	77	72	73	73	73	76	80	.
N. asteroides M-175	83	80	97	75	77	69	74	F
N. asteroides M-185	84	75	80	84	92	74	74	C‡
N. madurae C-637	60	75	68	65	63	52	74	.
N. leishmanii M-82	69	72	61	78	67	58	76	.
N. alba (Schoen) M-113	75	72	67	79	73	60	80	.
N. asteroides M-123	80	64	68	71	67	85	63	.

* A = *N. asteroides* (group A); R = *N. rubra* (Kyoto-II); F = *N. farcinica* (Kyoto-I); B = *N. brasiliensis*; C = *N. caviae*; Pe = *N. pelletieri*; M = *N. madurae*.

† As to the permissible limit of S-value, see Table 5.

‡ If an S-value was outside the permissible limit of 7 species but remained within a 5 % range from the permissible limit of a species, this species name was indicated with the double dagger. If an S-value was within the permissible low limit of a species, this species name was indicated as the species identified. Spaces remaining blank show that no species was identified.

Mean number of positive features for each group or species, intragroup-S-value for each group, and mean S-value to the HMO for the members of each group are shown in Table 2.

An S-value table of the HMOs is shown in Table 3, and a dendrogram prepared from it is shown in Fig. 4. Comparing the mean S-values to the HMO, which are shown in Table 2, and the S-values between the HMOs, which are shown in Table 3, it is conceivable that there are statistically significant differences between an intragroup-S-value to the HMO, which is shown on the right column in Table 2, and an intergroup-S-value (Table 3), and these 7 groups were considered to be distinct species.

Table 11. *Strains identified as Nocardia asteroides (Eppinger) Blanchard (Blanchard, 1896)*

Strain*	History
N. asteroides ATCC 9970	R. E. Gordon; C. W. Emmons (NIH) (9956); C. A. Perry
N. asteroides R-399	R. E. Gordon; C. W. Emmons (NIH) (9966)
N. asteroides R-860	R. E. Gordon; W. D. Jones, Jun.
N. asteroides R-443 (1)	R. E. Gordon; I. B. Christison; N. F. Conant (2338)
N. asteroides R-443 (2)	R. E. Gordon; I. B. Christison; N. F. Conant (2338)
N. asteroides W-3409	R. E. Gordon; *N. polychromogenes*; S. A. Waksman; H. L. Jensen
N. asteroides R-553	R. E. Gordon
N. caviae R-1351	R. E. Gordon
N. asteroides M-94	N. M. McClung; C. W. Emmons (9976)
N. asteroides M-124	N. M. McClung; R. E. Gordon (R-409); J. Caffey
N. asteroides M-128	N. M. McClung; R. E. Gordon (R-420 (1)); N. F. Conant (1004)
N. asteroides M-132	N. M. McClung; R. E. Gordon (W-3599); S. V. Keating
N. asteroides M-155	N. M. McClung; W. Jones (6M*)
N. asteroides M-161	N. M. McClung; D. Schneidau (306); pulmonary nocardiosis
N. asteroides M-206	N. M. McClung; S. McMillan; R. E. Gordon (R-421); N. F. Conant (1017)
N. asteroides M-10	N. M. McClung; var. *crateriforme* Bald. CBS
N. asteroides M-93	N. M. McClung; C. W. Emmons (NIH) (9935)
N. asteroides M-129	N. M. McClung; R. E. Gordon (W-3663); E. Haynes
N. asteroides M-156	N. M. McClung; W. Jones (8N*)
N. pretoriana M-194	N. M. McClung; D. Schneidau (368); S. T. Cowan (15)
N. corallina M-78†	N. M. McClung; ATCC 4273

* Species name is the name as received.
† Suggested to be a member of *N. asteroides* (see Table 10).

When two groups, *Nocardia asteroides* (group A) and *N. brasiliensis*, were compared, the HMO of these showed an S-value of 88 %, the highest among the intergroup-S-values (Table 3). Mean S-value for members of *N. asteroides* to its own HMO was significantly greater than mean S-value to the HMO of another species, *N. brasiliensis* (Table 4). On the other hand, mean S-value for the members of *N. brasiliensis* to its own HMO was significantly larger than that to the HMO of *N. asteroides* (Table 4). These results indicate that *N. asteroides* and *N. brasiliensis* are distinct from each other.

It was noticed that an S-value between two HMOs may differ from an intergroup-mean-S-value actually calculated on two groups. As shown in Table 3, S-value between the HMO of *Nocardia asteroides* (group A) and the HMO of *N. brasiliensis* was 88 %, and S-value between the HMO of *N. brasiliensis* and the HMO of *N. caviae* was 84 %. In contrast, when calculated with these groups directly, intergroup-mean S-value between *N. asteroides* (group A) and *N. brasiliensis* was $82{\cdot}9 \pm 3{\cdot}37$ % ($n = 49$)

(Table 5) and intergroup-mean S-value between *N. brasiliensis* and *N. caviae* was $84{\cdot}6 \pm 2{\cdot}62\,\%$ ($n = 42$) (Table 6). The former was significantly ($P = 5\,\%$) smaller than the latter. It is considered that this discrepancy is due to HMOs being a sym-

Table 12. *Strains identified as Nocardia farcinica Trevisan (Trevisan, 1889) (= group Kyoto-I = group B of N. asteroides)*

Strain*	History
N. madurae C-407	I. Uesaka; Arai; R. E. Gordon (R-407)
N. asteroides M-72	N. M. McClung; C. H. Drake; pulmonary nocardiosis
N. asteroides M-111	N. M. McClung; R. E. Gordon (R-504)
N. eppingeri M-112	N. M. McClung; R. E. Gordon (R-508)
N. asteroides M-131	N. M. McClung; R. E. Gordon (R-429); N. F. Conant (2123)
N. asteroides M-137	N. M. McClung; S. McMillan (N-31); brain abscess
N. asteroides M-138	N. M. McClung; S. McMillan (N-34)
N. asteroides M-139	N. M. McClung; S. McMillan (N-35)
N. asteroides M-144	N. M. McClung; S. McMillan (O-652)
N. brasiliensis M-146	N. M. McClung; S. McMillan (O-416)
N. asteroides M-158	N. M. McClung; Pinkerton, Texas (patient)
N. asteroides M-86	N. M. McClung; *N. farcinica* ATCC 3318
N. asteroides M-127	N. M. McClung; R. E. Gordon; *N. graminis* W-3601; G. J. Friou
N. asteroides M-133	N. M. McClung; S. McMillan (N-4)
N. asteroides M-158A	N. M. McClung; Pinkerton, Texas
N. asteroides M-162	N. M. McClung; D. Schneidau (308)
N. asteroides M-164	N. M. McClung; D. Schneidau (*312); ATCC 9504
N. asteroides M-167	N. M. McClung; D. Schneidau (320)
N. brasiliensis M-169	N. M. McClung; D. Schneidau (327); A. Batista (586)
N. asteroides M-177	N. M. McClung; D. Schneidau (343); leg and lung lesions
N. asteroides M-179	N. M. McClung; D. Schneidau (345)
N. asteroides M-181	N. M. McClung; D. Schneidau (347)
N. brasiliensis M-197	N. M. McClung; D. Schneidau (387)
N. brasiliensis M-198	N. M. McClung; D. Schneidau (390)
N. brasiliensis M-201	N. M. McClung; D. Schneidau (501); C. Lacaz (299)
N. asteroides M-205	N. M. McClung; S. McMillan (N-12)
N. asteroides M-186	N. M. McClung; D. Schneidau (353); A. Batista (702)
N. asteroides M-187	N. M. McClung; D. Schneidau (354); Salvin (2001)
N. asteroides M-189	N. M. McClung; D. Schneidau (356); (dog)
N. brasiliensis M-200	N. M. McClung; D. Schneidau (397); C. Lacaz (130)
N. madurae C-743	I. Uesaka; Arai; R. E. Gordon (R-743)
N. blackwellii M-81	N. M. McClung; ATCC 6846
N. asteroides M-130	N. M. McClung; R. E. Gordon (R-436); N. F. Conant (2249)
N. asteroides M-175	N. M. McClung; D. Schneidau (341)
N. asteroides ATCC 3318	R. E. Gordon: originally, *N. farcinica* ATCC 3318
N. asteroides W-3409B	R. E. Gordon; variant of W-3409 (*N. polychromogenes*; S. A. Waksman; H. L. Jensen)
N. asteroides R-784	R. E. Gordon; B. W. Lacey; A6 (NCTC)
N. asteroides M-163†	N. M. McClung; D. Schneidau; Henrici's strain A10A, var. *gypsoides*

* Species name is the name as received.

† Suggested to be a member of this species, *N. farcinica*. Remark: the first 30 strains, strain C-407 to strain M-200, are the strains first identified as the group Kyoto-I. Strains, ATCC 3318, W-3409B and R-784, belonged to the group B of *N. asteroides*.

bolized feature of a species, prepared by omitting characters appearing at low frequencies. Accordingly, an S-value between two HMOs is not a direct reflexion of an intergroup-mean S-value.

Three examples of comparison between groups are shown in Tables 5, 6 and 7. The

Table 13. *Strains identified as Nocardia brasiliensis (Lindenberg) Pinoy (Pinoy, 1913)*

Strain*	History
N. brasiliensis R-405	R. E. Gordon; Julia M. Coffey; J. B. Fischer; N. F. Conant (*N. mexicana*)
N. brasiliensis R-432	R. E. Gordon; *N. mexicana*; I. Christison; N. F. Conant
N. brasiliensis R-887	R. E. Gordon; J. L. Miranda (229)
N. brasiliensis R-1115	R. E. Gordon; L. F. Bojalil (N-30); Mycetoma
N. brasiliensis R-1117A	R. E. Gordon; L. F. Bojalil (variant of N-114)
N. brasiliensis R-1188	R. E. Gordon
N. brasiliensis R-1336	R. E. Gordon
N. brasiliensis C-851	I. Uesaka; Arai; R. E. Gordon (R-851)
N. brasiliensis M-199	N. M. McClung; D. Schneidau (393); C. Lacaz (602)
N. brasiliensis M-204	N. M. McClung; S. McMillan (N-2)
N. brasiliensis M-168	N. M. McClung; D. Schneidau (322); N. F. Conant (24); Waksman (3485); G. Ochoa (409)
N. brasiliensis M-145†	N. M. McClung; S. McMillan (O-410)

* Species name is the name as received.
† Suggested to be a member of *N. brasiliensis* (see Table 10).

Table 14. *Strains identified as Nocardia caviae (Erikson) Erikson (Erikson, 1935)*

Strain*	History
N. caviae R-416	R. E. Gordon; *N. asteroides*, W. C. Haynes (B-970)
N. caviae R-547	R. E. Gordon; *N. asteroides*, E. N. Azarowicz
N. caviae R-617	R. E. Gordon; E. N. Azarowicz
N. caviae R-1291	R. E. Gordon
N. caviae R-1315	R. E. Gordon
N. caviae R-1316	R. E. Gordon
Nocardia sp. M-54	N. M. McClung; E. Munch-Peterson
N. asteroides M-73	N. M. McClung; NRRL, Peoria
N. asteroides M-180	N. M. McClung; D. Schneidau (346); CDC, Atlanta (A-343)
N. asteroides M-166	N. M. McClung; D. Schneidau (318); CDC, Atlanta (A-343)
N. asteroides M-185	N. M. McClung; D. Schneidau (351); B. H. Webster (17292A)

* Species name is the name as received.

Table 15. *Strains identified as Nocardia pelletieri (Laveran) Pinoy (Pinoy, 1912)*

Strain*	History
N. pelletieri R-408	R. E. Gordon; *N. pelletieri* (Laveran) Pinoy; Julia M. Coffey (47293)
N. pelletieri R-755	R. E. Gordon; *N. pelletieri*; J. E. Mackinnon (IHM 614); Lab. de Parasit. Fac. Med. Paris
N. pelletieri R-755 var.	R. E. Gordon
N. pelletieri R-1352	R. E. Gordon
N. pelletieri R-1354	R. E. Gordon

* Species name is the name as received.

Table 16. *Strains identified as Nocardia madurae (Vincent) Blanchard (Blanchard, 1896)*

Strain*	History
N. madurae SAL I	R. E. Gordon
N. madurae R-431	R. E. Gordon; *N. madurae*; Isabel Christison; N. F. Conant (2177); A. González Ochoa (415)
N. madurae R-706	R. E. Gordon; *N. madurae*; C. W. Emmons (NIH) (9979)
N. madurae R-1091	R. E. Gordon; *Streptomyces albus*; H. D. Tresner (AO-928)
N. madurae R-1260	R. E. Gordon; *Streptomyces madurae*; F. Mariat, Paris (812)

* Species name is the name as received.

Table 17. *Frequency of positive characters appearing in each species of Nocardia*

	N. asteroides	*N. rubra*	*N. farcinica*	*N. brasiliensis*	*N. caviae*	*N. pelletieri*	*N. madurae*
No. of strains tested...	21	9	38	12	11	5	5
1. Gram reaction	21	9	38	12	11	5	5
2. Acid-fastness, complete	0	0	0	0	0	0	0
3. Acid-fastness, partial	21	8	35	12	10	5	5
4. Mycelium, permanent	2	0	9	0	1	5	5
5. Mycelium, temporary	21	9	38	12	11	5	5
6. Rod shape (with or without coccoids)	21	9	38	12	11	5	5
7. Cord formation (compact grouping)	21	9	38	12	11	5	5
8. Colonial morphology, rough	19	9	36	10	10	1	0
9. Colonial pigmentation	4	8	1	0	0	3	0
10. Photochromogenicity	0	0	0	0	0	0	0
11. Growth rate, rapid	20	9	38	12	11	0	0
12. Catalase	21	9	38	12	11	5	5
13. Nitrate reduction	8	0	0	0	6	2	2
14. Three-day arylsulphatase	0	0	0	0	0	0	0
15. Two-week arylsulphatase	8	0	0	0	2	0	0
16. Salicylate degradation	0	0	0	0	0	0	0
17. PAS degradation	0	0	0	0	0	—	—
18. Growth on 0·2 % PAS medium	21	9	38	12	11	—	—
19. Growth on NH_2OH medium (62·5 μg./ml.)	21	9	38	12	11	—	—
20. Growth on NH_2OH medium (125 μg./ml.)	17	9	35	9	11	—	—
21. Growth on NH_2OH medium (250 μg./ml.)	9	9	26	3	4	—	—
22. Growth on NH_2OH medium (500 μg./ml.)	3	4	5	2	1	—	—
23. Tolerance to 0·1 % picric acid	21	9	38	12	11	4	5
24. Tolerance to 0·2 % picric acid	20	9	38	12	11	3	5
25. Growth at 28°	21	9	38	12	11	5	5
26. Growth at 37°	18	8	38	11	11	5	5
27. Growth at 45°	0	2	35	0	11	3	4
28. Growth at 52°	0	0	6	0	0	0	0
29. Acetamidase	2	8	38	1	0	0	0
30. Benzamidase	0	0	27	0	0	0	0
31. Urease	16	5	37	12	11	0	0
32. Isonicotinamidase	0	0	0	0	0	0	0
33. Nicotinamidase	10	0	36	2	11	3	1
34. Pyrazinamidase	4	0	33	0	11	3	1
35. Salicylamidase	0	0	0	0	0	0	0
36. Allantoinase	1	0	0	0	7	0	0
37. Succinamidase	0	0	0	0	0	0	0
38. Malonamidase	0	0	0	0	0	0	0
39. Acetate (as C source)	21	9	38	12	11	5	5
40. Citrate (as C source)	12	9	7	12	0	0	3
41. Succinate (as C source)	20	9	35	12	11	0	4
42. Malate (as C source)	20	9	35	12	11	0	4
43. Pyruvate (as C source)	21	9	38	12	11	4	5
44. Benzoate (as C source)	1	1	0	0	0	0	0
45. Malonate (as C source)	1	1	0	0	0	0	1
46. Fumarate (as C source)	19	8	34	12	11	0	3
47. Acid from glucose	16	9	38	12	11	5	5
48. Acid from mannose	0	5	1	1	0	0	1
49. Acid from galactose	0	0	0	10	0	0	0
50. Acid from arabinose	0	0	0	0	0	0	5
51. Acid from xylose	0	0	0	0	0	0	4
52. Acid from rhamnose	0	0	34	1	0	0	5

Table 17 (*cont.*)

	N. asteroides	*N. rubra*	*N. farcinica*	*N. brasiliensis*	*N. caviae*	*N. pelletieri*	*N. madurae*
No. of strains tested ...	21	9	38	12	11	5	5
53. Acid from trehalose	0	5	7	9	0	0	4
54. Acid from raffinose	0	0	0	0	0	0	0
55. Acid from inositol	1	1	0	11	10	0	0
56. Acid from mannitol	0	9	0	1	11	0	5
57. Acid from sorbitol	0	9	0	0	0	0	0
58. Glycerol (as C source)	21	9	37	12	11	5	5
59. Glucose (as C source)	21	9	37	12	11	5	5
60. Mannose (as C source)	4	8	27	4	7	0	1
61. Galactose (as C source)	0	0	0	11	0	0	0
62. Arabinose (as C source)	0	0	0	2	0	0	5
63. Xylose (as C source)	0	0	0	1	0	0	5
64. Rhamnose (as C source)	0	0	34	1	0	0	5
65. Trehalose (as C source)	0	6	10	10	0	3	5
66. Raffinose (as C source)	0	0	0	0	0	0	0
67. Inositol (as C source)	2	1	0	12	11	0	4
68. Mannitol (as C source)	0	9	0	11	11	0	5
69. Sorbitol (as C source)	0	9	0	7	10	0	4
70. Fructose (as C source)	21	9	38	12	11	0	4
71. Sucrose (as C source)	0	6	1	0	0	0	3
72. Ethanol (as C source)	3	9	37	0	0	0	4
73. Propanol (as C source)	8	9	38	8	0	0	4
74. Propylene glycol (as C source)	1	5	30	0	0	0	0
75. 1,3-Butylene glycol (as C source)	0	1	36	1	0	0	0
76. 1,4-Butylene glycol (as C source)	0	0	3	0	0	0	0
77. 2,3-Butylene glycol (as C source)	4	4	37	0	0	0	0
78. L-Glutamate (as N-C sources)	19	9	38	12	11	5	5
79. L-Serine (as N-C sources)	0	0	0	7	9	0	0
80. Glucosamine (as N-C sources)	12	7	38	12	10	0	4
81. Acetamide (as N-C sources)	0	8	37	0	0	0	2
82. Benzamide (as N-C sources)	0	0	0	0	0	0	0
83. Monoethanolamine (as N-C sources)	1	9	37	0	10	0	0
84. Trimethylene diamine (as N-C sources)	0	2	4	0	0	0	0
85. L-Glutamate (as N source)	21	9	38	12	11	5	5
86. L-Serine (as N source)	16	9	7	11	10	0	5
87. L-Methionine (as N source)	18	9	9	8	6	0	5
88. Acetamide (as N source)	19	8	37	8	8	0	5
89. Benzamide (as N source)	3	1	6	1	0	0	1
90. Urea (as N source)	20	9	35	12	10	0	5
91. Pyrazinamide (as N source)	20	9	31	12	10	0	5
92. Isonicotinamide (as N source)	15	9	12	2	2	0	5
93. Nicotinamide (as N source)	20	9	31	12	10	0	5
94. Succinamide (as N source)	20	9	24	12	7	0	4
95. Nitrate (as N source)	20	9	32	12	10	0	5
96. Nitrite (as N source)	8	0	3	2	4	0	1
97. Nicotinic acid	0	0	0	0	0	0	0
98. Growth on TCH medium (10 μg./ml.)	21	9	38	12	11	—	—
99. Growth on salicylate medium (0·05 %)	21	9	38	12	11	—	—
100. Growth on salicylate medium (0·1 %)	21	9	37	12	11	—	—
Mean no. of positive characters per strain	42.0	56·4	52·1	48·8	49·9	20·8	47·6

comparisons, made by the method described by Tsukamura (1967*b*) showed that the groups tested were two different clusters.

To return to the problem of *Nocardia farcinica*, that is, group B of *N. asteroides*. Group Kyoto-I, consisting of 30 strains received from Dr Uesaka, formed one large

Table 18. *Strains not identified as any of 7 species*

Strain	History
N. coeliaca M-122	N. M. McClung; C. W. Emmons (NIH)
N. transvalensis M-193	N. M. McClung; D. Schneidau (366); R. E. Gordon (366); N.C.T.C.
N. tenuis convoluta M-203	N. M. McClung; D. Schneidau (551); A. Batista (189)
N. restrictus M-9	N. M. McClung; CBS; (= R-566)
N. bostroemi M-159	N. M. McClung; D. Schneidau (303); A. Batista (630); Inst. Pasteur, Paris (321); (= R-1011)
N. intracellularis M-170	N. M. McClung; D. Schneidau (328); M. L. Littman; Wiley
N. madurae M-172	N. M. McClung; D. Schneidau (333); N. F. Conant (2177); G. Ochoa
N. madurae C-637	I. Uesaka; Arai; R. E. Gordon (R-637); A. G. Ochoa (486)
N. leishmani M-82	N. M. McClung; ATCC 6855; lung disease and pericarditis
N. alba (schoen) M-113	N. M. McClung; R. E. Gordon (R-506); Inst. Pasteur, Paris
N. asteroides M-123	N. M. McClung; R. E. Gordon (W-3661); C. A. Payne; brain abscess

Table 19. *Typical strains for the species of Nocardia*

Species	Typical strain (species name as received is shown)	S-value to the HMO of its species (%)
N. farcinica (group Kyoto-I; group B of '*N. asteroides*')	*N. asteroides* M-133 (ATCC 23825)	99
	N. asteroides M-205 (ATCC 23826)	99
	N. asteroides M-175	97
N. rubra (group Kyoto-II)	*N. rubra* M-1	97
	N. globerula M-75	97
	N. lutea M-192	97
N. asteroides (group A of *N. asteroides*)	*N. asteroides* R-399* (ATCC 23824)	99
	N. asteroides M-129	97
	N. asteroides ATCC 9970 (Gordon)	95
N. brasiliensis	*N. brasiliensis* R-432	100
	N. brasiliensis R-887	98
N. caviae	*N. caviae* R-1315	99
	N. caviae R-1316	99
	N. caviae R-416	98
N. pelletieri	*N. pelletieri* R-1352	98
	N. pelletieri R-408	98
N. madurae	*N. madurae* SAL 1	95
	N. madurae R-706	94

* Proposed neotype strain deposited in the American Type Culture Collection, Rockville, Maryland, 20852, U.S.A.

cluster in numerical classification; characters of this group seemed to be similar to those of the group B of *N. asteroides* (*N. farcinica*). Twenty-two of the 30 strains had been received as *N. asteroides*, and the group Kyoto-I included strain M-86 of *N. asteroides*, which is the same as strain ATCC 3318 of *N. farcinica*.

Identification of the group B of *Nocardia asteroides* (that is, *N. farcinica*) as group Kyoto-I was carried out by determining S-values for three strains of the group B to various HMOs. These are shown in Table 8. Assuming that S-values to the HMO for individual members of a species show a normal distribution curve, it will be expected that *c.* 95 % of the members of a species will be included within the limits ((Mean) ± 2 × (Standard deviation)). Thus, a permissible low limit of the S-value for strains to be included in a species will be ((Mean) − 2 × (Standard deviation)) %; this limit is calculated from the data of Table 2 and is shown in Table 8.

Table 20. *Pattern of utilization for growth of nitrogen compounds as simultaneous nitrogen and carbon sources and pattern of amidases in the species of Nocardia*

		N. asteroides	*N. rubra*	*N. farcinica*	*N. brasiliensis*	*N. caviae*	*N. pelletieri*	*N. madurae*
	Ratio of type*...	17/21	6/9	32/38	12/12	9/11	5/5	4/5
Utilization as N and C sources	L-Glutamate	+	+	+	+	+	+	+
	L-Serine	−	−	−	±	+	−	−
	Glucosamine	±	+	+	+	+	−	+
	Acetamide	−	+	+	−	−	−	±
	Benzamide	−	−	−	−	−	−	−
	Monoethanolamine	−	+	+	−	+	−	−
	Trimethylene diamine	−	−	−	−	−	−	−
	Ratio of type*...	18/21	8/9	32/38	10/12	11/11	5/5	4/5
Amidase pattern	Acetamidase	− −	+	−	−	−	−	−
	Benzamidase	− −	−	±	−	−	−	−
	Urease	± +	±	+	+	+	−	−
	Isonicotinamidase	− −	−	−	−	−	−	−
	Nicotinamidase	− +	−	+	−	+	±	−
	Pyrazinamidase	− ±	−	+	−	+	±	−
	Salicylamidase	− −	−	−	−	−	−	−
	Allantoinase	− −	−	−	−	±	−	−
	Succinamidase	− −	−	−	−	−	−	−
	Malonamidase	− −	−	−	−	−	−	−

* (No. of strains showing the pattern indicated)/(no. of strains tested).

When S-values for the 3 strains of group B to various HMOs were determined, only S-values to the HMO of group Kyoto-I were within the permissible low limit (88·3 % or more); S-values to the other HMOs remained outside the limits (Table 8). Thus, strains ATCC 3318, W-3409B and R-784 were identified as a member of the group Kyoto-I.

Nomenclature of species. From the results obtained, 7 groups could be recognized in this study: (1) Kyoto-I = group B of *Nocardia asteroides*; (2) Kyoto-II; (3) group A of *N. asteroides*; (4) *N. brasiliensis*; (5) *N. caviae*; (6) *N. pelletieri*; (7) *N. madurae*. Of these, the nomenclature of the last four is no problem. In the following, nomenclature of the first three is discussed.

Strains of group A of *Nocardia asteroides* and group B of *N. asteroides* (= Kyoto-I) had been received bearing the name *N. asteroides*. These two groups, however, showed distinctly different clusters and were considered to be two different species. According

to the description of Gordon & Mihm (1957, 1959, 1962*a*), the group A seemed to be correctly named *N. asteroides* (Eppinger) Blanchard. This was discussed previously.

Group B (Kyoto-I) has been named *Nocardia farcinica* Trevisan for the following reasons: (*a*) Gordon & Mihm (1957, 1962*a*) recognized the priority of the name *N. farcinica* over the name *N. asteroides*, but they avoided the name *N. farcinica* because they considered that the name *N. asteroides* had already become established by usage; (*b*) the group containing strain ATCC 3318 was recognized as a distinct cluster clearly differentiated from other groups of *Nocardia*, including *N. asteroides* group A.

Table 21. *Distinguishing characters for the species of Nocardia*

Character	*N. asteroides*	*N. rubra*	*N. farcinica*	*N. brasiliensis*	*N. caviae*	*N. pelletieri*	*N. madurae*
Growth at 45°	−	−	+	−	+	+	+
Acetamidase	−	+	+	−	−	−	−
Benzamidase	−	−	+	−	−	−	−
Urease	+	+	+	+	+	−	−
Malate as C source	+	+	+	+	+	−	+
Succinate as C source	+	+	+	+	+	−	+
1,3-Butylene glycol as C source	−	−	+	−	−	−	−
Acid from arabinose	−	−	−	−	−	−	+
Acid from galactose	−	−	−	+	−	−	−
Acid from inositol	−	−	−	+	+	−	−
Acid from mannitol	−	+	−	−	+	−	+
Acid from rhamnose	−	−	+	−	−	−	+
Acid from sorbitol	−	+	−	−	−	−	−
Serine as N and C sources	−	−	−	+	+	−	−
Glucosamine as N and C sources	+	+	+	+	+	−	+
Acetamide as N and C sources	−	+	+	−	−	−	−
Monoethanolamine as N and C sources	−	+	+	−	+	−	−

The group Kyoto-II included 7 strains. Two strains were identified later, and a total of 9 strains of this group is listed in Table 9. Among these, the following 5 had been identified by Gordon & Mihm (1957) as *Mycobacterium rhodochrous*: (1) *N. rubra* M-1; (2) *N. erythropolis* M-8; (3) *N. globerula* M-75; (4) *N. lutea* M-192; (5) *N. polychromogenes* M-6. One strain, *N. minima* M-103, had been identified by them as *N. asteroides*. Accordingly, this group might be identified as *M. rhodochrous*, but it showed several discrepancies from the characters of that species as described by Gordon & Mihm (1957). It is possible that, since the strains used by us had been transferred through two or three investigators and had been maintained in Dr Uesaka's laboratory for 7 years, some strains might be mislabelled or have changed their characters. Considering this possibility, strain ATCC 13808 of *M. rhodochrous* received directly from the American Type Culture Collection was studied and compared with the above strains. ATCC 13808 showed an S-value of 86 % to the HMO of the group Kyoto-II, a value outside the permissible limit for the group. This result made us hesitate to identify this group as *M. rhodochrous*, and we preferred to name it, provisionally, *Nocardia rubra*. This name had appeared first among 8 names for

this group, *N. rubra*, *N. erythropolis*, *N. globerula*, *N. convoluta*, *N. minima*, *N. lutea*, *N. polychromogenes* and *N. rubropertincta* (*Bergey's Manual*, 1957; *Index Bergeyana*, 1966).

Identification of strains not forming a cluster by preliminary sorting

It is supposed that, when S-values to the HMO for individual members of a species show a normal distribution curve, *c.* 95 % of the members of the species are included to the range {(mean) $\pm 2 \times$ (standard deviation)}. The permissible low limit of the S-value to an HMO is set for each species at {(mean S-value to the HMO) $- 2 \times$ (standard deviation)} %. After establishing the permissible low limit for species, identification is made without subjective judgement (automatically) by determining S-values for the unknown strain to various HMOs. If an S-value to a HMO is within the permissible limit and S-values to the other HMOs are outside the permissible limits, the unknown strain is identified as a species of the first HMO. This method was applied to the strains received from Dr Uesaka and not forming a cluster in preliminary sortings, and the results are shown in Table 10. Adding the strains thus identified, the strains identified in the 7 species are shown in Table 9 and Tables 11 to 16, and the frequency of characters in the species summarized in Table 17.

Eleven strains remained unidentified (Table 18); some may be distinct species. S-values between these strains were less than 80 %, except in the following pairs: (1) *Nocardia intracellularis* M-170 and *N. bostroemi* M-159 (88 %); (2) *N. intracellularis* M-170 and *N. restricus* M-9 (84 %); (3) *N. leishmanii* M-82 and *N. alba* M-113 (87 %); (4) *N. madurae* C-637 and *N. coeliaca* M-122 (83 %).

Typical strains of the species. Strains showing a high similarity to the HMO are used as reference strains, and are shown in Table 19.

A simple method of identification

The principles of numerical taxonomy make an ideal basis for a method of identification, and a method of using HMOs has been presented in this paper. Laboratories not specialized for bacterial identification need a simpler method. In the identification of mycobacteria, two methods have been found useful, amidase pattern (Bönicke, 1962) and pattern of nitrogen compounds used as simultaneous nitrogen and carbon sources (Tsukamura, 1967*c*). By a combination of these two methods, identification is successful with most strains, and typical patterns of the species are shown in Table 20.

Schneidau (1963) stated that the amidase pattern was not as uniform in nocardias as in mycobacteria, and in the present study it was found that identification of nocardias was not successful by the use of the amidase pattern alone, but the test was useful in combination with utilization of nitrogen compounds as simultaneous nitrogen and carbon sources. A practical diagnostic table was prepared by a selection of distinguishing characters, with frequencies of more than 91 % in some species but in others at a frequency as low as 10 %. This is shown in Table 21, which included a few characters with a frequency less than 90 %.

Although both identification tables (Tables 20 and 21) include 17 tests, Table 20 could be more easily applied in practice. Table 21 contains more characteristic tests, and would provide a more accurate result.

DISCUSSION

Division of Nocardia asteroides into two species

The most important conclusion reached in this study is that the species *N. asteroides* can be divided into two species, *Nocardia asteroides* (Eppinger) Blanchard and *Nocardia farcinica* Trevisan (both in new senses). Among 112 strains received as *Nocardia* species, 21 strains of *N. asteroides* (in a new sense) and 38 strains of *N. farcinica* (in a new sense) were identified (Tables 11 and 12); the data of comparison between these two species are summarized in Table 22. Eighteen of 21 strains of the former and 28 of 38 strains of the latter had been named as *N. asteroides* when received.

Table 22. *Comparison between Nocardia asteroides (group A of N. asteroides) and N. farcinica (group B of N. asteroides; group Kyoto-I)*

Character	No. of strains showing a positive reaction* (%)		*N. asteroides* according to the description of Gordon & Mihm (1962)
	N. asteroides	*N. farcinica*	
No. of strains tested...	21	38	142
†Growth at 45°	0 (0)	35 (92)	(42)
†Acetamidase	2 (10)	38 (100)	.
†Benzamidase	0 (0)	27 (71)	.
Pyrazinamidase	4 (19)	33 (87)	.
†Acid from rhamnose	0 (0)	34 (90)	(32)
Rhamnose as C source	0 (0)	34 (90)	.
Ethanol as C source	3 (14)	37 (87)	.
Propylene glycol as C source	1 (5)	30 (79)	.
†1,3-Butylene glycol as C source	0 (0)	36 (95)	.
2,3-Butylene glycol as C source	4 (19)	37 (97)	.
†Acetamide as N and C sources	0 (0)	37 (97)	.
†Monoethanolamine as N and C source	1 (5)	37 (97)	.
Nitrate reduction	8 (38)	0 (0)	(88)
Two week-arylsulphatase	8 (38)	0 (0)	.

* Figures in parenthesis indicate percentage strains showing a positive reaction.
† Distinguishing characters shown in Table 12.

Relationships between species. Numerical classification of the strains received from Dr Gordon differentiated 6 clusters, (1) *Nocardia asteroides*, (2) *N. farcinica*, (3) *N. brasiliensis*, (4) *N. caviae*, (5) *N. pelletieri*, and (6) *N. madurae* (Fig. 1), and these results agreed well with the results obtained by classical methods, except that *N. asteroides* was divided into two species, *N. asteroides* (in a new sense) and *N. farcinica*. Numerical classification of the strains received from Dr Uesaka differentiated another cluster of *N. rubra*. Relationships between these species are shown in Table 3 as an S-value table of HMOs of these species and in Fig. 4 as a dendrogram.

The 100 characters used in this study were mainly composed of nutritional requirement tests and biochemical tests, and included only 9 morphological characters. Since morphological characteristics have been recognized as important in classical taxonomy of the genus *Nocardia*, we would like to add some comments on morphology. All strains received from Dr Gordon as *N. madurae* and *N. pelletieri* showed well-developed mycelium tending to fragmentation (designated permanent mycelium in this study). They showed only scant growth on Ogawa egg medium, possibly due to their sus-

ceptibility to malachite green (Uesaka, 1964), making it impossible to take into account in the S-value characters determined on this medium. These two species were distinct from other species in dendrograms (Fig. 2 and 4) and seemed to belong to a Streptomyces-like nocardia of Schneidau & Shaffer (1957). However, it has not been concluded whether or not these are *Streptomyces*.

Permanent mycelium was observed not only in these species but also, to some extent, in 16 strains of other species. Among these, 5 belonged to an unidentified group (Table 18) received as *N. madurae* C-637, *N. leishmanii* M-82, *N. alba* M-113, *N. asteroides* M-123 and *N. madurae* M-172. One belonged to *N. caviae* (M-180 received as *N. asteroides*), 2 belonged to *N. asteroides* (M-94 and M-161 received as *N. asteroides*), and 8 belonged to *N. farcinica* (M-72, M-158A, M-162, M-167, M-169, M-177, M-179 and M-181 received as *N. asteroides*). The presence of permanent mycelium seemed not to be sufficient to differentiate *N. pelletieri* and *N. madurae* from other species.

When observed by naked eye on glucose agar after incubation at 28° for 4 weeks aerial hyphae were observed at the following rates: *N. asteroides*, 5/21 (24 %); *N. farcinica*, 10/38 (26 %); *N. brasiliensis*, 2/12 (17 %); *N. caviae*, 1/11 (9 %); *N. rubra*, 0/9 (0 %); *N. pelletieri*, 0/5 (0 %); *N. madurae*, 0/5 (0 %); unidentified, 2/11.

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NOTE

After preparation of the manuscript (May 1968), the author was informed that there was a paper of Cerbón (1967) dealing with numerical taxonomy of *Nocardia*.

Recently, Magnusson & Mariat (1968) stated that *N. asteroides* seemed to be a separable species by an immunological method based on the specificity of delayed type skin reactions on guinea pigs. Their results are compatible with our results.

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Marked Delay in Excystment of *Schizopyrenus russelli* Cysts by *p*-Chloromercuric Benzoate and its Reversal with Reduced Glutathione

By A. K. RASTOGI, P. SAGAR AND S. C. AGARWALA
Central Drug Research Institute, Lucknow, India

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SUMMARY

p-Chloromercuric benzoate (*p*-CMB) caused marked delay in excystment of *Schizopyrenus russelli* cysts when incorporated in the aqueous extract of *Escherichia coli*, an excystment agent. Cysts treated with *p*-CMB first and then with reduced glutathione (GSH), excysted readily in the presence of bacterial extract, after the removal of *p*-CMB and GSH.

INTRODUCTION

Actively proliferating bacteria of an *Aerobacter* sp. and *Escherichia coli* and their aqueous extracts cause excystment of cysts of *Schizopyrenus russelli*; certain pure amino acids, within a suitable pH range, also cause excystment (Singh, Mathew & Anand, 1958). Further, the excystment-inducing property of glutamic acid and of aqueous extract of *E. coli* was completely inhibited by emetine hydrochloride. This inhibition was completely annulled after the removal of emetine (Imam, Dutta & Agarwala, 1968).

The present communication deals with the delay in excystment of *Schizopyrenus russelli* cysts caused by *p*-chloromercuric benzoate and its reversal with reduced glutathione.

METHODS

Preparation of viable sterile cysts of Schizopyrenus russelli. The cysts from a 'pure line' culture of the amoeba, growing with *Escherichia coli*, were used. Cysts free from living and dead bacteria were obtained essentially according to the method of Singh, Saxena & Iyer (1965). Non-nutrient agar, containing agar 3·5 and NaCl 0·5 (% w/v), pH 6·8 to 7·0, in 1 l Roux-type culture flasks was seeded with cysts (1×10^6) and a thick suspension of *E. coli* in 0·5 % (w/v) NaCl. After 12 to 15 days, at 25°, the cysts were harvested and washed with distilled water several times by centrifugation to get rid of most of the bacteria. They were then treated with N-HCl for 24 hr at room temperature to kill bacteria, active amoebae and partially formed cysts. The acid was removed by repeated washing with water. The thick suspension of cysts thus obtained was treated for a few minutes with a sterile solution of trypsin (2 %, w/v) to lyse dead bacteria completely. The cysts were washed quickly several times with distilled water to get rid of small particles and water-soluble products released from the dead bacteria. After testing the bacterial sterility of the cysts, they were diluted to 10×10^6/ml., counted with a haemocytometer.

Bacterial extract. Eighteen hours growth of *Escherichia coli* on nutrient agar (NaCl 0·5 %, peptone 1 %, Marmite 0·1 % and Lab-Lemco 0·3 %, pH 7·2) was harvested with physiological saline and washed twice by centrifugation. The pellet was weighed and, for every g. wet bacteria, 5 ml. of distilled water were added. The suspension was autoclaved at 121° for 15 min., thereafter it was centrifuged aseptically. The clear straw-coloured supernatant fluid (pH 6·5) was stored in a refrigerator and used as the excystment agent.

Excystment procedure. To 0·1 ml. of cyst suspension in a sterile culture tube (10 × 1·2 cm.) were added 0·7 ml. of bacterial extract, followed by 0·1 ml. *p*-CMB solution + 0·1 ml. water in experimental and 0·2 ml. distilled water in control experiment. The tubes were incubated in inclined position at 25°. Samples were taken at regular intervals and counts of the different stages of excystment were made with a haemocytometer. *Schizopyrenus russelli* forms a double-walled cyst and excystment takes place in two stages. First, the inner wall disappears and a small amoeba moves freely within the outer wall. In a successful case, the outer wall gives way and the amoeba emerges.

In pretreatment experiments the same quantity of cysts were incubated with 0·1 ml. of *p*-CMB solution and/or 0·1 ml. reduced glutathione (final volume 1 ml.) for 1 hr, after which cysts were washed 3 times with distilled H_2O and then studied for excystment in bacterial extract, as given earlier. Proper controls were run in all the experiments.

RESULTS

The data presented in Table 1 showed that *p*-CMB, at 10^{-3}M and 5×10^{-3}M, completely prevented the emergence of trophozoites from the cysts up to 6 hr. At 10^{-4}M the excystment in 4 and 6 hr was decreased. At 6 hr in the tubes with 10^{-4}M *p*-CMB,

Table 1. *Effect of p-chloromercuric benzoate (M) on the excystment of Schizopyrenus russelli cysts*

	2 hr			4 hr			6 hr		
Stage:	A*	B*	C*	A	B	C	A	B	C
Concn. (M)	Count (%)								
Control	43	19	38	15	22	63	10	25	65
5×10^{-3}	95	5	0	91	9	0	86	14	0
10^{-3}	90	10	0	89	11	0	82	18	0
10^{-4}	36	54	10	9	82	9	14	60	26

* A, Intact cysts; B, intermediary stage (trophozoite moving within the outer cyst wall); C, trophozoites.

60 % of the cysts were in the intermediary stage of excystment, but at higher concentrations of *p*-CMB there was a considerable delay in the appearance of intermediary stages and only 14 to 18 % cysts were in that stage. Table 2 shows that 81 % of cysts pretreated with *p*-CMB (10^{-3}M) for 1 hr were in the intermediary stage of excystment after 4 hr which increased to 93 % in 8 hr; but the trophozoites failed to hatch. *p*-CMB at 10^{-4}M, which decreased excystment when present throughout the experiment, had no permanent effect since excystment paralleled the control when *p*-CMB was removed.

Although *p*-CMB at 10^{-3}M and 5×10^{-3}M completely prevented the emergence of trophozoites from the cysts up to 6 to 8 hr, nearly all the cysts excysted in 18 to 24 hr, as in the control. Hence all the cysts were viable. The data presented in Table 3 show that, when cysts were treated with *p*-CMB and reduced glutathione (GSH) together, there was 90 % excystment in 4 hr, as compared with none when incubated with *p*-CMB alone. There was 76 % excystment in 4 hr when the cysts were first treated with *p*-CMB for 1 hr and then with GSH for 1 hr, as against 81 % in the control.

Table 2. *Effect of pretreatment of cysts of Schizopyrenus russelli with p-chloromercuric benzoate on their subsequent excystment*

The cysts were treated with *p*-chloromercuric benzoate for 1 hr, washed thrice with distilled water and then left for excystment.

	2 hr			4 hr			6 hr			8 hr		
Stage:	A*	B*	C*	A	B	C	A	B	C	A	B	C
Concn. (M)	Count (%)											
Control	60	8	32	13	21	66	2	8	90	1	4	95
10^{-3}	65	35	0	19	81	0	16	84	0	7	93	0
10^{-4}	72	4	24	34	7	59	6	3	91	4	0	96

* A, B, C as in Table 1.

Table 3. *Effect of reduced glutathione in reversing the delay in excystment of Schizopyrenus russelli cysts caused by p-CMB*

The cysts after each treatment were washed thrice with distilled water and then put for excystment.

	2 hr			4 hr		
Stage:	A*	B*	C*	A	B	C
Concn. (M)	Count (%)					
Control	34	4	62	14	5	81
Pretreated with *p*-CMB for 1 hr (10^{-3}M)	95	5	0	47	53	0
Pretreated with *p*-CMB (10^{-3}M) + GSH (2×10^{-3}M) for 1 hr	13	0	87	10	0	90
Pretreated with *p*-CMB (10^{-3}M) for 1 hr and then with GSH (2×10^{-3}M) for 1 hr.	45	14	41	24	0	76

* A, B, C as in Table 1.

DISCUSSION

p-CMB is a specific inactivator of −SH groups (Webb, 1966). The present studies definitely indicate involvement of such groups in the process of excystment of cysts of *Schizopyrenus russelli*. *p*-CMB presumably interferes with enzyme function during excystment and that interference is completely annulled by reduced glutathione. The initiation of excystment does not appear to be as sensitive to the action of *p*-CMB as the final emergence of the trophozoites.

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Degradation of Xanthine by *Penicillium chrysogenum*

By A. M. ALLAM AND TAHANY A. ELZAINY
Laboratory of Microbial Chemistry,
National Research Centre, Cairo, Egypt

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SUMMARY

Penicillium chrysogenum utilized the purines hypoxanthine, xanthine, uric acid and adenine as sole nitrogen sources but not the methylated purines caffeine and theobromine. Cell-free extracts of this organism contained the enzymes xanthine dehydrogenase, uricase, allantoinase, allantoicase and urease. Uric acid was degraded to allantoic acid by way of allantoin; allantoin was degraded to glyoxylic acid by way of allantoic acid. Xanthine dehydrogenase, uricase, allantoinase and urease were constitutive whereas allantoicase was inducible by xanthine or allantoin.

INTRODUCTION

Fungi utilize purines as a nitrogen source (DiCarlo, Schultz & McManus, 1951; Franke, Taha & Krieg, 1952; Wolf, 1955; Taha, Krieg & Franke, 1955; Taha & Sharabash, 1956; Sukhenko & Podgainaya, 1958; Kim & Wolf, 1961; Fukumoto, Watanabe & Yano, 1968; LaRue & Spencer, 1968). The utilization of these compounds presumably requires enzymes that catalyse the degradation of the purine skeleton to simpler compounds. Taha *et al.* (1955) reported xanthine oxidase in extracts of some fungal species. The enzyme uricase, which catalyses the degradation of uric acid to allantoin, has been shown to be present in many fungi (Brunel, 1937; Franke *et al.* 1952; Roush & Domnas, 1956; Laboureur & Langlois, 1967). The enzyme allantoinase, which catalyses the hydrolysis of allantoin to allantoic acid, was demonstrated in basidiomycetes (Brunel, 1937), *Aspergillus niger* (Brunel, 1931; 1939), *Candida utilis* and *Saccharomyces cerevisiae* (Lee & Roush, 1964). Allantoicase, catalysing the conversion of allantoic acid to glyoxylic acid and urea, was shown in *A. niger* (Brunel, 1939), *C. utilis* and *S. cerevisiae* (Domnas, 1962; Lee & Roush, 1964) and in *Penicillium notatum* and *P. citreo-viride* (Trijbels & Vogels, 1966). These enzymic studies do not give a complete picture of the degradation of purines in one single organism. The present work aims to elucidate the enzymic steps in the degradation of xanthine by extracts of *P. chrysogenum*.

METHODS

Organism. Penicillium chrysogenum was obtained from the centre of cultures of the National Research Centre of Egypt.

Media. The organism was cultivated and kept on slants of solid Czapek-Dox medium. This medium contained (% w/v): glucose, 3; K_2HPO_4, 0·1; $MgSO_4.7H_2O$, 0·05; KCl, 0·05; $NaNO_3$, 0·2; agar 2·0. The liquid medium used for growing the

organism was the same with the appropriate purine replacing $NaNO_3$ on an equivalent nitrogen basis. Purines and related compounds were sterilized by filtration but all other ingredients of the medium were autoclaved.

Culture and harvest of mycelium. Conidia were scraped from mycelium which had been grown on slopes for 7 days at 28° and suspended in cold sterile distilled water. One ml. samples of this suspension were used to inoculate 250 ml. Erlenmeyer flasks each containing 50 ml. sterile medium. The inoculated flasks were incubated at 28° for 3 to 4 days, then the mycelium was harvested by filtration, washed thoroughly with distilled water and finally blotted dry with absorbent paper.

Preparation of cell-free extracts. The blotted-dry mycelium was ground with about twice its weight of washed cold sand and extracted with 0·05 M potassium phosphate buffer (pH 7·4). The slurry was centrifuged at 8,000 rev./min. for 20 min. and the supernatant liquid used as the crude enzyme preparation. During this operation the containers and solutions were kept at 0°.

Preparation of potassium allantoate. This was prepared by the alkaline hydrolysis of allantoin (Young & Conway, 1942).

Enzyme assays. Xanthine dehydrogenase was assayed either by estimating the uric acid formed during the incubation of cell-free extracts with xanthine (Blauch & Koch, 1939), or by the anaerobic reduction of 2,3,5-triphenyltetrazolium chloride by the Thunberg tube technique and estimating the formazan formed (Kun & Abood, 1949). Uricase was assayed by measuring the disappearance of uric acid and the formation of allantoin during the incubation of extracts + uric acid; the allantoin formed was estimated by the method used by Franke *et al.* (1952). Allantoinase activity was measured by following the formation of allantoic acid when allantoin was incubated with extracts. Allantoic acid was estimated by the same procedure used for allantoin with the omission of alkaline hydrolysis. Allantoicase was assayed by following glyoxylic acid formation when potassium allantoate was incubated with the extracts. Glyoxylic acid was estimated by the procedure used for allantoin with the omission of alkaline and acid hydrolysis. Urease activity was determined by estimating the ammonia formed from urea using Nessler's reagent.

Protein determination. This was done by the method of Sutherland, Cori, Haynes & Olsen (1949).

Identification of products. Allantoin and allantoic acid were recognized by ascending paper chromatography on Whatman No. 1 paper and two different solvent systems: *n*-butanol + ethanol + water (4 + 1 + 1 by vol.) with R_F values of 0·13 and 0, respectively (the yellow spot of sprayed allantoic acid was located on the start line); phenol + 10 % sodium citrate (5:1, w/v), R_F values 0·41 and 0·17 (Sukhenko & Podgainaya, 1958). After drying the chromatograms at room temperature they were sprayed with Ehrlich's reagent (Smith, 1960); identifications were checked by co-chromatography with authentic standards. Glyoxylic acid was converted to the 2,4-dinitrophenylhydrazone (Smith & Smith, 1960), chromatographed on Whatman No. 1 paper with two different ascending solvent systems: *n*-butanol + ethanol + water (70 + 10 + 20 by vol.) where the R_F was 0·35 and *n*-butanol + ethanol + 0·5 N-NH_4OH (70 + 10 + 20 by vol.), R_F = 0·39 (Smith & Smith, 1960). The hydrazone of the authentic acid was prepared and run as control.

