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THE JOURNAL OF GENERAL MICROBIOLOGY

The *Journal* will publish accounts of original research in general microbiology, i.e. the study of bacteria, microfungi, microscopic algae, protozoa, and viruses in their biological activities and, more particularly, the fundamental aspects of the study of these forms, including structure, development, physiology, genetics, cytology, systematics and ecology. Writers of papers on a specialized aspect of their subject should describe their work so that its relevance to their own science and to microbiology in general will be apparent to readers who may be unfamiliar with the particular aspect.

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"Easy writing's curst hard reading."—*Richard Brinsley Sheridan*

"Easy reading's curst hard writing."—*The Editors, J. gen. Microbiol.*

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in English and should in general be divided into the following parts in the order indicated: (*a*) Summary: brief and self-contained; (*b*) Introduction; (c) Methods; (*d*) Results (illustrative protocols only should be included); (*e*) Discussion (if any), and general conclusions; (*f*) acknowledgements; (*g*) References.

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Symbols and Abbreviations. Authors should refer to current issues of *The Journal of General Microbiology* for information in this connection. Attention is particularly drawn to the following points: *c.* = circa or approximately; degrees Centrigrade are written, e.g. 100°, not 100° C.; hr., min., sec. (singular and plural); M = molar; m (milli-) = 10^{-3} and μ (micro-) = 10^{-6}; ml. (millilitre) should be used instead of c.c., and μg. (microgram) instead of γ; N = normal (of solutions); No. or no. = number. Ratios should be written 1:10; dilutions, 1/10.

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Descriptions of new species of cultivable microbes should not be submitted unless an authentic specimen of a living culture has been deposited in a recognized culture collection.

The word 'generation' should not be used synonymously with 'subculture'. For an agreed use of terms like strain, type, variant, phase, etc., see the International Bacteriological Code of Nomenclature, Section 1, Rules 7 and 8.

Except for good reasons, micro-organisms should be designated by the names used in the works listed below. When other authorities are followed, they should be cited whenever obscurity might result from their use.

MICROFUNGI. Ainsworth & Bisby's *A Dictionary of the Fungi*, 1954, 4th ed. (Kew: Imperial Mycological Institute.)

PLANT PATHOGENIC FUNGI AND PLANT DISEASES. *List of Common British Plant Diseases*, 1944. (Cambridge University Press.)

PLANT VIRUSES AND VIRUS DISEASES (1946). *Rev. appl. Mycol.* **24**, 513–56.

BACTERIA. Author's references in naming are at present accepted provided that the designation is unambiguous and conforms with the International Bacteriological Code of Nomenclature (1949; *J. gen. Microbiol.* **3**, 444) and the Opinions issued by the International Committee on Bacteriological Nomenclature. If desired a synonym may be added in brackets when a name is first mentioned.

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Processes in Cell Ensembles: Correlated Fluctuations and their Effects

By A. B. ZAHLAN
Physics Department, American University of Beirut, Beirut, Lebanon

(*Received* 14 *May* 1960)

SUMMARY

In the study of biological systems kinetic problems arise which are not significant in the kinetic systems of interest to physical chemists. One of these biological problems is the influence of correlated fluctuations in molecular properties on the macroscopic variables of the cell. It is demonstrated that, for an ensemble consisting of systems each of which includes two chemical compounds, there is a definite effect which originates in correlated fluctuations and is reflected in a broadened distribution of the concentration of chemical constituents. It is shown that a periodically varying temperature narrows this distribution. The narrowing is a function of the frequency at which the temperature is varying.

INTRODUCTION

This study is motivated by a number of observations recorded in the literature concerning the response of cell populations to temperature variation (Zeuthen, 1958; Scherbaum, 1960). By measuring any given property of any cell in a colony, one finds that the value of that property varies from cell to cell. Rahn (1932) attempted to explain these variations by assuming a step-wise growth of the chromosomes and by attributing equal probabilities for the addition of each successive gene. He then used this treatment to investigate the dependence of the spread of division time on the number of genes. Kendall (1948, 1952) investigated the role of variable generation time in the development of a stochastic birth process. Kendall defines a 'multiple-phase birth process' which holds that duplication of a cell follows after the cell has passed through a finite number of phases. Both Rahn's model and Kendall's model lead to the conclusion that individual generation times are independent of one another. This lack of correlation of the generation times is implicit in the models.

Differences frequently exist in the properties of cells in taxonomically homogeneous colonies. A possible cause is the statistical fluctuations in the concentrations of important constituents of which each cell contains only very few molecules. We will not consider such cases in the present study.

There is no detailed experimental study of induced synchrony by a cyclic variation of temperature. The limited studies available indicate that such a procedure sometimes leads to synchrony and sometimes does not. Synchrony and spread in generation time, though perhaps interdependent in the case of a cell, are two different and distinguishable things.

Correlated fluctuations in the velocity of reactions mediated by enzymes

Klotz and his co-workers (1957, 1958) have advanced the view that macromolecules are surrounded by a sheath of 'frozen water' which must be penetrated for the molecule to diffuse towards an active enzyme site. Klotz showed that if a molecule A diffuses through this sheath to the surface it must have induced some protein denaturation near the adsorption site. When molecule A undergoes some reaction on the surface of the enzyme it will diffuse from the site, but the succeeding molecule A′ diffusing to the surface will find this portion of the surface somewhat affected by the previous event. The condition of the surface is determined by random events and it is this condition which determines the reaction rate constant. Since these surface changes require a finite time, an event on the surface may influence succeeding events. Such a function of time is considered stochastic (Kittel, 1958; Uhlenbeck & Ormstein, 1954). The statistical fluctuations in the number of hydrogen bonds lead to fluctuations in both the entropy and the energy of activation for enzyme-controlled reactions.

In order to have correlated events, the velocity of reaction $K(t+\tau)$ associated with an enzyme E at time $(t+\tau)$ must be related to the velocity of reaction $K(t)$ at time t. This is possible when a reacting molecule diffuses to the reaction site and completes its chemical transformation in a time roughly equal to the correlation time of the function $K(t)$. Consider an enzyme E on whose surface the compounds A and B react at two different sites. If there is energy or charge transfer between the reacting sites (cf. Szent-Gyorgyi, 1957) then these reactions are coupled. The rate of one reaction is then controlled by the rate of the other reaction and the condition of the surface. These processes are stochastic in character and lead to correlated fluctuations. Our model of the cell is then one where thousands of chemical reactions are taking place, each of which is influenced by its environment in the manner discussed above. Every so often the cell duplicates itself and breaks up into two new cells. Since it is the cumulative effect of all of these reactions that leads to cell division it is clear that any fluctuations in these reactions will be reflected in the cell division time. That is, the cells of a population will not all have the same generation time (time between two divisions).

In this study we wish to demonstrate the role of correlated fluctuations in dephasing synchronized colonies and to show how a cyclic variation in temperature 'damps' such correlation effects. The total effect of temperature variation (specially when T is large) is more complex than is assumed here. A current study (by author, to be published) analyses these phenomena.

The treatment of a simple model which displays correlated fluctuations

Constant temperature. To demonstrate the validity of our model we have to write the equations which describe the space-time variations of all the chemical species in a cell, allow for diffusion of material across the cell wall, then let the rate constants vary in a stochastic manner and show that the cumulative effect of these correlated fluctuations in the rate constants leads to a broadening in the spectral distribution of various cell properties. At present it is impossible to carry out such a complete programme. Instead, in the following sections we shall analyse the simplest example of such a kinetic system, namely, an ensemble of systems each of which is

composed of two compounds (1, 2), each of which is changed into the other pseudo-unimolecularly:

$$E_1 + 1 \rightarrow 2; \quad E_2 + 2 \rightarrow 1.$$

We shall assume that these processes are controlled by two different enzymes and that because of environmental effects, the rate constants are time dependent and stochastically correlated.

We shall compute the first and second moments of the distribution of the number of molecules of each compound in the ensemble. This is simpler than finding the complete distribution function for each compound over the whole ensemble. The second moment shows the 'broadening' in the distribution function due to these random variations, in whose absence there would be no dispersion. When the temperature is made to vary periodically with time the distribution narrows; i.e. the second moment is diminished.

The two compounds are now allowed to be transformed pseudo-unimolecularly into each other at the rates $k_{12}(t)$, $k_{21}(t)$, where the $k(t)$'s are stochastic variables dependent on a catalytic process. Let p_1, p_2 be the mole fractions of each species; we have then

$$dp_1/dt = k_{12}\, p_2 - k_{21}\, p_1, \tag{1}$$

$$dp_2/dt = k_{21}\, p_1 - k_{12}\, p_2. \tag{2}$$

Define

$$p_1 + p_2 = 1, \tag{3}$$

$$k_{12} + k_{21} = \lambda. \tag{4}$$

We now analyse the rate constants into Fourier series:

$$k_{12}(t) = k_{12}^0 + \sum_n (A_n \sin nt + B_n \cos nt), \tag{5}$$

$$k_{21}(t) = k_{21}^0 + \sum_n (C_n \sin nt + D_n \cos nt), \tag{6}$$

where k_{12}^0 and k_{21}^0 are average values of the rate constants and (A_n, B_n, C_n, D_n) are all randomly distributed functions. We now assume that the random variable components average to zero within the time interval $0 \rightarrow t$ of interest to us, but not within times of the order of milliseconds. This means that a molecule could diffuse to a catalytic surface and depart from such a location within a time interval short compared to the duration of our experiment or to the rate of variation of the condition of the catalytic surface. This assumption simplifies our problem considerably, since now we can set

$$1/t \int_0^t \lambda\, dt = k_{12}^0 + k_{21}^0 = \lambda^0, \tag{7}$$

and our solution becomes:

$$p_1(t) = p_1(0)\, e^{-\lambda^0 t} + e^{-\lambda^0 t} \int k_{12}\, e^{\lambda^0 t}\, dt. \tag{8}$$

Let us now average over the ensemble A.

$$\therefore \langle p_1 \rangle_A = \langle p_1(0) \rangle_A\, e^{-\lambda^0 t} + e^{-\lambda^0 t} \int e^{\lambda^0 t} \langle k_{12} \rangle_A\, dt, \tag{9}$$

but

$$\langle k_{12} \rangle_A = k_{12}^0. \tag{10}$$

Since ensemble averages and time averages may be assumed to lead to the same results,

$$\lim_{t \rightarrow \infty} \langle p_1 \rangle_A = k_{12}^0/\lambda^0. \tag{11}$$

Now, instead of specifying the random variables (A_n, B_n, C_n, D_n) in more detail (such as a set of Gaussianly distributed functions) we follow Uhlenbeck & Ormstein's (1954) procedure and simply seek the second moment about the origin $\langle p_1^2 \rangle_A$.

On squaring the solution for $p_1(t)$ and averaging over the ensemble A, we find

$$\langle p_1^2 \rangle_A = \langle p_1^2(0) \rangle_A \, e^{-2\lambda^0 t} + e^{-2\lambda^0 t} \int\int e^{\lambda(\xi+\eta)} \langle k_{12}(\xi) k_{12}(\eta) \rangle_A \, d\xi \, d\eta. \tag{12}$$

One then finds

$$\langle p_1^2 \rangle_A = \langle p_1^2(0) \rangle_A \, e^{-2\lambda^0 t} + \frac{(k_{12}^0)^2}{2\lambda^0 \tau_1} (1 - e^{-2\lambda^0 t}) + \left(\frac{k_{12}^0}{\lambda^0}\right)^2 (1 - e^{-\lambda^0 t}) - \left(\frac{k_{12}^0}{\lambda^0}\right)(1 - e^{-\lambda^0 t}) \, e^{-\lambda^0 t} \tag{13}$$

and

$$\tau_1^{-1} = \int_{-\infty}^{\infty} \phi(w) \, dw, \tag{14}$$

where $\phi(w)$ is the correlation function, where $w = \xi - \eta$. Then

$$\lim_{t \to \infty} \langle p_1^2 \rangle_A = (k_{12}^0/\lambda^0)^2 + (k_{12}^0)^2/2\lambda^0 \tau_1 = \langle p_{1,0}^2 \rangle_A. \tag{15}$$

The significance of this result is that in a system where correlation effects are absent we should have

$$\langle p_1^2 \rangle_A = \langle p_1 \rangle_A^2. \tag{16}$$

Here, we have a broadened density function; the second moment is increased by

$$(k_{12}^0)^2/(2\lambda^0 \tau_1).$$

This is the variance of the distribution.

The effect of a sinusoidal variation of temperature

Let

$$T = T_0 + B \cos ft. \tag{17}$$

T is about 300°K. in most biological reactions while B is about 10°C.; therefore, the ratio T/B is about 30. One can then write

$$A \, e^{-E/RT} \simeq k(T_0) \left[1 + \frac{EB \cos ft}{RT_0^2} \right], \tag{18}$$

where

$$k(T_0) = A \, e^{-E/RT_0}. \tag{19}$$

This time variation of the temperature does not affect $\langle p_1 \rangle_A$. However $\langle p_1^2 \rangle_A$ changes to

$$\lim_{t \to \infty} \langle p_1^2 \rangle_A = \left(\frac{k_{12}^0}{\lambda^0}\right)^2 + \left(\frac{k_{12}^0}{2\lambda^0 \tau_1}\right) - \frac{2\lambda^0 f}{f^2 + \tau_{13}^2} \left(\frac{BA_{12}^0 G_{12}^0}{RT_0^2 \lambda^0}\right)^2 + \frac{BA_{12}^0 G_{12}^0 \lambda^0}{RT_0^2 \tau_{13} (\lambda^{02} - f^2)} \left[\sin 2ft - \left(\frac{f}{\lambda^0}\right) \cos 2ft \right] \tag{20}$$

where τ_1 is defined as before.

But

$$\tau_{13} = \tau_{11} + \tau_{12};$$

and τ_{11}, τ_{12} are defined by the correlation functions:

$$\langle A_{12}(\xi) \, A_{12}(\eta) \rangle = \langle A_{12}^2 \rangle \, e^{-\tau_{11} w} = (A_{12})^2 \, e^{-\tau_{11} w}, \tag{21}$$

$$\langle E_{12}(\xi) \, e^{-E_{12}(\xi)/RT_0} E_{12}(\eta) \, e^{-E_{12}(\eta)/RT_0} \rangle = (G_{12}^0)^2 \, e^{-\tau_{12} w}. \tag{22}$$

We note the existence of a 'forced motion' term $\{\sin 2ft - (f/\lambda^0)\cos 2ft\}$ due to the periodic variation in the temperature. We can eliminate this term by taking a time average over one cycle.

$$\langle \lim_{t\to\infty} \langle p_1^2 \rangle_A \rangle_{\text{one cycle}} = \langle p_{1,0}^2 \rangle_A - \frac{cf}{\tau_{13}^2 + f^2}, \tag{23}$$

where

$$C = (2/\lambda^0)\,(A_{12}^0 G_{12}^0 B/RT_0^2)^2. \tag{24}$$

Equation (23) displays a reduction in the variance induced by temperature variation. We will now give a brief analysis of this important result. The distribution will be narrowest for $f = \tau_{13}$. One can show that for all reasonable values of the constants involved the second moment will have a value intermediate between the case where no correlated fluctuations exist and where $f = 0$. Note that the narrowing goes to zero as f or B go to zero. The narrowing is a function of the parameters of the system, such as τ_{11}, τ_{12}, τ_{13}, A and G. This is why this approach might be fruitful in investigating complex molecular systems.

Generalization of the results for a specialized many component system

So far we have considered an ensemble of two enzyme systems (or cells). We now generalize these results to an ensemble of N-component systems. Each N-component system is now referred to as a 'cell' and the ensemble of such cells is referred to as a colony of cells. That is, if

$$p = (p_1, p_2, \ldots, p_N), \tag{25}$$

where

$$\sum_{i=1}^{N} p_i = 1, \tag{26}$$

$$\frac{d}{dt}\mathbf{p}(t) = \begin{pmatrix} k_{11} & \cdots & k_{1N} \\ \vdots & & \\ k_{N1} & \cdots & k_{NN} \end{pmatrix} \begin{pmatrix} p_1 & (t) \\ \vdots & \\ p & (t) \end{pmatrix}. \tag{27}$$

Let every cell in the colony be denoted by a superscript. Then $P^a(t)$ denotes the composition of the ath cell at time t. At any time t, one can determine the average molar concentration of any species (i, say) to be

$$p_{0\,i}(t) = 1/M \sum_{a=1}^{M} p_i^a(t) \quad (M = \text{no. of cells in colony}).$$

The lack of uniformity (synchrony) in the colony can be indicated by

$$\Delta_i(t) = \langle\, |\, P_i(t) - P_{0i}(t)\,|\,\rangle_a,$$

and

$$d^2 = \langle \overline{P_i - P_{0i}^2} \rangle_a.$$

As before, we want to show that the correlated fluctuations in the k_{ij}'s lead to an increase in the value of the second moment $d^2(t)$ and that a periodic temperature variation leads to a narrowing in the distribution, i.e. a decrease in the value of $d_i^2(t)$. The solution (Bellman, 1953) of such a system of first-order differential equation is of the type

$$\mathbf{p}(t) = \mathbf{C}\mathbf{Y}(t)\, e^{Bt},$$

where C is a constant matrix given by $P(0) = C$, and $Y(t)$ contains the time-dependent portion.

Comparing this equation with equation (8) one can observe the features necessary to contribute to the broadening of $\mathbf{p}(t)^2$ due to $\langle [Y(t)]^2 \rangle_a$ being broadened by correlated fluctuations in the same sense as k_{12} though this result is more complicated.

Similarly, temperature variation will induce a decrease in the value of $\langle [Y(t)]^2 \rangle_a$. This argument is naturally not rigorous. The more general solution of the problem will be postponed for a later publication.

Synchronization of cell colonies by periodic variation of light intensity

Zeuthen (1958) reviewed experimental observations on colony synchronization by periodic variation of light intensity. It is simple to reduce this problem to the one already discussed above. Consider the same ensemble of two-component systems except that the processes are now both catalytic and photochemical, i.e.

$$E_1 + h\nu_1 + (1) \xrightarrow{a_{12}} (2),$$

$$E_2 + h\nu_2 + (2) \xrightarrow{a_{21}} (1).$$

Then

$$dP_2/dt = a_{12}p_1I_1 - a_{21}p_2I_2,$$

$$p_1 + p_2 = 1,$$

therefore

$$dp_1/dt = a_{21}I_2 - \lambda p_1.$$

If I_1 and I_2 are constant we find

$$p_1 = p_1(0) \exp\left[-\int \lambda\, dt\right] + \exp\left[-\int \lambda\, dt\right] \int \left[a_{12}I_2 \exp\left[\int \lambda\, dt\right]\right] dt,$$

which is identical in form with equation (8). However, if (I_1, I_2) are time-dependent we obtain an equation similar to the one we were led to through equation (18); except for minor changes in the parameters involved in our equations (23, 24) we obtain similar results as before.

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Physiological Relationships of Rapidly Growing Mycobacteria

Adansonian Classification

By J. CERBÓN and L. F. BOJALIL
Unidad de Patología, Escuela de Medicina, UNAM, Hospital General, Mexico 7, D.F., México

(*Received* 24 *August* 1960)

SUMMARY

The physiological properties of a collection of rapidly growing acid-fast bacilli were analysed according to a simple mathematical method proposed by Sneath. Closer relationships were observed between *Mycobacterium smegmatis* and *M. phlei* than between either of them and *M. fortuitum*; all three species form natural groups. On the basis of the Adansonian classification two other small groups were found which probably are new species. Some unnamed strains which could not be included in any of the groups mentioned above were placed in the classification and collectively labelled irregular branch. A key for the rapid identification of the specific groups is described.

INTRODUCTION

The characterization and taxonomic position of mycobacteria have been a constant problem, with numerous proposals for an adequate classification taking into account different view-points. Cultural and physiological properties, cytochemical tests, pathogenicity and antigenic relationships have been considered by many authors (Frey & Hagan, 1931; Pinner, 1932; Thompson, 1932; Gordon, 1937; Gordon & Hagan, 1938; Uesaka, 1956; Parlett & Youmans, 1956, 1958; Kushner, McMillen & Senderi, 1957; Shepard, 1957; McMillen & Kushner, 1959; Vogel, 1959; Wayne, 1959; Bönicke, 1958, 1960).

Three well-characterized species are recognized among the rapidly growing acid-fast bacilli: *Mycobacterium smegmatis* (Trevisan), Lehmann & Neumann, *M. phlei* Lehmann & Neumann and *M. fortuitum* Cruz. Gordon & Smith (1953, 1955) reclassified into these three species many others which previously had been given other names. The present study aims at an analysis of the physiological properties and relationships of a number of strains whose gross and microscopic characters make them seem similar to established species of rapidly growing mycobacteria, as well as the determination of their taxonomic position by the method proposed by Sneath (1957*a*, *b*) which has given good results in similar studies (Hill, 1959; Bojalil & Cerbón, to be published).

METHODS

The strains studied are listed in Table 1. They were maintained in Löwenstein–Jensen medium (Wheeler, 1951) and checked for viability and purity by microscopic examination and subcultures in different media. Single cell cultures of the strains were used to test their physiological properties:

Table 1. *Strains of Mycobacterium studied*

No. in tables	Name	Source and number
1	*M. smegmatis*	Rutgers University, S. A. Waksman
2	*Mycobacterium* sp.	UPHG, A-57-439 lymph node
3	*M. phlei* st.	Tuberkulose Forschung Institute, Börstel, Bönicke, 169
4	*M. ranae*	National Trudeau Bank, W. Steenken Jr, Trudeau Labs.
5	*M. lacticola*	Escuela Nacional de Agricultura, Chapingo, México, Etchegaray
6	*M. ranae* 110	National Trudeau Bank, W. Steenken Jr., Trudeau Labs.
7	*Mycobacterium* sp.	607, Rutgers University, S. A. Waksman
8	*M. friedmanii*	ATCC 114
9	*Mycobacterium* sp.	599 Rutgers University, S. A. Waksman
10	*Mycobacterium* sp.	ATCC 65
11	*Mycobacterium* sp.	Soil, Escuela Nacional de Agricultura, Chapingo, México Etchegaray
12	*Mycobacterium* sp.	362, ATCC, received as *M. butyricum*
13	*M. stercoris*	ATCC, 281
14	*Mycobacterium* sp.	UPHG, sputum,* C–912
15	*Mycobacterium* sp.	UPHG, sputum, C–153
16	*Mycobacterium* sp.	UPHG, peritoneum,* A–57–429–1
17	*Mycobacterium* sp.	Facultad de Medicina de El Salvador, Alfonso Trejos, subcutaneous abscess
18	*Mycobacterium* sp.	UPHG, lung cavity, A–59–52–1*
19	*Mycobacterium* sp.	UPHG, lung cavity, B–57–1787*
20	*M. phlei*	Strains NTB and W, Trudeau Labs., W. Steenken Jr. and Rutgers University, S. A. Waksman
21	*Mycobacterium* sp.	UPHG, lung, B–59–637*
22	*Mycobacterium* sp.	UPHG, bronchial aspiration, H.I. 4955
23	*Mycobacterium* sp.	UPHG, spinal fluid, H.I. 5987
24	*Mycobacterium* sp.	UPHG, sputum, C–227*
25	*Mycobacterium* sp.	UPHG, sputum 1450
26	*Mycobacterium* sp.	UPHG, R.D. 25 sputum
27	*M. butyricum*	ATCC 357
28	*Mycobacterium* sp.	UPHG, RD.47[A] sputum
29	*M. fortuitum*	ATCC, 6841, Cruz original isolate, McMillen
30	*M. fortuitum*	Malta strain, from E. Agius. NCTC, 8573. Medical Research Council Unit, Oxford, England
31	*M. fortuitum*	*M. minetti*, strain of Penso, R. E. Gordon, R–480
32	*M. fortuitum*	Schumper—sputum, McMillen, 14
33	*Mycobacterium* sp.	UPHG, sputum,* Acapulco–343
34	*Mycobacterium* sp.	UPHG, sputum, Acapulco–103
35	*Mycobacterium* sp.	UPHG, sputum, Acapulco–102
36	*Mycobacterium* sp.	UPHG, sputum Acapulco–465
37	*Mycobacterium* sp.	Cornell University, Knaysi, received as *M. thamnopheous*
38	*Mycobacterium* sp.	Communicable Disease Centre, Chamblee Georgia, Kubica
39	*Mycobacterium* sp.	Escuela Nacional de Ciencias Biológicas, 58375
40	*Mycobacterium* sp.	Veterans Administration Hospital, 380, E. Runyon
41	*Mycobacterium* sp.	Veterans Administration Hospital, 518, E. Runyon
42	*Mycobacterium* sp.	Veterans Administration Hospital, 481, E. Runyon
43	*Mycobacterium* sp.	Cuba-29, Hepatic abscess, Cuba, Habana, Curbelo

UPHG, Unidad de Patología, Hospital General, México.
* Isolated together with tubercle bacilli.

Differential properties used: (1) *Acid* from glucose, galactose, mannose, fructose, lactose, maltose, sucrose, trehalose, melibiose, raffinose, L-arabinose, mannitol, sorbitol, dulcitol, *m*-inositol, erythritol and salicin. (2) *Utilization* of benzoate, citrate, succinate, tartrate, pyruvate and propionate. (3) *Temperatures* of growth 28°, 37°, 45°, 52°. (4) *Resistance* to 60° for 4 hr.

Gross and microscopic morphology, as well as the degree of acid-fastness, are not taken as differential criteria because of their high variability, even within one strain. The capacity for visible growth in 48–72 hr. in Löwenstein–Jensen medium, and in nutrient glycerinated agar was the basis for considering a strain as a rapid grower.

Acid formation from carbohydrates. The cultures were examined for acid production after 7 and 28 days' incubation at 37° on inorganic nitrogen agar, a modification of the medium of Ayers, Rupp & Johnson (1919) as described by Gordon & Smith (1953). Each carbohydrate was sterilized separately from the basal medium. Bromocresol purple was used as the indicator of acid production.

Utilization of organic acids. Modifications of Koser's citrate agar (Gordon & Smith, 1955) were made by combining 2 g. of the Na salts of benzoic, citric, succinic, pyruvic, or tartaric acids with saline solution buffered to pH 6·8, agar, 1·5 % (w/v) and phenol red as indicator. The alkaline colour of the indicator after incubation at 37° demonstrates the utilization of an organic acid as a carbon source.

Temperatures of growth. The cultures were inoculated on Löwenstein–Jensen and in Proskaur–Beck liquid medium (Youmans, 1946) and incubated in water baths at 28°, 37°, 45° and 52° for 2–4 weeks and examined at intervals for growth.

Resistance to 60° for 4 hr. The cultures were inoculated on Löwenstein–Jensen and Proskauer–Beck media previously heated to 60° and incubated in a water bath at that temperature for 4 hr. They were then cooled under running water and incubated at 37° for 2–4 weeks and examined for growth.

The method developed for the Adansonian classification, as well as the system for quantitative notation, were as described by Sneath (1957*b*).

RESULTS

The results obtained from the different tests were tabulated: a strain × strain (i × i) table was prepared from the similarity indexes obtained, which were expressed in percentages. Another (i × i) table was made by rearranging the strains in groups; the latter table is shown diagrammatically in Fig. 1. Consequently, a taxonomic tree was designed (Fig. 2), the branches of which may be considered as species. The three species previously characterized by Gordon & Smith (1953, 1955) remained as groups when subjected to Adansonian analysis. The diagram (Fig. 1) shows that *Mycobacterium smegmatis* and *M. phlei* (branch 1, groups 1 and 2) join together at a higher percentage S level than with *M. fortuitum* (branch 2, group 1). This indicates that these two species are more closely related than either is to the third. The distinctness of *M. phlei* is not very apparent in Fig. 1 because few strains were studied; but in Table 2 it is possible to appreciate that such distinctness exists. Intermediate strains joined independently to one or another of the three established species at variable S values. Also, one can observe the existence of two minor groups (branch 2, groups 2 and 3) related more to *M. fortuitum* than to *M. smegmatis* or *M. phlei*.

The branch named irregular is formed by strains that do not seem to be related to any of the other strains studied.

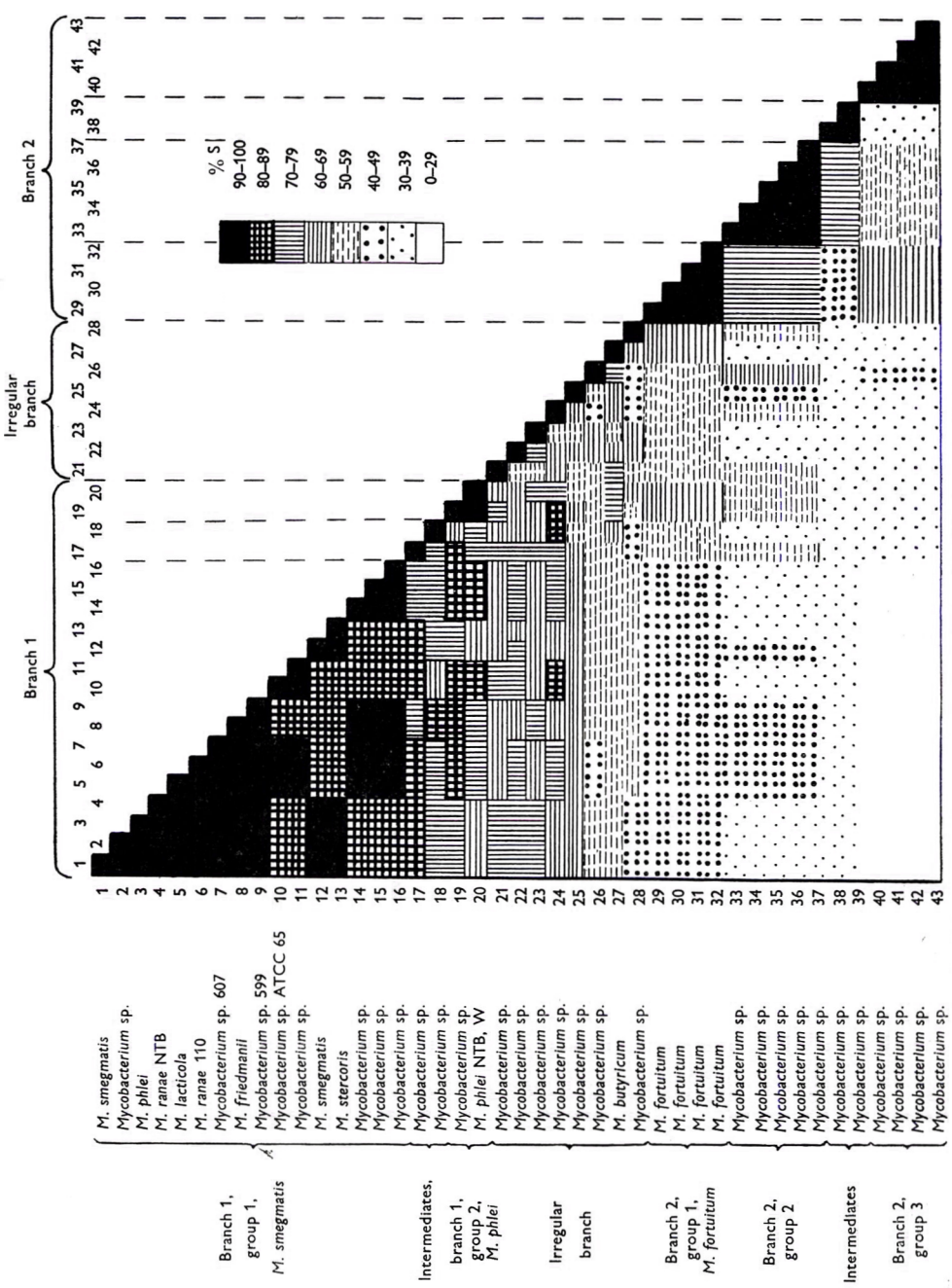

Fig. 1. Diagrammatic representation of the full % S value table.

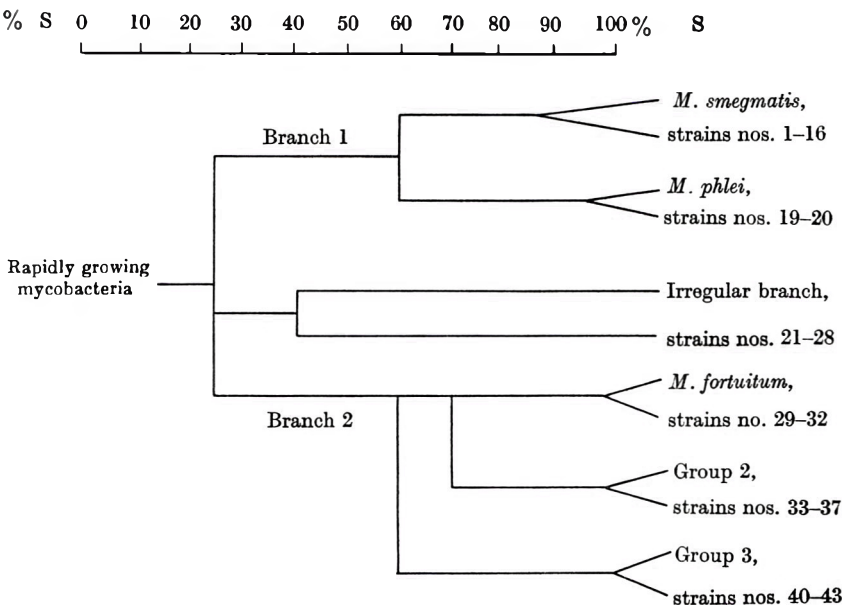

Fig. 2. Taxonomic tree of the rapidly growing mycobacteria. In another paper (Bojalil & Cerbón, to be published) the group 3 from branch 2 was named group 2*b*. The existence of a new group(group 2, branch 2) more closely related to *M. fortuitum* made necessary the above-mentioned change.

DISCUSSION

The three species *Mycobacterium smegmatis*, *M. phlei* and *M. fortuitum* constitute natural groups and can be accepted as logically classified. The Adansonian classification, on the other hand, clearly indicates that *M. smegmatis* and *M. phlei* are much more closely related to each other than they are to *M. fortuitum*.

Branch number 1 is formed by micro-organisms which in general are non-parasitic, capable of utilizing a great variety of carbohydrates. This group of micro-organisms was mostly isolated from soil, plants or animals, and includes *Mycobacterium smegmatis* and *M. phlei*.

Branch number 2 includes micro-organisms potentially parasitic, capable of utilizing only a limited number of carbohydrates, which were isolated mostly from pathological material or associated with these processes; *Mycobacterium fortuitum* belongs to this branch, as well as groups 2 and 3, which are definitely characterized. These groups 2 and 3 are small and include but a few unnamed strains. According to the Adansonian classification they are to be considered new species, since they show only a loose relation to pre-established rapidly growing species. On the other hand, these strains (groups 2 and 3) are grouped at high levels of similarity (S = 100 %). This is in contrast to many unnamed strains showing great disparity in their characters, which were included in the branch labelled irregular. Tests depending on subjective evaluation were not included. This makes the number of tests presented here seem small, but we believe that the results obtained by this Adansonian analysis are reliable.

However, it is premature to denote groups 2 and 3 as new species and a comparative study with other groups of mycobacteria is being made.

Table 2. *General properties of rapidly growing mycobacteria*

Property	Branch 1			Branch 2		
	Group 1 *M. smegmatis*	Group 2 *M. phlei*	Irregular branch	Group 1 *M. fortuitum*	Group 2	Group 3
Pigment on Löwenstein–Jensen medium	Pale-orange	Yellow	Yellow-orange	Pale-straw	Brilliant yellow	Pale-straw
Acid from:						
Glucose	+	+	±	+	+	+
Galactose	+	+	(±)	−	−	−
L-Arabinose	+	+	(±)	−	−	−
Xylose	+	+	+	−	−	−
Mannitol	+	+	+	(±)	+	−
Sorbitol	+	+	+	−	+	−
Dulcitol	+	−	(∓)	−	−	−
Mannose	+	+	+	+	+	+
Rhamnose	+	−	(∓)	−	−	−
Fructose	+	+	+	+	+	−
M-Inositol	+	−	(±)	−	−	−
Trehalose	+	+	(±)	+	+	−
Erythritol	+	−	−	−	−	−
Utilization of:						
Benzoate	+	−	(±)	−	−	−
Citrate	+	+	(±)	+	−	−
Succinate	+	+	+	+	+	+
Pyruvate	+	+	+	+	+	+
Propionate	+	+	+	+	+	+
Growth temperatures						
28°	+	+	+	+	+	+
37°	+	+	+	+	+	+
45°	+	+	(∓)	−	−	−
52°	−	+	−	−	−	−
Resistance to 60° 4 hr.	−	+	(∓)	−	−	−

+, Positive; −, negative; (±) the majority gave positive results; (∓) the majority gave negative results.

In the irregular branch, each strain could represent a specific group by itself. However, until there is particular interest in any of them they should be simply designated *Mycobacterium* sp., vigorously or poorly fermentative, thus indicating to which of the branches they are more closely related.

The following key (Fig. 3) may serve for the identification of the different groups. One frequently observes some variability in pigmentation and rate of growth in Löwenstein–Jensen medium on primary isolation; however, these properties (pigmentation and rate of growth) can be used for a preliminary identification in subcultures.

The characters listed in the key mentioned above (Fig. 3), are the most constant for each group. If a strain is aberrant in some of its properties, it may be classified incorrectly if the key alone is used; however, the same strain could be classified according to the properties listed in Table 2.

Studies of Gordon and her colleagues (1937, 1938, 1953, and 1955 principally) on acid-fast bacilli of relatively rapid growth at 28° and 37° in ordinary culture media, have allowed the separation of these micro-organisms into three main groups, especially on the basis of temperature relationships and carbohydrate utilization.

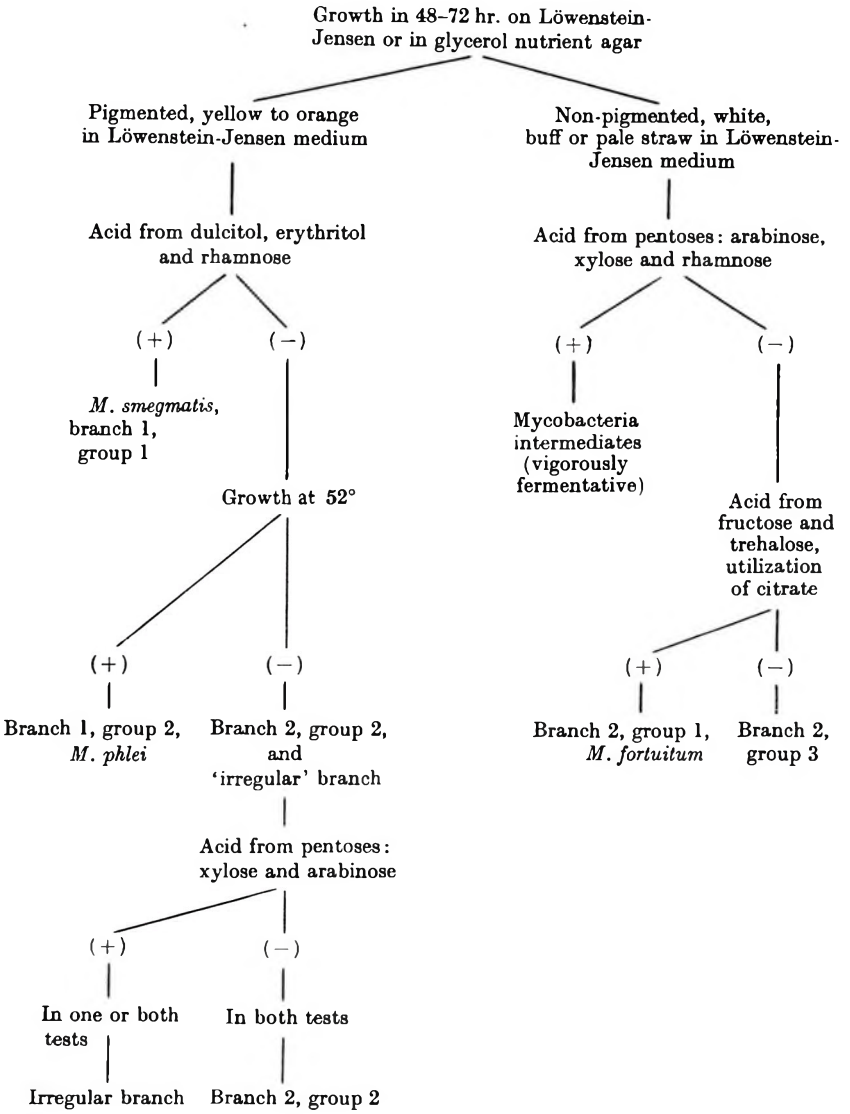

Fig. 3. Schema for the identification of rapidly growing mycobacteria.

The species *Mycobacterium smegmatis*, *M. phlei* and *M. fortuitum* are the only ones well characterized (up to the time of the latest reports). The following species, previously described as different, have been considered synonyms of one of these three species; *Mycobacterium berolinense* Bergey *et al.*, *M. butyricum* Bergey *et al.*, (Korn) Chester, *M. friedmanii* Holland, *M. graminis* Chester, *M. lacticola* Lehmann &

Neumann, *M. ranae* (Küster) Bergey *et al.*, *M. stercoris* Bergey *et al.*, and *M. aquae* Maië were considered as synonyms of *M. smegmatis*.

Mycobacterium giae Darzins and *M. minettii* Penso *et al.* were listed in the synonyms of *M. fortuitum*.

Groups 2 and 3 of the branch have been compared not only to those species mentioned above, but also to other species such as *Mycobacterium balnei*, *M. marinum*, *M. thamnopheous*, and *M. kansasi* and non-photochromogenic and scotochromogenic groups which will be referred to in separate reports. It should be noted, however, that group no. 3 of branch 2 differs markedly from the other studied organisms by its limited capacity to utilize sugars. This character is shown also by non-photochromogenic and photochromogenic micro-organisms. The latter, however, grow slowly, produce pigment and utilize different types of sugars (Bojalil, 1959; Bojalil y Cerbón, 1960). In particular photochromogenic Mycobacteria (including *M. marinum* and *M. balnei*) are capable of using fructose and occasionally galactose; but on the other hand, the non-photochromogenics do not utilize mannose.

Mycobacterium rhodochrous (Gordon, 1957) is not included in this study, it shows very weak acid-fastness and true filamentation and ramification in microcultures, features uncommon in the strains discussed in this paper, which relate it rather to the genus *Nocardia*. The amidase test (Bönicke, 1960) was negative for all strains except for those of *M. smegmatis* group for which it seems to be specific.

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Some Environmental Factors Affecting the Length of *Escherichia coli* Organisms in Continuous Cultures

By F. I. MACLEAN and R. J. MUNSON
Medical Research Council Radiobiological Research Unit, Harwell, Didcot, Berkshire

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SUMMARY

The lengths of organisms of *Escherichia coli* B/r grown in continuous culture in a glucose salts medium were measured over a wide range of population densities at 22° and 37°. In some cases the cultures were exposed to continuous gamma radiation at dose rates of 600 r./hr. at 22° and of 1000 r./hr. at 37°. The average length of the forms and the distribution with respect to length depended upon the temperature, population density and radiation dose rate. The growth rate was almost independent of population density over the range in which the average length of organism showed such a marked dependence. No significant differences in growth rate were observed as between unirradiated and irradiated cultures. Changes in average length of organisms are regarded as phenotypic responses to changes in growth conditions, some conditions favouring division and others inhibiting division amongst short organisms.

INTRODUCTION

One of the advantages of a continuous culture system for bacteria is that the organisms can be maintained in active division for many generations in an unchanging environment. Such a system is very suitable for the study of the production of spontaneous or induced mutants (Novick & Szilard, 1950) and has been used in our experiments on the effects of continuous gamma irradiation on actively growing cultures of *Escherichia coli*. The effect with which we are here concerned is the appearance of long filamentous forms in the bacterial population in both irradiated and unirradiated cultures. At the outset it seemed likely that these long forms might have some properties different from those of the 'normal' shorter bacteria, and that their relative proportions would affect the behaviour of the culture as a whole. For example, the radio-resistance of the long forms might differ from that of the short ones (Lea, Haines & Coulson, 1937) or the long ones might represent the emergence of a new strain (Hughes, 1953). Very long forms have been observed in cultures subjected to gamma radiation (Lea *et al.* 1937), X radiation (Alper, 1957), ultraviolet radiation (Witkin, 1947), sudden changes in osmotic pressure (Hinshelwood & Lodge, 1944), antibiotics (Fleming, Voureka, Kramer & Hughes, 1950) and growth inhibitors (Spray & Lodge, 1943). In all these cases the additional factor superimposed on the environmental conditions was responsible for the inhibition of normal cell division. Some inhibition of division must also occur in cultures grown

in broth in the usual way since large differences in length as between individual organisms are found. The average size of bacteria in batch cultures also depends upon the nutrient concentration and phase of growth, as shown by the systematic studies of Henrici (1928). Observations of long-organism formation in batch cultures cannot therefore be interpreted in a simple manner. The provision of an environment which remains unaltered over many generation times would appear to be a basal requirement for work in this and other fields where population changes occupy more than a very small number of generation times. A continuous culture system is admirably suited to this purpose.

Some observations on the relation between growth rate and bacterial size in continuous cultures have already been reported (Schaechter, Maaløe & Kjeldgaard, 1958; Herbert, 1959). Our own observations show a close correlation between the average size of organism and the proportion of abnormally long forms, each being dependent upon the population density, the temperature and radiation dose rate.

Growth rate and length distribution in continuous cultures

When *Escherichia coli* organisms are examined under the microscope different forms can be seen which might be appropriately described as normal rods, as chains or as filaments. Such a classification has not been attempted in the present work, and as a consequence we have found the need for an inclusive term to cover these and other forms. For convenience we have adopted the word 'cell' to cover any of these various forms. We count as one 'cell' any bacterium which is recognizably separate from its neighbours in liquid suspension and moves amongst them as one entity, although in the process it may suffer changes in internal configuration by Brownian movement.