RESULTS

Growth of Penicillium chrysogenum on different purines and other nitrogenous compounds. Table 1 demonstrates that allantoin yielded the best growth followed by hypoxanthine and adenine. Xanthine and uric acid supported growth which was more or less equal to that obtained on sodium nitrate. The methylated purines caffeine and theobromine were not utilized. Kim & Wolf (1961) found that methylated purines were not utilized by *P. chrysogenum* Q-176. The feeble growth obtained when ammonium chloride was sole nitrogen source might be attributed to the decrease of pH value of the culture medium due to utilization of the ammonium ions.

Table 1. *Growth of Penicillium chrysogenum on different purines and other nitrogenous compounds*

Triplicate culture flasks contained 50 ml. of Czapek-Dox medium in which $NaNO_3$ was replaced by a nitrogen-equivalent amount of each nitrogen source, incubated 8 days at 28°.

Nitrogen source	Mean mycelium dry weight (mg./flask)
Adenine	338·9
Hypoxanthine	345·0
Xanthine	249·9
Uric acid	274·5
Caffeine	0
Theobromine	0
Allantoin	392·3
Urea	220·0
$NaNO_3$	276·7
NH_4Cl	187·7

Table 2. *Xanthine dehydrogenase in extracts of Penicillum chrysogenum mycelium*

Duplicate Thunberg tubes under vacuum contained xanthine (where indicated), 45 to 50 μmoles; 2,3,5-triphenyltetrazolium chloride, 1·0 μmole; sodium pyrophosphate buffer, pH 8·6, 140 μmoles; extract, equiv. 3 to 4·4 mg. protein; total volume, 3·1 ml. (Expt. 1), 4·0 ml. (Expt. 2); temp. 37°; reaction time, 120 min.

Expt. no.		Additions	μg. Formazan formed
1	Xanthine-grown mycelial extract	Xanthine	145·3 ± 1·4
		—	53·6
2	Nitrate-grown mycelial extract	Xanthine	122·1 ± 3·3
		—	72·6 ± 6·6

Xanthine dehydrogenase. Table 2 shows xanthine dehydrogenase activity in extracts of *Penicillium chrysogenum* grown with xanthine or sodium nitrate. In the absence of xanthine the formazan formed ranged from 36 to 59 % of that produced when xanthine was present. Xanthine dehydrogenase was constitutive since it was present when the organism was grown with nitrate as sole nitrogen source. The data in Table 2 indicate that enzymic dehydrogenation of xanthine by extracts of the experimental organism is a slow process. This could be due to the partial loss of a certain requirement of the enzyme in the preparation of the extract. This point was tested by observing the formation of uric acid from xanthine: in comparable conditions to

those in Table 2 (but only 20 μmoles xanthine), only 0·25 μmole of uric acid was detected after 1·5 hr and 0·45 μmole after 3 hr. No significant uric acid was detected at the beginning of the reaction.

Uricase and combined uricase-allantoinase activities. Figure 1 shows the disappearance of uric acid catalysed by extracts of mycelia grown with xanthine or with nitrate; this shows that 7·9 μmoles of uric acid had almost disappeared in 150 min. with xanthine-grown mycelium. Analysis of this reaction mixture showed 7·12 μmoles

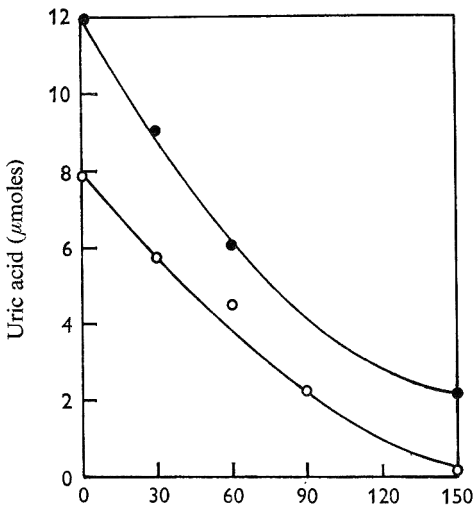

Fig. 1

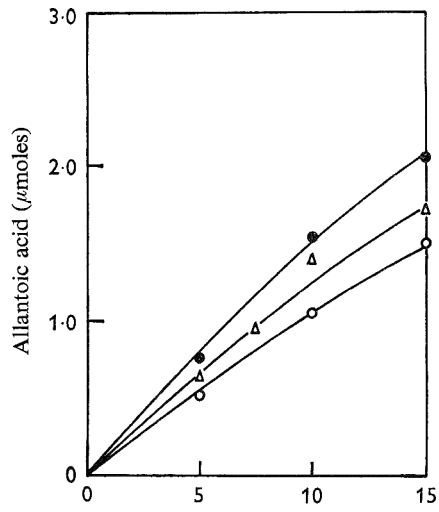

Fig. 2

Fig. 1. Disappearance of uric acid catalysed by extracts of *Penicillium chrysogenum* grown with xanthine (○) or nitrate (●). Reaction mixture contained uric acid, 7·9 to 12·0 μmoles; sodium pyrophosphate buffer, pH 8·6, 60 μmoles; extract, 9 to 10 mg. protein; total volume, 4 ml.; temp. 37°.

Fig. 2. Allantoinase in extracts of *Penicillium chrysogenum* grown with xanthine (○), allantoin (△) or nitrate (●). Reaction mixture contained allantoin, 2 to 2·5 μmoles/ml.; extract, equiv. 0·85 mg. protein/ml. (○), 1·1 mg./ml. (△), 2·25 mg./ml. (●); volume 4 to 8 ml.; temp. 37°.

allantoin, 1·05 μmoles allantoate, no uric acid or glyoxylate. The formation of allantoic acid indicated the presence of allantoinase which hydrolysed part of the allantoin formed to allantoic acid. Hence allantoin is an intermediate in the degradation of uric acid to allantoic acid. Glyoxylic acid was not detected; it seems that this acid was formed only when higher concentrations of allantoic acid were present. Figure 1 indicates that uricase is a constitutive enzyme in *Penicillium chrysogenum* since extracts prepared from mycelia grown on nitrate were active. Franke *et al.* (1952) showed that uricase is a constitutive enzyme in *Aspergillus niger* and *Alternaria porri*. On the other hand, Roush & Domnas (1956) reported that uricase is an inducible enzyme in *Torulopsis utilis* and Fukumoto *et al.* (1968) demonstrated that uricase was induced by uric acid in a Streptomyces species.

Allantoinase. Figure 2 shows the hydrolysis of allantoin to allantoic acid by extracts of mycelia grown with xanthine, with allantoin or with nitrate. The different rates were probably due to variations of the amounts of extract protein present in the

reaction mixture. These results indicate that the enzyme is constitutive in *Penicillium chrysogenum*. Lee & Roush (1964) reported that allantoinase was induced by uric acid, allantoin or allantoic acid in *Candida utilis* whereas this enzyme was constitutive in *Saccharomyces cerevisiae*.

Allantoicase. Figure 3 demonstrates the presence of this enzyme in extracts of xanthine-grown and allantoin-grown *Penicillium chrysogenum*. Urea, which is the other expected product of this reaction, was not determined because of a very active urease (see below).

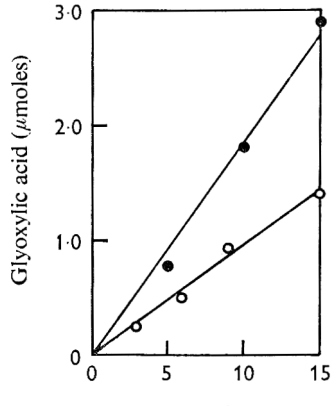

Fig. 3

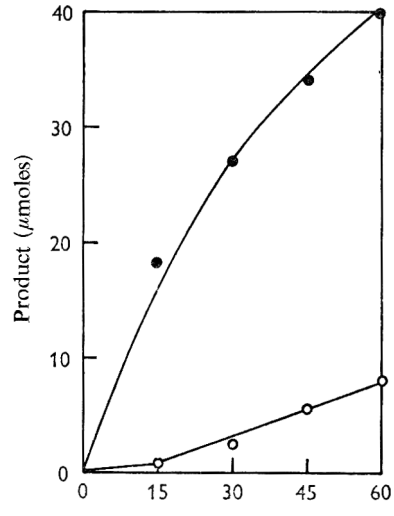

Fig. 4

Fig. 3. Allantoicase in extracts of *Penicillium chrysogenum* grown with xanthine (○) or allantoin (●) Reaction mixture contained potassium allantoate 32 μmoles; potassium phosphate buffer, pH 7·4, 100 μmoles; extract, equiv. 7·2 to 9·0 mg. protein; total volume, 3 ml.; temp. 37°.

Fig. 4. Combined allantoinase and allantoicase activities in extracts of *Penicillium chrysogenum* grown with xanthine. Reaction mixture contained allantoin, 200 μmoles; potassium phosphate buffer, pH 7·0, 100 μmoles; extract, equiv. 7·2 mg. protein; total volume, 5 ml.; temp. 37°. ●, Allantoate formed; ○, glyoxylate formed.

Trijbels & Vogels (1966) demonstrated allantoicase activity in extracts of *Penicillium notatum* and *P. citreo-viride* grown in an allantoin + yeast extract medium. The two penicillia were reported to be urease negative. These authors reported that ureidoglycollic acid is an intermediate in the degradation of allantoic acid to glyoxylic acid and urea. We tried to detect this intermediate in the reaction by their procedure making use of a neutral hydrolysis of ureidoglycollic acid to glyoxylic acid but unreproducible results were obtained.

Growing the experimental organism on nitrate yielded extracts lacking allantoicase activity. This shows that allantoicase is an inducible enzyme. Lee & Roush (1964) showed that, while allantoicase was induced by uric acid, allantoin or allantoic acid in *Candida utilis*, the same enzyme was constitutive in *Saccharomyces cerevisiae*.

Combined allantoinase and allantoicase activities. In this experiment allantoin was incubated with extracts of mycelia grown with xanthine as sole nitrogen source and

both allantoic and glyoxylic acids were determined. As shown in Fig. 4, glyoxylic acid started to appear in significant amounts after the formation of substrate amounts of allantoic acid. This experiment shows that allantoic acid is an intermediate in the degradation of allantoin to glyoxylic acid.

Urease. Figure 5 shows urease activity in extracts of this organism grown with xanthine. The enzyme is very active since about 90 μmoles of ammonia were formed in 12 min. Comparable urease activity was found in extracts of mycelia grown with allantoin or nitrate. Hence the enzyme is constitutive. The enzyme tolerated high concentrations of urea; in one experiment 300 μmoles of urea were added initially and fairly good enzymic activity was obtained.

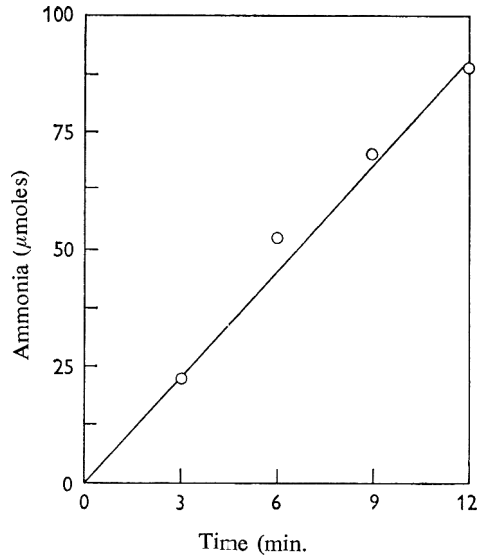

Fig. 5. Urease in extracts of *Penicillium chrysogenum* grown with xanthine. Reaction mixture contained urea, 60 μmoles; potassium phosphate buffer, pH 7·0, 100 μmoles; extract, equiv. 7·2 mg. protein; total volume, 3 ml.; temp. 37°.

DISCUSSION

Penicillium chrysogenum 65 grown on Czapek-Dox medium with xanthine as sole nitrogen source degraded xanthine by a multiple enzyme system as follows:

$$\text{Xanthine} \xrightarrow[\text{dehydrogenase}]{\text{xanthine}} \text{uric acid}$$

$$\text{Uric acid} \xrightarrow{\text{uricase}} \text{allantoin}$$

$$\text{Allantoin} \xrightarrow{\text{allantoinase}} \text{allantoic acid}$$

$$\text{Allantoic acid} \xrightarrow{\text{allantoicase}} \text{glyoxylic acid} + \text{urea}$$

$$\text{Urea} \xrightarrow{\text{urease}} \text{ammonia} + CO_2$$

From uric acid onwards, this degradation sequence is similar to that demonstrated in aerobic bacteria (Franke & Hahn, 1955; Campbell, 1955). The fate of glyoxylic acid, which is the other product of this degradation, needs further investigation; it could be further oxidized to oxalic acid by a system similar to that reported by Franke & Hahn (1955) in *Pseudomonas aeruginosa* or to formic acid and CO_2 as demonstrated by Campbell (1955), with extracts of an unidentified *Pseudomonas* sp. However, the possibility exists that this two-carbon compound could also be utilized in the synthesis of cellular compounds, e.g. the formation of glycine by the reductive amination of glyoxylic acid. It might also be utilized through the glyoxylate cycle if it is operating in this organism.

Xanthine dehydrogenase, uricase and allantoinase were constitutive enzymes but allantoicase seems to be inducible, being induced by xanthine or allantoin. The constitutive enzymes are presumably important to the organism; their combined activities may play a central role in the control of the concentration of free purines in the cell pool. On the other hand, when the organism is grown on the purine as its sole nitrogen source, all the enzymes, including allantoicase, are required to catalyse the complete degradation of the purine to glyoxylic acid and ammonia.

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Studies by Microelectrophoretic and Microscopic Techniques of the Sorption of Illite and Montmorillonite to Rhizobia

By K. C. MARSHALL

Department of Agricultural Science, University of Tasmania, Hobart, Tasmania 7001, Australia

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SUMMARY

Electrophoretic measurements indicated that with increasing concentrations of illite or montmorillonite the surfaces of bacteria became progressively enveloped by a layer of adsorbed clay. The amount of clay adsorbed per unit area of cell surface depended mainly on the surface ionogenic properties of the isolates of the Rhizobium species used. For any particular isolate, almost identical amounts of illite and montmorillonite were adsorbed per bacterium. Microscopic studies confirmed the presence of a clay envelope around the bacteria and provided some information on the mode of adsorption of the clays.

INTRODUCTION

Investigations of the electrophoretic mobility of bacteria in the presence of colloidal montmorillonite have indicated that sorption of the montmorillonite results in the formation of a clay envelope around the organisms (Lahav, 1962; Marshall, 1968*a*). More detailed information on the nature of bacterium/clay interactions has now been obtained by a comparison of the electrophoretic properties of bacteria + montmorillonite with those of bacteria + illite. The association of clays with bacteria has also been examined by fluoresence and electron microscopy.

METHODS

Organisms. The organisms used in electrophoretic studies were cultures of root-nodule bacteria with different ionogenic properties (Marshall, 1967). *Rhizobium trifolii* TAI and *R. lupini* UT 12 possess simple acidic (carboxyl) surfaces, whilst *R. trifolii* SU297B and *R. trifolii* SU298D have acidic groups along with some basic (amino) groups. These particular organisms were chosen because one from each group (*R. trifolii* TAI and *R. trifolii* SU298D) exhibited an electrophoretic mobility greater than that of Na-illite particles, whilst the mobilities of the remaining cultures (*R. lupini* UT 12 and *R. trifolii* SU297B) were considerably lower than that of the illite.

Rhizobium trifolii SU297B was used for the electron microscope study of rhizobium/clay interactions, but a culture of *Bacillus subtilis* was used for fluorescence microscopy since the larger bacilli facilitated observation and photography.

All *Rhizobium trifolii* isolates were grown on yeast + mannitol agar for 3 to 4 days at 28°; *R. lupini* UT12 was grown on the same medium at 28° for 10 to 12 days; *Bacillus subtilis* was grown on nutrient agar at 37° for 6 hr.

Clay preparation. Suspensions of homoionic Na-montmorillonite and Na-illite were

prepared from Volclay bentonite (American Colloid Co., Skokie, Illinois) and Fithian illite (Wards Natural Science Establishment, Rochester, N.Y.), respectively, as described by Lahav (1962). The concentrated clay suspensions were stored under refrigeration and appropriate dilutions were prepared as required.

Determination of electrophoretic mobility. Bacterial suspensions were prepared as described by Marshall (1968*a*). The electrophoretic mobility of the rhizobia in the presence or absence of clays was measured as described previously (Marshall, 1967), except that organisms were timed over a distance of 69 μ at the upper stationary level. Measurements were made with bacterial concentrations of about 10×10^6 rhizobia/ml. in an NaCl solution of ionic strength 0·05. More dilute rhizobial suspensions were used to determine 'limiting mobility' values (Marshall, 1968*a*).

Surface area of rhizobia. The length and diameter of the rhizobia (plus capsules where present) were determined by measurement of negatively stained preparations with a calibrated filar micrometer. The surface areas were calculated by assuming the rhizobia to be cylinders with flat ends.

Fluorescence microscopy. Washed *Bacillus subtilis* organisms were suspended in a solution of 0·01 % acridine orange in 0·1 M-phosphate buffer (pH 5·0) for 4 hr. The bacteria were then washed three times in phosphate buffer, and mixed with equal quantities of water or an aqueous suspension of Na-montmorillonite to give a final concentration of 100 μg. clay/ml. Wet and dry amounts of these preparations were examined under a Leitz Ortholux fluorescence microscope. The filters used were the 1 and 2 mm. UG1filters, a BG 38 heat-absorbing filter, and a K430 barrier filter. Exposure time was 3 min. with Kodachrome II film.

Electron microscopy. The samples were freeze-dried directly on collodion-carbon films on grids in order to avoid artifacts arising from surface tension effects during drying of the bacterium+clay suspensions on the grids (Kittrick, 1965). Any fluffy material on the grids after freeze-drying was carefully removed. Some specimens were shadowed with gold+palladium (60 % gold+40 % palladium). All observations were made with an AEI EM6 electron microscope at 60 kV.

RESULTS

Effects of clays on the electrophoretic mobility of rhizobia

The four Rhizobium isolates exhibited quite different and characteristic mobilities in the absence of any clay, but they all responded in a similar manner to increasing concentrations of Na-montmorillonite (Fig. 1A). The mobility of rhizobia of all cultures increased until they approximated that of the N-montmorillonite, suggesting that the observed rhizobial mobility actually is a measure of the mobility of a Na-montmorillonite envelope around each organism (Marshall, 1968*a*). Studies with Na-illite (Fig. 1B) also provided convincing evidence for the existence of such a clay envelope. Where the characteristic mobility of an isolate was lower than that of illite, the observed mobility increased with increasing clay concentration, as found with montmorillonite. With rhizobia possessing characteristically high mobilities, the observed mobility decreased with increasing clay concentration to a value about that of illite. Thus, the curves in Fig. 1B show very clearly that the rhizobial mobilities observed at high clay concentrations reflected the characteristic mobility of the clay adsorbed on the rhizobial surface.

For each rhizobial isolate, the amount of clay adsorbed per rhizobium was determined by the 'limiting mobility' method of Nevo, De Vries & Katchalsky (1955). Adsorption isotherms were obtained by plotting the amounts of illite or montmorillonite adsorbed per rhizobium at different mobilities, against the free or equilibrium concentrations of the respective clays (Fig. 2A, B). Inspection of these Langmuir-type adsorption isotherms revealed a range of degrees of saturation of clay adsorbed per rhizobium for the different cultures. The total amounts of either illite or mont-

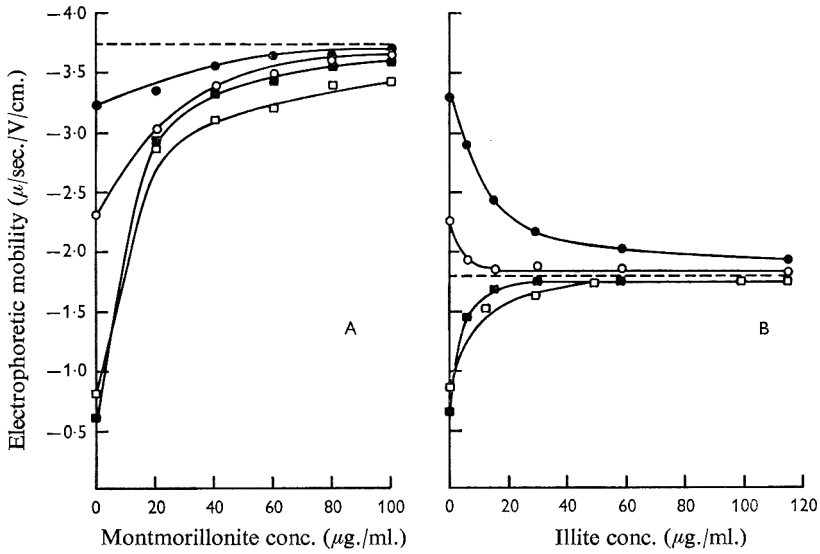

Fig. 1. Changes in the observed electrophoretic mobility of rhizobia with increasing clay concentration. ●—●, *Rhizobium trifolii* SU298D; ○—○, *R. trifolii* TAI; ■—■, *R. lupini* UT12; □—□, *R. trifolii* SU297B. A, broken line indicates mobility of Na-montmorillonite particles; B, broken line indicates mobility of Na-illite particles.

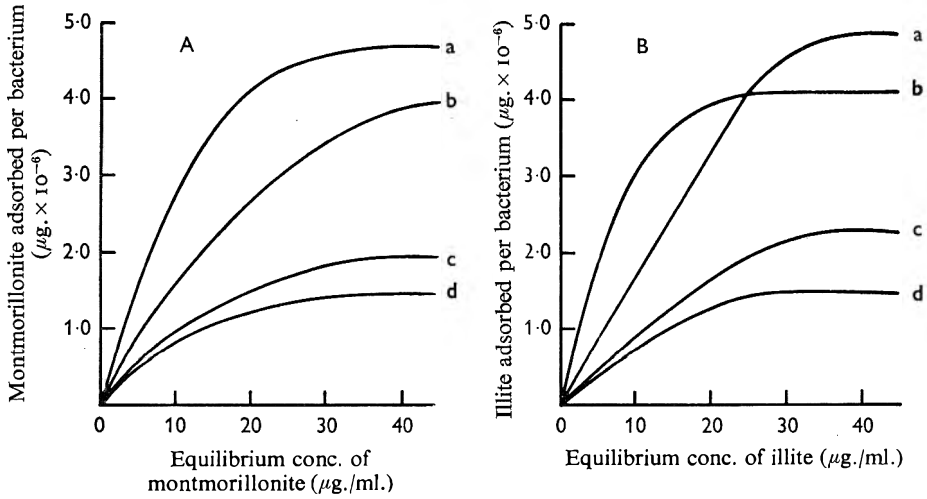

Fig. 2. Adsorption isotherms of A, Na-montmorillonite and B, Na-illite on: (a) *Rhizobium lupini* UT12; (b) *R. trifolii* TAI; (c) *R. trifolii* SU298D; (d) *R. trifolii* SU297B.

morillonite adsorbed per rhizobium for each individual isolate were remarkably similar (Table 1). As found previously with adsorption of montmorillonite (Marshall, 1968*a*), rhizobia with a simple carboxyl surface adsorbed about twice as much montmorillonite or illite per unit area of rhizobial surface as did rhizobia with a complex carboxyl + amino surface (Table 1).

Table 1. *Saturation levels of adsorbed Na-montmorillonite and Na-illite per bacterium and per unit area of bacterial surface on four isolates of Rhizobium*

Organism	Surface ionogenic groups	Surface area of bacteria (μ^2)	Montmorillonite adsorbed		Illite adsorbed	
			(μg./rhizobium)	(μg./μ^2)	(μg./rhizobium)	(μg./μ^2)
R. trifolii TA1	Carboxyl	6·82	$3{\cdot}98 \times 10^{-6}$	$0{\cdot}58 \times 10^{-6}$	$4{\cdot}10 \times 10^{-6}$	$0{\cdot}60 \times 10^{-6}$
R. lupini UT12	Carboxyl	9·24	$4{\cdot}70 \times 10^{-6}$	$0{\cdot}51 \times 10^{-6}$	$4{\cdot}90 \times 10^{-6}$	$0{\cdot}53 \times 10^{-6}$
R. trifolii SU297B	Carboxyl + amino	5·58	$1{\cdot}55 \times 10^{-6}$	$0{\cdot}28 \times 10^{-6}$	$1{\cdot}45 \times 10^{-6}$	$0{\cdot}26 \times 10^{-6}$
R. trifolii SU298D	Carboxyl + amino	8·17	$2{\cdot}03 \times 10^{-6}$	$0{\cdot}25 \times 10^{-6}$	$2{\cdot}30 \times 10^{-6}$	$0{\cdot}28 \times 10^{-6}$

Microscopic observation of bacterium-clay interaction

Because of the fine colloidal nature of the clays used it was not possible to observe directly by light microscopy an interaction between bacteria and the clays. An indirect demonstration of the presence of a clay coating around *Bacillus subtilis* organisms was achieved by observations under a fluorescence microscope of bacteria stained with acridine orange. The addition of a dilute suspension of Na-montmorillonite to a suspension of stained bacilli markedly decreased the intensity of the fluorescent image of the bacillia (Pl. 1, fig. 1, 2). This result suggested that clay particles partially coated the bacterial surface and interfered with the production of the fluorescent image.

A more direct observation of the mode of adsorption of clays on rhizobia was obtained by electron microscopy. Unshadowed and shadowed preparations of rhizobia in the absence of clays are shown in Pl. 1, fig. 3, 4. Unshadowed preparations of rhizobia + clay indicate that the Na-montmorillonite adsorbed around the rhizobia was much less electron-dense than the Na-illite (Pl. 2, fig. 5; Pl. 3, fig. 7), while shadowed preparations showed that this was a difference in the thickness of the individual clay particles (Pl. 2, fig. 6; Pl. 3, fig. 8). The electron micrographs show the distinct envelope of adsorbed clay around the rhizobia. There appeared to be more montmorillonite than illite adsorbed to each rhizobium, but each illite particle was very much thicker than the montmorillonite particle.

DISCUSSION

The suggestion that in suspensions of mixed clay + bacteria the outer surface of bacteria might become covered by adsorbed clay particles (Marshall, 1968*a*) has been confirmed by the electrophoretic and microscopic observations reported in the present paper. Such a clay envelope may be of significance in modifying the micro-environment around individual micro-organisms in certain natural soils (Marshall, 1964, 1968*b*; Stotzky & Rem, 1966). In addition to providing some indirect evidence

for the existence of a clay envelope around the bacteria, the studies on fluorescently labelled *Bacillus subtilis* indicated that even minute amounts of clay (100 μg./ml. of bacterial suspension) could interfere with the fluorescence image obtained with the u.v. microscope. This effect may explain the interference reported (Eren & Pramer, 1966; Schmidt, Eankole & Bohlool, 1968) in the use of the fluorescently-labelled antibody technique for the detection of specific micro-organisms in soils with high clay contents.

A feature of the sorption studies was the remarkable similarity between the amounts of illite and montmorillonite adsorbed per rhizobium for any particular isolate. It appears that the nature of the ionogenic surface of the root-nodule Rhizobium organisms was the most important factor in determining the amount of illite or montmorillonite adsorbed per unit area of rhizobial surface. Broadly speaking, the amount of clay adsorbed seems to be independent of the net surface-charge density (as indicated by the characteristic electrophoretic mobilities) either of the rhizobia or of the illite and montmorillonite. As noted previously (Marshall, 1968*a*), however, there is some relationship within each group of Rhizobium isolates with similar surface ionogenic properties between the electrokinetic charge of the rhizobia and the amount of clay adsorbed per unit area.

An attempt was made by Marshall (1968*a*) to explain the effect of ionogenic properties of bacteria on adsorption of clay by a consideration of the orientation of individual clay platelets (crystals) at the bacterial surface. It is obvious from the present electron micrographs that both the montmorillonite and illite clays were in a flocculated condition. Consequently, it is not possible to consider the sorption process solely in terms of the orientation of individual clay crystals at the surface of the bacteria. However, these electron micrographs do reveal that most clay particles were adsorbed on to the rhizobial surface in an edge-to-face, rather than a face-to-face, manner. This observation may help to explain the similarity in the amounts of illite and montmorillonite adsorbed per rhizobium, since any positive charge on the edge of the different clays is likely to be similar even though the net surface-charge density of the clays is very different. It does appear from observations with the electron microscope that a greater number of montmorillonite particles were adsorbed per rhizobium than illite particles. It must be noted, however, that the illite particles are much thicker than those of the montmorillonite, and it is feasible that the weights of the clays adsorbed per rhizobium were similar as indicated by the electrophoretic studies.

To have conditions in electron microscopy comparable to those used in the electrophoretic studies, the bacterium + clay suspensions were freeze-dried on grids to avoid artifacts arising from surface tension effects during drying. In natural soil environments, however, the accumulation of clay particles at the surfaces of bacteria as a result of surface tension during drying of the soil may be a normal situation. Such conditions are of some practical significance in soils where certain clay types apparently protect root-nodule bacteria from the detrimental effects of desiccation and high temperatures (Marshall & Roberts, 1963; Marshall, 1964).

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. *Bacillus subtilis* stained with acridine orange. Air-dried preparation, ultraviolet irradiation; × 1000.

Fig. 2. *B. subtilis* stained with acridine orange. Montmorillonite (100 μg./ml.) added. Air-dried preparation; u.v. irradiation; × 1000.

ELECTRON MICROGRAPHS

Fig. 3. *Rhizobium trifolii* SU297B without clay. Freeze-dried. Unshadowed; × 27,500.

Fig. 4. *R. trifolii* SU297B without clay. Freeze-dried. Shadowed with gold + palladium; × 34,500.

PLATE 2

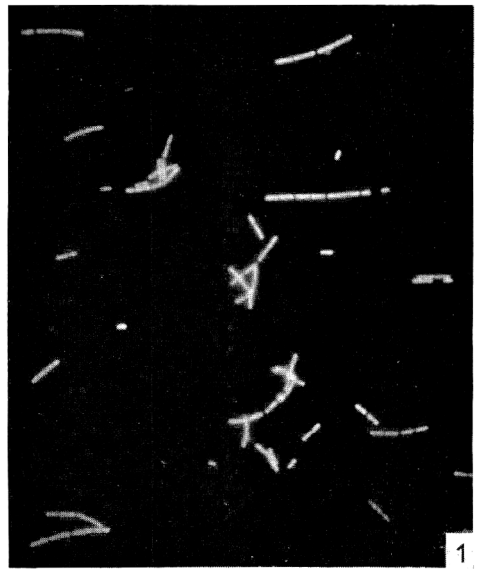
Fig. 5. *R. trifolii* SU297B plus montmorillonite (80 μg./ml.). Unshadowed; × 34,500.

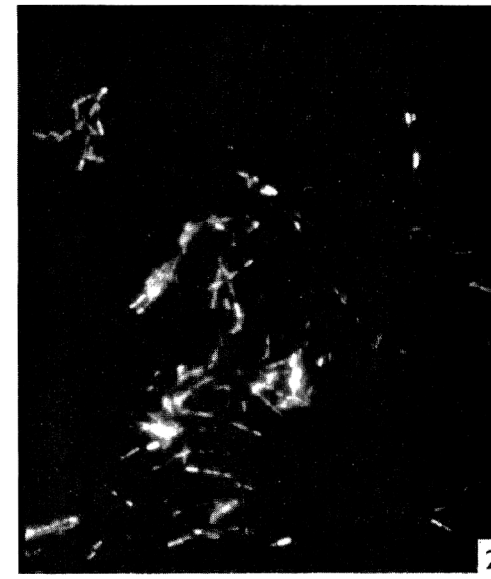
Fig. 6. *R. trifolii* SU297B plus montmorillonite (80 μg./ml.). Shadowed with gold + palladium; × 21,000.

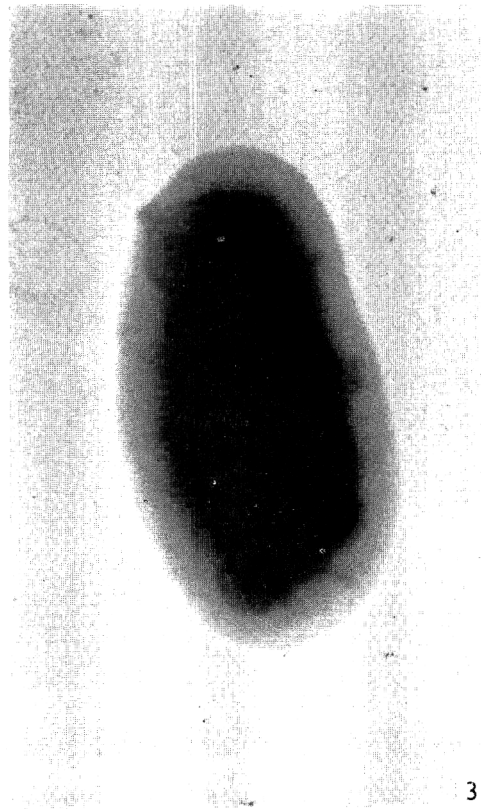
PLATE 3

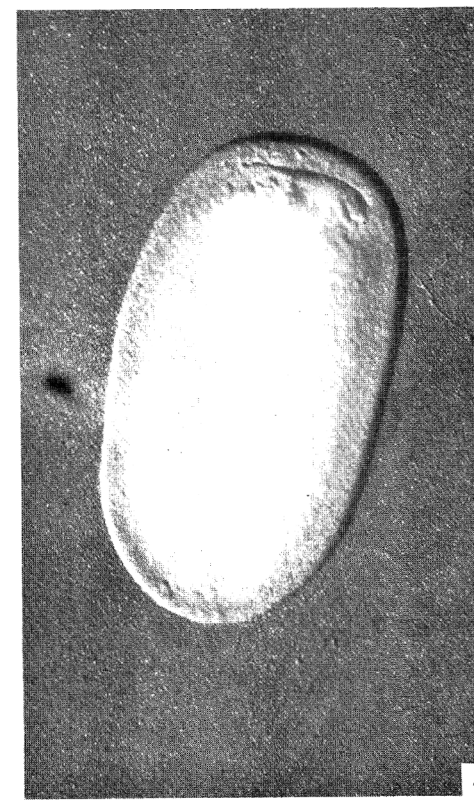
Fig. 7. *R. trifolii* SU297B plus illite (100 μg./ml.). Unshadowed; × 34,500.

Fig. 8. *R. trifolii* SU297B plus illite (100 μg./ml.). Shadowed with gold + palladium; × 43,000.









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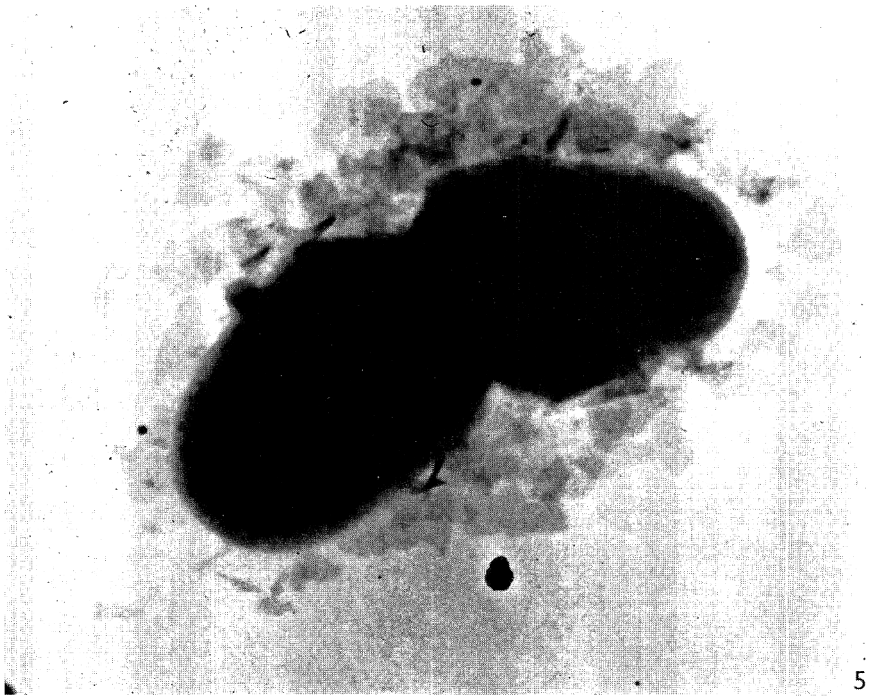

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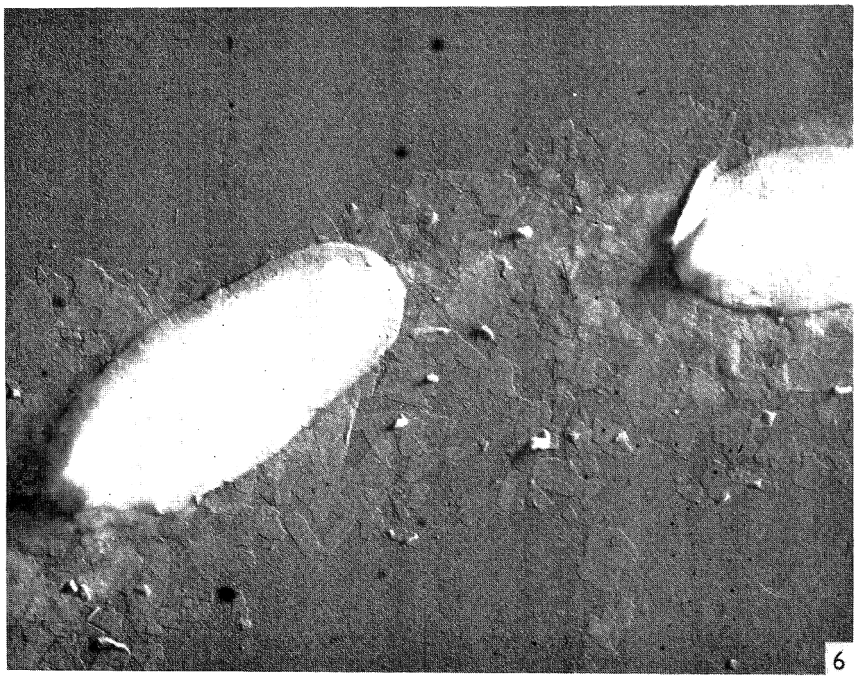

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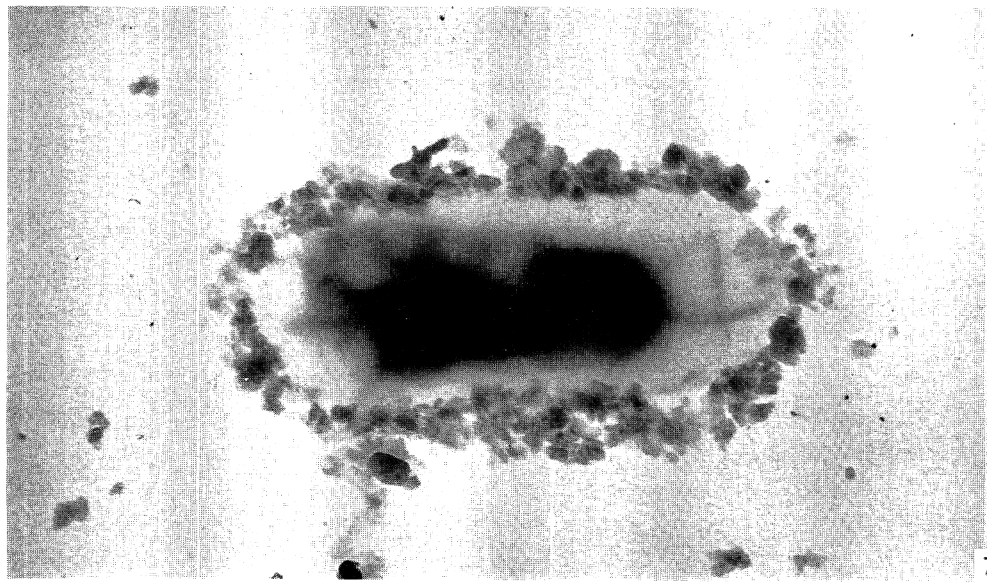

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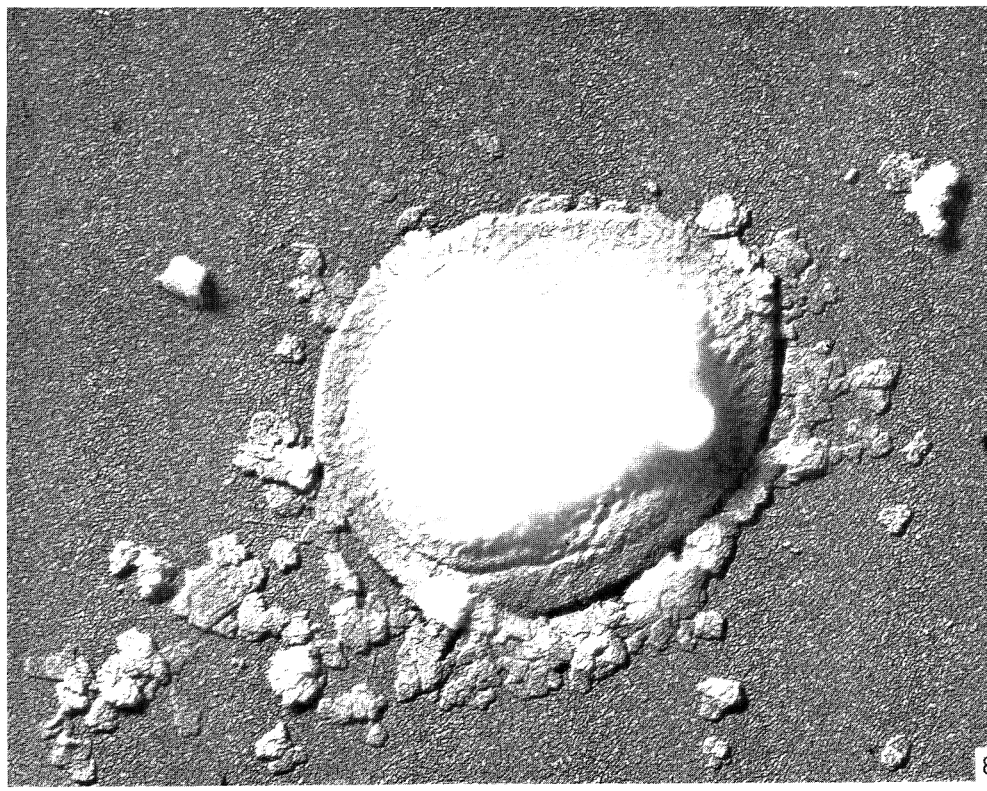

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Growth and Physiology of *Azotobacter chroococcum* in Continuous Culture

By H. DALTON* AND J. R. POSTGATE
A.R.C. Unit of Nitrogen Fixation, University of Sussex, Brighton, BN1 9QJ, Sussex

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SUMMARY

Azotobacter chroococcum (NCIB 8003) organisms, grown in continuous culture without fixed nitrogen, had chemical compositions at various dilution rates characteristic of nitrogen-limited populations. Fast-growing variants were selected for at high dilution rates; the efficiency of nitrogen fixation decreased with decreasing growth rate. In suitable media, carbon- and phosphate-limited populations were obtained and showed different compositions; they were very sensitive to inhibition by oxygen. Carbon-limited populations utilizing NH_4 under argon were not oxygen sensitive; they formed nitrogenase when they were N-limited. The chemical compositions of the various populations corresponded to theory for the nutritional state considered. Nitrogen fixation entrained a maintenance coefficient of 1·06 g. substrate/g. organism/hr compared with about 0·40 for ammonia assimilation. Assuming most of this maintenance was directed to respiratory protection of nitrogenase, an extrapolated maximum requirement of 4 moles ATP/mole N_2 fixed was observed. Attempts to repeat reports of (1) dependence of cytochrome pattern on nitrogen fixation and (2) increased efficiency of fixation with ultraviolet-irradiated N_2 were not successful with the strain of *A. chroococcum* used.

INTRODUCTION

Continuous culture is now an established technique for the study of microbial physiology. A body of work with heterotrophic aerobes such as *Klebsiella* (*Aerobacter*) *aerogenes* and *Torula utilis* in conditions of various nutrient limitations has established the profound effect of nutritional status on cell composition, enzymic activity and viability (Herbert, 1959; Malek & Fencl, 1966; Powell, 1967). The peculiar physiology of nitrogen-fixing bacteria makes them promising subjects for such studies, particularly the Azotobacteriaceae, which grow aerobically on simple media. Several papers making use of Azotobacter have been published; Malek (1952) and Macura & Kotkova (1953) investigated the morphological changes that occurred when *Azotobacter chroococcum* was cultivated in multi-stage continuous cultures; Marr & Marcus (1962) studied the kinetics of induction of mannitol dehydrogenase in carbon-limited and sulphate-limited cultures of *Azotobacter agilis*; Ierusalimsky, Zaitseva & Khmel (1962) and Khmel & Andreeva (1967) studied the behaviour of *Azotobacter vinelandii* in a variety of conditions and have determined the macromolecular composition of this organism at various growth rates; Zacharias (1963*a–c*) exposed nitrogen-fixing continuous cultures of *A. vinelandii* to u.v.-irradiated N_2 gas and observed an increase

* Present address: Department of Biological Sciences, Purdue University, Lafayette, Indiana 47907, U.S.A.

in the efficiency of nitrogen fixation (measured as mg. N fixed/g. glucose consumed); Aiba, Nagai, Nishizawa & Onodera (1967*a*, *b*) investigated the effect of carbon limitation on yield in nitrogen-fixing cultures of *A. vinelandii*, and, using Pirt's (1965) equation, obtained a value of 3·90 g. substrate/g. organism/hr for the maintenance coefficient.

These reports sometimes showed certain inconsistencies of detail and included no systematic study of the behaviour of a single strain in a variety of nutritional conditions. We report here a study of *Azotobacter chroococcum* in continuous cultures in which populations growing with and without fixed nitrogen were compared in three nutrient-limited states.

METHODS

Organism. Azotobacter chroococcum (NCIB 8003) was maintained on agar slopes of 'mannitol B' medium (Burk's medium as prescribed by Newton, Wilson & Burris (1953) with mannitol in place of sucrose) and stored at room temperature.

Growth conditions. Cultures were grown in 250 ml. chemostats of the design described by Baker (1968). Temperature was controlled automatically at 30°, and the pH value at 6·9 ± 0·1 in ammonia-grown cultures. No pH control was used with nitrogen-fixing cultures except with phosphate-limited populations, where the pH value was maintained at 6·9. A continuous culture vessel, capable of withstanding a pressure of 60 p.s.i., was constructed from Perspex (Imperial Chemical Industries Ltd.), the design and operation of which was similar to the system described by Baker (1968). Sterilization was effected by filling the vessel with 1 % β-propiolactone and leaving it overnight. It was then drained and flushed with 5 l. of sterile distilled water before admitting medium and inoculating.

Medium B_6 contained (g./l. dist. H_2O): mannitol, 10; K_2HPO_4, 0·64; KH_2PO_4, 0·16; NaCl, 0·2; $MgSO_4.7H_2O$, 0·2; $CaCl_2$, 0·1; plus these trace elements (mg./l.): $FeSO_4.7H_2O$, 2·5; H_3BO_3, 2·9; $CoSO_4.7H_2O$, 1·2; $CuSO_4.5H_2O$, 0·1; $MnCl_2.4H_2O$, 0·09; $Na_2MoO_4.2H_2O$, 2·5; $ZnSO_4.7H_2O$, 1·2; nitrilotriacetic acid, 100 (Nitrilotriacetic acid is not a source of nitrogen for these bacteria). The pH value was 7·4 ± 0·2 and the medium was autoclaved in 20 l. batches at 121° for 45 min. NH_4^+-limited medium contained the ingredients for mannitol B_6 medium plus 0·15 g./l. NH_4Cl, which was sterilized separately in concentrated solution and added later. Mannitol was decreased to 1·5 g./l. for nitrogen-fixing C-limited cultures and to 2·0 g./l. for C-limited cultures grown with NH_4Cl (1·5 g./l.). K_2HPO_4 was lowered to 0·1 mg./ml. for P-limited cultures, KH_2PO_4 was omitted and KCl (0·55 mg./ml.) was added to give an equivalent value of K^+ ion. Non-nitrogen-fixing P-limited cultures were grown with 1·5 g./l. NH_4Cl. Continuous cultures were run for at least four doubling times after a change in nutritional condition or dilution rate to allow re-establishment of a steady state; constant extinction and pH value were taken as preliminary indications of a steady state.

Cultures were accepted as limited by NH_4, mannitol or phosphate when (1) the organism concentration was halved when the concentration of nutrient in the inflowing medium was halved, (2) doubling the concentration of all other medium components did not alter the organism concentration. The first criterion proved inapplicable to the special case of nitrogen-gas limitation as discussed later in this paper. Aeration was tested by using a Clark type of oxygen electrode (Protech Advisory Services Ltd, 21

High St, Rickmansworth, Herts.) and was adequate in all the systems reported on here.