Suppose that at time t the number of cells/unit volume of a continuous culture is N and the dilution rate, i.e. the rate of flow divided by the capacity of the culture vessel, is D. If one assumes that the culture is always uniformly mixed then

$$\frac{dN}{dt} = N(\bar{\nu} - D), \tag{1}$$

where $\bar{\nu}$ is the apparent rate at which new organisms are 'born' or the number growth rate (Powell, 1956) for the whole population. If $\bar{\nu}$ is independent of N and of t, and if D can be regarded as constant during a time interval $t - t_0$, integration of (1) gives

$$\ln (N/N_0) = (\bar{\nu} - D)(t - t_0), \tag{2}$$

where N_0 is the population density at time t_0. Thus the graph of $\ln N$ against t should have a constant slope, the value of which will give $\bar{\nu}$ when D is known. In practice when D is kept constant and $\bar{\nu} > D$ initially, $\bar{\nu}$ will eventually decrease as N becomes large, due to exhaustion of the medium and other causes, but changes in $\bar{\nu}$ may also arise from changes in the average cell size or mass. For example, when the mass growth rate $\bar{\mu}$ of the whole culture (Powell, 1956) is constant and the average mass of the cells increases at the rate e the number growth rate will be $\bar{\mu} - \epsilon$.

The average diameters of *Escherichia coli* organisms under given cultural conditions are almost independent of their length (Deering, 1958; this paper, page 25), so it will be assumed in the following analysis that the mass of a cell is proportional to its length.

A continuous culture selects in favour of rapidly dividing cells from the time it is started, but several generation times may be required before the maximum and final growth rate is reached. During this early phase the distribution of generation times amongst the population changes (Powell, 1956), and consequently the distribution with respect to length usually changes also. Both distributions eventually become stable when the population density, temperature and other factors are kept constant and no further selection occurs. Such a culture will be called a steady-state culture, although the term has meaning only in relation to the property which is stable. In our case this is the length distribution, for which an infinite number of steady states seems possible, at least in theory.

The form of the length distribution in a bacterial population is determined by the time dependence of the processes of growth and division of the individual cells. Apart from studies of the distribution of generation times amongst a population (Powell, 1955, 1958) and some isolated observations of exponential growth of cells in which division had been inhibited by radiation (Lea *et al.* 1937; Deering, 1958) little is known about these processes in quantitative terms. It is possible that some information in this direction could be gained by constructing hypothetical models of the processes and comparing the length distributions predicted from them with those observed, but this will not be attempted here. Instead, we shall assume a simple exponential growth function and apply it to two special cases which represent extreme or limiting cases of the more general one.

In an unchanging environment the mass growth rate $\bar{\mu}$ of a population is constant so we shall assume, as a first approximation, that the growth rate of each cell is $\bar{\mu}$, i.e. each cell increases in length by the factor $e^{-\bar{\mu}t}$ in time t.

(1) In the first case suppose that all cells divide at a certain length $2l_1$, and that all daughter cells have an initial length l_1. Cells of length l_1 at time zero will have lengths given by

$$l = l_1 e^{\bar{\mu}\,dt} \tag{3}$$

at time dt. If at time zero there are $n(l_1)dl$ cells with lengths between l_1 and l_1+dl a fraction l_1/l of this number will have lengths between l and $l+dl$ after time dt provided they have not been washed away in the interval dt. Since the fraction surviving wash-out in this interval is $e^{-D\,dt}(= e^{-\bar{\mu}\,dt}$, since $D = \bar{\mu}$ in a steady-state culture) and the number of cells in any length range does not change with time, the number between l and $l+dl$ will be

$$n(l) = n(l_1)(l_1/l)e^{-\bar{\mu}\,dt}dl,$$

which by (3) can be written

$$n(l) = n(l_1)(l_1/l)^2 dl. \tag{4}$$

When $n(l)$ is plotted against l/l_1 using double logarithmic scales as in Fig. 7*a* the graph has a slope -2 for $2 \geqslant l/l_1 \geqslant 1$. Outside this length range $n(l)$ is zero. In any real culture all cells would not divide at precisely the same length nor would they divide to give two exactly equal daughters. The ends of the distribution would therefore not be sharp but rounded as in the experimental curve to which Fig. 7*a* has been fitted.

(2) In the second case suppose that cells do not divide at length $2l_1$, but continue to grow longer at the same rate $\bar{\mu}$ until they reach a length ql_1, where q is a large

positive integer. If they then divided into separate cells of length l_1, a steady-state culture would be possible and the length distribution for $q \geqslant l/l_1 \geqslant 1$ would have a slope of -2. This distribution is represented graphically by the line (*b*) of Fig. 7 for which q is assumed to be very large.

In passing, it may be noted that if divisions did not occur at one particular length but took place at all lengths greater than $2l_1$, with similar frequencies, the average cell length and the slope of the length distribution would have values between those for the two extreme cases (1) and (2). Thus the average length l_d at which cells pass through their first division would be correlated with the average length of the whole population and with the slope of their length distribution.

So far we have considered only steady-state cultures of cells behaving according to our simplified model. In practice it may not be possible to maintain the dilution rate at its steady-state value $D = \bar{\mu} = \bar{\nu}$ and it is therefore important to know whether short-term fluctuations in dilution rate are likely to influence the length distribution substantially. Let us suppose that a culture has been operated in a steady state with a dilution rate D_1 and that the dilution rate is suddenly increased to D_2. If l_d is independent of dilution rate, all cells will have the same decreased chance of surviving wash-out after time t, viz. $e^{(D_1-D_2)t}$. The change in dilution rate *per se* would therefore leave the length distribution unchanged. However, if the product $(D_1-D_2)t$ were sufficiently large, the cell density would change appreciably and a dependence of l_d upon cell density would result in an altered length distribution.

METHODS

In the experiments described below *Escherichia coli* B/r derived from a stock kindly supplied by Miss T. Alper was grown in a glucose salts medium (Lederberg, 1950) at pH 7·0 in a continuous culture system. Starter cultures were routinely grown overnight at 37° in minimal medium and samples of such cultures were used as inocula for continuous cultures. The culture vessels had capacities of approximately 9 ml. and each was supplied with air at the rate of 700 ml./hr.$^{-1}$ and with nutrient at a rate which could be adjusted by manual control of the hydrostatic head h (Fig. 1). The positions of the reservoir R and mixer M having been fixed the dilution rate fell by 1–3% over 24 hr., depending upon the actual dilution rate, the area of free liquid surface in the reservoir and the head h. Normally the system was adjusted to give unrestricted growth at constant or nearly constant population density. The apparatus in the form here described therefore functioned as a crude turbidostat (Bryson, 1959). A stirrer consisting of a permanent magnet encased in stainless steel driven at 300 rev./min. provided efficient mixing of medium and air. With its free-running tyre at one end and a fixed tyre at the other, the stirrer was subject to a frictional turning couple set up by its forced revolution and this caused it to rotate about its axis. This form of stirrer always remained free from adherent bacterial growth even after running continuously for as long as 2 weeks. Gamma radiation was provided by a ^{60}Co source of 8 curies kindly placed at our disposal by Dr G. J. Neary.

During an experiment samples were withdrawn from the culture vessels from time to time by means of Pasteur pipettes. These samples (vol. 10^{-2} ml.) were examined microscopically, with dark ground illumination and a Thoma Hawksley counting

chamber 25 μ deep. It was customary to record the total count (about 200) and also the numbers of 'long cells', i.e. cells longer than 8 μ, seen over the whole or part of the ruled area. When the complete length distribution of a sample was required the lengths of all cells (about 200) within a given area were measured.

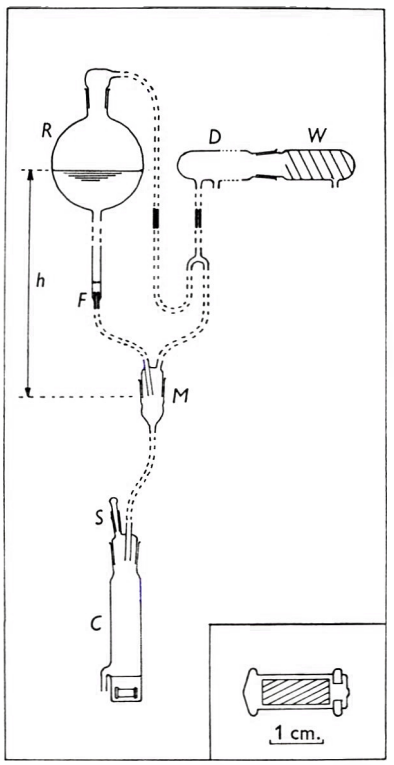

Fig. 1. Schematic diagram of the apparatus. Silicone rubber connexions between the glass components are shown by dashed lines. Heavy black lines indicate narrow tubes with appreciable flow resistance. C = culture vessel; S = sampling port; M = mixer; D = distributor of sterile humid air; W = cotton wool plug; F = filter; R = reservoir of medium. Inset, magnetic stirrer.

RESULTS

The results to be described relate to experiments in which two or three cultures were inoculated from the same starter culture and run simultaneously, usually at different population densities. The dilution rate of each culture was adjusted so that it was about equal to the growth rate. During the first few hours changes were observed in % long cells in the population and in the average cell length, but after several generation times both quantities reached apparently stable values. Figure 2 shows the changes in % long cells in typical cultures during this initial period. After the % long cells had become constant observations were continued during several generation times (usually at least 10) so that sufficient data relating to the length distribution of cells in the chosen steady state could be obtained. In the following paragraphs the results given are for cultures which had reached, or appeared to have reached, stable length distributions. However it must be emphasized that the

experiments were rarely continued for more than 24 hr. and therefore we have no evidence that the same effects would be found with much older cultures. The experimental errors in % long-cell values were also relatively large so that over the short periods covered by the observations it was not possible to determine whether there was a tendency for the % long cells to increase or decrease slowly.

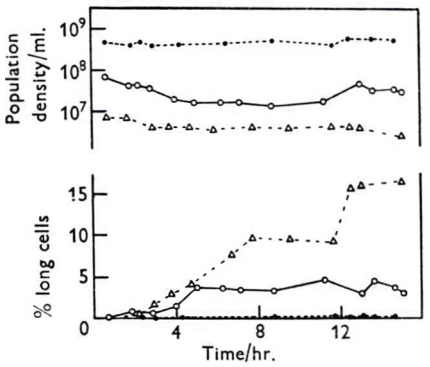

Fig. 2

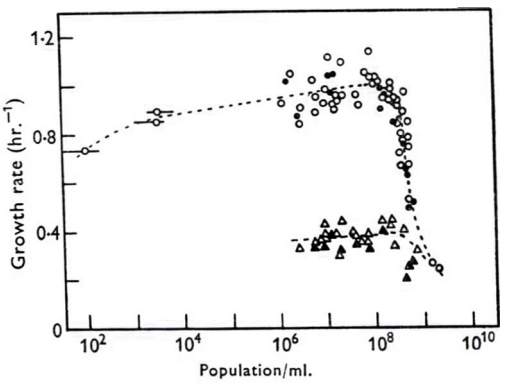

Fig. 3

Fig. 2. Changes in the proportion of cells which are long, i.e. greater than 8 μ during the first few hours in three continuous cultures inoculated simultaneously from the same starter culture and run at 37° at different population densities, namely: (●) 4×10^8/ml.; (○) 3×10^7/ml.; (△) 4×10^6/ml.

Fig. 3. The growth rates of cultures as functions of population density. The extent of the large variation in population density for the three most dilute cultures is indicated by horizontal lines. Unirradiated cultures at 22° (△) and 37° (○); irradiated cultures at 600 r./hr. at 22°, (▲) and at 1000 r./hr. at 37° (●).

Growth rates

The number growth rate, which becomes equal to the mass growth rate in a steady-state culture, was calculated from experimental data by using equation (2). All values of growth rate for cultures grown at 22 and 37°, with and without gamma irradiation, are plotted in Fig. 3. It was estimated that they were subject to errors of about 5 %, due mainly to temperature fluctuations and to errors in the measurements of culture vessel volumes. Although there were wide differences between the results from experiment to experiment, a definite progressive decrease in growth rate at 37° was evident at population densities greater than 2×10^8/ml. due presumably to partial anoxia or nutrient limitation. A similar trend appeared at 22° at a rather higher population density. At 37° observations were extended to population densities of about 10^4 to 10^2 cells/ml. by making viable counts (colonies at 72 hr. at 37° on salts glucose agar) instead of the usual total counts.

Although growth rates for irradiated cultures were somewhat smaller on the average than those for unirradiated cultures at the same population density, the differences are not statistically significant.

Proportion of long cells amongst population

Figure 4 shows that there was an almost unique relation between the average cell length and % long cells, particularly for unirradiated cultures. Since both of these

quantities are dependent upon the length distribution, it follows that either could be used to specify such a distribution.

At 37° the % long cells decreased towards zero as the cell concentration (population density) increased in the observed range, namely 10^6 to 10^9/ml. This trend could be readily demonstrated in a single experiment such as the one represented in Fig. 2 in which three cultures from the same inoculum were run simultaneously at different population densities. However, when the results from many experiments were pooled in one diagram (as in Fig. 5), it became clear that there was not a unique relation between % long cells and population density.

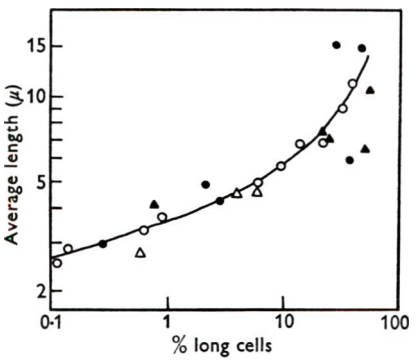

Fig. 4

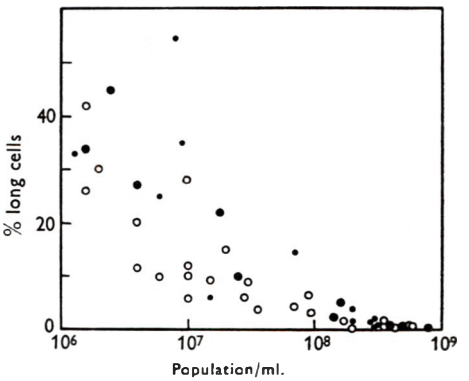

Fig. 5

Fig. 4. The average length of cells in a culture as a function of the % long cells. Points for irradiated and unirradiated cultures show no systematic differences. Symbols as Fig. 3.

Fig. 5. Observations from a large number of cultures showing the general form of dependence of % long cells upon population density at 37°. Points corresponding to cultures grown under continuous gamma irradiation at 1000 r./hr. (●), and 600 r./hr. (•) tend to lie above those for cultures grown in the absence of radiation (○), but the differences are not statistically significant.

The results of Figs. 3 and 5 taken together show that the greatest changes in % long cells occurred in the range of population densities below 2×10^8/ml. where the growth rate was almost constant. The average cell length in this population density range was therefore independent of the growth rate.

The effect of gamma radiation at dose rates up to 1000 r./hr. at 37° was tested by irradiating cultures after they had each reached an approximately steady state. The observed changes in the proportion of long cells were relatively small, and taken together did not give unequivocal evidence of an increase due to radiation at these dosages. The actual results are plotted in Fig. 5. Experiments were attempted at 43°, which is above the optimum temperature for growth, but reproducible results were not obtained.

The values of % long cells in unirradiated cultures at 22° were only about one-tenth of those values at 37° with the same population density. For this reason and also because fewer observations were made, the evidence for a dependence of % long cells on population density at 22°, similar to that of Fig. 3, was not conclusive. However, the presence of such a small proportion of long cells in an unirradiated

culture made the detection of a small increase due to gamma radiation correspondingly easier. The results of an experiment with cultures at two different population densities exposed to gamma radiation (600 r./hr.) are shown in Fig. 6. The increase in % long cells following irradiation was more marked at the smaller population density, the % long cells reaching a value typical of those for unirradiated cultures at 37°. Thus an increase in temperature and exposure to gamma rays produced similar changes in the proportion of long cells.

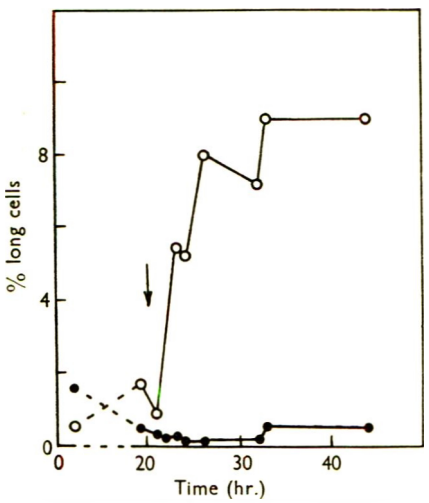

Fig. 6

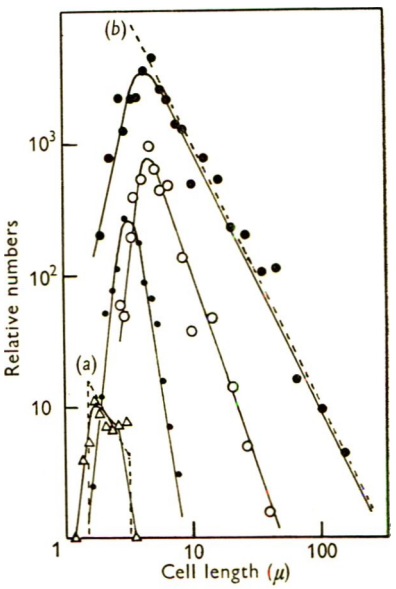

Fig. 7

Fig. 6. The effect of gamma irradiation at 600 r./hr. on % long cells in cultures at 22°, at high (3×10^8/ml., ●) and low (3×10^7/ml., ○) population densities. Irradiation commenced at 20·6 hr.

Fig. 7. The number of cells observed/unit interval of length plotted against length for each of four cultures at 37°. The population densities were 10^9/ml. (△), 4×10^8/ml. (▲), $1{\cdot}0 \times 10^7$/ml. (○) in the absence of gamma radiation, and $1{\cdot}2 \times 10^7$/ml. (●) at 1000 r./hr. gamma radiation. The points at longer cell lengths represent average values of the frequency over several adjacent unit length intervals. The graph (*a*) showing the distribution to be expected on the simplest assumptions (see text) has been fitted to one set of experimental points. The line (*b*) shows the expected distribution for a population in which cells only divide when they are very long.

The general form of the length distribution of cells of *Escherichia coli* strain B/r in continuous cultures is shown in Fig. 7. The number of cells with lengths between l and $l+dl$ divided by dl, which may be denoted by $n(l)$, is plotted against l, using double logarithmic scales. At lengths greater than the mode, the distribution curves became linear within the limits of experimental error. An analysis of all the data showed that the slope of the linear portion and the average cell length were correlated (coefficient 0·76, $P < 0{\cdot}001$) despite the wide range of conditions under which the cells were cultivated. It follows that the shapes of all distribution curves could be expressed in terms of the average length only, a conclusion independently

confirmed by the almost unique relation existing between the average length and % long cells (Fig. 4). The uppermost curve of Fig. 7 for a culture at 37° and irradiated at 1000 r./hr. is noteworthy in that the slope of its linear portion approximates to −2·0. This, on our simple model, is the smallest possible value of the average slope for a culture in a steady state and obtains when cells which have reached a certain length grow longer without further division.

Stability of cultures

Since a change in population density generally caused a change in length distribution, a culture could only become strictly stable when the dilution rate was maintained exactly equal to the growth rate. In these experiments this condition was only fulfilled at population densities greater than 2×10^8/ml. when the system behaved as a chemostat (Bryson, 1959) with the suboptimal growth rate (compare Fig. 2) adjusting itself to equality with the dilution rate. At population densities less than 2×10^8/ml. any error in the adjustment of the dilution rate caused a steady increase or decrease in population density (see Equation 2). Over the period of a few hours required for the establishment of a stable value of % long cells, changes in population density were relatively small, amounting to a factor of 2 or less, and the corresponding changes in % long cells to be expected on the basis of the trend in Fig. 5 were comparable to their experimental errors. The control of the population density can therefore be regarded as adequate for these experiments.

Although on some occasions the dilution rate was deliberately altered from its steady-state value by as much as 50% for periods of one or two generation times, changes in the population of long cells were always small and could be attributed to changes in population density. Possible complications in the interpretation of length distribution as due to sudden changes in dilution rate (as discussed earlier) have therefore been neglected.

Cell diameters

The diameters of living cells were estimated visually to the nearest 0·1 μ using a × 90 objective with phase contrast bright field illumination and with dark ground illumination. For 113 cells from one culture in the length range 2·5–30 μ the average diameters were $0{\cdot}93 \pm 0{\cdot}08$ μ with dark ground and $0{\cdot}78 \pm 0{\cdot}06$ μ with bright field. The difference between the two values can be ascribed to diffraction. When the measured diameters were divided into two equal groups about the median, the average diameters of the groups did not differ significantly.

Search for evidence of selection

The observations described about the dependence of average length upon temperature, population density and radiation could be explained in terms of phenotypic variation or of selection amongst clones of different average length. Experiments designed to distinguish between these two hypotheses showed that changes in the proportion of long cells due to changes in population density were reversible, whilst the proportions of long cells in cultures derived from single cells and from stock slopes did not differ significantly. An attempt to isolate a long-cell variant from a dilute culture at 37° was not successful, the progeny of single cells after 30 generation times showing no significant differences in average length despite the widely differing lengths of the parent cells.

DISCUSSION

Since we dealt exclusively with growing cultures it was not possible to determine whether the observed changes in length were due to selection or adaptation. Our failure to find evidence in our stock cultures for selection amongst two or more strains with very different tendencies to grow long forms has lead us to regard the observed changes as phenotypic responses to changing growth conditions. On this basis a change in average cell length of a population must be interpreted in terms of some change which tends to make all cells become longer or shorter. A change in average length implies a change in the length at which cells on the average divide; thus our experimental observations are broadly consistent with the simple model proposed above, if the average length at which cells divide is conditioned by the temperature, population density and the degree of gamma irradiation.

At 37° a change in population density from 10^6/ml. to 2×10^8/ml. produced no significant change in growth rate but a marked decrease in average cell length. A similar change in population density at 22° also resulted in a decrease in cell length, but again the growth rate remained constant. Thus, a dependence of average cell size upon growth rate which Schaechter *et al.* (1958) found with *Salmonella typhimurium* certainly does not hold for *Escherichia coli* in minimal medium at population densities less than 2×10^8/ml. On the other hand, when the results of Schaechter *et al.* (1958) for *S. typhimurium* are combined with similar ones for the dry weight of *Aerobacter aerogenes* and *Bacillus megaterium* (Herbert, 1959) and our own for *E. coli*, one finds that in all cases the forms of the dependence of size upon growth rate are almost identical in the region of suboptimal growth rates. We interpret this as indicating that the relation found by Schaechter *et al.* (1958) applies only when there is limitation of growth rate by partial exhaustion of the medium or limitation by accumulation of toxic substances.

The dependence of the % long cells upon population density found in the present experiments suggests that the bacteria alter their growth medium in such a way as to inhibit the formation of long cells. This dependence is consistent with the hypothesis that bacteria remove an inhibitor present in the minimal medium or with the alternative hypothesis that they excrete a metabolite into it. Since gamma radiation also affects the % long cells, particularly at 22°, the hypothetical inhibitor or metabolite may be a compound associated with a radiosensitive metabolic sequence.

We wish to thank Mr R. J. C. Hudson for the detailed design and construction of much of the apparatus.

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With 1 *plate*
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The Nature and Radiation Sensitivity of the Long Forms of *Escherichia coli* Strain B/r

By R. J. MUNSON and F. I. MACLEAN
Medical Research Council, Radiobiological Research Unit, Harwell, Didcot, Berkshire

(*Received* 26 *August* 1960)

SUMMARY

By suitable adjustment of growth conditions in continuous cultures, organisms of *Escherichia coli* B/r with average lengths covering a wide range were produced and studied. From X-ray survival data it was concluded that radiosensitive sites were distributed along the length of an organism at intervals of 1–1·5 μ, whilst nuclear staining by the HCl-Giemsa method showed chromatinic bodies at an average spacing of 1·2 μ. Thus each nuclear body appeared to be a radiosensitive site. Lysozyme treatment did not reveal evidence of transverse membranes apart from those at visible 'waists'. It was concluded that the longer organisms arose by the inhibition of the terminal stages of division. This inhibition did not affect the multiplication of the nuclear and cytoplasmic components, although there was considerable aggregation of nuclear material in irradiated organisms.

INTRODUCTION

When *Escherichia coli* strain B/r is cultivated in a glucose salts medium the average length of the organism ('cells' in sequel; see Maclean & Munson, 1961) can be altered by changing the temperature and the population density and also by gamma irradiation (Maclean & Munson, 1961). By adjustment of these environmental factors one could therefore readily compare the properties of *E. coli* strain B/r rods of different lengths, and investigate any specific effects associated with growth under gamma irradiation. From studies of X-ray survival and of the number and disposition of nuclear bodies and cell membranes, some progress has been made towards an understanding of the nature of the long forms and the effect of radiation on their division processes.

METHODS

The present experiments were carried out with *Escherichia coli* strain B/r cultivated in a continuous system in a glucose salts medium as described earlier (Maclean & Munson, 1961). Cultures were grown at 22° and 37° and in some experiments they were irradiated continuously with ^{60}Co gamma radiation at dose rates of 600 r./hr. at 22°, and 1000 r./hr. at 37°. Adjustment of the bacterial concentration (population density) provided a simple means of controlling the average length when the temperature and radiation dose-rate had been fixed. In each experiment growth was continued for at least 10 generation times under the selected conditions in order to allow the length distribution amongst the population to become stable. Samples were then drawn from the culture vessel with Pasteur

pipettes, one to be X-irradiated and the other for length measurements of 100 to 300 living cells. In some cases a third sample was taken for nuclear staining.

Survival after X-irradiation. The sample for X-irradiation was diluted to a population density of 10^5/ml. in ice-cold sterile minimal medium (Lederberg, 1950) prepared some hours previously. Samples of the suspension were put into small glass tubes cooled in ice and water, and exposed to X-ray doses of 0, 2, 5, 10 and 20 kr. delivered at a dose rate of 550 r./min. The radiation half value layer was 1·2 mm. copper. After irradiation 0·1 ml. samples were spread on minimal medium containing 2% (w/v) agar. The plating was carried out at room temperature as rapidly as possible and incubation at 37° was usually started within 30 min. of the end of the irradiation. Final counts of colonies were made after incubation for 72 hr. Amongst cultures which were not exposed to X-radiation the population of viable cells was not significantly less than unity ($0{\cdot}95 \pm 0{\cdot}21$).

Although our procedure of plating at room temperature was convenient it was open to the objection that recovery or restoration during the interval before the cells reached 37° might have altered the proportion of cells surviving and hence also the shapes of survival curves (Stapleton, Billen & Hollaender, 1953; Alper & Gillies, 1960). The ratio (colony count):(colony count at zero restoration time) which may be termed the restoration factor, was therefore measured at room temperature for restoration times up to 4 hr. The restoration factor increased progressively with dose of radiation and with restoration time up to about one generation time. There was, however, no evidence of a change in shape of survival curves, the smaller alterations in the surviving fractions after 30 min. restoration being equivalent merely to an expansion of the dose scale by a few %. We conclude that any errors in the estimation of surviving fractions due to partial restoration were of the same order as the overall errors due to other causes. No correction for restoration was therefore made.

Nuclear staining. Staining was carried out by a variation of the HCl-Giemsa method (Robinow, 1944). Smears were fixed in osmic acid vapour (3 min.), placed in Schaudinn's ethanol solution (1½ min.) and stored overnight in iodine + ethanol. The following day the slide was placed in N-HCl at 55°–60° (10 min.), washed with M/400 phosphate buffer (pH 7·0), stained with Giemsa (37° for 4 hr.) and dried and mounted in Canada balsam.

Digestion of bacterial DNA with DNA-ase. Two bacterial smears were made on one slide and these were dried and fixed in acetic acid + ethanol (1 + 3). On the following day a depression slide containing a solution of DNA-ase (DNA-ase, once recrystallized; Nutritional Biochemical Corpn.; 150 μg./ml.; $MgSO_4$, $1{\cdot}75 \times 10^{-3}$M in 0·06M-phosphate buffer, pH 7·2) was mounted over one smear and over the other smear a similar depression slide without DNA-ase. The slides were sealed with paraffin and incubated at 37° for 30 min. The depression slides were then removed and staining continued as above. The DNA-ase activity was tested by the method of McDonald (1955). No RNA-ase was detected by a modification (Dr J. E. Stanier, personal communication) of the method of Bernheimer & Steele (1955).

Preparation of osmotically sensitive spheres

Cell suspensions were filtered through a membrane filter (Oxoid) and washed with 10^{-3}M-phosphate buffer (pH 7·0) and then with 0·1M-2-amino-2-hydroxymethyl-

propane-1:3 diol (tris) buffer (pH 8·0). A few drops of a solution of lysozyme (100 μg. lysozyme/ml., Nutritional Biochemical Corpn., Cleveland, Ohio, U.S.A.; 200 μg. ethylenediaminetetra-acetic acid/ml., in 3×10^{-2}M-tris buffer (pH 8·0) and 0·5M sucrose; Mahler & Fraser, 1956) was applied to the paper and the cells brought into suspension by stirring with a platinum loop. A few loopfuls were then used to fill a depression slide (100 μ deep), which was sealed with a coverslip and cedar wood oil. The progress of the action of the lysozyme was then followed microscopically and photographs taken of the spheroplasts which had settled to the bottom of the depression.

RESULTS

Shapes of X-ray survival curves

The proportion of cells able to form colonies on minimal agar after X-irradiation was found to be a function of the dose of X-radiation and the average cell length; some typical results are shown in Fig. 1, where the logarithm of the surviving fraction is plotted against the dose of X-radiation. For the shortest cells the survival curve is almost exponential but as the average length increases the shoulders become more and more marked. By drawing smooth curves through the experimental points instead of the broken lines as in Fig. 1, the doses of X-radiation for any degree of survival (or killing) can be found. The shapes of the survival curves can then be conveniently specified in terms of the ratio of the X-irradiation doses at two arbitrarily chosen degrees of killing. It appears from Fig. 2 that the ratio of the doses for 90 and 50% killing, namely, LD90/LD50, depended mainly upon the average cell length and was independent of the cultural conditions within the rather wide limits set by the scatter of the points.

The graphs of Fig. 1 are similar in form to the family of survival curves which can be derived theoretically on certain simple assumptions which are formally equivalent to those of Lea (1946) and Atwood & Norman (1949). These assumptions are: (*a*) each cell consists of an integral number, r, of units which can be independently inactivated by radiation; (*b*) the probability of inactivation of a unit is $(1-e^{-\lambda D})$ where λ is its radiosensitivity and D is the radiation dose; (*c*) a cell remains viable in the sense that it can give rise to a colony provided that one or more of its units are not inactivated.

Whatever the nature of the units, it can readily be shown that the fraction S_r of cells with r units/cell which survive a dose D is given by

$$S_r = 1-(1-e^{-\lambda D})^r, \tag{1}$$

whence it follows that

$$\frac{\text{LD}90}{\text{LD}50} = \frac{\ln\left(1-(\frac{9}{10})^{1/r}\right)}{\ln\left(1-(\frac{5}{10})^{1/r}\right)}. \tag{2}$$

Comparison of the calculated values of the ratio LD90/LD50 for different values of r, shown on the scale to the right of Fig. 2 with the observed ratio LD90/LD50 for cells of different average length, indicates that r is approximately proportional to the average cell length. Thus $r = 8$ corresponds to a length of 12 μ, $r = 4$ to 6·5 μ, $r = 2$ to 3·5 μ, and $r = 1$ to approximately 2 μ. The survival data are therefore consistent with our assumptions if the units of which a cell is composed each occupy on the average about 1·5 μ of its length. Since it has been assumed that a colony

can arise from only one unit, each unit must contain one nucleus at least. It seems therefore likely that stained nuclei may prove to be useful markers of the assumed functionally separate cellular units.

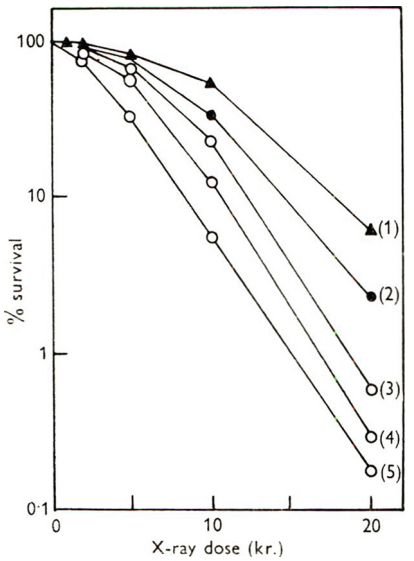

Fig. 1

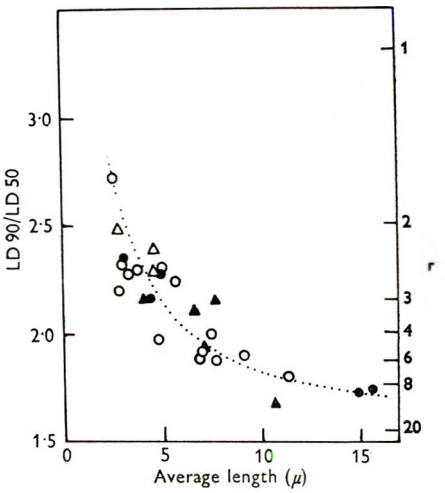

Fig. 2

Fig. 1. X-ray survival curves of *Escherichia coli* strain B/r of different average lengths. The average cell lengths and culture conditions were: curve (1), $\bar{l}$ = 11·0 μ; 22°, 600 r./hr.; curve (2), $\bar{l}$ = 16·1 μ; 37°, 1000 r/hr.; curve (3), $\bar{l}$ = 11·8 μ; 37°, no gamma radiation; curve (4), $\bar{l}$ = 7·3 μ; 37°, no gamma radiation; curve (5), $\bar{l}$ = 3·1 μ; 37°, no gamma radiation.

Fig. 2. The shape of X-ray survival curve of *Escherichia coli* strain B/r as a function of the average length. The shape is expressed in terms of the ratio of the doses for 90 % killing and 50 % killing. ○ = Cultures at 37° with no radiation; ● = cultures at 37° with gamma radiation (1000 r./hr.); △ = cultures at 22° with no radiation; ▲ = cultures at 22° with gamma radiation (600 r./hr.).

Nuclear staining and DNA-ase treatment

Plate 1, fig. 1, shows cells from a culture at 22° at a population density 7×10^8/ml. grown without gamma irradiation. The cells are very short and have only one or two nuclei. At 37° and a population density of 10^7/ml. (Pl. 1, fig. 2) the cells are generally much longer and contain several nuclei, mostly grouped in pairs. Under gamma irradiation cells grown at a similar population density at 22° (Pl. 1, fig. 3) and 37° (Pl. 1, fig. 4) have a completely altered appearance. The regular pattern has largely disappeared and the nuclear material occurs in large masses, often spaced irregularly. In some cases all the nuclear material is gathered into one or two areas and in others there appear to be no nuclei. Many of the shorter cells appear almost unaffected by the radiation.

Photographs of more than 1000 cells at a magnification of ×7070 were examined and the number of deeply-stained spots of diameter about 0·4 μ (which were assumed to be nuclei) and the cell length type recorded. In many cases spots of this size did not appear clearly separated from each other, and the number of nuclei scored was

then decided by subjective criteria such as the number of condensations within the deeply stained areas or by the lengths of the stained areas when no condensations were visible. The average lengths of cells containing 1, 2, 3, ..., nuclei grown under a variety of environmental conditions are shown in Fig. 3. Points for cultures grown at 22° and 37°, with and without gamma radiation, all lay close to one straight line through the origin. When one selects from the 1150 photographs those in which all nuclei are clearly visible (383) the points obtained also fit the same line.

Although gamma irradiation during growth had a characteristic effect on the appearance of stained preparations some difficulty was encountered in finding simple criteria by which the changed appearance could be expressed quantitatively. To minimize subjective errors it was decided that areas of staining should be scored rather than numbers of individual nuclei. At 37° significant differences between irradiated and unirradiated cultures were found for: (*a*) the proportion of cells having no stained areas (Table 1; $P < 0{\cdot}01$); (*b*) the proportion of cells having one central stained area only (Table 1; $P < 0{\cdot}01$); (*c*) the median lengths of cells having between 1 and 6 areas/cell ($P < 0{\cdot}01$). A comparison of preparations of cells treated with and without DNA-ase showed that the enzyme treatment removed most of the stain from the deeply stained areas which had been regarded as nuclei. There seems therefore no reasonable doubt that these were the regions which contained most of the DNA.

Table 1. *Proportions and average lengths of* Escherichia coli *strain B/r in cultures grown with or without gamma irradiation which show:* (1) *no nuclear stained areas;* (2) *one such central area only*

Continuous cultures at 37° and population densities between 10^6 and 2×10^8/ml. Dose rates zero or 1000 r./hr. Lengths are in mm. on prints at magnification ×7070.

Gamma radiation	Total no. cells observed	Cells with only one central stained area		Cells with no stained areas	
		No.	Av. length (μ)	No.	Av. length (μ)
–	548	6	17	3	14
+	457	40	22	25	18

Shrinkage in bacterial preparations and the average cell length/nucleus

It was found that the fixing, staining and mounting of the bacteria caused them to shrink in length and breadth. To find the average spacing of nuclei in a living cell it was therefore necessary to estimate the shrinkage factor. This was done in two ways: (*a*) by comparing the median values for the distributions of living and stained cells with respect to overall length; (*b*) by comparing the average lengths of segments into which cells were divided by visible 'waisting' in the living preparation and by incomplete cross-walls in the stained preparations. Consideration of the likely errors leads to the expectation that (*a*) would yield a falsely high value and (*b*) a falsely low value. The average values for the factor were: (*a*) $1{\cdot}67 \pm 0{\cdot}16$; (*b*) $1{\cdot}26 \pm 0{\cdot}08$. We have adopted the factor 1·5 as the best estimate.

The average distance between nuclei in the photographs, as given by the straight line of Fig. 3, is 0·57 mm.; allowing for shrinkage and magnification the actual distance is therefore $(1{\cdot}5 \times 0{\cdot}57)/7070$ mm., i.e. 1·21 μ. This is sufficiently near

the rough figure of 1·5 μ for the average length occupied by one radiosensitive unit as found from the data of Fig. 3 to suggest that the numbers of nuclei and radiosensitive units are equal.

Observations on cells treated with lysozyme

Under the action of lysozyme the cell wall loses its rigidity (Sistrom, 1958) and the remains of cytoplasm within the cell membrane tends to become spherical (= spheroplast) in media of suitable osmotic pressure. Visual observations of *Escherichia coli* strain B/r showed that a high proportion of the shorter cells became spherical after 20–40 min. treatment with the lysozyme solution described (Methods) at room temperature. The average diameter of the spheres appeared to be insensitive to changes in sucrose concentration over the range 0·2–0·5 M, but the observations were not sufficiently precise to rule out a slight dependence of size upon sucrose concentration. Cells initially composed of two segments changed to pairs of spheres which often remained in close contact, showing that although the membrane had divided, cell division was not complete. In a γ-irradiated culture a very long cell which was not segmented was seen to swell simultaneously at two points. One sphere grew more rapidly than the other and burst, whereupon the contents of the rest of the cell were rapidly lost through the open end. This observation suggested

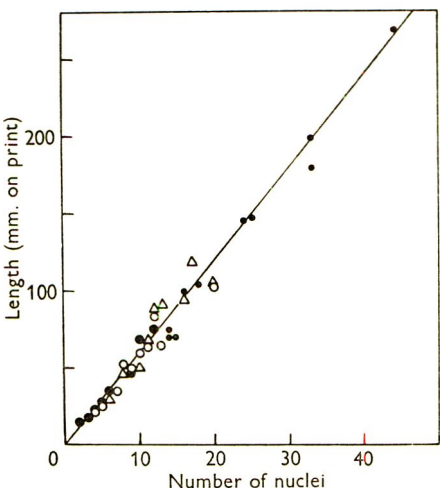

Fig. 3

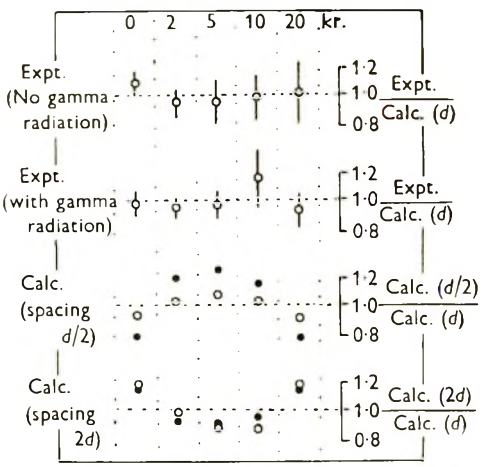

Fig. 4

Fig. 3. The relation between cell length and the number of nuclei for *Escherichia coli* strain B/r. Data from photographs. Observations for cells with a particular number of nuclei are pooled and the average length plotted. From all cells of unirradiated cultures, ○; from all cells of γ-irradiated cultures, △; from all cells of all cultures with less than 3 observations/point, •; from those cells of all cultures in which all nuclei are separately visible, ●.

Fig. 4. Test for systematic deviations of experimental points from calculated survival curves of best fit. At each X-ray dose deviations found in individual experiments were pooled and the mean deviation with its standard error plotted in the upper half of the figure. Data for γ-irradiated and unirradiated cultures are shown separately. Calculated curves were derived from the corresponding length distributions assuming that each radiosensitive unit occupies a cell length d (= 1·2 μ). The effect of a change in the assumed nuclear spacing by a factor of 2 either way is shown in the lower half of the figure for typical populations of short (●) and long (○) average length.

that the absence of constrictions of the cell wall was probably associated with absence of transverse septa.

Measurements on a large number of cells from one culture showed that the distributions with respect to volume before and after exposure to lysozyme were very similar except at large volumes, where the discrepancy was consistent with a relatively high probability of bursting. The average cell length, the average segment length and the average length of a rod of the same diameter as a normal cell and equal in volume to the average type, are recorded in Table 2 for three continuous cultures grown from the same parent culture and operated simultaneously. The close agreement between the figures in the last two columns implies that when a segment becomes spherical the change in volume must be relatively small.

Table 2. *Sizes of whole cells, segments and the osmotically-sensitive spheroplasts derived from them by lysozyme treatment*

For comparison purposes the average volume of the spheroplasts is expressed in terms of the length of a rod-shaped cell having the same volume.

Cultural conditions			Average cell length (μ)	Average segment length (μ)	Equivalent average length for spheroplasts (μ)
Temp.	Population density	Gamma radiation			
23°	$4-10^8$/ml.	—	2·7	2·0	2·2
23°	10^7/ml.	600 r./hr.	9·5	7·6	6·4
37°	10^7/ml.	—	5·7	3·7	4·1

Test of the hypothesis that the number of radiosensitive units/cell is equal to the number of nuclei

Although the evidence for the equality of the numbers of nuclei and radiosensitive units presented above is sufficient to justify it as a working hypothesis, a more searching test is desirable, for the following reasons. The data of Fig. 2 are concerned with survival in the range 10–100 %, which constitutes only part of the available experimental information. Also the relation between the ratio LD90/LD50 and the number of radiosensitive units/cell shown in Fig. 2 applies strictly only to a uniform population, whereas our cultures were markedly not uniform with respect to cell length (Maclean & Munson, 1961) and therefore not uniform with respect to the number of units/cell. A direct estimate of the average number of radiosensitive units/cell from the survival data could not be made by the method of Atwood & Norman (1949) because the numbers of nuclei/cell had distributions which deviated widely from a Poisson distribution. We have therefore tested our hypotheses by comparing the shape of the experimental survival curve for each culture with the corresponding shape of the calculated curve and looking for evidence of systematic discrepancies between them.

To calculate the survival curve for each culture it was first necessary to determine the distribution of the number of nuclei/cell. For each cell of length l the number of nuclei was taken as the integer nearest to l/d, where d is the average nuclear spacing (1·21 μ). If for each nucleus there were one unit of radiosensitivity λ and there were n_r cells with r nuclei in a sample of N cells, the surviving fraction would be

$$S = \sum^{r} n_r S_r / N, \tag{3}$$

where S_r is given by equation (1) and the summation is over all observed values of r.

Values of S have been calculated for a number of values of λD in equation (2) and they have been fitted to the observed surviving fractions ${}_0S$ at different doses D in the following way. Log ${}_cS$ has been plotted against log D on one piece of paper and log S against log λD on a separate piece of transparent paper. The two superimposed sets of points were then made to lie as nearly as possible on the same curve by a shift of one set parallel to the log D axis. The magnitude of this shift was a measure of log λ, since log λD = log λ + log D. The value of log λ for the best fit was found by the method of least squares. For cultures grown in the absence of gamma radiation a weight proportional to the square root of the number p_i of surviving cells (colonies) counted was assigned to each experimental point (the mean variance of replicate counts was $3p_i$) and the value of $\sum_i (\log {}_0S - \log S)^2$ found for different assumed values of λ. The minimum value of this sum, which corresponded to the best value of λ, was found by graphical interpolation. For cultures grown with gamma irradiation the variance of replicate counts increased with dose from $2p_i$ at zero dose to $9p_i$ at 20 kr. The weights assigned to points for different doses were adjusted accordingly and the rest of the fitting procedure carried out as already described.

If there were a systematic discrepancy between the shapes of the calculated curves of best fit and the observed ones, this would be expected to show up when the results of all experiments at each dose were pooled and the mean of the ratios ${}_cS/S$ plotted as a function of dose; Fig. 4 shows the results of this test. For 18 cultures grown in the absence of gamma radiation and for 9 grown under gamma irradiation the means of the ratios differed from unity by less than their standard deviations, indicating no significant discrepancy at any dose.

Some idea of the limits within which the average 'target' spacing could be fixed by the survival data alone was found by calculating surviving fractions for typical long (11 μ) and short (2·5 μ) populations with assumed 'target' spacings of $2 \times 1{\cdot}2$ μ and $0{\cdot}5 \times 1{\cdot}2$ μ. These fractions were then compared with those calculated for a spacing of 1·2 μ; the results are shown in the lower half of Fig. 4. In both cases the deviations from unity exceed the mean experimental ones, indicating that a spacing of 1·2 μ fits the experimental data better than either of the alternative spacings.

Radiosensitivity of the targets

Having found the calculated curve of the best fit for each culture, the value of λ followed at once. For cultures grown in the absence of gamma radiation at 22° and 37°, the average value of λ was $0{\cdot}349 \pm 0{\cdot}025$, whilst for cultures grown under gamma irradiation at 600 r./hr. at 22°, and 1000 r./hr. at 37°, it was $0{\cdot}289 \pm 0{\cdot}040$. These values are significantly different ($P < 0{\cdot}01$).

Lengths of bacterial segments

The number of segments into which each living cell was divided by visible 'waists' was noted when its length was measured under the microscope. The proportion of the population with 1, 2, 3, ..., segments varied from culture to culture, but the variations were not large and no correlation of the proportions with cultural conditions was evident. The length of the shortest cells in a culture was almost

Table 3. *Sizes of cell segments under different cultural conditions*

Average lengths are given for the whole population and for those cells which comprise the shortest 2 % of the population.

Cultural conditions			Average segment lengths for cells composed of			
			1 segment		2 segments	
Temp.	Population density (ml.)	Gamma radiation	All cells (μ)	Shortest 2 % (μ)	All cells (μ)	Shortest 2 % (μ)
23°	10^7–10^8	–	4·1	1·5	2·9	1·5
23°	10^7–10^8	+	7	1·8	5	1·8
37°	10^7	–	7	2·4	5	2·1
37°	10^7	+	14	1·6	10	1·8
37°	4×10^8	–	3·2	1·6	2·4	1·5
37°	4×10^8	+	3·5	1·4	2·9	1·5

independent of the cultural conditions, in contrast with the large dependence shown by the average length. This is illustrated in Table 3 in which data for cultures grown under similar conditions have been pooled. Results for cells with one segment and two segments are separately classified, the figures for the shortest cells being the average for 3–5 cells which comprised the shortest 2 % of each class. Although the choice of the fraction 2 % was arbitrary, these results show that the smallest bacteria may be presumed to have lengths in the range 1·5–2 μ at 'birth'.

DISCUSSION

Brownell (1955) reported an approximate equality between the average number of radiosensitive units/cell and the average number of nuclei/cell for comparatively short cells (1–4 nuclei/cell) of *Escherichia coli* strain B/r from batch cultures in minimal medium. The values of radiosensitivity given by Brownell's data, namely, 0·45 and 0·50, may be compared with our value of 0·35.

For cultures growing under γ-rays the doses received by cells during one generation time were in the region of 10^3 r., so only a very small % of cells should have failed to form colonies when plated (Fig. 1). Although the measured proportions of viable cells were not at variance with this expectation, the fact that the growth rate was not significantly decreased by γ-irradiation (Maclean & Munson, 1961) was the best evidence that radiation damage was not serious for the culture as a whole.

Our observations on cells treated with lysozyme indicate that one membrane normally encloses the contents of one segment and that cell division does not always take place as soon as the division of the cell membrane is complete. If there are transverse septa within segments they must be relatively weak, otherwise their presence would have been evident during the change of segment shape from rod to sphere.

It has been shown that the aggregation of chromatinic material in bacteria exposed to damaging agents such as ultraviolet radiation is dependent upon the salt concentration of the suspending medium (Whitfield & Murray, 1956). There are, however, diverse views about the significance of nuclear aggregation (Williams, 1959) so it would be premature to attempt to explain the role of gamma radiation in producing nuclear aggregation in *Escherichia coli* strain B/r. Whatever its cause,

nuclear aggregation in the γ-irradiated cells appeared to increase rather than to decrease the chance of surviving X-irradiation, so it may be presumed that undamaged nuclei can extricate themselves and initiate cell division. The absence of any transverse membrane within a segment would permit rapid diffusion of materials within the membrane and also perhaps a re-organization of aggregated nuclear material after the manner of fusion nuclei as envisaged by Bisset (1948).

The relative constancy in the length of the shortest segments of the living cells, grown under a variety of conditions (Table 3), shows that there was a preferred minimum length and that some cells of this length always arose despite a general inhibition of cell division in the population as a whole. The close correspondence between this minimum length (about 1·5 μ), the length/nucleus given by staining (1·2 μ) and the length occupied by one radiosensitive unit (1–1·5 μ) is strong evidence that a radiosensitive unit contains one nucleus and is functionally almost identical with the smallest cell of *Escherichia coli* strain B/r which can exist. In order that it should function independently each unit must have at least one nucleus and the average one must contain less than two; otherwise the experimental and calculated survival data could not be reconciled.

The uniformity in the spacing of nuclei along cells of all lengths shows that nuclear division proceeded in step with cytoplasmic growth at a constant rate over a wide range of population densities at 37° (compare Katchman, Spoerl & Smith, 1955). On the other hand, different population densities had a marked effect on cell division, one pair of transverse membranes being formed on the average for each pair of nuclei at high population densities and for each 4 or 5 pairs of nuclei at low population densities. Thus the 'terminal stage' of cell division, namely, the laying down of transverse cell membranes followed by segmentation and separation of the daughter cells was much more sensitive to environmental changes than was the rate of production of nuclear and cytoplasmic materials. The appearance of 'giants' among X-irradiated mammalian cells (Puck & Marcus, 1956) implies a similar inhibition of cell division. Moreover, with *Escherichia coli* strain B/r the wide range of cell lengths within one culture indicates that individual bacteria differ from each other in respect of cell division, although for the culture as a whole the number of nuclei produced/cross-wall has a definite characteristic value. This characteristic value is dependent upon the three environmental factors; temperature, population density and gamma-radiation dose rate. Since the effect of one factor can be modified by either of the others, each probably affects the same process (Maclean & Munson, 1961). The evidence provided by the present experiments leads us to conclude that this process is the terminal stage of cell division and that it can be inhibited almost completely without quantitatively affecting the continued multiplication of other cellular components. For an individual cell, completion of division may be simply a matter of random chance, although statistically one could assign to it a certain probability/unit time. According to this view no fundamental distinction can be made between cells of different lengths in the same culture.

We gratefully acknowledge the helpful advice of Dr K. A. Bisset on the technique of nuclear staining. We are also indebted to Miss T. Alper and Dr N. E. Gillies for allowing us to see their experimental data about 'restoration' before publication, and to Miss P. A. Jeffery for very able technical assistance.

Fig. 1

Fig. 2

Fig. 3

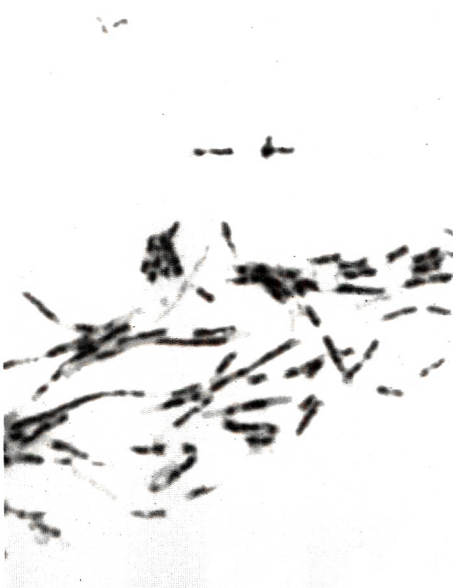

Fig. 4

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EXPLANATION OF PLATE 1

Escherichia coli strain B/r stained by HCl-Giemsa method and mounted in Canada balsam. Magnification, $\times 2520$.

Fig. 1. Cultivation at 22° at population density 7×10^8/ml.; no gamma radiation. Cells had one or two nuclei only.

Fig. 2. Cultivated at 37° at population density 10^7; no gamma radiation. Cells with 4 and 8 nuclei were most common, nuclei being usually in close pairs or groups of four.

Fig. 3. Cultivated at 22° at population density 2×10^7/ml. under gamma irradiation at 600 r./hr. Nuclear pattern much less regular than in fig. 2; there is some aggregation.

Fig. 4. Cultivated at 37° at population density 4×10^6/ml. under gamma irradiation at 1000 r./hr Nuclear pattern in longer cells very irregular; gross aggregation. Several cells appear to have no nuclei.

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Imidazole Compounds Accumulated by Purine Mutants of *Neurospora crassa*

By H. BERNSTEIN

Kerckhoff Laboratory of Biology,
California Institute of Technology, Pasadena, California, U.S.A.

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SUMMARY

A procedure is given for the detection of imidazole compounds accumulated in the mycelium of adenine mutants of *Neurospora crassa*. Of five such compounds detected, four have been tentatively identified. The distribution of these imidazoles among the mutants investigated allows a correlation between the adenine loci and the steps of purine biosynthesis.

INTRODUCTION

Extensive enzymic studies with avian liver systems have led to the elucidation of the complete pathway of adenosine-5′-monophosphate biosynthesis (Buchanan, 1958–59). The seven reaction steps following imidazole ring closure are shown in Fig. 1. From studies of the corresponding reaction pathway in *Neurospora crassa*, Giles, Partridge & Nelson (1957) have presented evidence that E mutants (Table 1) are blocked in the conversion of inosinic acid to adenylosuccinic acid ribotide. F mutants

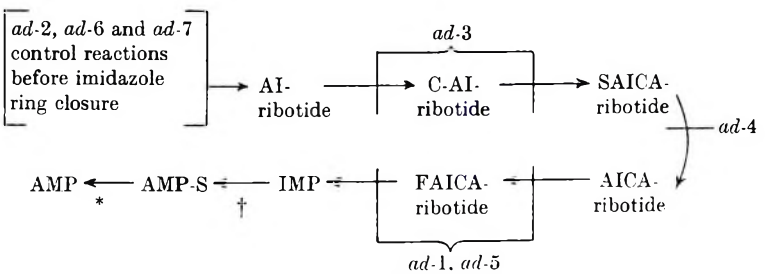

Fig. 1. Steps in purine biosynthesis controlled by adenine loci as judged from imidazole accumulation by mutants of *Neurospora crassa*.

Key: AMP, adenosine-5′-phosphate; AMP-S, adenylosuccinic acid ribotide; IMP, inosinic acid; FAICA-ribotide, 5-formamido-4-imidazolecarboxamide ribotide; AICA-ribotide, 5-amino-4-imidazolecarboxamide ribotide; SAICA-ribotide, 5-amino-4-imidazole-*N*-succinocarboxamide ribotide; CAI-ribotide, 5-amino-4-imidazolecarboxylic acid ribotide; AI-ribotide, 5-amino-imidazole ribotide.

* Giles *et al.* (1957) have shown that mutants at the *ad-4* locus are deficient in a de-acylase which splits both SAICA-ribotide and AMP-S.

† *ad-8* mutants were shown also by Giles *et al.* (1957) to be blocked in the conversion of IMP to AMP-S.

and mutant 44206 were also shown to lack activity for a bifunctional enzyme which catalysed the de-acylation of 5-amino-4-imidazole-*N*-succinocarboxamide ribotide and adenylosuccinic acid ribotide to 5-amino-4-imidazolecarboxamide ribotide

and adenosine-5′-phosphate, respectively. Dr T. French is reported (Buchanan, 1958–59) to have found in wild-type *N. crassa* all the purine biosynthetic enzymes present in the avian liver system. In the present work mutants representing several adenine loci of *N. crassa* were studied for their ability to accumulate imidazole compounds, intermediate in purine biosynthesis. All adenine mutants found to date have been allocated to eight loci (Barratt, Newmeyer, Perkins & Garnjobst, 1954; Giles *et al.* 1957); these are summarized in Table 1.

Table 1. *Summary of mutants of* Neurospora crassa *assigned to the eight adenine loci*

Locus	Mutants
ad-1	3254*
ad-2	70004, 27663, 20705
ad-3	35203, 38701, 38709, 45601, 68306, A†, B
ad-4	44206, 44415, F
ad-5	71104, J
ad-7	28610
ad-6	44411
ad-8	E

* Mutants with numerical designations came from the early mutant searches of Beadle & Tatum (1945).

† Letter designations were given by Giles *et al.* (1957) to groups of mutants related by heterokaryon complementation tests.

METHODS

Extraction of mycelia

The cultures of *Neurospora crassa* used as stocks, or as a source of conidial inocula, were grown in test tubes containing a complete medium described by Horowitz (1947). To obtain large quantities of mycelium, mutants were grown in carboys containing 10 l. of minimal medium (Beadle & Tatum, 1945) supplemented with limiting amounts of adenine sulphate (usually 30 μg./ml.). All mutants were grown at 25°, except the temperature sensitive strains (44206, 44415, 70004) which were grown at 35°. After 3–4 days of growth under forced aeration the mycelia were harvested by pouring the contents of the carboys through cheesecloth. The mycelial mats were placed in a Waring Blender and boiling water was added (about 1 l./100 g. wet mycelium). After disintegration the extracts were filtered through Whatman no. 1 filter paper on a Buchner funnel. The filtrates were then lyophilized overnight, leaving thick syrupy extracts in the flasks. Five to 10 ml. of water were sufficient to take up the extract from 100 g. mycelium. Residual material were separated by centrifugation and washed several times with 1 ml. portions of water which were added to the soluble fraction. The solutions contained all the imidazoles. The residues were discarded.

Detection of accumulated imidazoles

The redissolved extracts were spotted directly, or with dilution, on Whatman no. 1 paper and resolved by ascending chromatography in a variety of solvents. Isopropanol, water, and conc. aqueous NH_4OH (sp. gr. 0·880) in the volume ratios 70:20:10 and 70:40:10 were the most useful systems. After drying, the chromatograms were sprayed very lightly with diazosulphonic acid reagent (Ames & Mitchell,

1952), and again very lightly with 5% (w/v) aqueous Na_2CO_3 solution. By this procedure five distinct imidazole compounds not present in wild-type mycelium were detected in extracts from the mycelia of the mutant strains.

Isolation of Compounds I and II (*Table* 3)

To 14 ml. of the water soluble extract of 135 g. wet weight of mutant 44206 mycelium (see Methods) an equal volume of methanol was added. A precipitate was formed which was washed with 50% (w/v) methanol in water. The soluble fractions, containing virtually all of Compounds I and II, were combined to a total volume of 23·5 ml.

A Dowex exchange resin (1–X2, 200–400 mesh), from which the fines had been removed by repeated washings, was equilibrated with 2 M-formic acid. This was placed in a column 2·2 cm. in diam. to a volume of 170 $cm.^3$. Water at 3·5° was circulated through a jacket which surrounded the column. The flow rate of solvent was kept at about one drop every 15 sec. by maintaining the system under controlled positive pressure. A mixing vessel of 125 ml. capacity was included in the system to allow gradient elution according to the procedure of Thompson (1955). The column was first washed with distilled water and then the sample was added. For elution, the series of solutions listed in Table 2 were used in the order given. Successive solutions were added only after the pH value of eluted fractions did not change measurably. By using an automatic fraction collector, 5–10 ml. portions were collected. Compound II began to elute at pH 1·9 (solution 7); it came off the column in a total volume of 273 ml. After an additional 135 ml. of eluent had passed through, compound I began to appear and came through in the next 82 ml.

Table 2. *Solutions used for the serial elution of chromatographic columns*

No.	Solution	Molarity of NH_4^+	Molarity of anion	Measured pH value
1	Distilled water	.	.	.
2	Ammonium acetate buffer	0·4	1·2	4·28
3	Ammonium acetate buffer	0·4	1·0	4·40
4	Ammonium formate buffer	0·4	1·1	3·28
5	Acetic acid	.	1·0	2·53
6	Formic acid	.	1·0	1·97
7	Formic acid	.	6·0	1·17

Separation of Compounds III and IV (*Table* 3)

An extract of mutant 45601 containing both Compounds III and IV was subjected to a chromatographic procedure similar to that described above. Compound III was eluted by water very close to the front, and Compound IV came off soon after. Though separation was achieved, both fractions were impure. However, they were satisfactory for chromatographic comparisons.

Abbreviations

The following abbreviations, modified from Buchanan (1958–59), are used: AMP, adenosine-5′-phosphate; AMP-S, adenylosuccinic acid ribotide; IMP, inosinic

acid; FAICA-ribotide, 5-formamido-4-imidazolecarboxamide ribotide; AICA-ribotide, 5-amino-4-imidazolecarboxamide ribotide; SAICA-ribotide, 5-amino-4-imidazole-*N*-succinocarboxamide ribotide; CAI-ribotide, 5-amino-4-imidazolecarboxylic acid ribotide; AI-ribotide, 5-amino-imidazole ribotide.

RESULTS

The colour reactions and distribution of the five detected imidazoles among the mutants are detailed in Tables 3 and 4.

Table 3. *Colour reactions of accumulated compounds*

		Compounds				
		I	II	III	IV	V
Colour development	Before Na_2CO_3	Red-orange	Bright orange	Bright yellow	No colour	Yellow
	After Na_2CO_3	Fades to grey	Fades to grey	Grey spots—fades	Red	Blue
Probable identity		SAICA-ribotide	SAICA-riboside	AI-riboside	Unknown	AICA-riboside

Table 4. *Distribution of accumulated imidazoles*

		Compounds				
Locus	Mutants	I	II	III	IV	V
ad-1	3254	−	+	−	−	+
ad-2	70004	−	−	−	−	−
	27663	−	−	−	−	−
	20705	−	−	−	−	−
ad-3	35203	−	−	+	+	−
	38701	−	−	+	+	−
	38709	−	−	+	+	−
	45601	−	−	+	+	−
ad-4	44206	+	+	−	±	−
ad-5	71104	−	+	−	±	+
ad-6	28610	−	−	−	−	−
ad-7	44411	−	−	−	−	−

The following designations were used: +, compound present in mycelial extract; ±, compound present, but in low concentration; −, compound not detected.

Characterization

Compound I at pH 7 had an ultraviolet absorption peak at 269 mμ. By the method of Mejbaum (1939) it was shown to contain a pentose moiety. A sample of SAICA-ribotide generously provided by Dr T. French proved to be identical with Compound I, both in colour development and chromatographic mobility. When Compound I and the known SAICA-ribotide were hydrolysed by alkaline phosphatase each formed a product identical on chromatograms with Compound II.

Compound II at pH 7 absorbed maximally at 268 mμ, and was also shown to contain a pentose moiety. By using the procedure of Allen (1940) it was proved to lack a phosphate group. On hydrolysis at 105° with conc. HCl, for 15 hr. in a sealed

tube, Compound II yielded aspartic acid and glycine. The presence of aspartic acid on hydrolysis, the shape of the ultraviolet absorption curve, and position of the absorption maximum compared well with the properties reported for SAICA-ribotide (Gots & Gollub, 1957; Lukens & Buchanan, 1959*a*). The phosphate determination and the data from hydrolysis with alkaline phosphatase further showed that Compound II was SAICA-riboside.

Compound III formed an orange-red Bratton–Marshall reaction product (Bratton & Marshall, 1939) which absorbed maximally at 500–502 mμ. No absorption maximum in the ultraviolet region was detected. That Compound III failed to bind to an anion exchange resin argues against the presence of carboxyl or phosphate group. The Bratton–Marshall product of AI-ribotide is reported to be salmon-orange and to absorb maximally at 500 mμ (Lukens & Buchanan, 1959*b*). Also this compound is reported to have no ultraviolet absorption maximum above 210 mμ. All the properties observed for Compound III are consistent with its being AI-riboside.

The Bratton–Marshall reaction product of Compound IV was orange-red and absorbed maximally at 534–538 mμ. This corresponds to none of the maxima reported for the imidazoles involved in purine biosynthesis. However, since the concentration of this compound appeared to decrease as more care was taken in preparation of extracts, it seems likely that Compound IV was a reaction product of Compound III.

Ames & Mitchell (1952) reported that, of 16 compounds tested by their diazotization procedure, only 5-amino-4-imidazolecarboxamide (AICA) gave a blue reaction product. Subsequently AICA-ribotide was observed by the present author to give this blue colour. Compound V gave a colour reaction identical with that of the aglycone and AICA-ribotide. Chromatographic evidence suggested that Compound V was either AICA or AICA-riboside.

DISCUSSION

Knowing the distribution and probable identity of the detected imidazole compounds it is possible to correlate the adenine loci and the steps of purine biosynthesis (Fig. 1). Since *ad-2*, *ad-6* and *ad-7* mutants of *Neurospora crassa* can use IMP or hypoxanthine in place of adenine as a growth supplement (Mitchell & Houlahan, 1946) and yet accumulate no imidazole compounds, they can be assigned to steps preceding imidazole ring closure. This conclusion is in accord with an observation of Mitchell & Houlahan (1946). All *ad-3* mutants accumulate a distinctive purple pigment in their growth media. The purple pigment, which seems to have associated with it a 305 mμ ultraviolet absorption maximum, is probably a reaction product of Compound III. It was also shown by these authors that the double mutants of 35203 (*ad-3*) in conjunction with *ad-2*, *ad-6* and *ad-7* mutants, accumulate no purple pigment. The double mutant 35203, 44206 (*ad-4*) does accumulate the pigment. This again places the *ad-2*, *ad-6* and *ad-7* block before, and the *ad-4* block after, the reaction controlled by *ad-3*.

Colourless solutions of SAICA-riboside (Compound II) will turn red in time, especially at low pH values or on exposure to air. The reddish appearance of mutant 44206 (*ad-4*) mycelia is probably due to this effect. The percentage yield of SAICA-riboside and SAICA-ribotide in the dried mycelia of mutant 44026 can be calculated

from the observed optical densities at the ultraviolet absorption maxima and the extinction coefficient given by Lukens & Buchanan (1959*a*). The yields of the riboside and ribotide were found to be 2·1 and 0·023%, respectively, of the dry weight of its mycelia.

The two intermediates CAI-ribotide and FAICA-ribotide and the corresponding ribosides, known to be the least stable of the imidazoles, were not detected in any extracts.

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The Pure Culture of *Physarum polycephalum* on a Partially Defined Soluble Medium

By J. W. DANIEL and H. P. RUSCH
McArdle Memorial Laboratory for Cancer Research, Medical School, University of Wisconsin, Madison 6, *U.S.A.*

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SUMMARY

A wild strain of the multinucleate plasmodial myxomycete *Physarum polycephalum* was isolated in pure culture and grown on a medium consisting of 1% (w/v) Tryptone, 1% (w/v) glucose, 0·15% (w/v) yeast extract, 0·3% (w/v) $CaCO_3$, inorganic salts and a small amount of chick embryo extract. The organism may be grown with this medium either as a single large plasmodium on surface culture, or as a suspension of tiny plasmodia in submerged culture. From an initial inoculum of 1 ml. of a 3-day culture, the average plasmodial yield in a submerged culture was about 80 mg. dry weight/20 ml. medium at 72 hr. Growth occurred only in the presence of small amounts of an unidentified factor which was present particularly in chick embryo extract and foetal calf serum. An isolate of *P. polycephalum* was grown continuously on this medium for over four years without an appreciable decrease in growth rate. Under proper conditions a suspension of tiny plasmodia from shaken culture will fuse to form a single large surface plasmodium which exhibits synchronous mitosis.

INTRODUCTION

The myxomycete *Physarum polycephalum* possesses a number of characteristics which recommend it for studies of cellular activities: (i) mitoses are essentially synchronous in the multinucleate plasmodium (Howard, 1932); (ii) the plasmodia are sufficiently large to permit the correlation of changes in cell structure with changes in chemical content, since samples may be taken simultaneously and sequentially from a single plasmodium with a minimum disturbance to the organism; (iii) the stages of proliferation and differentiation (sporulation) are distinct and separable and may be controlled by the investigators (Daniel & Rusch, 1958).

Although the myxomycetes have been recognized for over a century (Schweinitz, 1822) most of the studies on these organisms have been morphological and taxonomic in nature. This limited study may be attributed to a general unawareness about this group of organisms but particularly to a lack of adequate methods of laboratory cultivation. Previous studies were conducted with impure cultures grown on oats or other natural substrates (Howard, 1931, 1932; Camp, 1936). Cohen (1939, 1941) and Sobels (1950) reported the isolation of pure cultures which grew on autoclaved yeast or oats, or in the presence of another organism, and Hok (1954) obtained growth on autolysed yeast preparations. Although pure cultures were apparently obtained, sustained growth was achieved only with insoluble natural media unsuitable for nutritional and biochemical investigations. This paper

presents methods for the growth of Physarum on a soluble medium free from other organisms (axenic culture) and describes the growth characteristics of the cultures. The morphological features of the organism grown under these conditions are described elsewhere (Guttes, Guttes & Rusch, 1961).

METHODS

Isolation. The culture of *Physarum polycephalum* used was isolated by permitting a plasmodium, reconstituted from the sclerotial stage (kindly furnished by Professor M. P. Backus, Department of Botany, University of Wisconsin) to migrate across sterile phosphate agar (pH 4·3) at 20°. Fragments of the arborescent plasmodium were cut out with a sterile knife, transferred to a similar sterile agar plate, and this procedure repeated four or five times with each isolate. Only plasmodial branches which had undergone maximum migration without intersecting their own paths were used. Such portions could be selected since plasmodia deposited an easily visible path of slime. To detect the presence of contaminating organisms, the plasmodia were then allowed to migrate on nutrient agar plates at pH 5 and at pH 6, and these fragments were transferred to these media a second time. The plasmodia were next transferred to nutrient agar plates (pH 5) streaked with sterile rolled oats and whole yeast, and incubated at 20°. The nutrient agar plates from which these plasmodia were removed were then incubated at 20° and 37°, and the migration path inspected for the appearance of colonies of microbial contaminants. When these plates showed no contaminants the plasmodia transferred from them were provisionally assumed to be pure and were transferred from the yeast + oats nutrient agar plates to Erlenmeyer flasks containing sterile oats, and incubated at 20°. These flasks were prepared by autoclaving 2 g. dry cereal-grade oats/500 ml. flask for 1 hr. at 121°. After being cooled, the oats were moistened with about 2 ml. sterile distilled water/g. oats. The transferred plasmodia were very small, and, because of this, growth on the moist oats could be observed only after 3–5 days. Transfers were made by loop, and care was taken to minimize damage to the fragments. Growth on this medium was rapid and profuse. After several transfers the organism was tested for contamination by dispersing plasmodial fragments of different ages in shaken and deep-tube static culture, by streaking on agar plates and by stab cultures at 21°, 28° and 37°, at pH 4 and 6. The media used were oat agar and a peptone Tryptone beef-extract yeast-extract agar, with and without glucose. After various periods of incubation the cultures were examined microscopically for evidence of contamination. In addition, direct microscopic examination of stained and unstained Physarum cultures was made. Samples of old degenerated cultures were also examined by the same procedures. The possibility that *P. polycephalum* may suppress the growth of contaminants by phagocytosis or by its reported antibiotic production (Sobels, 1950) is minimized, since Physarum degenerates in static submerged liquid culture at pH 6 or above. Although subsequent tests showed that penicillin and streptomycin, at 1000 and 100 units/ml., respectively, had little effect on the organism grown under the conditions employed, these antibiotics were not used for the initial isolation of the cultures.

Culture medium. Pure cultures of *Physarum polycephalum* were maintained on sterile rolled oats, as described in a preceding section, until a soluble medium was devised. The composition of the medium finally adopted for routine culture is given in Table 1.

Table 1. *Complete growth medium*

Component	Concentration (g./100 ml. medium)	Component	Concentration (g./100 ml. medium)
Tryptone (Difco)	1·0	$MnCl_2.4H_2O$	0·0084
Yeast extract (Difco)	0·15	$ZnSO_4.7H_2O$	0·0034
Glucose, anhydrous	1·0	Citric acid.H_2O	0·048
KH_2PO_4	0·20	HCl, concentrated	0·006 ml.
$CaCl_2.2H_2O$	0·06	Distilled water	to 100 ml.
$MgSO_4.7H_2O$	0·06	$CaCO_3$	0·30
$FeCl_2.4H_2O$	0·006	Chick embryo extract*	1·5 ml.

* Difco ampoule containing 2 ml. of a lyophilized 50 % extract reconstituted with 8·3 ml. distilled H_2O.

The following stock solutions were diluted to give the concentrations listed in Table 1: glucose, 20·0 % (w/v); Tryptone, 10·0 % (w/v); yeast extract, 4·0 % (w/v); KH_2PO_4, 4·0 % (w/v). All inorganic salts, as well as citric acid and HCl but not $CaCO_3$, were dissolved together so that 3·0 ml. of the concentrated solution in 100 ml. medium gave the final concentrations shown in Table 1. The above components were combined and autoclaved for 20 min. at 121°. A very pale yellow colour is characteristic of the concentrated salt solution freshly prepared with pure ferrous chloride. However, salt solutions which develop a marked increase in yellow colour, indicating the oxidation of ferrous to ferric iron, should not be used, since the organism requires ferrous iron. The stock solutions with a few drops of toluene added were stored in a refrigerator.

Calcium carbonate was autoclaved as a 10 % (w/v) suspension in distilled water for 1 hr. at 121°, cooled, and then added to this cooled medium. The pH value of the autoclaved suspension of $CaCO_3$ was approximately 8 to 8·5. Preparations of $CaCO_3$ of higher pH value made the final medium exceed the optimal value of pH 5 and were avoided.

The lyophilized embryo extract (Difco) was reconstituted with 8·3 ml. sterile distilled water/ampoule (equivalent to 2 ml. of 50 % freshly prepared non-lyophilized extract) and was added to the solution with a sterile syringe after the addition of $CaCO_3$. The complete medium, buffered at pH 5·0 with $CaCO_3$, was then dispensed in the desired sterile culture vessels, into which the organism was then inoculated. Care was taken to keep the $CaCO_3$ suspended while the medium was being dispensed.

Submerged agitated culture. Submerged cultures were prepared by allowing a plasmodial fragment to migrate from the wetted bottom of a tilted 500 ml. Erlenmeyer flask containing 20 ml. of growth medium, on to the liquid surface. The flask was then placed upright to float the piece of plasmodium and incubated without agitation for 48–72 hr. Care was taken not to submerge the organism, since this caused degeneration in static cultures. After the plasmodium had grown for about 48 hr., the flask was agitated at 170 reciprocations/min. with a stroke length of $1\frac{5}{8}$ in. and maintained at 21·5° ± 1°. The organism fragmented into tiny plasmodia (micro-

plasmodia) under such conditions and was maintained in shaken culture by serial transfer. Since growth may be retarded by prolonged exposure to ordinary room lighting, the cultures were kept in the dark except during periods of examination.

After several transfers satisfactory growth was obtained and a constant culture cycle established. Plasmodia from such cultures tended to adhere to the walls of the flask and had to be shaken down periodically to insure reproducible growth rates. Shaken cultures were routinely maintained in 500 ml. Erlenmeyer flasks containing 20 ml. medium. One ml. of a 72 hr. culture containing the equivalent of about 450 μg. plasmodial nitrogen, inoculated into 20 ml. medium, produced a culture of similar plasmodial concentration after 72 hr. of growth.

Surface culture. Surface cultures of a single plasmodium were grown on filter paper supported at the surface of the liquid medium on glass beads, either in Petri dishes or in Erlenmeyer flasks. Cultures were inoculated either with single fragments cut from other surface plasmodia or with suspensions of 2- to 3-day shaken cultures. Samples (1–2 ml.) of the latter were pipetted directly on to a dry filter paper (Whatman no. 40) and allowed for several hours to fuse into a single plasmodium before the medium was added below the filter paper. Earlier addition of the medium retarded fusion. About 13 ml. medium were required just to cover a layer of glass beads (about 3·5 mm. diam.) in a 9 cm. diam. Petri dish. When a larger surface plasmodium was desired, the culture was first centrifuged, resuspended in a volume of $CaCO_3$-buffered salts medium (pH 5) equal to the packed plasmodial volume, and dispersed in a thin layer on the filter paper. (This medium was prepared by diluting 3·0 ml. inorganic salts + citric acid + HCl concentrate to 100 ml. with H_2O, autoclaving, and buffering with 2 ml. sterile 10% (w/v) $CaCO_3$ suspension; see Medium, Table 1.) Surface cultures carried in Petri dishes could not be used for the prolonged maintenance of the Physarum in pure culture because of their susceptibility to contamination, but such cultures could be maintained in 500 ml. Erlenmeyer flasks.

Agar cultures of the organism were prepared by autoclaving dry granular agar evenly distributed over the bottom of the desired culture vessel for 1 hr. and, after cooling, adding 5·7 ml. medium/g. agar. These cultures were inoculated with loop-transferred fragments of plasmodium or with suspensions of microplasmodia from agitated cultures.

Stock cultures. Cultures were maintained routinely in shaken flasks and on the surface of granular agar in Erlenmeyer flasks. Each of two cultures was inoculated into triplicate cultures. Unopened cultures from two previous transfers were retained as reserve inocula. The shaken cultures were transferred every 3 days, and the surface cultures every 6–7 days.

The isolates now in use have been maintained continuously in various types of culture for more than 4 years and are periodically re-examined for purity. Particular attention must be paid to aseptic technique, since the relatively slow multiplication rate of Physarum enables many microbial contaminants to establish themselves without being readily detected for a number of transfers.

In addition to the original isolate, a few specimens of *Physarum polycephalum* obtained from other sources have also been isolated, and, apart from some characteristic differences, all have grown under the conditions described in this paper.

Analytical procedures. The growth of *Physarum polycephalum* was estimated by the measurement of the total trichloroacetic acid-precipitable nitrogen and of the dry weight. Nitrogen was estimated by the microKjeldahl method of Johnson (1941). Centrifuged plasmodia were suspended in a small volume of distilled water and precipitated with a volume of 8% (v/v) trichloroacetic acid (TCA) in acetone (TCA + acetone; 8·0 ml. of 100% (w/v) TCA diluted to 100 ml. with acetone) equal to the volume of the plasmodial suspension. After standing overnight at 5°, the suspension was centrifuged, the clear supernatant fluid saved for determination of the yellow plasmodial pigment, and the precipitate suspended and dissolved in 0·3 N-NaOH, samples of which were used for nitrogen analysis. Small amounts of the mucoprotein-like substances present in the medium of cultures older than 2·5 days and insoluble in TCA + acetone were unavoidably included in the analysis of these samples. Dry weight was determined as the weight of residue of washed plasmodia dried for 24 hr. at 115°.

The amount of yellow pigments was determined by reading suitable dilutions of the TCA + acetone supernatant fluid at 415 mμ in a cell of 1 cm. light path (E_{415}) in the Beckman DU spectrophotometer. The results were expressed as total absorption/20 ml. shaken culture or of a single 2 g. oat culture. The pigment has absorption maxima at approximately 385 mμ in dilute aqueous alkali and at approximately 415 mμ in aqueous TCA + acetone. The nature of the yellow pigments is under investigation by Professor F. M. Strong and associates (Biochemistry Department, University of Wisconsin; see C. F. Dresden, 1959).

Hydrogen-ion concentrations were determined with the Beckman model G pH meter.

RESULTS

Preliminary studies demonstrated that submerged rather than surface culture of *Physarum polycephalum* was the more useful form for determining nutritional and growth characteristics. In submerged culture growth was more rapid, more readily measured, and was easier to maintain free from contamination. This type of culture was also basically more useful, since the very small plasmodia so obtained readily fused, when pipetted on to filter paper, to yield large plasmodia suitable for study of synchronous growth.

Submerged agitated culture

Typical growth curves obtained in submerged culture for two different isolates with the Tryptone glucose medium are given in Fig. 1. The average plasmodial yield from 100 ml. medium at 3–4 days was equiv. to 0·42 g. dry weight, with an average microKjeldahl-N value of 8·7% and a dry weight of 18·7%.

To determine the optimal concentrations of glucose and Tryptone for growth, the amounts of these components were varied independently of each other in the basal medium (Fig. 2). The results indicated that 1·0% (w/v) of each was the most suitable for routine use. The small increase in yield achieved by increasing either one or both components to 1·5% (w/v) did not warrant the relatively large (50%) increase in amount of substrate. Glucose and Tryptone each at 2·0% (w/v) depressed yields considerably even when the optimal pH 5 was maintained. In the absence of glucose the maximal yield was 29% of that obtained with 1% (w/v) glucose. The change in slope of the variable-Tryptone curve suggests that Tryptone

may also serve as a precursor of products in addition to those required for plasmodial growth. The appearance of a viscous acid and 'Sevag-precipitable' product in the medium of cultures older than 60 hr. containing 1 % (w/v) or more Tryptone supports this possibility. (An ethanol-insoluble fraction isolated from Sevag-precipitated culture filtrates was found by Professor M. Heidelberger, private communication, to be related to certain groups of pneumococcal capsular antigens.) Tryptone could be replaced by enzymic (Difco) or acid (General Biochemicals, Inc.) casein hydrolysates, peptone (Difco), or protone (Difco).

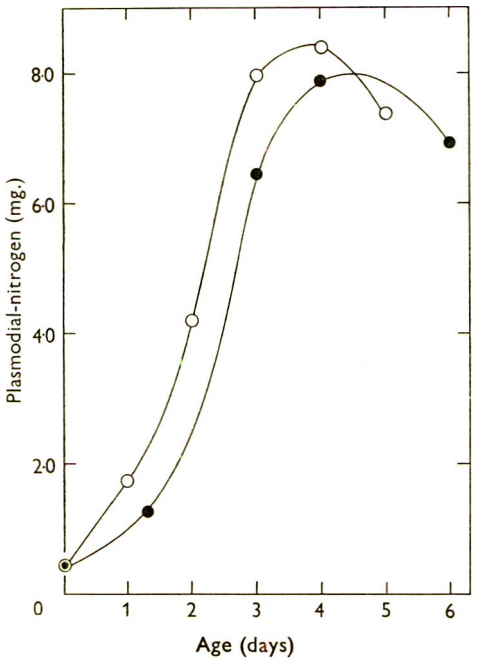

Fig. 1

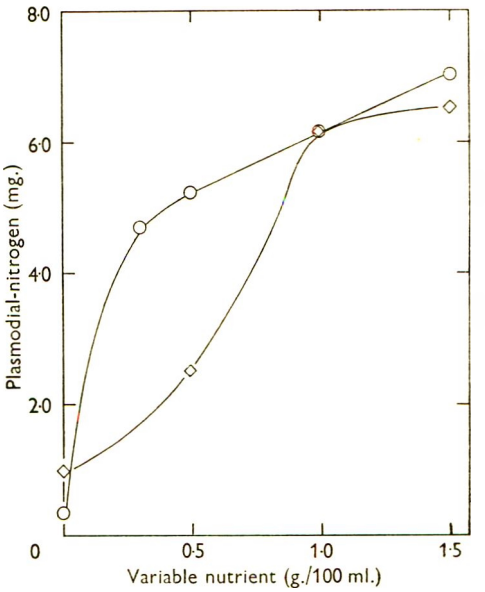

Fig. 2

Fig. 1. Growth of *Physarum polycephalum* in submerged culture on a semi-defined medium. —○—, —●—, represent the growth of isolates made 2 years apart from the same sclerotium. Cultures contained 20 ml. medium and 1·0 ml. inoculum/500 ml. Erlenmeyer flask and were incubated at 21–22°, agitated at 170 reciprocations/min. Plasmodial-N represents the average microKjeldahl-N of duplicate 21 ml. cultures. —●—, 4-day inoculum; —○—, 3-day inoculum.

Fig. 2. The effect of glucose and Tryptone concentration on the growth of *Physarum polycephalum*. —◇—, Tryptone, 1·0 g./100 ml. medium (Table 1), glucose concentration varied; —○—, glucose, 1·0 g./100 ml. medium, Tryptone concentration varied. Cultures contained 20 ml. medium and 1·0 ml. (equiv. 400 μg.-N) of a 4-day inoculum/500 ml. Erlenmeyer flask, harvested after 4 days growth in submerged culture at 21–22° and 170 recip./min.; plasmodial-N represents the average microKjeldahl-N of duplicate 21 ml. cultures.

The medium listed in Table 1 failed to support growth in the absence of chick embryo extract. The results in Fig. 3 show this requirement and clearly indicate that no growth occurred without embryo extract; the small yield obtained was essentially equivalent to the inoculum added. The flattening of the curve at maximum growth suggests that the active fraction of embryo extract was a micronutrient and not a gross nitrogenous source. The reconstituted embryo extract

listed in Table 1 contained about 0·6 mg. N/ml., while the complete medium contained about 1·7 mg. N/ml. Neither the pH value nor the concentrations of salts, yeast extract, glucose or Tryptone were growth-limiting in these experiments.

Of a large number of natural products and pure compounds tested, only chick embryo extract, foetal calf serum, and foetal calf erythrocyte haemolysate showed high growth-promoting activity. Less activity was found in chick and beef sera and in human umbilical cord serum. Although whole yeast and oat flakes also supported growth, various extracts of these materials were inactive.

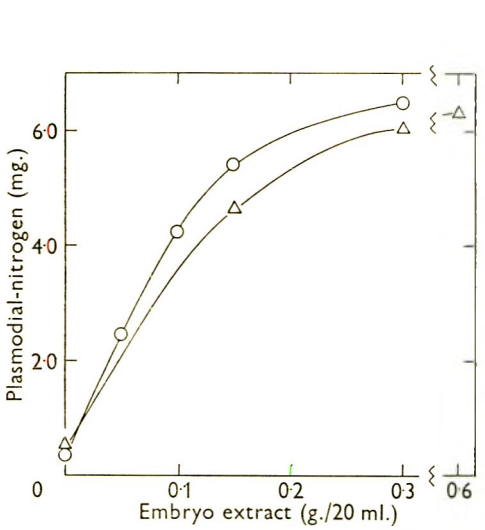

Fig. 3

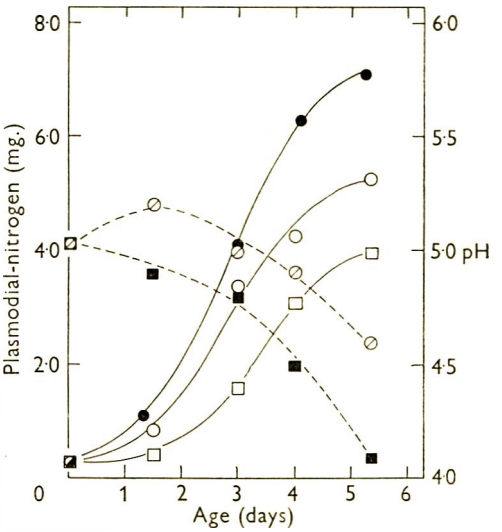

Fig. 4

Fig. 3. Embryo extract requirement for the growth of *Physarum polycephalum*. —○—, submerged cultures, 20 ml. medium/500 ml. Erlenmeyer flask, 1·0 ml. (equiv. 350 μg.-N), 4-day inoculum; cultures harvested after 4 days incubation at 21–22° and 170 recip./min. —△—, surface cultures, 20 ml. medium absorbed by 3 g. agar (Difco, granular), dry-autoclaved in 500 ml. Erlenmeyer flasks, loop-inoculated with a small plasmodial fragment (equiv. about 470 μg.-N)/flask; harvested after 10 days static incubation at 21–22° by floating plasmodium off agar surface. Chick embryo extract prepared in manner described for medium; plasmodial-N represents the average microKjeldahl-N of duplicate 21 ml. cultures.

Fig. 4. Effect of $CaCO_3$ and phosphate buffers on pH value of culture and plasmodial yield. All cultures contained 50 ml. medium/500 ml. flask and 2·5 ml. inoculum (equiv. 300 μg.-N)/ml. and were incubated at 21–22° at 170 recip./min.; plasmodial-N represents the average microKjeldahl-N of duplicate 21 ml. cultures. —●—, N for 0·3 % $CaCO_3$ buffer; —○—, N for 0·2 % $CaCO_3$ buffer; – – –⊘– – –, pH value for 0·2 % $CaCO_3$ buffer; —□—, N for cultures buffered with KH_2PO_4 (adjusted pH 5) and added to give a medium concentration equivalent to 1·5 % (w/v) KH_2PO_4, $CaCO_3$ omitted. – – –■– – –, pH value for phosphate-buffered cultures; sterile phosphate buffer added to medium after autoclaving.

Under otherwise optimum conditions, maximum growth was obtained when the soluble medium was maintained at pH 5 with calcium carbonate. The acid production accompanying growth and the necessity of using a buffer to obtain a maximum yield are shown in Fig. 4. The smaller buffering capacity of the phosphate at pH 5, as compared with that of 0·2 % (w/v) $CaCO_3$, permitted a steady decrease

in pH value which resulted in a 24% decrease in growth. When the amount of calcium carbonate was increased from 0·2 to 0·3%, the culture remained at pH 5 for 5 days (not shown in Fig. 4) and a yield of 3·55 mg. N/20 ml. was obtained. Since the optimum pH value of 5 was maintained well past the time of peak yield (3–4 days), cessation of net growth resulted either from a depletion of nutrients or from an increase in growth-inhibiting metabolites.

With citric acid buffer (0·5%, w/v, adjusted to pH 5) there was only 49% as much growth as with 0·3% (w/v) $CaCO_3$. Acetic acid buffer (0·5%, w/v, adjusted to pH 5) inhibited growth completely. Potassium phosphate buffers at pH 4 and 6·8 supported only 30 and 2%, respectively, as much growth as was obtained with the same buffer at pH 5. When cultures were maintained on a medium buffered with 1·5% (w/v) KH_2PO_4 adjusted to pH 5, and free from added calcium, a requirement for Ca^{++} could be demonstrated (Fig. 5). The concentration of magnesium present in the growth medium (Table 1) did not replace Ca^{++}.

The growth behaviour as a function of culture volume is shown in Fig. 6. The yield was substantially improved by the better aeration provided by the smallest (20 ml.) volume. However, the interpretation is complicated for a coenocytic organism since the growth rate may also be influenced by the degree to which plasmodial fragmentation is affected by agitation. In addition to the effect of culture volume, the rate and type of agitation obtained on various shakers also influenced both aeration and fragment size. The extent to which the mechanical characteristics of these shakers contributed to differences in growth rate is being examined. Submerged cultures grown at atmospheric pressure under nitrogen showed no net growth.

Decreasing the size of the inoculum delayed the time required to reach the peak yield. As summarized in Table 2, the use of an inoculum equivalent to 450 μg.-N resulted in the most rapid average duplication time ($t_{av.}$) and did not unduly prolong the time (a) of peak yield (y). A relatively small volume of inoculum also decreased the amount of culture fluid carried with each transfer.