Analytical procedures. Organism concentration (mg. dry wt/ml. culture), mannitol concentration, nitrogen content, cytochrome spectra and oxygen concentration were determined by previously described methods (Dalton & Postgate, 1968). Phosphate was determined by the method of Baginski, Foa & Zak (1967) with K_2HPO_4 as standard. Protein was determined with the Folin reagent based on Lowry, Rosebrough, Farr & Randall (1951) with bovine albumin serum (Sigma Chemical Co) as standard. Poly-β-hydroxybutyrate was determined by the method of Law & Slepecky (1961) as modified by Stockdale, Ribbons & Dawes (1968) with purified polymer from Azotobacter and sodium DL-β-hydroxybutyrate as standards. Ribonucleic acid was determined by the orcinol reaction (Morse & Carter, 1949) with yeast ribonucleic acid (Sigma Chemical Co.) as standard. Deoxyribonucleic acid was estimated by the method of Burton (1956) with calf thymus deoxyribonucleic acid (Sigma Chemical Co.) as standard. Polysaccharide was determined with the anthrone reagent based on a method devised by Mr P. J. Phipps (M.R.E., Porton, Wilts.) with glucose as standard.

Nitrogenase activity. Acetylene is reduced to ethylene by nitrogenase (Dilworth, 1966; Schöllhorn & Burris, 1967), with a specific activity about three times that observed for N_2 fixation by similar preparations. Acetylene reduction thus provides a rapid assay method for nitrogenase since the ethylene produced can be detected by vapour phase chromatography. Continuous culture samples (2 ml.) were transferred to 25 ml. conical flasks, gassed with different argon:oxygen mixtures (0·01 to 0·4 atm. O_2) for 2 min., and sealed with a Suba-Seal cap. Acetylene (1 ml.), freshly prepared from calcium carbide and water, was injected into the flask which was then transferred to a water bath at 30° with a shaking rate of 25 strokes/min. The flasks were set up as rapidly as possible because prolonged manipulation caused the activity of the population to decline. Two-ml. samples were taken from the flask by first injecting 2 ml. of the appropriate argon + oxygen mixture and then withdrawing a 2 ml. gas sample with an argon-flushed hypodermic syringe and needle. The sample was analysed for hydrocarbons in a Pye 104 gas-chromatography instrument fitted with a flame ionization detector, a 5 ft. Porapak R column of 4 mm. internal diameter at 45° with N_2 as carrier gas at a flow rate of 60 ml./min. The instrument was calibrated quantitatively for ethylene by injecting measured volumes of the gas accurately diluted with argon. The concentration of ethylene was taken as proportional to the peak height.

RESULTS

Growth in nitrogen-fixing conditions

Azotobacter chroococcum was grown in medium B_6 without ammonia and steady states were established at various dilution rates. Figure 1 illustrates the influence of dilution rate on organism concentration: the dry-weight organism/ml. culture increased dramatically with decreasing growth rate. This pattern was obtained in three runs with this organism and the organism concentrations were reproducible within 10% between runs 18 months apart. The pattern was obtained in two runs with a 100 ml. culture vessel and *Azotobacter vinelandii*; Dr D. Herbert and Dr D. W. Tempest also obtained such a pattern with nitrogen-fixing *A. vinelandii* of uncertain nutrient limitation (personal communication).

The curve also differed from theoretical (see Herbert, 1959) in that wash-out was not abrupt: steady states of low cell concentration were obtained at dilution rates between 0·3 and 0·4 hr^{-1}. This phenomenon was due to selection of fast-growing variants at the high flow rates because, when continuous culture was interrupted by removing 90 % of the culture and replacing it with fresh medium, the rate of growth of the 'batch' culture so obtained increased with increasing dilution rate. The doubling time of batch cultures obtained in this manner from a population growing at $D = 0{\cdot}15\ hr^{-1}$ was 2·56 hr, identical with that of a batch culture from a slope on mannitol B_6 agar. From a dilution rate of 0·33 hr^{-1} the doubling time in a batch culture was 1·97 hr.

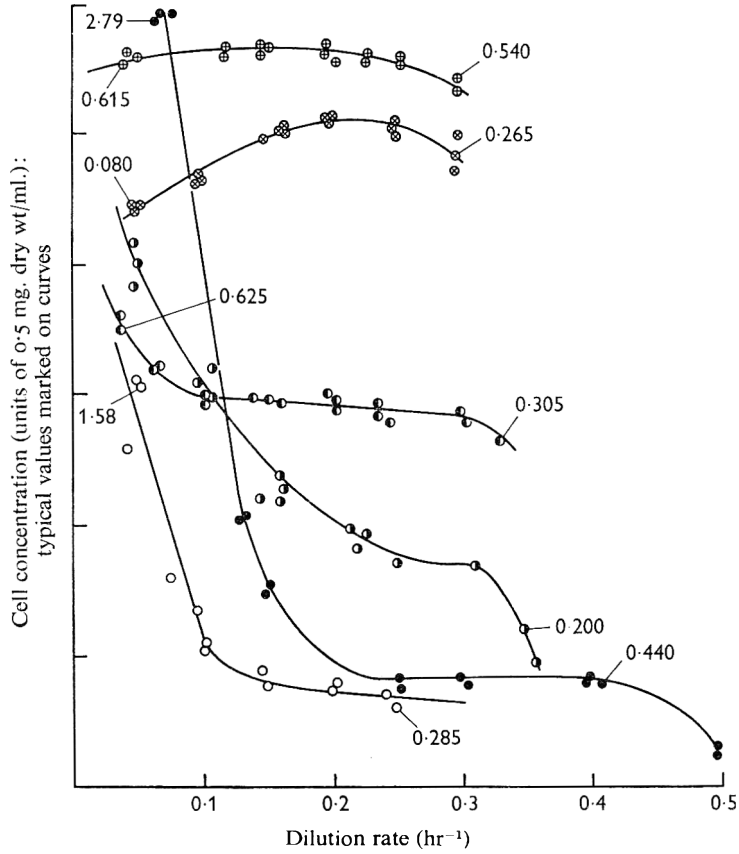

Fig. 1. Relations of cell concentration to dilution rate in continuous cultures of *Azotobacter chroococcum* of various nutritional types. Reasons for accepting the 'N_2-limited' conditions are given in the text. The ordinate is in units of 0·5 mg. dry wt organisms/ml., but the curves have been spaced out for clarity of presentation; cell concentrations at certain positions on the curves are therefore quoted. Only two series were run into the 'wash-out' stage. ◑, N_2-limited, N_2-fixing; ⊗, mannitol-limited, N_2-fixing; ○, phosphate-limited, N_2-fixing; ◐, NH_4 limited, NH_4-assimilating; ⊕, mannitol-limited, NH_4-assimilating; ●, phosphate-limited, NH_4-assimilating.

Table 1 illustrates the effect of dilution rate on macromolecular composition. Much of the increased cell mass at low growth rates was due to accumulation of polysaccharides and poly-β-hydroxybutyrate; RNA increased with increasing growth rate as it does with all other micro-organisms studied (Herbert, 1959). The efficiency of nitrogen

fixation in terms of carbon consumed increased with increasing growth rate (Table 2). Mannitol was normally detectable in samples from the culture vessel, usually in amounts exceeding 3 mg./ml.

The pattern of organism concentration against dilution rate and the presence of appreciable amounts of mannitol in the culture fluid indicated that the culture was not carbon-limited. This was confirmed by altering the mannitol concentration from 1 to 2 % at $D = 0{\cdot}1$ and $0{\cdot}2\ hr^{-1}$: no change in organism concentration occurred. Doubling the concentrations of other constituents of medium B_6 had no effect on cell concentration at these dilution rates, hence a component of the atmosphere must have

Table 1. *Selected data on macromolecular composition of Azotobacter chroococcum in continuous culture*

Values as % dry weight organism; figures in parentheses indicate upper limits.

	Dilution rate (hr^{-1})	Protein	Poly-saccharide	PHB	RNA	DNA	Sum
N-limited, N_2-fixing	0·05	39·0	19·8	28·2	0·7	2·3	96·3
	0·10	40·0	10·3	27·1	6·9	2·7	87·0
	0·22	62·0	7·8	8·1	21·0	1·9	100·8
C-limited, N_2-fixing	0·05	54·6	(3·2)	(0·4)	7·1	—	—
	0·15	58·2	6·1	—*	16·8	2·4	—
	0·20	63·7	5·4	3·1	16·8	1·8	91·3
P-limited, N_2-fixing	0·05	37·8	10·0	31·1	7·8	1·2	87·9
	0·15	63·1	12·8	9·4	8·7	3·1	97·1
	0·20	56·2	8·4	—	13·6	2·5	—
N-limited, NH_4-assimilating	0·04	63·0	18·7	12·8	5·3	—	—
	0·15	69·5	8·1	8·5	12·8	—	—
	0·20	65·1	5·4	0·0	23·4	—	—
C-limited, NH_4-assimilating	0·05	57·5	6·8	3·9	5·4	—	—
	0·15	57·1	8·9	2·6	10·2	2·0	80·8
	0·27	63·4	5·1	—	13·8	1·8	—
P-limited, NH_4-assimilating	0·05	41·2	5·2	35·0	7·1	2·1	90·6
	0·16	58·0	12·0	1·0	12·1	3·4	86·5
	0·30	68·0	4·0	0·0	15·0	—	—

* — = not done

Table 2. *Efficiency of nitrogen fixation by A. chroococcum in continuous culture*

Analyses were performed on samples from steady state growth in air; the culture was N_2-limited at all growth rates (see text).

Dilution rate (hr^{-1})	μg. N fixed/mg. mannitol consumed
0·05	12·2–13·6
0·10–0·11	17·5–18·0
0·15–0·16	28·4–30·2
0·20–0·22	37·6–39·1
0·30	36·0

been limiting. Increasing the atmospheric pO_2 to 0·42 atm. at $D = 0{\cdot}2\ hr^{-1}$ did not alter the organism concentration but the amount of mannitol consumed increased from 2·1 mg./ml. to 4·9 mg./ml.; an increased stirring rate at $pO_2 = 0{\cdot}2$ atm. similarly increased the mannitol consumption without altering the organism concentration. Oxygen electrode measurements, with electrodes calibrated for molarity of dissolved oxygen in distilled water, showed an ambient oxygen concentration in the culture of

about 20 μM; when this was increased to above 45 μM, either by vigorous stirring or high pO_2, growth of the culture was inhibited as we reported earlier (Dalton & Postgate, 1968). Hence, by elimination, nitrogen was the growth-limiting nutrient.

For reasons given in the Discussion section it is intrinsically unlikely that such physical factors as stirring rates, pN_2 and solubility of N_2, limited access of nitrogen to the organism; this possibility was nevertheless tested by growing cultures at various pN_2 values using argon as diluent and keeping the pO_2 at 0·2 atm. At $D = 0{\cdot}16\ hr^{-1}$ the organism concentration remained constant at 0·66 mg. dry wt/ml. between pN_2 values of 0·8 and 0·29, decreased to 0·61 at $pN_2 = 0{\cdot}19$ and 0·41 at $pN_2 = 0{\cdot}09$. An apparatus was designed to grow the bacteria in continuous culture under high pressures (it will not be described in detail here since the findings in this context were negative) and the steady state organism concentration was found to be constant (1·61 to 1·71 mg. dry wt/ml. at $D = 0{\cdot}06\ hr^{-1}$) at total pressures of 1·5, 2·15 and 3·2 atm. of N_2 containing sufficient O_2 to keep the ambient concentration at 17 to 20 μM (at high pressures the pO_2 required to do this was less than 0·2; when a pO_2 of 0·2 was sustained at high pressures oxygen inhibition occurred). Hence the cell concentration was independent of pN_2 from 0·3 to 3 atm. despite nitrogen being physiologically the growth-limiting substrate; the question whether the population can be properly referred to as 'nitrogen-limited' is raised in the Discussion; for the purposes of this publication we shall call them 'N_2-limited'.

K_m of continuously multiplying cells. Extrapolation of the figures relating pN_2 to organism concentration gave a K_m for nitrogen fixation by actively multiplying bacteria of 0·07 atm., higher than most recorded values for Azotobacter (0·02 to 0·05 atm.: Wilson & Roberts, 1954; Parker & Scutt, 1960), but in close agreement with Strandberg & Wilson's (1967) value of 0·066 atm. obtained with $^{15}N_2$.

Test of irradiated N_2. Zacharias (1963*a–c*) observed that u.v.-irradiated nitrogen was fixed more efficiently (in terms of carbon consumed) than non-irradiated gas by continuous cultures of *Azotobacter vinelandii*. To test for a comparable effect with *A. chroococcum* a quartz tube, 30 × 2·5 cm., was attached to the gas-inlet port and N_2 was irradiated while passing along the tube at about 200 ml./min. by a lamp emitting 30 watts at 253·7 nm set 3 mm. distant from the tube before mixing to pN_2 of 0·8 with oxygen. No significant change in efficiency of nitrogen fixation or organism concentration occurred in 2- or 3-day steady states at $D = 0{\cdot}21\ hr^{-1}$, whether the lamp was on or off.

Growth with ammonium as nitrogen source

Since nitrogen fixation was limiting growth in the cultural condition just described, comparable continuous cultures were established under an argon + oxygen mixture of pO_2 0·2 atm. with NH_4Cl as growth-limiting substrate; automatic pH control was used to compensate for acidity generated as NH_4^+ was assimilated; with 1·4 mM-NH_4Cl the cell concentration was 0·21 mg./ml. at $D = 0{\cdot}2\ hr^{-1}$ and was doubled at 2·8 mM-NH_4Cl; the former concentration was adopted to obtain the plot of organism concentration against growth rate in Fig. 1; the form of the curve is very similar to that shown by nitrogen-fixing populations. Table 1 lists representative analyses illustrating comparable trends in polysaccharide, poly-β-hydroxybutyrate and RNA contents. The ambient oxygen concentration was about 15 μM; the population did not show oxygen sensitivity of the kind reported by Dalton & Postgate (1969) for nitrogen-fixing cultures, though increasing the oxygen concentration to 52 μM caused a 25% lowering of the organism concentration at $D = 0{\cdot}105\ hr^{-1}$.

Nitrogenase content of NH_4-limited cells. Ammonium is known to prevent synthesis of nitrogenase but, since the ammonium concentration in an ammonia-limited continuous culture is vanishingly small, ammonium repression might not operate in such conditions. Figure 2 shows that whole cells assayed by the acetylene reduction technique showed 'nitrogenase' activity and illustrates that activity depended on pO_2; sufficient O_2 to allow growth and metabolism was necessary, but too much was inhibitory. Substitution of nitrogen for the argon in the gas phase of the continuous culture enhanced the cell concentration considerably, but decreased the specific 'nitrogenase' activity; the population decreased and its activity increased on returning to argon again (Fig. 3). The values used to plot Fig. 3 refer to optimal pO_2 values in the test system; these differed according to the density of the population being tested.

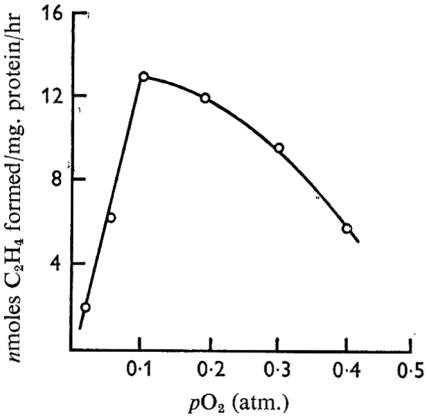

Fig. 2

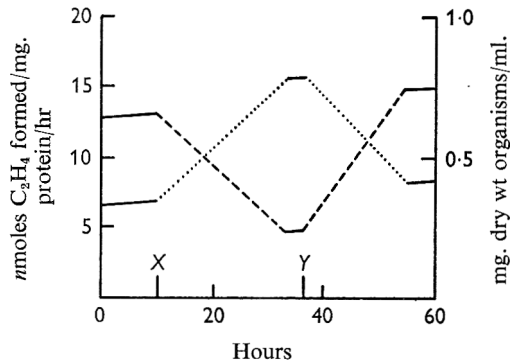

Fig. 3

Fig. 2. Acetylene reduction by *Azotobacter chroococcum* grown in NH_4-limited continuous culture under argon + oxygen: effect of pO_2. Samples from continuous culture were gassed with argon + oxygen mixtures and sealed in conical flasks, C_2H_2 was injected to start reaction. Rates of ethylene production were obtained by gas chromatography of samples at timed intervals. For details see text.

Fig. 3. 'Nitrogenase' activity of ammonium-limited *Azotobacter chroococcum* in continuous culture. An ammonium-limited continuous culture was growing under A + 20 % (v/v) O_2 in a steady state at D. The atmosphere was changed to air at *X* and back to argon + oxygen at *Y*. Ethylene production rates were then at the optimal pO_2 value for the population being tested. (See Fig. 2). ..., bacterial concentration; ---, acetylene-reducing activity.

Carbon-limited populations

Nitrogen-fixing, carbon-limited cultures. The establishment of steady states in mannitol-limited continuous cultures was difficult because of the oxygen-sensitivity reported by Dalton & Postgate (1968); inhibition of growth by over-aeration occurred readily, and was associated with formation of a black pigment. Steady states were successfully established in which the concentration of mannitol and no other component of the environment determined the cell concentration when the stirring rate was adjusted so that the dissolved oxygen did not exceed 23 μM; for all experiments reported here the stirring rates were adjusted manually to maintain the oxygen concentration at between 5 and 10 μM at a pO_2 of 0·2 atm.

Figure 1 includes a plot of bacterial concentration against dilution rate. Unlike the comparable curve for N_2-limited populations, a sharp wash-out was observed and the

yield declined dramatically at low dilution rates. Table 1 records some data on the organism composition: polysaccharide or poly-β-hydroxybutyrate were low and did not accumulate at low dilution rates; more RNA appeared the faster the cells grew. These findings are consistent with the normal behaviour of carbon-limited populations in a chemostat.

Ammonium-grown, carbon-limited cultures. No difficulty from oxygen inhibition was experienced in establishing mannitol-limited cultures under argon + 20 % (v/v) oxygen; 3 g. NH_4Cl/l. provided excess NH_4^+ in the presence of 2 g. mannitol/l.; pH control was necessary. Stirring was adjusted to produce an oxygen concentration of about 40 μM. The curve relating cell concentration to dilution rate (Fig. 1) is typical of that of a normal carbon-limited aerobic organism with little significant deviation from linearity. (The wash-out range above $D = 0{\cdot}3$ hr^{-1} was not studied.) It differed markedly from the pattern obtained when the population was fixing nitrogen. On the other hand, the data on cell composition (Table 1) show similar trends.

Phosphate-limited populations

Nitrogen-fixing, phosphate-limited cultures. Mannitol B_6 medium with K_2HPO_4 lowered but with K^+ restored to its normal molarity as described in Methods gave phosphate-limited populations according to our usual criteria. The populations showed oxygen sensitivity comparable to carbon-limited, nitrogen-fixing cultures (and again showed pigment formation when over-aerated); and the pH value tended to fluctuate because of the lower buffering power. To obtain the data plotted in Fig. 1 the dissolved oxygen was held at about 10 μM under $pO_2 = 0{\cdot}2$ atm. by manual control of the stirring rate, and automatic pH control to 6·9 was used. The pattern of organism concentration against dilution rate resembled that of N_2-limited population but Table 1 shows, as one might expect on theoretical grounds, that the increased dry weight at low dilution rates was due largely to storage of poly-β-hydroxybutyrate, not polysaccharide, which requires phosphate for its deposition. RNA showed the usual pattern.

Ammonium-grown, phosphate-limited cultures. A comparable chemostat was set up with 3 g. NH_4Cl/l. in the medium and an atmosphere of A + 20 % (v/v) O_2. It was phosphate-limited according to the usual criteria and showed no abnormal sensitivity to aeration; oxygen was held about 25 μM and pH at 6·8. Fig. 1 shows the curve relating organism concentration to dilution rate; it was essentially similar to that for nitrogen-fixing population but the yield (wt organism/wt P) was higher and wash-out did not occur until $D = 0{\cdot}5$ hr^{-1}. Table 1 illustrates that poly-β-hydroxybutyrate was again the main storage product responsible for deviation from linearity at low dilution rates.

Morphological observations

Morphological changes consistent with those reported by other workers using *Azotobacter vinelandii* were observed in all these experiments and will not be reported in detail. Generally speaking, azotobacters tended to be coccoid at dilution rates from 0·15 to about 0·3 hr^{-1}, large and bacillary from 0·3 hr^{-1} to wash-out. Below 0·15 hr^{-1} long, filamentous forms appeared and were common at 0·03 hr^{-1}. N_2-, NH_4^+- and particularly PO_4-limited populations showed pronounced clear areas under phase contrast at low dilution rates. These stained with Sudan Black and were presumably poly-β-hydroxybutyrate.

Cytochrome contents of Azotobacter

Table 3 illustrates the relative peak heights of standard suspensions of *Azotobacter chroococcum* obtained in various nutrient conditions. Peaks of cytochromes a_1, a_2, b_1, c_4 and c_5 were seen but insufficient cytochrome *o* (Jones & Redfearn, 1967) was produced to register. No evidence for an association of cytochrome content with nitrogen fixation (Lisenkova & Khmel, 1967) was obtained, though modest variations with nutritional status occurred, notably a high content of cytochromes *a* in the N-limited populations. The carbon-limited population was examined under high purity commercial A, H_2, N_2 and O_2 in an attempt to detect oxidation of the cytochrome peaks by N_2 (Wilson, 1958). None was found, though O_2 produced the theoretical oxidation of all components.

Table 3. *Cytochrome content of Azotobacter chroococcum from various nutrient limitations*

Peak heights relative to c_4+c_5 taken as 100.

			Nutrient limitation			
Cytochrome	N_2	NH_4	C (N_2-grown)	C (NH_4^+-grown)	PO_4 (N_2-grown)	PO_4(NH_4^+-grown)
a_2^{α}	31·5	31·4	6·5	16·7	11·5	7·5
a_1^{α}	22·6	18·2	6·5	6·7	1·8	1·5
b_1^{α}	93·6	90·3	78·8	76·3	80·4	74·6
b_1^{β}	63·5	67·4	59·0	61·2	64·8	63·4
$(b+c)^{\gamma}$	275·0	225·0	128·0	572·0	435·0	276·0
O.D. $(c_4+c_5)^{\alpha}$	0·266	0·258	0·279	0·214	0·190	0·206

DISCUSSION

Nitrogen-limitation in Azotobacter

Nutrient limitations in continuous cultures are usually operational characters: the type of nutrient limitation (carbon/energy, nitrogen source, sulphur source etc.) is imposed by the experimenter and determined by (1) dependence of cell concentration on the concentration of growth-limiting substrate, and (2) independence of cell concentration from moderate changes in the concentration of any other substrate. When the growth-limiting nutrient is relatively insoluble (e.g. with oxygen) the first criterion applies to the concentration of dissolved substrate; such limitations can be detected empirically if, when the flow of liquid medium is discontinued, the cell concentration increases. *Azotobacter chroococcum* growing in mannitol B_6 medium showed the characteristics of an organism limited by N_2: independence of other components, increasing cell density with decreasing dilution rate, continuation of growth when medium flow stopped. But if the first criterion is applied strictly, difficulties arise. At $D = 0{\cdot}2\ hr^{-1}$, for example, the oxygen solution rate was about 70 mmole O_2/l./hr, which, allowing the pN_2 and solubility of N_2, is equivalent to a nitrogen solution rate of 140 mmole N_2/l./hr. From the nitrogen content of the culture the nitrogen fixation rate was about 0·67 mmole N_2/l./hr, so obviously physical access of N_2 to the bacteria was not limiting and this fact is reflected in the K_m value of 0·07 atm. obtained here. It follows that some intrinsic property of the organisms leads to N_2-limitation at all

growth rates tested in the routine medium. This situation is probably also true of *A. vinelandii* and can be extended to the majority of batch cultures of either species. These, in conventional media, will normally be N_2-limited until they reach a sufficient density to become oxygen-limited. Daesch & Mortenson (1968) described continuous cultures of *Clostridium pasteurianum* in which growth continued when the flow of medium ceased which, though they did not interpret their results in this way, is evidence that they were N_2-limited in the sense used here.

We have no information on the physiological 'bottleneck' which leads to N_2-limitation in these organisms, but it is clearly of survival value since carbon- and phosphate-limitations lead, when nitrogen is being fixed, to excessive sensitivity to oxygen. Dalton & Postgate (1968) presented evidence that the oxygen sensitivity of nitrogen-fixing Azotobacter, which is almost absent from ammonia-grown populations but which is most marked in carbon- and phosphate-limitation, is an expression of respiratory protection of nitrogenase. Nitrogenase was regarded as damaged or inactivated by oxygen, but the organism's respiration normally scavenges oxygen and keeps it away from the enzyme. Thus the sensitivity of nitrogen-fixing Azotobacter depends upon the population's capacity to respire oxygen away; this will be high with nitrogen-limited populations because of the excess of carbon substrate available; it will be low with carbon-limited populations and may be so with phosphate-limited populations. Thus the carbon-limited populations which we obtained were probably in a state of fine balance between nitrogen limitation and oxygen inhibition; the physiological meaning of the term 'carbon-limited' becomes doubtful when limitation by one metabolic patterning (C) may lead to intoxication of a second pathway (N) by a substrate for the third (O).

Maintenance coefficients in Azotobacter

Deviations from linearity in curves relating yields to dilution rates of carbon-limited continuous cultures are attributed to 'maintenance' factors (Herbert, 1959). From Fig. 1 it is clear that the 'maintenance coefficient' (Powell, 1967) of carbon-limited, nitrogen-fixing populations of *Azotobacter chroococcum* is dramatically different from that of comparable ammonia-utilizing organisms. Plots of $1/Y$ against $1/D$ (Pirt, 1965) from the data in Fig. 1 gave a maintenance coefficient of 1·06 g. mannitol/g. dry. wt organism/hr when fixing nitrogen compared with about 0·04 for ammonia-grown cells and with values of 0.055 for *Escherichia coli* (Schulze & Lipe, 1964) and 0·094 for *Aerobacter cloacae* (Pirt, 1965). Aiba *et al.* (1967*a*) reported a value of 3·90 for *A. vinelandii*. These very large maintenance coefficients are presumably mainly expressions of 'respiratory protection': carbon substrate diverted to prevent access of oxygen to the nitrogenase system.

ATP consumed in nitrogen fixation

At a given growth rate, the yield of carbon-limited continuous cultures fixing N_2 differed from the yield of one using NH_4^+ by an amount representing two factors: the carbon substrate expended in respiratory protection and that required to generate ATP specially for nitrogen fixation. This difference decreased with increasing growth rate, as would be expected if the need for respiratory protection declined. By plotting the reciprocal of the difference in yield against the reciprocal of the dilution rate and extrapolating to a hypothetical 'infinite' growth rate, a yield difference corresponding

to minimum respiratory protection can be obtained which, using the widely accepted value of 10·5 for Y_{ATP} (Bauchop & Elsden, 1960), may be translated into ATP available for nitrogen fixation. Extrapolation of this kind gave a value of between 4 and 5 moles ATP available/mole N_2 fixed. Cell-free preparations consume about 15 moles ATP/mole N_2 (Silver, 1967); it follows that growing organisms are much more efficient in their ATP economy. This calculation errs on the side of over-estimation, because Y_{ATP} is not likely to be much less than 10·5 and may well be greater with an aerobe; moveover, extrapolation used assumes that the whole extrapolated yield difference represents ATP for nitrogen fixation and some may not. The value of 4 to 5 mole ATP/mole N_2 with Azotobacter contrasts with about 30 mole ATP/mole N_2 reported by Daesch & Mortenson (1968) for *Clostridium pasteurianum.*

Efficiency of nitrogen fixation. The efficiency of nitrogen fixation is sometimes expressed as mg. N fixed/g. carbon source consumed, and Jensen (1953), on the basis of batch culture analyses indicating efficiencies of 10 to 15 mg. N/g., decided that the contribution of free living Azotobacter to the soil economy was negligible since carbon sources capable of supporting significant nitrogen fixation in agricultural terms were not available. However, in chemostat culture, particularly at low pO_2 values, efficiencies of 30 to 40 mg. N fixed/g. carbon were obtained, which may be compared with the efficiency of incorporation of ammonia by a normal heterotroph (*Aerobacter aerogenes* incorporated about 75 mg. N/g. glycerol, calculated from data of Postgate & Hunter, 1962). Soil is not always well aerated and its micro-environments generally resemble more closely nutrient-limited chemostats than laboratory batch cultures; it may be that the contributions of Azotobacter to soil fertility have been under-estimated.

Characteristics of Azotobacter in continuous culture. The dependence of chemical composition on nutritional status which is general among micro-organisms (Herbert, 1961) followed the usual lines once the condition of N_2-limitation in 'ordinary' culture was recognized. N-limitation, via either N_2 or NH_4^+, leads to increased synthesis of non-nitrogenous polymers at low growth rates and the steady state cell concentration increased with decreasing growth rate. RNA increased with growth rate in all conditions of nutrient limitation. DNA/cell remained constant within experimental error; protein varied consistently with the proportion of other polymers.

Selection of mutants of high growth rate during 'wash-out' was originally considered an improbable event (Herbert, Elsworth & Telling, 1956) but there is now evidence that continuous culture at low growth rate favours the selection of variants of low μmax; the fast-growing variants obtained here may thus have originated by a reverse process.

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Release of Hydrolytic Enzymes from Cytoplasmic Particles of Solanum Tuber Tissues During Infection by Tuber-rotting Fungi

By D. PITT AND CLARE COOMBES
Department of Botany, University of Exeter, Exeter, Devon

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SUMMARY

Histochemical evidence showed that infection of tuber tissues and tissue-culture cells of *Solanum tuberosum* by *Phytophthora erythroseptica*, *Phytophthora infestans* and *Fusarium caeruleum* caused swelling and disruption of host cytoplasmic particles containing acid phosphatase, esterases and proteases. Heavy diffuse cytoplasmic staining for acid phosphatase was a consistent feature of infection by all three fungi, but staining reactions for esterases and proteases showed much less diffuse staining and a lesser degree of particle swelling. Biochemical assays showed that acid phosphatase was liberated from the particulate fraction to the supernatant fluid fraction of infected callus cells; acid ribonuclease behaved similarly. The biochemical evidence suggested that esterase activity of infected cells remained associated with a sedimentable fraction from cell homogenates. Assay methods for proteolytic enzymes did not confirm the histochemical evidence of a particulate localization of these enzymes in healthy or infected callus tissues. An excess recovery of ribonuclease from tissues infected with *P. infestans* and *F. caeruleum* was found; the significance of this is discussed. Attempts to separate a particulate fraction rich in hydrolases from homogenates of healthy or infected potato tubers were unsuccessful.

INTRODUCTION

The spherosomes of plant cells have affinities with the lysosomes of animal cells (Gahan, 1965; Matile, Balz, Semadeni & Jost, 1965; Walek-Czernecka, 1965; Pitt, 1968). Balz (1966) and Semadeni (1967) have provided convincing evidence that the spherosomes represent organelles equivalent to the lysosomes of animal cells. Although lysosomes have been implicated in a number of cellular processes (de Duve, 1963) particular attention has been directed to their role in animal cell pathology (Allison & Mallucci, 1965; Mallucci & Allison, 1965; Bernheimer & Schwartz, 1964; Johnson, 1968). Histochemical observations (Pitt & Coombes, 1968) have shown that during infection of Solanum tissues by *Phytophthora erythroseptica* there was swelling and disruption of lysosome-like host-cell particles, accompanied by increased diffuse cytoplasmic staining for acid phosphatase and esterase. The present work adopts a primarily biochemical approach complemented with relevant histochemical observations to discover whether lysosomal damage occurring as a symptom of pink-rot disease of potato tubers, revealed by predominantly histochemical methods in earlier work (Pitt & Coombes, 1968), is a general feature of infection of potato tubers and callus tissues by other tuber-rotting fungi.

METHODS

Infecting agents. The isolate of *Phytophthora erythroseptica* Pethybr. was that used earlier (Pitt & Coombes, 1968). *Fusarium caeruleum* (Lib) Sacc. was isolated from a tuber of *Solanum tuberosum* cv. Arran Pilot showing symptoms of dry-rot disease. The culture of *Phytophthora infestans* (Mont.) de Bary, Race 1:4, was kindly supplied by Professor N. F. Robertson (University of Hull).

Solanum. Potato tubers cv. Majestic were grown from the stock used in earlier experiments (Pitt & Coombes, 1968), and callus cultures were obtained and maintained by the methods of Ingram & Robertson (1965).

Inoculation of callus cultures and tubers. Callus tissues were grown as previously reported (Pitt & Coombes, 1968) and inoculated with a disc of the appropriate fungal culture. Cultures of *Phytophthora erythroseptica* and *Fusarium caeruleum* were grown on malt extract agar and those of *P. infestans* on sliced green-bean agar (Ingram & Robertson, 1965). Potato tubers were wound-inoculated with discs of agar cultures of *P. erythroseptica* or *F. caeruleum*, then wrapped in greaseproof paper and incubated in polythene bags at 20° for 1 week and 4 weeks, respectively, before examination. Half tubers were inoculated with sporangial suspensions derived from plate cultures of *P. infestans* and incubated in damp chambers for 3 to 4 days.

Microscopy. Observations were made with a Leitz Ortholux microscope equipped as previously described (Pitt & Coombes, 1968).

Histochemical methods

Tissue culture material or tuber tissues were fixed in Baker's formol-calcium or neutral formalin for 16 hr at 0° to 4°, washed in distilled water and sectioned, where appropriate, on a freezing microtome at 25 μ to 50 μ. All observations were repeated with fresh callus tissues and fresh hand-cut tuber sections. Tests for proteolytic enzymes were done on unfixed material only.

Acid phosphatase. The standard coupling azo dye method of Grogg & Pearse (1952) was normally used, with results checked by other methods (Pitt & Coombes, 1968).

Esterases. Methods included those used in earlier work (Pitt & Coombes, 1968), with α-naphthyl acetate coupled with Fast blue B salt (G. T. Gurr, Ltd.) as the routine procedure.

Proteolytic enzymes. Leucine aminopeptidase activity was located in fresh hand-cut sections of healthy and infected potato tuber tissues and in unfixed tissue culture cells by the method of Nachlas, Crawford & Seligman (1957) with L-leucyl-β-naphthylamide (Koch-Light Laboratories Ltd.) as a substrate and incubation times of 60 to 90 min. at 37°. This method was also used with some of the artificial substrates proposed by Nesvadba (1962), which included tyrosine-β-naphthylamide (Koch-Light. Ltd.) and glycyl-β-naphthylamide hydrochloride (Koch-Light, Ltd.).

Biochemical methods

Attempts were made to prepare a lysosomal fraction from fresh potato tuber tissue by grinding tissues cooled to 0° in a mortar with washed sand and ice-cold 0·25 M-sucrose. The homogenate was filtered through cheese cloth, centrifuged for 10 min, at 2000 *g* at 0°, the sediment discarded and the supernatant fluid further centrifuged for 20 min. at 35,000 *g* at 0°. Enzyme assays showed high acid phosphatase, esterase

and ribonuclease (RNase) activities in the supernatant fluid fractions from healthy or diseased tubers, but negligible activity in the particulate fractions. Because of the frequent difficulties encountered in the preparation of active sub-cellular particulate fractions from storage tissues many modifications were made to the extraction medium; these included the addition of tris buffer (pH 7·1), 1 mM-EDTA, 0·001 M-cysteine hydrochloride and 0·001 M-ascorbic acid, but all lysosomal fractions were low in hydrolase activity. No protease activity was found in either the supernatant fluid or particulate fractions with natural or artificial substrates with an extraction medium of 0·25 M-sucrose with or without tris buffer (pH 7·1). Since all efforts to isolate an active lysosomal fraction from potato tubers by using a range of sucrose concentrations up to 0·5 M failed, the biochemical methods were applied to homogenates of healthy and infected callus tissues from which lysosomal fractions rich in hydrolase activity were readily prepared. Healthy and infected callus tissues were homogenized, after appropriate incubation periods, by the technique of Pitt & Coombes (1968) whereby minimum disruption of the fungal component occurred. The progress of infections was followed microscopically and infected and control calluses were harvested and homogenized separately for 4 min. in 0·25 M-sucrose by using a Pyrex glass homogenizer rotating at approximately 200 rev./min. at 0° to 4°. Experiments with tissues infected with *Phytophthora erythroseptica* and *P. infestans* showed that centrifugation of the homogenate for 10 min. at 2000 ***g*** at 0° deposited most of the unbroken cells, nuclei, cell debris and partially broken fungal mycelium. The deposit was discarded and the supernatant fluid was further centrifuged for 20 min. at 35,000 ***g*** at 0° to give a clear supernatant fluid and a pellet consisting of a homogeneous particulate fraction rich in mitochondria and lysosomes. It was necessary with homogenates derived from calluses infected with *Fusarium caeruleum* to give a first centrifugation for 10 min. at 3000 ***g*** to sediment out the smaller mycelial fragments arising from this septate mycelium. Attempts to improve the stability of the lysosomal component of tissues by homogenizing in 0·25 M-sucrose containing 1 mM-EDTA and buffered at pH 7·1 with 0·1 M-tris buffer were unsuccessful. Consequently all cell fractionations of tissue culture material were made in 0·25 M-sucrose. The pellet from the second centrifugation was resuspended and made up to volume with 0·25 M-sucrose at 0° and enzyme determinations done on the supernatant fluid and lysosomal fractions as follows.

Acid phosphatase. The method of Berthet & de Duve (1951) was used as described by Pitt & Coombes (1968), with β-glycerophosphate as the substrate and an incubation time of 20 min.

Esterase. The method was that of Nachlas & Seligman (1949), in which enzyme preparations were incubated with α-naphthyl acetate in 0·05 M-veronal buffer (pH 7·4) for 20 min. at 20°. An ice-cold solution of Fast blue B salt was added with shaking followed by the addition of trichloroacetic acid to 1 % (w/v). The coloured complex was then extracted with ethyl acetate, the organic layer separated after centrifugation at 1000 ***g*** for 5 min., and extinction readings made at 520 nm. A standard curve was obtained by using known concentrations of α-naphthol coupled with Fast blue B salt and subjected to the above procedure.

Acid ribonuclease: 0·5 ml. of 0·1 M-acetate buffer (pH 5·0) + 0·05 ml. of 1 % (w/v) yeast RNA (highly polymerized; British Drug Houses) + 1 ml. of enzyme preparation were incubated for 1 hr at 37°. The reaction was stopped by adding 1·5 ml. of 0·25 %

(w/v) uranyl acetate in 4 % (w/v) perchloric acid. The precipitated RNA was removed by centrifugation for 10 min. at 1000 *g* and the supernatant fluid was diluted 1/4 with distilled water. The differences in extinctions at 260 nm between incubated and non-incubated samples were corrected for non-enzymic degradation of RNA.

Acid deoxyribonuclease (DNase). The method for RNase was used but with 0·5 % (w/v) calf thymus DNA (highly polymerized, British Drug Houses) replacing RNA as substrate.

Protease. All attempts to assay aminopeptidase activity by the hydrolysis of artificial substrates, including β-leucyl, β-glycyl or β-tyrosylnaphthylamide as proposed by Nesvadba (1962), were unsuccessful over a wide range of pH values and in the presence of several known activators of these enzymes. Similarly no protease activity was found in cell fractions by using natural substrates (denatured haemoglobin, casein, egg albumin). The phenol colour method and the procedures found successful by Matile (1964) and Matile *et al.* (1965), who worked with Neurospora and lysosomal fractions from corn and tobacco seedlings, respectively, were used.

Protein determinations. Soluble proteins in enzyme preparations were determined colorimetrically with the Folin phenol reagent by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Total hydrolase activities of Solanum callus cells were determined after homogenisation in 0·25 M-sucrose containing 1 % Triton X-100 or after freezing and thawing the calluses several times before homogenization. By using such determinations for comparison it was found that about 30 % of acid phosphatase, 40 % of esterase and 70 % of RNase activities were sedimentable by the fractionation procedure adopted. Experiments showed that the enzyme activity of cell fractions from calluses of different ages varied appreciably; but several assays on calluses of similar size and age and on the same callus showed agreement within ± 10 %. To minimize such variation, calluses of the same age and size were used for acid phosphatase and esterase determinations, since at least 2 to 3 g. fresh weight of tissue were required for each determination and it was not possible to obtain regularly calluses of 5 to 6 g. fresh weight to permit sampling of healthy and infected tissue from the same callus. Furthermore, the rate of spread of a fungal pathogen through a callus varies and permits only an arbitrary assessment of the extent of infection. However, since each RNase assay required about 1 g. fresh weight of callus tissue it was possible to obtain at least three samples from each callus during the course of infection, thus eliminating some variation between calluses.

Staining reactions of infected and uninfected tissues of Solanum

Fixed callus cells and tuber sections of healthy and infected potato tissues were stained for acid phosphatase and esterase. All observations were repeated with unfixed tissues. Since the method for aminopeptidase is only applicable to living material observations were confined to fresh tissues.

Acid phosphatase. Uninfected tuber tissues and tissue culture cells, either fixed or unfixed, contained numerous particles which stained brown by the standard coupling azo dye method with incubation for 15 min. at 37°. Staining of fresh and fixed callus tissues and tuber sections revealed that infection by *Phytophthora erythroseptica*,

P. infestans and *Fusarium caeruleum* resulted in swelling and disruption of host cell lysosomes, accompanied by greatly increased cytoplasmic staining. The time sequence of swelling and disruption of these particles varied with the pathogens used. Thus, callus cells infected with *P. erythroseptica* showed maximum particle swelling and diffuse cytoplasmic staining 3 to 4 days after infection. Infection with *P. infestans* and *F. caeruleum* produced comparable reactions at 5 and 7 days after inoculation, respectively. The spread of the pathogens within tubers was also variable with *P. erythroseptica* and *P. infestans*, having comparable effects on host cell lysosomes 1 week after inoculation, whereas similar effects were generally detectable 4 to 6 weeks after tuber infection with *F. caeruleum*.

Esterase. The responses to esterase staining of living and fixed callus cells and tuber sections showed that uninfected tissues contained small discrete particles which stained black when using a substrate of α-naphthyl acetate accompanied by negligible cytoplasmic staining. Esterase staining of tissue culture cells and tuber tissues infected with *Fusarium caeruleum* and *Phytophthora infestans* gave results substantially similar to those reported by Pitt & Coombes (1968) following infection of potato tuber tissues and callus cells with *P. erythroseptica*. Such staining of fixed and fresh tissues showed swelling of host-cell lysosomes to two or three times their size in healthy tissues, accompanied by a small but definite increase in diffuse cytoplasmic staining. The time sequence of swelling and disruption of infected tissues closely followed that found for the various pathogens by using the acid phosphatase staining procedure.

Aminopeptidase. The response of fresh callus cells and tuber sections to staining for aminopeptidases indicated that such activity in healthy tissues was largely confined to cytoplasmic particles similar in size, shape and distribution to the lysosome-like particles containing acid phosphatase, esterases and β-D-galactosidase reported by Pitt & Coombes (1968). Infection of callus tissue and potato tubers by all three pathogens resulted in responses similar to those observed by staining for esterase and acid phosphatase.

Enzyme assays of cell fractions of infected and healthy tissue culture cells of Solanum

The histochemical results indicated that all three pathogens caused disruption of the lysosome-like particles within the host cytoplasm. Confirmation that such disruption resulted in the liberation of considerable amounts of the hydrolases was obtained by enzyme assays for acid phosphatase and acid RNase of the supernatant fluid (S) and the particulate (P) fraction of healthy and infected callus tissue cell homogenates. Table 1 shows that acid phosphatase activity of the particulate fractions of callus tissues infected by all three pathogens was appreciably less than for the appropriate control. Infection by *Phytophthora infestans* and *Fusarium caeruleum* resulted in large increases in soluble enzyme activity. Such an increase was less pronounced following infection by *P. erythroseptica*.

The results in Table 2 conflict with the histochemical findings in showing increases in the esterase activities of particulate fractions derived from infected cells. These results are discussed later.

Although there is no suitable method for locating RNase activity, there is abundant biochemical evidence that this enzyme, along with DNase, is associated with animal cell lysosomes, and Matile *et al.* (1965) showed biochemically that RNase is associated with the particulate fraction from corn and tobacco seedlings. Table 3 shows that

RNase activity of callus cells was associated with the particulate fraction and was liberated to the supernatant fluid in infected tissues. The high RNase activities of the cell fractions permitted the use of small quantities of tissue for each assay, thus allowing three enzyme determinations to be made for each host/parasite combination on

Table 1. *Solanum callus tissue infected with Phytophthora erythroseptica, P. infestans or Fusarium caeruleum*

Acid phosphatase activity in particulate (P) and supernatant fluid (S) fractions of potato callus tissue.

	Activity of fractions. Inorganic phosphate released (μg. P_i/20 min./mg. protein)		Total activity (P+S fractions) in fractions (%)	
Treatment	P	S	P	S
P. erythroseptica				
Uninfected	3·073	2·300	57·19	42·81
4-Day infected	1·040	2·314	31·01	68·99
P. infestans				
Uninfected	5·259	3·900	57·42	42·58
$5^1/_2$-Day infected	2·600	8·971	22·48	77·52
F. caeruleum				
Uninfected	3·055	1·430	68·11	31·89
$4^1/_2$-Day infected	1·985	4·875	28·94	71·06

Table 2. *Solanum callus tissue infected with Phytophthora erythroseptica, P. infestans or Fusarium caeruleum*

Esterase activity in particulate (P) and supernatant fluid (S) fractions of potato callus tissue.

	Activity of fractions. α-naphthol liberated (mg. α-naphthol/20 min./mg. protein)		Total activity (P+S fractions) in fractions (%)	
Treatment	P	S	P	S
P. erythroseptica				
Uninfected	0·115	0·328	26·04	73·96
3-Day infected	0·380	0·256	59·77	40·23
P. infestans				
Uninfected	0·121	0·392	23·63	76·37
5-Day infected	0·250	0·583	30·00	70·00
F. caeruleum				
Uninfected	0·126	0·386	24·57	75·43
$4^1/_2$-Day infected	0·234	0·347	40·29	59·71

tissues derived from the same callus. The third observation in each series shows a decline in the activities of the supernatant fluid fractions at late stages of infection. The results in Tables 1, 2 and 3 are typical of many similar experiments. The methods used did not show a particulate localization of DNase activity, although some activity was found in the supernatant fluid fractions of healthy and infected cells. No protease activity was found in the particulate or supernatant fluid fractions of healthy or infected callus cells.

Table 3. *Solanum callus tissue infected with Phytophthora erythroseptica, P. infestans or Fusarium caeruleum*

RNase activity in particulate (P) and supernatant fluid (S) fractions of potato callus tissue

Treatment	Enzyme activity of fractions. Change in extinction/hr/mg. protein	
	P	S
P. erythroseptica		
Uninfected	1·376	0·890
5-Day infected	1·301	1·244
7-Day infected	0·301	0·373
P. infestans		
Uninfected	1·630	0·901
4-Day infected	1·484	3·20
6-Day infected	1·230	1·310
F. caeruleum		
Uninfected	1·298	0·838
6-Day infected	1·110	5·146
7-Day infected	1·010	4·206

DISCUSSION

It is well established (Gahan, 1965, 1967; Matile *et al.* 1965; Walek-Czernecka, 1965) that higher plant cells contain organelles having affinities with the lysosomes of animal cells. Balz (1966) and Semadeni (1967) considered that the spherosomes of plant cells are equivalent to animal cell lysosomes. The present histochemical observations confirm the view of Pitt & Coombes (1968) that infection of potato tuber tissues by *Phytophthora erythroseptica* results in the liberation of lysosomal enzymes (and extends this concept to other lysosomal enzymes) during infection of potato tissues by other tuber-rotting fungi. Substantial biochemical evidence now supports these observations and shows a decrease in acid phosphatase and RNase activities in the particulate fractions of infected cell homogenates accompanied by increases in the activities of these enzymes in the supernatant fluid fractions. However, infections of callus cells by *P. infestans* and *Fusarium caeruleum* resulted in a massive increase in RNase activity within the supernatant fluid fractions amounting to an excess recovery of the enzyme as determined from assays of total enzyme activity in healthy callus cells. Such excess recovery of lysosomal hydrolases following homogenization of plant tissues was reported by Matile *et al.* (1965), but in the present work there is a possibility that some of this activity in the soluble phase may have arisen from fungal secretions. Furthermore, it is likely that at least a part of the enzyme activity of the particulate fraction may have arisen through release during homogenization of some fungal lysosomes, which are known to contain enzymes similar to those of higher plant lysosomes (Pitt, 1968). But, since the RNase determinations for each host/parasite combination were done on the same callus and since a gradual decrease in enzyme activity of the particulate fractions still occurred with progressive infections, it is clear that there was a net loss of such activity from the particulate fractions. This indicated that any fungal contribution to the activity of the latter was smaller than the amount released from the host lysosomes.

The esterase activity of the particulate fractions of infected cells was greater than

that of the healthy controls, suggesting the possibility that this enzyme was not released during infection. This was not supported by the histochemical evidence, although there is always the possibility that some of the remaining esterase activity of infected cells may have been within particles which were not resolved by the microscopic methods used. However, the behaviour of lysosomal esterase during cell fractionation (Matile *et al.* 1965) and during lysosomal staining (Holt, 1963) is a matter of conjecture. It has been suggested that lysosomal esterase may not be liberated during lysosomal damage since it is a structural component of the lysosome, or alternatively that it is readily released and becomes attached to other cell particles of different densities which would affect its sedimentability. Furthermore, hydrolysis of the α-naphthyl acetate substrate by lipase, which is not believed to be a lysosomal enzyme, could explain the soluble esterase activity in healthy tissues, since according to Matile *et al.* (1965) esterase does not occur in a free form, at least in corn and tobacco. Thus there is no satisfactory explanation for the distribution of esterase activity in infected Solanum tuber cells at the present time. Elucidation awaits the use of differential substrates and gradient-density techniques.

The failure to detect proteolytic activity using a wide range of conditions and substrates is surprising in the light of the excellent histochemical localization obtained in callus tissues and tuber sections, and the biochemical evidence for a particulate distribution shown by Matile *et al.* (1965), although it is possible (Matile *et al.* 1965) that inhibitors of acid protease and possibly other hydrolases gain access to such enzymes during the cell fractionation procedure.

Thus the histochemical evidence from infected Solanum tubers and the biochemical and histochemical evidence from callus tissues strongly indicate that infection by a number of tuber-rotting fungi results in the release of lysosomal enzymes from host plant lysosomes. Whether such infections involve *de novo* synthesis of such enzymes by the host or the pathogen or merely a redistribution of preformed enzymes is difficult to determine in the presence of an indeterminate and continuously increasing fungal component which cannot be separated from the infected host tissues. The present studies, as with many of the observations on animal tissues (Mallucci & Allison, 1965; Ballard & Holt, 1968), do not indicate whether disruption of such particles is a primary cause of cell death. However, Weissman, Kaiser & Bernheimer (1963) showed that streptococcal haemolytic toxins caused release of lysosomal enzymes from granular fractions of rabbit tissues, and that this preceded liberation of malic dehydrogenase from the mitochondria within such preparations. Similar observations made on intact Solanum cells would be necessary to confirm a primary role of lysosomal damage in tissue infection and cell damage.

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Effects of Inhibitors of Sterol Synthesis on Growth of Sordaria and Phytophthora

By C. G. ELLIOTT
Botany Department, University of Glasgow, Glasgow W.2

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SUMMARY

Four hypocholesteremic compounds, SKF 3301-A, SKF 525-A, SKF 16467-A and AY 9944, inhibited the growth of *Sordaria fimicola*; SKF 2314, SKF 7732-A_3 and SKF 7997-A_3 did not. All seven compounds inhibited the growth of *Phytophthora cactorum*. The inhibition of growth of *S. fimicola* by SKF 3301-A and AY 9944 was annulled by certain unsaturated fatty acids but not by sterols; with *P. cactorum* the inhibition was annulled by oleic acid + cholesterol.

With *Sordaria fimicola* sub-inhibitory concentrations of SKF 3301-A caused growth which showed two phases, distinguished by an abrupt change in growth rate. At the position of the hyphal front when growth rate changed a ring of perithecia was formed. During the second period, when growth was slower, perithecia were formed in rings. The number of perithecia formed could be at least twice that in the drug-free controls. With AY 9944 no perithecia were produced at concentrations sub-inhibitory to vegetative growth. On media with SKF 3301-A or AY 9944 and oleic acid, few perithecia were formed despite good vegetative growth; with SKF 3301-A, but not AY 9944, the addition of cholesterol as well increased perithecial production.

INTRODUCTION

Sterols have been shown to be required for sexual reproduction in the oomycetes (Hendrix, 1964; Haskins, Tulloch & Micetich, 1964; Elliott, Hendrie, Knights & Parker, 1964; Leal, Friend & Holliday, 1964), and they are also required for the production of normal sporangia and zoospores (Chee & Turner, 1965; Hendrix, 1965). Sterols do not appear to be essential for vegetative growth, which can take place in sterol-free medium, although these fungi appear not to synthesize them (Elliott *et al.* 1964; Hendrix, 1966; Schlosser & Gottlieb, 1966). The role of sterols in their metabolism can thus be studied simply by adding the sterol to the medium.

The higher fungi, however, synthesize sterols, and it was thought that some indication of the function of sterols here might be obtained by the use of compounds which inhibit sterol synthesis (hypocholesteremic compounds). If sterols have a specific role in reproduction, these compounds would perhaps have different effects on vegetative and reproductive growth. The ascomycete *Sordaria fimicola* was chosen for such an investigation. It is homothallic and forms perithecia readily but has no asexual spores; thus attention is more readily directed to effects on sexual stages. It was confirmed that this organism synthesizes sterols (B. A. Knights & C. G. Elliott, unpublished

observations). The effect of the compounds on the oomycete *Phytophthora cactorum* was studied for comparison.

The hypocholesteremic compounds tested were β-diethylaminoethyl-diphenyl-propyl acetate hydrochloride (SKF 525-A); 2,2-diphenyl-1-(β-dimethylaminoethoxy)-pentane hydrochloride (SKF 3301-A); 2,2-diphenyl-pentanoic acid (SKF 2314);

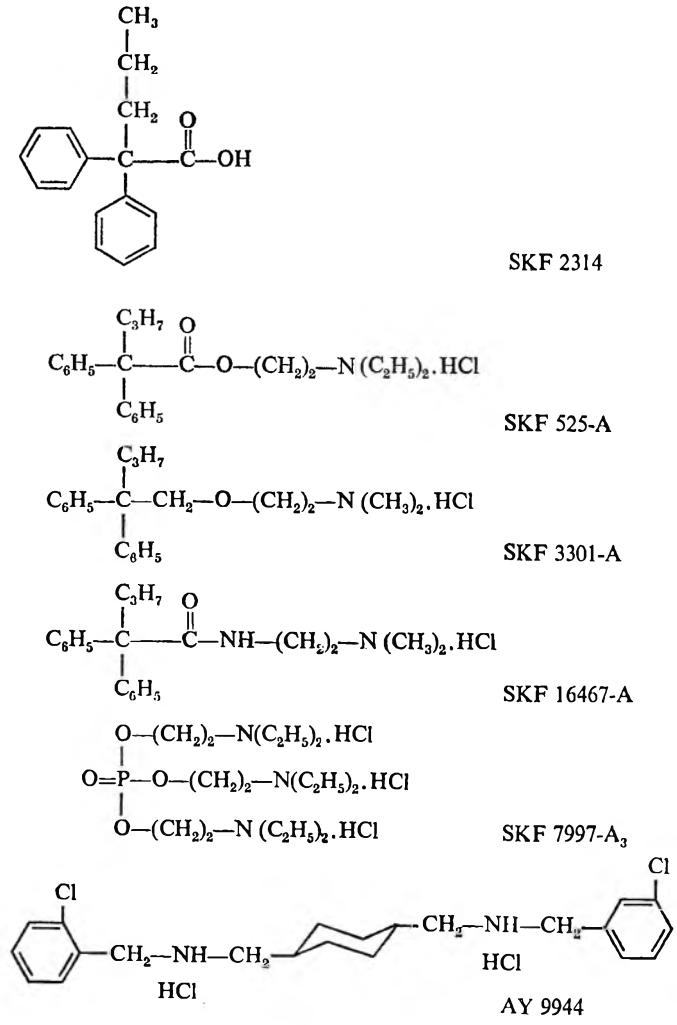

Fig. 1. Structure of hypocholesteremic compounds tested.

N-dimethylaminoethyl-α,α-diphenyl valeramide hydrochloride (SKF 16467-A); tris-(2-dimethylaminoethyl)-phosphate trihydrochloride (SKF 7732-A_3) and its diethyl analogue (SKF 7997-A_3); *trans*-1,4-bis(2-chlorobenzylaminoethyl)cyclohexane dihydrochloride (AY 9944). The structure of these compounds is shown in Fig. 1. Work on mammalian tissue has shown that SKF 525-A and SKF 3301-A act before the formation of squalene (Holmes & Bentz, 1960; Holmes & Di Tullio, 1962). SKF

7732-A_3 and SKF 7997-A_3 principally inhibit the conversion of lanosterol to zymosterol (Holmes & Di Tullio, 1962). AY 9944 inhibits the saturation of the double bond at position 7; 7-dehydrocholesterol cannot be converted to cholesterol (Chappel *et al.* 1964; Horlick, 1966). It has also been shown that SKF 7997-A_3 inhibits sterol synthesis in the flowering plant *Xanthium pensylvanicum* (Bonner, Heftmann & Zeevaart, 1963).

METHODS

Fungi. The strain of *Sordaria fimicola* used was obtained from Professor C. T. Ingold, Birkbeck College, London. The strain of *Phytophthora cactorum* was IMI 21168, obtained from the Commonwealth Mycological Institute, Kew.

Media. Sordaria fimicola was grown on a medium containing: glucose, 2·5 g.; $NaNO_3$, 1·0 g.; KH_2PO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·25 g.; trace element solution, 1 ml.; thiamine hydrocholoride, 2 mg.; biotin, 4 μg.; water, 1 l. *Phytophthora cactorum* was grown on a medium containing: sucrose, 10 g.; L-asparagine, 1·0 g.; K_2HPO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·25 g.; trace element solution, 1 ml.; thiamine hydrochloride, 1 mg.; water, 1 l. The trace element solution contained: $Na_2B_4O_7.10\,H_2O$, 88 mg.; $CuSO_4.5\,H_2O$, 393 mg.; $Fe_2(SO_4)_3.9\,H_2O$, 910 mg.; $MnCl_2.4\,H_2O$, 72 mg.; $Na_2MoO_4.2\,H_2O$, 50 mg.; $ZnSO_4.7\,H_2O$, 4403 mg.; ethylenediaminetetraacetic acid disodium salt, 5 g.; water, 1 l. Difco Bacto-agar (1 %, w/v) was used to solidify the media.

The hypocholesteremic compounds were dissolved in sterile water; SKF 2314 was dissolved in 0·1 M-NaOH. Sterols and fatty acids were dissolved in diethyl ether. These substances were added to the medium after it was autoclaved and while still hot.

Both fungi were grown at 24° in the dark in 9 cm. diameter Petri dishes containing 16 ml. medium. The inoculum was a disc 5 mm. diameter cut from mycelium growing on drug-free medium. Growth was usually measured as colony diameter; in some experiments the weight of mycelium was also determined by melting down the agar cultures in an autoclave, rinsing the mycelium in hot water and drying (Timnick, Lilly & Barnett, 1951). Perithecia were counted in two opposite sectors (occasionally four) of about 23 angular degrees in each Petri dish.

RESULTS

Observations on Sordaria fimicola

Compound SKF 3301-A. When an inoculum disc cut from growth on drug-free medium was placed on medium containing SKF 3301-A, the hyphae after the initial lag phase grew at a constant rate for some 40 hr (Fig. 2), during which period the growth rate was related to drug concentration, but then the growth rate rather abruptly changed and growth continued at a constant but much slower rate (Fig. 2). (A slight change in growth rate occurred also in the controls at about the same time in many experiments.)

Perithecia were mature in the controls in 7 days and were more or less evenly distributed over most of the Petri dish (Pl. 1, fig. 1). At 7 days, the colonies on media with SKF 3301-A 20 mg./l. or more did not fill the Petri dish, but at this time they bore perithecia densely crowded in a ring towards the outer part of the colony. With further incubation more perithecia were formed during the continued slow growth,

and these perithecia were distributed in definite rings. At 20 mg./l. the innermost ring was sharply defined, those beyond less so (Pl. 1, fig. 2), but the rings became more sharply defined with increasing concentration of the drug up to 50 mg./l. (Pl. 1, fig. 5). The distance between successive rings corresponded to about 3 days growth. The innermost ring was formed at the position of the hyphal front at the time of abrupt change in growth rate. The perithecia were so densely crowded in the innermost ring that even at 7 days the greatest numbers of perithecia per Petri dish were found not in the controls but in media with SKF 3301-A 20 mg./l. (Table 1). With continued perithecial production during the slow growth on drug-containing media, the numbers of perithecia continued to increase, as shown in Table 1. At high concentrations of SKF 3301-A (e.g. 70 mg./l.) very few perithecia were formed, but the fungus produced a considerable amount of brown pigment. No growth from the inoculum disc occurred at SKF 3301-A 120 mg./l.

Table 1. *Sordaria fimicola. Counts of perithecia in media with SKF 3301-A; means of two counts in each of six Petri dishes, the same dishes being counted on two occasions*

Concentration of SKF 3301-A mg/l.	Age of culture (days from inoculation)	
	7	19
0	63 ± 4·0	86 ± 5·0
10	79 ± 8·5	99 ± 7·2
20	81 ± 9·7	158 ± 9·1
40	55 ± 6·9	149 ± 13·8
60	9 ± 3·9	26 ± 5·3

Compound SKF 525-A. The growth curves of *Sordaria fimicola* on media with various concentrations of this compound resembled those of Fig. 2, but the change of growth rate after the initial more rapid phase was not so marked. The effect of concentration of the compound on the initial growth rate is shown in Fig. 3. Growth rate did not fall off continuously with increasing concentration. The break in the curve (Fig. 3) corresponded to a change in the appearance of the margin of the colony. At 40 mg./l. and above the main hyphae continued to elongate for a considerable time, but the growth became progessively more and more diffuse, the hyphae being sparsely branched and widely spaced. At 30 and 20 mg./l., the edge of the colony was much more definite.

At 50 mg./l. only very small perithecial primordia were seen, and they were few and well scattered. At 40 mg./l. the perithecia were more numerous, but they reached a maximum diameter of in most dishes of about 150 μ (normal size in the controls 300 to 350 μ) and ostioles were not developed. (Some mature perithecia were, however, found in a few of the dishes in each experiment.) The perithecia and primordia occurred in more or less definite rings, but the rings were less clearly defined than with SKF 3301-A. At 30 and 20 mg./l. numerous perithecia matured; there were also many smaller immature perithecia which reached a later stage of development than the abortive perithecial initials in the controls. The number of mature perithecia at these concentrations was also greater than in the controls.

Compound SKF 16467-A. The inhibitory concentrations of this compound were

considerably higher than with SKF 3301-A and 525-A, as shown in Fig. 3. As with 3301-A, there was an abrupt change in growth rate with subinhibitory concentrations (e.g. 75 mg./l.) when the mycelium had covered about half the Petri dish, and perithecia were produced in a definite ring. Beyond this ring perithecia were scattered, rather

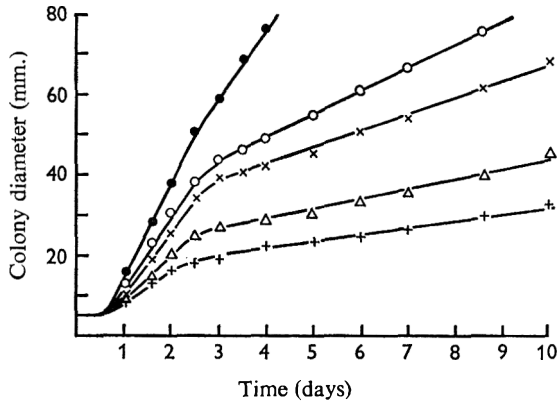

Fig. 2. Growth of *Sordaria fimicola* in media with various concentrations of SKF 3301-A. ●—●, 0 mg./l.; ○——○, 20 mg./l.; × — ×, 30 mg./l.; △—△, 50 mg./l.; + — +, 70 mg./l.

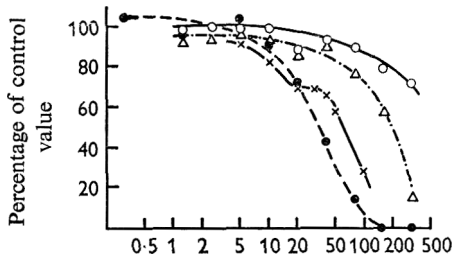

Fig. 3

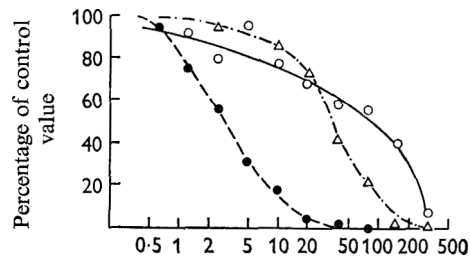

Fig. 4

Fig. 3. Effect on initial linear growth rate of *Sordaria fimicola* hyphae of varying concentrations of hypocholesteremic compounds. ○—○, SKF 2314; △—·—△, SKF 16467-A; × — ×, SKF 525-A; ●— —●, SKF 3301-A.

Fig. 4. *Phytophthora cactorum*. Growth (as dry weight after 14 days) in media with various concentrations of hypocholesteremic compounds. ○—○, SKF 2374; △—·—△, SKF 16467-A; ●— —●, SKF 3301-A.

than in definite rings. At 100 mg./l. and higher concentrations the number of perithecia was decreased though some were found even at 200 mg./l., but a great many tiny perithecial initials were produced, especially in the region corresponding to the definite ring of perithecia formed at lower concentrations.

Compounds SKF 2314, SKF 7732-A_3 and SKF 7997-A_3. These compounds had little effect, growth rate being reduced by only 30% at a concentration of SKF 2314 of 320 mg./l. (Fig. 3), and by less than this with 7732-A_3 and 7993-A_3. There were no effects on perithecial production at this concentration with any of the three.

Compound AY 9944. At concentrations of this compound above 10 mg./l. a curious puffing of the margin of the colony took place when growth rate was slowing down

(Pl. 1, fig. 6). Sometimes a single hypha extended for some distance through the medium and then produced a dense mass of much branched mycelium. The effects on perithecial production were strikingly different from SKF 3301-A etc., in that at concentrations of the drug which partially inhibited vegetative growth there was no increase in the numbers of perithecia produced. Indeed, perithecia were very few at any concentrations where the mycelium did not rapidly fill the Petri dish; e.g. at AY 9944 25 mg./l. the initial growth rate was reduced by 20 % but no perithecia were formed (Pl. 1, fig. 6). In such colonies the central part was darkly pigmented, though the edge was often pale.

Table 2. *Sordaria fimicola. Effect of fatty acids etc. annulling growth inhibition by SKF 3301-A*

Upper figures, increases in colony diameter (mm.) during a 24 hr period commencing 48 hr after inoculation. Lower figures, these values, relative to the control value.

Substance added	Concentration mg./l.	Concentration of SKF 3301-A (mg./l.) 0	50	100
None	—	29·7 ± 0·71 100	5·5 ± 0·22 19	4·0 ± 0·52 13
Oleic acid	200	25·7 ± 0·67 87	20·7 ± 2·37 70	6·7 ± 0·71 22
	800	27·8 ± 0·31 94	26·7 ± 0·49 90	19·7 ± 2·88 66
Linoleic acid	200	24·3 ± 0·67 82	21·3 ± 1·20 72	10·7 ± 1·31 36
	800	13·5 ± 1·38 46	16·2 ± 1·33 54	13·3 ± 1·41 45
Methyl linoleate	200	28·2 ± 0·17 95	12·3 ± 0·49 42	5·7 ± 1·33 19
	800	25·8 ± 0·60 87	11·8 ± 0·65 40	7·7 ± 0·88 26
Elaidic acid	200	31·7 ± 0·56 107	7·0 ± 0·26 24	3·8 ± 0·31 13
	800	30·8 ± 0·70 104	10·5 ± 1·02 35	4·3 ± 0·42 15
Stearic acid	200	27·0 ± 1·00 91	7·8 ± 1·27 26	2·8 ± 0·31 10
	800	27.3 ± 0·92 92	8·2 ± 0·57 27	3·8 ± 0·54 13
Cholesterol	100	26·0 ± 0·94 88	6·0 ± 0·48 20	3·3 ± 0·33 11

Annullment of the effects of compounds SKF 3301-A and AY 9944 on growth of Sordaria fimicola. As shown in Tables 2 and 3, cholesterol was ineffective in annulling the effects of compounds SKF 3301-A and AY 9944 on hyphal growth rate, but oleic acid (*cis*-octadec-9-enoic acid) was highly effective. Linoleic acid (octadec-9,12-dienoic acid) was rather toxic, but when allowance was made for its toxicity it was evident that it could also annul the effects of both compounds. Methyl lineolate and elaidic acid (*trans*-octadec-9-enoic acid) were less effective. Elaidic acid appeared to be about half as effective as oleic acid (compare Kodicek & Worden, 1946). Stearic acid (octadecanoic acid) was ineffective. Ergosterol was also ineffective. These results are based on measurements of colony diameter over periods of up to 24 hr during the initial phase of

more rapid growth. Provided an adequate amount of unsaturated fatty acid was supplied, growth in drug+media continued uninterruptedly to the edge of the dish.

The effects of oleic acid and cholesterol on perithecial production in media-containing hypocholesteremic compounds were studied; but first, the effects of oleic acid and cholesterol alone must be mentioned. In media to which only oleic acid was added the surface hyphae had a curious waviness (Pl. 1, fig. 7). There were cushion-like

Table 3. *Sordaria fimicola. Effect of fatty acids etc. annulling growth inhibition by AY 9944*

Increase in colony diameter during a 10½ hr period com mencing 37 hr after inoculation, relative to the control value.

Substance added	Concentration (mg./l.)	Concentration of AY 9944 (mg./l.)		
		0	100	300
None	—	100	73	7
Oleic acid	400	97	86	25
	1200	94	92	70
Linoleic acid	400	41	50	49
	1200	28	35	39
Methyl lineolate	400	81	61	8
	1200	75	41	2
Elaidic acid	400	86	65	28
	1200	89	75	42
Stearic acid	1200	86	76	5
Cholesterol	400	86	50	1
	1200	85	44	8

Table 4. *Sordaria fimicola. Effect of SKF 3301-A, oleic acid and cholesterol on numbers of perithecia in 7-day-old cultures*

Mean count from four sectors in each of four Petri dishes.

Concentration of SKF 3301-A (mg.l.)...	0		30		90	
Concentration of oleic acid (mg./l.)...	0	500	0	500	0	500
Concentration of cholesterol (mg./l.)						
0	134	127	72*	7	0	6
100	138	119	55*	99	0	31

* After a further 5 days with continued growth of the colonies these counts increased to 180 and 156 respectively. With other treatments the growth of the colonies was completed in 7 days.

Analysis of variance

	D.f.	Mean square
Main effects: SKF 3301	2	233692.8 **
Oleic acid	1	150.5 NS
Cholesterol	1	12513.0 **
First-order interactions:		
SKF 3301/Oleic acid	2	4931.8 NS
SKF 3301/Cholesterol	2	6372·1 NS
Cholesterol/Oleic acid	1	19926·8 **
Second-order interactions	2	15420·1 **
Between replicate Petri dishes	36	1645·8 **
Counts within dishes	144	625·7

** Significant at 1 % level; NS, not significant.

aggregates of hyphae, darkly pigmented, and raised above the surface of the agar, and these sometimes bore numerous perithecia; but in the main the surface of the culture was bare of perithecia, these being numerous only at the extreme edge of the dish against the glass. The effect of cholesterol alone was slight, but its addition to media with SKF 3301-A 30 or 35 mg./l. (no oleic acid) made the zonation of perithecia less pronounced; also perithecial production was somewhat accelerated in the presence of cholesterol, the perithecia being formed outside the first ring before there were any outside it in the medium without cholesterol.

Table 5. *Sordaria fimicola. Effect of AY 9944, oleic acid and cholesterol on numbers of perithecia in 11-day-old cultures*

Mean count from two sectors in each of 6 Petri dishes.

Concentration of AY 9944 (mg./l.)	0			30			60		
Concentration of oleic acid (mg./l.)	0	300	600	0	300	600	0	300	600
Concentration of cholesterol (mg./l.)									
0	59	86	83	13	99	78	0	52	73
100	66	65	86	3	75	100	0	17	37

Analysis of variance

	D.f.	Mean square
Main effects: AY 9944	2	37614·7**
Oleic acid	2	55923·7**
Cholesterol	1	6069·6*
First-order interactions:		
AY 9944/Oleic acid	4	8404·9**
AY 9944/Cholesterol	2	2344·5 NS
Oleic acid/Cholesterol	2	3681·5*
Second-order interactions	4	1862·5 NS
Between replicate Petri dishes	90	1197·3**
Counts within dishes	108	753·6

* Significant at 5 % level; ** at 1 % level; NS, not significant.

Results for oleic acid and cholesterol with SKF 3301-A are shown in Table 4. The addition of oleic acid alone to media with SKF 3301-A resulted in few perithecia (Pl. 1, fig. 8); the number was increased if cholesterol was added (Pl. 1, fig. 9), the increase being significant as shown by the second-order interaction in Table 4.

The results with compound AY 9944 were however different. In six experiments there was either no increase in the numbers of perithecia with the addition of cholesterol to the medium with AY 9944 and oleic acid (Table 5), or an increase which was comparable with that observed in the controls without AY 9944. The second-order interactions, which were significant in the SKF 3301-A experiments, were not significant. In Table 5 there is a significant main effect of cholesterol, but this is due to the *decrease* in the numbers of perithecia in the treatments with cholesterol.

Observations on Phytophthora cactorum

Compounds SKF 2314, 525-A, 3301-A, 16467-A, and AY 9944 all inhibited the growth of *Phytophthora cactorum*, the effective concentrations being less than for *Sordaria fimicola* (Fig. 4; cf. Fig. 3). SKF 7732-A_3 and 7997-A_3 were also inhibitory at low concentrations, but adaptation to them occurred, as shown in Fig. 5. When

mycelium growing on SKF 7997-A_3 was transferred to fresh drug-containing medium (4 mm. disc inoculum cut from the hyphal front), it did not immediately grow, but did so after a lag period similar to that observed in the first place. The lag period was the same on both freshly made medium and medium kept at 24° for 14 days.

The effectiveness of oleic acid and cholesterol in annulling the inhibition of growth due to compounds SKF 3301-A and AY 9944 has been examined (Table 6). In the absence of drug, the effect of oleic acid alone was to give extremely rapid growth of the hyphae, but the colony was much less dense than on basal medium. Addition of

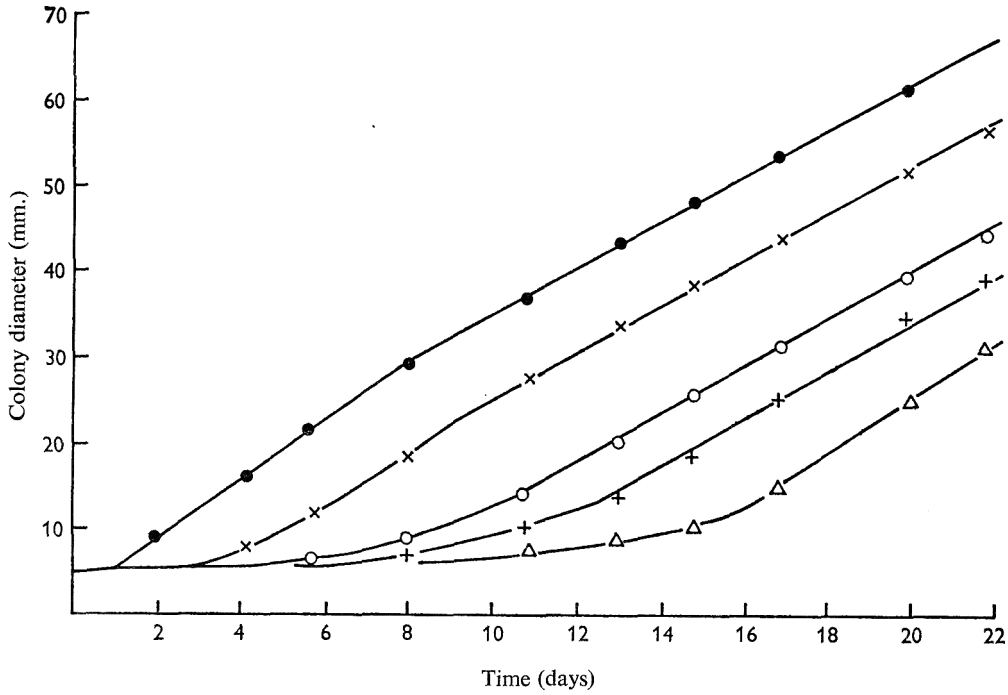

Fig. 5. *Phytophthora cactorum*. Growth in medium with various concentrations of SKF 7997-A_3. ●—●, 0 mg./l.; × — ×, 1 mg./l.; ○—○, 2 mg./l.; +—+, 3 mg./l.; △—△, 4 mg./l.

cholesterol with the oleic acid increased colony density. On media with SKF 3301-A or AY 9944 and oleic acid, hyphae grew out from the inoculum, but the growth was exceedingly sparse. However, the addition of cholesterol as well restored the appearance of the colony virtually to normal. Cholesterol by itself seemed to have some effect except at the higher concentration of SKF 3301-A. In the treatments marked with an asterisk in Table 6 there was much variation from dish to dish, in some cases growth hardly starting from the inoculum while in others a large very dense growth occurred.

The fact that cholesterol had to be added with the oleic acid to annul the effects of the inhibitors might be attributed to a need to detoxify the oleic acid. Unsaturated fatty acids are toxic to lactobacilli, but this toxicity can be annulled by a number of compounds, notably sterols (Kodicek, 1949) and Tween 40 (Williams, Broquist & Snell, 1947). Hendrix, Norman & Apple (1966) considered oleic acid to be toxic to

Phytophthora, but found the toxicity could be annulled by cholesterol and tocopherol. Tween 40 did not appear to be an effective replacement for cholesterol in my experiments.

Table 6. *Phytophthora cactorum. Effect of oleic acid and cholestero on growth in media with SKF 3301-A or AY 9944*

Diameter (mm.) (upper figure) and dry wt (mg.) (middle figure) and density (lowest figure) after 9 days incubation. The inoculum was of diameter 5 mm. and dry wt approx. 0·8 mg. Means of 6 values. The 6 mycelia of each treatment were weighed together. Density = (100 × dry wt)/(diameter^2).

Concentration of oleic acid (mg./l.)...	0		500	
Concentration of cholesterol (mg./l.)...	0	35	0	35
No inhibitor	31·7 ± 0·13	35·5 ± 0·92	70·7 ± 2·44	70·2 ± 1·44
	17·0	22·7	33·8	55·0
	1·69	1·80	0·68	1·12
SKF 3301-A				
24 mg./l.	13·5 ± 0·50	12·5 ± 2·41*	59·2 ± 1·50	62·3 ± 1·31
	0·7	4·7	3·5	41·7
	—	3·01	0·10	1·07
88 mg./l.	7·8 ± 0·78	6·3 ± 0·67	54·5 ± 1·33	34·7 ± 6·36*
	0·2	0·8	4·7	18·0
	—	—	0·16	1·50
AY 9944				
24 mg./l.	17·3 ± 0·21	19·5 ± 4·14*	53·7 ± 1·56	62·8 ± 2·17
	0·8	12·9	5·2	42·7
	—	3·16	0·18	1·08
88 mg./l.	15·3 ± 2·14	12·8 ± 1·70	31·2 ± 3·82*	29·8 ± 5·82*
	0·7	4·2	1·5	14·0
	—	2·56	0·15	1·58

* Note high variance.

DISCUSSION

The compounds SKF 3301-A and 16467-A have in common the two groups $(C_6H_5)_2(C_3H_7)$— and $—(CH_2)_2N(CH_3)_2$, and SKF 525-A has $(C_6H_5)_2(C_3H_7)$— and $—(CH_2)_2N(C_2H_5)_2$; the central parts of the molecules are however different (Fig. 1). All three compounds inhibited the growth of *Sordaria fimicola*, but their effects were characteristic. SKF 3301-A was effective at lower concentrations than 16467-A, but with both a marked change of growth rate occurred when the mycelium had grown part of the way across the Petri dish. Only SKF 3301-A produced the marked zonation of perithecia during the latter period of slower growth; with SKF 16467-A there were many more abortive perithecia. Abortive perithecia were also numerous with SKF 525-A, and this compound produced marked thinning of the edge of the colony. Neither of the compounds SKF 2314, which has only the $(C_6H_5)_2(C_3H_7)$— group, nor SKF 7997-A_3, which has only $—(CH_2)_2N(C_2H_5)_2$, was inhibitory. All these compounds were inhibitory to *Phytophthora cactorum*, the toxic concentrations being lower than for *S. fimicola*. The effects of these compounds on several bacteria and fungi were described by Aaronson & Fulco (1968) and on the ciliate *Ochromonas danica* by Aaronson (1966).

The inhibition of vegetative growth of *Sordaria fimicola* by the compounds SKF 3301-A and AY 9944 was annulled by unsaturated fatty acids but not by sterols. It is possible that the sterols, being present as crystals dispersed through the agar, could not be taken up by this fungus, although sterols so presented to *Phytophthora cactorum* are effective. Nevertheless, while the effects of two other compounds which inhibit sterol synthesis, triparanol and benzmalecene, on growth of various ciliates and flagellates could be annulled by both sterols and unsaturated fatty acids (Aaronson, Bensky, Shrifrine & Baker, 1962; Holz, Erwin, Rosenbaum & Aaronson, 1962), the delay in division induced by triparanol in synchronous cultures of *Tetrahymena pyriformis* could be prevented by oleic acid and not by sterols (Holz *et al.* 1962). Again, oleic acid but not sterols annulled the effect of SKF 525-A and 3301-A on *Ochromonas danica* (Aaronson, 1966). Aaronson (1964, 1965) found that the effects of triparanol and benzmalecene on several micro-organisms were more effectively annulled by oleic acid than by ergosterol or squalene (though not in all cases; Aaronson & Fulco, 1968); saturated acids were ineffective. These and my results are difficult to explain unless the compounds inhibit processes other than sterol synthesis. The fact that growth of *Phytophthora cactorum* is inhibited by hypocholesteremic compounds is significant, as Phytophthora appears not to synthesize sterols. Compound SKF 525-A is in fact known to affect other processes in mammalian systems; the hydrolysis of procaine, for example, is inhibited competitively, and demethylation of *o*-anisole non-competitively (Netter, 1962).

Although the addition of oleic acid to medium with compounds SKF 3301-A or AY 9944 resulted in vigorous hyphal growth of Sordaria, it did not lead to normal reproductive competence. Perithecia were few and irregularly distributed, and the mycelium developed much brown pigment. It would appear that oleic acid repairs the lesions induced in processes essential for vegetative growth more effectively than in those required for reproduction. It may be significant that the addition of cholesterol to media with intermediate amounts of SKF 3301-A (30 to 35 mg./l.) speeded up perithecium formation and decreased the definition of zonation, but the effect was slight. More definitely, the addition of cholesterol to media with oleic acid and SKF 3301-A improved reproductive competence. This was not observed with AY 9944, but the latter compound was manifestly different in its action from the SKF compounds; particularly, it did not lead to an increase in perithecial production when present in the medium at concentrations sub-inhibitory to vegetative growth. That sterols are involved in the effects of SKF 3301-A and similar compounds on reproductive phases was strikingly shown by the work of Nelson, Huisingh & Webster (1967) with the heterothallic ascomycete *Cochliobolus carbonum*. They applied SKF 3301-A to partly grown cultures at various times after mating, and found that perithecial development was inhibited; this inhibition was annulled by the addition of various sterols.

I am indebted to Messrs Smith, Kline and French Laboratories, Welwyn Garden City, Hertfordshire, and Philadelphia, Pennsylvania, and to Imperial Chemical Industries, Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire, for supplying the hypocholesteremic drugs. I also wish to thank Professor P. W. Brian, F.R.S., for his interest in this work and for many suggestions.

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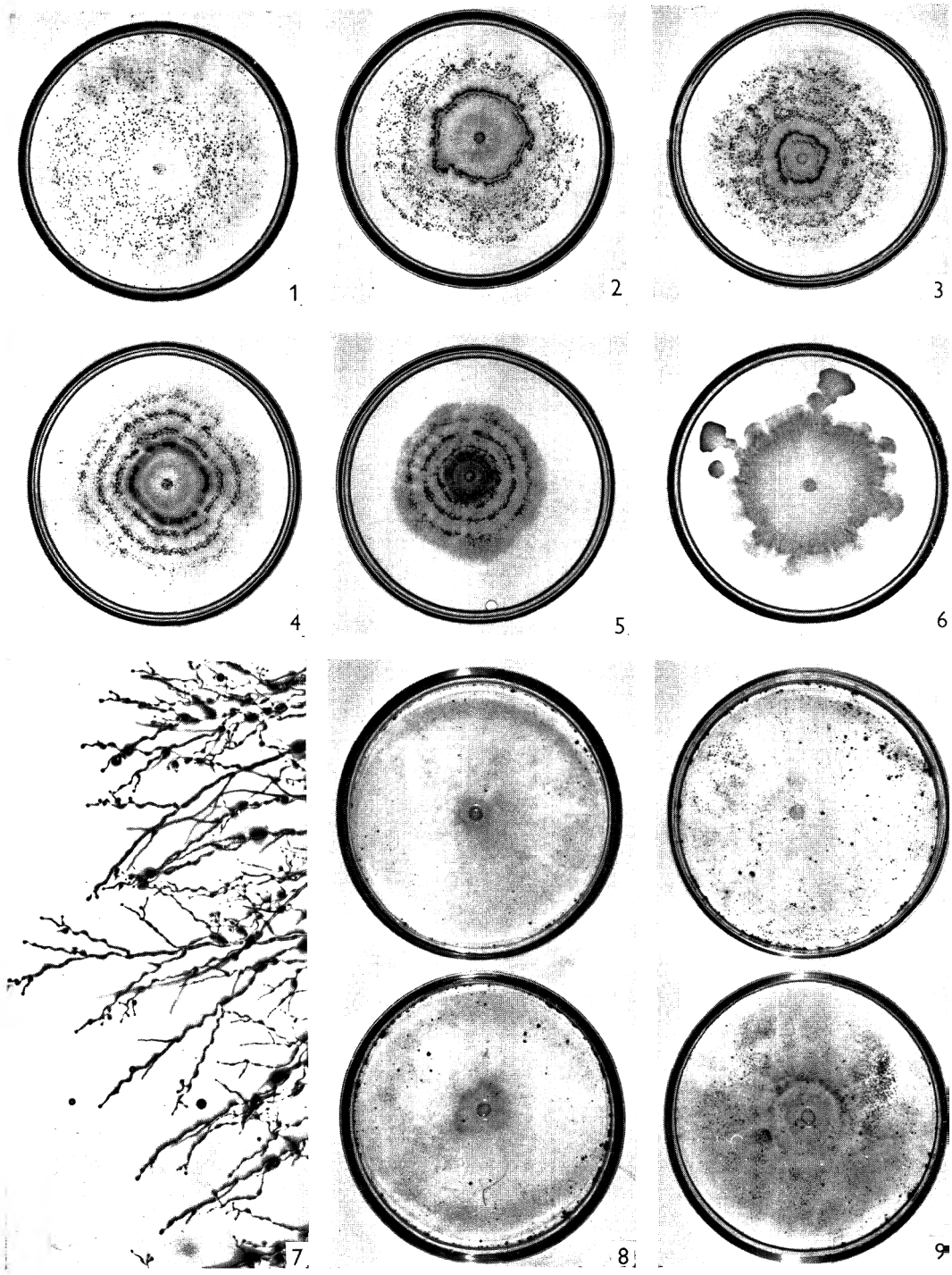

C. G. ELLIOTT

(*Facing p.* 343)

EXPLANATION OF PLATE

Sordaria fimicola

Fig. 1. Control culture on drug-free medium.

Fig. 2. SKF 3301-A, 20 mg./l.

Fig. 3. SKF 3301-A, 30 mg./l.

Fig. 4. SKF 3301-A, 40 mg./l.

Fig. 5. SKF 3301-A, 50 mg./l.

Fig. 6. AY 9944, 25 mg./l.

Fig. 7. Hyphae on medium with oleic acid.

Fig. 8. SKF 3301-A, 70 mg./l. + oleic acid, 500 mg./l.

Fig. 9. SKF 3301-A, 70 mg./l. + oleic acid, 500 mg./l. + cholesterol, 100 mg./l.

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Sedimentation Properties of Ribonucleic Acid from *Rhodopseudomonas spheroides*

By LYDIA BORDA, M. H. GREEN AND M. D. KAMEN
Departments of Chemistry and Biology, University of California at San Diego, La Jolla, California 92037, *U.S.A.*

(*Accepted for publication* 18 *February* 1969)

SUMMARY

Phenol-purified RNA was prepared from *Rhodopseudomonas spheroides* and characterized by sucrose-gradient centrifugation. The patterns obtained indicate that *R. spheroides* possesses a ribosomal RNA complement similar to that of *Escherichia coli*. This is contrary to previous claims that the 23S component is missing. This species of ribosomal RNA was demonstrated to be more labile than the corresponding component in *E. coli* and variations in yields produced by changes in extraction procedures are described.

INTRODUCTION

Although sedimentation profiles of ribosomes obtained from many species of bacteria invariably exhibit 50S and 30S subunits, which yield respectively 23S and 16S RNA molecules (Osawa, 1968), it has been reported (Lessie, 1965) that the facultative photoheterotroph, *Rhodopseudomonas spheroides*, does not contain the 23RNA species, which implies that this bacterium is unique in that it can build all its ribosomes from the 16S species alone. However, some data indicating that *R. spheroides* does in fact contain the usual ribosomal subunits have appeared (Lessie, 1965; Friedman, Pollara & Gray, 1966). In addition, the sedimentation profiles of *Rhodospirillum rubrum* and other facultative photoheterotrophs have revealed both types of RNA with sedimentation characteristics quite similar to those obtained from *Escherichia coli* (Lessie, 1965; Yamashita & Kamen, 1968). In the course of researches on polynucleotide metabolism in *R. spheroides*, we have had occasion to examine RNA composition in this organism, including not only the strain investigated originally but also two others—one of which is a uracil-requiring mutant—and report herewith that in all cases the 23S RNA component can be isolated, but that its lability is pronounced. This fact appears to be the source of the present contradictions in previous reports.

METHODS

Organisms

The strains of *Rhodopseudomonas spheroides* used were: no. 2.4.1 (originally from the collection of Professor C. B. van Niel); another culture of no. 2.4.1 obtained from Dr S. Kaplan; and no. 5–11, a uracil-requiring mutant of strain GA, obtained from Dr J. Lascelles. The *Escherichia coli* used was laboratory strain K12-W3110.

Growth of Organisms

Rhodopseudomonas spheroides was grown photosynthetically, from a 1/10 (v/v) inoculum, in medicine bottles, under strong illumination, at 30 to 32°, in the glutamate medium of Lascelles (1959). For radioactive labelling, the medium was supplemented with 0·5 μc/2 μg./ml. of either tritiated or ^{14}C-uracil. Organisms were grown mostly to about the second half of the logarithmic phase, although on occasion organisms in early stationary phase were used with no apparent effect on results. *Escherichia coli* was grown with forced aeration, at 37°, for about five generations in K medium (Green, 1966), supplemented with 0·5 μc ^{14}C-uracil/2 μg./ml. All organisms were harvested by centrifugation. As explained in the Results section, it was found necessary to avoid storage by freezing and to use these suspensions immediately for RNA isolation and characterization.

Preparation of phenol-purified RNA from Rhodopseudomonas spheroides

Organisms were washed once with the appropriate buffer (see Results) and re-suspended, at about $\frac{1}{10}$th of their original volume, in buffer containing 10 μg. DNase (Worthington DNase I, electrophoretically purified) per ml. The suspensions were chilled on ice and passed through a pre-cooled French pressure cell at 5000 to 9000 p.s.i. They were then left at room temperature for 10 to 15 min. for degradation of the DNA. Sodium dodecylsulphate (SDS) was added to a final concentration of 0·5 to 1% and the mixture extracted three times with freshly distilled phenol equilibrated with the buffer used in extraction. The aqueous phase of the last extraction was precipitated with twice its volume of ethanol, in ice, for about one half hour. When the extinction was to be measured, the precipitates were washed twice with ether to remove traces of phenol, then dried with air. The precipitates were dissolved in small volumes of SSC (0·15 M-NaCl, 0·015 M-Na citrate).

Preparation of phenol-purified RNA from Escherichia coli

The bacteria were washed once with 5×10^{-3} M-tris-Cl, pH 7·3, containing 10^{-2} M-$MgSO_4$. They were resuspended in the same buffer at $\frac{1}{25}$th of their original volume, and lysozyme was added to a concentration of 100 μg./ml. The bacteria were then frozen and thawed three times, over a period of 40 hr. After the last thawing DNase was added to a final concentration of about 20 μg./ml., and degradation allowed to proceed at room temperature for 15 min. The preparation was made 1% in SDS and stirred well. It was then extracted three times with freshly distilled phenol equilibrated with the above buffer. The aqueous phase from the last extraction was precipitated with twice its volume of ethanol. After centrifugation, the precipitates were dried with air and dissolved in a small volume of SSC.

Sucrose gradient centrifugation

Linear, 27 ml. gradients of 5 to 20% w/v sucrose in 0·01 M-tris-Cl + 0·1 M-NaCl, pH 7·5, were prepared with the aid of an apparatus similar to that of Britten & Roberts (1960). The gradients were left to equilibrate in the cold (4°) for at least one half hour and up to 24 hr. A small sample (0·05 to 0·2 ml.) of the phenol-purified RNA preparations was layered on top of the gradient and centrifugation performed in

a Model L or a Model L-2 Beckman ultracentrifuge, using the SW 25·1 swinging bucket rotor (24,000 rev./min., 15 hr, $T = 3°$). One ml. fractions were collected from the tubes after puncture in the usual fashion. Radioactive samples were precipitated with cold trichloracetic acid (TCA), filtered over Schleicher and Schuell type B-6 membrane filters, and counted in a scintillation counter. Unlabelled samples were assayed by their extinction at 260 mμ (nm.).

RESULTS

It was found that *Rhodopseudomonas spheroides* possessed an RNA complement similar to that of *Escherichia coli*, i.e. the ribosomal RNA had two components that corresponded to the 16S and 23S RNAs. However, the 23S RNA was labile with yields varying under different conditions of extraction.

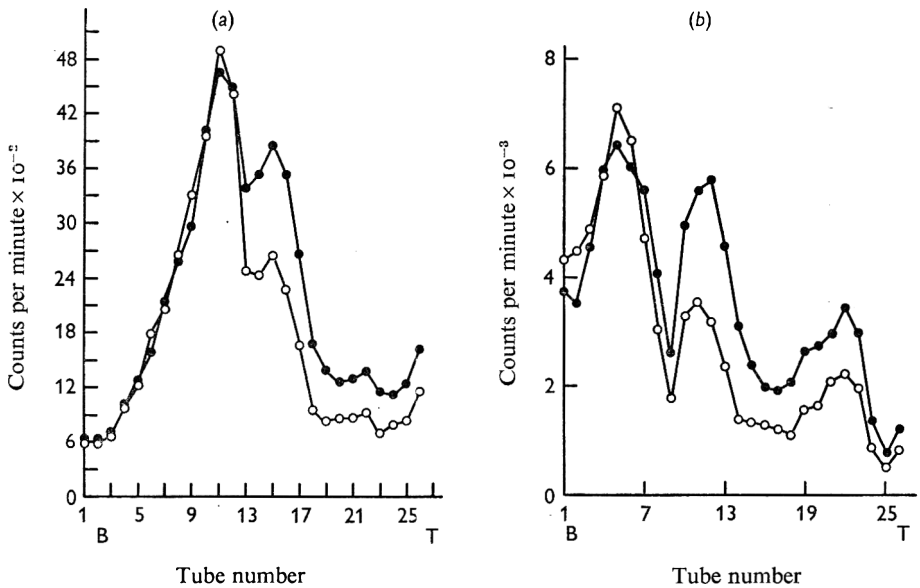

Fig. 1(*a*). Sucrose gradient pattern of a mixed preparation of *Rhodopseudomonas spheroides* and *Escherichia coli* RNA, in the absence of Mg^{2+}, *R. spheroides* no. 2.4.1 was grown on ^{3}H-uracil; *E. coli* was grown on ^{14}C-uracil. The two cultures were mixed; RNA was phenol purified, precipitated with alcohol and dissolved in SSC. A portion of this preparation was layered on top of a 5–20% (w/v) sucrose gradient in 10^{-2} M-tris-Cl, (pH 7·5), + 0·1 M-NaCl. —○—, ^{14}C (*E. coli* RNA); —●—, ^{3}H (*R. spheroides* RNA).

(*b*) Sucrose gradient pattern of a mixed preparation of *R. spheroides* and *E. coli* RNA in the presence of 10^{-2} M-Mg^{2+}. The RNA preparation in Fig. 1*a* was dialysed against 5×10^{-3} pHM-tris-Cl (pH 7·5), + 10^{-2} M-$MgSO_4$ and analysed as in Fig. 1*a* except that the gradient contained also 10^{-2} M-Mg^{2+}. —○—, ^{14}C (*E. coli* RNA); —●—, ^{3}H (*R. spheroides* RNA)

In one experiment *Rhodopseudomonas spheroides* was grown on ^{3}H-uracil and *Escherichia coli* on ^{14}C-uracil, as described in the Methods section. After measuring the TCA precipitable counts, the two cultures were mixed so as to contain an approximately equivalent amount of the two isotopes, and RNA was extracted from the mixed cultures by the usual procedure (see Methods); the buffer used was 0·005 M-tris-

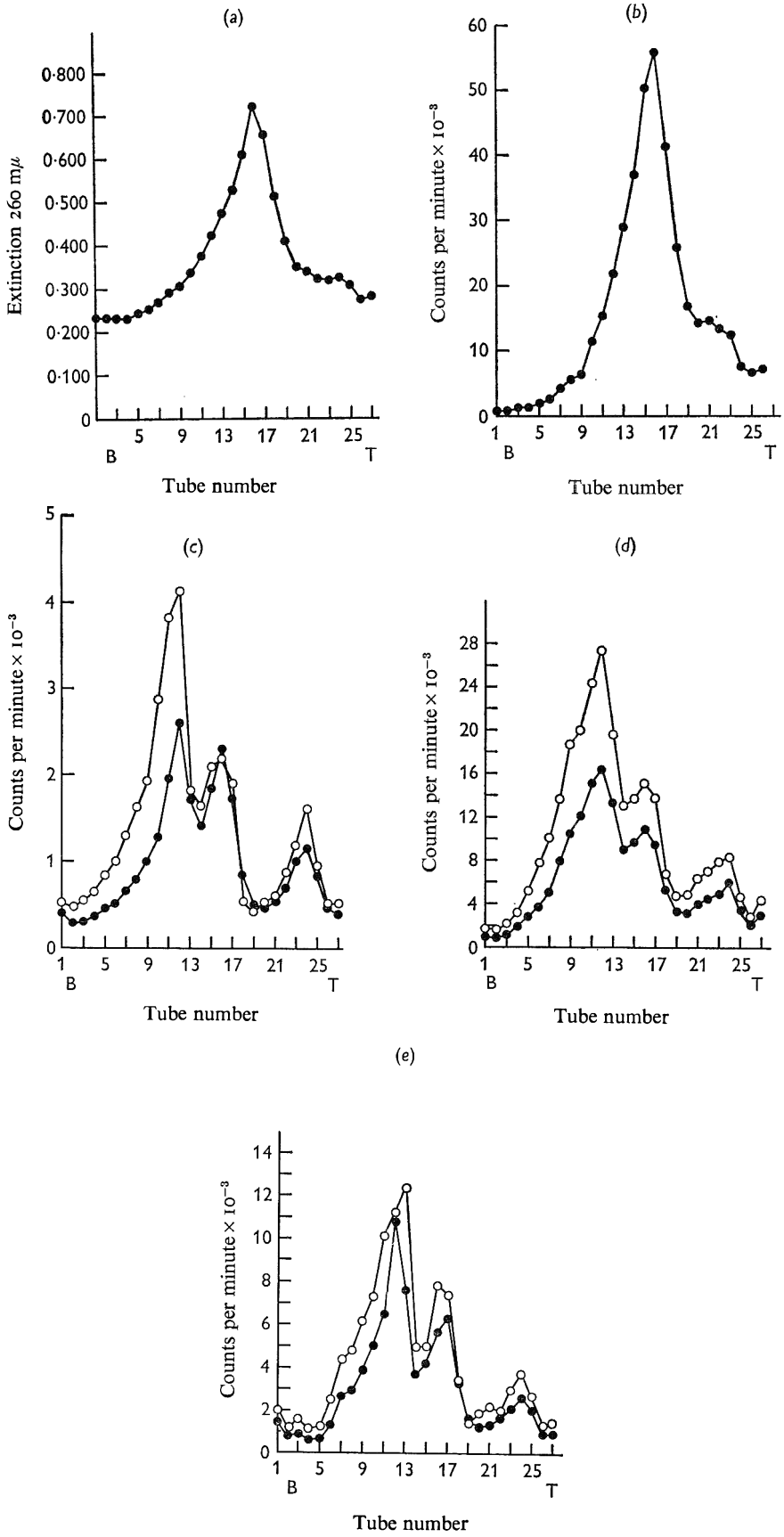

Fig. 2. For legend see opposite page.