The data of Table 3 summarize the relation between duplication time and pigment formation with this medium. From a comparison of the calculated N/pig. values tabulated against duplication times observed directly from the nitrogen curves, it is apparent that during the period of most rapid growth (0·5 ml. inoculum, at 0–28 hr.) pigment formation was relatively depressed. This was followed by a period of decline in growth rate, during which pigment formation abruptly increased. When the growth rate was constant but somewhat slower than the maximum (2·5 ml. inoculum, 0–28 hr.) the N/pig. ratio remained approximately constant and was followed after 46 hr. by a period during which growth declined and pigment formation rose rapidly. In each case the relative plasmodial pigment content was lowest (highest N/pig. ratio) at approximately the time of most rapid growth. The N/pig. ratio appeared to remain constant only when the duplication time was also constant. Studies in progress in this laboratory on the properties of the pigment indicate that the *in vitro* formation of a leuco form under reducing conditions may also occur *in vivo*. The N/pig. ratio may then reflect either the synthesis or oxidation-reduction ratio of the pigment.

The tendency of non-growing or slowly-growing plasmodia to accumulate pigment is a useful measure of the capacity of a medium to support growth. Fig. 7 shows the

data of Fig. 2 plotted as a function of pigment content. The increasing yields correlate with increasing N/pig. ratios but not necessarily with increased growth efficiency (g. plasmodium/g. substrates). Oat-grown plasmodia also continued to produce pigment (Fig. 8) for a considerable period after the termination of growth at 6 days as indicated by the increasing N/pig. ratio.

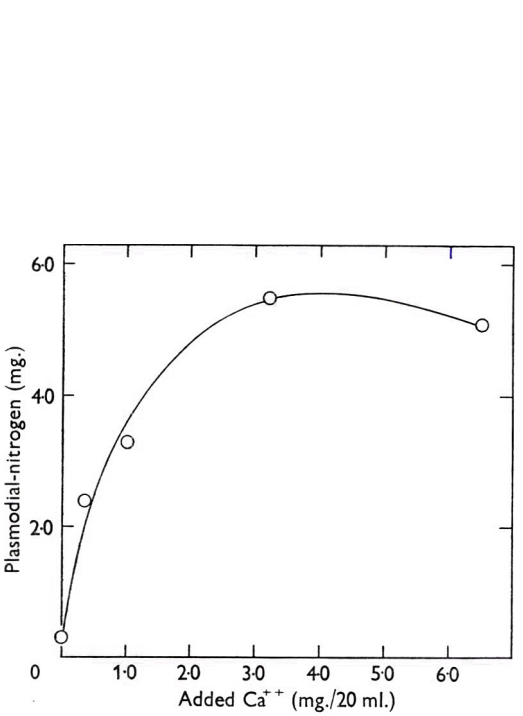

Fig. 5

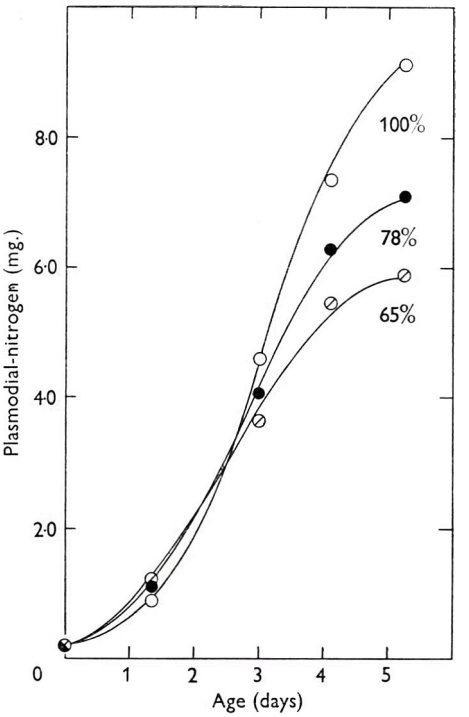

Fig. 6

Fig. 5. Ca^{++} requirement for growth of *Physarum polycephalum*. Medium prepared as described in the text with the following exceptions: the phosphate buffer as described in Fig. 4 replaced the $CaCO_3$ buffer; $CaCl_2.H_2O$ was omitted and added back to individual flasks to give indicated Ca^{++} concentrations; 'added Ca^{++}' corrected for Ca^{++} contained in inoculum. Inoculum: 1·0 ml. (equiv. 356 μg.-N)/culture, grown 4 days on above phosphate-buffered medium containing the $CaCl_2.H_2O$ concentration indicated in Table 1. Cultures containing 20 ml. medium/500 ml. Erlenmeyer flask were harvested after incubation for 4 days at 21–22° and 170 recip./min. The yield for zero added Ca^{++} was obtained from the third transfer of the culture on the phosphate-buffered medium with no added Ca^{++}. Plasmodial-N represents the average microKjeldahl-N of duplicate 21 ml. cultures.

Fig. 6. The effect of varying the culture volume on the plasmodial yield. Volume of medium/500 ml. Erlenmeyer flask: —⊘—, 100 ml.; —●—, 50 ml.; —○—, 20 ml. Cultures inoculated with 1·0 ml. (equiv. 200 μg.-N) of a 4-day culture (21 ml.)/20 ml. medium, incubated at 21–22° on an Eberbach reciprocal shaker having a 1⅝ in. stroke at 170 recip./min. Plasmodial-N represents the average microKjeldahl-N of duplicate 21 ml. cultures or calculated to a 21 ml. volume; % represents % of yield obtained with 20 ml. volume at 126 hr.

The amount of slime produced by the plasmodium also increased as indicated by increase in viscosity of the culture medium toward the end of the growth period, but no quantitative determinations were made.

Table 2. *Effect of inoculum size on growth rate*

Inoculum (I) (μg.-N/20 ml.)	Yield (y) (μg.-N/20 ml.)	Age (a) (hr.)	Duplication time ($t_{av.}$) (hr.)	Factor (k)
242	7100	114	25·8	1·3
450	7640	88	20·0	1·0
726	7040	80	23·8	1·2
1210	7160	69	28·3	1·4

I = inoculum, equiv. μg. microKjeldahl-N in 20 ml. culture; 1·0 ml. = 450 μg.-N.
y = peak yield in μg. microKjeldahl-N/20 ml. culture.
a = age of culture at peak yield.
$t_{av.}$ = average plasmodial duplication time in hr. as defined by $t_{av.} = \frac{a}{3{\cdot}3 \log y/I}$.
k = factor expressing the average time required for doubling of plasmodial growth relative to that required for a culture having an inoculum of equiv. 450 μg.-N.

Table 3. *Relation between duplication time and pigment formation*

Inoculum* (ml.)		Age of culture (hr.) 0	16	28	46	69
0·5	Duplication time (hr.)	14	16	18	22	—
	N/pig.	13·7	20·5	27·0	14·0	12·0
2·5	Duplication time (hr.)	19	19	20	—	—
	N/pig.	13·7	14·5	14·8	12·6	8·0

* ml. culture (equiv. 500 μg.-N/ml.) added to a submerged culture containing 20 ml. medium.
N/pig. = $\frac{\mu\text{g.-plasmodial-N}}{E_{415}}$/20 ml. culture.

Surface cultures

Plasmodia also grew very well on the surface of filter paper resting on glass beads at the surface of the nutrient medium. Under optimal conditions, a 1 ml. inoculum of a 3-day submerged culture, after fusing into a single plasmodium grew to a maximum diameter of 7 cm. in 3 days. Such plasmodia grew as thin compact circular disks. During maximal growth, the organism did not migrate but expanded peripherally; however, when the nutrients were exhausted or were replaced by a non-nutrient medium, the plaque-like culture quickly changed into a highly motile arborescent network. The growth of the plasmodium on the surface of nutrient agar was slower than that obtained on nutrient-moistened filter paper, and assumed a loose web-like appearance. Plasmodia floating on the surface of nutrient media also grew satisfactorily, but great care was required to prevent submersion of the organism.

DISCUSSION

The method of pure culture described in this paper has permitted the controlled production of *Physarum polycephalum* in sufficient quantity for critical studies on growth and morphogenesis. As far as the authors are aware, this report provides the first description of such growth conditions for a plasmodial myxomycete in axenic culture. The growth of *P. polycephalum* on a completely soluble medium refutes the conjecture often found in the literature that this plasmodial species requires particles or whole organisms for its nutrition.

The composition of the growth medium differs principally from that of media developed for other micro-organisms in that it contains embryo extract. Although a large number of soluble natural media were tested, the addition of embryo extract was always found necessary to obtain sustained growth. The required factor in embryo extract apparently occurs in few, if any, commercially processed soluble natural materials such as are usually tested for growth activity, and it was not present in various extracts of oats prepared in our laboratory, although rolled oats

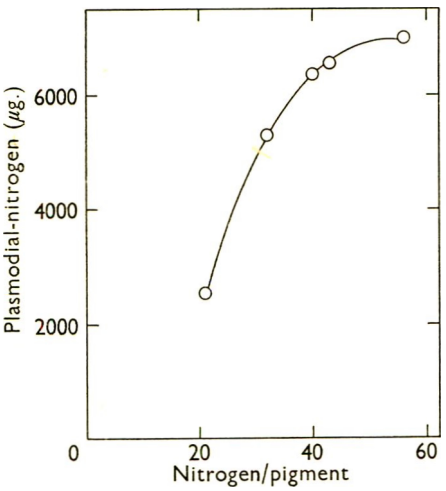

Fig. 7

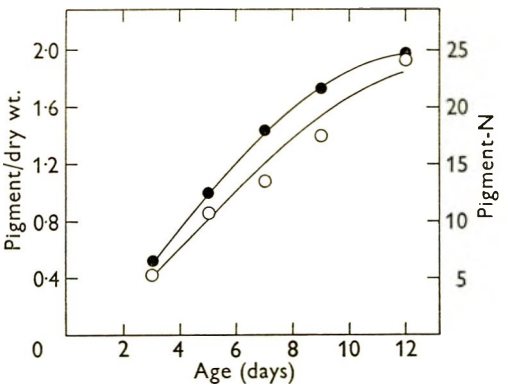

Fig. 8

Fig. 7. The relation between the nitrogen and pigment content of cultures grown on media of different Tryptone and glucose concentrations. Points represent nitrogen/pigment (N/pig.) for data shown in Fig. 2. Cultures containing 20 ml. medium and 1·0 ml. (equiv. 400 μg.-N) of 4-day inoculum were incubated for 4 days at 21–22° and agitated at 170 recip./min.

$$\frac{\text{Nitrogen}}{\text{Pigment}} = \left(\frac{\text{N}}{\text{pig.}}\right) = \frac{\mu\text{g. microKjeldahl-N}}{\text{total } E_{415}} \Big/ 20 \text{ ml. culture.}$$

Fig. 8. The relation between the pigment content and the nitrogen and dry weight of plasmodia grown on oats. Triplicate oat cultures, 2 g./500 ml. Erlenmeyer flask, were each inoculated with a fragment from a similar 4-day oat culture, and incubated at 21–22° without agitation; 'oat-free' plasmodial samples were removed for analysis at the times indicated.

$$—○— = \frac{\text{Pigment } (E_{415})}{\text{Dry weight (mg.)}}\ ; \quad —●— = \frac{\text{Pigment } (E_{415})}{\text{Nitrogen (N) } (\mu\text{g.})}\ .$$

Ratios represent average values for samples from triplicate cultures.

served as an excellent growth medium. The factor is readily extracted from chick embryo, several blood sera and erythrocytes. However, it cannot be replaced by the large number of pure metabolites so far tested. Embryo extract and blood serum have also been widely used for the growth of mammalian cells *in vitro* and, although the optimum concentration required for the growth of *P. polycephalum* is much lower, the resulting yield of organism is greater than for mammalian cells.

Although the maximum growth rate so far obtained with *Physarum polycephalum* on this medium is considerably less than that of many micro-organisms, the maximum yield is relatively high (equiv. 0·42 g. dry wt./g. glucose) and comparable to

the yields obtained with the aerobic filamentous fungi. However, *P. polycephalum* can also use Tryptone, to a small extent, as well as glucose, as an energy source. As the growth rate of ageing cultures declines, two readily detected products accumulate in the medium: a Sevag-precipitable viscous material, and the yellow plasmodial pigments. Estimation of the nitrogen content of these products is in progress to evaluate the efficiency of Tryptone for plasmodial growth as opposed to the formation of these by-products.

Physarum polycephalum, previously considered to grow only on surfaces as a large single plasmodium, can now be grown in submerged culture as a suspension of multinucleate microplasmodia. Such cultures facilitate the reproducible handling of this organism at any stage of growth under a variety of nutritional conditions, and expedite the preparation of uniform surface plasmodia in which synchronous mitoses can be most readily investigated. The small plasmodia from shaken cultures readily fuse under proper conditions within several hours, without appreciable deterioration, to form large (1–2 g.) plasmodia. This unique capacity of integrating rapidly and efficiently large numbers of small independent protoplasmic units into a single large stable plasmodium with little or no apparent loss of cellular material suggests the presence of a highly active and unusual cell interfacial boundary. Preliminary electron micrographs obtained by Dr I. B. Sachs in this laboratory confirm the presence of a very thin cell boundary and the absence of a grossly structured cell wall.

The mitotic synchrony first recognized by Howard (1932), and which occurs in plasmodia as large as 20 cm.^{2} (Guttes *et al.* 1961) recommends *Physarum polycephalum* as an ideal material for studies requiring synchronous growth because: (*a*) synchronous mitosis is a natural and cyclic event (thus objections raised to the use of cell populations synchronized under conditions resulting in unbalanced cell multiplication or short-lived synchrony do not apply); (*b*) growth through a number of mitotic cycles can be studied in a single plasmodium or 'cell'. The advantage of such plasmodia for biochemical investigations was recently illustrated by the demonstration with conventional procedures that DNA is synthesized during a short period immediately following mitosis (Nygaard, Guttes & Rusch, 1960). Synchrony in this organism is probably dependent to a large extent on the rapid metabolic communication afforded by the rhythmic cytoplasmic flow among all areas of a single plasmodium.

Observations made during the present study indicate that migration of the plasmodium occurred only when the nutrient medium was suboptimal. Since protoplasmic streaming occurs in both migrating and non-migrating plasmodia, it appears that a decreased growth rate favours a directional net flow of the organism. The cause of streaming is unknown, but is thought to be associated with myxomyosin, a contractile protein (Kamiya, Nakajima & Abe, 1957; Ohta, 1954). This actomyosin-like protein was first detected by Loewy (1952) and further studied by Ts'o, Bonner, Eggman & Vinograd (1956).

The application of these present cultural methods also facilitates a more critical study of the life cycle. Vegetative plasmodia grown in agitated culture and fused into large surface plasmodia may be induced to sporulate under appropriate conditions of pure culture; the metabolic patterns which characterize this change are now being studied.

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The Survival of Stationary Phase *Aerobacter aerogenes* Stored in Aqueous Suspension

By R. E. STRANGE, F. A. DARK and A. G. NESS
Microbiological Research Establishment, Porton, Wiltshire

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SUMMARY

The survival characteristics of washed stationary phase *Aerobacter aerogenes* organisms suspended in buffered sodium chloride solution and stored at room temperature, or at 37° with aeration, depended on the medium used for growing the bacteria. Populations of bacteria harvested from tryptic meat broth or tryptone glucose medium remained almost completely viable for longer periods than bacteria from a simple ammonium salt + mannitol medium in which carbon was limiting. Analyses of washed freeze-dried preparations of freshly harvested bacteria showed that the amounts of protein, carbohydrate and ribonucleic acid present varied according to which of the above media was used for growth. During the initial stages of storage at 37°, when the viability of the population remained apparently unchanged, a progressive loss in bacterial dry weight occurred, due to degradation of these cell constituents. Endogenous glycogen was degraded and oxidized; bacteria which contained glycogen survived well. However, the addition of glucose to suspensions stored under aerobic or anaerobic conditions did not favour survival. Utilization of substances made available by degradation of various endogenous macromolecular constituents may be an important factor concerned with the survival of bacteria in unfavourable environments.

INTRODUCTION

Harrison (1960) showed that population density affected the survival of bacteria held under growth conditions in the absence of added nutrients. Products from dead or dying bacteria are utilized by the living organisms, allowing maintenance of viability or division to occur. It is possible that another important factor concerned with bacterial survival is the ability of a bacterium to maintain itself by utilizing some of its own internal constituents. The problem of energy reserve substances in bacteria was reviewed by Wilkinson (1959); he considered that these substances are probably homoglycans (glycogen), poly-β-hydroxybutyrate, other lipids (triglycerides) and polyphosphate. The accumulation and breakdown of internal glycogen was studied in *Escherichia coli* (Holme & Palmstierna, 1956*a–c*; Dagley & Dawes, 1949) and in yeast by Stier & Stannard (1935–36). Degradation of other macromolecular constituents occurs in bacteria maintained in the absence of added nutrients. For example, Holden (1958) reported that when *Lactobacillus arabinosus* was incubated in phosphate buffer at 37°, ribonucleic acid (RNA) was degraded, resulting in the release of ultraviolet (u.v.) absorbing substances into the

medium; a similar phenomenon was investigated in yeast (Higuchi & Uemura, 1959). Turnover of protein occurs in *Escherichia coli* maintained at growth temperature in the absence of nutrients (Mandelstam, 1958*a*, *b*; Mandelstam & Halvorson, 1960). We have studied the survival of stationary phase *Aerobacter aerogenes* organisms suspended in buffer solution and have investigated gross changes in the concentrations of endogenous RNA, protein and carbohydrate.

METHODS

The strain of *Aerobacter aerogenes* used was obtained from Professor Sir Cyril Hinshelwood's laboratory.

Media. (*a*) Defined medium usually contained (g./l.): NaH_2PO_4, 0·6; $(NH_4)_2HPO_4$, 5·95; K_2SO_4, 1·75; mannitol, 10; and salt mixture (5 ml.). Salt mixture was prepared by dissolving MgO (10 g.), $CaCO_3$ (1 g.), ZnO (0·16 g.), $FeCl_3.6H_2O$ (2·7 g.) and $MnCl_2.4H_2O$ (0·4 g.) in water (200 ml.) containing conc. HCl (50 ml.) and diluting to 1 l. with water. The final medium was sterilized by autoclaving (120°, 15 min.). (*b*) Tryptic meat broth, prepared from bullock's meat, contained 2·5 mg. nitrogen/ml. (*c*) Tryptone glucose (Wade, 1961). A concentrate was prepared by mixing three solutions (*A*, *B*, and *C*) together. *Soln. A*: 2·8 kg. of Tryptone (Oxo Ltd., London) were mixed with 1·05 l. of 2M-K_2HPO_4 and made up to 19·6 l. with water. The solution was adjusted to pH 9·3 with NaOH, brought to 100° for 5 min., then filtered (Green's filter-paper no. 904). Citric acid (112 g.) was added, the solution adjusted to pH 7·6 with HCl and the volume re-adjusted with water to 19·6 l. The solution was autoclaved in volumes of 2·8 l. at 115° for 30 min. *Soln. B*: 406 g. NaCl, 140 ml. M-ferric citrate, 14 ml. M-$CaCl_2$ and 560 ml. M-$MgSO_4$ were made up to 2·8 l. with water. Volumes of 750 ml. were autoclaved at 115° for 20 min. *Soln. C*: 2·8 kg. glucose were made up to 5·6 l. with water and the solution autoclaved in volumes of 1 l. at 115° for 10 min. The concentrate was prepared by mixing 2·8 l. of soln. *A*, 750 ml. of soln. *B* and 1 l. of soln. *C* together, and stored at 2–5°. The concentrate was diluted with sterile water (5 vol.), the final medium containing 1·8% (w/v) glucose.

Cultural conditions and harvesting. Organisms were grown at 37° in an apparatus (culture volume 0·5 or 3 l.) essentially as described by Elsworth, Meakin, Pirt & Capell (1956) and used as a batch culture vessel. The medium in the culture vessel was seeded with a suspension of organisms grown for 8 hr. at 37° in a flask containing the same medium, aerated by rocking on a shaker; the initial concentration in the culture vessel was about 10^8 living bacteria/ml. medium. Filtered air was passed through the impeller at 1 ml./min./ml. culture and the pH value was continuously maintained at 7·2. Cultures reached the stationary phase in 6 hr. or less with media described above. Bacteria were harvested by centrifugation, washed twice with buffer solution and resuspended at about 10^{10} bacteria/ml. in the same buffer solution. The solution used for washing and resuspending the organisms contained NaCl (0·13M) and $KH_2PO_4 + K_2HPO_4$ (0·02M-PO_4); final value pH 6·5.

Bacteria counting procedures. Total bacterial counts were made with a Thoma chamber and dark ground illumination (average of three determinations on each sample). Organisms within one-fifth of the ruled area were counted and samples were diluted with buffered saline (pH 6·5) so that the number present was 250–500. The

results obtained when 20 determinations were made on one sample varied between 298 and 368 (mean, 328; standard deviation, ±21). Counts of viable bacteria were made by spreading 0·2 ml. sample (diluted by successive tenfold dilutions with buffered saline to contain 500–1000 viable organisms/ml.) on the dried surface of each of five nutrient agar plates. Colonies were counted after incubation for 24 hr. at 37°. When the dilution and plating procedure was repeated 20 times with one suspension, the number of colonies/ml. counted varied between 689 and 852 (mean, 777; standard deviation, ±57). A direct determination of the % viable bacteria in a suspension was made by the slide culture method of Postgate, Crumpton & Hunter (1961) with tryptic meat broth agar and dark ground illumination. Microcolonies and undivided organisms were counted after incubation for about 3·5 hr. at 37°. When the % viability of one suspension was determined twelve times by this technique, results varied between 93·5 and 96·8 (mean, 95·7; standard deviation, ±0·90).

Storage of suspensions of Aerobacter aerogenes. Sterility precautions were taken during the preparation, storage and sampling of bacterial suspensions. For storage at 37° with aeration, a suspension of washed freshly harvested organisms (usually 500 ml.; about 10^{10} bacteria/ml.; dry weight about 2·5 mg./ml.) at pH 6·5, was transferred to a sterilized gas wash bottle (1 l.; Dreschel pattern) held in a water bath at 37°. Washed filtered air was passed (0·2 l./min.) into the suspension through a sintered glass disk and the effluent air allowed to escape through a water reflux condenser fitted with a cottonwool filter. During the storage period, the pH value of the suspension was measured at intervals and, when necessary, adjusted to pH 6·5 by the slow addition of N-NaOH or N-HCl. Samples were removed at intervals for determinations of total and viable counts, % viability by the slide culture method, dry weights and chemical analyses. Samples for chemical analysis were centrifuged to separate the bacteria and the supernatant liquid was filtered through a Millipore bacteriological filter. The deposit of bacteria was washed with buffered saline by centrifugation and resuspended in water to known volume at a concentration of about 5 mg. dry weight/ml.; bacterial suspensions and filtered supernatant fluids were stored in the frozen state when not analysed immediately.

Analytical methods. Nucleic acids were determined in washed freeze-dried bacteria by the method of Schmidt & Thannhauser (1945); results were expressed in terms of nucleic acid phosphorus. Loss of RNA from *Aerobacter aerogenes* during storage was followed with the Schneider (1945) method applied to bacteria separated from suspensions by centrifugation; pentose in the hot trichloroacetic acid (TCA) fraction was estimated by the colorimetric method described by Morse & Carter (1949) or by the Dische & Borenfreund (1957) method, and converted to RNA by comparison of the colour obtained with that given by a known weight of yeast RNA (P, 8·1%; assumed to be 85% RNA). Bacterial pentose excluding pyrimidine pentose was determined with the Bial reaction (Morse & Carter, 1949) applied directly to a suspension; dried ribose (Laboratory Reagent Grade from The British Drug Houses Ltd., Poole) was used as the standard. Hexose interfered in the method and allowance was made for interfering carbohydrate in *A. aerogenes* by determining total carbohydrate in the bacterial suspension with the sulphonated resorcinol method of Devor, Conger & Gill (1958); dried ANALAR glucose from The British Drug Houses Ltd. was used as the standard. Light absorption was measured at 500 mμ

and, at this wavelength, ribose gave 96–102% of the colour given by an equal weight of glucose. In the Bial method, light absorption was measured at 660 mμ and glucose gave 11–15% of the reading given by an equal weight of ribose. If x = 'true glucose' (μg./ml.), y = 'true pentose excluding pyrimidine pentose' (μg./ml.), a = total carbohydrate found (μg./ml.) and b = pentose found (μg./ml.), then $x+0{\cdot}99y = a$ and $0{\cdot}13x+y = b$; interference factors for x and y were determined for each batch of analyses. The results obtained for 'true pentose excluding pyrimidine pentose' by solving the equations, were close to values obtained for purine pentose in the hot TCA fraction (Schneider, 1945) and, in many experiments, RNA loss from suspensions was determined by application of the Bial and resorcinol methods. Total phosphorus in freeze-dried bacteria was determined by the method of King (1932) and total nitrogen by a Kjeldahl method after reduction of the sample with hydriodic acid and red phosphorus (Friedrich, 1933) in a Kjeldahl flask. Protein was determined by means of a Biuret method after the bacteria had been dissolved by heating in N-NaOH for 5 min. at 100° (Stickland, 1951); dried bovine plasma albumin (N, 15·9%) from Armour and Co. Ltd., Hampden Park, Eastbourne, was used as standard. Ammonia was determined by nesslerization after distillation of the sample in a Markham still with alkaline phosphate + borate buffer (Tracey, 1952). Keto acids were estimated by the method of Friedmann & Haugen (1943); individual keto acids were identified according to the paper chromatographic method of El Hawary & Thompson (1953). Sugars were identified by paper chromatography on Whatman no. 1 paper with ethyl acetate + pyridine + water (2 + 1 + 2, v/v) as solvent (Jermyn & Isherwood, 1949) and aniline phthalate as spray reagent (Partridge, 1949). For the paper chromatographic detection of amino acids, butanol + acetic acid + water (4 + 1 + 5, v/v) was used as solvent and ninhydrin as the spray reagent. Free amino acids were extracted from bacteria by heating suspensions in distilled water (20 mg. dry wt./ml.) for 20 min. The extract was separated by centrifugation and desalted by passage through a column of Amberlite IR-120 (H form). The column was washed with water and the amino acids eluted with pyridine water (10%, v/v). The eluate was concentrated to small volume and chromatographed. Free amino acids released into the suspending medium during storage were detected after the suspension was freed from organisms by centrifugation and filtration through a Millipore filter. The filtrate was desalted in an electrolytic desalting apparatus, reduced to a small volume and chromatographed. Ultraviolet absorption was measured in a Unicam quartz spectrophotometer, model S.P. 500, with a 1 cm. light path. The dry weight of a suspension was determined by centrifuging a measured volume, washing the deposit once with saline (NaCl, 0·85%, w/v) containing HCHO (3%, w/v) and once with water, and drying the deposit at 100° for 16 hr.

Isolation of glycogen from Aerobacter aerogenes *harvested from tryptone glucose medium.* Freeze-dried organisms (1·5 g.) suspended in water (50 ml.) were disintegrated in the presence of ballotini beads (size 12) and capryl alcohol in a Mickle (1948) tissue disintegrator. The homogenate was centrifuged at 6000 *g* for 30 min. and the supernatant liquid separated. TCA solution (25%, w/v) was added to the cold supernatant liquid to a concentration of 2·5% (w/v) TCA and the precipitate separated by centrifugation. The supernatant liquid was neutralized by addition of solid $NaHCO_3$ and dialysed against running distilled water at 2° for 24 hr. The sac

contents were concentrated to 16 ml. under reduced pressure and treated with 3 volumes of ethanol and 2–3 drops saturated ethanolic potassium acetate solution. After standing for 24 hr. at 2° the precipitate was isolated by centrifugation, washed with ethanol and dried *in vacuo*. The yield was 140 mg. (= 9·3% dry wt. bacteria); carbohydrate content, 85% (as glucose). Further fractionation with ethanol resulted in a fraction containing 97% carbohydrate (as glucose) which, after acid hydrolysis and paper chromatography, gave one spot corresponding in position to glucose. The isolated material gave an opalescent solution in water and reacted with iodine solution to give a reddish violet colour.

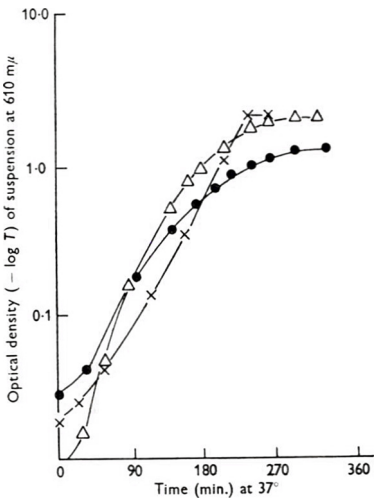

Fig. 1. Growth curves for *Aerobacter aerogenes* in defined medium (×), tryptic meat broth (●) and tryptone glucose medium (△).

RESULTS

Growth curves for *Aerobacter aerogenes* grown in defined medium, tryptic meat broth and tryptone glucose medium in the fermenter are shown in Fig. 1. Growth was determined by turbidity measurements at 610 mμ in a Coleman Junior spectrophotometer with the appropriate medium as blank and, when necessary, the sample and blank were diluted with buffered saline (pH 6·5) so that the optical density reading was not greater than 0·25. The shape of the growth curve depended on the medium used and with the defined medium the change from the exponential phase into the stationary phase was sharp whereas, with either of the complex media, the turn was more gradual. Bacteria were harvested after growth had ceased for 0·5 hr. unless otherwise stated. The viability of washed suspensions of bacteria freshly harvested from any of the three media was high (93–99%) according to the slide-culture method of Postgate *et al.* (1961).

The Postgate *et al.* method determines only the % viability of a suspension and not the total number of bacteria present. It was important in the present work, to know whether the total number of organisms in a given suspension changed during storage and, for this reason, total and viable counts were determined. In our hands,

the coefficients of variation for the results of slide-culture viability determinations, total counts and viable counts were ±0·95, ±6·4 and ±7·4% respectively (see Methods). Evidently results for the % viability of suspensions determined by the slide-culture method were more reliable than those derived from a combination of total and viable counts, provided the generation time of individual organisms did not exceed the incubation period allowed (3·5 hr.). A comparison of the results for % viability of an aerated suspension of *Aerobacter aerogenes* during storage at 37° as determined by the slide-culture technique and from viable and total counts is shown in Table 1. The values obtained with the Postgate *et al.* method were usually higher than those derived from the other methods. On the other hand, when the % viabilities of more aged suspensions were determined, it was frequently found that values by the slide culture method were lower than those derived from total and viable counts. This was probably related to the long lag phase of a proportion of an aged population. The incubation period of slide cultures could not be extended much beyond 3·5 hr. otherwise large colonies from single organisms with short generation times overgrew the non-growing bacteria. The shapes of survival curves plotted with data obtained by either the slide-culture technique or by total and viable counts were similar for periods during which biochemical changes occurring within the bacteria were studied.

Table 1. *Comparison of the results obtained for the % viability of an aerated suspension of stationary phase* Aerobacter aerogenes *during storage at* 37° *determined* (*a*) *by the method of Postgate* et al. (1960); (*b*) *from total and viable counts*

Organisms harvested from tryptic meat broth were washed twice with sodium chloride + potassium phosphate solution (pH 6·5) and resuspended in the same solution.

Time of storage (hr.) ...	0	16	24	40	48	64	72	88
(1) Total cell count $\times 10^{-10}$	1·13	1·11	1·27	1·16	—	—	—	1·17
(2) Viable cell count $\times 10^{-10}$	0·98	0·95	0·90	0·96	0·95	0·75	0·73	0·60
(3) % viability ((2)/(1) × 100)	87	86·5	71	83	—	—	—	51
(4) % viability by the method of Postgate *et al.* (1961)	97·5	98	96	90	87	78	68	59

Some of the factors which affected survival of washed bacteria were investigated. In the pH range 4–9 with Michaelis (1931) constant ionic strength buffers, the optimum value for survival of suspensions (about 10^{10} organisms/ml.) stored at 20–22° under nitrogen was near pH 6·5; in the range pH 5–8 with orthophosphate buffers (0·02 M PO_4) in sodium chloride (0·13 M) solutions, a similar pH optimum was found with suspensions stored under these conditions or at 37° with aeration. A solution of buffered sodium chloride (pH 6·5) was used routinely for washing and suspending bacteria. This solution appeared to be non-toxic since bacterial suspensions stored at 20–22° under air or nitrogen survived almost completely for days and in some cases for weeks. It was found that at 37° with aeration, the initial bacterial concentration affected survival of a population. Initial concentrations of less than 10^7 organisms/ml. multiplied to 10^6–10^7/ml. within 70 hr. This is in agreement with the work of Garvie (1955) who found that *Escherichia coli* multiplied in buffer solution prepared from specially purified salts dissolved in distilled water. Above a

concentration of 10^7–10^8 bacteria/ml. significant multiplication did not occur and the length of the initial period of almost complete survival decreased as the concentration of organisms was increased to 10^{10}/ml. A concentration of about 10^{10} organisms/ml. was used routinely because this avoided maintenance of a significant proportion of the population by contaminating nutrients present in the buffer and also was convenient for subsequent chemical analyses. The shape of survival curves for suspensions of bacteria, particularly those harvested from defined medium, stored at a concentration of about 10^{10}/ml. sometimes showed evidence of regrowth; this was probably due to the utilization of material obtained from dying or dead organisms (Harrison, 1960). Regrowth was decreased when bacterial suspensions in cellophan sacs were dialysed against buffered saline. The survival of bacteria depended on the growth phase of the organisms at the time of harvesting. Survival curves for bacteria harvested from defined medium during the late exponential growth phase and at different times in the stationary phase (Fig. 2) showed that extension of the stationary phase under growth conditions favoured survival. The composition of the medium used for growing the bacteria affected their subsequent survival in buffered saline. Organisms harvested from tryptone glucose medium survived best and those harvested from tryptic meat broth usually survived better than bacteria grown in carbon-limiting defined medium. This was true during storage at 37° with aeration (Fig. 3), or at 20–22° under nitrogen; under the latter conditions the initial periods of almost complete survival were about 15, 10 and 4 days for organisms harvested from tryptone glucose medium, tryptic meat broth and carbon-limiting defined medium, respectively.

Analyses of bacteria grown in different media. Analyses of well-washed freeze-dried stationary phase bacteria harvested from the three media are shown in Table 2. On a dry-weight basis, bacteria grown in tryptone glucose medium contained 3–4 times as much carbohydrate (as glucose) excluding pentose and about half as much RNA as bacteria grown in the defined medium. Bacteria grown in tryptic meat broth contained a relatively large amount of protein and less RNA than did bacteria from the defined medium. Poly-β-hydroxybutyric acid (Lemoigne, 1927; Forsyth, Hayward & Roberts, 1958) was not detected in chloroform extracts of 1 g. amounts of freeze-dried bacteria which had been grown in any of these media. Besides differences in chemical analyses the bacteria varied in average weight according to whether they were grown in defined or complex medium (Table 2). Stationary phase bacteria harvested from defined medium were metabolically more active than bacteria from tryptone glucose medium. For example, Q_{O_2} (μl./hr./bacterium) values for washed bacteria during the period when the rate of oxygen uptake was constant, were 5·2 and $2{\cdot}36 \times 10^{-9}$ for organisms from the defined medium and from the tryptone glucose medium, respectively; in the presence of mannitol (1 %, w/v) Q_{O_2} values of 2·07 and $0{\cdot}64 \times 10^{-7}$, respectively, were obtained.

The high carbohydrate content of bacteria harvested from tryptone glucose medium (Table 2) was due to the presence of glycogen; this was isolated from the organisms (see Methods). Significant amounts of glycogen were not found in homogenates of organisms harvested from carbon-limiting defined medium or from tryptic meat broth.

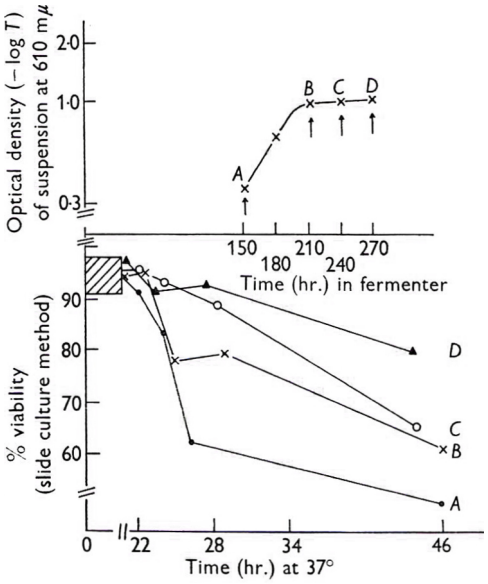

Fig. 2

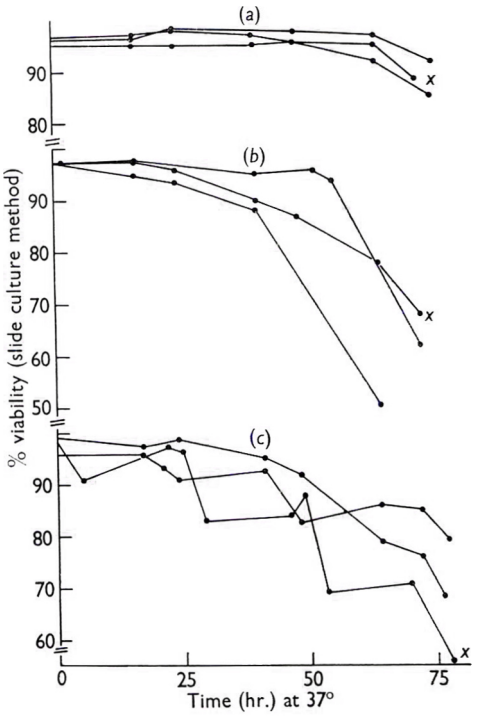

Fig. 3

Fig. 2. Survival of *Aerobacter aerogenes* harvested from defined medium during the exponential and stationary phases of growth. Arrows on upper curve indicate when samples (*A–D*) were taken. Lower curves show survival of washed bacteria from samples *A–D* during storage in buffered saline (pH 6·5) at 37° with aeration. Viability determined by the method of Postgate *et al.* (1961). Viability of all four suspensions determined at 0, 1, 2, 3, 4, 5 and 20 hr. was 91–98 %; this is indicated by the hatched area.

Fig. 3. Survival curves for *Aerobacter aerogenes* harvested 0·5 hr. after cultures had reached stationary phase in: (*a*) tryptone glucose medium; (*b*) tryptic meat broth; (*c*) defined medium. Washed bacteria were stored in buffered saline (pH 6·5) at 37° with aeration. Curves marked '*x*' and Figs. 4–9 refer to the same experiments (see text).

Table 2. *Analysis of washed freeze-dried stationary phase* Aerobacter aerogenes *harvested from different media*

Medium	Average dry wt./ bacterium (g. × 10^{13})	Total N	Total P	Total protein	Total carbohydrate excluding pentose	Total pentose*	RNA P	DNA P
		(g./100 g.)						
Defined	3·47	13·6	2·77	66	5·8	5·6	1·66	0·31
Tryptone glucose	2·42	11·6	1·95	60	15–20	3·7	0·87	0·38
Tryptic meat broth	2·25	13·9	2·14	72	3·8	4·0	1·09	0·49

* Excluding pyrimidine pentose

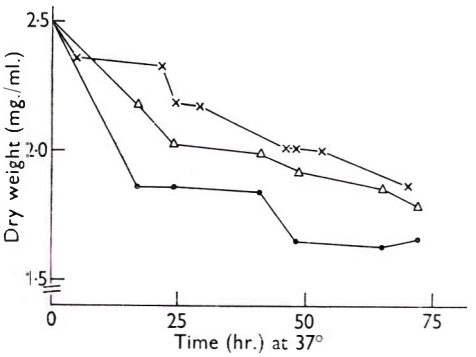

Fig. 4. Loss of dry weight.

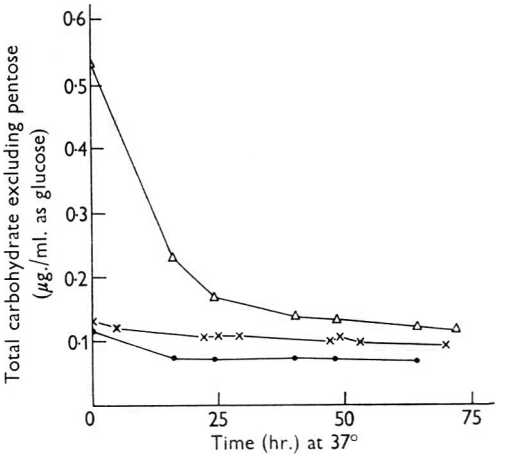

Fig. 5. Loss of glycogen.

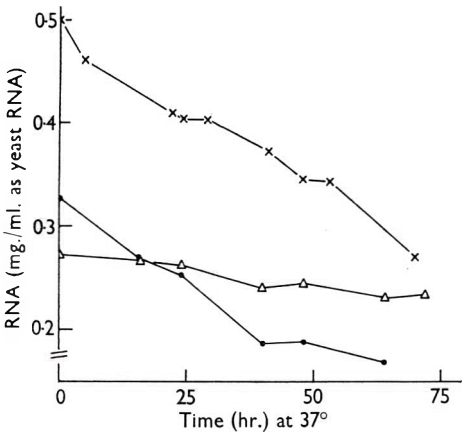

Fig. 6. Loss of RNA (determined by the Schneider, 1945, method).

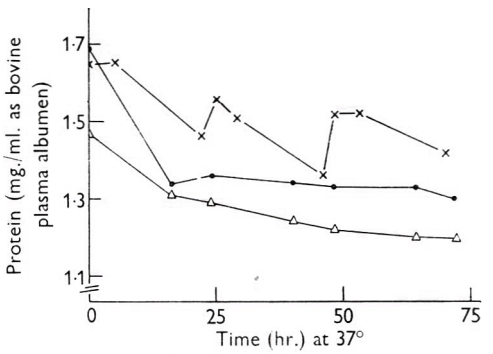

Fig. 7. Loss of protein.

Figs. 4–7. Loss of macromolecular constituents from washed *Aerobacter aerogenes* during storage in buffered saline (pH 6·5) at 37° with aeration. Bacteria harvested after 0·5 hr. in stationary phase from tryptone glucose medium (△), tryptic meat broth (●) and defined medium (×).

Losses of protein, RNA and glycogen from stationary phase bacterial suspensions

Suspensions of washed bacteria (about 10^{10}/ml.) freshly harvested from the three media were incubated at 37°, with aeration, in gas wash bottles. Samples were removed at intervals for determinations of pH value, total bacterial count, viability and chemical analysis. The data shown (Figs. 4–9) are those for one typical experiment with bacteria harvested from each of the three media; in each case the results were adjusted for a dry weight of 2·5 mg. organisms/ml. at $t = 0$. Survival curves referring to the suspensions investigated are shown in Fig. 3*a–c* (×). Considering first bacteria harvested from tryptone glucose medium, their initial viability of 95% was maintained for at least 65 hr. (slide-culture method; Fig. 3*a*, ×). At 0, 16, 24, 48 and 64 hr., the total counts were 1·23, 1·22, 1·20, 1·30 and 1·18 and viable counts

0·94, 0·98, 0·97, 0·93 and $0·96 \times 10^{10}$ organisms/ml., respectively. Thus the substantial loss of dry weight which occurred in 65 hr. (about 25%; Fig. 4) apparently represented a loss from living bacteria. The dry-weight loss was due mainly to losses of glycogen (0·41 mg./ml.; Fig. 5), RNA (0·04 mg./ml.; Fig. 6) and protein (0·27 mg./ml.; Fig. 7). Endogenous glycogen appeared to be metabolized by the bacteria because (1) the carbohydrate content of the supernatant fluid from the suspension did not increase significantly during storage; (2) the keto acid content of the organisms increased about fourfold during the first few hours of storage and then decreased to near the original value in 17 hr. The keto acid increase was due mainly to the formation of pyruvic acid. During the period when endogenous keto acid concentration increased, the rate of glycogen loss was high (Fig. 5). The suspension of bacteria harvested from tryptic meat broth had an initial viability of 97·5% and this was maintained for about 40 hr. (slide-culture method; Fig. 3*b*, ×); the respective total and viable counts for this suspension are shown in Table 1. Again there was a loss of dry weight during storage (about 25% in 40 hr.; Fig. 4) mainly due to losses of RNA (0·14 mg./ml.; Fig. 6), protein (0·35 mg./ml.; Fig. 7), and total carbohydrate excluding pentose (0·04 mg./ml.; Fig. 5). Survival curves for bacteria harvested 0·5 hr. after growth had stopped in carbon-limiting defined medium were usually less regular than those for organisms from the complex media (Fig. 3*c*); the initial viability of the suspension discussed here was 96%, and this was more or less maintained for 40 hr. (Fig. 3*c*, *x*). At 0, 17, 24, 41 and 64 hr., total counts were 0·70, 0·80, 0·70, 0·73, 0·74 and viable counts 0·59, 0·54, 0·68, 0·59, $0·40 \times 10^{10}$ organisms/ml., respectively. The loss of dry weight (about 20%, Fig. 4) which occurred in 40 hr. was mainly due to losses of RNA (0·15 mg./ml.; Fig. 6), protein (0·17 mg./ml.; Fig. 7) and total carbohydrate excluding pentose (0·03 mg./ml.; Fig. 5). In further experiments, the disappearance of RNA from these bacteria was shown to be most rapid during the first 8 hr. (average loss, about 2·0%/hr.) and the rate decreased during the next 16 hr. (average loss, about 1·0%/hr.).

In parallel with the losses of endogenous constituents there was an excretion into the surrounding medium of material which included ammonia (Fig. 8) u.v.-absorbing substances (Fig. 9) and traces of free amino acids. Bacteria harvested from tryptic meat broth which initially contained the largest amount of protein (68–72% dry weight), excreted the largest amount of ammonia. Since the total nitrogen of RNA lost from this suspension during storage for 40 hr. was equivalent to about 22 μg./ml. and the ammonia-N excreted was 65 μg./ml. it seems probable that some of the ammonia was released as a result of protein degradation. During storage, the concentration of free amino acids in the bacteria or in the surrounding medium did not change sufficiently to explain the protein loss as being due to proteolysis alone. Bacteria harvested from defined medium, from which a considerable amount of RNA was lost (Fig. 6), excreted the largest amount of u.v.-absorbing material; some of this was re-absorbed, presumably by living organisms (Fig. 9). The absorption spectrum of the excreted material showed a broad flat peak extending from 248 to 257 mμ, similar to that of the material excreted by *Lactobacillus arabinosus* and investigated by Holden (1958). Analysis of this material by ion-exchange resin chromatography and paper ionophoresis, kindly done by our colleague Mr H. E. Wade, showed that the u.v.-absorbing substances were mainly free bases with a relatively high proportion of hypoxanthine. The presence of hypoxanthine suggested

that, during storage, adenine nucleotides were degraded, resulting in the release of ammonia and ribose. Since the concentration of pentose, as well as endogenous RNA, decreased, it is evident that ribose was metabolized. Similar losses of RNA, glycogen and protein occurred when bacterial suspensions were held at 20–22° under nitrogen, but at a slower rate.

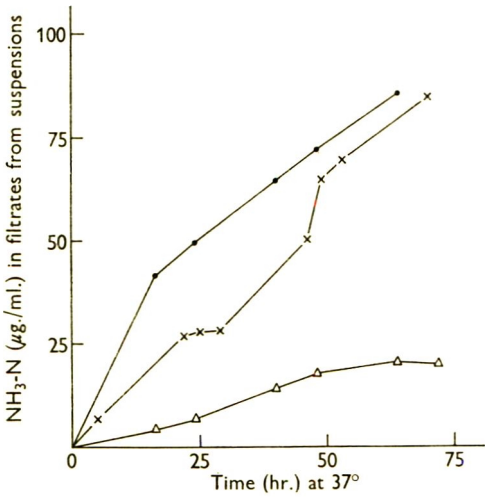

Fig. 8

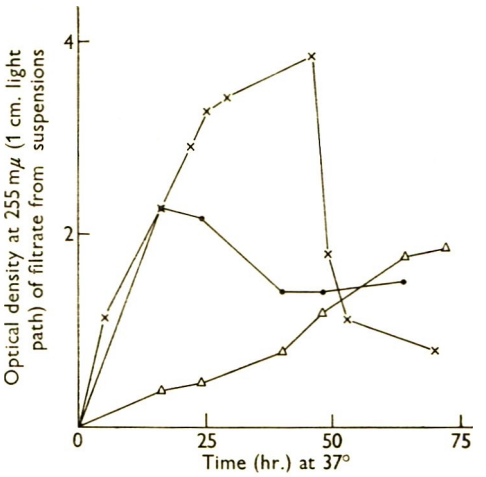

Fig. 9

Fig. 8. Excretion of ammonia by stationary phase *Aerobacter aerogenes*. Growth media, conditions of storage and symbols as for Fig. 4.

Fig. 9. Excretion of u.v.-absorbing substances by stationary phase *Aerobacter aerogenes*. Growth media, conditions of storage and symbols as for Fig. 4.

Synthesis of endogenous glycogen by bacteria grown in defined medium

The defined medium described in Methods contained 1% (w/v) mannitol and 0·12% (w/v) nitrogen as ammonium salt. Growth was limited by the mannitol and when this had been utilized about 55% of the nitrogen had been used. When the ammonium salt concentration was decreased by 75%, nitrogen became limiting and, as reported by Holme (1957), glycogen was laid down in the organisms during the stationary phase. The results of a typical experiment are shown in Fig. 10. Bacteria were harvested after 4 hr. in the stationary phase from both nitrogen-limiting medium and the normal carbon-limiting medium, and their survival characteristics were compared. The glycogen-containing bacteria survived better than did those without reserves of glycogen.

Effect of added glucose on the survival of bacteria in buffered saline

Stationary phase bacteria freshly harvested from tryptic meat broth were washed by centrifugation and suspended at a concentration of about $0{\cdot}6 \times 10^{10}$ viable organisms/ml. in buffered saline (pH 6·5). Glucose (to 0·45%, w/v) was added and the suspension incubated at 37° with aeration. During incubation the pH value of

the suspension was controlled by the addition of NaOH. During a 4 hr. incubation period, samples were removed for viability determinations and chemical analyses. It was found that about 5% of the added glucose was turned into cellular glycogen and about 60% was metabolized (Fig. 11). During the incubation period, 50% of the bacteria became non-viable; apparently this was due to the presence of glucose, for similar suspensions without added sugar remained completely viable during the experimental period. When similar suspensions containing added glucose were incubated at 37° under anaerobic conditions (nitrogen) the Pasteur effect was evident, i.e. the substrate was fermented much more rapidly. No cellular glycogen was formed under these conditions. Again about 50% of the population died during a 4 hr. incubation period.

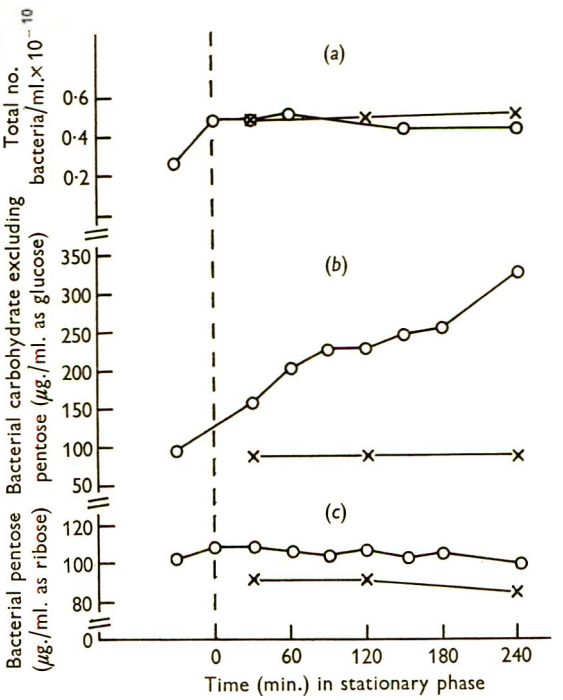

Fig. 10. Synthesis of endogenous glycogen by *Aerobacter aerogenes* grown in defined medium. Bacteria grown in nitrogen-limited medium (○); (*a*) total count, (*b*) total carbohydrate excluding pentose in washed cells, (*c*) total pentose in washed organisms. Some results for bacteria grown in carbon-limited defined medium are shown for comparison (×).

DISCUSSION

In the present work, populations of stationary phase organisms of *Aerobacter aerogenes* were grown in a culture vessel under controlled conditions and, in a given medium, their survival characteristics, mean mass and amounts of protein, ribonucleic acid and carbohydrate did not vary greatly from batch to batch. Suspensions of bacteria grown in this way had high initial viabilities which were maintained for periods of hours or days, according to the medium used for growth and the conditions of storage. During this period, degradative changes occurred within the bacteria resulting in the loss of up to 25% of dry weight. Products made available

by these degradative processes were metabolized by the living organisms and it appeared that their viability was maintained as a result of this. Once a proportion of a bacterial population dies, a comparatively large amount of nutrient material from the corpses may eventually become available to the survivors, thus allowing some of them to grow and divide. When division occurs the population is no longer the original one; in any case chemical changes found to occur after this are difficult

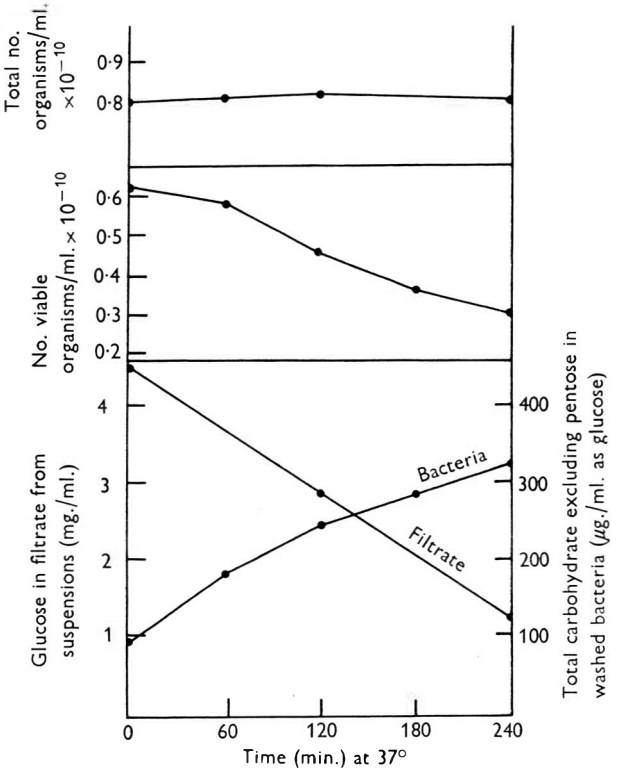

Fig. 11. The effect of exogenous glucose on the survival of *Aerobacter aerogenes* suspensions stored at 37° with aeration. Bacteria harvested from tryptic meat broth were washed and resuspended in buffered saline (pH 6·5). Glucose (4·5 mg./ml.) was added at zero time.

to interpret since they are occurring in viable and/or dead bacteria. The phenomenon of re-growth in suspensions of *A. aerogenes* held at growth temperature in the absence of nutrients was studied by Harrison (1960). It may be prevented to some extent by dialysing suspensions in cellophan sacs; with such suspensions the survival curve usually showed an initial period of almost complete survival followed by a period of nearly exponential death. This appears to substantiate the hypothesis that bacteria may survive in an unfavourable environment at the expense of endogenous constituents to the stage when further utilization affects cell integrity.

Results of previous studies concerned with the role of glycogen in bacteria and yeasts suggest that in these organisms the substance does not always fulfil the criteria of an energy reserve as it does in animals (Holme, 1957). For example,

Stier & Stannard (1935–36) showed that the glycogen of yeast cells decreased slowly when they were suspended in a carbon-free medium, but it was not fermented. Holme (1957) reported that utilization of internal glycogen by *Escherichia coli* occurred during rapid growth in a complete medium. In bacteria starved of an organic carbon source but supplied with nitrogen, no increase in nitrogen-containing compounds occurred in spite of the fact that 6–8 % of the dry weight of the organisms was glycogen. The fact that bacteria lose internal glycogen during growth and division does not necessarily affect the hypothesis that glycogen serves as a reserve material for stationary phase organisms in an unfavourable environment. Also the fact that bacteria containing 6–8 % glycogen, when put into nitrogen-containing medium, did not synthesize nitrogen-containing compounds is perhaps not so important as whether or not the organisms survived. On this aspect no data are given by Holme and his colleagues. Holme & Palmstierna (1956*d*) reported other experiments in which the utilization of glycogen-carbon for synthetic purposes was shown and, as pointed out by Wilkinson (1959), in these experiments internal glycogen did fulfil the criteria of an energy-reserve substance. The results of our survival studies showed that stationary phase organisms containing glycogen survived well in an unfavourable environment. It was found that when cell-free extracts of mechanically disrupted *Aerobacter aerogenes* were incubated at 37° with purified glycogen (see Methods) and buffer (pH 7·2), glucose was released. No free glucose was detected in control tubes containing glycogen without extract or extract without glycogen, incubated in parallel. Thus *A. aerogenes* had the mechanism for degrading the polysaccharide and the energy derived from glucose metabolism may have contributed towards the better survival characteristics of glycogen-containing bacteria. However, when glucose was added to an aerated suspension of organisms in buffered saline at 37°, endogenous glycogen equivalent to about 5 % of the added sugar was formed in 4 hr. and most of the remaining glucose was metabolized. During the incubation period about 50 % of the population lost their viability. It is possible that the metabolism of a relatively large amount of glucose in the absence of a nitrogen source caused the degradation or denaturation of internal substances, such as enzymes, which could not be replaced.

It has been shown that the ribonucleic acid of stationary phase *Lactobacillus arabinosus* stored in buffer at 37° is degraded (Holden, 1958); in our experiments with *Aerobacter aerogenes* a similar process occurred which had no immediate effect on viability. Stephenson & Trim (1938) showed that washed suspensions of *Escherichia coli* were able to degrade adenine nucleotides and that the products of the reaction were hypoxanthine, inorganic phosphate, ammonia and ribose; ribose did not accumulate but was fermented. Eggleston & Krebs (1959) extended this work. We found that when cell-free extracts of *A. aerogenes* were incubated with adenosine at 37° and pH 8·4, ammonia and ribose were released and the peak of the reaction mixture absorption spectrum minus the absorption spectrum of the extract shifted from 257 to 247 mμ, the latter being the wavelength of maximum absorption of hypoxanthine. Similarly cytidine was converted to uracil on incubation with cell extract. The excretion of free bases, including hypoxanthine, by suspensions of viable *A. aerogenes* suggests that the degradation of ribonucleic acid may provide the bacteria with readily metabolizable sources of carbon (ribose) and nitrogen (ammonia).

Data about protein turnover in *Escherichia coli* maintained at growth temperature in the absence of nutrients are available from the reports of Mandelstam (1958*b*) and Mandelstam & Halvorson (1960). The rate of protein-degradation was equal to the rate of protein synthesis at about 5%/hr. It was suggested that the pool of amino acids made available by this process was used for the production of inducible enzymes to cope with changed chemical environments (Mandelstam, 1956; Pollock, 1958). The data were obtained for suspensions incubated for up to 4 hr. The results of the present work show that in an unfavourable environment for longer periods, degradation of protein in *Aerobacter aerogenes* was not in equilibrium with synthesis and apparently went beyond the amino acid stage, resulting in the release of ammonia. This loss of protein did not immediately affect viability, and in some cases up to 25% of the total cell protein was lost before significant deaths occurred in the population. It is evident that the concentrations of major constituents such as protein, ribonucleic acid and glycogen in bacteria vary according to the medium used for their growth and that these substances may be present in excess of the amounts needed for immediate use. It is difficult to prove unequivocally that utilization of these substances allows cells to survive, but the shape of the survival curve obtained in the present work, showing an initial lag period before the population started to die, could be explained on this basis.

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Analysis of a Syntrophic Growth of *Lactobacillus plantarum* and *Streptococcus faecalis*

By N. R. KRIEG* and M. J. PELCZAR, Jun.

Department of Microbiology, University of Maryland, College Park, Maryland, U.S.A.

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SUMMARY

Syntrophism was demonstrated between strains of *Lactobacillus plantarum* and *Streptococcus faecalis* in a chemically defined medium which lacked phenylalanine and pteroylglutamic acid (PGA); however, the presence of *p*-aminobenzoic acid (PABA) was essential. Enumeration of the two organisms in syntrophic growth was found to be possible by the use of Trypticase soy agar + 0·25% (w/v) glucose at pH 9·0 as a selective medium for *S. faecalis*. It was found that *S. faecalis* predominated in the early stages of the syntrophic growth, but that *L. plantarum* eventually predominated. Characterization by a bioautographic technique of the phenylalanine-active factor synthesized by *S. faecalis* and required by *L. plantarum* indicated that the factor was not identical with phenylalanine or shikimic acid. Microbiological assays of the culture fluid from cultures of *L. plantarum* indicated that three types of folic acid compounds were synthesized: (1) oxygen-stable compounds which supported the growth of *Pediococcus cereviseae*; (2) oxygen-labile compounds which supported the growth of *P. cereviseae*; (3) oxygen-stable compounds which supported the growth of *S. faecalis* but not of *P. cereviseae*.

INTRODUCTION

Syntrophic growth (mutual feeding) of *Lactobacillus plantarum* strain 17-5 with *Streptococcus faecalis* strain R was first reported by Nurmikko (1954). In a chemically defined medium which lacked phenylalanine (required by *L. plantarum*) and pteroylglutamic acid (PGA; required by *S. faecalis*), neither organism grew alone; but in mixed culture, abundant growth of both organisms occurred. It was suggested that this syntrophic growth occurred because each organism synthesized the omitted growth factor required by the other, i.e. phenylalanine was synthesized by *S. faecalis* and PGA was synthesized by *L. plantarum*. Koft & Morrison (1956) reported much the same phenomenon. Judge (1958) studied this and other examples of syntrophism, and analysed the culture filtrates of *S. faecalis* to determine the nature of the phenylalanine-active substance synthesized and present in the culture filtrate. A one-dimensional ascending chromatographic technique was used, and the presence of a spot, corresponding to the position of phenylalanine, was demonstrated with a chemical indicator.

* Present address: Department of Bacteriology, Virginia Polytechnic Institute, Blacksburg, Virginia, U.S.A.

The purpose of the present investigation was directed toward a further analysis of this system of syntrophic growth, and can be divided into two parts: (1) population aspects of the syntrophism, (2) the nature of the factor(s) synthesized by each of the two organisms involved in the mutual growth promotion.

METHODS

Organisms. The two organisms used in the syntrophic growth experiments were *Lactobacillus plantarum* strain 17-5 (American Type Culture Collection, Washington 7, D.C., U.S.A., ATCC No. 8014) and *Streptococcus faecalis* strain R (ATCC No. 8043). For the microbiological assay of folic acid-like compounds, the following organisms were used, in addition to *S. faecalis*, namely, *Lactobacillus casei* (ATCC No. 7469) and *Pediococcus cereviseae* (ATCC No. 8081). All cultures were maintained in stabs of Micro assay culture agar (BBL; Baltimore Biological Laboratory, Inc., Baltimore 18, Maryland, U.S.A.) in the refrigerator: new transfers were made every 2–3 weeks.

Media. The defined medium used was that described by Nurmikko (1954) except for variations in the phenylalanine, *p*-amino benzoic acid (PABA) and PGA content. The composition of this medium is also described in an earlier report from this laboratory (Krieg & Pelczar, 1960). The term basal medium, as used here, refers to Nurmikko's medium minus *p*-aminobenzoic acid (PABA), pteroylglutamic acid (PGA) and phenylalanine.

Analysis of syntrophic growth. Lactobacillus plantarum and *Streptococcus faecalis* were grown separately in the basal defined medium supplemented with 200 μg. phenylalanine, 0·2 μg. PABA and 0·01 μg. PGA/ml. After incubation for 18 hr. the cultures were centrifuged, washed twice with sterile physiological saline, and then resuspended in sterile distilled water to an optical density of 0·150 at 420 mμ as measured in a 16 mm. cuvette with a Bausch and Lomb Spectronic 20 colorimeter. These suspensions were then further diluted 1/10 in sterile distilled water. These dilutions were used as inocula (0·1 ml./tube containing 5·0 ml. medium) and, respectively, contained about $6{\cdot}5 \times 10^6$ viable organisms of *S. faecalis*, or $7{\cdot}5 \times 10^6$ of *L. plantarum*/ml. The scheme of inoculation (single cultures and cultures in admixture) to assess the effect of PGA, PABA and phenylalanine is evident from Fig. 1. It can be seen that the variations in media concerned the phenylalanine, PABA, and PGA content, and variations in inocula concerned the use of single cultures of each organism or both organisms inoculated together into the same medium. All incubations were at 35°, for various times (Fig. 1) in an air incubator. Growth was measured turbidimetrically at 600 mμ in 16 mm. optically matched screw-capped tubes.

In experiments where the two syntrophic organisms were enumerated separately from the mixed culture (Table 1), the inoculum was prepared as described above except that the concentrations of PABA and PGA were changed to 0·002 and 0·05 μg./ml., respectively. Growth was measured by three methods: direct count, colony count, turbidimetrically (Table 2).

Analysis of Lactobacillus plantarum *culture medium for folic acid activity.* For the production of the folic acid-like factor(s) synthesized by *Lactobacillus plantarum*, 5·0 ml. basal medium supplemented with 200 μg. phenylalanine and 0·05 μg. PABA/ml. were inoculated from the stock stab culture. After incubation for 24 hr.,

a second transfer was made to the same medium. After a further incubation for 18 hr., 1·0 ml. of the culture was added to 300 ml. of the same medium supplemented with an excess of PABA (1 mg./ml.). After incubation for 24 hr. the culture was divided into two portions: one portion was untreated, while ascorbic acid (10 mg./ml.) was added to the other. Both portions were centrifuged to remove the organisms and the supernatant fluids adjusted to pH 6·7 with a measured quantity of 2 N-NaOH. The two portions of supernatant fluid were kept frozen at $-10°$ until assayed for folic acid activity; the portion which contained ascorbic acid was held in an atmosphere of argon.

Assays with *Pediococcus cereviseae* were made with the medium of Sauberlich (1949); 6·0 ml. of assay medium were used per tube. When assays were performed with the ascorbic acid-containing portion, 1·33 mg. ascorbic acid was added to each ml. of single-strength assay medium, which was adjusted to pH 6·3. Besides being used for the assay, this ascorbate medium was used as a diluent for the ascorbic acid-containing portion described above. All media, after all additions to tubes, were autoclaved at 112° for 5 min. The inoculum culture consisted of the second of two consecutive serial 24 hr. broth cultures grown in the assay medium supplemented with 1 mμg. leucovorin/ml.; the culture was centrifuged, resuspended in 5 ml. sterile physiological saline and diluted 1/100 with sterile saline. One drop of this dilution served as inoculum for each assay tube. (Leucovorin was obtained from the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, U.S.A., in solution in ampoules.) Incubation was for 24 hr. at 35°, and growth was determined turbidimetrically at 600 mμ. For the aerobic *P. cereviseae* assay a second method, which yielded results almost identical with those given by the first method described, was used; this second assay was performed with the medium and method described in the *Difco Manual* (1953). Assays with *Streptococcus faecalis* were also performed by using the medium and method described in the same publication (*Difco Manual*, 1953). Assays performed with the ascorbic acid-containing portion were treated as described for the *P. cereviseae* assay.

Assays with *Lactobacillus casei* were made by using the basal medium of Nurmikko (1954); 6·0 ml. media were used per tube. The medium was autoclaved at 112° for 5 min., with the glucose added before autoclaving. The inoculum was prepared like the *Streptococcus faecalis* inoculum. Growth was recorded turbidimetrically after incubation for 60 hr.

In each assay method, standard curves were prepared by using leucovorin, and the results were expressed in terms of leucovorin activity.

Analysis of Streptococcus faecalis *culture medium for phenylalanine activity.* For production of the phenylalanine-active factor synthesized by *Streptococcus faecalis*, 5·0 ml. of the basal medium, supplemented after autoclaving with 0·05 μg. PGA/ml., were inoculated from the stock stab culture. After 24 hr. a second transfer was made to the same medium, and after a further 18 hr., one loopful was transferred to 100 ml. of the same medium.

The concentration of the phenylalanine-active factor was accomplished in the following way. After incubation of *Streptococcus faecalis* for 24 hr. in the medium described, the organisms were removed by centrifugation, the supernatant fluid adjusted to pH 6·7 with 2 N-NaOH and evaporated to dryness at 60° in a 1 l. beaker. The residue was redissolved in a minimum quantity of water and extracted with

100 ml. 95% (v/v) ethanol in water, followed by a second extraction with 50 ml. 95% (v/v) ethanol. The ethanolic extract was evaporated to dryness at 60°, redissolved in a minimal amount of water, and preserved at −10° until assayed for activity.

Bioautography of the ethanolic extract for phenylalanine activity was accomplished in the following way, a modification of a method described by Block, Durrum & Zweig (1958). The concentrate was applied in a band to Whatman No. 1 chromatography paper; after drying, it was developed by an ascending technique with the solvent acetone + water + urea (60 + 40 + 0·5; v, v, w). After drying, the paper was again developed with 0·04 N-HCl at right angles to the first direction so that the phenylalanine-active factor was concentrated at the top of the paper. The paper was dried and then placed face down on basal medium solidified with 1·5% (w/v) agar and supplemented with 0·05 μg. PABA/ml., previously seeded with *Lactobacillus plantarum*. The paper strip was removed after 1 hr., and the plates incubated overnight.

Conventional methods of 'spotting' were tried on numerous occasions without success, probably because of the interference with the migration of the test compounds by the high concentration of other solutes in the concentrate. Even with the present method, however, when compounds such as shikimie acid were chromatographed from pure solution, in contrast to being incorporated into the ethanolic extract concentrate, a considerable difference was noticed between the R_f values. When applied from pure solution, the shikimic acid migrated much further than when incorporated in concentrate. Therefore, special care was taken to avoid the comparison of R_f values of pure compounds with the R_f values of the phenylalanine-active factor in the concentrate.

RESULTS

Figure 1 presents the results obtained when various mixtures of phenylalanine, PABA and PGA were supplied for the growth of *Lactobacillus plantarum* and *Streptococcus faecalis*, when grown separately and in mixed culture. It can be seen that syntrophic growth occurred in the medium which lacked phenylalanine and PGA but was supplemented with PABA (designated by code 19 in Fig. 1). In media of the same composition (Fig. 1; codes 5 and 12), neither organism alone grew significantly during the incubation periods recorded. Syntrophic growth was not observed when PABA was not present in the medium.

Growth of *Lactobacillus plantarum* in the absence of phenylalanine eventually occurred upon prolonged incubation. The time at which such growth appeared was quite variable, but generally occurred within 3–5 days. Experiments wherein the size of the inoculum was decimally decreased indicated that after incubation for 120 hr., even those tubes which had been theoretically inoculated with one *L. plantarum* organism eventually exhibited growth in the basal medium supplemented with PABA but lacking phenylalanine. These results indicated that carry-over of phenylalanine in the inoculum or in the organisms of the inoculum was not a factor in this delayed response.

In the present study a direct microscopic counting procedure, as by using the Petroff–Hauser counting chamber, could not be used to follow the growth of each

of the two organisms in syntrophic culture, because of the inability to distinguish microscopically in this technique the organisms of *Lactobacillus plantarum* from those of *Streptococcus faecalis*, On solid media, such as microassay culture agar (BBL), *L. plantarum* was found as long rods; but in the basal medium supplemented with phenylalanine and PABA, the organism occurred as short rods or cocci. In micro-inoculum broth (BBL) stained smears made up to 18 hr. revealed short rods or cocci; after 25 hr. they revealed predominantly long rods.

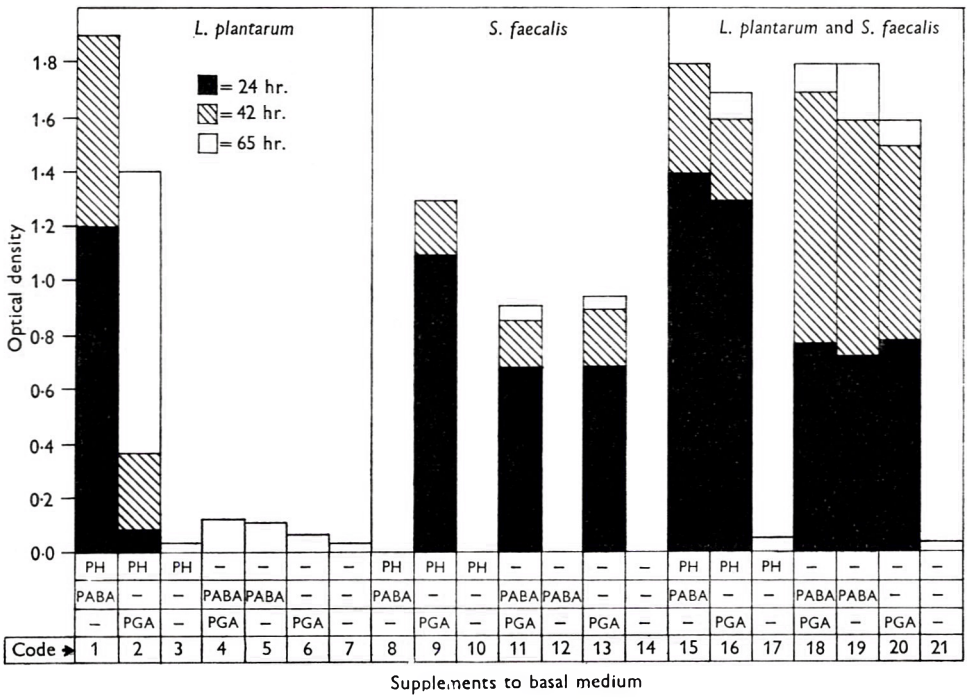

Fig. 1. The effect of phenylalanine, PABA, and PGA in the nutrition of *L. plantarum* and *S. faecalis* at 35° in a chemically defined medium. The medium to which the compounds were added is the basal medium referred to in the text under Methods. PH = phenylalanine, 200 μg./ml.; PGA = pteroylglutamic acid, 0·01 μg./ml.; PABA = *p*-aminobenzoic acid, 0·2 μg./ml. Code designations 1 through 21 identify various supplements and inocula.

Trypticase soy agar (BBL) supplemented with 0·25 % (w/v) glucose at pH 9·0 was found to serve as a selective medium for the growth of *Streptococcus faecalis*. The same medium at pH 7·3 supported the growth of both *S. faecalis* and *Lactobacillus plantarum*. The selective medium was used to determine the actual numbers of each organism during a period of syntrophic growth. The results (Table 1) indicate that *S. faecalis* predominated in the culture up to 26 hr., but that after 46 hr. it decreased markedly so that *L. plantarum* eventually predominated. The discrepancies apparent as between the results obtained by the three methods of enumerating the total population can be attributed to: (1) differences in size of organisms at different times; (2) the presence of non-viable, as well as viable, organisms as incubation was prolonged.

Table 1. *Growth, as measured turbidimetrically, microscopically, and by the standard plate count, of* Lactobacillus plantarum *and* Streptococcus faecalis *in pure and mixed cultures*

Growth was in basal medium (see Methods) lacking phenylalanine and PGA, and supplemented with 0·002 μg. PABA/ml. Incubation at 35°.

The standard colony count for *S. faecalis* was determined by plating the syntrophic culture in Trypticase soy agar supplemented with 0·25 % (w/v) glucose and adjusted to pH 9·0.

The total (*S. faecalis* and *L. plantarum*) colony count was determined with the same medium at pH 7·3. The difference between the two counts represented the standard colony count for *L. plantarum*.

Incubation time (hr.)	Pure cultures		Both cultures
	S. faecalis	*L. plantarum*	
	Optical density (600 mμ)		
22	0·00	0·05	0·37
46	0·00	0·05	1·50
51	0·00	0·06	1·70
74	0·00	1·70	1·70
	Petroff–Hauser total count (cells/ml.)		
22	—	—	1,630,000,000
46	—	—	5,000,000,000
51	—	—	4,500,000,000
74	—	—	9,000,000,000
	Standard plate counts from syntrophic culture		
22	795,000,000	35,000,000	830,000,000
46	30,000,000	2,605,000,000	2,635,000,000
51	< 1,000,000	2,694,000,000	2,694,000,000
74	< 1,000,000	1,830,000,000	1,830,000,000

In preliminary assays for the presence of the phenylalanine-active factor synthesized by *Streptococcus faecalis*, the ethanolic extract of the culture medium from a 24 hr. culture of *S. faecalis*, and the ethanol-insoluble residue remaining after this extraction, were added in different amounts to sterile paper disks; these were placed on the surface of solidified basal medium supplemented with PABA (but lacking phenylalanine), which had been previously seeded with *Lactobacillus plantarum*. The presence of the phenylalanine-active factor was found to reside in the ethanolic extract, as exhibited by the growth response around the corresponding disk. A bioautograph was prepared in the manner previously described, on which the ethanolic extract alone was compared with the extract to which phenylalanine had been added. It was evident that the difference in the positions of phenylalanine and of the phenylalanine factor in the extract ruled out the possibility that the factor was identical with phenylalanine. The factor was a faster moving compound than phenylalanine in the solvent system used.

A second bioautograph was prepared in which pure shikimic acid was compared with extract containing added shikimic acid. The pure shikimic acid was used to demonstrate that it was possible to obtain a 'spot' with the concentration used. However, the appearance of two 'spots' on the system when extract + shikimic acid was tested, indicated that the factor synthesized by *Streptococcus faecalis* was not identical with shikimic acid. The fact that shikimic acid migrated more slowly

when incorporated in the extract, than when applied from pure solution, was also demonstrated. Nevertheless, the appearance of the shikimic acid 'spots', although different in position, clearly identified the known compound. The 'spot' of low density, that of the excreted factor, was similar in position and size to that exhibited in the preceding bioautographic experiment. The difference in positions of the two shikimic acid 'spots' may be attributed to interference with migration of the compound by the high concentration of other solutes in the extract.

The results of microbiological assays of the fractions prepared from the culture medium of *Lactobacillus plantarum* cultures, obtained as previously described, are presented in Table 2. The quantitative aspect of these results has significance with respect to the nature of the folic acid-like compound(s) synthesized and excreted into the medium by *L. plantarum*, in the light of recent developments in the field of folic acid metabolism. This topic is discussed below.

Table 2. *Folic acid activity present in* Lactobacillus plantarum *culture medium after* 24 *hr. growth, as assayed by* Pediococcus cereviseae, Streptococcus faecalis, *and* Lactobacillus casei *with leucovorin as reference standard*

Organism	Type of assay	Sample no. 1	Sample no. 2
		Equiv. mμg. leucovorin/ml. culture fluid	
P. cereviseae	Aerobic	3·7	2·9
	Anaerobic*	14·8	11·6
L. casei	Aerobic	9·9	10·2
S. faecalis	Aerobic	11·2	10·1
	Anaerobic	24·8	22·3

* Anaerobic = addition of 1·33 mg. ascorbic acid/ml. assay medium, with adjustment of medium to pH 6·3.

DISCUSSION

The failure to demonstrate syntrophic growth of *Lactobacillus plantarum* with *Streptococcus faecalis* in the absence of added PABA was in contrast to the results obtained by Nurmikko (1954) who reported syntrophic growth of the two organisms in a medium lacking PABA (designated code 21 in Fig. 1), although the growth was clearly slower than in a medium which lacked phenylalanine and PGA. Only when the purines and uracil were omitted, in addition to PABA, were the two organisms unable to grow together. In the present investigation, the medium contained the purines and uracil; but in the absence of PABA, syntrophic growth did not occur, even on prolonged incubation.

Although growth of *Lactobacillus plantarum* in the presence of phenylalanine, but not of PABA or PGA, did appear after prolonged incubation, within the first 3–5 days independent requirements for phenylalanine and PABA could be demonstrated. This observation is also in contrast to the results of Nurmikko (1954), who reported that the requirement for PABA could be effectively replaced by phenylalanine, that this replacement could be inhibited by sulphonamides, and that increasing concentrations of phenylalanine were effective in overcoming the

inhibiting action of the sulphonamides. When one considers that *L. plantarum* has long been used as an assay organism for PABA in a medium containing Norite-treated acid-hydrolysed casein (Lewis, 1942), it does not appear likely that there could be a replacement of PABA by phenylalanine.

In microbiological assays involving tetrahydroPGA, it has been found that the pH value of the medium is important when the standard curve is based upon response to leucovorin (Donaldson & Keresztesy, 1959). Therefore, at pH 6·3 (the pH value used in the anaerobic assays shown in Table 2), the activity of the oxygen-labile compounds assayed in the *Pediococcus cereviseae* system, and represented by the difference between the aerobic and anaerobic *P. cereviseae* assays, may be much higher than is indicated, if tetrahydroPGA be assumed to be the major component of this fraction.

The assumption is made, for purposes of this discussion, that differences in pH value will not affect compounds other than tetrahydroPGA; otherwise, comparison becomes impossible because of the use of different pH values for aerobic and anaerobic assays, or because of the use of different organisms. Therefore, with this assumption, the kinds of folic acid compounds (in a general way only) can be deduced from the results of Table 2. Moreover, the concentrations of each of the different folic acid-like entities synthesized by *Lactobacillus plantarum* can also be determined as summarized in Table 3.

Table 3. *Summary of types of folic acid-like compounds synthesized by* Lactobacillus plantarum *as calculated from microbiological assay data*

Type of folic acid compounds	Sample no. 1 (μg./ml.)	Sample no. 2 (μg./ml.)
1. Oxygen-stable, for *P. cereviseae* (e.g. leucovorin)	3·7*	2·9*
2. Oxygen-stable and oxygen-labile, for *P. cereviseae* (e.g. leucovorin + tetrahydroPGA)	14·8*	11·6*
3. Oxygen-labile, for *P. cereviseae* (e.g. tetrahydroPGA)	(14·8 less 3·7) = 11·1†	(11·6 less 2·9) = 8·7†
4. Oxygen-stable, for *L. casei* (e.g. leucovorin) + PGA	9·9*	10·2*
5. Oxygen-stable, for *L. casei* but not stimulatory for *P. cereviseae* (e.g. PGA)	(9·9 less 3·7) = 6·2†	(10·2 less 2·9) = 7·3†
6. Oxygen-stable, for *S. faecalis* (e.g. leucovorin) + PGA	11·2*	10·1*
7. Oxygen-stable, for *S. faecalis* but not stimulatory for *P. cereviseae* (e.g. PGA)	(11·2 less 3·7) = 7·5†	(10·2 less 2·9) = 7·2†
8. Oxygen-stable and oxygen-labile, for *S. faecalis* (e.g. PGA + leucovorin + tetrahydroPGA)	24·8*	22·3*
9. Oxygen-labile, for *S. faecalis* (e.g. tetrahydroPGA)	(24·8 less 11·2) = 13·6†	(22·3 less 10·1) = 12·2†

* Experimentally determined value (see Table 2). † Calculated from experimental data.

The values presented in Table 3, it may be noted, agree very well with theoretical calculations. For example, the oxygen-stable fraction stimulatory for *Pediococcus cereviseae* + the oxygen-labile fraction stimulatory for *P. cereviseae* + the oxygen-stable fraction stimulatory for *Streptococcus faecalis* but not for *P. cereviseae*, should

be equivalent to the sum total of all the folic acid activity present in the sample. This value was determined experimentally by assay with *S. faecalis*, under anaerobic conditions, to be 24·8 or 22·3 μg./ml. of spent medium (Table 2). This can be determined by the following scheme:

	Sample no. 1 (μg./ml.)	Sample no. 2 (μg./ml.)
Oxygen-stable fraction	3·7	2·9
Oxygen-labile fraction	11·1	8·7
Oxygen-stable fraction (stimulatory for *S. faecalis* but not for *P. cereviseae*)	7·5	7·2
Total activity of sample on a theoretical basis	22·3	18·8

It may be noted that the values for the oxygen-labile fraction for *P. cereviseae* and the oxygen-labile fraction for *S. faecalis* (Table 3) are nearly equal, which would indicate that the same chemical entities, e.g. tetrahydroPGA, are being assayed Since there appears to be little difference between values for the oxygen-stable fraction determined by assay with *Lactobacillus casei* (but having no activity for *P. cereviseae*) and the oxygen-stable fraction for *S. faecalis* (having no activity for *P. cereviseae*), it may be concluded that here again the same chemical entities, e.g. PGA, are being assayed.

From these observations, it would appear that three kinds of folic acid compounds are present in 24 hr. spent medium of *Lactobacillus plantarum*: (1) a fraction which is oxygen-stable and supports the growth of *Pediococcus cereviseae* (such as leucovorin, leucovorin-anhydride, or ^{10}N-formyltetrahydroPGA); (2) a fraction which is oxygen-labile and supports the growth of *P. cereviseae* (such as tetrahydroPGA or dihydroPGA); (3) a fraction which is oxygen-stable and does not support the growth of *P. cereviseae* but which is active for *Streptococcus faecalis* and *Lactobacillus casei* (such as PGA, ^{10}N-formylPGA, folylglutamate, thymine, thymidine, ^{10}N-formyldihydroPGA). It would also appear that pteroic acid and ^{10}N-formylpteroic acid are not present, since these compounds are active for *S. faecalis* but not for *L. casei*.

The foregoing generalizations cannot be considered as proved by the evidence presented, but merely offer a reasonable explanation based on the recent developments concerning folic acid metabolism. As mentioned, much more information needs to be obtained about the effect of pH value on compounds other than tetrahydroPGA when the response is measured with reference to leucovorin standard curves. In addition, more data are needed about the quantitative response of different assay organisms to these compounds when interpreted in terms of the response of the assay organisms to leucovorin.

The elimination of phenylalanine, as being identical with the factor synthesized by *Streptococcus faecalis*, differs from the observation reported by Judge (1958). However, since Judge used filtrates of cultures which had been concentrated *in vacuo* 5- to 10-fold, and since these materials were applied to chromatograph paper in a single 'spot' in 5–10 μl. quantities, it is quite possible that interference with the migration of amino acids occurred in a manner similar to that found here for shikimic acid, namely, because of a large concentration of other solutes in the concen-

trate. Since ninhydrin was used as the indicator, all α-amino acids should have been detected. The system used in the present investigation used a biological indicator, the growth of *Lactobacillus plantarum*, the other member of the syntrophic pair, which specifically indicated the position of the phenylalanine-active factor on the agar plate.

The demonstration that neither phenylalanine nor shikimic acid was identical with the factor synthesized by *Streptococcus faecalis* and responsible for supporting the growth of *Lactobacillus plantarum*, suggests the possibility that the factor may be one of the compounds which have been suggested or demonstrated as intermediates in the biosynthesis of phenylalanine. Of such compounds (*a*) tyrosine cannot replace the phenylalanine requirement of *L. plantarum*, and (*b*) quinic acid and phenyl pyruvic acid cannot replace the phenylalanine requirement of *L. plantarum* (Nurmikko, 1954). Other possibilities about the nature of the substance involved are 5-dehydroshikimic acid, 5-dehydroquinic acid and shikimic acid-5-phosphate; these, however, were not explored.

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The Relationship Between Activity and Cell-wall Permeability in Dried Baker's Yeast

By L. I. K. EBBUTT
The Distillers Co., Ltd., Glenochil Research Station, Menstrie, Clackmannanshire

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SUMMARY

A close relationship has been found between the baking activity of dried yeast and the yeast cell volume after reconstitution; other things being equal, the cell volume is in turn dependent upon the permeability of the cell wall. It appears that the total observed loss of activity occasioned by drying yeast may be attributed to increased cell-wall permeability.

INTRODUCTION

In the production of dried baker's yeast a strain of *Saccharomyces cerevisiae* is used and the moisture content is decreased from about 70% to 7–8% by a drying process which may be one of several alternatives. Drum drying, in which the pressed yeast is placed in a revolving drum and dried in a current of hot air, is one method used. The product shows markedly improved storage properties. Whereas pressed yeast loses much of its baking activity after 1 week at room temperature, dried yeast may be stored for several months without appreciable deterioration. When required for use, dried yeast is simply added to the desired quantity of water and a suspension of rehydrated cells is obtained after a few minutes; this process is commonly referred to as reconstitution. Even the most favourable drying conditions, however, cannot produce a dried yeast which retains all the activity which it originally possessed in the pressed state; a greater or lesser proportion of the original activity is lost on drying, the extent of the loss depending on the strain of yeast used, the drying conditions and the method by which the yeast was propagated. As a general rule, the higher the initial activity the greater the loss suffered on drying.

It has been known for some time that dried yeasts become permeable to small molecules and ions, as shown by the work of Meyerhof & Kaplan (1951), Herrera, Peterson, Cooper & Peppler (1956), and Rothstein, Jennings, Dennis & Bruce (1959). While this is suggestive of a connexion between cell permeability and activity, the extent of the relationship has not been investigated. It has indeed been suggested that a decrease in permeability occurs on drying, resulting in a slower release of enzymes to the medium (Proskuryakov & Operysheva, 1956). It is the intention in this paper to show that the relationship between dried-yeast activity and cell-wall permeability is so close as to suggest that the latter is the main determining factor in dried-yeast performance; a rapid method for assessing the over-all permeability of the yeast cell wall is also given.

Throughout the work described here, cell-volume measurements were used as an indication of the over-all permeability of the cell wall. In order to appreciate the validity of this assumption it is necessary to consider the osmotic equilibrium of the

yeast cell. The normal healthy yeast cell consists of a mass of protoplasm enclosed by a structural cell wall together with the plasma membrane. In this respect it resembles most plant cells, although in plants the cell wall is relatively rigid so that when the cell is plasmolysed the membrane detaches itself from the cell wall and shrinks along with the cytoplasm, leaving the cell wall unaltered in size and shape. It has been shown in this laboratory, however, that a linear relationship exists between cell volume and external osmotic pressure up to 10 atmospheres, i.e. there is Hookean behaviour within this range; beyond this point the relationship is non-linear, the cell volume decrease for a given osmotic pressure being less than expected. Nevertheless, the yeast cell volume progressively decreases with increasing osmotic pressure up to at least 25 atmospheres. It is thus assumed that the cell wall is flexible, and that true plasmolysis does not occur, up to an external osmotic pressure of at least 10 atmospheres. Within this range the osmotic pressure inside the cell must always be greater than that outside, equilibrium being established when the external osmotic pressure plus the tension within the cell wall balances the internal osmotic pressure. For any yeast suspended in a given liquid the cell volume will thus be a function of the internal osmotic pressure, this being the integrated effect of all the soluble cell components within the plasma membrane. If the cell now becomes permeable to a certain range of molecular species these will no longer contribute to the internal osmotic pressure and the cell will immediately react and restore equilibrium by assuming a smaller volume with a consequent decrease in cell-wall tension; this change will take place before the molecular species concerned has diffused from the cell. The wider the range of molecular species to which the cytoplasmic membrane becomes permeable, the greater will be the diminution in cell volume. Cell volume may therefore be taken as a measure of the over-all permeability of the membrane.

It was shown by Eirich, Bunzl & Margaretha (1936) that the Einstein equation relating the viscosity of a suspension to the volume fraction of the particles was valid for yeast cells in dilute suspension, i.e. the viscosity is independent of the size distribution. For more concentrated cell suspensions the Einstein equation does not apply, but other workers have produced equations which are claimed to be valid e.g. Mooney (1951), Orr & Blocker (1955), Ting & Luebbers (1957). These equations all differ to a greater or lesser degree but they all verify the fact that the viscosity is a function of volume fraction only.

It was found in this laboratory that no electroviscous effect was apparent under the conditions used, possibly due to the high cell concentration or to the fact that when commercial baker's yeast is suspended in water a number of ionic species are found to be leached from the cells in small concentrations; the ionic strength is probably sufficient to neutralize the electroviscous effect. A calibration curve relating viscosity and cell volume fraction could then be prepared from which the latter could be directly determined. The average dry matter content (DM) of pressed yeast is 27 % so this volume was adopted as the standard to which estimations were adjusted, enabling valid comparisons to be made.

The cell volume fraction at 27 % DM (ϕ_{27}) was obtained from the following relationship

$$\phi_{27} = \frac{Vs\,\phi_s}{(Vs - E') + W(DM/27 - 1)},$$

where: Vs = vol. of yeast suspension on which measurements were taken; ϕ_s = cell volume fraction of suspension; E' = vol. of water added to prepare suspension; W = wt. of yeast sample used; DM = dry matter content of yeast sample.

Once the values of ϕ_{27} for two different yeast samples, or for the same sample before and after a given treatment, have been determined, the ratio between the values will correspond to the ratio between the mean cell sizes, assuming that the dry matter/cell remains constant.

This method for the determination of cell volumes was evaluated and chosen because of the need for greater precision than may be obtained with the centrifuged cell-volume technique; the accuracy of the viscosity measurement was such that changes in cell volume fraction of $\pm 0{\cdot}05\,\%$ could be detected. Moreover, an instantaneous reading is obtained and a continuous record of any progressive change may be made.