Cl, (pH 7·3) + 0·01 M-$MgSO_4$ ("TM"). Part of the final preparation (in SSC) was analysed on a sucrose gradient (Fig. 1*a*). Another portion was dialysed against TM buffer and then analysed on a sucrose gradient containing 10^{-2} M-$MgCl_2$ (Fig. 1*b*). The resulting fractionation pattern clearly revealed RNA complements similar to those of *E. coli*, i.e. three peaks corresponding to the 4S, 16S and 23S species. Moreover, *R. spheroides* RNA, like *E. coli* RNA, sedimented more rapidly in the presence of Mg^{2+}, but the three components were again clearly visible. However, the ratio of 23S to 16S RNA in *E. coli* was approximately 2:1, as expected for an equal number of 50S and 30S ribosomes (because 23S RNA has twice the molecular weight of 16S RNA), while for *R. spheroides* the ratio was lower. Since all operations were carried out on the mixed cultures, it was apparent that 23S RNA in *R. spheroides* was more labile.

In the course of a large number of RNA extractions from the three different cultures of *Rhodopseudomonas spheroides* used, the relative amounts of 23S and 16S ribosomal RNA were found to vary, ranging from no apparent 23S to a ratio of 23S/16S of 1·7 in the best preparation obtained (see Fig. 2*a–e*).

The phenol-purified RNA preparations degraded randomly with time when stored at 4°. At −20° they were more stable, but a decrease in the 23S to 16S ratio was still noted. It appeared that the 23S RNA was sensitive to extraction conditions. Further experiments were performed to evaluate the effects of different parameters in the extraction procedure.

(*a*) *Effect of* Mg^{2+} *concentration.* As this factor was known to affect the 23S to 16S ratio in *Escherichia coli* (Midgley, 1965*a*), we tried extractions in 5×10^{-3} M-tris-Cl, (pH 7·3), buffer with varying amounts of Mg^{2+} from none up to 10^{-2} M-$MgSO_4$. In general, slightly higher yields of 23S relative to 16S were obtained in the absence of Mg^{2+}, but the over-all yields of RNA extracted in this manner were from 2 to 10 times lower than when Mg^{2+} was present. Therefore, when it is not essential to have intact 23S RNA, as in preparations used for hybridization assays, we recommend extraction in the presence of Mg^{2+}.

(*b*) *Phenol.* It was found essential for both *Rhodopseudomonas spheroides* and *Escherichia coli* that phenol be freshly distilled. Various methods for storage of distilled phenol were tried, such as maintenance at 4° or −10°, presence or absence

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Fig. 2. Sucrose gradient patterns of several preparations of RNA from *Rhodopseudomonas spheroides*. The gradient was 5 to 20 % (w/v) sucrose in 10^{-2} M-tris-Cl, (pH 7·5) + 0·1 M-NaCl. ^{14}C labelled RNA from *E. coli* was prepared separately and was used in some of the experiments as a marker.

(*a*) *R. spheroides* suspension used for RNA extraction had been stored in frozen state for several months.

(*b*) *R. spheroides* suspension used was kept suspended in 0·005 M-tris-Cl, (pH 7·5) + 10^{-2} M-$MgSO_4$, at 4°, for 48 hr, before breaking it and extracting the RNA.

(*c*) RNA from *R. spheroides* 5-11, a uracil auxotroph obtained from Dr J. Lascelles. —○—, ^{14}C (*E. coli* RNA); —●—, ^{3}H (*R. spheroides* RNA).

(*d*) RNA from *R. spheroides* no. 2.4.1 obtained from Dr S. Kaplan. —○—, ^{14}C (*E. coli* RNA); —●—, ^{3}H (*R. spheroides* RNA).

(*e*) One of the better RNA preparations obtained from the culture of *Rhodopseudomonas spheroides* no. 2.4.1 that was used in most of this work. —○—, ^{14}C (*E. coli* RNA); —●—, ^{3}H (*R. spheroides* RNA).

of buffer, use of nitrogen and avoidance of light. None of these practices, however, singly or together, proved to be entirely reliable, and it was concluded that phenol should be freshly distilled and used within 24 hr.

(*c*) *Previous history of cells.* Little or no 23S RNA was obtained from organisms that had been frozen for several months or kept for 48 hr at 4° in the extraction buffer. In this context it is to be noted that previous negative findings involved the use of frozen organisms (Lessie, 1965).

DISCUSSION

The characteristics of *Rhodopseudomonas spheroides* RNA may be correlated with those of *Escherichia coli* ribosomal RNA. Thus, studies of the latter have shown that, when extraction conditions are not optimal (Midgley, 1965*a*), or upon mild alkaline treatment (Midgley, 1965*b*), 23S RNA can be degraded, producing predominantly RNA of the 16S class. However, *E. coli* 23S RNA exhibits only one 3′-terminal residue (Midgley, 1965*c*) and one 5′-terminal residue (Takanami, 1967) per molecule, indicating it is not a simple dimer of the 16S RNA. Our extractions of RNA from mixed cultures of *R. spheroides* and *E. coli* produce *E. coli* ribosomal RNA in almost the theoretical ratio, whereas the 23S component from *R. spheroides* is obtained in relatively low yield. This observation might argue for some unusually fragile linkage(s) in the *R. spheroides* 23S RNA which may be particularly susceptible to nuclease attack. Observations on the liberation of various non-covalently bound enzymes from mitochondrial membranes by freezing and thawing (Egger & Rapoport, 1963) support our suggestion that previous negative findings (Lessie, 1965) were due at least in part to the use of frozen cells. One may note also that in these negative experiments even the *E. coli* RNA preparation exhibited a ratio of 23S to 16S of 1 : 1 instead of the theoretical 2 : 1. We may conclude that the composition of ribosomal RNA in *R. spheroides* is much like that of *E. coli* and other bacteria and requires no supposition of uniqueness, at least in sedimentation behaviour.

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The Occurrence of Natural Antibodies to Rumen Bacteria

By M. ELISABETH SHARPE, M. J. LATHAM AND B. REITER

National Institute for Research in Dairying, Shinfield, Reading, Berkshire

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SUMMARY

The sera of 11 ruminants and one horse contained relatively high titres of agglutinating antibodies against strains of anaerobic bacteria isolated from the bovine rumen. They were not detected in the sera of non-ruminants, i.e. pig, rabbit, guinea pig, rat and man. A close relationship existed between motility of rumen organisms and their ability to evoke detectable antibodies, although antibodies to both flagellar and somatic antigens were produced. The antibodies to the rumen organisms were highly specific and of the IgM type. They occurred in bovine colostrum at the same level as in the blood serum, and were transferred to the calf via the colostrum. After 1 month they had disappeared from the calf serum but between 5 and 8 months later antibodies of a similar activity were detected. In addition, antibodies to the non-motile *Bifidobacterium bifidus* were found in the sera of the young calf.

INTRODUCTION

In the absence of infection or overt stimulation with exogenous antigens, antibodies have been demonstrated in the sera of animals and man which are regarded as natural antibodies (Boyden, 1966). The intestinal flora is considered to be the most important source of antigenic stimulation for the production of such antibodies, providing a small but unremitting source of antigens.

Attempts have been made to define patterns of natural antibodies against Enterobacteriaceae (Gibson, 1930; Landy & Weidanz, 1964) amongst the sera of different animal species. Although it was not possible to make clear-cut distinctions between the animals, there were indications that each species might have its own pattern. It has been suggested (Reiter & Oram, 1967) that in ruminants conditions are particularly favourable for the formation of natural antibodies from rumen organisms. The specialization and high concentrations of organisms within the rumen might give rise to a greater specificity and to a higher titre of antibodies in the sera of these animals.

This paper is concerned with agglutination titres in bovine sera against anaerobic rumen bacteria, the patterns of antibody reactions of different animal sera against these organisms, and the appearance of such antibodies in the bovine colostrum and their transfer to the calf serum.

METHODS

Samples of well-mixed rumen contents were removed from a fistulated cow (A), and organisms isolated by the anaerobic techniques of Hungate (1950). Primary

isolation was made in roll tubes using a slightly modified medium 10 of Caldwell & Bryant (1966). Two stock mineral solutions were prepared (1) K_2HPO_4 0·6% w/v; (2) NaCl 1·2%, $(NH_4)_2SO_4$ 1·2%, KH_2PO_4 0·6%, $CaCl_2$ 0·12%, $MgSO_4 7H_2O$ 0·25%, and a stock mixture of volatile fatty acids: acetic 17 ml., propionic 6 ml., *n*-butyric 4 ml., isobutyric 1 ml., DL-α-methyl-*n*-butyric 1 ml., isovaleric 1 ml., *n*-valeric, 1 ml. The composition of the medium was as follows (% w/v final concentration): glucose, 0·05; cellobiose, 0·05; soluble starch, 0·05; maltose, 0·05; yeast extract, 0·05; trypticase, 0·2; resazurin, 0·0001; haemin, 0·0001; agar, 2·0; cysteine-HCl and sodium sulphide each, 0·015; sodium carbonate, 0·4; volatile fatty acid mixture, 0·45 (v/v); mineral solutions 1 and 2, 7·5 (v/v) each; adjusted to pH 6·7 to 6·8 with N-NaOH. The cysteine-HCl + sodium sulphide mixture and the sodium carbonate were added separately as sterile solutions to the freshly autoclaved medium. Cleland's reagent, a protective reagent for SH groups (Cleland, 1964), was also added (to a final concentration 0·0001 M) in some instances to help poise the medium at the low Eh value required for growth of some of the rumen organisms. Further cultivation was done on agar slopes and in broth of a similar composition but containing 0·2%, w/v, each of glucose, cellobiose and maltose and without starch (medium M10).

All media distribution and cultural work was done in an atmosphere of oxygen-free CO_2.

Isolations and counts of total viable organisms were made from 3 rumen samples taken before, and 1 and 14 days after, a change of diet from 100% hay to 100% flaked maize.

Sample No.	Cows' diet	Viable organisms/g. rumen contents
1T	100% hay	$2{\cdot}12 \times 10^9$
2T	100% flaked maize	$2{\cdot}0 \times 10^9$
7T	100% flaked maize	$3{\cdot}5 \times 10^{10}$

Colonies were picked from appropriate roll tubes onto slopes of medium M10, were cultured in M10 broth and preserved on slopes of M10 at −60°.

Isolation of Escherichia coli. These organisms were isolated from the rumen contents of two cows B and C on 100% hay diet, using conventional anaerobic conditions (McIntosh & Fildes jar) and MacConkey media. Counts were estimated by mean probable numbers (m.p.n.), and were positive at 10^{-3} dilutions of rumen contents, but not at 10^{-4}.

Laboratory strains of Enterobacteriaceae. Six human strains of *Escherichia coli* NCTC 8603, 9026, 9703, 9705, 9707, 9855. Ten strains of *Klebsiella aerogenes* isolated previously from bovine mastitis cases and from straw bedding by Dr C. M. Cousins (N.I.R.D.).

Identification of organisms

Anaerobic rumen bacteria. Isolates were presumptively classed in a number of genera and species by morphology, motility, Gram reaction, production of gas from glucose, final pH value in glucose broth, H_2S production and breakdown of cellulose and starch (Hungate, 1966). Lactobacilli were identified by physiological and biochemical tests (Sharpe, Fryer & Smith, 1966), using media containing 0·02% (w/v) cysteine and anaerobic cultivation by conventional anaerobic methods. The tests described by Shattock (1963) were used to identify *Streptococcus bovis*; *Escherichia coli* strains were identified by morphology and IMVIC tests.

Serological tests

Suspensions for agglutination tests. Anaerobic organisms were grown for 18 hr anaerobically in 100 ml. medium M10, centrifuged, washed and half the organisms suspended in 0·85% saline + 0·2% formaldehyde. The other half was suspended in 0·85% saline and heated to 100° for 1 hr to destroy flagellar antigens. Extinctions were adjusted to a value of 0·55 (1 cm. cell, filter 58) on the Hilger Biochem. Absorptiometer. Lactobacilli were grown anaerobically in medium MRS (de Man, Rogosa & Sharpe, 1960) + 0·02% (w/v) cysteine; *Escherichia coli* and *Streptococcus bovis* were grown aerobically in yeast glucose broth. All cultures were divided into two portions and suspensions treated in a similar manner to those of the anaerobic bacteria.

Agglutination tests. Results were read after overnight incubation at 50°.

Agglutinin absorption tests. Sera were absorbed with an equal volume of formol treated or heat-treated (100° for 1 hr) bacterial suspensions (extinction 1·4, 1 cm. cell filter 58).

Animal sera. Sera were obtained from: cows A, B, C and D (fistulated experimental cows); cow E from a different part of the country; cows F, G and H from the Institute herd; two goats from the Institute herd, housed and kept quite separately from the cows; one rabbit, one guinea pig, two rats, one pig (all Institute experimental animals); one sheep and one horse from a different part of the country. Sera were preserved with 0·01% w/v thiomersalate and stored at −20°.

Preparation of antisera in rabbits. Rabbits were inoculated intravenously every 3 to 4 days with an 18 hr culture of washed formaldehyde-treated organisms for a course of eight injections.

Transfer of naturally occurring antibodies from cow to calf. Samples of sera and of the secretion of the non-lactating udders were taken from three cows three weeks before parturition. In addition samples of the first colostrum, of the milk 3 weeks after calving and of the cows' sera 6 weeks after calving were examined. Serum was taken from each calf before the first feed with colostrum and at monthly intervals for 9 months.

Gel filtration. Cow serum and colostrum were filtered on Sephadex G-200, equilibrated with 0·1 M-KCl + 0·01 M-tris buffer + HCl, (pH 7·5).

RESULTS

Agglutination titres of anaerobic rumen bacteria in serum of cow A

Table 1 shows the agglutination reactions of 70 rumen strains isolated from cow A, when titres as high as 320 were sometimes obtained. The close correlation between motility of an organism and its ability to react with the antibodies in the serum can be seen. Agglutinins to flagellar antigens were detected in 37 of the strains and agglutinins to somatic antigens in 17 out of 38 strains examined. With the non-motile strains practically no agglutination reactions were observed. Three strains of non-motile lactobacilli were not agglutinated whereas a relatively high titre was present against the motile strain.

Agglutination titres of anaerobic rumen organisms with sera of different animals

Some of the rumen isolates from cow A were then tested against the sera of five other cows, two goats, rabbit, two rats, pig, horse and sheep (Table 2). The sheep, one cow and the horse were from different environments, the other sera came from animals housed at the Institute. The same organisms were agglutinated in a similar pattern and to similar titres against both flagellar and somatic antigens with the sera of the cows, the goats, the sheep and the horse. The sera of the other animals however showed only an occasional weak reaction with rumen organisms.

Table 1. *Agglutination reactions of rumen bacteria isolated from cow A with serum from cow A*

	Formol-treated organisms			Heat-treated organisms		
Genus or species	No. of strains examined	No. of strains in which motility observed	Titres	No. of strains	No. of strains in which motility observed	Titres
Butyrivibrio	15	14	40–320	7	7	40–160
Selenomonas	12	12	40–160	6	6	20–80
Bacteroides	12	0	< 20	7	0	< 20
Streptococcus bovis	10	0	< 20	8	0	< 20
Streptococcus bovis	2	0	20–40	.	.	.
Lactobacillus	3	0	< 20	3	0	< 20
Lactobacillus	1	1	160	1	1	80
Ruminococcus	2	0	< 20	1	0	< 20
Spirillum	1	1	20	.	.	.
Succinivibrio	1	1	80	.	.	.
Lachnospira	1	1	80	1	1	40
Unclassified	8	7	20–160	2	2	20–40
Unclassified	2	0	< 20	2	0	< 20
	70			38		

. = not tested.

Agglutination titres of anaerobic rumen organisms with human sera

Eleven of 12 human sera tested had no agglutinins for selected rumen organisms: the exception agglutinated to a titre $> \frac{1}{20}$ the somatic antigens only, of 2 Butyrivibrio strains, 2 Lachnospira and one Lactobacillus strain.

Agglutination reactions of anaerobic rumen bacteria isolated from a different cow

Organisms isolated from cow D in the same way as from cow A were characterized and motility noted. Of 14 strains examined 12 gave characteristic patterns of agglutination similar to those of isolates from cow A, when tested with sera from three cows, one goat, horse and rabbit. Two motile strains behaved somewhat differently in that they gave no reaction with the goat and cow A sera although they gave a strong reaction with the sera of cows D and F and with horse serum.

Agglutination reactions of Escherichia coli

Sixteen strains isolated from the rumen of cows B and C showed agglutination patterns against cow A serum similar to those of the motile anaerobic rumen bacteria

Table 2. *Agglutination reactions of 14 strains of rumen bacteria isolated from cow A, tested against sera from different animals*

Strain	Treatment of organisms	Titre of serum: Five cows A, D, E*, F, G	Two goats 1, 2	Horse*	Sheep*	Two rats, one rabbit	Guinea pig	Pig
†Butyrivibrio 1T18	F	160–640	160–320	320	160	—	40	—
	T	40–640	80–160	160	160	—	20	—
†Butyrivibrio 1T44	F	80–640	320	160	160	.	.	.
	T	40–160	80	80	80	.	.	.
†Butyrivibrio 2T70	F	80–640	160–320	320	80	.	.	.
	T	40–320	80	160	80	.	.	.
†Selenomonas 1T60	F	80–320	80–160	160	40	—	—	—
	T	40–320	20–40	160	20	—	—	—
†Selenomonas 1T68	F	40–320	20	80	20	—	—	—
	T	40–320	20	160	20	—	—	—
†Lachnospira 1T24	F	80–320	160	160	80	—	—	—
	T	40–160	80	160	40	—	—	—
†Lactobacillus 2T59	F	40–320	80	80	80	—	40	80
	T	40–640	40	320	80	—	20	20
Bacteroides 1T11	F	—	—	—	—	—	—	—
Bacteroides 1T14	F	—	—	—	—	—	—	—
Bacteroides 1T31	F	—	—	—	—	—	—	—
Streptococcus bovis A2/3	F	—	—	—	—	—	—	—
Streptococcus bovis B3/9	F	—	—	—	—	—	—	—
L. acidophilus 7T25	F	—	—	—	—	—	—	—
Bifidobacterium bifidus 7T4	F	—	—	—	—	—	—	—

*, Animal from a completely different environment; †, motility observed; F, formol treated; T, heat treated; —, titre of less than 20; ., not tested.

Table 3. *Agglutination titres against rumen bacteria, found in blood serum, dry period secretion, colostrum and milk of a cow and in the blood serum of its calf*

Organism		Titres in cow H of: Serum before calving	Dry period secretion	Colostrum	Milk 3 weeks after calving	Serum 6 weeks after calving	Before colostrum	Five days after colostrum	Titres in calf H serum at: One month	Four months	Six months	Nine months
Butyrivibrio 1T18	F	320	5120	320	80	320	—	40	—	20	—	320
	T	80	2560	80	40	160	—	80	—	—	—	40
Butyrivibrio 1T35	F	40	640	80	—	20	—	40	—	—	—	40
	T	40	320	40	—	40	—	80	—	—	—	20
Selenomonas 1T60	F	160	1280	80	20	80	—	80	—	40	20	160
	T	40	1280	80	20	80	—	—	—	—	—	80
Selenomonas 1T68	F	40	640	80	—	20	—	40	—	—	40	40
	T	40	320	40	—	20	—	20	—	—	20	20
Lachnospira 1T24	F	160	320	160	.	320	—	160	—	—	80	40
	T	160	640	80	.	160	—	—	—	40	40	80
	T	160	640	80	.	160	—	—	—	40	40	80
Bacteroides 1T11	F	—	—	—	—	—	—	—	—	—	—	—
Lactobacillus 2T59	F	160	640	80	—	160	—	80	—	40	160	640
	T	160	320	40	—	40	—	—	20	—	20	40
Lactobacillus 7T25	F	—	—	—	—	—	—	—	—	—	—	—
S. bovis A2/3	F	—	—	—	—	—	—	—	—	—	—	—
B. bifidus 2T4	F	.	.	.	—	—	.	.	.	—	—	—
E. coli BC3	F	.	.	.	.	160	.	.	.	—	—	—
	T	.	.	.	.	160	.	.	.	—	—	20

F, Formol-treated cells; T, heat-treated cells; —, titre of less than 20; ., not done.

except that no agglutination occurred with the sheep serum but it did occur with the pig serum. Four human strains were not agglutinated by the sera, and two were only weakly agglutinated. Ten non-motile *Klebsiella aerogenes* strains were not agglutinated.

Serological relationships between strains of rumen bacteria

Absorption tests showed that the serum of cow A had natural agglutinins specific for many of the organisms, despite some sharing of antigens. Thus there were four different serotypes in five Butyrivibrio strains, with variations in both somatic and flagellar antigens; 3 Selenomonas strains had different antigens, but 2 Lachnospira strains appeared identical. Injection of the organisms into rabbits induced specific somatic and flagella agglutinins.

Retesting serum from cow A

Eleven months later another sample of serum was taken from cow A. During this time the cow had been on several different diets and at the time of sampling was out at pasture. The pattern of agglutination against rumen organisms was similar to that found with the previous sample of serum.

Transfer of naturally occurring antibodies from the cow to the calf

Table 3 shows the agglutination titres found in one of the three experiments with a cow and its calf. The level of antibodies in the secretion of the non-lactating udder was approximately 8-fold that of the blood serum, whereas in the colostrum the levels were the same as the serum. In the milk 3 weeks after parturition only a very low level of antibodies could be detected. In serum, 6 weeks after calving the level was similar to that found three weeks before calving. The concentration of these antibodies in the serum of a pregnant cow was therefore the same as in the lactating cow.

No antibodies were detected in the calf (J) serum before colostrum was given, whilst after feeding colostrum for 5 days agglutination titres were detected. However at 1 month, on a diet of milk and roughage, these passively acquired antibodies had disappeared and even at 3 months the calf had manufactured only traces of its own antibodies against these strains.

In the two other experiments (Cows F and G) the titres of antibodies in the serum after parturition were similar to the levels in the colostrum. In the milk, levels were again very low. With the two calves K and L the results were similar to calf J except that in one of them (K) the level of absorbed antibodies was much lower than in the other calves although the titres in the mother's serum were as high as in the other two cows. With calf L, which was on a milk diet for 2 months, results were obtained similar to those from the other two on milk and roughage diets.

At 4 months traces of antibodies which agglutinated some of the rumen strains were observed in all three calves. At 6 months the titres had risen, particularly in calf K, where titres against all except one of the normally positive rumen strains varied from 80 to 320. Eventually, after 9 months, agglutinin patterns similar to those in adult ruminants became established.

To investigate whether the calves developed any antibodies against their own rumen organisms, samples of rumen contents were taken by stomach tube from calves K

Table 4. *Agglutinating titres of sera of two calves K and L aged 5 months, against organisms isolated from their own rumens*

Agglutination reactions of strains isolated from calf K with calf serum K					Agglutination reactions of strains isolated from calf L with calf serum L				
			Titre of serum					Titre of serum	
Strain	Motility	Presumptive identifications	Formol-treated	Heat-treated	Strain	Motility	Presumptive identifications	Formol-treated	Heat-treated
A18	+	Selenomonas	40	80	P2	+	Selenomonas	80	< 20
A27	+	Selenomonas	80	40	P19	+	?	40	40
A36	+	?	20	< 20	P7	+	?	< 20	< 20
A30	+	Butyrivibrio	< 20	< 20	P26	+	?	< 20	< 20
A35	+	Butyrivibrio	< 20	< 20	P30	+	?	< 20	< 20
A40	+	Butyrivibrio	< 20	< 20	P36	+	?	20	< 20
A2	−	*B. bifidus*	80	40	P8	−	*B. bifidus*	320	20
A12	−	*B. bifidus*	320	80	P28	−	?	640	160
A45	−	*B. bifidus*	320	< 20	P34	−	*B. bifidus*	80	80
A1	−	?	< 20	< 20	7 strains	−	?	< 20	< 20
A5	−	Bacteroides	< 20	< 20					
A39	−	Bacteroides	< 20	< 20					
A46	−	Bacteroides	< 20	< 20					

< 20, Titre less than 20; ?, strain not identified; F, formol-treated cells; T, heat-treated cells.
−, non-motile; +, motile

and L at 5 months of age, organisms were isolated by anaerobic techniques and tested against the calves' own sera. Identification of these organisms was by microscopic appearance and presence or absence of motility only. Table 4 shows that there was not the same correlation between motility and ability to react with antibodies as with the sera of adult ruminants. No agglutinins were observed for some of the motile strains, including some selenomonds, whilst relatively high titres occurred with some non-motile strains, in particular some *Bifidobacterium bifidus* strains. No antibodies to Bacteroides were observed in calf K, where these organisms were among the majority flora. It thus appears that motility is not a necessary factor to stimulate the formation of antibodies in the calf against its own rumen strains.

Fractionation of serum and colostrum from cow H

One of the physical criteria of natural antibodies is that they are predominantly present in the IgM fraction of immunoglobulins, apparently because of the very low level, but continuous antigenic stimulation (Michael & Rosen, 1963). Cow serum was chromatographed on Sephadex 200 and the fractions of the first two peaks tested against a Butyrivibrio strain and a Selenomonas strain. All the agglutinating activity for both flagellar and somatic agglutinins appeared in the fraction which was completely excluded from the gel, being contained in the front peak. No agglutinins were found in the later fractions, indicating that the antibodies were of the IgM type, and confirming the findings of previous workers (Landy & Weidanz, 1964). Similar results were obtained with colostrum, which also contained all the agglutinins in the high molecular fractions of the first peak.

DISCUSSION

The agglutinin titres obtained in bovine serum against rumen bacteria substantiated our suggestion that the high concentration of these organisms might evoke a strong antigenic stimulus. A sensitive bactericidal test (Landy & Weidanz, 1964) has been used to detect the presence of natural antibodies against Gram-negative bacteria, but the relatively high titres obtained with agglutination tests against the rumen bacteria made such tests unnecessary. The high specificity of the natural agglutinating antibodies in ruminants was shown by absorption tests where, with only 10 strains of three different genera, the presence of eight different serotypes was demonstrated. Specificity is further illustrated by the absence of agglutinins against a human *Escherichia coli* strain although they were detected against a rumen *E. coli* strain. The presence of antibodies against the indigenous rumen flora differentiate the ruminants and the horse from other mammals such as the rat, rabbit, guinea pig, pig and man. Horse serum may contain the same antibodies as the ruminant because microbial digestion of plant residues takes place in the caecum which probably contains similar organisms to those in the rumen (Davies, 1964).

The site of antigenic stimulation against these rumen organisms is unknown. Rumen organisms are transferred almost continuously from the rumen to the omasum and abomasum (Smiles & Dobson, 1956) so that only small numbers of whole organisms pass to the small intestine. The low pH of about 2·0 in the abomasum would probably destroy most of the survivors, except lactobacilli and coliforms (Smith, 1965). No information is available on the obligate anaerobic flora of the adult bovine intestine.

The presence of antibodies in the sera against rumen strains of *Escherichia coli*, which were only present in very small numbers in the rumen, suggests that they colonize the intestine where antigenic stimulation could occur. Microbial cells may also be translocated across the gut wall and eventually enter the lymph nodes (Wolochow, Hildebrand & Lamanna, 1966; Grys, 1966). Such translocation might also occur in the rumen wall.

The reason for the selective stimulation in the adult ruminant of antibodies against motile strains only of rumen organisms is not yet clear. Lovell (1932) detected flagellar agglutinins against Salmonella in 67% of animals examined (horses, cattle, sheep, pigs) so that flagellar antibodies may be widespread. Flagellins are not destroyed by pepsin and trypsin but are rendered soluble at pH 2·0 (Kobayashi, Rinker & Koffler, 1959; Stocker & Campbell, 1959). Solubilized flagellins are antigenic (Fitch & Winebright, 1962), and could therefore be a source of antigenic stimulation in the intestine. *Butyrivibrio fibrisolvens* (Bryant & Small, 1956*a*) is monotrichous; *Selenomonas ruminantium* (Bryant, 1956) has a tuft of lateral flagella; *Lachnospira multiparvus* (Bryant & Small, 1956*b*) has one lateral flagellum. The single flagellum or laterally placed flagella are unlikely to be fixed over the whole cell surface in formolized cultures, and some of the somatic antigens might also be taking part in agglutination.

In the calf strong agglutinins to the non-motile Gram-positive *Bifidobacterium bifidus* were present. These bifidus organisms are present in much larger numbers in the calf; they were isolated as part of the majority flora in the rumen of all three calves examined aged 3, 4 and 5 months respectively. The calf has therefore been subjected to much greater exposure to bifidus than has the adult animal.

As natural antibodies are reported to be of the IgM type, it was to be expected that the agglutinins specific for the rumen organisms would be IgM. The lactating bovine mammary gland is not very permeable to immune globulins, although low agglutinin titres against streptococci, staphylococci and coliforms have been detected in milk (Reiter & Oram, 1967). During the dry period the gland becomes selective and concentrates the immunoelectrophoretically fast globulins of IgG (Murphy, Aalund, Osebold & Carroll, 1964; Pierce & Feinstein, 1965). However IgM has been detected by immunoelectrophoresis in colostrum and in calf serum after feeding colostrum (Popovici & Jurenkova, 1967; Baglioni & Fioretti, 1967). Dr W. J. Penhale ((Royal Dick) School of Veterinary Studies, Edinburgh, personal communication and 1965) has made quantitative studies of the concentration of IgM in colostrum and its absorption into the calf serum using purified IgM, IgG; he also found that IgM was absorbed through the calf intestines.

The specific agglutinins against rumen organisms were found to be sensitive markers of the transfer of IgM to the secretion and colostrum and then to the calf serum. Our results show that IgM antibodies against rumen organisms were present in the same concentration in the colostrum as in the blood serum, indicating that there was no selective concentration of this type of immunoglobulin in the udder. The increased titres in the non-lactating secretion compared with those in blood serum are due to physical concentration because of the re-absorption which takes place during drying off. Our results also confirm that in the calf IgM is freely taken up through the gut.

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Antimicrobial Proteins Isolated from the Teat Canal of the Cow

By K. G. HIBBITT, C. B. COLE
Institute for Research on Animal Diseases, Compton, Newbury, Berkshire
AND B. REITER
National Institute for Research in Dairying, Shinfield, Reading, Berkshire

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SUMMARY

Proteins which inhibited the growth of two strains of *Staphylococcus aureus* and one strain of *Streptococcus agalactiae* were isolated from the teat canal keratin of the cow. Electrophoresis of the proteins on polyacrylamide gels suggested that they were basic. They were separated into six bands at pH 3·0 but gave only two bands at pH 4·5 and two lines of precipitin after gel diffusion and immunoelectrophoresis at pH values between 4·5 and 8·5. In the presence of the anionic polymers DNA or heparin, complexes were formed which were not inhibitory of bacterial growth. The isolated proteins and the whole teat canal keratin were completely free from lysozyme. Their role is discussed in relation to the natural defence mechanisms of the teat canal.

INTRODUCTION

The teat canal is generally regarded as a barrier to artificial and natural infections of the mammary gland (Murphy & Stuart, 1953; Fincher, Hodges, Murphy & Morse, 1956; Plastridge, 1958). Pathogens placed beyond the teat canal into the teat cistern invariably lead to infection (Murphy & Stuart, 1954; Newbould & Neave, 1965; Hibbitt & Jones, 1967). The teat canal may function as a mechanical barrier due to its efficient sphincter muscles but, in addition, it has been reported to possess antimicrobial properties which can be attributed to the sebum-like material, called teat canal keratin, which probably originates from the heavily keratinized squamous epithelium (Murphy, 1959). Teat-canal keratin was reported to consist of up to 90% lipid and has been shown to inhibit the growth of streptococci *in vitro* (Adams & Rickard, 1963), but the lipid content of the keratin and its role in natural resistance has now been disputed (Treese, Morse & Levy, 1966). More recently cationic proteins have been isolated from teat-canal keratin and they strongly inhibited the growth of staphylococci and streptococci isolated from mastitic udders *in vitro* (Hibbitt & Cole, 1968).

This paper describes some of the properties of the proteins isolated from teat canal keratin.

METHODS

The isolation of antimicrobial protein fractions from teat-canal keratin. Teat-canal keratin was obtained from lactating and dry cows of the Institute herd by the 'reaming' procedure described by Murphy & Stuart (1953); larger amounts came from cows slaughtered at the abattoir. All keratin samples were stored at $-20°$ until used.

The keratin was extracted by a procedure based on the method of Zubay & Wilkins (1962). Approximately 300 mg. tissue were homogenized for 2 min. in 25 ml. 0·15 M-NaCl in an MSE blender. The homogenate was centrifuged at 12,000 *g* (av.) for 20 min. and the supernatant fluid discarded. The precipitate was resuspended in 25 ml. glass-distilled water, and an equal volume of 0·5 M-HCl containing 0·2 M-barium acetate was added slowly and mixed thoroughly. The mixture was rehomogenized for 1 min. in the barium acetate solution, stirred for 15 min. and centrifuged at 20,000*g* (av.) for 30 min. The supernatant fluid was dialysed against four changes of 5 l. glass-distilled water for 48 h. After dialysis the proteins were freeze-dried and chromatographed on a carboxymethylcellulose column equilibrated with an 0·1 M-acetate buffer (pH 4·2); after exhaustive washing with the buffer they were eluted with 0·2 M-HCl. The eluted proteins which appeared as a single peak were dialysed and freeze-dried. All operations were done at 4°.

Disc electrophoresis. The proteins were separated by electrophoresis in polyacrylamide gels at pH 3·0 and pH 4·5 by the procedure described by Narayan, Narayan & Kummerow (1964). A buffer consisting of 0·1 M-tartaric acid + 0·1 M-formic acid containing 0·01 M-EDTA and 6 M-urea was used for the separation at pH 3·0. For the experiments at pH 4·5 the same concentration of EDTA was added to an 0·1 M-acetate-buffer.

Preparation of proteins for assay. Approximately 2·0 mg. protein were shaken in 4 ml. 0·01 M-citric acid + Na_2HPO_4 buffer (pH 7·0) containing 0·1 M-NaCl. The undissolved protein was sedimented at 1500 *g* for 10 min. and the protein content of the supernatant determined spectrophotometrically (Layne, 1957). The volume of this solution was finally adjusted to give a protein concentration of 150 μg./ml.

Organisms. Cultures of two strains of *Staphylococcus aureus* (42D and 52B phage type) were grown in Oxoid nutrient broth and *Streptococcus agalactiae* S13 (Pattison, 1948) in Todd–Hewitt medium. After incubation at 37° for approximately 6 hr they were harvested while still in their logarithmic growth phase and diluted in physiological saline to give a count between 50 and 100 organisms/0·02 ml. This bacterial suspension could be stored at 4° for up to 2 days without deterioration.

Antimicrobial assay. The teat-canal proteins were assayed for inhibition of the test organisms by the procedure described by Hirsch (1958), with the exception that the colonies were counted in a Hannay counting chamber.

Lysozyme assay. Lysozyme was assayed by the procedure describe by Shugar (1952).

Preparation of antisera. Rabbits were immunized by a course of four intramuscular injections, given at weekly intervals, and consisting of 10 mg. of antimicrobial protein extracted from teat-canal keratin in 0·5 ml. of complete Freund's adjuvant (Difco). Three weeks after the last inoculation the sera were harvested.

Immunoelectrophoresis. The micro method of Scheidegger (1955) was used with 0·02 M-veronal, 0·004 M-EDTA buffer (pH 8·6) or 0·05 M-KH_2PO_4 buffer (pH 4·5). The applied potential was 75 V/cm. for 1 hr.

RESULTS

The antimicrobial proteins extracted in this study constituted approximately 4–5 % (wet weight) of the teat canal keratin. These proteins which are soluble in distilled water and dilute acids were subjected to polyacrylamide gel disc electrophoresis. At pH 4·5 the proteins were resolved into two principal bands which moved towards the cathode. At pH 3·0, however, the proteins moved more rapidly towards the cathode and were resolved into 6 bands. The ultraviolet absorption curve (Fig. 1) of the extracted proteins showed a characteristic pattern for material with minimal nucleic acid contamination, a major absorption peak occurring at 276 mμ.

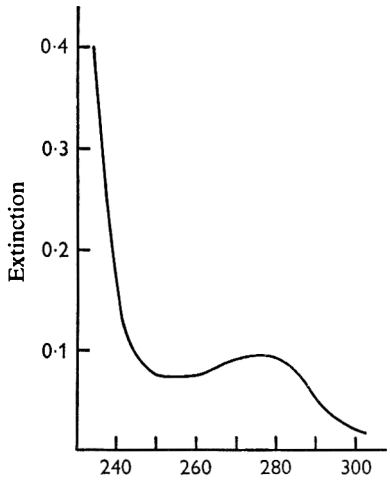

Fig. 1

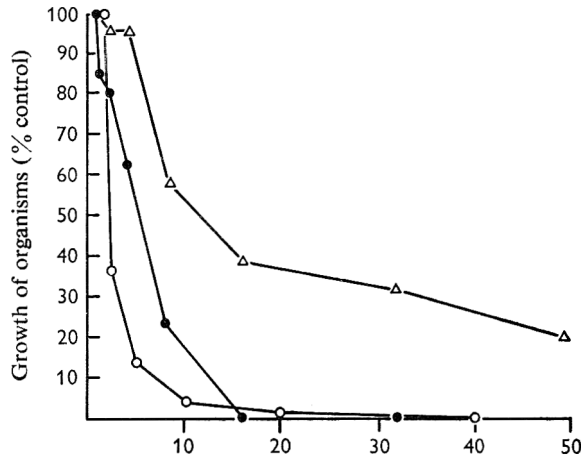

Fig. 2

Fig. 1. The ultraviolet absorption spectrum of antimicrobial proteins isolated from teat-canal keratin.

Fig. 2. The inhibitory effect of proteins isolated from teat canal keratin on the growth of micro-organisms. ○--○, *Staphylococcus aureus* 42D; ●—●, *Staphylococcus aureus* 52B; △—△, *Streptococcus agalactiae* S13.

Inhibition of staphylococci and streptoccoci by teat canal proteins. The effect of various concentrations of the teat canal proteins on the growth of the test organisms is shown in Fig. 2. The growth of the two strains of *Staphylococcus aureus* was inhibited by 50 % at a concentration of 2–5 μg. protein/ml. while *Streptococcus agalactiae* was inhibited at 11·5 μg./ml.

Removal of inhibition by anionic polymers. Electrophoresis had indicated that the proteins were basic; it was considered, therefore, that anionic polymers would combine with the teat-canal proteins and destroy their biological activity. The proteins were incubated at 37° for 15 min. with the anionic polymers DNA and heparin and assayed for their inhibitory activity. Table 1 shows that the inhibition of staphylococcal growth by the teat canal proteins at a concentration of 8 μg./ml. was progressively destroyed by increasing concentrations of both anionic polymers with complete destruction occurring at 3·2 μg./ml.

Absence of lysozyme in the teat canal protein preparations. Although staphylococci and streptococci are not susceptible to lysozyme (also a basic protein) in the sense that the cells are lysed, it is known that lysozyme can decrease the viable count of staphylococci (Kern, Kingkade, Kern & Behrens, 1951). As this enzyme is known to be widely distributed in biological fluids and tissues it was thought to be of some interest to test the protein preparations from the teat canal for their lysozyme content. There was no lysozyme activity in the teat canal protein preparation or in the original keratin (Table 2).

Table 1. *The effect of DNA and heparin on the antibacterial activity of the teat canal proteins*

Organism:	Treatment	Growth (%)
*Staphylococcus aureus.**	Nil	100·0
	Incubated† with cationic proteins	0·0
	Incubated with cationic proteins complexed‡ with:	
	0·4 μg. DNA	0·0
	0·8 μg. DNA	0·7
	1·6 μg. DNA	49·2
	3·2 μg. DNA	100·0
	0·2 μg. heparin	0·0
	0·4 μg. heparin	2·1
	0·8 μg. heparin	5·0
	1·6 μg. heparin	69·2
	3·2 μg. heparin	100·0

* Approximately 70 colony forming units of *Staphylococcus aureus* were incubated for 30 min. in 1 ml. 0·01 M-citric acid + Na_2HPO_4 buffer (pH 7·0) then grown in Oxoid nutrient broth containing 1 % Davis agar.

† *Staphylococcus aureus* was incubated for 30 min. in 1 ml. of the buffer described above containing 8 μg. of the cationic proteins.

‡ The protein complex was prepared by incubating 8 μg. of the cationic proteins with DNA or heparin in 1 ml. of the buffer described above at 37° for 15 min.

Table 2. *Test for lysozyme content of whole teat canal keratin and partially purified antimicrobial proteins*

	Lysozyme (units/mg. protein*)
Standard lysozyme	8600
Whole teat canal keratin	0
Antimicrobial proteins from teat canal keratin	0

* One unit of activity is equivalent to a decrease in absorbency at 450 mμ of 0·001 per min. at pH 7·0 and 25°.

Gel-diffusion tests. The agar gel-diffusion test which is normally performed at pH 7·0 suggested initially some degree of homogeneity of the protein since it showed only one line of identity at a concentration of 2·5 mg./ml. against homologous antiserum. When the concentration of the protein was decreased to 1·25 mg./ml. there was an indication that two precipitin lines may have been present. This result was not sufficiently clear to record; on the other hand two lines could be expected since two bands were also identified in the electrophoresis studies at pH 4·5.

Immunoelectrophoresis. The double line which was thought to be present in the gel diffusion test at the lower concentration was confirmed by immunoelectrophoresis at pH 8·6 (Fig. 3) since two arcs of precipitation were formed against the antiserum. Immunoelectrophoresis is normally not performed at a low pH because of the danger of precipitating the serum proteins and the possible streaking of the protein. Therefore it was impossible to study the effects of the antisera on the protein fractions separated at pH 3·0. On the other hand, at pH 4·5, the pH value which separated the proteins into two principal bands on polyacrylamide gel, two arcs were formed which moved towards the cathode (Fig. 4).

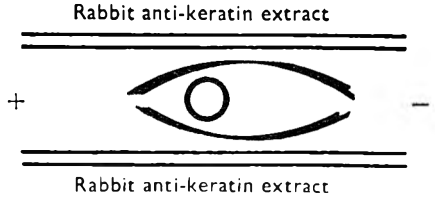

Fig. 3. Immunoelectrophoresis of teat canal antibacterial protein at pH 8·6.

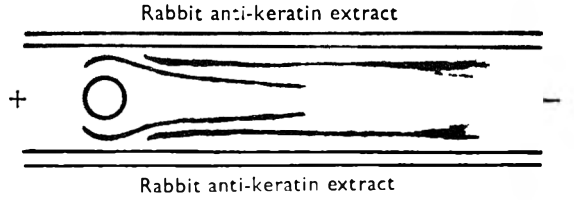

Fig. 4. Immunoelectrophoresis of teat canal antibacterial protein at pH 4·5.

DISCUSSION

An investigation of the natural methods of protecting the bovine mammary gland against the invasion of micro-organisms may lead to a better understanding of the reasons why animals vary in their susceptibility to mastitis. The bacteriostatic activity of the lipids in the teat-canal keratin (Adams & Rickard, 1963) is possibly only one of the many factors involved in natural defence since cationic proteins isolated from various tissues have been shown to possess bactericidal properties *in vitro* (Scarnes & Watson, 1957; Hirsch, 1958; Zeya & Spitznagel, 1966). It is therefore conceivable that the cationic proteins isolated from the teat canal keratin may act *in vivo* as part of the protective mechanism against invasion of pathogens.

The behaviour of the isolated proteins during electrophoresis on polyacrylamide gels suggested that they contain a predominating number of positively charged groups. This possibility was further supported in the experiment, where it was shown that the antimicrobial activity of the proteins was lost when they were pretreated at pH 7·0 with negatively charged substances such as DNA or heparin. The positively charged proteins of teat-canal keratin would in all probability also be linked electrovalently to negatively charged components such as other proteins or nucleic acids, with a consequent loss of activity. This was borne out in a single experiment, not recorded in this paper, when it was found that isolated whole keratin had no anti-

microbial activity. However, in a living teat canal, proteins are being synthesized continuously and would be free to bind to any negatively charged material including micro-organisms.

The complete absence of lysozyme in the isolated proteins and in whole teat-canal keratin was not surprising since Padgett & Hirsch (1967) were unable to demonstrate its presence in tears, saliva, nasal exudates and peritoneal leucocytes obtained from a group of ten cattle. Furthermore, the morphological changes produced by lysozyme on *Staphylococcus aureus* were markedly different from those produced by the proteins isolated from teat-canal keratin (McMillan & Hibbitt, 1969).

Although six bands of protein were separated on polyacrylamide gels at pH 3·0, at pH 4·5 only two principal bands were observed. The resolution of closely related positively charged proteins would be less marked at higher pH values as their isoelectric points are approached. In the immunological studies therefore, which were necessarily carried out on the teat canal proteins at higher pH values, the separation of only two antigens is understandable.

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The Effect of Antimicrobial Proteins on the Fine Structure of *Staphylococcus aureus*

By W. G. MACMILLAN AND K. G. HIBBITT
Institute for Research on Animal Diseases, Compton, Newbury, Berkshire

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SUMMARY

Changes in the morphology of staphylococci incubated with antimicrobial cationic proteins isolated from the teat canal of the cow were studied with the electron microscope. Morphological changes in similar organisms deposited in the teat canals of a healthy cow for 24 hr and subsequently recovered were also examined. In both instances changes were observed in the cell wall and plasma membrane. Accumulation of a dense layer of finely fibrillar material on the surface of the organism was also observed.

Calf thymus histone, lysozyme, protamine sulphate and poly-L-lysine also produced morphological changes in staphylococci *in vitro*. The changes induced in staphylococcal morphology following incubation with calf thymus histone closely resembled those produced by the materials of teat canal origin. Incubation with lysozyme induced lysis of limited regions of the cell wall; incubation with protamine sulphate and poly-L-lysine produced an agranular cytoplasm and the loss of nuclear areas.

INTRODUCTION

In an investigation of factors associated with protection of the bovine mammary gland against bacterial invasion, Hibbitt & Cole (1968) and Hibbitt, Cole & Reiter (1969) isolated antimicrobial proteins from the keratinous layers of the teat canal. These proteins were inhibitory to strains of *Streptococcus agalactiae* and *Staphylococcus aureus* capable of producing mastitis.

In a review on antimicrobial tissue factors Scarnes & Watson (1957) described the binding of cationic proteins to the surface of micro-organisms, but the cause of death of the organism was not determined. Hirsch (1958) studied the effects of histones on microbial growth and suggested that the adsorbed proteins may damage the osmotic barrier of the cell wall, thereby impairing respiration. In a more recent study of the antimicrobial cationic proteins isolated from lysosomes of guinea-pig polymorphonuclear leucocytes Zeya & Spitznagel (1966) suspected a disturbance of cell respiration associated with damage to the cell membrane.

In the experiments described above, little attention was paid to the detailed morphological changes produced in the micro-organisms by the growth-inhibitory proteins. The following experiments were designed to study the changes produced in a strain of *Staphylococcus aureus* by a known antibacterial substance, penicillin, and by various cationic polymers including calf thymus histone, protamine sulphate, poly-L-lysine, lysozyme and a protein fraction isolated from the teat canal keratin of the cow.

METHODS

Materials. Teat canal proteins were prepared as described previously (Hibbitt *et al.* 1969). The calf thymus histone fraction F2A was purchased from the Sigma (London) Chemical Co. Ltd., the protamine sulphate and poly-L-lysine from British Drug Houses Ltd., and egg-white lysozyme from Worthington Biochemical Corporation. Penicillin G was supplied by Glaxo Laboratories Ltd..

The treatment of bacterial suspensions with cationic proteins. The effect of various cationic proteins was studied on cultures of *Staphylococcus aureus* (42D phage type) isolated originally from the mammary gland of a cow suffering from mastitis. A 5·0 ml. nutrient broth (Oxoid) culture of the organism was incubated for 16 hr at 37° then centrifuged at 2000 *g* for 15 min. The sedimented organisms were washed twice by resuspending in isotonic saline (0·15M) and centrifuging to remove all traces of the broth. The organisms were finally suspended in 2·0 ml. isotonic saline containing the protein under investigation at a concentration of 250 μg./ml. After a further period of incubation at 37° for 1 hr the suspensions were centrifuged at 2000 *g* for 15 min. and the pellets sectioned and studied in the electron microscope. In the control experiments the organisms were incubated in isotonic saline alone. Penicillin G was added to the saline to 250 μg./ml. when the effect of this antibiotic was being studied.

Deposition of staphylococci within the teat canal. In an *in vivo* experiment to study the morphological changes which occur in pathogenic staphylococci inoculated into the teat canal, approximately 4×10^7 organisms in 0·1 ml. isotonic saline were deposited in the teat canal of a lactating cow at a point 3 mm. from the external orifice by the procedure of Hibbitt & Jones (1967). The suspension of organisms was prepared by centrifuging 100 ml. of an overnight nutrient broth culture at 2000 *g* for 20 min. and resuspending the pellet in 10 ml. isotonic saline. The cow was milked immediately before the organisms were injected; after the injection, the cow was left unmilked and then slaughtered 24 hr later. The teats were removed, unwanted tissue dissected away and portions of the teat canals prepared for electron microscopy.

Electron microscopy. Pellets of the treated staphylococci and portions of the teat canals were fixed in ice-cold 1% osmium tetroxide at pH 7·3 (Millonig, 1961) for 4 hr. The fixed materials were washed in a 70% (v/v) methanol in water, dehydrated in absolute methanol and embedded in Araldite. Sections were cut with diamond knives on a Reichert ultramicrotome, stained with uranyl acetate, as described by Stempak & Ward (1964), and examined in an A.E.I. EM6 electron microscope.

RESULTS

Normal staphylococci. A detailed study was made of the fine structure of normal organisms to control the effects of various antimicrobial proteins on the morphology of staphylococci. Plate 1, fig. 1 shows a normal organism after a 60 min. incubation period in isotonic saline. A series of concentric light and dark layers, similar to those described by Suganuma (1965), were seen at the periphery of the organism. The cell wall was composed of two dense layers with an intervening less densely stained layer (layers *a* to *c* Pl. 1, fig. 1). Adjoining the cell wall was a less densely stained layer which constituted the middle layer of the bacterial plasma membrane (of which the outer and inner densely stained layers were confluent with the cell wall and cytoplasm respectively,

and were not separately identified). The organisms illustrated in Pl. 1, fig. 1, were dividing, their cytoplasm was finely granular with occasional discrete larger granules, identified by Glauert (1962) as ribosomes. The nuclear areas (*n*) were represented by the less dense areas in the central parts of the dividing cell.

The effect of antimicrobial proteins isolated from the teat canal on staphylococci. Organisms (Pl. 1, fig. 2) incubated with teat canal antimicrobial proteins showed anatomical changes. One organism possessed a relatively intact cell wall, although its outermost densely staining layer appeared to be absent; the other organisms did not have a discrete cell wall. In all cases the outermost layers of the organisms appeared to be surrounded by a layer of fibrillar material.

Morphological changes in staphylococci recovered from the teat canal. In the previous figure, the deviations from normal in the morphology of staphylococci were produced by the action of an antimicrobial substance in an *in vitro* experiment. However, changes were also observed in the organisms in an *in vivo* experiment in which the organisms were placed in the teat canal and recovered 24 hr later. In most cases the changes resembled those produced by teat canal antimicrobial protein (Pl. 1, fig. 3); the organisms possessed a densely staining cytoplasm but lacked a discrete cell wall. The outermost layer of each cell wall was surrounded by a layer of densely staining fibrillar material.

The effect of calf thymus histone on staphylococci. Cocci incubated with calf thymus histone F2A (Pl. 1, fig. 4) resembled those treated with teat canal antibacterial proteins. The nuclear areas were reduced in size or in some cases had disappeared completely. The triple-layered plasma membrane had ruptured in places and the cell wall was altered retaining its trilaminar structure in certain regions only. The outer surface appeared to be covered by a relatively dense layer of finely fibrillar material. Damage to the cell wall varied from a relatively intact, but altered, structure to an organism completely lacking a wall.

The effect of lysozyme on staphylococci. The incubation of staphylococci with egg-white lysozyme resulted in a densely stained cell wall, the profile of which was decreased in thickness in some regions. In some cases, the cell wall and plasma membrane had ruptured leading to an extrusion of cytoplasm (Pl. 2, fig. 5).

The effect of protamine sulphate and poly-L-lysine on staphylococci. Protamine sulphate and poly-L-lysine produced very similar effects on cultures of staphylococci (Pl. 2, fig. 6, 7). In both cases the cell wall remained intact, with a faint deposit of electron dense material on the outer surface. The cytoplasm was agranular and nuclear areas were absent. Membranous bodies (mesosomes) were associated with the cell wall and with the septum betwen dividing organisms.

The effect of penicillin on staphylococci. Organisms treated with penicillin (Pl. 2, fig. 8) were greatly damaged. The densely stained cell wall was ruptured in several places as was the plasma membrane. Residual cytoplasm was fibrous and clumped.

DISCUSSION

An investigation of the action of cationic proteins isolated from teat canal keratin on the morphology of staphylococci was made following *in vitro* studies which demonstrated a marked antimicrobial activity (Hibbitt, & Cole, 1968; Hibbitt *et al.* 1969). In the previous studies which included experiments with the anionic dye Fast Green

FCF at pH 8·0, it was suspected that the cationic protein fractions became attached to the surface of the micro-organisms before inhibiting their growth. It appeared these bound proteins may have produced some morphological changes which led to the death of the micro-organism. In the present study marked morphological changes were observed in organisms incubated with teat canal proteins. These changes closely resembled those observed in the *in vivo* studies reported above.

Although the antibacterial activity of cationic proteins was not a new finding, less is known of their action upon the fine structure of micro-organisms. The morphological changes produced in staphylococci by protamine sulphate, poly-L-lysine and lysozyme showed little resemblance to those produced by the teat canal proteins and calf thymus histone. Experiments in which staphylococci were incubated with penicillin allowed a comparison to be made with a substance which had a known action on cell walls.

Particular attention was paid to the effects of lysozyme on staphylococci, in accordance with its possible function as a natural protective mechanism of the body. Dubos (1945) has pointed out that it may fulfil an important function in preventing potentially pathogenic organisms from establishing themselves in a host. Kern, Kingkade, Kern & Behrens (1951) observed no marked changes in the morphology of staphylococci incubated with lysozyme (3 mg./ml.) for 6 hr at 37°, apart from a gradual decrease in electron-density at the periphery of the cell. Nevertheless, they reported a substantial decrease in the viable count. The concentration of lysozyme used in the present experiment, which was considerably less than that used by the above workers, caused some damage to the cell walls and plasma membranes of the organisms. This finding is consistent with the suggestion of Salton (1957) that lysozyme produces a slow and incomplete digestion of the cell wall of *Staphylococcus aureus*.

Our studies suggest that the presence of antimicrobial proteins in the teat canal might enable it to function not only as a mechanical barrier to invading pathogenic micro-organisms, but as a region where these organisms may be actively destroyed.

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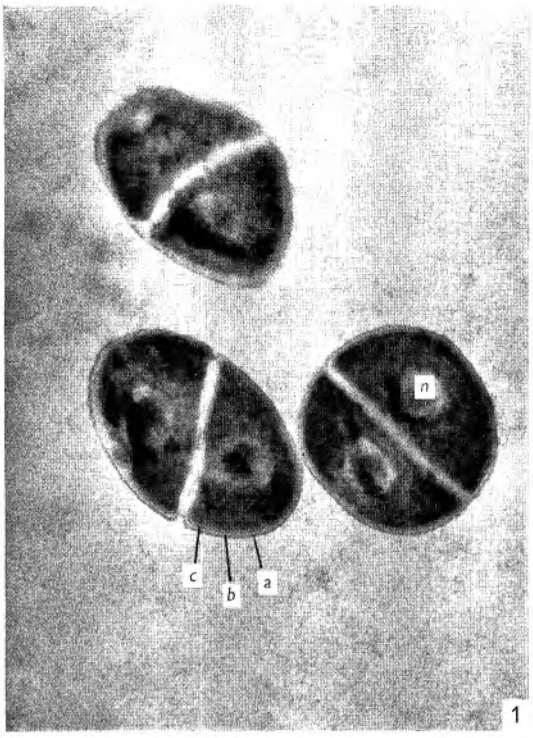

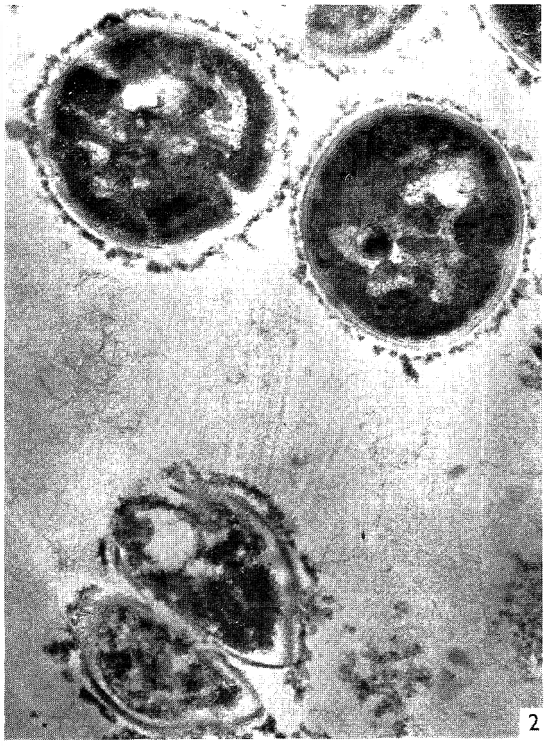

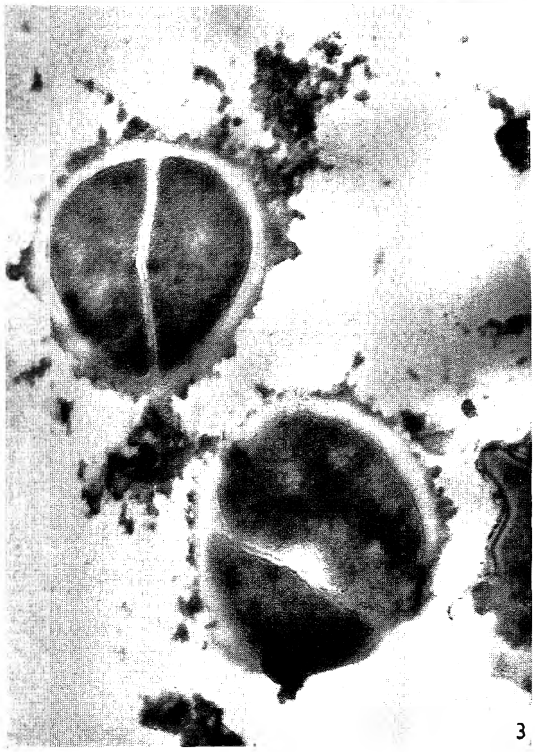

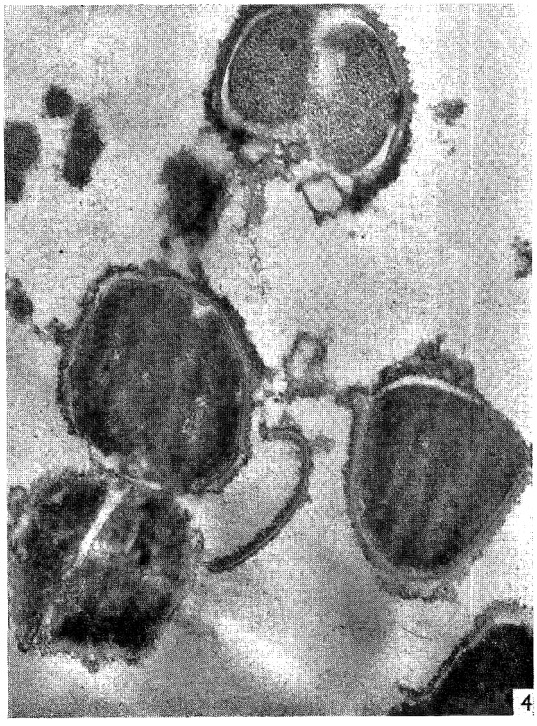

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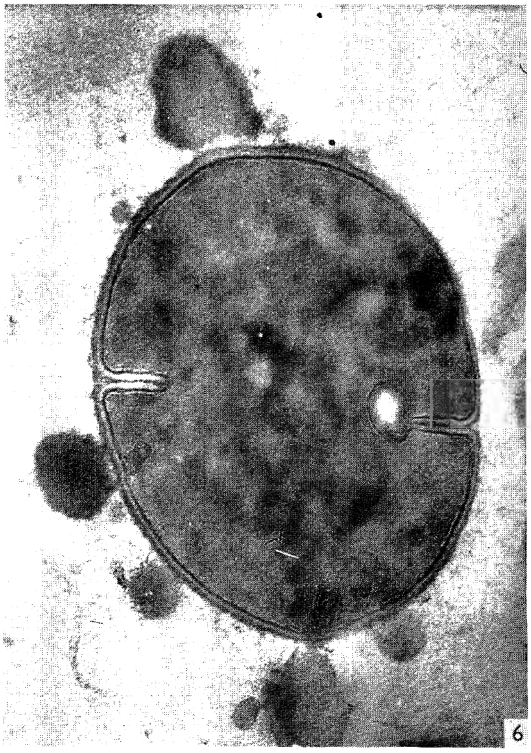

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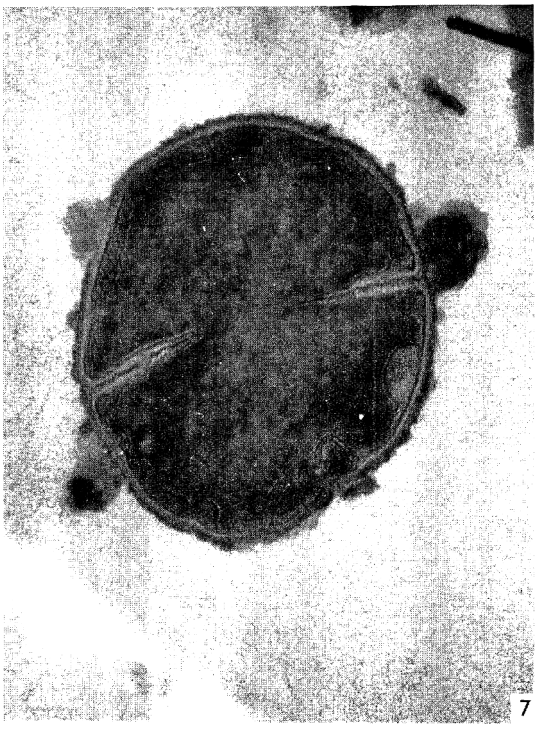

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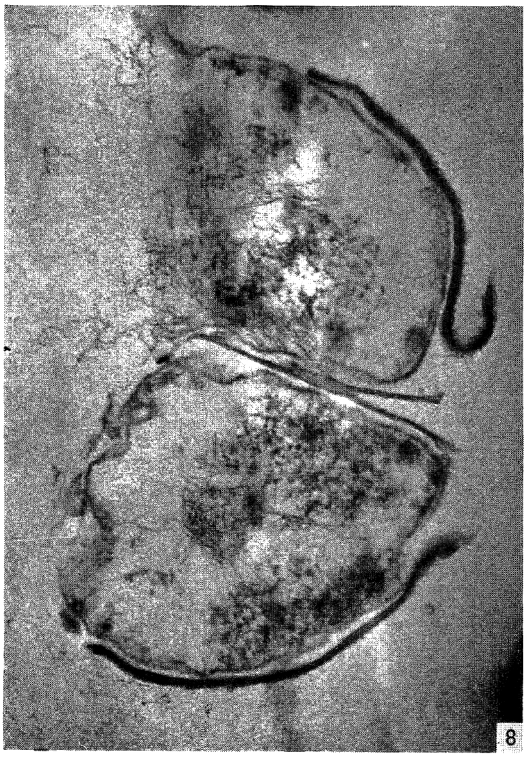

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Normal *Staphylococcus aureus*. × 56,000. (*a*) Outer densely staining layer. (*b*) Median less densely staining layer. (*c*) Inner densely staining layer. *n*, nuclear region. (*a*), (*b*) and (*c*) constitute the bacterial cell wall. The less densely staining layer inner to (*c*) denotes the middle layer of a triple layered plasma membrane of which the outer and inner layers are confluent with the cell wall and cytoplasm respectively.

Fig. 2. Staphylococci treated with antimicrobial proteins isolated from teat canal keratin (250 μg/ml.). × 64,000.

Fig. 3. Staphylococci recovered after 24 h in the teat canal of the cow. × 80,000.

Fig. 4. Staphylococci treated with calf thymus histone F2A (250 μg./ml.). × 51,000.

PLATE 2

Fig. 5. Staphylococci treated with lysozyme (250 μg./ml.). × 54,000.

Fig. 6. Staphylococci treated with protamine sulphate (250 μg./ml.). × 110,000.

Fig. 7. Staphylococci treated with poly-L-lysine (250 μg./ml.). × 100,000.

Fig. 8. Staphylococci treated with penicillin (250 μg./ml.). × 97,000.

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Aminotransferase Activity of Hartmannella (Culbertson Strain A–1) Grown Axenically

By M. M. HUSAIN AND V. K. MOHAN RAO
Division of Biochemistry,
Central Drug Research Institute, Lucknow, India

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SUMMARY

Cell-free extracts of trophozoites of Hartmannella (Culbertson strain A–1), grown axenically, catalysed transamination between 2-oxoglutarate and a number of amino acids. Aspartic acid, alanine and serine showed the maximum activity. The L-alanine-2-oxoglutarate aminotransferase (E.C. 2.6.1.2) was purified about 15-fold by adsorption with calcium phosphate gel and elution with 0·2 M-phosphate buffer. Stability during storage, pH optima in different buffers K_m values for alanine and 2-oxoglutarate, and the role of pyridoxal-5-phosphate are reported. *p*-Chloromercuribenzoate, cyanide and metal-chelating agents markedly inhibited the enzyme. Among dicarboxylic acids, maleic acid had the maximum inhibitory effect. Amoebicidal drugs and antibiotics had no marked inhibitory effect on the enzyme.

INTRODUCTION

Certain strains of the free-living amoebae belonging to the genus *Hartmannella* (Acanthamoeba) produce meningoencephalitis in mice and monkeys and are supposed to cause the same disease in man (Culbertson, Smith, Cohen & Minner, 1959; Patras & Andujar, 1966; Butt, 1966). The growth, nutritional requirements and metabolic activities (Adam, 1959, 1964; Band, 1959, 1961, 1962; Neff, 1957) of some of these strains have also been studied. The patterns of the proteolytic activities of Hartmannella (Ambrosioni, 1952; Bianchini, 1953*a*, *b*) and Acanthamoeba (Jarumilinta & Maegraith, 1961*a*, *b*) have been reported, using different substrates. In the intermediary metabolism of proteins and in the formation of pool amino-acids, transaminases play an important role. In view of this, the aminotransferase (E.C. 2.6.1.2) activity present in crude cell-free extract of trophozoites of Hartmannella has been partially purified and characterized. The action of certain amoebicidal drugs and antibiotics has also been studied and the results are presented in this communication.

METHODS

Source of enzyme. Pathogenic Hartmannella strain A–1, obtained from Dr C. G. Culbertson, was cultured axenically in medium containing Difco Proteose-peptone (1 %, w/v), Oxoid tryptone (1 %, w/v) and sodium chloride (0·5 %, w/v), pH 6·8, in 1 l. Roux flasks. After 6 days at 37°, trophozoites were harvested and washed three times with glass-distilled water. The washed cells were suspended in water and

homogenized in a Potter–Elvehjem glass homogenizer at 4°. The homogenate was centrifuged at 3000 rev./min. in a MSE refrigerated centrifuge for 30 min. The resulting opalescent crude enzyme extract was purified over calcium phosphate gel.

Assay of enzyme activity

Colorimetric method. Glutamate-pyruvate transaminase (GPT) was estimated by the method of Reitman & Frankel (1957). In a typical assay system, 0·2 ml. of the enzyme was incubated for 60 min. with 2 μmole 2-oxoglutarate, 200 μmole DL-alanine and 100 μmole Sørensen phosphate buffer (pH 7·4); total volume 1·2 ml. The reaction was stopped by addition of 1 ml. of 0·02% (w/v) 2,4-dinitrophenylhydrazine in 2 N-HCl. After 20 min. colour was developed by the addition of 10 ml. of 0·4 N-NaOH and the intensity of the colour was read at 505 mμ in a Beckman DU model spectrophotometer. Enzyme units were calculated from a standard curve for the extinction of dinitrophenyl-hydrazones of pyruvate in conditions similar to those of enzyme assay.

Chromatographic method. Wherever the above method could not be applied, paper chromatography was used for estimating the glutamate formed during transamination. The chromatogram was run for 16 hr in *n*-butanol + acetic acid + water (4 + 1 + 1 v/v) and the amino acid spots, after developing with ninhydrin, were quantitated according to the method of Giri, Radhakrishnan & Vaidyanathan (1952). In a typical assay, 0·5 ml. of reaction mixture contained 1 to 20 μmole 2-oxoglutarate, 1 to 10 μmole DL-alanine and 20 μmole phosphate buffer, (pH 7·4). The reaction was run for 4 hr at 37°, stopped by heating 5 min. in a boiling water bath and the chromatograms were run using 0·02 ml. reaction mixture. Independent experiments (without enzyme) showed that under these conditions non-enzymic transamination did not occur due to heating.

Estimation of protein. Protein in the extract was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine plasma albumin as standard.

Preparation of calcium phosphate gel. Calcium phosphate gel was prepared according to the method of Keilin & Hartree (1938) and about 8-months old gel was used for adsorption purposes.

Chemicals and reagents. DL-alanine and 2-oxoglutarate (B.D.H.) and pyridoxal-5-phosphate (E. Merck) were used. All other reagents were of analytical grade.

Samples of paromomycin sulphate (Parke Davis (India) Ltd.), chlortetracycline hydrochloride (Aureomycin: Cyanamide (India) Ltd.), cycloheximide (Actidione: Upjohn Co., U.S.A.), 4-carbamidophenyl arsonic acid (Carbarsone: Burroughs Wellcome and Co. (India), Private Ltd.), chloroquine phosphate and emetine hydrochloride (Bengal Immunity Co. Ltd.), iodochlorhydroxyquin (Enterovioform: CIBA of India Ltd.) and chlorhexidine dihydrochloride (I.C.I.) were employed in these studies.

RESULTS

Preliminary screening of the crude extract of trophozoites of Hartmannella for aminotransferase activity, using 20 common amino acids as amino-group donors and the chromatography technique, revealed that 2-oxoglutarate was a more effective amino group acceptor than pyruvate or oxalacetate. Results with some of the active amino acids are given in Table 1.

Purification of the enzyme. Table 2 illustrates partial purification of the enzyme activity by calcium phosphate gel adsorption and elution by different molar concentrations of phosphate buffer. The most active enzyme protein was eluted by 0·2 M-phosphate buffer. Due to a paucity of material further purification was not attempted. In the following experiments about 15-fold purified enzyme was utilized for the estimation of GPT activity.

Table 1. *Aminotransferase activity of cell-free extracts of Hartmannella (Culbertson strain A–1) in 2-oxoglutarate system*

Donor amino acid	μmole glutamic acid formed per mg. protein
L-Alanine	10
L-Aspartic acid	13
L-Cysteine	3
DL-Isoleucine	3
L-Methionine	0·7
L-Serine	8
L-Valine	6

Reaction conditions: 0·2 ml. of cell-free enzyme extract (43 μg. protein), 10 μmole 2-oxoglutarate, 10 μmole L-amino acid (20 μmole in the case of DL-amino acid), 20 μmole Sørensen phosphate buffer (pH 7·4), total volume 0·5 ml; incubation 4 hr at 37°. 0·02 ml. of reaction mixture was used for the paper chromatographic estimation of glutamic acid formed.

Table 2. *Purification of alanine-2-oxoglutarate aminotransferase activity of Hartmannella (Culbertson strain A–1)*

Step	Vol. (ml.)	Protein (mg.)	Activity units* × 100	Specific activity	Fold purification
Crude extract	7	18·9	126	666	1
Addition of calcium phosphate gel	7	—	—	—	—
Unadsorbed fraction	7·6	4·9	—	—	—
Elution with phosphate buffer pH 7·4					
0·05 M	5	2·6	—	—	—
0·05 M	5	1·6	—	—	—
0·1 M	5	1·2	42·5	3,541	5·5
0·1 M	5	0·8	30	3,750	5·7
0·2 M	5	1·1	95	8,636	13
0·2 M	5	0·5	55	11,000	16·5

* One unit of enzyme produces 1 mμmole pyruvate in 60 min. at 37°. Reaction conditions: 0·2 ml. enzyme, 2 μmole 2-oxoglutarate, 200 μmole DL-alanine, 100 μmole Sørensen phosphate buffer (pH 7·4), total volume 1·2 ml; incubation 60 min. at 37°.

Properties of the enzyme

Stability. The partially purified enzyme solution was distributed into two tubes which were stored separately at 4° and −10° and assayed for GPT activity at intervals. Enzyme stored at 4° progressively lost more than half of its activity in 20 days, while freezing the enzyme extract at −10° for 2 days and thawing resulted in the loss of more than half of its activity.

Coenzyme requirement. Enzyme preparations, one dialysed overnight at 4° and

the other undialysed, were incubated with various concentrations of pyridoxal-5-phosphate for 15 min. and their activities determined. Figure 1 demonstrates that the coenzyme dissociated to some extent from the apoenzyme and higher concentrations of coenzyme inhibited the GPT activity.

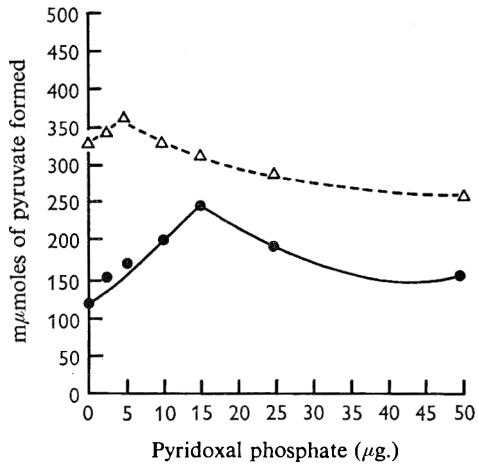

Fig. 1. Cofactor requirement of alanine-2-oxoglutarate aminotransferase of Hartmannella (Culbertson strain A–1). The enzyme was preincubated with pyridoxal-5-phosphate (0 to 50 μg.) for 15 min. Reaction conditions: 0·2 ml. enzyme (8 μg. dialysed protein, 11 μg. undialysed protein), 2 μmole 2-oxoglutarate, 200 μmole DL-alanine, 100 μmole Sørensen phosphate buffer (pH 7·4), total volume 1·2 ml.; incubation 60 min. at 37°. ●—●, Enzyme dialysed; △ – – △, undialysed.

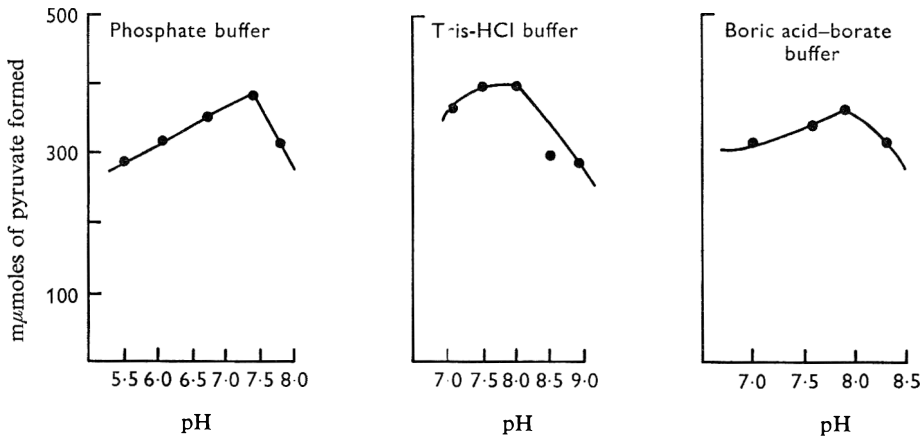

Fig. 2. Effect of pH on the alanine-2-oxoglutarate aminotransferase activity of Hartmannella (Culbertson strain A–1). Reaction conditions: 0·2 ml. enzyme (21 μg. protein), 2 μmole 2-oxoglutarate, 200 μmole DL-alanine, 100 μmole phosphate, tris-HCl, or boric acid-borate buffers, total volume 1·2 ml., incubation 60 min. at 37°. Enzyme preincubated 15 min. with 5 μg. pyridoxal-5-phosphate.

Time–activity relationship. Thirty μg. protein incubated for 15 min. with 5 μg. pyridoxal phosphate (conditions otherwise as for Fig. 1) formed pyruvate linearly for 45 min. and reached a maximum by 60 min.

Enzyme concentration. In similar experimental conditions the relationship between the amount of pyruvate formed and enzyme protein was linear between 7·5 and 60 μg.

Effect of pH. The optimum pH in phosphate buffer was at 7·4, in tris-HCl buffer in the range 7·5–8·0 and in borate buffer at 7·9 (Fig. 2).

K_m values for substrates. Amounts of glutamic acid formed at different concentrations of alanine and 2-oxoglutarate were estimated. The K_m value for alanine was $2{\cdot}22 \times 10^{-3}$ M (Fig. 3) and for 2-oxoglutarate $7{\cdot}1 \times 10^{-3}$ M (Fig. 4).

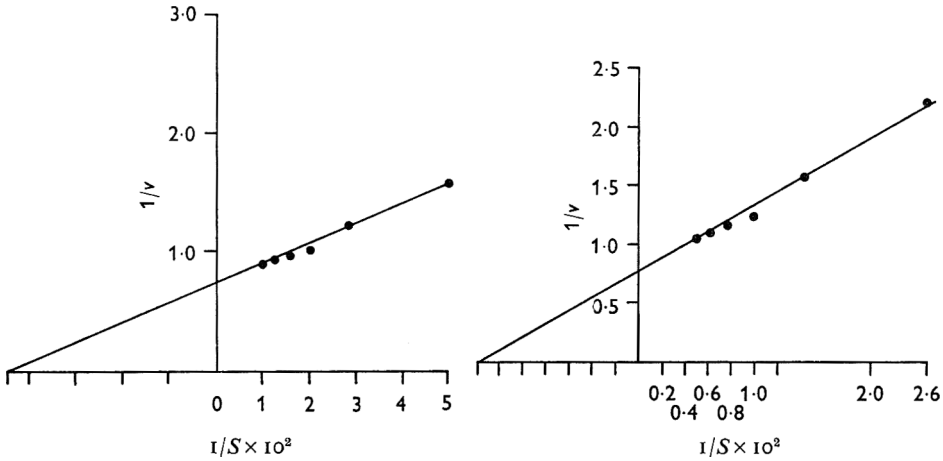

Fig. 3. Determination of K_m value for alanine. Reaction conditions: 0·2 ml. enzyme (96 μg. protein), 10 μmole 2-oxoglutarate, 0 to 10 μmole DL-alanine, 20 μmole Sørensen phosphate buffer, total volume 0·5 ml.; incubation 4 hr at 37°. 0·02 ml. reaction mixture was used for paper chromatographic estimation of glutamic acid formed.

Fig. 4. Determination of K_m value for 2-oxoglutarate. Reaction conditions: 0·2 ml. enzyme (72 μg. protein), 10 μmole DL-alanine, 0 to 10 μmole 2-oxogluterate, 20 μmole Sørensen phosphate buffer, total volume 0·5 ml.; incubation 4 hr at 37°. 0·02 ml. reaction mixture was used for paper chromatographic estimation of glutamic acid formed.

Effect of inhibitors. The actions of some common inhibitors on the enzyme activity are summarized in Table 3. Carbonyl agents such as hydrazine sulphate inhibited the enzyme by a quarter, while hydroxylamine hydrochloride inhibited the enzyme by about half. Metal chelating agents, *o*-phenanthroline, 8-hydroxyquinoline and α,α'-dipyridyl, markedly inhibited the enzyme, while EDTA had a slight stimulatory effect. Salts of heavy metals such as copper sulphate and mercuric chloride completely inactivated the enzyme. Structural analogues of pyridoxal-5-phosphate, namely isonicotinic acid hydrazide (INH) and pyridine-3-sulphonic acid at 5×10^{-3} M, inhibited the enzyme by only about one-fourth to one-fifth. Neither of these inhibitions could be annulled by 10 μg. of the cofactor. Most of the enzyme activity was inhibited by 10^{-3} M sodium cyanide. The sulphydryl reagent *p*-chloromercuribenzoate (*p*CMB) at 10^{-4} M completely inhibited the enzyme, while 10^{-3} M-iodoacetate had no effect on the enzyme activity. The inhibitory action of *p*CMB could not be annulled by 5×10^{-5} M-cysteine. Cysteine at 10^{-2} M concentration, at neutral pH, inhibited the enzyme completely, but at 5×10^{-5} M it activated the enzyme by a quarter.

Effect of dicarboxylic acids. Fumarate, malate, succinate had a slight inhibitory

Table 3. *Effect of activators and inhibitors on alanine-2-oxoglutarate aminotransferase activity of Hartmannella (Culbertson strain A–1)*

Activator/Inhibitor	Concentration (M)	Inhibition (%)*
Hydroxylamine hydrochloride	1×10^{-4}	−54
Hydrazine sulphate	1×10^{-4}	−25
Sodium iodoacetate	1×10^{-4}	Nil
Sodium cyanide	1×10^{-3}	−95
Isonicotinic acid hydrazide (INH)	1×10^{-2}	−22
Pyridine-3-sulphonic acid	1×10^{-3}	−96
p-Chloromercuribenzoate (pCMB)	1×10^{-4}	−100
8-OH-quinoline	1×10^{-2}	−88
EDTA	1×10^{-2}	+8
α,α'-Dipyridyl	1×10^{-2}	−61
o-Phenanthroline	1×10^{-2}	−91
Sodium succinate	1×10^{-2}	−6
Sodium fumarate	1×10^{-2}	−15
Sodium malate	1×10^{-2}	−22
Sodium maleate	1×10^{-2}	−52
Sodium oxalate	1×10^{-2}	+10
Copper sulphate	$3{\cdot}6 \times 10^{-4}$	−100
Mercuric chloride	5×10^{-4}	−100
Cysteine hydrochloride	1×10^{-2}	−100
*p*CMB	5×10^{-5}	−100
Cysteine hydrochloride	5×10^{-5}	+26
*p*CMB + cysteine hydrochloride	5×10^{-5} each	−100
Pyridine-3-sulphonic acid	5×10^{-3}	−29
Pyridine-3-sulphonic acid + 10 μg. pyridoxal-5-phosphate	5×10^{-3} each	−30
INH	5×10^{-3}	−17
INH + 10 μg. pyridoxal-5-phosphate	5×10^{-3} each	−20

0·2 ml. inhibitor/activator or water (control) was incubated with 0·2 ml. enzyme for 15 min. and then 1 ml. of the buffered substrate (containing 2 μmole of 2-oxoglutarate and 200 μmole of DL-alanine and 100 μmole Sørensen phosphate buffer at pH 7·4) was added. The reaction was then run for 60 min. at 37°.

* −, Inhibition; +, Activation.

Table 4. *Effect of amoebic drugs and antibiotics on alanine-2-oxoglutarate aminotransferase of Hartmannella (Culbertson strain A–1)*

Drugs	Inhibition (%)
Cycloheximide	3
Chlortetracycline hydrochloride	14
4-Carbamidophenyl arsonic acid	20
Chlorhexidine dihydrochloride	34
Chloroquine phosphate	11
Emetine hydrochloride	11
Iodochlorhydroxyquin	11
Paromomycin sulphate	5

0·2 ml. of enzyme (36 μg. protein) was preincubated for 15 min. with 0·2 ml. of respective drug (1 mg./ml.) or water (control) and the reaction was run as described in Table 3.

effect on the enzyme, while oxalate had a slight stimulatory influence. Only maleate inhibited the enzyme by half.

Effect of antiamoebic drugs and antibiotics. The action of some antiamoebic drugs and antibiotics on the GPT of Hartmannella are presented in Table 4. Chlorhexidine dihydrochloride inhibited the enzyme by one-third. None of the antibiotics inhibited the enzyme markedly. Among the other potent amoebicidal drugs, 4-carbamidophenyl arsonic acid inhibited the enzyme to the maximum extent of one-fifth, while the remaining three drugs, namely emetine hydrochloride, chloroquine phosphate and iodochlorhydroxyquin, inhibited it by about one-tenth.

DISCUSSION

Just like in the aminotransferase system of *Entamoeba histolytica* (Mohan Rao & Dutta, 1966), 2-oxoglutarate was the most active amino-group acceptor compared with pyruvate and oxalacetate in the Hartmannella system. Valine, asparagine, leucine and aspartic acid have been reported to be the most active amino-group donors to 2-oxoglutarate in *E. histolytica* system, but in the Hartmannella aspartic acid, serine and alanine were most active. Serine was actively transaminated by Hartmannella, whereas *E. histolytica* failed to transaminate serine under similar experimental conditions. While Hartmannella poorly transaminated leucine, the same amino acid was actively transaminated by *E. histolytica* (Mohan Rao & Dutta, 1966). Amoebicidal drugs and antibiotics had almost the same type of effect either on Hartmannella or *E. histolytica* (Mohan Rao & Dutta, 1966) enzymes. In both these enzyme systems the maximum inhibition of about 30 % was effected by chlorhexidine dihydrochloride.

Higher concentrations of pyridoxal-5-phosphate inhibited either dialysed or undialysed enzyme extracts of Hartmannella. Similar findings were reported with the aminotransferase systems of *E. histolytica* (Mohan Rao & Dutta, 1966), animal tissues (Roberts & Bregoff, 1953) and human intestines (Ramaswamy & Radhakrishnan, 1964).

The inhibition caused by some dicarboxylic acids, especially by maleate, may be explained on the basis of an earlier observation (Jenkins, David & Irwin, 1959) that the enzyme forms an inactive complex with these acids.

The K_m value for alanine for the aminotransferase activity of Hartmannella compares favourably with those reported for cotton seeds (Turano, Bossa, Fasella & Fanelli, 1966) and equine erythrocytes (Balasarswathi & Krishna Murti, 1967).

The above discussion focuses some of the differences in the properties of aminotransferase activities of the two pathogenic amoebae, one belonging to free-living and the other to parasitic groups of organisms.

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Delayed Multiplication of Newly Capsulated Transformants of *Haemophilus influenzae* Detected by Immunofluorescence

By B. W. CATLIN AND V. R. TARTAGNI

Department of Microbiology, Marquette School of Medicine, Milwaukee, Wisconsin 53233, *U.S.A.*

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SUMMARY

Capsular transformation of *Haemophilus influenzae* strain RD was examined at the cellular level by a semi-quantitative method. Type b capsular antigen synthesized by transformants was detected by its reaction with type b antibody conjugated to fluorescein isothiocyanate. Deoxyribonucleate (DNA) preparations from type b capsular transformants of strain RD elicited somewhat higher frequencies of transformation than preparations from type b clinical isolates. *Str-r* markers on these DNAs conferred resistance to a concentration of 500 μg. dihydrostreptomycin sulphate/ml. For a given DNA, the frequency of transformation to streptomycin resistance was at least 100 times higher than that to capsular synthesis. The time course of expression of the two phenotypes by DNA-treated bacteria was examined in broth in which the generation time of the total population was about 50 min. Resistant transformants began to appear 25 min. after initiation of treatment and capsulated bacteria were observed after 40 min. Both properties continued to be expressed until about 120 min. Thereafter, the behaviour of recipients of the two kinds of genetic markers differed. The rate of increase of the *str-r* transformants became equal to that of the total population, and in a subculture diluted 1/20 they increased 20-fold during subsequent incubation for 150 min. The number of capsulated transformants, on the other hand, increased less than two-fold over the period 120–330 min.

INTRODUCTION

The capsules of *Haemophilus influenzae* are composed of various immunologically distinct compounds (Leidy, Hahn, Zamenhof & Alexander, 1960), those of type b being polyribophosphate (Rosenberg & Zamenhof, 1961). The colonies produced by capsulated *H. influenzae* are smooth and highly iridescent. Occasionally, spontaneous genetic changes occur which result in inability to produce capsules, and these mutants can be detected by the lack of iridescence of their colonies (Pittman, 1931). From a type d *H. influenzae* clinical isolate, Alexander & Leidy (1951) selected such a non-capsulated, non-iridescent strain, RD, and showed serologically that it failed to synthesize capsular antigen. By transformation, however, a fraction of the population of recipient strain RD may regain the ability to produce capsules and, accordingly, the typical colonial iridescence. The serological specificity of the transformant capsules depends on the specific genetic information supplied, being type d or type b in accordance with the capsular type of the DNA-donor (Alexander & Leidy, 1951). Furthermore,

the production of new capsular types (ab or ad) by transformants of strain RD was demonstrated (Leidy, Hahn & Alexander, 1953).

Investigations of capsular transformation were hampered, however, by inability to examine the kinetics of transformation at the cellular level. The low frequencies of transformation prohibited direct observation and enumeration of bacteria which developed capsules following phenotypic expression of the information specified by the entering DNA molecules. Although various methods have been employed in previous studies of capsular transformation, they have all dealt exclusively with those altered bacteria which are capable of multiplying to produce recognizable transformant clones (e.g. Alexander & Leidy, 1951; Ravin, 1954; Bernheimer, Wermundsen & Austrian, 1967).

Immunofluorescence techniques now available provide a means of tracing specific bacterial antigens (e.g. Sell, Cheatham, Young & Welch, 1963; Biegeleisen, Marcus, Dawson & Cherry, 1965; Cole, 1965).

We have used immunofluorescence to examine the type b capsular antigen which is synthesized soon after the non-capsulated *Haemophilus influenzae* recipients are treated with transforming DNA. To aid in analysing and interpreting the kinetics of the reaction, streptomycin resistance was followed in parallel tests. This familiar reference marker provided valuable quantitative data on competence of the recipient bacteria and genetic activities of the DNA preparations, as well as on times of phenotypic expression and multiplication of the *str-r* transformants.

METHODS

Growth factors. The X and V requirements of *Haemophilus influenzae* were satisfied by solutions of haemin (recrystallized, Nutritional Biochemicals Corp., Cleveland, Ohio) and nicotinamide adenine dinucleotide (NAD, P. L. Biochemicals Inc., Milwaukee, Wis.). Haemin was dissolved (200 mg. in 10 ml. of 0·1 M-NaOH, with subsequent addition of water to give a final volume of 100 ml.) and was autoclaved at 121° for 15 min. Biological activity of this haemin solution tended to deteriorate at 4°, as noted (Catlin, 1958), but was preserved for more than 2 years at −18°. The stock solution of NAD (5 mg./ml. water) was sterilized by filtration (Millipore, 0·45 μ, type HA membrane). These solutions were stored in small volumes in the frozen state until used.

Media. Three kinds of medium were used. HIY + HD was composed of heart infusion broth (Difco) with yeast extract (Difco, used at 0·3 %, w/v); before use, it was supplemented aseptically with solutions of glucose (final concentration, 0·1 % w/v) together with haemin and NAD (final concentration of each, 10 μg./ml.). BHI + HD broth was brain heart infusion (Difco) supplemented with haemin and NAD (10 μg./ml.). The stock solution for Levinthal broth was prepared as described by Alexander (1965) except that human blood was used; this was combined with an equal volume of BHI supplemented with 5 μg. NAD/ml. (no haemin was added). HIY + HD agar had the same composition as the liquid but was solidified with 1·0 % (w/v) Ionagar no. 2 (Oxoid) for hard agar plates, or with 0·5 % Ionagar no. 2 for soft agar overlays.

Antibody. Haemophilus influenzae type b antiserum for reference purposes was obtained from the Biological Reagents Section of the National Communicable

Disease Center (Atlanta, Georgia). Also, type b antiserum was obtained commercially from Hyland Laboratories (Los Angeles, California), and a portion of this was conjugated with fluorescein isothiocyanate by Hyland. This immunofluorescent reagent was used to check the capsules of cultures used for DNA extraction and for immunization.

Antiserum for preparation of additional fluorescein-tagged antibody (FTA) was obtained by immunization of albino rabbits. The suspension of strain SB sm 1000 used as antigen was freshly prepared each second week from 6 hr BHI + HD cultures, care being taken to obtain well-capsulated bacteria. They were sedimented by centrifugation at 3°, and were washed and resuspended in chilled phosphate-buffered saline (pH 7·2) containing 0·5 % (v/v) formaldehyde solution. Precautions emphasized by Alexander (1965) and Alexander, Leidy & MacPherson (1946) were observed to minimize degeneration of the capsular antigen.

After precipitation with ammonium sulphate, the antibody was conjugated to fluorescein isothiocyanate (Baltimore Biological Laboratories) as described by Cherry, Goldman, Carski & Moody (1960). Sephadex G25 (medium grade, Pharmacia Fine Chemicals, Inc.) was used for removal of unreacted fluorescein, as recommended by Gordon, Edwards & Tompkins (1962). Polyethylene glycol 20,000 (Fisher Sci. Co.) was used to concentrate the conjugate (Kohn, 1959). The final product was filtered through a Millipore membrane (0·45 μ).

Strains. The seven strains of *Haemophilus influenzae* used in this work are characterized in Table 1. All showed typical nutritional requirements; that is, growth occurred on HIY + HD agar, but not on HIY agar with haemin only or NAD only. Strains RD and SB sm 1000 (Santo) were provided by Miss Grace Leidy, and have been described (Alexander & Leidy, 1953). Strains designated *str-r*, and referred to as streptomycin-resistant, were isolated by growth in HIY + HD broth or agar which contained dihydrostreptomycin sulphate (DHS, Pfizer) 500 μg./ml. Methods similar to those described for Neisseria (Catlin & Cunningham, 1964), were used to obtain the single-step spontaneous *str* mutants, and to preserve the strains by freezing.

All capsulated bacteria gave a positive Quellung reaction in type b antiserum (from NCDC), and a brilliant capsular stain with the immunofluorescent reagent. Young colonies of capsulated strains showed a marked iridescence when viewed with strong light obliquely transmitted, as described by Pittman (1931). Typical iridescent colonies observed with the aid of × 9 magnification were subcultured after 14–18 hr incubation. Each of the strains appeared to be relatively homogeneous as determined by FTA-staining of the capsules, except RD(b)BR *str-r*. This strain, even after repeated single-colony isolation, usually showed a few (less than 0·1 %) bacteria with narrow capsules in addition to the typical heavily capsulated rods (Pl. 1, fig. 3).

Transforming DNA. Preparations were made from cultures of five strains (Table 1). Cultures in HIY + HD broth containing 200 μg. DHS/ml. were incubated at 37° with shaking for 5–6 hr. They were harvested by centrifugation at 3° before attaining maximal turbidity (to reduce the likelihood that mutants involving altered capsulation might be selected). The sedimentated bacteria were washed twice in SSC (a solution containing 0·15 M-NaCl + 0·015 M-sodium citrate), and were resuspended in SSC and frozen.

When thawed, the bacteria were lysed with sodium dodecyl sulphate (0·5–1·0 %, w/v) in the presence of Pronase (1·0 mg./ml., as described by Berns & Thomas, 1965). The

nucleic acids were precipitated from the lysate with ethanol, and were dissolved in SSC. DNA was partially purified by methods described earlier (Catlin & Cunningham, 1964), and the concentration was determined by the diphenylamine reaction.

Competent cultures of strain RD. The use of Nephelos Flasks (Bellco Glass, Inc. Vineland, N.J.) containing 25 ml. volumes of broth permitted rapid periodic readings of extinction at 650 mμ without risk of contaminating the growing cultures. Warmed BHI+HD broth was inoculated with bacteria from an overnight culture to give

Table 1. *Strains of Haemophilus influenzae*

Strain	Use	Appearance of colony	Distribution and origin of type b antigen	Response to streptomycin (500 μg./ml.)
RD	Recipient	Non-iridescent, moist, grayish translucent	Absent	Susceptible (3 μg. streptomycin/ml. inhibits growth)
SB sm 1000 (Santo)	Source of DNA preparation 1; antigen for immunization	Iridescent, opaque	Present as capsule: spinal fluid isolate	Resistant
MCH9311, type b *str-r*	Source of DNA preparation 2	Iridescent, opaque	Present as capsule: spinal fluid isolate; see Pl. 1, fig. 1	Resistant, by single-step mutation
RD(9311) *str-r* R	FTA-staining	Non-iridescent, very rough, greyish, opaque	Present as patches, not entire capsule; see Pl. 6	Resistant, by transformation
RD(b) *str-r*	Source of DNA preparation 3	Iridescent, opaque	Present as capsule: transformant elicited by DNA preparation 1	Resistant, by single-step mutation
RD(b)FA *str-r*	Source of DNA preparation 4	'Faded' iridescent, slightly translucent	Present as capsule: transformant elicited by DNA preparation 3; see Pl. 1, fig. 2	Resistant, by single-step mutation
RD(b)BR *str-r*	Source of DNA preparation 5	'Bright' iridescent, opaque	Present as capsule: transformant elicited by DNA preparation 3; see Pl. 1, fig. 3	Resistant by single-step mutation

an initial extinction 0·060–0·090. The flask was shaken at 37° until the extinction reached 0·400–0·600; the generation time was about 35 min. Then the flask was tilted so that the culture ran into the side-arm (cuvette) where it was held at 37° for 75 min. (non-aerated period). Lastly the culture was tilted back into the flask and shaken at 37° for 30 min., at which time the bacteria numbered $3–6 \times 10^9$ colony-forming units/ml. Warm glycerol was added to give a concentration of 15% (v/v), and the culture was distributed into 8 ml. screw-cap tubes and frozen at minus 60°.