METHODS

Measurement of viscosity. Viscosity was measured with a 'Poisemeter' electric viscometer manufactured by Messrs Dobbie McInnes Ltd. (Glasgow). The instrument consists essentially of a constant speed electric motor driving a drag member within the fluid to be measured. Changes in viscous drag cause changes in the current flowing in the motor circuits and these changes are indicated on a scale calibrated in angular degrees. The indication obtained varies approximately as the logarithm of the dynamic viscosity of the fluid being measured. Good reproducibility can be obtained. Errors due to variations in mains voltage or frequency are compensated within the instrument.

Calibration of viscometer. In order to obtain a calibration curve relating scale reading or viscosity to cell-volume fraction it was necessary to determine the external water content of a sample of pressed yeast, since the distribution of water in this material may be such that about 25 % of the total is outside the cells (White, 1954). This determination was done by the inulin method (Conway & Downey, 1950*a*). It was known from previous experience that it was desirable to operate within the range of cell volume fractions of 0·50–0·57; above this range the suspension is too viscous to be readily handled, while with more dilute suspensions the sensitivity of viscosity to changes in cell volume diminishes rapidly. Yeast (450 g.) was made into suspension with sufficient water (210 ml.) to give a cell-volume fraction of approximately 0·57. Distilled water was added in 5 ml. amounts and the viscosity read after each addition. All measurements were carried out at 20°. The external water content of the yeast used was found to be 14·3 % (v/w), from this and the volume of the suspension the volume fraction could be calculated for each dilution by the relationship $\phi_s = 1-(E+E')/V_s$, where E = vol. of external water in 450 g. pressed yeast, E' = total vol. of water added at each dilution, and ϕ_s = volume fraction of the suspension.

Preparation of samples. For an accurate viscometer reading about 600 ml. of yeast suspension was required; this was provided by the suspension of 450 g. pressed yeast in 200 ml. water or 200 g. dried yeast in 500 ml. water. These usual quantities were, however, frequently varied for special trials. All samples were brought to 20° before measurement.

Drying technique. The experiments described in this paper were carried out with

a yeast strain of high activity which is not normally used for dried yeast production (standard DCL baker's yeast). This enabled a wide range of activities to be studied since this material was found to give very variable results on drying. Pressed yeast (1000 g.) was forced through a die by means of a hydraulic ram to yield a mass of long, vermicelli-like strands of extruded yeast (1/20 in. diameter), and was then transferred to a drying drum, 16 in. in length and 9 in. diameter, constructed from sheet steel. The drum was rotated at 24 rev./min. and dry air at 50° passed through at a flow rate of 5 foot^3/min. The interior of the drum was fitted with three 'flights', each 16 in. $\times$ 1$\frac{1}{2}$ in., to lift the yeast to near the top of the drum and then allow it to fall through the air stream. During drying the long strands of yeast were rapidly broken down to small pellets. Drying was continued until the moisture content had fallen to 8%, this taking usually about 16 hr.

Examination of the permeability of cells to specific substances. Pressed yeast (450 g.) was suspended in 180 ml. water and adjusted to pH 5·0. The temperature was brought to 20° and the viscosity adjusted to a particular reference point on the viscometer scale by the further addition of water; 600 ml. of this suspension were then measured into a clean dry vessel. The cell-volume fraction of this suspension was then 0·599, i.e. 335 ml. cells and 265 ml. water. Ten ml. of a solution containing such a weight of the substance under examination as to make 275 ml. of 0·25 osmolar solution were pipetted into the suspension and the reading observed after 2 min. and at such intervals thereafter as was considered necessary. The relative cell volume (RCV) i.e. cell volume after addition/cell volume before addition was then obtained from a previously prepared graph. When the substance under examination did not pass through the cell wall it would, of course, exert an osmotic effect and the observed viscosity would be much lower than that calculated for the addition of 10 ml. water alone; moreover, the reading would be constant. For a substance which passed through the cell wall and took a measurable time to do so, the viscosity would show the same initial decrease, but as the substance slowly diffused into the cell the cell volume and viscosity would increase until diffusion equilibrium was attained, when the internal and external concentrations were equal and the RCV had risen to 1·0. Finally, a substance which immediately entered the cell would exert no osmotic action and the relative cell volume would remain at 1·0, the viscosity of the solution decreasing only by the addition of the 10 ml. liquid. A similar procedure was used for dried yeasts.

Determination of yeast activity. For this test the standard fermentometer apparatus as described by Burrows & Harrison (1959) was used. Yeast (1·5 g.) was suspended in about 100 ml. water at 37·5° and allowed to stand for 15 min. at about 18°; 62·5 ml. of 10·8% (w/v) sodium chloride were then added to the suspension and the volume made up to 250 ml. Of this suspension 15 ml. were pipetted into a fermentometer bottle and left for 15–20 min. in the fermentometer bath; 20 g. flour was then added and mixed thoroughly. Thirteen minutes after the addition of flour the system was closed to the atmosphere and the volume of gas evolved after 3 hr. recorded. The observed gas volume was corrected to 20° and 760 mm. Hg. pressure and the yeast activity expressed as ml. gas evolved/g. dry matter.

Determination of yeast dry matter. An accurately weighed sample of about 1 g. was dried at 105° for 6 hr. in a laboratory oven and re-weighed after cooling in a desiccator. The dry matter was calculated from the loss in weight.

RESULTS

Comparison between pressed and dried yeasts

Cell volume. A microscopic examination of the cells of pressed yeast before drying and of the cells as reconstituted after drying revealed no morphological difference other than the fact that the cells after drying appeared smaller. This observation was confirmed by measurement of the cell-volume fraction. This was found to be 0·811 for a sample of pressed yeast; after drying to 8 % moisture and reconstitution, a ϕ_{27} value of 0·526 was obtained. Hence the mean cell volume after drying was only 65 % of that before drying.

Effect of sodium chloride. Two reasons for the decrease in cell volume on drying seemed possible: either an increase in the permeability of the cell wall or an alteration of the mechanical properties of the wall may have occurred. To distinguish between these possibilities it was necessary to examine directly the permeability of dried yeast. It is well known that the inner region of the yeast cell wall is impermeable to sodium chloride under normal circumstances although the compound will permeate throughout the outer region (cf. Conway & Downey, 1950*b*; Malm, 1947; Kotyk & Kleinzeller, 1958). This was verified by the addition of sodium chloride to a sample of standard yeast cream to make a 0·125 M-solution in the external water. The relative cell volumes obtained were 0·9543, 0·9540 and 0·9530 for three different yeast samples, the viscosity remaining constant for periods up to at least 24 hr. This experiment was repeated on two samples of dried yeast, reconstituted at 37·5°, from different laboratory dryings. With both samples it was found that the RCV remained at 1·0, apparently indicating that the sodium chloride passed immediately through the cell wall. It could still be argued, however, that the mechanical properties of the cell wall had been altered by drying to such an extent that it was capable of withstanding the osmotic forces. To test this possibility, the extent to which the sodium chloride had penetrated the cell was estimated directly by titration of the filtrate from a cell suspension containing a known weight of sodium chloride. From a knowledge of the cell-volume fraction it was readily calculated that the salt had penetrated through 60 % of the cell volume, assuming that only simple diffusion was involved.

Permeation by other substances. The effect of a number of other substances on reconstituted dried yeast was examined. These included: urea, thiourea, malonamide, acetamide, glucose, sucrose, hexamethylenetetramine, peptone. Each of these substances was found to pass directly through the cell wall, whereas in the case of pressed yeast they either did not penetrate the wall or passed through at a relatively slow and measurable rate. It was of interest to note that the cell wall of the strain of pressed yeast used was completely impermeable to hexamethylenetetramine, even after prolonged exposure; Ørskov (1945) found that this substance diffused slowly through the cell wall of a different strain. This indicates that different strains of yeast can show different permeability relationships.

Reconstitution of dried yeast at different temperatures

The activity of dried yeast is greatly influenced by the temperature of reconstitution (Peppler & Rudert, 1953), 37·5° being considered the optimum by many authorities, although this value is not critical within a few degrees. Two identical

samples of dried yeast were reconstituted, one at 37·5° and the other at 8°. The values of ϕ_{27} were found to be 0·582 and 0·513, respectively, as compared with 0·801 for the same yeast before drying. The permeability to urea of the original yeast and of the two reconstituted samples was also examined. It was found that diffusion equilibrium was reached in 9 hr. with the pressed yeast, 1·5 hr. with the sample reconstituted at 37·5°, while diffusion equilibrium was instantaneous with the final sample reconstituted at 8°.

Autofermentation

It was found that the reconstitution of dried yeast in the quantity and concentration required for viscosity determination gave rise to vigorous autofermentation which persisted for 1 to 2 hr. Moreover, although no measurements of gas evolution were taken, it was obvious that the more active the yeast, the more vigorous the fermentation. Viscometer measurements were normally postponed until gassing had ceased. The substrate for this autofermentation is not known, but no fermentable carbohydrate was detected in the filtrate from a freshly reconstituted dried yeast. It appears that the increased permeability of the cell wall creates a certain amount of disorder within the cell and in the cell-wall region, whereby enzymes are allowed to come into contact with substrates which would not normally be available in the intact healthy cell. The view held is that a 'perfect' dried yeast would show no autofermentation; slightly damaged yeasts, however, exhibit vigorous autofermentation, indicating that there is still a good capacity for fermentation although the disarrangement of the cell is sufficient to bring normally separated enzymes and substrates into close proximity. As the amount of damage becomes more extensive, the over-all fermentative capability decreases through the loss and dilution of essential co-enzymes etc., and this effect more than counterbalances the increased substrate availability. By the use of iodoacetic acid as an inhibitor of the autofermentation it was shown that a decrease in the permeability of the cell wall took place during this period. Dried yeast was reconstituted at 37·5° in water containing 0·2% (w/v) iodoacetic acid; the ϕ_{27} value was 0·571. A further sample of the same yeast was reconstituted in the normal manner and 0·2% (w/v) iodoacetic acid added to this suspension after autofermentation had ceased; the ϕ_{27} value was then 0·637. The two samples were examined for permeability to urea in the usual way. It was found that the cell permeability corresponded to the difference in cell volumes, diffusion equilibrium taking about 1 hr. for the sample to which iodoacetic acid was added immediately, and 4 to 5 hr. for the other.

Relationship between cell volume and activity

The determination of the cell volume and permeability of a number of dried yeast samples showed that they did not all behave in the manner previously described, i.e. were fully permeable to all substances for which measurement was possible. This was found to apply only to yeasts of very poor quality. Yeast samples of higher activity were examined as they became available and it was found that they exhibited a variety of permeability effects according to their activity. Very active samples were completely impermeable to sodium chloride but permitted the passage of urea fairly rapidly; other samples of lesser activity were almost instantaneously permeable to urea and allowed sodium chloride to diffuse at a measurable

rate. It had been hoped to find some substance which could be used in a standard permeability test and which would show a measurable but variable permeation rate with all yeast samples, i.e. never becoming zero or infinite with any sample. It became apparent, however, that such an ideal could not be attained and it was at this stage that the cell volume was adopted as a measure of the over-all permeability of the cell.

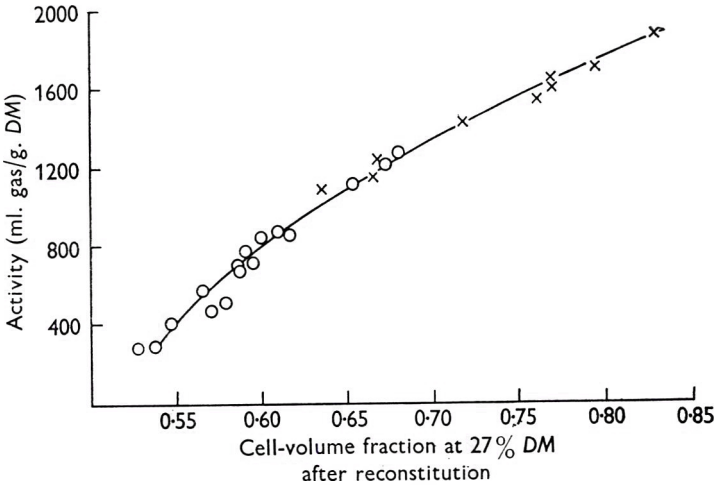

Fig. 1. Relationship between activity of pressed yeast and cell-volume fraction at 27 % dry matter (*DM*) after reconstitution. ○, Fully dried samples; ×, samples removed at intervals during drying.

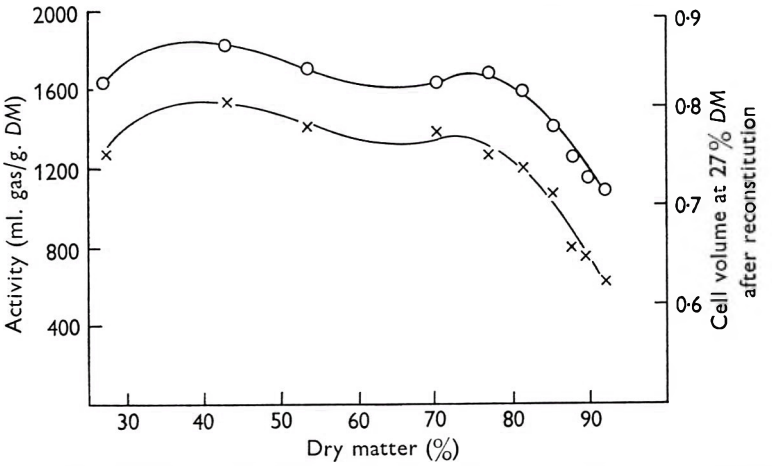

Fig. 2. Relationship between activity, cell-volume fraction and dry matter during the course of drying. ○, yeast activity; ×, cell-volume fraction at 27 % dry matter (*DM*) after reconstitution.

A number of laboratory dryings of different batches of yeast were carried out and the dried products examined for cell volume and activity. The results of this series are given in Fig. 1 (circled points) where it will be seen that there is a very close relationship between these variables. As an extension of this, the cell volume and activity were measured at intervals during the drying process in one case (Fig. 2).

The cell volume curve was found to be an exact duplicate of the activity curve. When these results were transferred to Fig. 1 (crosses) they formed a smooth extension to the curve already obtained.

To prove that the diminution in cell volume and the increased permeability of the cell were not merely incidental consequences of the loss in activity, a number of identical samples of cream prepared from pressed yeast were incubated for various times at 45° and the cell-volume fraction then measured after cooling to 20°. A portion of each suspension was filtered after incubation and the activity and dry matter of the pressed samples determined. It was shown thereby that the activity of yeast could be almost completely destroyed without the cell volume being significantly affected (Fig. 3).

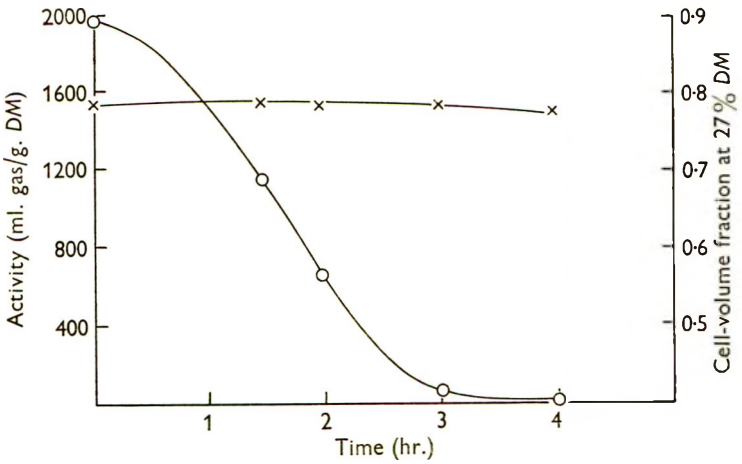

Fig. 3. Effect of incubation of yeast cream at 45°. ○, yeast activity; ×, cell volume fraction at 27 % dry matter (*DM*).

Throughout the investigations mentioned in this paper, the pH value of reconstituted dried yeast suspensions was routinely measured. There was a fairly good correlation between pH value and activity; suspensions of poor dried yeasts had a pH value of 5·9–6·2 while the better yeasts gave suspensions of lower pH value. This observation is a further reflection of the increased permeability of poor dried yeasts, since the internal pH value of the strain used is about 6·0–6·2.

DISCUSSION

The results presented here serve to illustrate the fact that the permeability of the cell wall is of primary importance in dried yeast research. From the evidence collected it does not appear that any other hypothesis need be invoked to account for the disparity between the activity before and after drying. The correlation between activity and cell volume is sufficiently good to indicate that the former is a function principally of the latter. It was originally thought that heat inactivation of enzymes might play a part, but it seems probable that such an effect is insignificant as compared with the activity loss brought about by increased permeability of cell wall. The view now held is that during drying, no matter how low the moisture content may be diminished, there is no damage to the fermentation system of the

yeast provided that the drying temperature is not excessively high. From approximately 80% dry matter onwards, however, there is a progressive disarrangement of the cytoplasmic membrane. The damage only becomes apparent when the yeast is resuspended in water and loss of essential components occurs.

The work of Herrera *et al.* (1956) is of particular interest in relation to the results presented here. In studying the differences in activity caused by reconstituting dried yeast at various temperatures it was found that 75% of the cell diphosphopyridine nucleotide (DPN) was extracted at 4–5°, as compared with 15% at 43°. These authors suggested that the difference might be accounted for in terms of the rate of rehydration of the cytoplasmic membrane, this process being slow at a low temperature and comparatively fast at the higher temperature of 43°. It is possible that the increase in cell volume reported here as occurring during the autofermentation period was the final stage of such a process. It is to be hoped that future work may yield more definite information about the composition and physical state of the cytoplasmic membrane; this knowledge would be of great value in solving problems associated with drying yeast and other micro-organisms.

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The Sodium and Potassium Content of Non-Halophilic Bacteria in Relation to Salt Tolerance

By J. H. B. CHRISTIAN and JUDITH A. WALTHO
Commonwealth Scientific and Industrial Research Organization, Division of Food Preservation, Homebush, New South Wales, Australia

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SUMMARY

The sodium and potassium contents of cells of 32 strains of non-halophilic bacteria were determined after growth in a standard medium of low salt content. The salt tolerance of each strain was assessed by determining the lowest water activity at which growth occurred in sodium chloride medium. Among the strains examined the potassium contents varied about fivefold and were positively correlated with salt tolerance.

INTRODUCTION

Bacteria differ greatly in ability to grow in high concentrations of sodium chloride. The growth of some strains is prevented by 2–3% salt, but some halophilic bacteria proliferate in media saturated with sodium chloride. These halophiles were shown by Baxter & Gibbons (1956) to contain enzymes which were active in solutions of very high ionic strength. No explanation has been advanced for the large differences in salt tolerance found among non-halophilic bacteria.

Cells of *Salmonella oranienburg* respiring in concentrated solutions accumulated much more potassium than in dilute media (Christian, 1955), suggesting that potassium largely controlled the water activity (a_w) within the cell. Further, the salmonellas are relatively intolerant of high salt concentrations or low a_w (Christian & Scott, 1953) and contain little potassium (Barber, 1931; Christian, 1958), while the staphylococci are very salt tolerant (Maitland & Martyn, 1948; Scott, 1953) and are very rich in potassium (Barber, 1931). The present paper presents results which support the hypothesis that salt tolerance in non-halophiles is related to ability to accumulate potassium within the cells.

METHODS

Organisms. Thirty-two strains of bacteria were studied, comprising: twelve Gram-positive cocci (*Leuconostoc citrovorum*; *Micrococcus lysodeikticus*, *M. roseus*; two *Sarcina* spp.; *Staphylococcus aureus*, *Staph. albus*, *Staph. citreus*; *Streptococcus cremoris*, *Strep. faecalis*, *Strep. lactis* and an unidentified coccus); fifteen Gram-positive spore-forming rods (two *Bacillus cereus*, *B. megaterium*, *B. mycoides*, *B. subtilis* var. *niger*, nine unidentified strains); five Gram-negative strains (*Escherichia coli*, *Flavobacterium* sp., *Pseudomonas fluorescens*, *Salmonella oranienburg*, *Vibrio metchnikovi*).

Salt tolerance. Salt tolerance was determined at pH 7·0 in brain + heart infusion

broth, sodium chloride being added to provide the desired values of a_w in steps of 0·01. Ten ml. volumes of medium were dispensed in 100 ml. screw-cap bottles which, after inoculation with 0·02 ml. of a 16 hr. broth culture, were incubated at 30° for 28 days. The lowest a_w value which supported growth was determined by observation of turbidity.

Ion content. For analysis, organisms were grown in 100 ml. brain + heart broth (0·993 a_w) in 250 ml. Erlenmeyer flasks shaken at 30°. On reaching the stationary phase of growth, each culture was centrifuged to give two equal cell pellets. One pellet was weighed before and after drying at 105° (giving dry weight and water content) and the other pellet was extracted with cold trichloroacetic acid. Extracts were analysed for sodium and potassium by flame photometry.

Interstitial space was determined by phosphate dilution (Mitchell & Moyle, 1956) in centrifugates of four Gram-positive cocci, one Gram-positive rod and one Gram-negative rod. Large differences were found, probably because of differences in packing fractions as between strains. However, when internal water was calculated from these values, the mean and standard error for the six strains was 1·50 ± 0·03 ml./g. dry weight. This figure was applied to all strains, and, the dry weight and total water content of the centrifugate and the water content of the medium for each preparation being known, the interstitial volume could be calculated. The supernatant fluid, and hence the interstitial medium, contained 163 m-equiv. sodium and 25 m-equiv. potassium/l. The amounts of these ions in the interstitial volume were calculated and subtracted from the values for whole pellets. These net values for sodium and potassium contents were expressed as μ-equiv./100 mg. dry wt. cells. All values presented are means from at least two experiments.

RESULTS

The relationship between the potassium content of cells grown in standard medium and the lowest a_w value which supported growth of the same strains in media adjusted with sodium chloride is shown in Fig. 1. For the thirty-two strains the correlation coefficient was −0·89 ($P < 0·001$). An unidentified coccus, growing to 0·84a_w, was much more salt-tolerant than the staphylococci (lower limits of 0·89 to 0·88a_w) yet contained no more potassium. If this organism was excluded the correlation coefficient was −0·91 ($P < 0·001$). Potassium content was directly related to salt tolerance, increasing by about fivefold as the minimum a_w supporting growth decreased from 0·975 to 0·84.

An inverse relationship often exists between cellular contents of sodium and potassium. The increase in salt tolerance which accompanied enhanced potassium content might thus be associated with a decrease in cell sodium. This was generally the case for Gram-positive cocci and Gram-negative rods (Fig. 2*a*). However, at a given sodium content, the rods were less salt tolerant than the cocci. The most salt-tolerant coccus again behaved exceptionally. On the other hand, in Gram-positive sporing rods, sodium content increased appreciably with decrease in limiting a_w value (Fig. 2*b*). The pronounced scatter of values in Fig. 2*b* may result from uptake of sodium during harvesting and preparation for analysis. When only the lowest sodium contents recorded for each strain are plotted, the increase in sodium with decreasing a_w value is more marked. Figure 2 shows also that some strains had internal sodium concentrations lower than in the growth medium. These strains

were amongst the most salt-tolerant cocci and Gram-negative rods, and among the least tolerant of the *Bacillus* spp.

The differences between the two groups of bacteria are further demonstrated in Fig. 3, where potassium to sodium ratios are plotted as a function of the minimum a_w value supporting growth. The steep rise in this ratio as salt tolerance increased in cocci and Gram-negative rods contrasts with its virtual constancy for *Bacillus* spp.

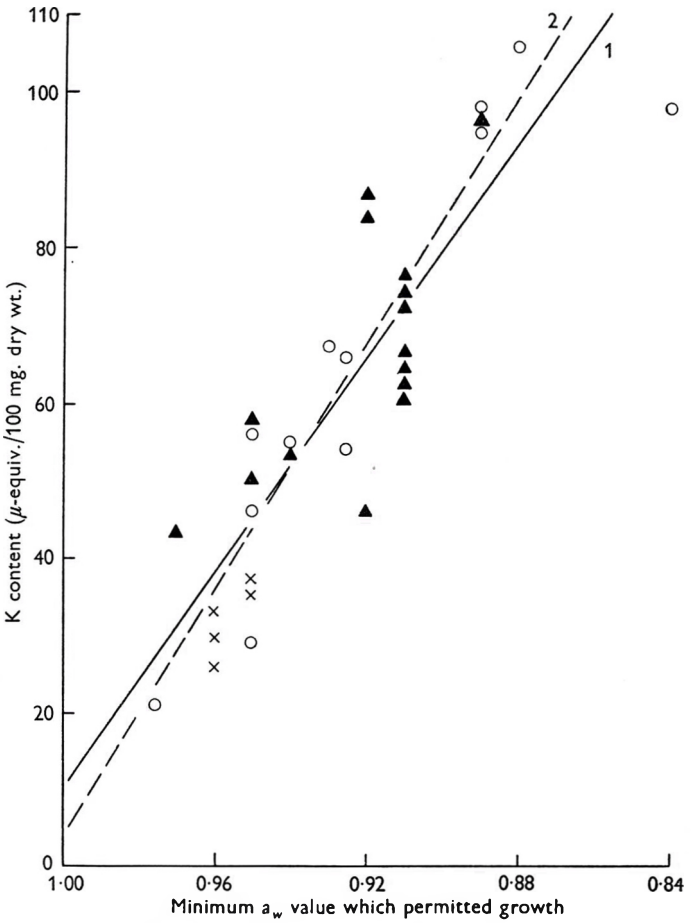

Fig. 1. Potassium content of bacteria in relation to the minimum a_w value which permitted growth. Analyses were performed on organisms grown in basal medium (0·993a_w) under standard conditions, and the minimum a_w values for growth were determined in basal medium adjusted to the desired a_w value by addition of sodium chloride. ○, Gram-positive cocci; ▲, *Bacillus* spp.; ×, Gram-negative rods. (1) Regression line for all organisms; (2) regression line excluding data for the most salt-tolerant coccus.

Table 1 shows the concentration gradients of sodium and potassium across the cell boundaries of two cocci which differed greatly in salt tolerance. Internal concentrations were calculated on the basis of 1·50 ml. cell water/g. dry wt. It is clear that between strains the internal concentrations of each ion may vary greatly with respect to the external concentration of the same ion and with respect to the internal concentration of the other.

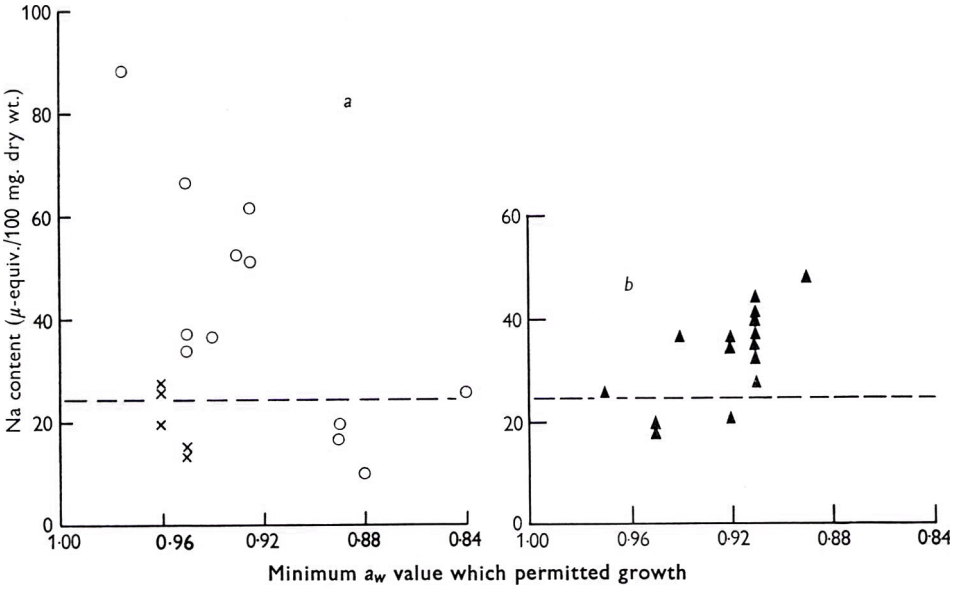

Fig. 2. Sodium content of bacteria in relation to the minimum a_w value which permitted growth. Conditions as for Fig. 1. The broken line indicates the value at which concentrations of sodium in cells and basal medium were equal. (*a*) ○, Gram-positive cocci; ×, Gram-negative rods. (*b*) ▲, *Bacillus* spp.

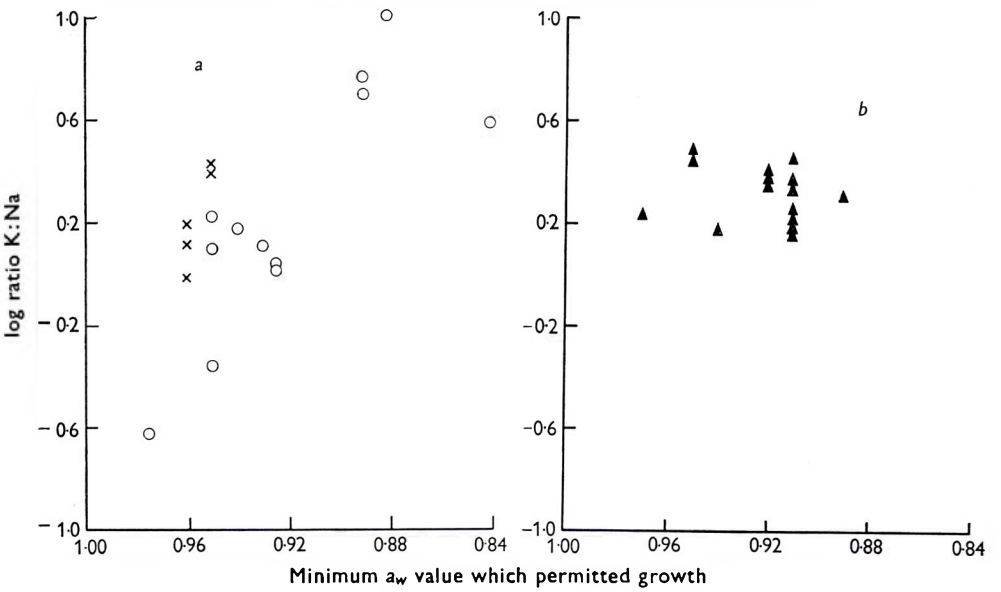

Fig. 3. Logarithms of potassium:sodium ratios in bacteria in relation to the minimum a_w values which permitted growth. Conditions as for Fig. 1. (*a*) ○, Gram-positive cocci; ×, Gram-negative rods. (*b*) ▲, *Bacillus* spp.

Table 1. *Apparent concentrations of sodium and potassium in cocci which differed greatly in salt tolerance*

Organism	Minimum a_w for growth	Sodium concentration (mM)		Ratio cell Na	Potassium concentration (mM)		Ratio cell K	Ratio cell K
		cells*	medium	medium Na	cells*	medium	medium K	cell Na
Leuconostoc citrovorum	0·975	586	163	3·6	140	25	5·6	0·34
Staphylococcus aureus	0·88	64	163	0·39	708	25	28·3	11·1

* Concentrations calculated on the basis of 1·5 ml. internal water/g. dry wt. cells.

DISCUSSION

The correlation observed between potassium content and salt tolerance holds for such a wide range of bacterial strains that it seems likely to be general among non-halophilic species. However, it must be stressed that this study has been confined to strains growing under one set of environmental conditions. The relationship may not hold for yeasts, since Takada (1956) found that after growth in low-salt medium a yeast adapted to tolerate high salt concentrations contained no more potassium (or sodium) than the parent strain.

The unidentified coccus grew at a sodium chloride concentration above 4M. Such salt tolerance is unusual in a non-halophilic bacterium, and exceeds that of the halophilic *Vibrio costicolus* (Smith, 1937). The water relations of this coccus deserve further study.

Two assumptions were made in calculating net sodium and potassium contents. The barriers to the movement of sodium, potassium and phosphate were assumed to be identical, and a value of 1·5 ml. internal water/g. dry wt. was applied in determining interstitial space for all preparations. Insufficient amounts of potassium were present in the interstitial space for the results to be affected appreciably by errors in these assumptions, but estimates of net sodium content might be subject to considerable variation. However, this would not alter the distribution of strains into those containing higher or lower concentrations of sodium than existed in the growth medium, nor would it affect the conclusion that the lowest sodium contents were found in the most salt-tolerant cocci and the least salt-tolerant *Bacillus* spp. The different relationships between sodium content and salt tolerance in cocci and Gram-positive rods suggest that sodium content is not important for salt tolerance in non-halophilic bacteria. This may be related to the finding that in *Salmonella oranienburg* maintenance of the internal value for sodium was not dependent on the a_w value of the environment, while retention of potassium and of substances which absorbed in the ultraviolet region demanded a relatively low a_w value (Christian, 1958), as did retention of amino acids by *Escherichia coli* (Britten, 1956). The basis of the relationship between potassium content and salt tolerance is obscure. If a high potassium content implies a high concentration of internal solutes, salt-tolerant bacteria will possess low internal a_w. This property would impart resistance to dehydration and plasmolysis, thus overcoming one of the deleterious effects of a concentrated salt environment.

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In vivo Studies of Methanogenesis in the Bovine Rumen: Dissimilation of Acetate

By R. A. OPPERMANN,* W. O. NELSON and R. E. BROWN
Department of Dairy Science, University of Illinois, Urbana, Illinois, U.S.A.

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SUMMARY

The introduction of sodium acetate-2-^{14}C into a bovine rumen resulted in the *in vivo* labelling of the rumen gases and volatile fatty acids. The relative isotope concentration in substrate and products indicated that a maximum of 5·6% of the methane and 11% of the carbon dioxide in rumen gas might have been derived from the methyl carbon of acetate when the substrate was added to the rumen 18 hr. after the animal had been fed a normal ration. A maximum of 3·2% of the methane and 4·2% of the carbon dioxide might have been derived from the methyl carbon of sodium acetate-2-^{14}C when this substrate was introduced into the rumen immediately after the animal had been fed. The addition of sodium acetate-1-^{14}C to the rumen 18 hr. after feeding indicated that 2% of the methane and 10% of the carbon dioxide was derived from the carboxyl carbon of the substrate. Most of the derived radioactivity of the volatile acids of the rumen was found in the butyric acid fraction, although smaller amounts appeared in propionic acid and the volatile fatty acids with a chain length of greater than 4 carbons.

INTRODUCTION

The biochemical reactions mediated by the known species of methane bacteria (Barker, 1956) and the abundance of various methanogenic substrates in rumen fluid suggest that several pathways may be involved in rumen methane formation, as illustrated in Fig. 1. Beijer (1952) detected a *Methanosarcina* sp. in the goat rumen, and Oppermann, Nelson & Brown (1957) demonstrated *Methanobacterium formicicum* and a methanogenic acetate-utilizer biochemically resembling *Methanobacterium söhngenii* in enrichment cultures from cattle rumen contents. Nelson, Oppermann & Brown (1958) reported that rumen enrichment cultures stabilized to valeric or butyric acid contained methane bacteria which biochemically resembled *Methanobacterium suboxydans*. Smith & Hungate (1958) isolated and characterized a new species *Methanobacterium ruminantium* which is present in the bovine rumen in great abundance. Thus, with the exception of the oxidation of propionic acid, there is evidence that the rumen contains bacteria capable of generating methane by the pathways shown in Fig. 1. The numerous unsuccessful attempts to obtain the utilization of propionic acid by rumen enrichment cultures (Oppermann *et al.* 1957; Nelson *et al.* 1958) are consistent with the observations of McNeill & Brown (1954), and strongly suggest that propionate is not a substrate for rumen methanogenesis.

* Present address: Nalco Chemical Co., Chicago, Illinois, U.S.A.

The abundance of evidence relative to the *in vitro* reduction of carbon dioxide to methane by rumen organisms (Carroll & Hungate, 1955; Smith & Hungate, 1958) and the efficiency of the *in vivo* reduction of carbon dioxide to methane by the rumen microflora (Kleiber, 1953) support the hypothesis that hydrogen and carbon dioxide are the chief substrates which give rise to methane in the rumen. However, the existence of acetate-utilizing methane bacteria in the rumen (Beijer, 1952; Oppermann *et al.* 1957; Nelson *et al.* 1958; Yahiro, 1959) suggests that some methane may arise from this source as it does in the anaerobic fermentation of certain industrial wastes (Buswell, 1936). The present investigation was conducted to measure the extent of the *in vivo* conversion of acetate to methane in the bovine rumen.

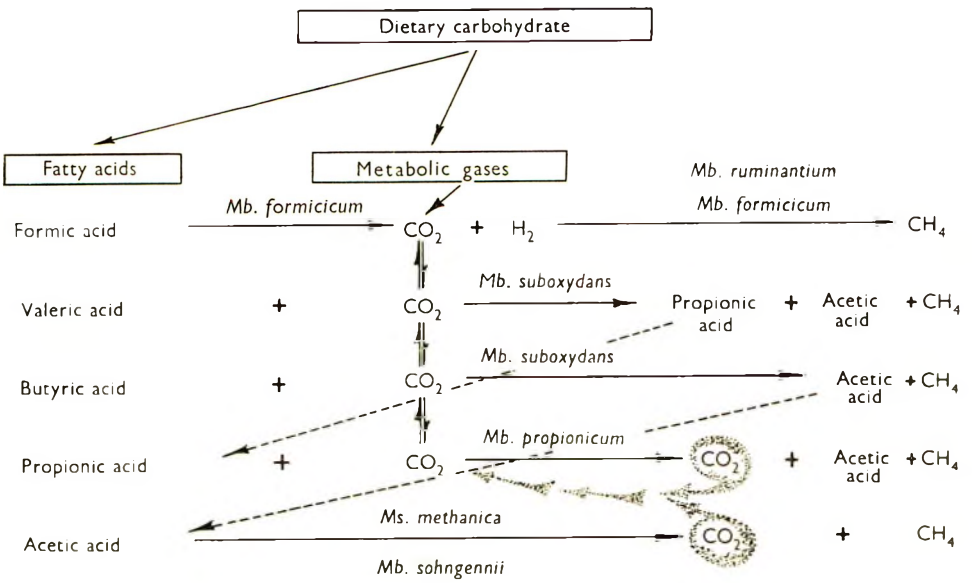

Fig. 1. Theoretical pathways of rumen methanogenesis.

METHODS

In vivo *methods*. The dissimilation of acetic acid with concomitant methanogenesis was studied in a rumen-fistulated dairy cow maintained on a daily intake of 24 lb. of alfalfa hay and 5 lb. of a 14% protein concentrate mix. The apparatus used for the collection of rumen fluid and gas samples is schematically represented in Fig. 2. Samples of the rumen gas were forced into the Douglas bag by normal rumen contractions. Liquid samples were obtained by use of a sidearm test tube and an aspirator bulb.

The radioactive substrate was added to the rumen 18 hr. after the animal had been fed the standard roughage + concentrate ration, except in one trial as will be noted. This period in the rumen digestion cycle was postulated to be the most favourable for acetate utilization because: (*a*) the approximate 1:1 ratio of carbon dioxide to methane found at this period (Nelson, Brown & Kingwill, 1960) approaches the actual (Oppermann *et al.* 1957) and theoretical (Buswell, 1936) composition of gas obtained from the methanogenic dissimilation of acetate; (*b*) the

acetate concentration in rumen fluid is relatively constant at this period (Brown, 1954); (*c*) formate and carbon dioxide, which may be preferentially utilized and/or be inhibitory to acetate utilization (Oppermann *et al.* 1957), would be at negligible (formate) and minimum (carbon dioxide) concentrations.

Sodium acetate-^{14}C, labelled in either the 1 or 2 position, was dissolved in 500 ml. distilled water and added to the rumen through the fluid sampling line. The line then was flushed with from 1 to 1·5 l. distilled water. An evacuated Douglas bag was connected to the gas-sampling line and the gas collected. At the end of each time period, the bag containing the sample was removed and an evacuated bag attached.

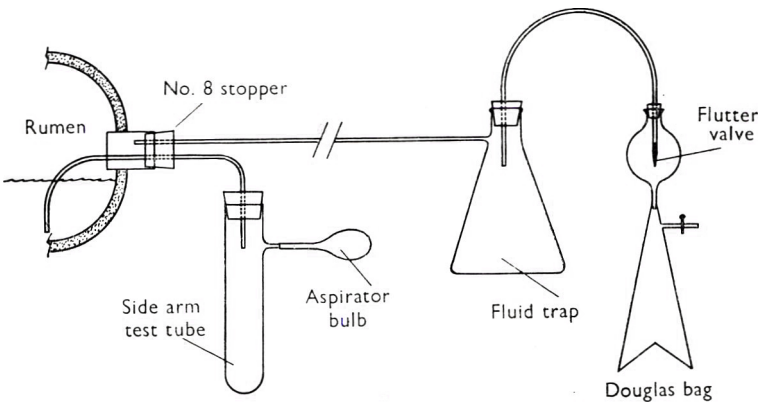

Fig. 2. Schematic representation of apparatus for collection of samples of rumen gas and fluid.

The sodium acetate-2-^{14}C was purchased from commercial sources and the sodium acetate-1-^{14}C was synthesized via carbonation of methyl magnesium iodide in the Radiocarbon Laboratory of the University of Illinois.

Chemical methods. A 50 ml. sample of the rumen gas was analysed by absorption-combustion procedures in a Burrell (Model 35-802) apparatus before fractionation of the remainder of the sample in a gas absorption chain. Carbon dioxide was trapped in a 0·25 N solution of Primene 81-R (a mixture of tertiary alkyl primary long chain C_{12}–C_{14} amines; Rohm & Hass Co., Philadelphia, Pa.) in methanol (Oppermann, Nystrom, Nelson & Brown, 1959) contained in a large test tube fitted with a sintered glass gas-diffusion tube. Oxygen was absorbed in the second vessel which contained alkaline pyrogallol prepared by dissolving 15 g. pyrogallic acid and 113 g. potassium hydroxide in 100 ml. water. The effluent gas from the absorption train, containing primarily methane and some nitrogen, was subjected to catalytic combustion in the Burrell apparatus.

The carbon dioxide derived from catalytic combustion of methane was absorbed in 0·25 M-Primene contained in a special vessel attached to the Burrell apparatus. The gas was passed through the vessel repeatedly until complete absorption had taken place. The volume of carbon dioxide absorbed was recorded and the % methane present in the original gas sample calculated.

Volatile fatty acids (VFA) present in the rumen were determined by a modification of the chromatographic method of Neish (1949) as described in a previous

report by Oppermann *et al.* (1957). The rumen fluid samples were preserved for analysis by acidification with 10N-sulphuric acid and were immediately frozen for storage until analysis could be made.

Carbon-^{14}C determinations. When rumen fluid samples were chromatographed, three 1 ml. samples were taken, before titration, from three successive 5 ml. samples collected from the top of each volatile fatty acid peak and placed in 13 ml. of scintillation fluid. The remainder of the eluate was titrated and the amount of fatty acid present calculated on the basis of the original volume. The scintillation fluid was prepared by dissolving 3 g. 2:5-diphenyloxazole (PPO) in a litre of high purity toluene. The fatty acid solutions were counted at 810 V. (tap 3) using discriminator settings of 10–50 V. and 50–100 V. on a Tri-Carb liquid scintillation spectrometer (Model 314X, Packard Instrument Co., LaGrange, Ill.).

The absorbent for all carbon dioxide-^{14}C samples was either 0·25M or M-Primene 81-R. Usually 1 ml. of absorbent solution containing the trapped carbon dioxide-^{14}C was added to 13 ml. of scintillation fluid and counted at 890 V. (tap 4) using discriminator settings for 10–50 V. and 50–100 V. in the scintillation counter. Carbon dioxide-^{14}C was determined at a higher tap number than the fatty acids because of the quenching caused by methanol in the Primene solutions (Oppermann *et al.* 1959).

Table 1. *Distribution of ^{14}C from sodium acetate-2-^{14}C added* to the rumen 18 hr. after feeding. Trial* 1

Time after addition of ^{14}C (hr.)	Volatile fatty acids: Acetate (SA)†	Acetate (m-mole/100 ml.)	Propionate (SA)	Propionate (m-mole/100 ml.)	Butyrate (SA)	Butyrate (m-mole/100 ml.)	Gases: CO_2 (SA)	CO_2 (%)‡	CH_4 (SA)	CH_4 (%)
¾	629§	3·65	0	0·71	56	0·81	—	—	—	—
1	479	3·70	0	0·68	72	0·77	8·7	1·82	1·1	0·24
2	370	3·49	31	0·60	115	0·74	8·9	2·28	3·5	0·91
3	398	3·09	37	0·49	163	0·72	19·5	4·91	5·5	1·38
4	448	3·49	55	0·60	150	0·74	19·2	4·31	7·5	1·68
5	332	3·05	56	0·58	146	0·76	21·6	6·50	12·6	3·80
6	296	2·49	57	0·49	125	0·48	8·8	2·98	4·1	1·39

* 1 mc. of sodium acetate-2-^{14}C (SA 0·74 mc./m-mole).

† Specific activity $= \frac{\text{Dis./min.}}{\text{m-mole}} \div 1000.$

‡ Percentage derived from substrate $= \frac{\text{SA } CO_2 \text{ or } CH_4}{\text{SA acetate}} \times 100.$

§ Each value mean of three or more observations.

RESULTS

In the first trial 1 millicurie (mc.) of sodium acetate-2-^{14}C having a specific activity (SA) of 0·74 mc./mmole was introduced into the rumen 18 hr. after feeding, and samples of rumen fluid and gas were collected hourly over a 6 hr. period. As shown in Table 1, the methane and carbon dioxide, as well as propionic and butyric acid became labelled soon after the addition of the acetate-2-^{14}C. The specific activity and the total concentration of the acetate decreased at a moderate rate throughout

the trial. The amount of ^{14}C in propionic acid increased slightly while the total quantity of this acid decreased slowly. Carbon-^{14}C concentration in butyric acid increased during the first 3 hr. and decreased gradually thereafter. The total amount of butyric acid in the rumen fluid remained relatively constant during the first 5 hr. before dropping sharply during the sixth hour. The specific activity of the carbon dioxide and methane in the rumen gas increased during most of the experimental period. As the specific activity of each gas gradually increased the % of the gas derived from acetate also increased.

Although the values of radioactivity encountered in the volatile fatty acids, carbon dioxide, and methane were somewhat lower in the second trial, the data followed the same general pattern. However, as shown in Table 2, radioactivity was not detected in the propionic acid fraction and ^{14}C was detected in butyric acid only in the early median portion of the trial.

Although these experiments show that some of the label from acetate was recovered *in vivo* in rumen methane, the simultaneous labelling of propionic and butyric acid and carbon dioxide complicated the elucidation of the role of acetate in methane production because the methane-^{14}C could have been derived by reduction of $^{14}CO_2$, or by direct cleavage of the acetate-2-^{14}C, or both.

The calculation of the relative isotope concentration (Block, Clark & Harary, 1954) or % conversion, i.e.:

$$\frac{\text{specific activity methane-}^{14}\text{C at } T \text{ time}}{\text{specific activity acetate-2-}^{14}\text{C at } T \text{ time}} \times 100 = \% \text{ conversion},$$

shows that maximum values of only 3·8 and 5·6 % of the methane in trials 1 and 2, respectively, might have been derived from the methyl group of acetate even if it be assumed that labelled butyric acid and/or carbon dioxide-^{14}C did not participate in the methanogenesis, and that all of the methane-^{14}C recovered arose from the

Table 2. *Distribution of ^{14}C from sodium acetate-2-^{14}C added* to the rumen 18 hr. after feeding. Trial 2*

Time after addition of ^{14}C (hr.)	Volatile fatty acids: Acetate (SA)†	Acetate (m-mole/100 ml.)	Propionate (SA)	Propionate (m-mole/100 ml.)	Butyrate (SA)	Butyrate (m-mole/100 ml.)	Gases: CO_2 (SA)	CO_2 (%)‡	CH_4 (SA)	CH_4 (%)
½	439§	3·43	0	0·84	0	0·50	1·6	0·36	1·1	0·25
1	439	3·41	0	0·91	99	0·37	2·2	0·52	0·3	0·06
2	403	2·53	0	1·07	132	0·41	4·2	1·04	1·5	0·37
3	330	3·28	0	0·85	179	0·34	6·8	2·06	3·9	1·19
4	245	3·19	0	0·85	139	0·40	3·8	1·54	9·0	3·66
5	186	2·86	0	0·69	0	0·44	12·2	6·55	1·1	0·60
6	176	2·09	0	0·84	0	0·27	10·5	5·94	1·5	0·88
7	152	3·11	0	0·78	0	0·38	16·9	11·11	8·5	5·57

* 1 mc. of sodium acetate-2-^{14}C (SA 0·74 mc./m-mole).

† Specific activity $= \frac{\text{Dis./min.}}{\text{m-mole}} \div 1000.$

‡ Percentage derived from substrate $= \frac{\text{SA } CO_2 \text{ or } CH_4}{\text{SA acetate}} \times 100.$

§ Each value mean of three or more observations.

direct cleavage of acetate, the intact methyl group of this substrate being incorporated into methane in accordance with the findings of Buswell & Sollo (1948), Stadtman & Barker (1949) and Pine & Barker (1956).

Calculation of the relative isotope concentration in acetate as precursor and carbon dioxide as product indicates that acetate participated in reactions which led more readily to the formation of carbon dioxide than to the formation of methane. In trial 1 a maximum of 6·5% and in trial 2 a maximum of 11% of the carbon dioxide was derived from the labelled carbon which originally resided in acetate-2-^{14}C. (Comparable values for methane were 3·8 and 5·6% for trials 1 and 2, respectively.)

Whilst the specific activities of carbon dioxide and methane in trial 2 were rather erratic, in general, the relationship of the specific activity curves of carbon dioxide-^{14}C and methane-^{14}C in trials 1 and 2 is that of two products derived from a single precursor. Should there be a decrease in carbon dioxide-^{14}C in such a manner that its curve crosses that of methane, or an increase in the specific activity of methane so that its curve crosses or touches that of carbon dioxide, then the relationship between the two would be that of precursor (carbon dioxide) to product (methane) (Aranoff, 1956). The point where the specific activity curves cross would be the maximum specific activity of the product. The experimental time period was not extended long enough to determine this final relationship. However, considering the trends of the data, the curves which start as two products probably would end in a new precursor-product relationship.

Table 3 summarizes an experiment in which 1 mc. of acetate-2-^{14}C (SA 7·5 mc./m-mole) was added to the rumen 30 min. after feeding to determine the effect of maximum rate of rumen fermentation on the incorporation of methyl carbon from acetate into other products of rumen digestion. These data show that the specific activity of acetic acid decreased rapidly during this period as a result of the increased production of acetate in the rumen fermentation. In contrast to the previous trials, the labelling of propionic and butyric acid was more pronounced and the higher volatile fatty acids (greater than C_4) were labelled throughout the experimental period. The specific activity of butyric acid decreased during the first 2 hr., increased during the next 2 hr., and then decreased during the remainder of the trial. The concentration of propionic acid-^{14}C increased throughout most of the experiment and began to decrease only at the end of the sampling time. The methyl carbon of acetate could account for a maximum of 3·2% of the methane and a maximum of 4·2% of the carbon dioxide. While the % of methane derived from the methyl group of acetate was in the general range of values obtained in the previous experiments, the % of carbon dioxide-^{14}C was much smaller because of the increased production of carbon dioxide from other sources during this period of maximum activity of the rumen microbiota.

The results from the preceding experiments relative to the contribution of methyl-labelled acetate to rumen methanogenesis and carbon dioxide production suggested an experiment to determine the contribution of the carboxyl group of acetate to the formation of these two gaseous products of rumen digestion. The experimental conditions used were as before except that 2 mc. of acetate-1-^{14}C (SA 2·25 mc./m-mole) were added to the rumen 18 hr. after feeding, as in the first trial.

Table 3. *Distribution of ^{14}C after addition* of sodium acetate-2-^{14}C to the rumen $\frac{1}{2}$ hr. after feeding*

Time after addition of ^{14}C (hr.)	Volatile fatty acids: Acetate (SA)†	Acetate (m-mole/100 ml.)	Propionate (SA)	Propionate (m-mole/100 ml.)	Butyrate (SA)	Butyrate (m-mole/100 ml.)	V.F.A. > C_4 (SA)	V.F.A. > C_4 (m-mole/100 ml.)	Gases: CO_2 (SA)	CO_2 (%)‡	CH_4 (SA)	CH_4 (%)
$\frac{1}{2}$	5620§	6·68	0	1·58	736	1·36	247	0·20	3·2	0·06	2·5	0·05
1	1060	6·30	35	1·81	580	1·52	237	0·26	2·1	0·29	3·4	0·33
2	643	6·75	38	1·72	504	1·27	372	0·26	3·6	0·56	8·6	1·34
3	543	7·87	58	1·67	649	1·42	362	0·35	7·2	1·33	6·3	1·17
4	481	5·98	69	2·08	716	1·51	438	0·30	8·7	1·81	5·4	1·13
5	343	7·73	67	2·14	597	1·47	374	0·29	9·4	2·75	5·6	1·65
6	271	7·88	78	1·83	537	1·44	269	0·26	9·4	3·50	6·9	2·54
7	215	8·15	65	2·02	434	1·44	335	0·28	8·9	4·16	7·0	3·24

* 1 mc. of sodium acetate-2-^{14}C (SA 7·5 mc./m-mole).

† Specific activity = $\frac{\text{Dis./min.}}{\text{m-mole}} \div 1000$.

‡ Derived from acetate = $\frac{\text{SA } CO_2 \text{ or } CH_4}{\text{SA acetate}} \times 100$.

§ Each value mean of three or more observations.

Table 4. *Distribution of ^{14}C from sodium acetate-1-^{14}C added* to the rumen 18 hr. after feeding*

Time after addition of ^{14}C (hr.)	Volatile fatty acids: Acetate (SA)†	Acetate (m-mole/100 ml.)	Propionate (SA)	Propionate (m-mole/100 ml.)	Butyrate (SA)	Butyrate (m-mole/100 ml.)	V.F.A. > C_4 (SA)	V.F.A. > C_4 (m-mole/100 ml.)	Gases: CO_2 (SA)	CO_2 (%)‡	CH_4 (SA)	CH_4 (%)
$\frac{1}{2}$	8757§	2·74	0	0·58	59	0·54	0	0·19	1	0·01	5·6	0·06
1	1866	1·60	0	0·60	212	0·66	0	0·05	17	0·92	0·4	0·02
2	1168	3·19	67	0·69	281	0·58	129	0·23	26	2·20	1·2	0·11
3	1035	2·69	79	0·48	277	0·54	0	0·21	39	3·76	1·9	0·18
4	941	3·35	85	0·69	335	0·58	291	0·25	51	5·36	8·8	0·94
5	795	2·86	96	0·67	464	0·49	211	0·22	70	8·85	9·3	1·18
6	640	2·72	108	0·57	392	0·44	0	0·24	64	10·09	9·1	1·43
8	595	2·63	116	0·51	247	0·40	0	0·29	56	9·49	11·7	1·97

* 2 mc. of sodium acetate-1-^{14}C (SA 2·25 mc./m-mole).

† Specific activity = $\frac{\text{Dis./min.}}{\text{m-mole}} \div 1000$.

‡ % = $\frac{\text{SA } CO_2 \text{ or } CH_4}{\text{SA acetate}} \times 100$.

§ Each value mean of three or more observations.

The results (summarized in Table 4) show that the carbon dioxide was much richer in ^{14}C than the methane. The data also show other differences from the preceding experiments, in that the labelling of butyric acid continued for 5 hr. instead of 3 hr., and the specific activities were higher, even when corrected for the addition of twice as much isotope. The fatty acids with chain lengths greater than C_4 also became labelled in some instances. The propionic acid labelling pattern was similar to that of the first experiment. The differences in the labelling of the various volatile fatty acids suggests that the method of incorporation of carboxyl carbon differs from that of the methyl carbon of acetate. It was calculated that the carboxyl group of acetate-^{14}C gave rise to a maximum of 2% of the methane and 10% of the carbon dioxide encountered during the experimental time period.

DISCUSSION

Although bacteria which convert acetate to methane and carbon dioxide are readily established in enrichment cultures of bovine rumen contents, the data presented in this report demonstrate that only a small portion of rumen methane was derived from acetate and suggest that these organisms were present in small numbers. This observation is in accord with the report (Nelson, Brown & Kingwill, 1960) that the addition of *d*-limonene to the rumen *in vivo* in concentrations which were toxic to acetate-utilizing methane bacteria *in vitro* (Crane, Nelson & Brown, 1957) did not appreciably alter the ratio CO_2:CH_4 in rumen gas. Beijer (1952) previously had observed that methane was not formed from acetate by rumen contents *in vitro* during short term incubation.

The active participation of acetate-1-^{14}C and acetate-2-^{14}C in reactions leading to the labelling of other higher volatile fatty acids in the rumen pool in these experiments is in agreement with the observations of Gray, Pilgrim, Rodda & Weller (1952) who reported that most of the derived radioactivity was found in the butyrate fraction of rumen fluid after the addition of acetate-1-^{14}C to the rumen of a sheep. The data presented here extend these observations to include the participation of acetate not only in volatile fatty acid synthesis but also in methanogenesis and in the formation of carbon dioxide in the rumen.

The data demonstrating the participation of acetate-2-^{14}C in reactions leading to the *in vivo* labelling of other volatile fatty acids and carbon dioxide in the rumen are in direct contrast to the *in vitro* fermentation of this substrate. When the acetate-2-^{14}C used in the *in vivo* trials was fermented *in vitro* by acetate-stabilized rumen enrichment cultures, essentially all of the radioactivity of the gas resided in the methane and only trace quantities of ^{14}C were found in the carbon dioxide.

It is evident that the participation of acetate in various metabolic reactions in the rumen forestalls any precise measurement of the direct incorporation of the intact methyl group of acetate-2-^{14}C into methane. However, it is possible to ascribe maximum values for this reaction in terms of the experimental values. Calculation of the relative isotope concentration indicates that a maximum of 3·8 and 5·6% of the methane might have arisen from the intact methyl group. Since the carboxyl carbon of acetate-1-^{14}C gave rise to 2% of the methane and 10% of the carbon dioxide, it can be postulated that the acetate molecule contributed from 5·8 to 7·6% of the rumen methane and from 16 to 21% of the carbon dioxide generated

in the rumen during the fasting experimental period. The contribution of acetate-2-^{14}C to carbon dioxide and methane formation during the period of maximum rumen activity immediately after the ingestion of a ration is somewhat less than the above range of values.

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Yeasts from the Bovine Rumen

By R. T. J. CLARKE
Plant Chemistry Division, Department of Scientific and Industrial Research, Palmerston North, New Zealand
and MARGARET E. di MENNA
Soil Bureau, Department of Scientific and Industrial Research, Wellington, New Zealand

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SUMMARY

Yeasts belonging to the genera *Candida* and *Trichosporon* have been isolated in small numbers from the rumen of fistulated cows. Similar yeasts were not found on samples of feed material. The yeasts present fell into two groups dependent on the diet of the cow from which they were isolated.

INTRODUCTION

The yeast flora of the lower intestinal tract of cattle was studied by Parle (1957) and by Van Uden & do Carmo Sousa (1957); but in spite of the similarity of the rumen environment to that of the lower intestinal tract, rumen yeasts have received little attention. A rumen yeast flora has not been reported previously although the work of several authors has suggested its existence. Ingram & McGaughey (1948) isolated '*Candida*-like' organisms from the rumen of sheep and Cunningham & Brisson (1955) noted an alcoholic fermentation in the stomachs of lambs on a high glucose diet. Lubinsky (1957, 1958) listed yeasts as food for many species of ciliate protozoa from the rumens of reindeer, sheep and goats. Rumen yeasts may not play an important part in the rumen fermentation, but from the veterinary point of view they may be of importance as a reservoir of infection. Yeast infections of the bovine udder are relatively frequent and have been caused by species of *Candida*, *Trichosporon* and *Pichia*. The present investigation into the rumen yeast flora was prompted by the isolation of *Candida albicans* from the rumen during a preliminary survey (Clarke, 1960).

METHODS

The rumen contents of five cows, one normal (no. 34) and four with rumen fistulas (no. 1, 90, 293, 294) were sampled at intervals between January 1959 and February 1960. All cows were fed hay during the winter months (April–August). During the spring and summer (September–February) all cows, with the exception of cow no. 1 which was hay-fed, were given fresh red clover (*Trifolium pratense* L.). As stall feeding could be carried out only on working days (Monday to Friday inclusive), the cows were turned out to pasture on Saturdays and Sundays. In spring and summer the pasture was predominantly red clover and in winter predominantly ryegrass (*Lolium perenne* L.).

Samples of rumen contents were removed from fistulated cows by an operator who wore sterile rubber gloves. The non-fistulated cow was sampled by using a sterile oesophageal tube, with suction.

The pasture, fresh clover and clover hay on which the cows were feeding were each sampled on two occasions. Hay samples were taken with aseptic precautions and clover and pasture samples were cut with sterile box shears.

All samples were plated on glucose peptone agar (4%, w/v, glucose; 1%, w/v, peptone; 2%, w/v, agar; adjusted to pH 4). Serial dilutions of the samples to be plated were made by the method of di Menna (1957). All cultures of rumen samples were incubated at 39°, but in addition two from hay-fed cows and parallel cultures from the non-fistulated cow were incubated at room temperature. Cultures of hay and pasture samples were incubated both at room temperature and at 39°. After 3–6 days of incubation all yeast-like colonies were picked off for identification from plates where the colonies were well separated.

Yeast cultures were maintained on the following medium (%, w/v): Malt Extract (Difco) 0·3; Yeast Extract (Difco) 0·3; Neopeptone (Difco) 0·2; glucose 1; in tap water. The yeasts obtained were identified by the criteria of Lodder & Kreger-van Rij (1952).

RESULTS

Rumen isolates

Particulars of the rumen samples and of the yeasts isolated are given in Table 1. Nine species belonging to three genera were identified from the 134 isolates examined. Viable counts made during the isolation procedure ranged from 80 to 13,000 organisms/g. rumen contents. Particulars of the species isolated are given below.

Candida spp. Ten isolates of *Candida albicans* (Robin) Berkhout were recovered from two clover-fed cows and one from a hay-fed cow. Two of the isolates were tested for pathogenicity by intravenous inoculation into rabbits. Both killed rabbits within 48 hr. and were recoverable from the animals' kidneys. Four isolates of *C. tropicalis* (Cast.) Berkhout, four of *C. krusei* (Cast.) Berkhout and two of *C. rugosa* (Anderson) Diddens & Lodder were recovered from two clover-fed cows. One isolate of *C. rugosa* was obtained from a hay-fed cow.

Trichosporon spp. Sixteen isolates of *Trichosporon cutaneum* (de Beurm., Gougerot & Vaucher) Ota were recovered from two cows, both fed on hay. These isolates resembled those of *T. cutaneum* type II described by Lodder & Kreger-van Rij (1952, p. 624) in that cultures on solid media were cream, dull, dry, becoming folded, but not hairy. They differed in that assimilation of maltose was not apparent on solid carbon-source auxanograms and was extremely weak in liquid carbon-source auxanograms. Cultures of two of these isolates were sent to Dr N. J. W. Kreger-van Rij (Yeast Division, Centraalbureau voor Schimmelcultures) who considered them to be variants of *T. cutaneum*.

Eighty-four isolations of *Trichosporon sericeum* (Stautz) Diddens & Lodder were made from four cows, seventeen from a clover-fed cow and the rest from hay-fed cows. Twenty-six of the isolates were recovered from room temperature cultures. In the key to the genus *Trichosporon* Lodder & Kreger-van Rij (1952) separate *T. sericeum* from *T. capitatum* Diddens & Lodder by its reduced mycelium and relatively strong assimilation of galactose. The eighty-four strains isolated here produced mycelium readily under aerobic conditions but also assimilated galactose

Table 1. *Yeasts isolated from the bovine rumen*

Feed	Temperature of isolation (°C)	Cow (no.)	Date	Organisms*								
				C. albicans	*C. tropicalis*	*C. krusei*	*C. rugosa*	*T. cutaneum*	*T. sericeum*	*R. glutinis*	*R. mucilaginosa*	*R. macerans*
				No. of isolates								
Clover	39	294	13. i. 59	.	.	1	2	.	.	.	.	.
Clover	39	294	12. ii. 59	5	.	.	.	.	.	.	.	.
Clover	39	294	16. ii. 59	4	.	.	.	.	.	.	.	.
Clover	39	294	17. ix. 59	.	.	.	.	.	7	.	.	.
Clover	39	294	28. x. 59	.	.	.	.	7	8	.	.	.
Clover	39	294	26. i. 60	.	2	1	.	.	2	.	.	.
Clover	39	293	16. ii. 59	.	2	2	.	.	.	.	.	.
Clover	39	293	1. ii. 60	1	.	.	.	.	.	.	.	.
Clover	39	34	27. xi. 59	.	.	.	.	.	.	.	.	.
Clover	*c.* 19	34	27. xi. 59	.	.	.	.	.	.	1	.	2
Hay	39	90	15. vii. 59	.	.	.	.	.	5	.	.	.
Hay	39	294	19. v. 59	.	.	.	1	6	8	.	.	.
Hay	39	293	29. v. 59	.	.	.	.	1	.	.	.	.
Hay	39	293	3. vii. 59	.	.	.	.	.	27	.	.	.
Hay	*c.* 19	293	3. vii. 59	.	.	.	.	2	26	2	3	.
Hay	39	1	1. ii. 60	1	.	.	.	.	6	.	.	.
Hay	*c.* 19	1	1. ii. 60	.	.	.	.	.	.	3	.	1
	Total			11	4	4	3	16	84	6	3	3

* *C* = Candida; *T* = Trichsporon; *R* = Rhodotorula.

strongly on solid auxanographic medium. Authentic strains of *T. sericeum* and *T. capitatum* were obtained from the Centraalbureau voor Schimmelcultures and compared with the bovine isolates. As the results from this comparison were inconclusive, it was decided to allocate the bovine isolates to the longer established species, *T. sericeum*.

Rhodotorula spp. *Rhodotorula glutinis* (Fres.) Harrison, *R. mucilaginosa* (Jorg.) Harrison and *R. macerans* Frederikson were isolated from three cows. All these isolations were made at room temperature. *R. glutinis* and *R. macerans* were the only yeasts isolated from the non-fistulated cow (34).

Hay and pasture isolates

Six species belonging to three genera were identified from the twenty-one isolates obtained from hay and pasture samples. All the yeasts recovered from the hay and pasture samples were from cultures incubated at room temperature; none could be isolated at 39°. No yeasts were recovered from the clover samples. Particulars of the species isolated are given below.

Cryptococcus laurentii (Kufferath) Skinner and *C. luteolus* (Saito) Skinner were recovered from pasture samples.

Torulopsis ingeniosa di Menna was recovered from pasture. *Rhodotorula glutinis* (Fres.) Harrison was recovered from pasture and both *R. macerans* Frederikson and *R. mucilaginosa* (Jorg.) Harrison were found on hay.

DISCUSSION

In view of the similarity of the anaerobic fermentations in the bovine rumen and lower intestinal tract, the presence of yeasts in the rumen is not surprising. With the exception of *Candida albicans*, those yeasts isolated here from cultures incubated at 39° are represented among those found in the bovine caecum by Van Uden & do Carmo Sousa (1957), and fall into their second group of yeasts: facultative saprophytes of warm-blooded animals. It is evident that the yeasts isolated here are true rumen inhabitants since yeasts which could grow at 39° were not isolated from the samples of feed material, and the rumen yeasts were not represented among those considered as part of the normal flora of the leaves of pasture plants (di Menna, 1959). The yeasts isolated here from hay and pasture samples were similar to those found by di Menna (1959). Of these, only *Rhodotorula* spp. were isolated from the rumen, and then only at room temperature.

The yeasts isolated from the rumen at 39° fall into two groups, fermenting and non-fermenting yeasts. The presence of either group appears to be dependent upon the diet of the cows. Except for one isolate of *Candida albicans* all the yeasts isolated from hay-fed cows were of the non-fermenting type. This isolate of *C. albicans* was made from cow no. 1 only a few days after it was returned to a hay diet. All the other fermenting yeasts were recovered from cows fed on fresh clover. This division into fermenting and non-fermenting yeast flora may be dependent on the soluble sugar content of fresh clover and clover hay. The soluble sugar content of fresh red clover may reach as high as 9 % of the weight, calculated on a dry-matter basis, while that of clover hay is usually considerably lower depending on the conditions at the time of harvesting (Dr R. W. Bailey, personal communication).

The failure to isolate yeasts other than *Rhodotorula* species from the non-fistulated cow was probably the result of having to use an oesophageal tube for sampling. The samples collected were always small and from an undetermined part of the rumen and reticulum. From experience with fistulated cows it is known that it is difficult to obtain a representative sample of rumen liquor because of layering of the rumen contents.

The numbers of yeasts found in the rumen are too low for them to be important in the provision of soluble fermentation products although they undoubtedly contribute to the total microbial protein available to the animal.

Our thanks are due to Dr N. J. W. Kreger-van Rij (Yeast Division, Centraalbureau voor Schimmelcultures, Delft, Netherlands) for her identification of the *Trichosporon cutaneum* cultures.

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The Influence of Environment on Antigen Production by *Pasteurella pestis* Studied by Means of the Continuous Flow Culture Technique

By S. J. PIRT, E. J. THACKERAY and R. HARRIS-SMITH
Microbiological Research Establishment, Porton, Salisbury, Wiltshire

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SUMMARY

The yields of three antigens of *Pasteurella pestis* (fraction I, antigen 4, V antigen) in continuous flow culture over a range of temperature and pH values have been determined quantitatively. The production of some other antigens was followed qualitatively. The production of fraction I, V antigen and antigen 4 was greatest at 37° and little or none at 28°. The production of antigen 4 required an environmental pH below 6·9. The production of antigen 5 showed oxygen dependence at 28° but not at 37°. In transitions from one antigenic state to another the amounts of some antigens in the organism passed through maxima. Selection against types with the V and fraction I antigens occurred during growth at 37°, but not at 28°. Selection against types with antigen 4 did not occur.

INTRODUCTION

To produce organisms of constant antigenic composition, quantitatively and qualitatively, strict control of environment would seem to be necessary. We became interested in this problem because of the need to control the antigenic composition of *Pasteurella pestis* in studies of the immunogenicity and virulence of this organism. Continuous flow culture rather than batch culture was used because of the possibility of maintaining a given environment indefinitely in the continuous system. Consideration of the differences between a batch process and a continuous process leads one to expect that the two techniques may give organisms of different antigenic composition. This may result because, in the course of the batch process, especially one which requires a complex medium (as pathogenic bacteria usually do) there may be environmental changes (in available nutrients or in physical factors such as pH value) unless adequate control equipment is available. Different antigens may be produced at different stages in batch culture; *P. pestis* provides an example of this (Crumpton & Davies, 1956). On the other hand, in a continuous flow culture one can select any environment from that of the log phase to the stationary phase and maintain it indefinitely, but one may then find that, because the organisms grow in only one environment, not all the antigens can be produced at once. The way out of this difficulty should be to use a multi-stage process (Pirt & Callow, 1959; Callow & Pirt, 1961). Data about these problems is provided by the present work. The lack of control over, and the variation of conditions in batch culture may explain why, with rare exceptions, the published work on the antigenic

composition of micro-organisms does not indicate that variation in environment is a reliable way of altering the antigenic composition. The problem of the effect of environment was taken up by Lacey (1953) who found that reversible changes in the antigenic composition of *Haemophilus* spp. could be induced by variation in the proportions of Na, Mg, Cl and SO_4 ions, and in the temperature of the medium. Ogburn, Harris & Harris (1958) used a continuous flow technique to show that an extracellular antigen of a haemolytic streptoccocus required an acid pH value for its formation. Striking examples of the effects of environment on antigenic composition have been found with *Paramecium aurelia* (Beale, 1954) where the antigenic composition is a function of the temperature, the inorganic salt composition of the growth medium and the amount of nutrients supplied.

The earliest recognition of the effect of an environmental factor on antigen production by *Pasteurella pestis* seems to have been that of Schütze (1932) who found that the envelope antigen (now known as fraction I) was produced at 37° but not at 26°, although earlier Rowland (1914) had recognized the temperature dependence of envelope formation. For an historical review of the antigenic analysis of *P. pestis* reference should be made to Crumpton & Davies (1956). These authors, by application of gel diffusion analysis, revealed the presence of seven new antigens. One of these antigens was antigen 4, later characterized in greater detail and shown to be of immunogenic importance (Crumpton & Davies, 1957). The requirements for antigen 4 production seemed to be exacting because, although produced in an aerated broth galactose medium at 37°, the antigen did not appear in the organisms until 16 hr. after the initiation of growth. This antigen was also absent from organisms grown as surface cultures on broth agar. An explanation of this behaviour is provided by our results. Further development of knowledge about *P. pestis* antigens came from the recognition by Burrows & Bacon (1956) of the V and W antigens as characteristic of the virulent strains. The present work has three parts: quantification of the effects of pH value and temperature on antigen production; a description of some transitional stages when the antigenic composition is changed by the environment; some effects of variation and selection on antigenic composition in long-term cultures and their control by the environment.

METHODS

For descriptions of methods other than those given below reference should be made to Pirt & Callow (1958). The method of dry wt. determination was used with formolized samples (HCHO concn. 3%, w/v).

Continuous flow-culture technique. The culture apparatus was based on that of Elsworth, Meakin, Pirt & Capell (1956) and Callow & Pirt (1961), but modified to ensure safety in work with these pathogenic organisms. The medium flow rate was 0·1 culture volumes/hr. (dilution rate $0{\cdot}1 \pm 0{\cdot}005$ hr.$^{-1}$); hence the generation time of the organisms was 6·9 hr. in the steady state.

The pH value was controlled by the automatic addition of 2N-NaOH or 2N-H_2SO_4 (Callow & Pirt, 1956); the pH controller (Fielden Ltd., Paston Road, Wythenshawe, Manchester) controlled within $\pm 0{\cdot}15$ pH units. Temperature was controlled within $\pm 0{\cdot}5°$: changes in pH value and in temperature were made gradually at rates of 0·1 pH unit or 1°/4 hr.

Media. The liquid medium contained (g./l.): casein acid hydrolysate (Oxoid) 15·0; L-cysteine monohydrochloride, 0·32; glycine, 1·5; glucose, 12·0; KH_2PO_4, 0·85; $Na_2HPO_4.2H_2O$, 8·0; trisodium citrate dihydrate, 3·48; $MgSO_4.7H_2O$, 0·6; $FeSO_4.7H_2O$, 0·03; $MnSO_4.4H_2O$, 0·02; $CaCl_2$, 0·0224. The final pH value was 7·4. Sterilization was by autoclaving (126° for 20 min.); the glucose and cysteine solutions added were sterilized separately.

Either Alkaterge-C (Commercial Solvents Corp., Terre Haute, Indiana, U.S.A.) 50 % (v/v) in liquid paraffin, or silicone antifoam B (Midland Silicones, Ltd) 25 % (w/v) was added as an antifoam.

The solid medium contained agar with tryptic digest of meat to which 5 % of its volume of Fildes peptic digest of sheep's blood (Mackie & McCartney, 1948) was added.

Organisms. The avirulent Tijwidej smooth (TS) strain of *Pasteurella pestis* and the virulent L37 strains were used. The stock cultures were maintained as freeze-dried cultures or in stab cultures on the solid medium at 4°.

Preparation of inoculum. A slope of the solid medium was inoculated from the stock culture. The slope culture was transferred to a conical flask containing 12·5 ml. liquid medium with the following changes: KH_2PO_4, 3·2 g./l.; Na_2HPO_4, 13·6 g./l.; casein hydrolysate 10 g./l.; glucose replaced by galactose, 10 g./l.; glycine and $CaCl_2$ omitted. These changes were used principally to increase the buffer capacity and to restrict the growth to be more in keeping with the amount of available oxygen. After 24 hr. gentle reciprocal shaking (throw 0·6 in., 100 cycles/min.) the culture was transferred to 100 ml. of the same medium in a 2 l. bottle and shaken gently for 24 hr. when the culture was used to inoculate the continuous flow culture, 2 l. in volume. Initially, to ensure growth, excess aeration had to be avoided; for the first 2 or 3 generations a sulphite oxidation value of 5 mmole O_2/l./hr. was adequate, with no air flow through the culture vessel.

Antigenic analysis. The organisms were prepared by centrifuging down and drying with acetone at −20°. The supernatant liquid was examined after it had been freed from organisms by membrane filtration.

Qualitative antigenic analysis was carried out by the Ouchterlony agar gel diffusion method as described by Crumpton & Davies (1956) . Photographic records were made at about the fourth day. For the detection of V antigen the micro gel diffusion method of Mansi (1958) with 4 mm. between wells was used for economy of antiserum and antigens. The diffusions for qualitative analysis were carried out at ambient temperature (*c.* 22°). For the identification of antigens other than V antigen a standard line pattern obtained with TS organisms and antiserum provided by Dr D. A. L. Davies (this Department) was used. For the detection of V antigen a standard line pattern showing V and W antigens only was obtained with serum and organisms provided by Dr T. W. Burrows (this Department). The organism preparations of Davies & Burrows are referred to as the standard batch culture preparations.

Methods of quantitative antigenic analysis were developed by using the micro gel diffusion method of Mansi (1958) with specific antisera. The method consisted of comparing the highest dilution of the standard batch culture preparation of antigen which would give a line on the plate not coincident with the antigen well, with the highest dilution of the sample which did likewise. The unit of antigen was defined as the amount present in 1 mg. of the standard batch culture preparation; the units

of antigen/mg. sample were calculated from the dilutions. The factor between antigen dilutions was 2. The distance between wells on the plate was 2 mm. Fraction I, antigen 4 and V antigen were estimated in this way. The diffusion temperature for the quantitative assays of fraction I and antigen 4 was 37° for 4 and 3 hr., respectively. The diffusion of the V antigen was at *c.* 22° for 18 hr.

RESULTS

Growth of the organism

The average yield of either strain when there was an excess of available oxygen was 5·5 mg. dry wt./ml. at 28° and 3·7 mg. dry wt./ml. at 37°. This difference between the yields expresses quantitatively the known fact that growth of *Pasteurella pestis* is poorer at 37° than at 28°. The mean total number of organisms/mg. dry wt. was $2{\cdot}5 \times 10^9$.

With an excess of available oxygen the oxygen uptake rate was 29 mmole/l./hr. at either temperature. All the results with the TS strain were obtained with excess of available oxygen, that is, with respiration unlimited by oxygen supply. In work with the L37 strain the oxygen uptake rate was limited to 9 mmole/l./hr. when it was found that, with one exception noted below, an excess of oxygen was not essential to the production of the antigens in which we were interested. Limitation of the aeration had the advantage of making foam control easier. This restriction of the oxygen supply decreased the dry wt. yield of organism by 35 %.

The yield of organism was independent of the pH value in the range pH 6·3–7·3. The TS strain was grown with undiminished yield at pH 5·9 and 37°. An advantage of using pH control instruments was that glucose could be used as a carbon source. Previous workers, without using this control method, found that acid production from glucose soon stopped growth; this difficulty was previously overcome by using galactose as a carbon source.

Apart from the effect on the yield of organism, increase in the temperature from 28° to 37° also caused a severe shock to the culture. This shock, which was particularly marked in growing cultures supplied with an excess of oxygen, was characterized by a high death-rate of the organisms, the appearance of much cell debris, much foaming, and decreases in dry wt. and oxygen uptake to much below the eventual steady-state values. This shock was largely avoided when growth was limited by the oxygen supply.

Production of antigen 4

The production of antigen 4 was dependent on pH value and temperature. The pH value dependence is illustrated in Fig. 1. Production of antigen 4 was completely suppressed at pH values above 6·9. The optimum pH value for antigen 4 production was pH 5·9, at which value the amount of antigen present in the organisms was six times greater than the amount in the standard batch culture preparation. The supernatant fluid contained a large amount of the antigen, about twice the amount found in the organisms. The dialysed solids of the supernatant fluid at pH 5·9, we are informed by Dr M. J. Crumpton, were about 20 % pure antigen 4, which means that this fluid was the richest source of antigen.

The effect of pH value on antigen 4 production by the L37 strain was the same as with the TS strain. The range of yields of the antigen by the two strains are given in

Table 1. The yields of antigen 4 are compared at pH 6·3 instead of at the optimum pH value of 5·9, because we found it convenient to work in the range pH 6·3–7·3 and thus to decrease the time necessary for the pH changes. The effect of temperature on antigen 4 production is shown in Table 2. At 28°, a small amount of the antigen was produced at pH 6·3; the production was 20 times greater at 37°. At 28° and pH 7·3 antigen 4 was undetectable. Antigen 4 production in the culture was characterized by a marked increase in the sedimentation time of the organisms; this is in agreement with the finding of Crumpton & Davies (1957) that this antigen stabilizes a suspension of the organisms.

Production of fraction I and V antigen

Fraction I production was independent of environmental pH value in the range pH 6·3–7·3, but was dependent on the temperature (Table 3). At 28° only trace

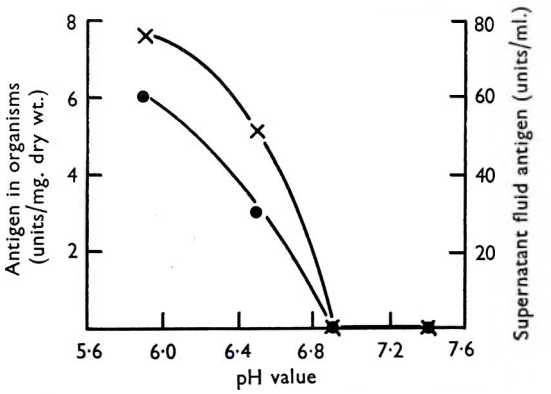

Fig. 1. Antigen 4 production by the TS strain of *Pasteurella pestis* as a function of pH value at 37°. ●, Antigen in organism; ×, supernatant fluid antigen. The values were obtained in the same culture. In this experiment the medium was modified in that the concentrations of casein hydrolysate, glycine and glucose were increased by 33%; and $CaCl_2$ was omitted.

Table 1. *Comparison of antigen 4 production by the TS and L37 strains of* Pasteurella pestis *at high and low pH values at* 37°

Pooled results from four different cultures of each strain; each set of conditions was reproduced two or more times.

Strain of *P. pestis*	pH 7·3	pH 6·3
	Antigen in organism (units/mg. dry wt.)	
TS	< 0·06	0·5–2·0
L37	< 0·06	1·0–1·0
	Antigen in supernatant fluid (units/ml.)	
TS	< 1·28	2·6–12·4
L37	< 1·28	2·6–10·2

The very high values for antigen 4 production reported in Fig. 1 are not comparable with those above because of important differences in media used.

Table 2. *Effect of temperature on antigen 4 production at pH 6·3 by* Pasteurella pestis

Strain of *P. pestis*	Growth temperature 28°	Growth temperature 37°
	Antigens in organisms (units/mg. dry wt.)	
TS	0·125	2·0
L37	0·03	1·0
	Antigen in supernatant fluid (units/ml.)	
TS	2·56	10·2
L37	< 1·28	5·1

The values for each strain are from the same culture, those for 28° being obtained before those for 37°

Table 3. *Effect of temperature on fraction I production by* Pasteurella pestis

Strain of *P. pestis*	Growth temperature 28°	Growth temperature 37°
	Fraction I in organisms (units/mg. dry wt.)*	
TS	0–0·03	0·25–0·50
L37	0–0·06	0·125–0·50
	Fraction I in supernatant fluid (units/ml.)*	
TS	0–0·03	1·28–2·26
L37	< 0·32	0·32–1·25

* Maximum amount in steady states. Pooled results of four cultures of each strain: each set of conditions was reproduced two or more times.

amounts of the antigen were produced; at 37° the antigen was produced in large amount, though the maximum fraction I content of organisms was only about a half of that of the standard batch culture preparation. About 50 % of the total fraction I antigen content of the culture was present in the supernatant fluid.

V antigen was not detected in organisms grown at 28°. At 37° a maximum V antigen content of 1 unit/mg. dry wt. was obtained; this equals that of the standard batch culture preparation. None of this antigen was detected in the unconcentrated supernatant fluid. The V antigen production was constant over the range pH 6·3–7·3.

Production of other antigens

We also identified in our samples antigens 1, 2, 5 and 8 as defined by Crumpton & Davies (1956). Although the amounts of these antigens varied from time to time, their synthesis, with the exception of antigen 5, could not be correlated with any particular conditions. The W antigens of the virulent organism accompanied the V antigen but, judging by the line intensity in the diffusion analysis, were present in very small amount.

The synthesis of antigen 5 seemed to be oxygen dependent at 28° with the L37 strain (the TS strain was not examined in this respect). Organisms grown at 37° gave a strong antigen 5 line in gel diffusion whether or not growth was limited by oxygen. But at 28° a strong antigen 5 line was only produced with excess of available oxygen. Crumpton & Davies (1956) reported that antigen 5 production was highly dependent on temperature; this would appear to be true only when the growth is oxygen limited.

Rate of change in antigenic composition with change in environment

It is known that after a substrate change there must often be a period of adaptation while the metabolism of the organism adjusts itself to this change. Here we are concerned with the adaptation to a change in the physical environment such as that of temperature or pH value. There also arose the practical question of how quickly could a change in temperature or pH value be made without adverse effects on the organism. Generally we found that rates of change in temperature of 1° and of pH value of 0·1 unit, every 4 hr., were tolerated.

In the following discussion it may be borne in mind that, in a continuous culture in a steady state, the rate of antigen production per unit weight of organisms (q_{ag}) may be derived from the antigen concentration by the equation which relates rate of product formation with product concentration:

$$q_{ag} = \frac{\text{antigen concn.} \times D}{x},$$

where D is the dilution rate and x the concentration of organism. Hence, if D and x are constant, q_{ag} is directly proportional to the antigen concentration.

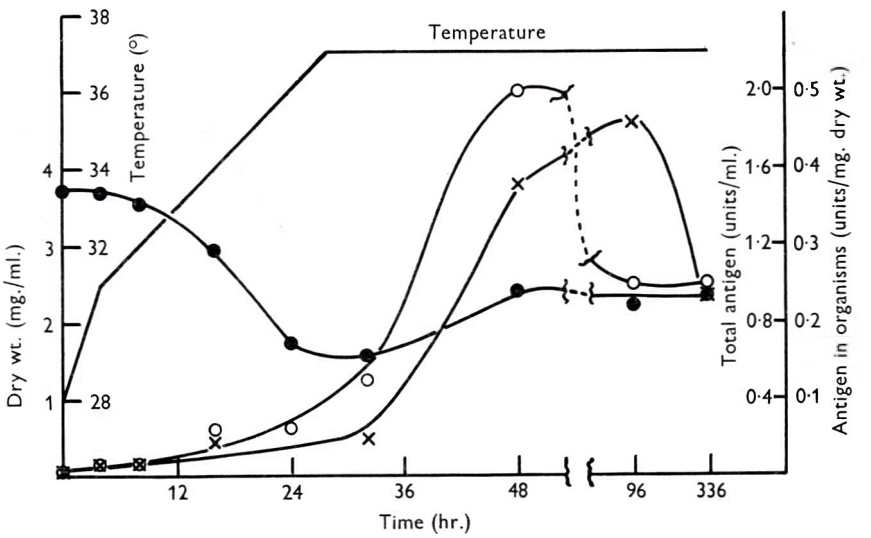

Fig. 2. Fraction I antigen production by strain L37 of *Pasteurella pestis* as a function of time on raising the temperature from 28° to 37°. ○, Antigen in organism; ×, total antigen (supernatant fluid + organism); ●, dry wt. of organisms (growth was oxygen limited).

Changes in the fraction I content and in the dry wt. of organism as functions of time on raising the temperature from 28° to 37° are shown in Fig. 2. The main features of the transition are that the dry wt. quickly responded to the temperature change and passed through a minimum before it adjusted itself to the new steady-state value. The production of antigen fraction I, however, did not respond as rapidly as the dry wt. but increased rapidly only when the temperature reached 37°. The amount of fraction I antigen in the organisms passed through a maximum. This may be attributed to an initial retardation of diffusion of the antigen from the organism into the supernatant fluid; it was not due to a decrease in the rate of antigen synthesis since the total amount of antigen present increased when the organism antigen content decreased. The fall in total antigen observed between 96 and 336 hr. may be attributed to selection of fraction I− organisms, that is, organisms without the ability to produce fraction I.

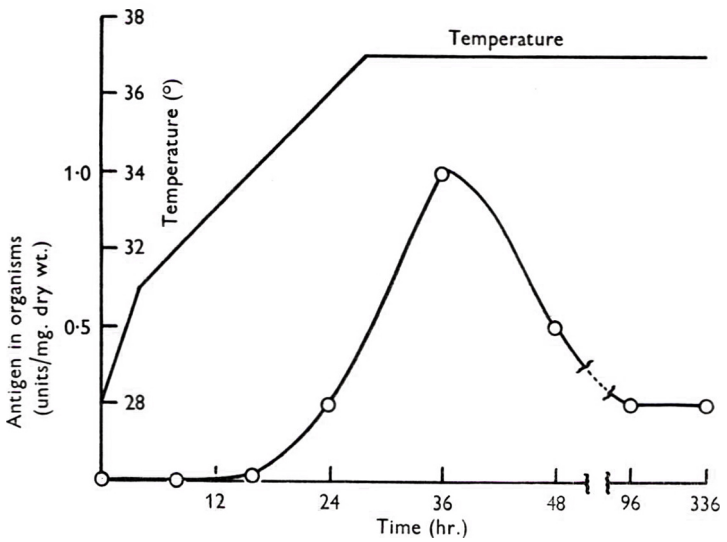

Fig. 3. V antigen production by strain L37 of *Pasteurella pestis* as a function of time on raising the temperature from 28° to 37°.

The transition to V antigen production is shown in Fig. 3. When the temperature exceeded 34° the production of V antigen rose rapidly (in 4 generation times) from zero to maximum. Unlike antigen fraction I, V antigen was not detected in the supernatant fluid. To account for the peak value in the V antigen content it seems necessary to postulate that the initial rate of antigen production exceeded the steady-state rate.

The transition to antigen 4 production is shown in Fig. 4. The transition began when the pH value fell below 6·9. With decrease in pH value the antigen production rate rose rapidly to the maximum. Again, the results show that in the transition the antigen content of the organisms passed through a maximum in a way similar to the transition to fraction I antigen production. The reversibility of the transition to antigen 4 production is illustrated in Fig. 4. On raising the pH value from 6·3 to 7·2 antigen 4 production stopped and the residual antigen was diluted out.

Maintenance of antigen synthetic abilities; variation and selection

An organism, which, given the right environment, is able to produce fraction I antigen is termed a fraction I+ organism; an organism which has lost this ability is termed a fraction I− organism. The terms V+ organism and V− organism are derived similarly.

During cultivation at 37° the organisms gradually lost their abilities to produce both fraction I and V antigens as shown in Tables 4 and 5. These changes we

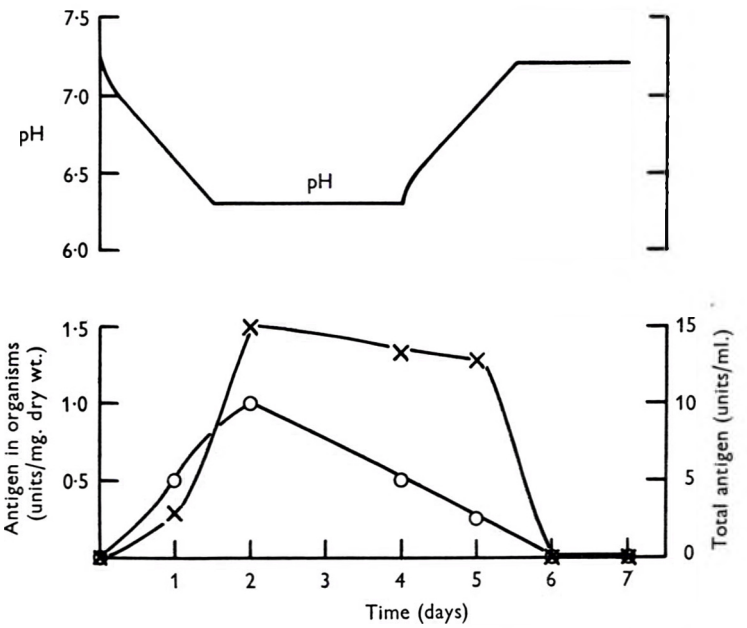

Fig. 4. Antigen 4 production by strain TS of *Pasteurella pestis* as a function of time with pH changes. ○, antigen in organism; ×, total antigen (supernatant fluid+organism). The dry wt. concn. was 2·6 mg./ml. (in this medium the casein hydrolysate, glycine and glucose concentrations were decreased by one-third).

attribute to selection against fraction I+ and V+ organisms. In the L37 culture (Table 4) there was a 75% decrease in the concentration of fraction I antigen, and consequently in the rate of production, in 59 generations (69th to 128th). Selection against V+ organisms was slower than that against fraction I+ organisms.