Transformation tests. A tube of competent culture of strain RD was thawed at 37° and immediately diluted 1/5 in BHI+HD broth or in double-strength Levinthal broth. The diluted culture was drawn up into a pipette and rapidly expelled, and this was repeated about 20 times to decrease cellular aggregation; one volume was then added to one volume of pre-warmed DNA solution (diluted in BHI+HD). The moment of mixing bacteria with DNA was taken as time zero; thereafter, the mixture was incubated in a water bath at 36·5°. After 20–30 min. deoxyribonuclease

(crystalline. Worthington Biochemical Corp.) was added to give a concentration of 10 μg./ml. (details in Catlin, 1960). Samples were removed periodically for assays of transformation to capsular synthesis and to streptomycin resistance. Suitably diluted bacteria were spread over the surfaces of DHS-containing HIY + HD hard agar for kinetic studies of phenotypic expression. For other studies involving the *str-r* marker, bacteria were taken from broth about 30 min. after time zero. A dilution was inoculated into a 20 ml. volume of DHS-free soft agar at 44°, and 4 ml. samples of the mixture were immediately pipetted on supporting layers of hard agar (20 ml. volumes, pre-dried); after 5 min. the plates were transferred to a 36° incubator. The transformants were challenged 3·5 hr after time zero by adding the antibiotic in a top layer (6 ml. of soft agar containing 2500 μg. DHS/ml. to give a final concentration of 500 μg./ml.). Similarly in each test the number of colony-forming units/ml. of recipient bacteria was assayed in DHS-free agar. A control test with deoxyribonuclease-inactivated DNA was included in every experiment. Colonies were counted after incubation of plates for 2–3 days.

Microscopic preparations. For the semi-quantitative assay of capsulated bacteria in a transformation reaction culture, 5 μl. (delivered with a Drummond Microcap, Kensington Sci. Corp., Oakland, Cal.) was spread uniformly on a clean microscope slide (0·96–1·06 mm. thickness) and dried. For tests of microcolony-formation, a loopful of the DNA-treated bacteria was spread on the surface of HIY+HD or Levinthal agar. After incubation for 1–5 hr, a 2 cm. square block of agar was cut out and inverted on a cleaned slide; the agar was immediately flipped off leaving a film which dried rapidly and maintained the spatial arrangements of the bacteria.

Films were fixed by immersion in absolute methanol for 10 min. soon after preparation and preferably stained the same day; but when this was impracticable they were stored in a tightly covered box in the refrigerator. Slides were never blotted, but were dried by evaporation. Staining was done by the method recommended by Cherry *et al.* (1960) and Biegeleisen *et al.* (1965). The FTA reagent was applied for 30 min., poured off and the slide was rinsed for 5 min. in each of two baths of phosphate-buffered saline (pH 7·2) followed by water. The preparation was dried and mounted with pH 7·2 buffered-glycerol under a no. 1 22 × 30 mm. coverslip. Stained slides were refrigerated until examined. For assays of number of capsulated transformants, the smear was scanned with the oil-immersion objective for a period of exactly 30 min. using a standard search pattern.

Fluorescence microscopy and photography. FTA-stained preparations were examined using a Leitz Labolux III fluorescence microscope equipped with a dark-field condenser D 1·20. BG–38, BG–12, and OG–1 filters were used. An Osram HBO 200 high-pressure mercury burner was used and, alternately, a low-voltage tungsten lamp, which was valuable for revealing artifacts or for assessing the possible significance of unusual forms observed with ultraviolet radiation. Photographs were made using a fluorite oil-immersion objective, NA 1·32 with iris diaphram, and × 10 eyepiece, with a Leitz MIKAS microattachment and camera for 35 mm. film. Kodak Tri-X film was developed in UFG developer at 21° for $6\frac{1}{4}$ min.

RESULTS

Transforming activities of DNA preparations

The response of one competent population to various DNA preparations is given in Table 2. DNA preparations 3–5, extracted from capsular transformants of strain RD, were more active than DNAs from the clinical isolates. DNA preparation 1, which we used for developmental work on the capsular transformation method, characteristically elicited the fewest capsules.

Table 2. *Transformation of Haemophilus influenzae strain* RD *by deoxyribonucleate preparation from different donors*

Mixtures of recipient bacteria, $3{\cdot}7 \times 10^8$/ml., and DNA in Levinthal broth were incubated at 36·5°; deoxyribonuclease was added after 20 min., and incubation was continued. Samples taken at 30–60 min. were assayed by the agar-overlay technique to determine resistance to 500 μg. DHS/ml.; 5 μl. samples were taken at 150 min. for microscopic preparations (see Methods).

DNA donor strain	DNA conc. (μg./ml.)	Transformants	
		Str-r (no./ml.)	Capsular*
SB sm 1000 (preparation 1)	1·0	4,184,000	88
	0·01	779,600	26
	0·0001	8,750	0
MCH9311 *str-r* (preparation 2)	1·0	4,487,000	214
	0·01	830,600	21
	0·0001	7,790	0
RD(b) *str-r* (preparation 3)	1·0	5,920,000	152
	0·01	794,000	70
	0·0001	8,460	0
RD(b)FA *str-r* (preparation 4)	1·0	5,014,000	275
	0·01	714,500	29
	0·0001	8,650	0
RD(b)BR *str-r* (preparation 5)	1·0	5,504,000	165
	0·01	678,000	50
	0·0001	7,330	1

* Number counted during a 30 min. period of standard search.

The assay technique for capsulated bacteria is only semi-quantitative. Although the microscopic preparation contained all the transformants present in a 5 μl. sample, some of these would not be counted during the allotted time. Nevertheless, the counts indicate that, under these test conditions, the frequency of transformation to capsular synthesis was only about 1 % that to streptomycin resistance. In pneumococcus, also, the ratio of type I capsular transformants to *str-r* transformants is about 1 % (Bernheimer *et al.* 1967). However, this apparent concordance may be accidental as the methods differ significantly in respect to the stage at which transformation is detected. In the pneumococcal system capsular transformants are recognized only after they have undergone considerable multiplication; any which are unable to divide normally may be overlooked. In the *Haemophilus influenzae* system the genetic event is detected at the cellular level, and within 2 hr. However, as will be shown in the next section, the multiplication of these newly capsulated bacteria was affected, and some may not have formed macrocolonies during subsequent incubation.

Transformation to capsular synthesis was related to the concentration of DNA (Table 2), and capsulated bacteria were never found in control preparations of recipient strain RD treated with deoxyribonuclease-inactivated DNA. The response of competent bacteria to eight different concentrations of DNA was examined in experiments with DNA preparations 2 and 4. Results for both *str-r* and type b antigen markers corresponded to those for streptomycin resistance reported by Alexander, Leidy & Hahn (1954) and by Goodgal (1961). Capsulated bacteria were observed in tests of 0·0001 μg. DNA/ml., and the number rose in proportion to the concentration up to about 0·1 μg. DNA/ml. A ten-fold higher DNA concentration elicited only a 20–25 % increase of capsulated forms.

Phenotypic expression

To examine the kinetics of capsular transformation of strain RD, and to permit comparisons with another genetic marker, periodic samples of a recipient population cultivated in broth continuously for 5·5 hr were tested. After treatment with 1·0 μg. DNA/ml. for 20 min., deoxyribonuclease was added. Total number of bacteria, number of bacteria resistant to DHS (200 μg./ml. and 500 μg./ml.) and number of capsulated bacteria were determined. Three different DNA preparations (2, 4 and 5) were examined in similar experiments.

Figure 1 shows the time course of the increase of streptomycin-resistant transformants and of the total population (generation time about 50 min. during the first 2 hr) after adding DNA (preparation 5) from the transformant strain RD(b)BR *str-r*. No transformants were found after treatment for 5 min. Beginning at about 25 min. there was an initial rapid increase in numbers of bacteria which were able to form colonies when spread directly on DHS-containing agar. About 7×10^6 *str-r* transformants per ml. were present after incubation of the original reaction mixture for 145 min. Thereafter, the rate of increase of the *str-r* transformants was about the same as that of the total population (doubling times 70–75 min. over the period 145–220 min.).

During the early stages of expression of the streptomycin-resistant phenotype, transformants were not equally resistant to DHS. Alexander & Leidy (1953) had found that resistance to 10 μg. streptomycin/ml. began to develop within 15 min., but that resistance to 1000 μg./ml. appeared only after some delay. In our tests (Fig. 1) new transformants which had become resistant to 200 μg. DHS/ml. appeared to require about 5 min. to increase their resistance to 500 μg./ml. Thus, in 1·0 ml. of the 30 min. sample, 1420 transformants produced colonies on agar containing 200 μg./ml., but only 180 grew out on 500 μg./ml.; whereas in the 35 min. sample, 1410 were resistant to 500 μg./ml. This intervening time may be required for a stage in the modification of ribosomes (Luzzatto, Apirion & Schlessinger, 1968; Stuart & Ravin, 1968). Bacteria sampled after 120 min. incubation no longer showed a significant difference of degree of resistance, which suggested that phenotypic expression was essentially complete at this time. This was corroborated by the sharp deflection of the curve representing increase of *str-r* transformants which occurred regularly at 120–145 min. in each of three experiments. Furthermore, a subculture of the original reaction mixture diluted 1/20 in fresh pre-warmed broth to stimulate continued multiplication showed similar growth rates of *str-r* transformants and total bacteria (open symbol-curves Fig. 1).

The time course of phenotypic expression of capsulation during incubation of broth cultures could not be followed with the same precision as streptomycin resistance because of the lower reproducibility inherent in the method of preparing and scanning the microscope slide preparations. In six different experiments samples were taken at 5 min. intervals over the period 35 to 60 min., and the FTA-stained preparations

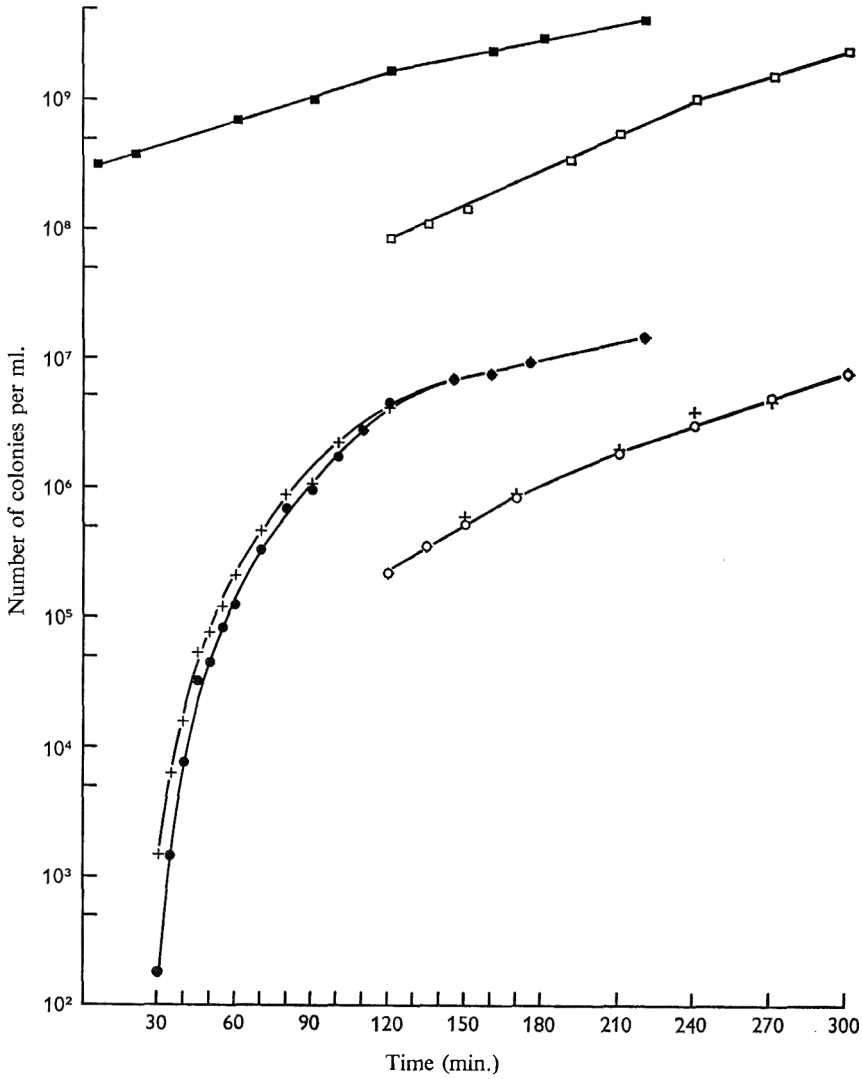

Fig. 1. Increase of streptomycin-resistant transformants and of the total population during cultivation in BHI+HD broth of strain RD treated with 1·0 μg. DNA preparation 5/ml. Deoxyribonuclease was added after 20 min. A subculture representing a 1/20 dilution of the original culture was made after 120 min. Symbols: ■, colonies on DHS- free agar plated with sample of the undiluted culture; □, colonies on DHS-free agar plated with sample of the diluted culture; + (left-hand curve), colonies on agar containing 200 μg. DHS/ml. from undiluted culture; + (right-hand curve), colonies on agar containing 200 μg. DHS/ml. from diluted culture; ●, colonies on agar containing 500 μg. DHS/ml. plated with sample of the undiluted culture; ○, colonies on agar containing 500 μg. DHS/ml. plated with sample of the diluted culture.

were each examined systematically for one or two 30 min. periods of the standard search. Fluorescent capsules were first detected 40 and 50 min. after exposure to DNA preparation 2, 40 and 45 min. after exposure to DNA 4, and 45 and 50 min. after exposure to DNA 5. Cultures then showed a progressive, though erratic, increase in number of capsulated bacteria over the next 75 min. After 120 min. total elapsed time of incubation, phenotypic expression of capsulation was nearly complete. Thereafter, unlike the *str-r* transformants, the number of capsulated bacteria tended to remain approximately the same, within a two-fold range of experimental variation, during subsequent incubation for 3 hr. Figure 2 shows results of assays of the same population which was examined in Fig. 1. Diluting the original culture after 120 min. incubation (open circles) reduced the number of capsulated organisms found. Samples taken at the time of preparation of this subculture yielded 186 for the undiluted culture and 2·5 for the 1/20 dilution. In subsequent samples the number of capsulated organisms in the diluted culture fluctuated within the range 14 to 44, and within the range 118 to 225 for the parallel undiluted culture. These values represent capsulated units; the bacteria present in an unseparated pair or chain were counted as one because of difficulties of interpretation (for example, Pl. 3, fig. 24, 25, 30–38). Their number was tallied, however, and did not exceed 15% of the number of single capsulated bacteria. In contrast, a few transformants began to multiply normally during this period, as revealed by rare microcolonies containing 9 to 16 capsulated bacteria on impression smears of 4 hr agar cultures (Pl. 2, fig. 12). The data, however, show only a minor tendency for replication of the capsulated bacteria, as opposed to the replication of *str-r* transformants.

Corresponding results, though with a lower yield of transformants, were obtained in a similar experiment performed with DNA (preparation 2) from a type b clinical isolate. As before, *str-r* transformants were unequally resistant to DHS during the early stages of expression. Thus, the 35 min. sample contained 1040 transformants/ml. resistant to 200 μg. DHS/ml., but only 240/ml. resistant to 500 μg./ml.; 5 min. later 1050 transformants/ml. were resistant to 500 μg./ml. After 120 min. total incubation the transformants were equally resistant, and they numbered $2{\cdot}0 \times 10^6$/ml. (compared to $4{\cdot}6 \times 10^6$/ml. for the experiment shown in Fig. 1); the total population at this time (both experiments) had reached $1{\cdot}7 \times 10^9$ colony-forming units/ml. The numbers of transformants to capsular synthesis found in samples of this culture and in a 1/20 dilution are given in Fig. 3. They were lower than in the experiment plotted in Fig. 2, as expected from the lower frequency of *str-r* transformation elicited by this DNA. Too few capsulated bacteria were present in the diluted culture for accurate counts, which ranged from 8 down to zero for samples taken at 180 and 300 min. However, they confirm the previous finding of minimal increase.

Thus, the transforming DNA initiated the process of synthesis of type b antigen, but multiplication of many of these newly capsulated bacteria was arrested. In view of their failure to divide during this 5·5 hr period of incubation, and the possibility that some may not subsequently produce macrocolonies, they will be referred to as capsular 'transformants' in the following discussion.

Properties of capsular 'transformants'

We originally supposed that the processes of initiating competence and freezing and thawing might partially synchronize the bacteria such that, after a limited

exposure to transforming DNA, the sites of initiation of capsule production might be revealed in newly transformed bacteria. Actually, transitional forms were rare. A few non-capsulated bacteria in samples taken after 35 to 50 min. incubation exhibited polar or subpolar dots, one or more rarely two per organism. Although their fluorescence was stronger than that of the weakly stained wall, the intensity was insufficient for photography. Most early capsules were entire but narrow, and stained with only moderate brilliance (e.g. Pl. 3, fig. 15, 16, 25, 26 and Pl. 4, fig. 46, 47).

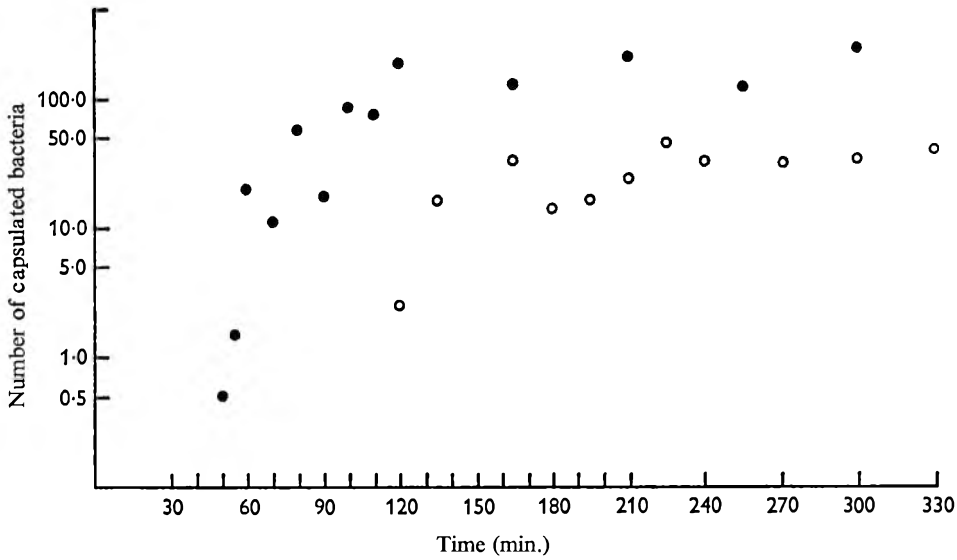

Fig. 2. Capsulated bacteria found during incubation of the undiluted (●) and the diluted (○) transformation cultures of the experiment shown in Fig. 1.

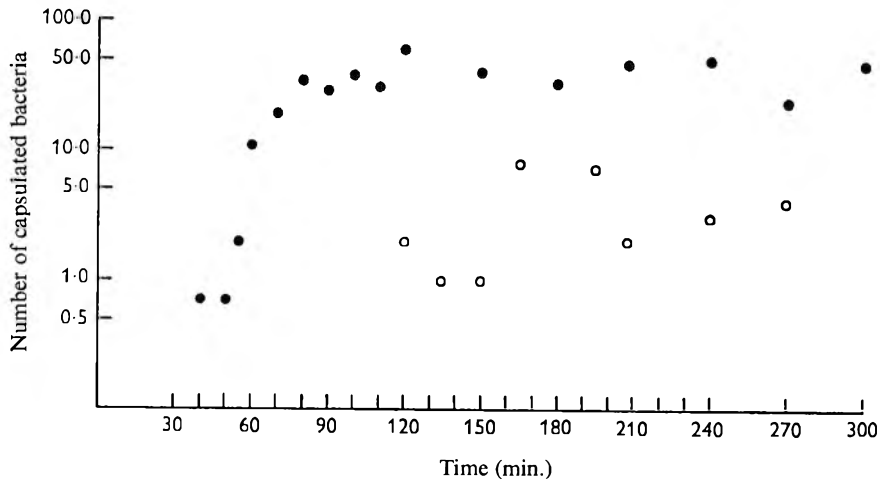

Fig. 3. Transformants to capsular synthesis found during cultivation in BHI+HD broth of strain RD which was treated with 1·0 μg. DNA preparation 2/ml. Deoxyribonuclease was added after 20 min. A subculture representing a 1/20 dilution of the original culture was made after 120 min. Symbols: ●, capsulated bacteria in undiluted culture; ○, capsulated bacteria in diluted culture.

During phenotypic expression, and subsequently, the capsular 'transformants' showed considerable morphological diversity. As each represented the culmination of a separate genetic event, it is premature now to attempt to reconstruct a sequence of events based on observations of forms in successive samples of a transformation culture. Accordingly, photographs are presented to document this diversity.

A few of the capsular 'transformants' multiplied to produce pairs or short chains of bacteria, which tended to remain attached, even in preparations of broth cultures. Commonly their capsules fluoresced with unequal brilliance, suggesting a mother–daughter relationship (Pl. 2, fig. 10; Pl. 3, fig. 23–25, 31). Other 'transformants' which had undergone growth without division showed similar inequalities of capsule production (Pl. 3, fig. 35–37; Pl. 5, fig. 62, 66). Some filamentous forms were entirely capsulated (Pl. 4, fig. 56), but others were not (Pl. 4, fig. 42, shows a dispersion of capsular material). Still other 'transformants' were observed in which capsular synthesis had diminished or had ceased altogether, while growth and wall-formation continued; these showed a polar or bipolar conservation of the capsule (e.g. Pl. 4, fig. 44–45, 51–53; Pl. 5, fig. 72–73).

A pair of bacteria of which only one was capsulated was seen in a 70 min. sample (Pl. 4, fig. 48–49). As the total number of bacteria had more than doubled at this time, different interpretations are possible. The DNA might have altered only one of two 'chromosomes' present in a single competent bacterium, and at division these were segregated into separate bacteria. Or, the DNA may have entered one bacterium of a pre-existing pair; it was earlier recognized that in the competent population a colony-forming unit is composed of between 1·6 and 2 bacteria (Goodgal & Herriott, 1961). Another possibly similar example, observed after longer incubation (Pl. 4, fig. 43), was a chain composed of multiple unseparated capsulated and non-capsulated units.

Among the capsular 'transformants' elicited by DNA (preparation 2) from a type b clinical isolate were forms (10 to 15% of the total) which showed bright fluorescent embossments which appeared to be incomplete capsules (Pl. 5). The DNA donor strain itself exhibited normal capsules (Pl. 1, fig. 1), and young colonies on HIY + HD agar were highly iridescent when viewed with oblique transmitted light. However, the strain was genetically unstable, and upon further incubation, rough areas developed along the margins of some colonies. Subcultures of such areas produced very rough, non-iridescent colonies, as vividly described by Pittman (1931). Smears of these R mutants revealed pleomorphic rods, some of which showed fluorescent embossments resembling those of the 'transformants' (and possibly similar to the forms noted by Sell *et al.* 1963). Furthermore, the same kind of non-iridescent very rough colonies arose during subculture of iridescent transformant colonies isolated from DNA preparation 2-reaction cultures.

The type b antigen of one such very rough isolate (designated RD(9311) *str-r* R; see Pl. 6) was synthesized according to specifications received from strain MCH9311. That the embossments were typically fluorescent suggested that the serological specificity of the type b antigen was normal. However a more critical evaluation of the specificity of the FTA reaction could be made using the one-step fluorescence-inhibition test (Goldman, 1957). This was done on preparations of 5 hr cultures of strain RD(9311) *str-r* R, and on the capsulated DNA donor strain and a rough variant derived from it. The fluorescence of all capsular antigen was greatly decreased in tests where the combining sites were blocked by unlabelled type b antibody (from

NCDC). Control preparations with undiluted normal rabbit serum substituted for the unlabelled antiserum in the mixture with the FTA reagent showed brilliant fluorescence of the embossments as well as of the capsules of the donor strain. Thus the identity of the fragmentary capsular material as type b antigen was verified.

The capacity of these bacteria to produce type b antigen was evidently dissociated from the capacity to produce an intact capsular structure. Another example of a genetic difference which affected the morphology of the capsule without altering the antigenic specificity of the capsular material was detected by differences of colonial iridescence. 'Bright' (BR) colonies were distinguished from FA colonies present in the same transformation reaction culture by a greater brilliance of iridescence observed with oblique transmitted light (Table 1). The FTA-stained capsules of the BR colony transformants were typically more massive than those of the FA colony transformants (Pl. 1, fig. 2, 3), the distinction was more conspicuous with young cultures on agar than in broth, possibly owing to solubility of the capsular antigen (noted by Pittman, 1931). The genetic basis of these differences remains to be established.

DISCUSSION

The culture of non-capsulated streptomycin-susceptible recipient bacteria examined in the experiment presented in Fig. 1 and 2 had a generation time of about 50 min. during the first 2 hr of incubation. *Str-r* transformants began to appear 25 min. after initiation of DNA treatment. Capsulated bacteria were observed at 40 to 50 min., and for the next hour both kinds of transformant phenotypes continued to be expressed. Thereafter, the recipients of the two kinds of genetic markers differed in their behaviour. The rate of increase of *str-r* transformants after 2 hr incubation became equal to that of the total recipient population. In contrast, the number of capsulated bacteria increased only slightly during incubation of the same culture over the period 120 to 330 min.; some became filamentous during this time signifying a disturbance of cellular division. Furthermore, although iridescent colonies composed of capsulated bacteria were present on assay plates, the number did not approach that expected on the basis of the estimated ratio of capsulated to *str-r* transformants (0·1 to 1·0 %).

An adequate interpretation of these observations in molecular terms will require further knowledge, including information on the question of whether or not the locus determining type b antigen is integrated at this time. Integration of the *str* locus has been shown to occur soon after uptake of donor DNA by the RD strain of *Haemophilus influenzae* (Voll & Goodgal, 1961; Notani & Goodgal, 1966). Explanations which may account for the two- to three-generation lag in multiplication of *str-r* transformants cannot be invoked for the much longer delay which we observed for the capsular 'transformants'. Not only were their replicative activities disturbed, but in many instances synthesis of type b antigen apparently diminished or ceased. Plates 2 to 5 show examples of various capsular 'transformants' which had grown and synthesized new wall material without producing a corresponding amount of capsular antigen. These findings bear some resemblance to abortive transduction of Salmonella in which phage-introduced bacterial DNA fails to be integrated but persists as a functional, non-replicating fragment passing in a unilinear manner from the recipient to only one daughter bacterium at each division (see, Demerec & Ozeki, 1959; Stocker, 1963).

We thank Miss Grace Leidy for information and strains, and Miss Mary Nimlos for assistance with immunofluorescence techniques. This study was supported by Public Health Service grant AI 02353; V.T. was aided by a grant from the Marquette University Committee on Research. Part of this work was included in a thesis submitted by V.T. to the Graduate School, Marquette University, in partial fulfilment of the requirements for the M.S. degree.

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EXPLANATION OF PLATES

Photomicrographs of *Haemophilus influenzae* preparations stained with fluorescein isothiocyanate-conjugated anti-type b globulin. Ultraviolet (u.v. 20 to 45 sec. exposure) or tungsten light (60 to 65 sec. exposure) was used with a dark-field condenser. Magnification of all bacteria approximately × 2900.

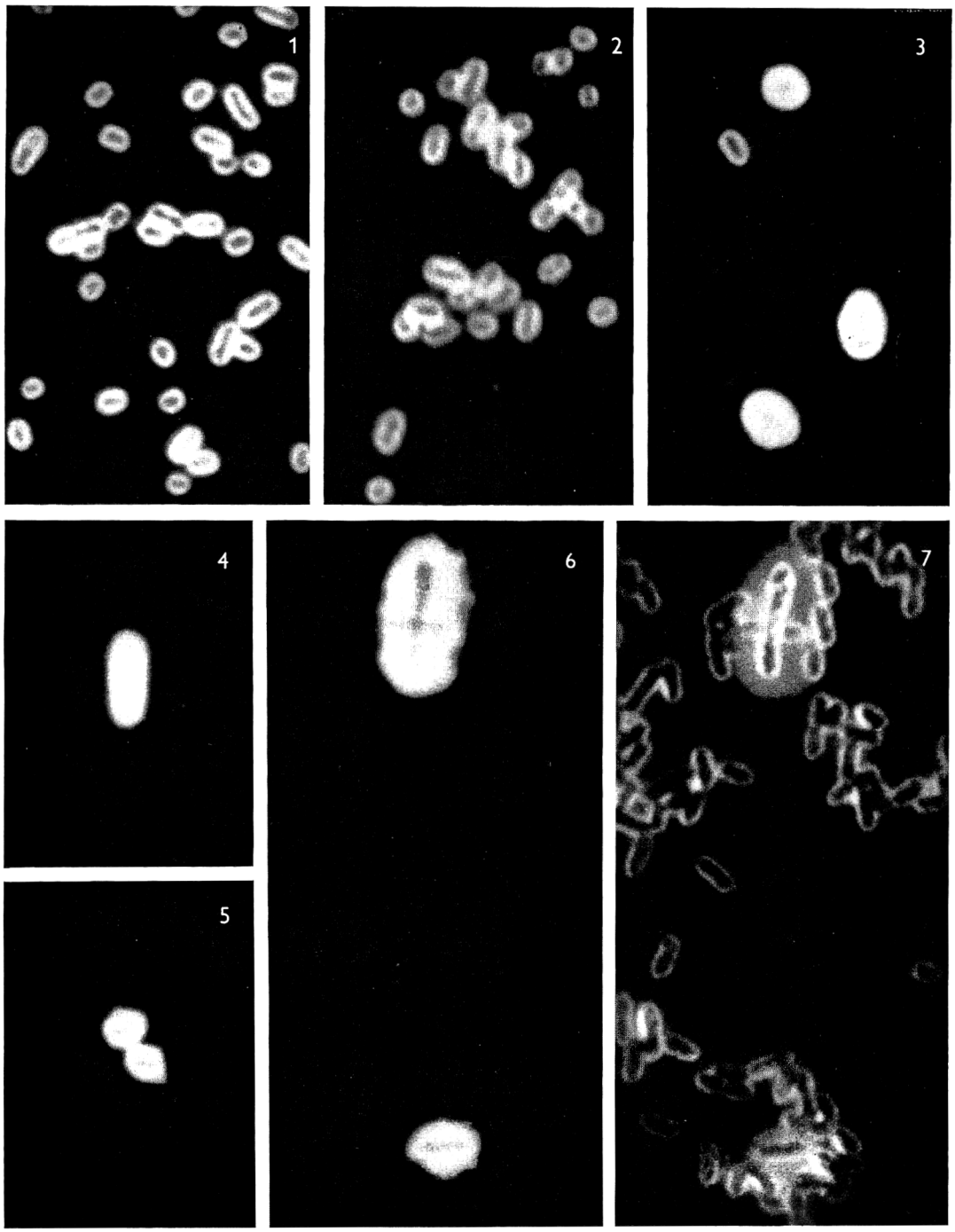
PLATE 1

Fig. 1–3. Pure cultures of DNA-donor strains incubated 6·5 hr on HIY+HD agar. U.v., 25 sec. Fig. 1, MCH 9311 type b *str-r*, source of DNA 2. Fig. 2, RD(b)FA *str-r*, source of DNA 4. Fig. 3, RD(b)BR *str-r*, source of DNA 5.

Fig. 4–7. Capsular 'transformants' in impression preparations; strain RD was treated 30 min. with DNA 5, 1·5 μg./ml., and subsequently incubated on HIY+HD agar. Fig. 4, 5, 110 min. total incubation, u.v. 30 sec. Fig. 6, 225 min. total incubation; bacteria photographed with u.v., 25 sec.; Fig. 7, tungsten exposure of the same bacteria shows that peripheral notches of the voluminous capsules are deformations due to the abutting non-capsulated bacteria (RD recipients whose margins appear bright due to dark-field illumination).

PLATE 2

Fig. 8–14. Capsular 'transformants' in impression preparations; strain RD was treated 30 min. with DNA 4, 1·5 μg./ml., and subsequently incubated on HIY + HD agar. Fig. 8, 105 min. total incubation; bacteria photographed with u.v., 40 sec.; Fig. 9, tungsten exposure of the same bacteria shows the capsulated organism located in the corresponding position together with non-capsulated (dark-field illuminated) forms. Fig. 10, 210 min. total incubation; bacteria photographed with u.v., 35 sec.; Fig. 11, tungsten exposure of the same bacteria shows that crowding by non-capsulated bacteria is responsible for the notched margin of the capsule at right, but not for the different intensity of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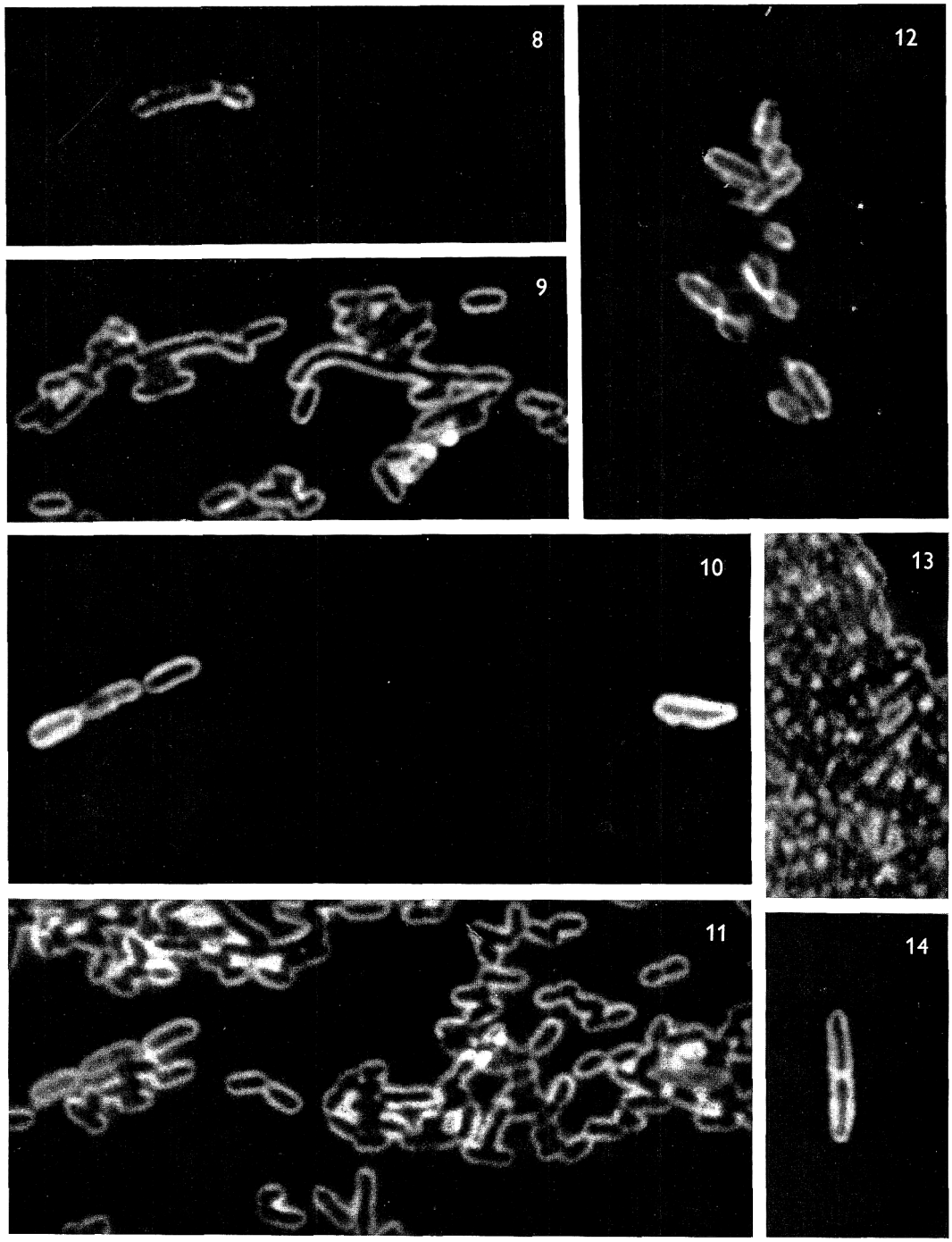
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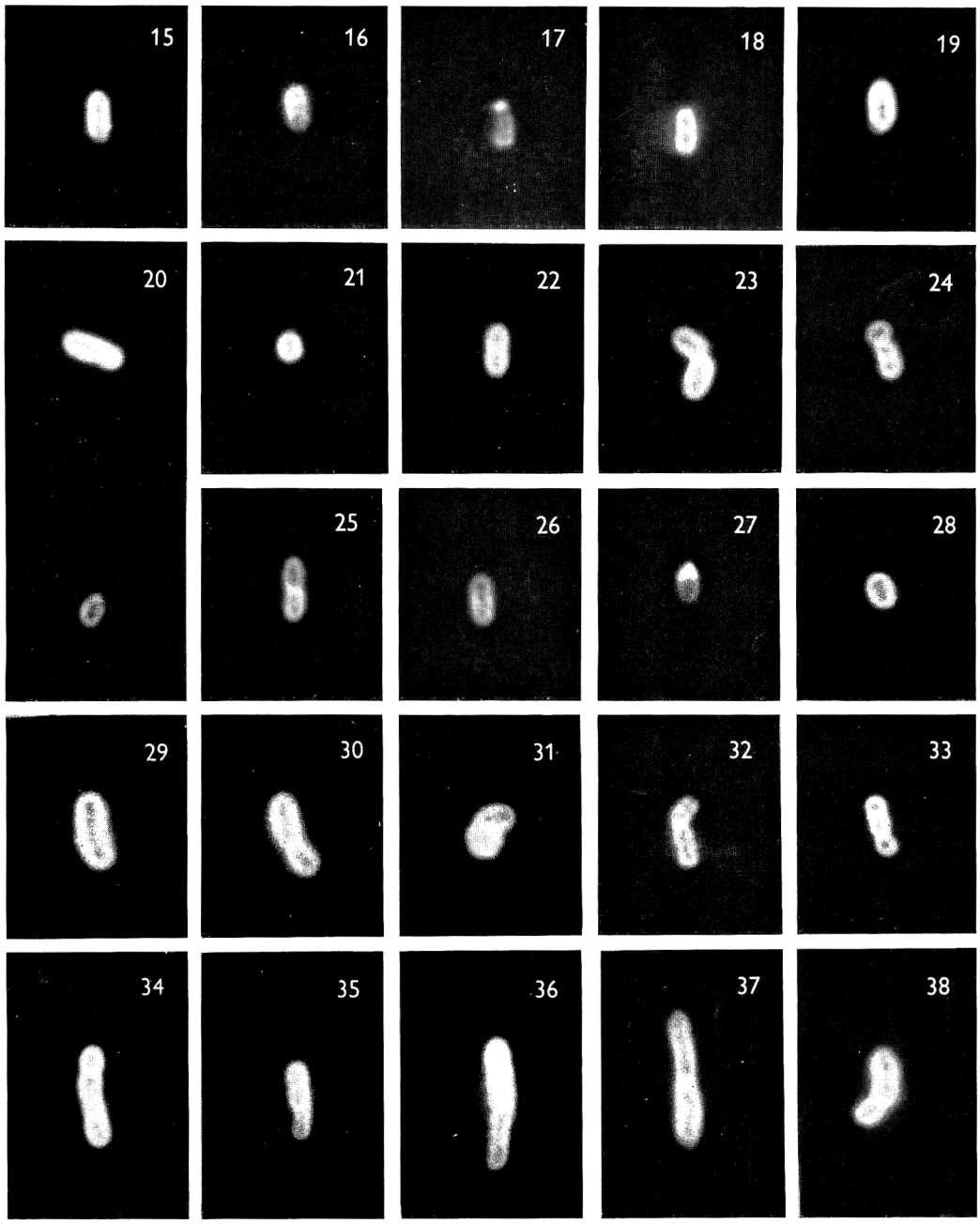
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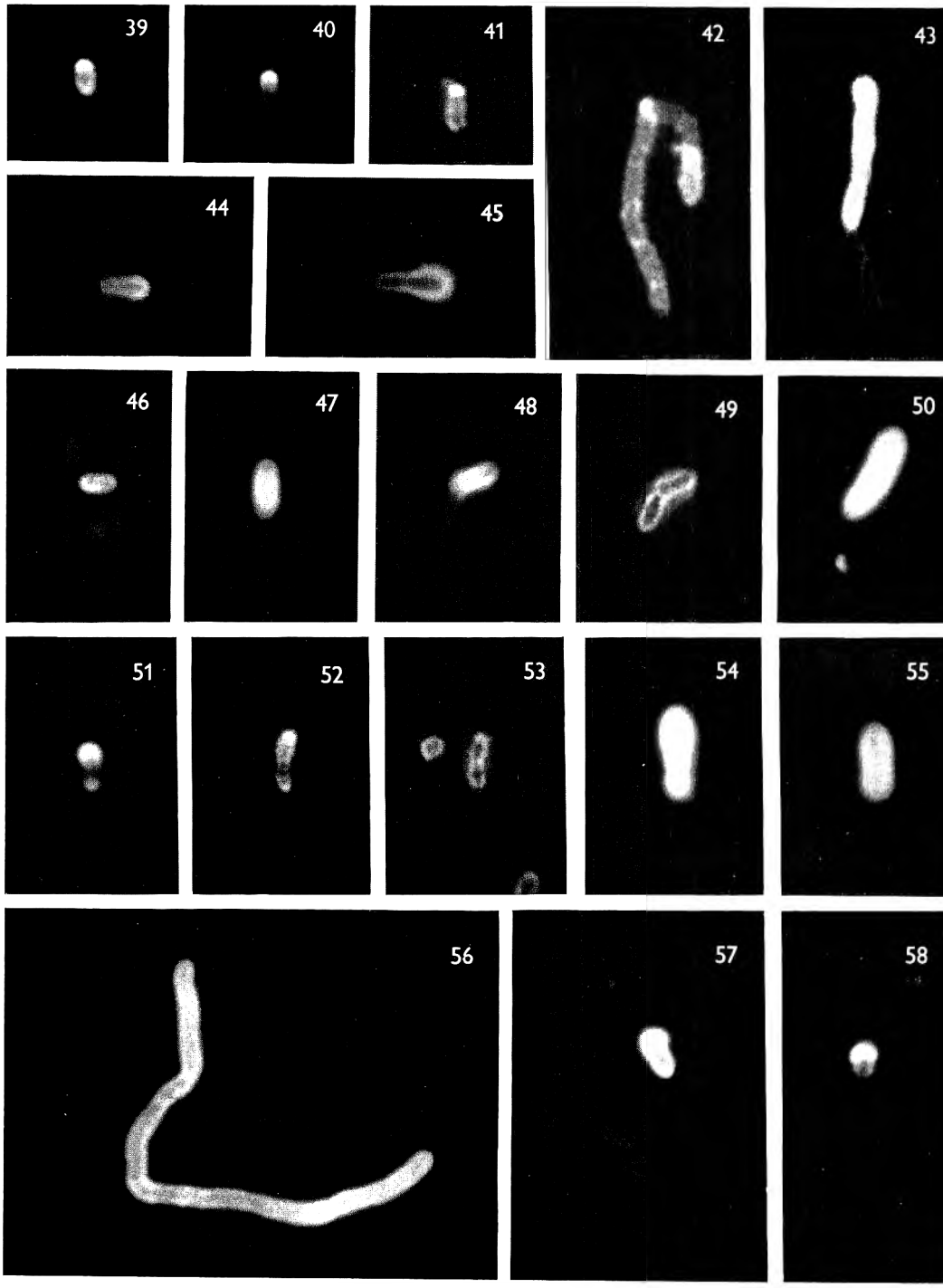
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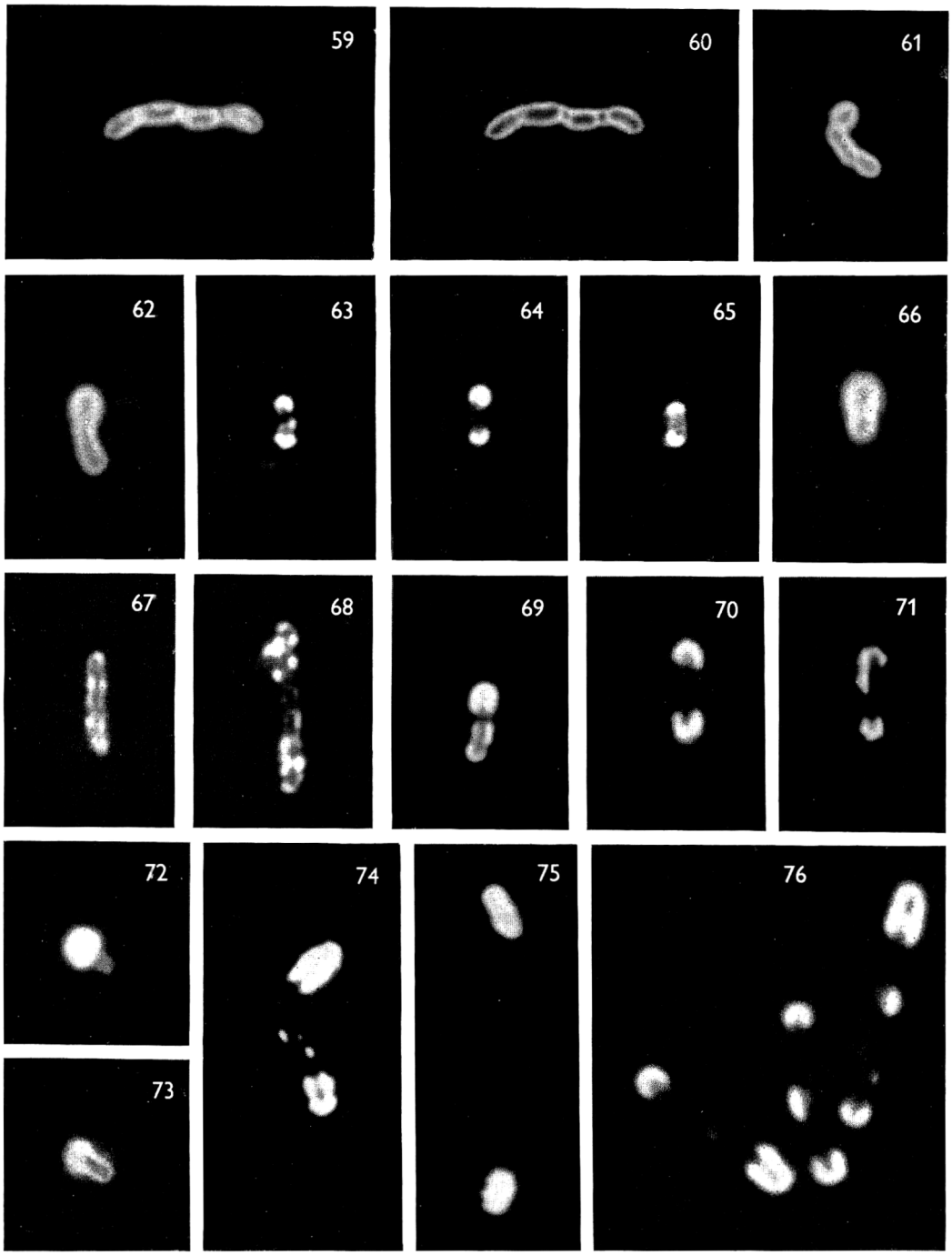
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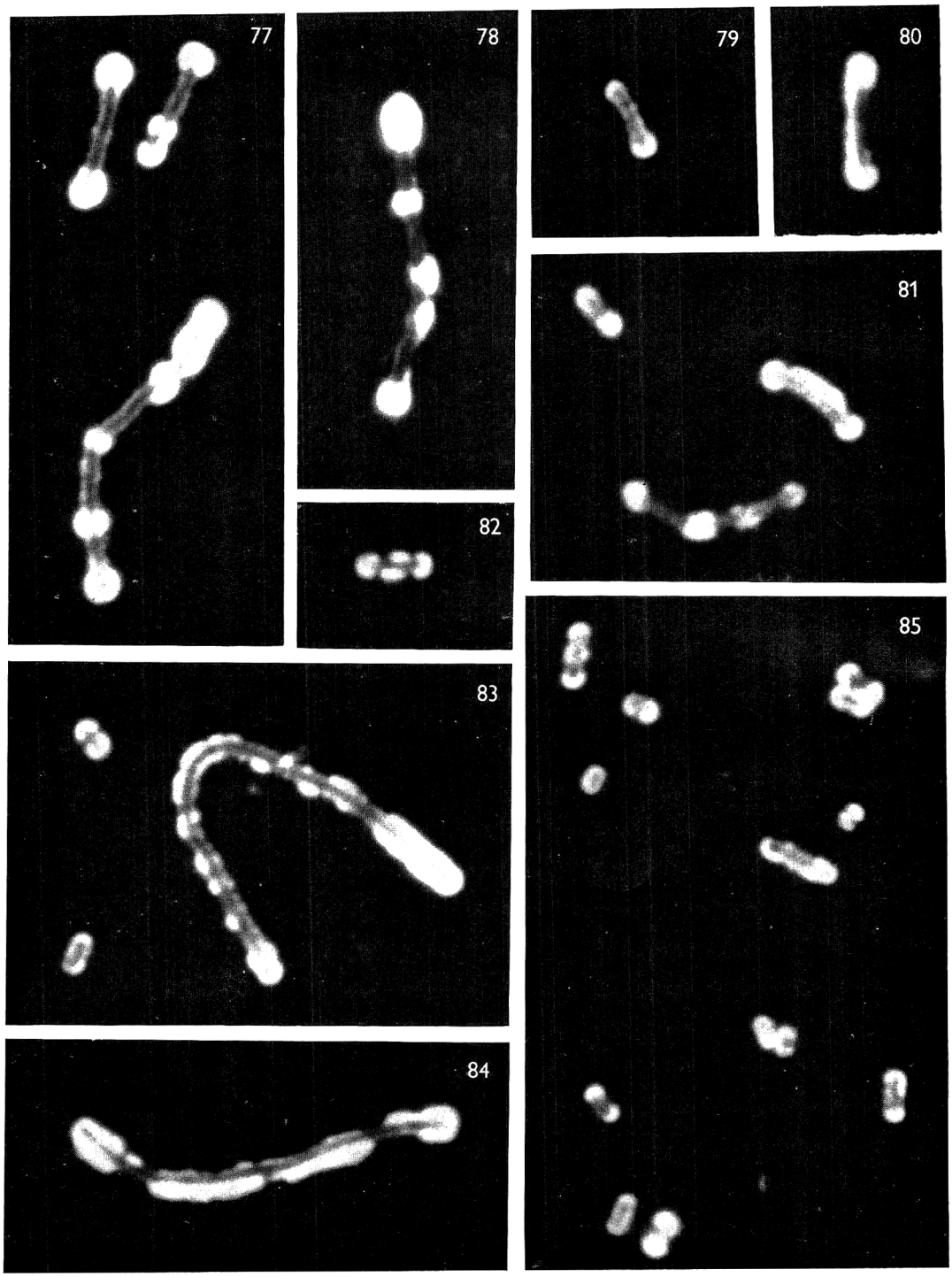
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fluorescence of one capsulated bacterium in the chain at left (which contained only three bacteria). Fig. 12, Microcolony of capsular transformants after 240 min. total incubation; u.v., 30 sec.; Fig. 13, tungsten exposure of a portion of the same field showing confluent growth of non-capsulated bacteria. Fig. 14, 105 min. total incubation; u.v., 40 sec.

PLATE 3

Capsular 'transformants' of strain RD elicited by treatment for 20 min. with 1·0 μg. of DNA/ml., except 0·01 μg./ml., Fig. 34; sampled after continuous cultivation in broth. U.v. exposures for all except Fig. 18. Fig. 15–16. DNA 4; cultivation in Levinthal broth. Fig. 15, 50 min. incubation. Fig. 16, 55 min. incubation.

Fig. 17–18. DNA 4; cultivation in BHI+HD broth for 90 min. Fig. 17, u.v., 45 sec.; Fig. 18, tungsten exposure of the same bacterium.

Fig. 19. DNA 4; 105 min. incubation in BHI+HD broth.

Fig. 20–24. DNA 4; 240 min. incubation in Levinthal broth.

Fig. 25–38. DNA 5; cultivation in BHI+HD broth. Fig. 25, 26, 60 min. incubation. Fig. 27–36, 120 min. incubation. Fig. 37, 38, 210 min. incubation.

PLATE 4

Fig. 39–45. Capsular 'transformants' elicited by treatment with DNA 5 in the experiment plotted in text-fig. 2. U.v. exposures. Fig. 39, 135 min. total incubation, diluted culture. Fig. 40, 195 min. total incubation, diluted culture. Fig. 41, 42, 240 min. total incubation, diluted culture. Fig. 43–45, 255 min. incubation of undiluted culture.

Fig. 46–58. Capsular 'transformants' elicited by treatment with DNA 2 in the experiment plotted in text-fig. 3. U.v. exposures for all except Fig. 49 and 53. Fig. 46, 40 min. incubation of undiluted culture. Fig. 47, 55 min. incubation of undiluted culture. Fig. 48, 70 min. incubation of undiluted culture; one capsulated organism with attached non-capsulated bacterium, photographed with u.v.; Fig. 49, tungsten exposure of the same pair. Fig. 50, 110 min. incubation of undiluted culture; a large capsulated bacterium lies above a small one with only a unipolar fluorescent cap. Fig. 51, 110 min. incubation of undiluted culture. Fig. 52, 120 min. incubation of undiluted culture, u.v.; Fig. 53, tungsten exposure of the same organism. Fig. 54, 120 min. incubation of undiluted culture. Fig. 55, 180 min. incubation of undiluted culture. Fig. 56, 57, 195 min. total incubation, diluted culture. Fig. 58, 225 min. total incubation, diluted culture.

PLATE 5

Capsular 'transformants' of strain RD elicited by treatment for 20 min. with various concentrations of DNA 2. U.v. exposures for all except Fig. 73.

Fig. 59–68. Sampled after continuous incubation in BHI+HD broth. Fig. 59, 240 min. incubation, 0·3 μg. DNA/ml.; capsulated chain of organisms photographed with u.v., 35 sec.; Fig. 60, the same chain rephotographed with a second 35 sec. exposure with u.v. revealing additional detail and reduced fluorescence. Fig. 61–63, 240 min. incubation, 0·3 μg. DNA/ml. Fig. 64–68, 210 or 300 min. incubation, 1·0 μg. DNA/ml.

Fig. 69–76. Impression preparations of cultures on HIY+HD agar. Fig. 69–71, 240 min. total incubation, 1·0 μg. DNA/ml. Fig. 72, 180 min. total incubation, 1·0 μg. DNA/ml.; bacterium with a large unipolar fluorescent cap, u.v.; Fig. 73, tungsten exposure of the same bacterium. Fig. 74, 270 min. total incubation, 0·01 μg. DNA/ml. Fig. 75, 270 min. total incubation, 1·0 μg. DNA/ml.; two separate normally capsulated bacteria. Fig. 76, 270 min. total incubation, 0·15 μg. DNA/ml.

PLATE 6

Fig. 77–85. Pure cultures on HIY+HD agar of strain RD(9311) *str-r* R, a very rough colonial isolate. U.v. Fig. 77–79, 4 hr incubation. Fig. 80–85, 23 hr incubation.

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Viable Bacteria Inside the Rumen Ciliate *Entodinium caudatum*

By R. W. WHITE

Biochemistry Department, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

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SUMMARY AND INTRODUCTION

Although bacteria are visible in Gram-stained smears of rumen entodinomorphid protozoa, no successful attempts to culture these bacteria have been reported. Gutierrez & Davis (1959), using oligotrich protozoa from the calf rumen, cultured 3 to 10 *Streptococcus bovis* per protozoon, after disruption by crushing between glass slides. The purpose of the present study was to investigate the viable bacterial flora of *Entodinium caudatum* which had originated from the sheep rumen, and had been maintained under constant conditions *in vitro* for over 6 years (Coleman, 1958, 1960). Part of the work has been reported previously (White, 1966).

METHODS

The standard medium used for the enumeration, isolation, and maintenance of the bacteria from the disrupted protozoa was that of Bryant & Robinson (1961), containing 0·05, 0·65 or 1·25 g. of agar per 100 ml., for liquid medium, counting medium, or roll bottles, respectively. The reducing reagent contained per 100 ml: $Na_2S.9H_2O$, 2·5 g., L-cysteine 2·5 g., adjusted to pH 11 and autoclaved under N_2 at 115° for 20 min.; 0·1 ml. of this with 0·4 ml. of sterile 8 % (w/v) Na_2CO_3, and any other test substances in a volume of 0·1 to 0·5 ml. were added to 9 ml. volumes of medium at 48°. Equilibration done with CO_2 freed from O_2 by passage over heated copper, and containers sealed with rubber stoppers. For the preparation of inoculum dilutions, sterile diluting fluid consisting of the standard medium described above, but without agar and with water substituted for rumen fluid was used.

Sodium azide was dissolved in sterile water at × 100 the defined final concentration, before use.

Conventional unsealed media used for anaerobic culture were freshly prepared, and incubated under 5 % CO_2 + 95 % H_2 (v/v). Aerobic bacteria were maintained on Oxoid nutrient agar and broth (Oxoid Division of Oxo Ltd., Thomas House, Queen Street Place, London, E.C.4). All cultures were incubated at 39°.

Extracts for streptococcal grouping were prepared by the acid extraction method of Lancefield (1933). Tests for production of arginine dihydrolase and for deamination of phenylalanine were as described by Whitfield (1967), for lysine and ornithine decarboxylases and the production of visible gas at 44° according to Cowan & Steele (1965). Motility was observed in hanging-drop preparations from liquid cultures taken as soon as visible growth occurred. Suspensions of bacteria from the water of condensation of agar slope cultures were mixed with indian ink and examined for capsule

formation. Flagella and fimbriae were observed with the electron microscope using the negative staining technique. Three day old cultures in nutrient broth containing 0·5% (w/v) glucose and in the lactate medium of Sundman & Björksten (1958) were examined by gas/liquid chromatography for the production of volatile fatty acids, by the procedure of Emery & Koerner (1961).

The terms 'aerobe' and 'aerobic' are used to define bacteria which grow freely in the presence of gaseous oxygen, whether or not they also grow well under anaerobic conditions. An 'anaerobe' is defined as an organism which cannot grow in the presence of gaseous oxygen.

The protozoa were supplied by G. S. Coleman, they were cultured and inoculum suspensions prepared first as described by Coleman (1960, 1964), then transferred to sterile tubes, washed three times in sterile Coleman (1960, 1964) buffer solution containing 0·1% (w/v) L-cysteine, on the centrifuge, and resuspended at a population density of 2 to 5×10^5/ml. One ml. of this suspension, free of extracellular bacteria, was placed in a sterile corked 2 in. × ½ in. tube, immersed to the depth of the contained suspension in the water in a three-pint ultrasonic cleaning bath driven by an 80 kcyc./sec., 80 W. generator (Kerry Ltd., Chester Hall Lane, Basildon, Essex). The bath was tuned to maximum cavitation before immersing the protozoal suspension. The number of viable bacteria was determined before and after sonic treatment of the protozoa by serial tenfold dilution followed by inoculation in triplicate into the standard Bryant & Robinson solid counting medium contained in modified Carrel (1923) flasks having a 12 mm. deep circular chamber of 10 to 11 ml. capacity and a straight neck, made to specification by Wesley Coe (Cambridge) Ltd., Scotland Road, Cambridge. After 3 days incubation, colony counts were done on flasks showing more than 10 and less than 300 colonies. Counts of protozoa before and after disruption, were done by suitable dilution in 0·02 M-iodine and all protozoa in 0·1 ml. counted under the phase-contrast microscope.

RESULTS

The effect of increasing times of sonic treatment on the number of protozoa and of viable bacteria per protozoon is shown in Fig. 1. From this the 5 sec. period of treatment was chosen and used routinely on 30 samples of the protozoa taken at approximately weekly intervals; the number of viable bacteria obtained was $21{\cdot}8 \pm 8{\cdot}2$ per protozoon. Before sonic treatment the number of viable bacteria per protozoon was always very close to 1, as would be expected.

In a series of 30 triplicate viable bacterial counts at various levels of broken protozoa, the scatter of reciprocals of mean counts were all within 20% and mainly within 10% (Fig. 2).

Most of the bacteria found were Gram-negative aerobes which were readily isolated on nutrient agar plates but rapidly over-grew the less numerous organisms in roll bottles. To overcome this for isolation purposes, sodium azide was found to be the best of a number of selective inhibitors, including antibiotics, which were tried. Sodium azide 500 μg/ml., with seed dilutions of 10^{-2} and 10^{-3} was used in roll bottles of the standard medium. Under these conditions, to achieve satisfactory reduction, as judged by decolorization of the resazurin, the concentration of L-cysteine was raised to 0·5 mg./ml. (Margherita & Hungate, 1963). From these roll bottles,

single colonies were seeded in the standard liquid medium and examined. A number of each morphological type found were again twice put through roll bottles and single colonies picked off, examined for purity and for identification. Nine species of bacteria were isolated, placed in groups for convenience, as follows:

Aerobic Gram-negative bacteria. From 69 strains, two kinds emerged designated 17G 1 and 17G 5A. Organism 17G 1 is a member of the genus *Klebsiella* because of lactose fermentation, capsule formation, production of lysine decarboxylase, absence of motility and inability to form indole it is assigned to *K. aerogenes* species on the basis of dulcite fermentation, negative methyl-red and positive Vosges-Proskauer reaction. No fimbriae were found and the 44° test was positive. Cowan & Steele

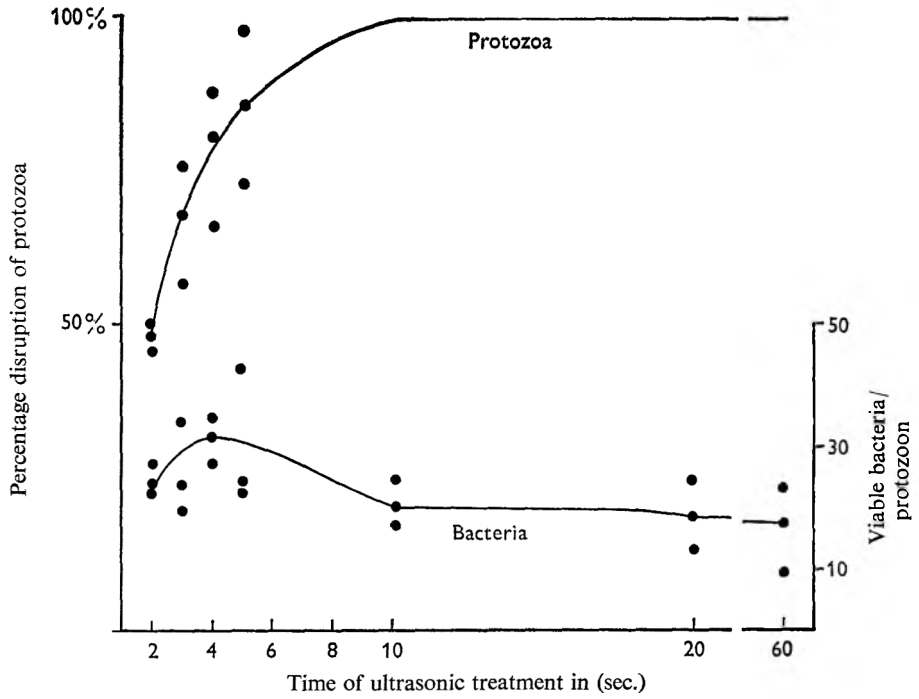

Fig. 1. Relationship of the degree of disruption of *E. caudatum* with numbers of released bacteria viable after treatment by bath ultrasonic treatment for 2 to 60 sec.

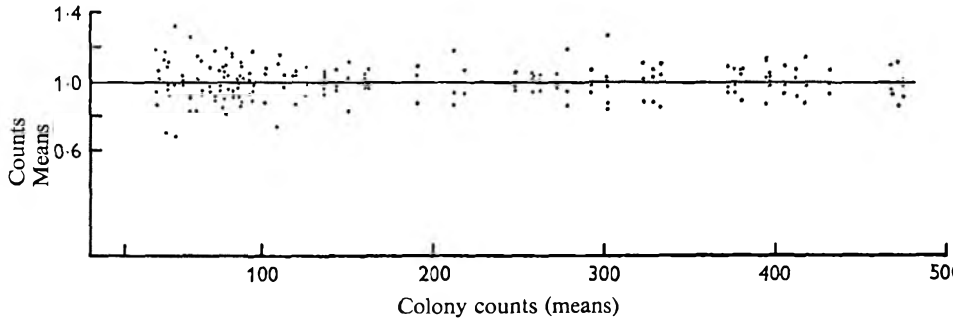

Fig. 2. Reciprocals (counts/means) of bacterial colony counts (triplicate) from disrupted *E. caudatum*, plotted to show variations around means over a range. Results from 30 experiments.

(1960) reported that 50 % strains were 44° positive and predicted the occurrence of non-fimbriate varieties. Organism 17G 5A was identifiable as a member of the genus *Proteus* because of motility with peritrichous flagella, and the ability to deaminate phenylalanine (Whitfield, 1967). It was assigned to *P. mirabilis* on the basis of sucrose fermentation, decarboxylation of ornithine and failure to produce indol.

Anaerobic Gram-negative bacteria. Two kinds were isolated, designated 7P 1 and 22G 33C. Organism 7P 1 was a small curved rod, motile with a single very long subpolar flagellum. It was fermentatively active, neither produced nor utilized lactate, and produced butyric acid freely from glucose (Table 1). It is assigned to the genus *Butyrivibrio* with no species identification. Bryant (1959) reported that only a proportion of butyrivibrio strains of ruminal origin fermented cellulose. Hungate (1966) placed strains not producing lactate in a new species *B. alactacidigens*, however, the pattern of substrate fermentation he described was not exhibited by 7P 1. Organism 22G 33C, a small slender rod, initially grew slowly in groups, but after repeated subculture good growth of separate bacteria occurs. As a non-motile bacterium, which hydrolysed casein, produced small amounts of lactic acid, but a trace of acetic acid only from glucose (Table 1) this organism is assigned to the genus *Bacteroides*. Absence of succinic acid production from glucose, and of amylolytic activity remove it from the commonly described rumen species; no species identification is offered.

Table 1. *Production of volatile fatty acids by isolated bacteria (m-moles per 100 ml. substrate)*

Strain	Substrate	Acetic	Butyric	Propionic
7P 1	Glucose	0	0·31	0·03
	Lactate	0	0	0
22G 33C	Glucose	0·06	0	0
	Lactate	0·03	0·02	0
15N 2*	Glucose	0·34/0·29	0·59/0·31	0/0
	Lactate	0·54/0·52	0·66/0·55	0/0
41GT16 2*	Glucose	0·19/0·43	0/0	1·10/1·25
	Lactate	0·11/0·24	0/0	0·50/0·68

* Two strains tested.

Gram-positive bacteria. Two kinds were isolated, designated 15N 2 and 41GT 16 2. Organism 15N 2 was frequently encountered closely associated with the Butyrivibrio 7P 1, from which it was difficult to separate even in single colony picks. It was a pleomorphic bacterium, bifid forms occurred, and agar slopes of the media described by Sundman, Björksten & Gyllenberg (1959) supported good growth of clubbed and irregular rods under anaerobic conditions only. It was fermentatively relatively inactive, utilized lactate freely and produced quantities of butyric acid from glucose or lactate (Table 1), and is a *Butyribacterium*. It differs from the single species member, *B. rettgeri* (Barker & Haas, 1944) in not surviving 60° for 30 min.

Organism 41GT 16 2 was a pleomorphic rod which grew very slowly in the standard medium to form tangled masses. Agar slopes of the Sundman *et al.* (1959) media supported growth of clubbed and beaded rods and on their 'G' agar and 'C.Y.E.' agar slopes this occured equally well under aerobic or anaerobic conditions; this was therefore an 'aerobe'. The organism survived 60° for 30 min., grew freely at pH 5·0, and pro-

duced lactic acid and large amounts of propionic acid from glucose (Table 1). It is assigned to the genus *Propionibacterium* (*Bergey's Manual*, 1948) with no species identification.

Streptococci. Three organisms were found, designated 15N 1, 16N 18 and 20G 1. None showed haemolysis in horse blood agar plates or survived 60° for 30 min., all three displayed haemolysins in a tube test and grew at pH 9·6. Extracts of organisms 16N 18 and 20G 1 reacted with grouping sera of Lancefield groups D and N. Organism 16N 18 was aerobic, grew at 45° and fermented raffinose, organism 20G 1 was anaerobic and failed to grow at 45° or to ferment raffinose. Both are assigned as strains of *Streptococcus bovis*. Perry, Newland & Briggs (1958) recorded the occurrence of cross-reactions between Lancefield groups D and N in strains of *S. bovis* from the rumen. Organism 15N 1 was a small streptococcus, extracts of which reacted with serum of Lancefield group G only. It failed to grow at 45° or to ferment raffinose; it produced ammonia from arginine. No species definition is offered.

Numerical relationships. To establish relationship between total numbers of intracellular and extracellular viable bacteria samples of Coleman's (1958, 1962) surrounding protozoal medium taken from his protozoa cultures were examined by the same bacterial counting method and medium as for the disrupted protozoa. Viable bacterial counts of between 98 and 267×10^5/ml. were obtained in eleven experiments in which the counts of protozoa were between 22 and 31×10^3/ml. From this can be calculated a figure of approximately 20 times as many viable bacteria per protozoon outside as inside the protozoa.

Attempts were also made to determine the ratio of the fast growing aerobic bacteria to the other organisms inside the protozoa by parallel aerobic and anaerobic counts, and by parallel roll bottle counts, with and without sodium azide. The first method gave a ratio of 3 to 1, the second method 13 to 1.

The ratio of *Klebsiella aerogenes* to *Proteus mirabilis* in the protozoa was determined by using the sensitivity of the latter to chloramphenicol determined on the isolated strains, when a concentration of 50 μg/ml. decreased numbers of *K. aerogenes* from 418 to 2×10^7, whereas the *P. mirabilis* was unaffected. Aerobic counts on the disrupted protozoa suspension were decreased by 30% in the presence of chloramphenicol, this was taken to indicate a ratio of approximately 2 to 1, *K. aerogenes* to *P. mirabilis*.

DISCUSSION

The bacterial flora in Coleman's protozoa culture tubes have become established over a long time, and under maintenance conditions which allow free entry of adventitious organisms, so that the environment has determined the pattern. Some of the bacteria isolated notably the Butyrivibrio and Bacteroides might have originated from the rumen. The *Klebsiella aerogenes* and *Proteus mirabilis* exerted a powerful reducing effect in the protozoa cultures.

Coleman (1964) showed that bacteria were engulfed and rapidly digested by his *Entodinium caudatum* in culture, and that differences in survival were not related to any obvious character or to the source of the bacteria. Coleman & Hall (1966) suggested that bacteria ingested by the protozoa might be taken whole into the cytoplasm and broken down there.

No real evidence emerges of recognizable colonization of the interior of the protozoa

by bacteria. If these were nidated inside, wide fluctuations in numbers might be expected, according to how far colony masses had become variably dispersed during disruption of the protozoa. The maximum possible number of the most numerous bacterium per protozoon can be calculated as 7 *Klebsiella aerogenes*, which is too low to interpret unequivocally as colonisation. It is most likely that the viable bacteria cultured were those which had been swept in by the protozoal cilia, possibly into an antrum but which had not progressed into the body of the protozoa at the time when these are disrupted.

Thanks are due to Drs P. F. V. Ward and P. Kemp for gas/liquid chromatograph estimations of volatile fatty acids, to Dr E. Munn for electron micrographs, to Mr G. A. Embleton for the insertion of permanent rumen cannulae in sheep, and to Mrs M. E. Black for invaluable technical assistance. Dr G. S. Coleman supplied his cultures of *E. caudatum* and much helpful criticism.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its fifty-fourth General Meeting at the University College of South Wales and Monmouthshire, Cathays Park, Cardiff, on 2 and 3 January 1969. The following communications were made:

ORIGINAL PAPERS

The Bacterial Metabolism of Flavonoids: Hydroxylation of Taxifolin. By A. M. JEFFREY, M. KNIGHT and W. C. EVANS (*Department of Biochemistry and Soil Science, University College of North Wales, Bangor*)

A bacterium isolated from rat faeces has been shown to produce taxifolin (3′,4′,5,7-tetrahydroxyflavanonol) and protocatechuic acid when grown on (+)-catechin (3′,4′,5,7-tetrahydroxyflavanol) (Farr, I. S. & Evans, W. C., unpublished experiments). This communication describes the hydroxylation of taxifolin to dihydrogossypetin (3′,4′,5,7,8-pentahydroxyflavanonol) by extracts of this organism.

Cell-free extracts prepared from bacteria grown on (+)-catechin catalysed the disappearance of taxifolin, measured both by decrease in E_{325} and by the colour reaction with Zn + HCl (Pew, J. C. (1948), *J. Am. chem. Soc.* **70**, 3031). Activity was dependent on oxygen and NADH; NADPH could partially replace NADH.

Enzyme preparations, fractioned by ammonium sulphate precipitation and chromatography on Sephadex G-200 and DEAE-cellulose consumed 1 mole of oxygen and 1 mole of NADH per mole of taxifolin, with no detectable acid production. With these preparations, the disappearance of the spectral peak at 325 mμ was followed by the appearance of peaks at 302 and 383 mμ. If the reaction were carried out in deionized water rather than phosphate buffer only one peak (λ max. 331 mμ) was found; this spectrum could be rapidly converted to the former one, non-enzymically, by addition of Mn^{2+}, Fe^{3+} or Cu^{2+}.

The material absorbing at 331 mμ was isolated from a scaled-up reaction mixture and purified by thin-layer chromatography. The products of sodium amalgam reductive fission of this compound (Hurst, H. M. & Harborne, J. B. (1967), *Phytochem.* **6**, 1111) were identical on thin-layer chromatography to those obtained from taxifolin. Oxidation with bismuth carbonate (Mahesh, V. B. & Seshadri, T. R. (1948), *Proc. Ind. Acad. Sci.* **41**A, 210) gave a compound with identical chromatographic properties, and ultraviolet spectral characteristics (both in ethanolic solution and in presence of zirconium oxychloride) to gossypetin. Infrared and ultraviolet spectra and characteristic colour reactions are all consistent with the products of the enzymic reaction being dihydrogossypetin.

We are grateful to the African Territories Wattle Industry Fund for supporting this work.

A Heat-stable Nicotinamide Adenine Dinucleotidase from *Pseudomonas fluorescens*. By I. H. MATHER and M. KNIGHT (*Department of Biochemistry and Soil Science, University College of North Wales, Bangor*)

NADases have been isolated and purified from a variety of microbial and mammalian sources (e.g. Gopinathan, K. P., Sirsi, M. & Vaidyanathan, C. S. (1964), *Biochem. J.* **91**, 277; Swislocki, N. I. & Kaplan, N. O. (1967), *J. biol. Chem.* **242**, 1083). They are usually glycohydrolases, catalysing the hydrolysis of the nicotinamide-ribose linkage to yield nicotinamide and ADP-ribose. Some microbial enzymes, such as those from *Mycobacterium tuberculosis*

(Gopinathan, Sirsi & Vaidyanathan, 1964) and *M. butyricum* (Kern, M. & Natale, R. (1958), *J. biol. Chem.* **231**, 41) are relatively heat-stable and appear to have heat-labile inhibitors associated with them.

An apparently similar NADase-inhibitor system has been found in extracts of *Pseudomonas fluorescens* KB1. The enzyme is extremely stable to heat, while the inhibitor is heat-labile. Extracts were therefore activated by heating at 100°. Activity was maximal after 3 min. at 100°, and decreased by about 30 % over 30 min.

When activity was measured over a 5 min. incubation period, the enzyme had an apparent optimum temperature of 37°; it was inactive above 60°. Since the preparations were made by boiling it appears that heat-denaturation is reversible. The enzyme has a broad pH optimum between pH values of 5 and 10; below pH 3·6 it is inactive. NADP is hydrolysed at the same rate as NAD; the reduced forms are not attacked.

The products have been shown to be nicotinamide and ADP-ribose. The enzyme is inactivated by incubation with trypsin, is excluded from Sephadex-G25 and precipitated by ammonium sulphate. The enzyme has been purified in six stages to a single band on acrylamide gel electrophoresis.

The Response of Natural Marine Populations to Abrupt Changes in Substrate Concentrations. By R. W. GRAY and P. J. LEB. WILLIAMS (*Department of Oceanography, University of Southampton*)

A radiochemical method capable of following the oxidation of individual organic compounds in natural waters has been developed (Williams, P. J. leB. & Askew, C. (1968), *Deep-Sea Res.* **15**, 365). This method has been used to study the response of natural heterotrophic populations to sudden increases in the concentration of organic compounds. Seawater samples were taken from a point 22 miles offshore in the English Channel and from an estuary—Southampton Water. To these samples 0·1, 500 and 5000 μg./l. of ^{14}C-labelled amino acids were added, and their respiration followed over a period of 48 hr by the liberation of radioactive carbon dioxide. The turnover of amino acids was more rapid in the estuarine water (16 hr) than in the sample from offshore (190 hr). In both waters the addition of 500, and 5000 μg./l. of amino acids caused a pronounced increase in turnover time. This increase, however, was temporary; in the estuarine water after 20 to 30 hr the heterotrophic population increased its activity and reduced the turnover time of amino acid oxidation to a value approaching the original one. In the offshore water the lag was longer, at the 500 μg./l. level the turnover of amino acids returned to the original value after about 36 hr, whereas at the 5000 μg./l. level the turnover time was still well below the original after 48 hr.

Immuno-specific Extraction of Non-Specific Inhibitor from Anti-viral Serum. By R. G. C. GALLOP, G. S. PLATT & B. T. TOZER (*Microbiological Research Establishment, Porton Down, Salisbury, Wiltshire*)

Many animal sera are known to contain non-specific inhibitors of arbovirus haemagglutination. The methods most commonly used to remove this inhibitor prior to the haemagglutination inhibition test for specific antibody (Clark, D. H. & Casals, J. (1958), *Am. J. Trop. Med. Hyg.* **7**, 561) are cumbersome and not always effective (Holden, P., La Motte, L. C. & Shriner, R. B. (1965), *Science, N.Y.* **147**, 169; Holden, P., Muth, D. & Shriner, R. B. (1966), *Am. J. Epidemiol.* **84**, 67) or may remove the specific antibody (Gardamovich, S. Y., McKler, L. B. & Kahaltayeva, G. G. (1967), *Acta Virol.* **11**, 114).

We have isolated a fraction of normal guinea-pig serum containing the non-specific inhibitor of arbovirus haemagglutination. A specific anti-serum to this inhibitor has been prepared in rabbits and its use in the removal of non-specific inhibitor from immune guinea-pig serum by an immuno-specific technique has been demonstrated.

The isolation of the inhibitor was effected by a simple two-stage process, ultracentrifugation followed by gel filtration of the least dense fraction through Sephadex G200. The inhibitor was found in the early fractions, and was free of gamma-globulin. An anti-serum to this fraction was produced in rabbits by the method of Freund & McDermott (1942), and rabbit non-specific inhibitor removed from it by ultracentrifugation.

A volume of this treated serum equivalent to 0·1 ml. of original serum was found to be effective in removing all non-specific inhibitors from 0·1 ml. of immune guinea-pig serum without affecting the specific antibody titre, whether the antibody was present as IgM or IgG. A certain degree of species specificity is apparent.

Part of this work is the subject of Patent Application no. 376/2/68.

β-Ketoadipate CoA-transferase in *Neurospora crassa*. By R. B. CAIN (*Microbiology Group, Department of Botany, University of Newcastle upon Tyne*)

The metabolism of β-ketoadipate by bacteria involves the synthesis and subsequent cleavage of β-ketoadipyl-CoA (Katagiri, M. & Hayaishi, O. (1957), *J. biol. Chem.* **226**, 439) but earlier studies with *Neurospora crassa* (Ottey, L. & Tatum, E. L. (1957), *J. biol. Chem.* **229**, 77) suggested that the mechanism in this fungus was essentially a hydrolytic cleavage even though a requirement for CoA was demonstrated. Although they established that most fungi and bacteria were biochemically identical in their utilization of β-ketoadipate Cain, R. B. *et al.* (1968, *Biochem. J.* **108**, 797) found no transferase in vanillate-induced *N. crassa*.

A detailed examination of induced wild-type and 'aromaticless' mutant strains has now revealed that *N. crassa* resembles other fungi in possessing β-ketoadipate CoA-transferase (EC 2.8.3.6). The spectrophotometric assay of this enzyme at 305 mμ, pH 8·0, depended upon critical quantities of substrates and mycelial extract and could be reliably followed by expanding the extinction scale 10-fold. The techniques originally used by Ottey & Tatum (1957) did not detect activity. Furthermore, these authors prepared their extracts in mannitol-phosphate buffer; the transferase, however, was strongly inhibited (72 %) by phosphate.

Transferase activity was Mg^{2+}-dependent, highly specific for succinyl-CoA and β-ketoadipate and was readily reversed by succinate. The transferase was strictly inducible in wild-type mycelia but large amounts were present in the 'aromaticless' mutant grown on glucose + aromatic amino acid-supplement. The transferase and the other enzymes of the protocatechuate pathway appeared to be induced and repressed co-ordinately.

The transferases of both wild-type and mutant were identical as indicated by their elution from Sephadex G-200, their thermal denaturation at 45° and their sensitivity to adipate inhibition In these properties, they closely resemble transferase III of *Moraxella calcoacetica* (Canovas, J. L. & Stanier, R. Y. (1967), *European J. Biochem.* **1**, 289).

The Measurement of Antibody to *Mycoplasma gallisepticum* by the Metabolic-inhibition Technique. By D. TAYLOR-ROBINSON (*M.R.C. Clinical Research Centre, Harvard Hospital, Salisbury*) and D. M. BERRY (*Glaxo Laboratories Ltd., Greenford, Middlesex*)

Antisera to *Mycoplasma gallisepticum* strains were prepared in rabbits, turkeys and chickens. The response of individual rabbits or birds varied widely. Using rabbit antisera, antigenic differences were detected between strains so that they could be divided into at least three subtypes. Antibody titres were lower in turkeys than in rabbits, and lower still in chickens. The assumption that the most suitable mycoplasma strain to use for detecting antibody was that used to inoculate the birds was incorrect. Antibody was often only detectable by using a heterologous strain in the metabolic-inhibition test. Such inability to measure antibody was not overcome by the addition of fresh guinea-pig, turkey or chicken sera to the tests. These findings may be relevant in the measurement of antibody to other mycoplasma species.

SYMPOSIUM: MICROBES IN HEAVY INDUSTRY

Microbes in Mining Operations. By J. R. POSTGATE (*A.R.C. Unit of Nitrogen Fixation, University of Sussex*)

Micro-organisms are involved at three main stages in mining industry.

Ore formation

'Bog iron' deposits formed by iron bacteria are of historical interest but are now largely exhausted; various iron organisms are involved, *Sphaerotilus* being commonest. Sulphate-

reducing bacteria appear responsible for the deposition of several sulphide ores—iron (as hydrotroilite or pyrites), copper, lead and possibly others—the formation of pyritic fossils being typical of the process that accompanies their action: a concentration gradient in this direction of the sulphide zone, caused by precipitation of the metal sulphide, favours selective accumulation of the metal. In many cases the role of sulphate-reducing bacteria in metal sulphide deposition needs checking by isotope fractionation, which has shown unequivocally their role in the formation of most natural sulphur deposits. Microbiological sulphur formation has been adopted industrially in countries in which natural sulphur is rare, expensive or running out; microbial processes for concentrating rare metals might be developed with advantage. Peat and coal formations involve the participation of microbes but the details are not understood.

Extraction of ores

The leaching of pyritic ores by *Thiobacillus ferro-oxidans* and related species is now well established. Though this process yields a proportion of ochre ('ferric hydroxide'), which is used in the paint industry, its main value lies in the concomitant extraction of copper from pyritic deposits, particularly the copper-rich chalcopyrite. The copper is recovered from such effluents by adding scrap iron; zinc and manganese may also be leached in the process. The organism is mesophilic, aerobic, autotrophic, acidophilic and highly copper-resistant. The ferrous iron of the pyrites is oxidized to ferric and may then react with other elements which in consequence may also be leached. Recent patents are much concerned with the removal of uranium by the transformation $U^{IV} + 2\,Fe^{III} \rightarrow U^{VI} + 2\,Fe^{II}$. Vanadium and molybdenum are solubilized in a similar fashion. These processes provide a cheap but slow method of upgrading low-grade ores at acid pH values; in the uranium and copper extraction processes, patent files give evidence that a fairly complex microbiological technology is developing.

Leaching may remove unwanted contaminants from minerals and a process has been developed for upgrading coking coal by using *T. ferro-oxidans* to lower its pyrites content.

Effluent control

Acid mine waters are corrosive to pumping machinery and damaging to surrounding soils and the flora and fauna of rivers. The acid, H_2SO_4, arises from the action of *T. ferro-oxidans* on pyritic strata newly exposed to air in coal and gold mines. The water, when neutralized, is highly ferruginous and not usually acceptable by water authorities. Control of acid production by microbial inhibitors is rather short-term; promising claims have been made for the use of antagonistic microflora from naturally non-acid areas. Sulphate-reducing bacteria have been proposed for the removal of iron from ferruginous waters since, in appropriate conditions, the ferrous sulphide formed is magnetic and thus readily removed.

Micro-organisms and the Petroleum Industry. By J. F. WILKINSON (*Department of General Microbiology, University of Edinburgh*)

The importance of microbiology to the petroleum (including natural-gas) industry lies both in negative or defensive aspects (e.g. contamination of oils, etc.) which will not be considered in this contributions and in positive or offensive aspects. The latter can be divided into their use in prospecting for new oil or natural gas fields and in hydrocarbon fermentation processes.

(1) *Prospecting*. The value of microbiological prospecting depends on two premises:

(*a*) The hydrocarbon-utilizing micro-organisms occur naturally at higher concentration when a seepage from underground deposits of oil and natural gas is present. It is therefore important that the organisms should be obligate hydrocarbon-utilizers and that the particular hydrocarbon or hydrocarbons being used as the carbon source in media used for counting should not be produced in the surface layers.

(*b*) There is a direct spatial relation between such surface concentrations of hydrocarbons and the corresponding underground deposits. In practice and for geological reasons, this is often not the case and in general microbiological prospecting has not found favour with the oil companies.

(2) *Hydrocarbon fermentation.* Since the ability to attack hydrocarbons is widespread among micro-organisms (bacteria and fungi) and since certain fractions of crude oil or natural gas can represent the cheapest form of organic carbon available, much work has been done on hydrocarbon fermentations as a means for the production of cheap sources of nutrients (and especially protein) for animal and human nitrition. Interest has centred on three main types of substrates.

(*a*) *Gas oil.* Here the micro-organisms act as fractionating agents, removing the *n*-paraffins from the gas oil. In this way the micro-organisms can be considered as a useful by-product of the dewaxing process. It is possible to carry out the process in a non-aseptic continuous culture but problems do arise in separation of the microbial cells (usually yeasts) from the non-utilized fractions of the gas oil. This is particularly important if the micro-organisms are to be used as a feedstock.

(*b*) *n-alkanes.* The *n*-paraffins are separated from gas oil by a non-microbiological dewaxing process. The substrate for growth is therefore more expensive but it is possible to obtain complete utilization in growth and hence the separation processes used in the gas oil process are no longer required.

Although bacteria can be used for both these processes, interest has centred on yeasts from the point of view of ease of handling and harvesting, lack of toxic components and resistance to virus attack.

(*c*) *Natural gas.* The only methane-utilizing micro-organisms to be isolated appear to be bacteria, although a wide range of different morphological types can be obtained. They are mainly obligate methane or methanol utilizers and since their study is still relatively in its infancy, it is difficult to be certain of their potentiality.

Most of the interest up to now has centred on the production of bulk protein, although all the systems can theoretically be extended to the synthesis of any metabolite by the use of appropriate over-producing mutants.

Microbes in Metal Manufacture and Fabrication. By E. C. HILL

In the production of steel and aluminium sheet and in their fabrication into finished articles, petroleum products are inevitably involved, not only as lubricant oils but also as coolant emulsions. In machining, grinding, pressing, drawing and extruding metal parts, oils or oil emulsions are widely used in systems of from 5 to 100,000 gallons which are often ideal for the growth of micro-organisms. The organisms only grow actively in the aqueous phase, and if carried by agitation into the oil phase survive for a few hours only (Hill, Evans & Davies (1967), *J. Inst. Petrol.* **53**, 280), and plug pipes and filters. Populations in the order of 10^9/ml. are easily attained, and not infrequently final levels of 5 % wet wt microbial cells have been recorded.

The inevitable rapid chemical changes in the oil formations are reflected in their progressive failure in engineering terms. Other industrial metal-working fluids, particularly plating solutions, suffer similar massive infections.

Oil emulsion freshly put into works use is contaminated immediately by hundreds of thousands of organisms per ml. The source of these organisms normally arises in the works from slimes from the previous fouled emulsion. In continuously used well-aerated systems at close to ambient temperatures growth is rapid and species of *Pseudomonas*, *Alcaligenes* and *Achromobacter* dominate. Systems not well aerated, intermittently used, or with a slow turnover, also contain anaerobic organisms, particularly *Clostridium nigrificans* and facultative anaerobes. Rolling-oil emulsions are maintained at an elevated temperature which suppresses most of the organisms found in cooler systems, and *Nocardia* sp. then dominate.

The exact microbiology of any system will depend on the rate of aeration, the temperature, pH, the availability of the nutrient elements required and the presence of effective inhibitors. In normal emulsions there is an excess of carbon for microbial requirements and a deficiency of nitrogen, phosphorus and perhaps sulphur. These elements may be supplemented by sandwiches, cigarette ends, urine, etc.

Engineering significance of microbial growths

The most obvious visible evidence of microbial growth is the microbes themselves. They appear as *slimes* and *sediments* wherever they are allowed to accumulate, and are also removed from systems as deposits in centrifugal or paper swarf separators. Also obvious are the objectionable smells due to degradation products, usually accompanied by darkening colour.

The chemical changes which take place may affect lubricating properties and viscosity; and alter additives which induce 'bite', increase oil film strength, inhibit corrosion and stabilize emulsions.

Thus an emulsion progressively changes from finely dispersed oil droplets in water to a coarse dispersion and eventually free oil separates.

High microbial activity in oil emulsions causes the following. (*a*) Short oil life: costly oil changes; time loss during changes; excessive topping up with oil to maintain the emulsion strength. (*b*) Poor surface finish during machining and rolling; roll-slip in rolling steel and aluminium; corrosion on steel sheet (Hill & Pemberthy, (1968), *Metals and Materials* **2**, 359). (*c*) Fouled grinding wheels; wheel burn; high wheel dressing costs. (*d*) Excessive solid loads on filters and clarifiers; slimes and deposits. (*e*) Rapid corrosion after machining. (*f*) Smells and discolorations.

Straight oils in use often have an adequate water content for bacterial growth and oil becomes turbid and slimey. Infected bearing-oils often form the reservoir of infection for emulsions used as coolants.

The study of the infected systems has fallen into three major categories.

(*a*) Relationship between engineering practice and microbial infection followed by recommendations to minimize the potential hazard (Hill (1967), *Engng. Mater. Des.* **10**, 1924). (*b*) Oil formulation practice at least to ensure that the components do not form an ideal diet for the organisms. (*c*) Physical or chemical methods to inhibit microbial growth. Considerable success has been achieved in this field with biocides to kill existing infections and biostats to minimize re-infection. Additives must be carefully screened to ensure having no adverse engineering features and safe in use. (*d*) Works trials with regular microbiological examination to assess progress. (*e*) The development of simple tests to enable ordinary works laboratory staff to assess rapidly the daily level of microbial infection and biocide additions (Hill, Davies, Pritchard & Byrom (1967), *J. Inst. Petrol.* **53**, 275). In a rolling mill, oil savings alone may amount to tens of thousands of pounds a year, with substantial additional bonuses in time-saving, product quality and less shut-down time as previously outlined. In machining operations the bonuses are in less 'down-time', less 'scrap', increased tool life, consistent surface finish and freedom from corrosion.

Microbes in Waste Water Purification. By S. JACKSON and D. E. F. HARRISON (*Water Pollution Research Laboratory, Stevenage (Ministry of Technology)*)

Among the principal problems in water pollution, aggravated by silting due to sedimentation of suspended solids present in effluents, is diminution of the dissolved-oxygen concentration by biochemical oxidation of organic polluting matter, although the directly toxic effects of some inorganic and organic substances on river life is also a matter of serious concern.

The essence of sewage purification processes is to expose sewage (after settling out much of the suspended solids) to prolonged contact with large populations of micro-organisms, under well-aerated conditions to bring about the biochemical oxidation before discharge to surface waters. The inoculation of the process is from the sewage itself or from the air. The predominant bacteria in treatment plants are Gram-negative rods (Pseudomonads and Achromobacteraceae) (Allen, L. A. (1944), *J. Hyg., Camb.* **43**, 424) and in the form of very coherent flocs (activated sludge) or slimes (percolating filters), the latter being also rich in fungi. The presence of protozoa is important for the removal of free-swimming bacteria to clarify the effluent (Curds, C. R. *et al.* (1968), *Wat. Pollut. Control* **67**, 312). Numerous coliform bacteria are present in sewage, but only small numbers of pathogenic organisms can be detected. Very large proportions are removed in producing an effluent which satisfies the usual quality standards for biochemical oxygen demand and suspended-solids content, but the residual count of coliforms is still usually of the order of 10^4/ml.

Sewage is much weaker than conventional bacteriological media. A typical domestic sewage in this country (Painter, H. A. *et al.* (1961), *J. Inst. Sew. Purif.* p. 302) contains about 600 mg./l. of carbon as organic compounds. About half of this is settled out at the primary sedimentation stage. Of the rest, which is subjected to biochemical oxidation, only one-third is dissolved, with two-thirds present in the suspended solids. The principal constituents of the suspended solids are higher fatty acids and their esters, and proteins. Contact of this particulate matter with the bacteria evidently involves adsorption, and its degradation is probably less easy than that of the soluble organic matter. About half the dissolved carbon consists of sugars, with considerable amounts of organic acids. About 20 mg./l. of anionic surface-active agents is present, half in solution, half adsorbed on the suspended solids. These are largely alkyl benzene sulphonates; the manufacture of these has been modified during the last few years to minimize the extent of branching in the alkyl chains, in order to make them susceptible to biochemical oxidation. Much the most important nitrogenous constituent is ammonium ion, present at a concentration of about 50 mg./l.

A full account of the process of biochemical oxidation of such a complex mixture by a comparably complex mixture of organisms is clearly a major challenge. The treatment processes are run at long retention times, to achieve maximum oxidation of organic matter with minimum productivity of microbial sludge. A bacterial population of much higher activity could be envisaged, and this appears to offer scope for increasing the rate of sewage treatment. It is, however, important that this should not be achieved at the cost of heavy production of surplus microbial sludge of a character difficult to sediment and to de-water. Further, when removal of ammonia is required by the river authority, a comparatively low rate of operation is obligatory to avoid wash-out of the rather slow-growing nitrifying bacteria. Sometimes a compromise is effected by employing two successive stages of treatment, the first at a high rate of flow in a compact unit, the second at a lower rate in a plantof larger volume.

Anaerobic digestion is widely used as part of the treatment of the thin sludges arising from primary settlement of sewage and from biological treatment. Large sewage works usually employ continuous digestion in closed tanks heated to about 35° C. The methane produced is collected and used as fuel. Several species of methane bacteria are involved in the final production of methane from simple substrates; the bacteria which produce these simple substrates by degradation of more complex compounds have been little studied (Harkness, N. (1966), *J. Inst. Sew. Purif.* p. 542).

Many industrial waste waters containing organic compounds are purified by using aerobic biological processes. Even the liquors arising from carbonization of coal, containing phenol, thiosulphate, and thiocyanate can be successfully treated by the latter method, yielding an activated sludge containing Thiobacilli (Hitchinson, M. & White, D. (1964), *J. appl. Bact.* **27**, 244). Strong organic wastes, e.g. from meat processing, are sometimes treated by anaerobic digestion.

Microbiological Research in the Electrical Power Industry. By JOAN E. RIPPON (*Central Electricity Research Laboratories*)

All power stations use large quantities of water for cooling purposes. Microbial multiplication may occur throughout this system and microbes are thus a potential hazard in the electricity supply industry. A list of the unwanted activities of microbes in a power-station might include the following. (*a*) Slime formation in the cooling water system, notably the condensers. (*b*) Production of hydrogen sulphide in parts of the cooling system, resulting in corrosion. (*c*) Decay of the timber packing of the cooling towers. (*d*) Slime formation in the alkaline water of nuclear fuel element cooling ponds. (*e*) Postulated breakdown of inhibitors in turbine oils.

Slime formation in the condensers varies considerably in quantity from station to station and is usually controlled by chlorine injection without recourse to the microbiologist's assistance. Corrosion of the condenser tubes with sulphide deposition occurs at a few stations, and it has been thought that this might be due to the activities of sulphate-reducing bacteria.

A less readily removed slime is that which has occurred in some nuclear-element cooling ponds. The spent fuel rods are stored under water to allow for some radioactive decay before

they are sent to Windscale for processing. Demineralized water at a pH greater than 10 is used to prevent corrosion of the magnox fuel cans. Despite the radioactivity, and the fact that the water is continuously treated to keep down impurities, micro-organisms have flourished in some stations to the extent of producing masses of very tenacious slime. Investigation has shown this slime to be bacterial in origin, with mainly orange or pink pigmented Gram-negative rods predominating. Although at first sight the strains from different stations appear similar, they show many slight differences on further examination. They are unreactive in most laboratory tests but antibiotic sensitivity has proved useful in trying to group them. It seems probable that in the radioactive environment mutation is taking place, making comparison of the strains very difficult.

Bacteria have been used to advantage as tracers for following water movements in the Blackwater estuary, *Serratia marcescens* has been used and it has been possible to follow the movements of the culture over 3 days, during which time a patch of water up to 9 miles in length has been marked.

Modern coal-fired power-stations produce eight million tons of pulverized fuel ash a year, and although increasing use of this material is made by the construction industries, a residue is still left for disposal. Work by, among others, Cope, of Leeds University (Ph.D. thesis, 1961), showed that certain crops could be grown on p.f.a. despite the undesirable properties of high alkalinity, high levels of boron and poor structure.

Investigation has shown that most soil micro-organisms will tolerate an ash environment and a considerable microflora will build up over the years. Ash lacks organic material and any addition of organic material will improve plant growth. Experiments are now in progress using waste organic materials with the addition of selected micro-organisms to improve plant growth. Experiment has shown improved growth of barley following the addition of a cellulose decomposing fungus to ash/sewage sludge or ash/grass mixtures. Field trials are now in progress.

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