At 28° selection against fraction I+ and V+ organisms did not occur. It was possible to grow the organism for any length of time (at least up to 111 generations) at 28° without affecting the ability to produce fraction I antigen when the temperature was raised to 37°. Ability to produce V antigen was unaffected by cultivation of the organism at 28° for at least 55 generations.

The antigen 4 production by the culture over a long period is shown in Table 5, which shows that there was no significant loss in the ability to produce antigen 4 after 210 generations. Hence antigen 4 production may be classed as a stable function of the culture under the conditions used.

Practically all the antigen synthetic functions other than those for fraction I and V antigens seemed to be stable over long periods under the conditions used, judging by the qualitative analyses of line patterns in gel diffusion. In TS cultures after about 150 generations at different pH values and temperatures, ten antigens of the original twelve were detected; fraction I antigen was one of the missing components. Similarly, in L37 cultures, thirteen antigens of the original sixteen were retained, fraction I and V antigens being two of the missing components.

Table 4. *Maintenance of antigen synthetic abilities over long periods by strain L37 of* Pasteurella pestis

Culture age (hr.)	No. of generations	Steady-state concentrations: Fraction I antigen in organisms (units/mg. dry wt.)	Steady-state concentrations: V antigen in organisms (units/mg. dry wt.)
455	69	0·25	0·25
695	104	0·25	0·25
790	118	0·125	0·50
862	128	0·062	0·25
1462	215	< 0·007	0·125

The temperature was 37° from the 55th to the 128th generation, and from the 166th to the 215th; otherwise it was 28°.

Table 5. *Maintenance of antigen synthetic abilities over long periods by strain TS of* Pasteurella pestis

Culture age (hr.)	No. of generations	Steady-state concentrations: Fraction I antigen in organisms (units/mg. dry wt.)	Steady-state concentrations: Antigen 4 in organisms (units/mg. dry wt.)
214	33·5	0·25	N.R.
238	36·9	0·25	N.R.
286	44	0·06	0·50
310	47	0·06	N.R.
363	55	< 0·007	N.R.
622	92	< 0·007	2·0
646	96	< 0·007	1·0
670	99	< 0·007	2·0
1030	151	< 0·007	0·5
1438	210	< 0·007	0·5

The temperature was 37° from the 16th to the 55th generation, from the 89th to the 151st, and from the 197th to the 210th; otherwise it was 28°.

The pH value of the culture was 6·3 when antigen 4 production was determined and either pH 6·3 or 7·3 when fraction I and V antigens were determined.

N.R. means no result.

DISCUSSION

Conditions for producing various antigens. A pH value below 6·9 seems essential for the production of antigen 4, this fact and the pH–time curves for growth in batch cultures (unpublished work of Ross, Hakes & Herbert of this Laboratory) explain the apparently exacting requirements for antigen 4 production which Crumpton & Davies (1956) observed. When galactose is the carbon source it takes about 16 hr. for the pH value to fall below 6·9 from the initial value 7·5; this

accounts for the time lag before the appearance of antigen 4 in the cultures of Crumpton & Davies. The absence of antigen 4 from broth agar cultures may be attributed to the absence of galactose or glucose from the medium since, in the absence of the sugar, there is no acid production and the pH value remains too high for antigen 4 production.

The identity between the conditions necessary for the change to antigen 4 production, and those which Aronson & Bichowsky-Slomnicki (1960) found to cause a marked lowering of the electrophoretic mobility of *P. pestis*, suggests that antigen 4 may be the surface component postulated by Aronson & Bichowsky-Slomnicki to explain the change in electrophoretic mobility. However, one piece of evidence seems at variance with this interpretation, namely that the change in electrophoretic mobility was observed to occur not only with the TS, L37 and other strains producing antigen 4, but also with the TRU strain which Crumpton & Davies (1956) classified as a non-producer of antigen 4. This discrepancy may be explained by the presence in the TRU organism of a substance derived from antigen 4 by a slight molecular change sufficient to destroy the antigen specificity but not sufficient to change the electrophoretic properties of the molecule.

The results of Crumpton & Davies (1957) show that antigen 4 is important in the production of immunity to *Pasteurella pestis* by the live TS strain in mice. If production of immunity in this way involves some *in vivo* production of antigen 4 it must mean that the organism finds some environment *in vivo* where the pH value is less than 6·9, that is, considerably lower than the so-called 'physiological' value, pH 7·4.

The qualitative differences in antigenic composition which can be induced by environmental changes make it appear that this is a technique which deserves to be used more in antigenic analysis in the same way as genotypic variants are used. Clearly we have not studied all the environmental changes of interest. In particular it would be of value to study the effect of inorganic salt changes in view of their effects on the antigenic composition of Haemophilus (Lacey, 1953) and Paramecium (Beale, 1954). Variation in antisera to organisms grown in differently controlled environments is another aspect which requires study, for example, to see whether new antigens are produced.

Transition from one antigenic state to another. It is important to notice that the highest concentrations of some antigens may arise not in any steady states but in the transition from one steady state to another. Practical use might be made of this finding to produce the highest possible yields of some antigens. It may also help to account for the differences between the antigenic composition of organisms grown in batch culture and that of organisms grown in a continuous steady-state culture, since transitional states may occur in batch culture.

Evolution in continuous flow cultures. In long-term cultures (the longest used here was 2300 hr.; 330 generation times) such as we have been studying, selection of variants is a problem to be reckoned with. It has been previously reported (Taylor, 1933) that *Pasteurella pestis* loses virulence more rapidly by *in vitro* culture at 37° than at 27°. An explanation of this is provided by the observation of Burrows (1960) that V− and W− organisms have a selective advantage over their V+ and W+ counterparts, when mixed cultures are incubated at 37°, though not at 28°. Our observations on selection in continuous flow cultures confirm the observations of

Burrows and, furthermore, show that fraction I− organisms have a selective advantage over the fraction I+ organisms at 37°; this gives an added reason why virulence is lost during *in vitro* culture at 37°. If it be desired both to preserve the abilities to synthesize V and fraction I antigens and to produce these antigens in a continuous flow process, it would seem to require more than one stage. The organisms would have to be grown at 28° to prevent selection against V+ and fraction I+ organisms and then transferred to a second stage at a higher temperature for the production of the desired antigens.

We thank Drs D. A. L. Davies, M. J. Crumpton and T. W. Burrows for initiating us into antigenic analysis of *Pasteurella pestis*, and for the supply of standard antigen preparations and antisera.

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The Effect of Formaldehyde on the Shape of Bacterial Flagella

By E. LEIFSON

Stritch School of Medicine and Graduate School, Loyola University, Chicago, Illinois, U.S.A.

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SUMMARY

Addition of formalin (40% formaldehyde) to a concentration of 5–10% (v/v) to a bacterial culture before flagellar staining may change the shape of the flagella as seen in the stained preparation. With many bacteria, if not a majority, the formalin has no effect on the flagellar shape. The coiled, the straight, the small-amplitude and the typical undulant flagellar shapes evidently are mainly due to formalin fixation. The effect of formalin on flagellar shape may have taxonomic significance and should be recorded. Formalin fixation is advantageous in flagellar staining in that it kills the bacteria and makes for nicer preparations, especially when the bacteria are capsulated.

INTRODUCTION

For the past decade the author has routinely added 5–10% (v/v) of formalin (40% formaldehyde solution) to all bacterial cultures before washing and flagellar staining. The original reason for this was to kill the bacteria and lessen the danger when working with pathogenic cultures. At the time the formalin procedure was adopted the flagella on the bacteria under study did not show any difference in appearance as a result of the formalin treatment. The consequent assumption that formalin had no effect on flagellar shape will here be shown to be incorrect. In his *Atlas of Bacterial Flagellation* (Leifson, 1960) the author described the various flagellar shapes which may be observed in stained preparations of formalin-fixed cultures. The most common shape observed is that of a fairly uniform wave with a wavelength: amplitude ratio of about 4:1. The author labelled this the 'normal' shape. From published darkfield observations such as those of Pijper & Abraham (1954) and from electronmicrographic studies (Stocker, 1956) it is evident that the usual shape of bacterial flagella is helical. When the flagella dry on the glass surface the helix becomes flattened and the familiar wave shape results. In addition to the normal flagella many bacteria, and most peritrichously flagellate types, may have flagella of wavelength about one half that of the normal flagella. The author has labelled this the 'curly' type. The curly type has also been observed by Pijper in his studies of living bacteria. With many bacteria the normal → curly variation appears to have a genetic basis; in others a change of pH value can cause the change of wavelength. Aside from the normal and curly flagellar shapes the various other recorded shapes such as 'coiled', 'straight', 'small amplitude' and 'undulant' would appear to be mainly a result of formalin fixation.

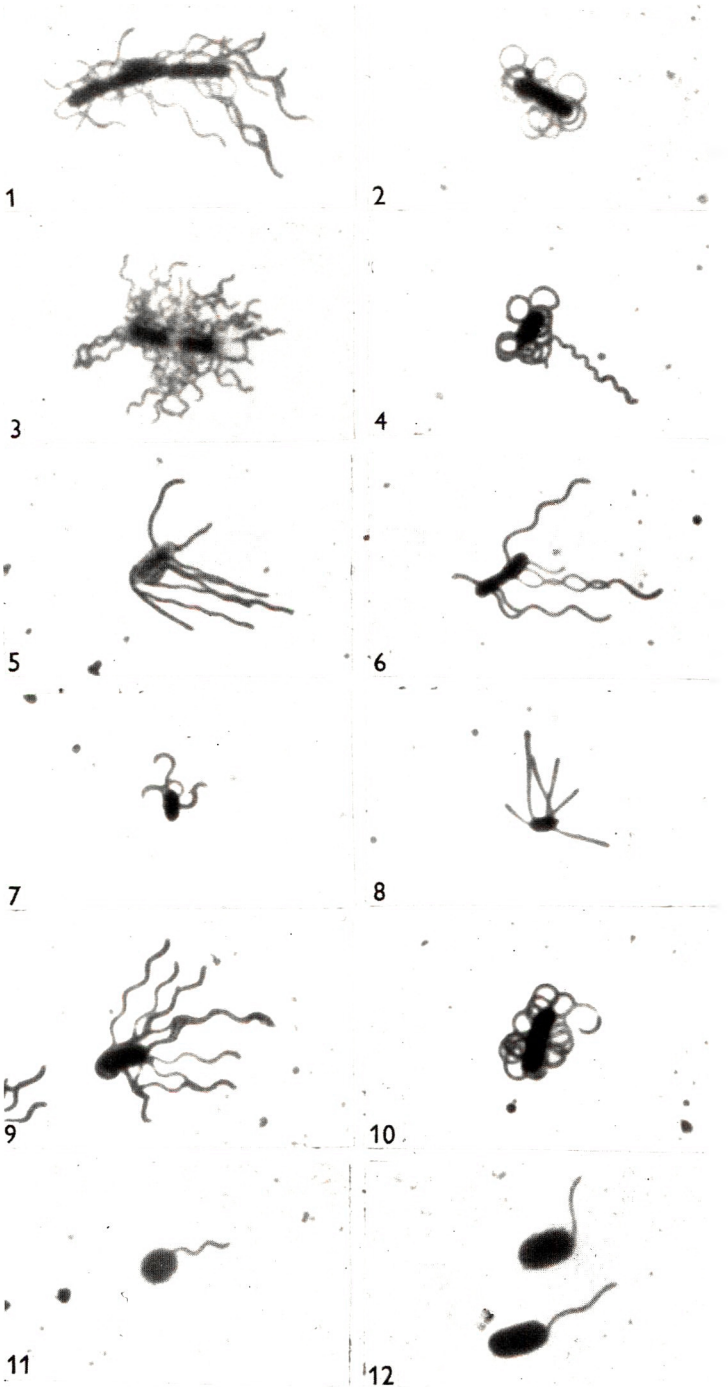
Formalin-fixed cultures of several types of bacteria show flagella of the coiled shape. Most Serratia strains show normal flagella in unfixed preparations and coiled flagella in formalin-fixed preparations (Pl. 1, figs. 1, 2). A smaller proportion show curly flagella in unfixed preparations and coiled flagella in formalin-fixed preparations (Pl. 1, figs. 3, 4). The only exception ever observed is a strain of *Serratia indica* (NCIB 2847) with small amplitude and straight flagella in unfixed preparations, and normal flagella in formalin-fixed preparations (Pl. 1, figs. 5, 6). A strain of Listeria (B-3D) showed coiled flagella in formalin-fixed preparations and normal flagella in unfixed preparations. The same is true for a culture originally labelled *Aeromonas liquefaciens* (Kluyver L-418) as illustrated in Pl. 1, figs. 9 and 10. A few strains of polar multitrichous pseudomonads have shown coiled flagella in formalin-fixed preparations and normal flagella in unfixed preparations. The flagella of some bacteria may show some coiling in unfixed preparations, but the coiling is invariably enhanced by formalin fixation.

Among peritrichous flagellated bacteria formalin-fixation only rarely produces the straight or small amplitude type of flagella. An example of this is shown in Pl. 1, figs. 7 and 8 with Listeria (B-3A). Incidentally it might be worth noting that this strain of Listeria and one other showing small amplitude flagella are practically non-motile, which indicates some flagellar abnormality. However, bacteria with typical normal flagella after formalin fixation but without apparent motility also exist. Among the polarly flagellate bacteria straight and stiff flagella have occasionally been observed following formalin fixation. Partially straight or undulant flagella are more frequently observed following formalin fixation, particularly among the marine pseudomonads (Pl. 1, figs. 11, 12). These undulant flagella which result from the formalin treatment must not be confused with flagella of long wavelength and large amplitude often found on polarly flagellate bacteria and which are normal for these bacteria.

DISCUSSION

For the sake of clarity a description of flagellar shape should always include a statement about the method of fixation, whether with formalin or other chemical or none. Preferably both the unfixed and the fixed shapes should be recorded. Formalin fixation is of considerable value in flagellar staining: pathogens are killed; flagella of slime-producing and capsulated bacteria are more readily stained after formalin fixation. The latter is particularly striking with halophilic marine bacteria. The differences in the effect of formalin on the shape of bacterial flagella may have taxonomic significance and the terms 'formalin sensitive' and 'formalin insensitive' may be suggested.

When the flagellar shape is normal or curly after formalin fixation the probability is very small that unfixed preparations will show a different shape. When the flagellar shape is coiled, straight, small amplitude or typically undulant after formalin fixation, the probability is great that the unfixed preparation will show flagella of normal or curly shape.



E. LEIFSON

(*Facing p.* 133)

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EXPLANATION OF PLATE

Effect of formalin fixation on the shape of the flagella of Serratia and three other types of bacteria. Leifson flagella stain, × 2000.

Figs. 1, 2. *Serratia kielensis* (NCIB 4619). In fig. 1 is shown the unfixed preparation with normal flagella and in fig. 2 the formalin fixed preparation with coiled flagella.

Figs. 3, 4. *Serratia marcescens* (Smith). In fig. 3 is shown the unfixed preparation with curly flagella. In fig. 4 is shown the formalin fixed preparation with coiled flagella and one curly flagellum.

Figs. 5, 6. *Serratia indica* (NCIB 2847). In fig. 5 is shown the unfixed preparation with small amplitude and straight flagella and in fig. 6 the formalin fixed preparation with normal flagella. The flagellar shape of this strain of Serratia is unique in the author's experience.

Figs. 7, 8. *Listeria monocytogenes* (B-3A). In fig. 7 is shown the unfixed preparation and in fig. 8 the formalin fixed preparation. The flagella become straight and stiff as a result of the formalin fixation.

Figs. 9, 10. *Aeromonas* (*Aerobacter*) *liquefaciens* (Kluyver L-418). In fig. 9 is shown the unfixed normal flagella and in fig. 10 the formalin fixed coiled flagella.

Figs. 11, 12. *Pseudomonas* sp. (halophilic marine type, Colwell). In fig. 11 is shown the unfixed normal flagellum and in fig. 12 the formalin fixed undulant flagella. This is typical of many but not all of marine pseudomonads.

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The Use of the Thionin Blue Sensitivity Test in the Examination of Brucella

By W. J. B. MORGAN
Central Veterinary Laboratory, Weybridge, Surrey

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SUMMARY

A total of 171 cultures of Brucella, mostly isolated in this country from milk and foetal material, was examined for sensitivity to thionin blue as well as to basic fuchsin and thionin, and for agglutination in monospecific sera. Standard strains of *Brucella abortus*, *B. melitensis* and *B. suis* grew in the presence of thionin blue in the concentration studied. Three groups of *B. abortus* were found, however, which were sensitive to thionin blue. These were: (*a*) strains resembling the vaccine strain *B. abortus* strain 19; (*b*) dye sensitive strains (*B. abortus* type II Wilson), (*c*) a previously undescribed group. Cultures of this last group required added CO_2 for growth, grew on basic fuchsin and were virulent for guinea-pigs. The significance of these results is briefly discussed.

INTRODUCTION

McLeod (1944) reported that *Brucella abortus* strain 19 would not grow on media containing thionin blue in concentrations that would allow the growth of other strains of *B. abortus*. This observation was confirmed by Levine & Wilson (1949) and Cruickshank (1954). The latter author also pointed out that strains of *B. abortus* type II Wilson (Huddleson, 1955) did not grow on thionin blue media. These strains are known to be very sensitive to dyes and require the presence of serum or Tween 40 for growth (Huddleson, 1956; Sackman, 1957; Morgan, 1960). The purpose of the present paper is to record the results of the examination of Brucella cultures for sensitivity to thionin blue.

METHODS

Organisms. Most of the organisms studied were isolated at this Laboratory from foetal material or milk; the remainder had been sent in for confirmation of identity. Except for recently isolated cultures, they were all checked for dissociation by using obliquely transmitted light (Henry, 1933) and only smooth cultures were studied.

Media. Serum glucose agar was used for the propagation of cultures and as basal medium for the addition of dyes. The composition of this medium has been described (Morgan, 1960).

Basic fuchsin (supplied by Pharmaceutical Laboratories National Aniline Division, Allied Chemical and Dye Corporation, New York) was added to the melted and cooled medium just before pouring plates to give a concentration of 1/25,000.

Thionin (Allied Chemical and Dye Corp.) was used at a concentration of 1/50,000. Initially, three concentrations of thionin blue (British Drug Houses Ltd., London) were used, namely, 1/500,000, 1/1,000,000, 1/2,000,000. Later however only the 1/500,000 concentration was used for routine use. Stock solutions of the dyes were made and steam sterilized. All new batches were checked for activity against known strains. All plates were incubated overnight at 37° before use and no plate was used which had been stored for longer than one week in the refrigerator. The three F.A.O./W.H.O. reference strains *Brucella abortus* strain 544, *B. melitensis* strain 16M and *B. suis* strain 1330, as well as *B. abortus* strain 19 were always included in each series of tests.

All cultures were tested for the requirement of additional CO_2 (above that present in air) for growth, as soon as possible after isolation. Two slopes were inoculated with each isolate; one was incubated aerobically at 37° and the other in an atmosphere containing 10% (v/v) added CO_2. Slopes were examined after 4 days of incubation.

For the dye sensitivity tests, cultures were incubated for 48 hr. in the CO_2 enriched atmosphere and the growth suspended in buffered saline (pH 6·8) to give a concentration of approximately 3×10^9 organisms/ml. One loopful of the suspension was streaked five times on each of the dye plates, without recharging the loop, using a quarter plate per suspension. The loop was sterilized between each plate. All the plates were incubated in the CO_2 enriched atmosphere and read after 5 days of incubation at 37°. The results for each dye were interpreted as negative or 1+ to 5+ depending on whether growth had occurred on 1 or all 5 streaks.

Serological test. Monospecific sera for *Brucella abortus* and *B. melitensis* were prepared from rabbit sera by absorption with the heterologous antigen. One loopful of the serum, diluted 1/5 in phenol saline, was placed on a slide and the culture emulsified. The suspension had not to be too concentrated, otherwise agglutination was delayed. Occasionally the results of slide agglutination tests were confirmed by the tube agglutination test with monospecific sera. Suspensions of the standard strains of *B. abortus* and *B. melitensis* were always included as controls.

RESULTS

The pattern of results obtained with the three F.A.O./W.H.O. reference strains of Brucella, together with *Brucella abortus* strain 19 (the vaccine strain), is shown in Table 1. *Brucella abortus* strain 19 did not grow at concentrations of 1/500,000 and 1/1,000,000 of thionin blue; the reference strains *B. abortus*, *B. melitensis* and *B. suis* did grow at these concentrations. At the 1/2,000,000 concentration of thionin blue, *B. abortus* strain 19 did grow, but in all cases gave only 2+ to 3+ values. *Brucella melitensis* and *B. suis* also showed less growth at the 1/500,000 concentration of thionin blue. A number of recently isolated Brucella cultures was examined, using the three concentrations of thionin blue; a concentration of 1/500,000 was eventually chosen for routine use, since this gave clear-cut results.

In Table 2 are given the results of the examination of 171 cultures of Brucella, together with their source, and it is apparent that there were three groups of *Brucella abortus* which did not grow on thionin blue at concentrations which allowed the growth of the majority of the strains. Of these, the largest group (15·2% of

Table 1. *The results of examining standard strains of Brucella*

Strain	CO_2 requirement	Growth on serum agar plus: Basic fuchsin 1/25,000	Thionin 1/50,000	Thionin blue 1/500,000	Thionin blue 1/1,000,000	Thionin blue 1/2,000,000	Agglutination in monospecific abortus/melitensis sera	
B. abortus strain 544 (reference strain)	+	5+	−	5+	5+	5+	+	−
B. abortus strain 19 (vaccine strain)	−	5+	−	−	−	2+	+	−
B. melitensis 16M (reference strain)	−	5+	5+	3+	5+	5+	−	+
B. suis 1330 (reference strain)	−	−	5+	2+	5+	5+	+	−

Table 2. *The results of examining* 171 *Brucella isolates*

Organism	CO_2 requirement	Growth on serum glucose agar plus: Basic fuchsin (1/25,000)	Thionin (1/50,000)	Thionin blue (1/500,000)	Slide agglutination with monospecific abortus/melitensis sera		No. of strains tested	Source distribution of strains
Typical *B. abortus*	+	5+	−	5+	+	−	118	51 foetal, 67 milk
Aerobic *B. abortus*	−	5+	−	5+	+	−	2	2 milk
B. abortus type II (Wilson)	+	−	−	−	+	−	26	19 milk, 7 foetal
B. abortus Vaccine strain 19	−	5+	−	−	+	−	8	3 foetal, 5 milk
B. abortus Thionine blue sensitive	+	5+	−	−	+	−	7	5 foetal, 2 milk
B. abortus/melitensis	+	5+	−	5+	−	+	2	2 foetal
B. abortus thionin-resistant	−	5+	5+	5+	+	−	1	1 foetal (Holland)
B. melitensis	−	5+	5+	3+	−	+	7	2 foetal, 5 milk

all cultures studied) belonged to the type II (Wilson). Such strains are known to be sensitive to most dyes. A second group, comprising 4·7 % of all strains examined, behaved biochemically exactly like the vaccine strain 19 *B. abortus*; these were recovered both from foetal material and from milk.

The third group (4·1 % of total isolates examined) consisted of strains that did not grow in presence of thionin blue but differed from *Brucella abortus* strain 19 in requiring additional CO_2 for growth; but they did grow in presence of basic fuchsin, thus differing from *B. abortus* type II (Wilson). Such strains were also recovered from milk and foetal material. Most of the strains (69 %) were typical of *B. abortus* in all their reactions, but two aerobic strains were also recovered. The other cultures consisted of *B. melitensis* (4·1 %) and *B. abortus/melitensis* (2·1 %). One strain of thionin-resistant *B. abortus* was received (from Holland).

Cultures obtained from guinea-pigs and the milk of cows experimentally infected with *Brucella abortus* strain 19, and with the thionin blue sensitive strains of *B. abortus* in all cases behaved exactly like the strains used for inoculation, thus confirming their stability *in vivo*.

At the 1/500,000 concentration of thionin blue, a few colonies were observed with strain 19 and with some of the CO_2 requiring thionin blue sensitive strains. After subculture on serum agar these resistant colonies gave profuse growth (5+ readings), indistinguishable from those of the standard *Brucella abortus* 544 on thionin blue at a dilution of 1/500,000.

DISCUSSION

Widespread vaccination of cattle with *Brucella abortus* strain 19 is carried out in this and other countries, and many workers have drawn attention to the need for a simple and reliable method for the differentiation of the vaccine strain from other strains of *B. abortus*. However, no single character can be singled out as characteristic of the vaccine strain only, and it is obvious that the thionin blue sensitivity test is not pathognomonic for strain 19. In addition to *B. abortus* strain 19 and *B. abortus* type II (Wilson), a third group has now been found, also sensitive to thionin blue. Cultures of this group differ from strain 19 in that they need added CO_2 for growth. Preliminary experiments at this laboratory on the virulence of cultures of the thionin blue sensitive group of *B. abortus* have shown that these are as virulent as the standard 544 strain. Virulence tests were based on the duration of bacteraemia in guinea-pigs (Cruickshank, 1957).

Since *Brucella abortus* strain 19 does not appear in the milk of cows after subcutaneous inoculation (Taylor & McDiarmid, 1949) the origin of strains recovered both from milk and foetuses, and indistinguishable from strain 19, is a matter for conjecture. Some of these strains, together with strain 19, have been examined for virulence in guinea-pigs, and all have been of equally low virulence. The only conclusion appears to be that either strain 19 can appear in the milk and foetal material in some cases, or that strains occur in the field that are indistinguishable from strain 19, by our present methods.

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5-Aminoimidazole and its Riboside from Biotin-Deficient Yeast

By J. D. WOODWARD* and C. RAINBOW

Department of Applied Biochemistry, University of Birmingham

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SUMMARY

A purified preparation of the 'amine' which accumulated in the medium during the growth of a strain of *Saccharomyces cerevisiae* (yeast 47) under conditions of partial biotin-deficiency contained two compounds, one of which was chromatographically and electrophoretically identical with synthetic 5-aminoimidazole. Both compounds gave identical colours on diazotization and coupling in the Bratton & Marshall reaction and on treatment with the Pauly imidazole reagent. The other, and major, component of purified 'amine' was shown to be 5-aminoimidazole riboside by its conversion to the free base and ribose on incubation with a bacterial nucleosidase. Synthetic 5-aminoimidazole gave analytical figures for formate, NH_3-N and total-N similar to those previously obtained for purified 'amine'. Evidence was obtained that resting organisms of a mutant *Escherichia coli* converted 'amine' and synthetic 5-aminoimidazole to 5-amino-4-imidazole-carboxamide. One of the adenine derivatives which accumulated instead of 'amine' and hypoxanthine in a medium supplemented with aspartate was tentatively identified as thiomethyladenosine.

INTRODUCTION

Previous papers (Chamberlain, Cutts & Rainbow, 1952; Chamberlain & Rainbow, 1954; Lones, Rainbow & Woodward, 1958) described the accumulation of diazotizable amine ('amine') and hypoxanthine in culture filtrates of *Saccharomyces cerevisiae* (yeast 47) after growth in a biotin-deficient defined medium which contained L-methionine. 'Amine' accumulation was invariably associated with the formation of a pink pigment by the organism. Addition of adenine or aspartate to the medium suppressed the production of this pigment and the accumulation of 'amine' and hypoxanthine. These results were regarded as indicating that a derangement in adenine biosynthesis was conditioned by biotin deficiency. Preliminary studies on a purified preparation indicated that 'amine' was 5-aminoimidazole riboside. In the present paper, evidence is presented which, in conjunction with that already reported, identifies beyond reasonable doubt the chief components of purified 'amine' as 5-aminoimidazole and its riboside, the latter being the major component.

* Present address: Department of Physiological Chemistry, University of Reading.

METHODS

Preparation of 'amine'. Purified 'amine' was prepared from culture filtrates of *Saccharomyces cerevisiae* (yeast 47) grown on a defined medium containing DL-methionine (500 μg./ml.) and D-biotin (0·02 mμg./ml.). Details of the organism, its maintenance and cultivation, and of the purification of 'amine' were as given by Lones *et al.* (1958), except: (*a*) the final eluate was concentrated by lyophilization; (*b*) by using proportionately larger ion-exchange resin columns, a total of 2 l. culture filtrate, yielding 28–38 mg. crystalline material, was handled. These preparations are referred to as purified 'amine' and were used as the chief experimental material in this work.

Preparation of synthetic 5-aminoimidazole. An aqueous solution of 5(4)-aminoimidazole was prepared freshly as required by catalytic reduction of 5(4)-nitroimidazole (Rabinowitz, 1956).

Experiments with suspension of organisms. Resting suspensions of *Escherichia coli* B 96 were used, according to the procedure described by Gots (1950), to demonstrate the conversion *in vivo* of 'amine' to 5(4)-amino-4(5)-imidazolecarboxamide, which accumulates with this purineless mutant. The organism was grown for 24 hr. at 37° in 200 ml. portions of a medium consisting of glucose (0·2%, w/v), tryptone (2%, w/v), and hypoxanthine (20 μg./ml.). The organisms were harvested by centrifugation, washed twice with 0·85% (w/v) saline and resuspended in 1 ml. 0·85% (w/v) saline.

Complete reaction mixtures (6·1 ml.) contained 0·13 M-phosphate buffer (pH 7·2; 3 ml.), 0·02 M-glucose (3 ml.), purified 'amine' solution (20 μl.) and organism suspension (0·1 ml.). Reaction mixtures were incubated for 3 hr. at 37°, after which the organisms were centrifuged down, and the clear supernatant fluids submitted to the Bratton & Marshall diazotization procedure (see below). The absorption maxima of the colours thus produced were determined spectrophotometrically.

Preparation of bacterial nucleosidase. The bacterial nucleosidase used in this work was an unpurified extract of *Lactobacillus brevis* L 4. This organism was grown on 500 ml. portions of the medium described by Chamberlain & Rainbow (1954) except that glucose was omitted and maltose monohydrate (2%, w/v) and $CH_3COONa.3H_2O$ (0·4%, w/v) were included. After incubation at 28° for 24 hr., the organisms were harvested (centrifuge), washed twice with 1% (w/v) KCl solution, resuspended in 5 ml. of that solution, and then disrupted in a tissue disintegrator (H. Mickle, Gomshall, Surrey) with ballotini beads (0·2–0·3 mm. diameter). During the process the temperature was kept below 5° by alternating cycles (5 min.) of shaking and cooling in ice. The disrupted suspension was poured off from the beads and centrifuged at 2° to remove cell debris. The supernatant fluid was used immediately, or stored at −20° at which temperature it retained its nucleosidase activity for at least 2 months. This extract readily hydrolysed adenosine to adenine and ribose.

Analytical methods

Diazotizable amine. This was determined by the colour produced in the Bratton & Marshall diazotization and coupling procedure as described by Chamberlain & Rainbow (1954).

Pauly reaction. This was performed on solutions in accordance with the modification suggested by Koessler & Hanke (1919) or on paper by the modification introduced by Ames & Mitchell (1952).

Ribose. In solutions this was determined by the orcinol method of Drury (1948). On paper chromatograms and electrophoretograms, free ribose was detected by aniline hydrogen phthalate and compared with authentic D-ribose as marker. Combined ribose was detected directly on paper by the lead tetra-acetate method of Buchanan, Dekker & Long (1950) and, after elution, by the orcinol method.

Purine and purine derivatives. These were detected on paper chromatograms and paper electrophoretograms by ultraviolet photography. After elution, absorption spectra were determined in an S.P. 500 quartz spectrophotometer (Unicam, Cambridge).

Paper chromatography. A conventional descending solvent technique, with Whatman no. 3 or no. 4 paper and (unless otherwise stated) the butanol + acetic acid + water (125 + 30 + 125) solvent of Woiwod (1949), was used.

Paper electrophoresis. This was carried out in 0·05 M-buffer solutions, with 22 in. strips of Whatman no. 3 paper in an enclosed strip technique. Voltages of 2000–3000 V. were usually used.

RESULTS

Chromatographic and electrophoretic behaviour of purified 'amine'

When subjected to paper chromatography or paper electrophoresis samples (about 500 μg.) of purified 'amine' gave two major diazotizable spots (see also Lones *et al.* 1958). Table 1 summarizes some of the properties of the components thus separated.

Table 1. *Properties of two diazotizable materials separable from purified 'amine'*

R_F values are given for descending chromatograms on Whatman no. 4 paper with solvent *n*-butanol + acetic acid + water (125 + 30 + 125). Migrations are quoted as cm. moved in 0·05 M-acetate buffer (pH 5·0) when 3000 V. was applied for 50 min. Pentose was tested for by the lead tetra-acetate and the orcinol reactions; phosphate by Allen's (1940) method.

Component	R_F value	Electrophoretic migration	Bratton & Marshall colour ($\lambda_{max.}$; mμ)	Pauly colour	Pentose	Phosphate
Major	0·33	22·5	Orange-red (500)	Yellow	Present	Absent
Minor	0·46	37·7	Orange-red (500)	Blue	Absent	Absent

The results indicated that the slower-moving compound (chromatographically; electrophoretically) was an imidazole riboside derivative, while the faster-moving one was the corresponding free base, as Lones *et al.* (1958) had found. The riboside was quantitatively the major constituent. Supporting evidence was obtained by electrophoresis at different pH values. At more acid pH values, the ionization of the diazotizable amino group was enhanced and migration increased: at less acid pH values, the opposite behaviour was observed (Table 2). The results with borate buffer (pH 10) were particularly significant. This buffer has been used for the electrophoretic separation of sugars (Foster, 1952), the borate ion complexing with

the free hydroxyl groups of the sugar and giving good migrations towards the anode. In borate buffer the migration of the riboside was reversed, while the free base continued to migrate towards the cathode but at a much decreased rate because of the suppression of ionization of the amino group at pH 10. Ascending chromatography in a solvent consisting of 85% (w/v) ammonium bicarbonate in water (Hems, 1959) also reversed the order in which the diazotizable spots separated. This behaviour was consistent with the proposed relationship between the two substances, the presence of the ribose moiety conferring on the riboside more hydrophilic properties.

Table 2. *Electrophoretic behaviour of the diazotizable constituents of purified 'amine' preparations*

Spots (20 μl.) were applied to Whatman no. 4 paper and migration measured after 50 min. application of 3000 V.

	Migration			
	Riboside		Free base	
Buffer	(cm.)	direction	(cm.)	direction
Formate (pH 3·5)	31·5;	to cathode	51·5;	to cathode
Acetate (pH 5·0)	22·5;	to cathode	37·7;	to cathode
Phosphate (pH 7·0)	10·3;	to cathode	15·7;	to cathode
Borate (pH 10·0)	11·3;	to anode	6·4;	to cathode

Identity of the free-base component of 'amine' preparations with 5(4)-aminoimidazole

Synthetic 5-aminoimidazole gave a strong orange-red Bratton & Marshall colour with an absorption maximum (500 mμ) identical with that given by purified 'amine' preparations. The chromatographic mobilities of the synthetic material and the free-base constituent of 'amine' were identical, as were their electrophoretic migrations at pH values 3·5, 5·0, 7·0 and at pH 10·0 (in borate buffer). In the Pauly reaction, the synthetic material and the free-base component gave identical blue colours.

The ultraviolet absorption spectrum of the purified 'amine' used in this work differed slightly from that reported by Lones *et al.* (1958) in that the feeble peak at 238 mμ was absent. The elimination of this peak may have been the result of the somewhat improved method of 'amine' preparation used.

Samples of synthetic 5-aminoimidazole were hydrolysed and then analysed for formic acid, ammonia and total-N by the methods described by Lones *et al.* (1958). For two different preparations the molar ratios of formate: NH_3-N: total-N (referred to NH_3-N as 2) were 1:2:4 and 0·93:2:4. Considering the great instability of 5-aminoimidazole and that only unpurified aqueous solutions were available for analysis, these values compared fairly well with the theoretical ratio of 1:2:3, and with the ratios obtained by Lones *et al.* (1958) for purified 'amine'. The possible presence of small quantities of unchanged nitroimidazole may be a cause of the high figures for total-N in the synthetic material.

Enzymic hydrolysis of the riboside component

Portions of purified 'amine' solution containing about 400 μg. were incubated for 24 hr. with 0·5 ml. *Lactobacillus brevis* L4 extract. After concentration *in vacuo* at room temperature, samples of the reaction mixtures, together with reference spots of D-ribose and synthetic 5-aminoimidazole, were chromatographed and examined for diazotizable compounds and for free ribose. The chromatograms showed that extensive but not complete hydrolysis of 'amine' had occurred, the amount of free-base component and of free ribose had increased, and the amount of riboside component had diminished. These changes did not take place when boiled L4 extract was substituted for unboiled extract. Under the above conditions adenosine, guanosine, inosine, xanthosine and cytidine underwent extensive hydroiysis in 3 hr. by the bacterial extract, whereas a much longer period (12 hr.) was required before the riboside component of 'amine' underwent appreciable hydrolysis. This slow rate of action may reflect the pseudo-nucleoside structure of the 'amine' riboside substrate, in which an imidazole derivative takes the place of the 'enzymically preferable' purine or pyrimidine moiety of normal nucleosides.

Table 3. *Conversion of 'amine' to a new diazotizable substance by* Escherichia coli *B*96

The complete reaction mixture contained: 0·13 M-phosphate buffer (pH 7·2; 3·0 ml.); 0·02 M-glucose (3·0 ml.); purified 'amine' solution (about 600 μg. in 20 μl.); 0·1 ml. washed suspension of *E. coli* B96. The colour was that obtained when the Bratton & Marshall procedure was applied to the supernatant fluids after reaction had proceeded for 3 hr. at 37°.

Reaction mixture	Colour	
	Visual	($\lambda_{max.}$ mμ)
Complete	Purple	520
'Amine' omitted	Colourless	—
Organisms omitted	Orange-red	499
Phosphate omitted	Orange-red	499
Glucose omitted	Orange-red	500
Complete + boiled organisms	Orange-red	499

Conversion of 'amine' to 5-amino-4-imidazole carboxamide in vivo

The change in colour developed on diazotization and coupling with the Bratton & Marshall reagents has been used as an index of the conversion of 5-aminoimidazole to 5-amino-4-imidazolecarboxamide (Love & Gots, 1955; Friedman & Moat, 1958). The changes which occurred in the reaction mixture when freshly prepared 'amine' solutions were incubated with washed suspensions of *Escherichia coli* B96 are recorded in Table 3 which shows that, in a complete system for which living organisms, glucose and inorganic phosphate were all essential, there was produced diazotizable material giving a purple colour, quite distinct from the orange-red colour given by 'amine' itself. The absorption maximum of this material (520 mμ) which gave the purple colour was nearer that of 5-amino-4-imidazolecarboxamide (525 mμ) than of 'amine' (500 mμ) so that the change observed was consistent with

a substantial conversion of 'amine' to 5-amino-4-imidazolecarboxamide. Similar changes occurred when synthetic 5-aminoimidazole was substituted for 'amine' in the complete reaction system. No purple colour resulted when 'amine' or synthetic 5-aminoimidazole was omitted from the reaction mixtures, i.e. the changes did not represent new synthesis of 5-amino-4-imidazolecarboxamide.

The complete conversion of 'amine' or synthetic 5-aminoimidazole to 5-amino-4-imidazolecarboxamide, as indicated by azo colour with an absorption maximum of 525 mμ, was not obtained even when complete reaction mixtures were incubated for longer periods. However, artificial mixtures of 'amine' and synthetic 5-aminoimidazole gave colours having a single absorption maximum intermediate between the maxima given by either component singly, the actual value depending on the relative proportions of the components. These results are consistent with the belief that 'amine' is 5-aminoimidazole (or a near derivative) and that it undergoes transformation to 5-amino-4-imidazolecarboxamide by living *Escherichia coli* B96.

'Amine' and pigment formation

Purified 'amine' was a pale greenish yellow crystalline solid which slowly darkened with the formation of deep purplish pigments which settled out of solution in small flocculent masses even during storage at 0–4° *in vacuo* in the dark. This darkening and precipitation of pigments was most marked in relatively concentrated aqueous solutions (5–20 μg./ml.) and was always associated with loss of diazotizability. On chromatographic or electrophoretic development on paper, 'amine' left a trail of apparently similar purplish pigments; solutions of synthetic 5-aminoimidazole behaved similarly. The properties and manner of formation of these pigments indicate them to be substances of high molecular weight formed by polymerization involving the primary (diazotizable) amino group of 5-aminoimidazole. Hunter & Hlynka (1941) produced evidence that pigment production from 5-aminoimidazole involved deamination followed by oxidative condensations; during the course of the present work it was observed that a remarkable series of yellow-orange and red zones appeared during the chromatographic development of relatively large amounts of synthetic 5-aminoimidazole. All these substances were less mobile than 5-aminoimidazole itself and may well have represented different stages of polymer formation. The readiness with which 'amine' undergoes transformation into pigments suggests that the pink pigment characteristic of *Saccharomyces cerevisiae* yeast 47 when it is accumulating 'amine' is derived from 'amine' itself.

Formation of thiomethyladenosine by Saccharomyces cerevisiae, *yeast* 47

Lones *et al.* (1958) showed that the addition of L-aspartate prevented the accumulation of 'amine' and hypoxanthine during the growth of *Saccharomyces cerevisiae* yeast 47 on their medium II; instead, adenine derivatives accumulated. One of these compounds was tentatively identified as thiomethyladenosine as follows. Concentrations of the adenine-containing compounds prepared according to Lones *et al.* (1958) were submitted to separation by paper chromatography; the fastest-moving ultraviolet-absorbing spot (R_F value about 0·8) was located, cut out and eluted. Various eluates were prepared and the material in them was shown to have the

following properties: (*a*) it gave positive reactions in the modified nitroprusside test of Csonka & Denton (1946), and in Feigl's (1939) test for the =C=S or ≡C—SH linkage; (*b*) on hydrolysis with 2 N-H_2SO_4 at 100° for 2 hr. it yielded a single purine derivative chromatographically and spectrophotometrically identical with adenine; (*c*) its ultraviolet spectrum in 0·1 N-HCl was indistinguishable from that of authentic adenosine ($\lambda_{max.}$ 257 mμ); (*d*) on electrophoresis in 0·05 M-phosphate buffer (pH 7·0) it migrated slowly towards the cathode (0·066 cm.hr.$^{-1}$/V.cm.$^{-1}$ as compared with 0·070 cm.hr.$^{-1}$/V.cm.$^{-1}$ quoted for thiomethyladenosine by Baddiley, Cantoni & Jamieson, 1953); (*e*) it gave a positive but weak response in the orcinol pentose test; this accords with the report of Baddiley *et al.* (1953) that only 32% of the pentose present in thiomethyladenosine was detected after 40 min. heating in this test; (*f*) it contained no phosphorus detectable by Allen's (1940) method after preliminary incineration with 60% (w/v) perchloric acid. The absence of phosphate was also confirmed by its electrophoretic behaviour.

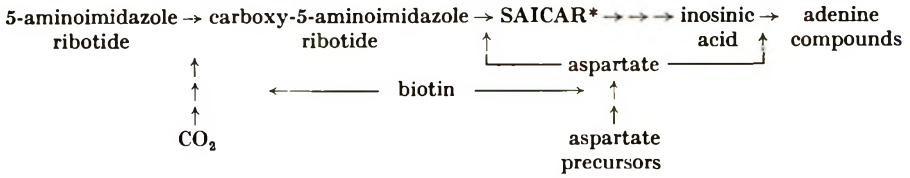
DISCUSSION

Taken in conjunction with previous work (Chamberlain & Rainbow, 1954; Lones *et al.* 1958) the present work establishes the identity of the diazotizable amine accumulated by *Saccharomyces cerevisiae* yeast 47 under conditions of biotin-deficiency as a mixture of 5-aminoimidazole riboside and a smaller quantity of the aglycone. It was not possible to determine whether the free base was present as such in the culture filtrate or whether it arose by hydrolysis during purification since the Bratton & Marshall test did not distinguish between riboside and free base. The presence of traces of other diazotizable substances is not excluded.

The role of 5-aminoimidazole as an intermediate in purine biosynthesis at the nucleotide level is now well substantiated (Love & Gots, 1955; Levenberg & Buchanan, 1956; Lukens & Buchanan, 1959). In the light of this knowledge, the accumulation of 'amine' hypoxanthine by yeast 47 may be interpreted as due to a block in aspartate synthesis conditioned by biotin-deficiency. First, the conversion of inosinic acid to adenylic acid involves the amino group of aspartate (Abrams & Bentley, 1955). Secondly, the conversion of 5-aminoimidazole ribotide to 5-amino-4-imidazole(*N*-succinylo)-carboxamide ribotide via the intermediate 5-amino-4-imidazolecarboxylic acid ribotide involves both aspartate and the incorporation of CO_2 (Lukens & Buchanan, 1959). Biotin is known to be involved in aspartate metabolism in yeast (Winzler, Burk & du Vigneaud, 1944) and in bacteria (Lichstein & Umbreit, 1947; Broquist & Snell, 1951) and in bacterial CO_2 fixation (Broquist & Snell, 1951). Hence, biotin deficiency may well cause hypoxanthine, 5-amino-imidazole and its riboside to be excreted as the respective degradation products of the inosinic acid and 5-aminoimidazole ribotide (or the readily decarboxylated carboxy-5-aminoimidazole ribotide) which accumulate initially within the cell.

A supply of exogenous aspartate removes these biotin-dependent blocks in purine biosynthesis, and then adenine-containing compounds accumulate instead of 'amine' and hypoxanthine. The presence of thiomethyladenosine as one of these compounds is readily explained since it is a degradation product of S-adenosyl-methionine, which accumulates in yeast cells grown on methionine-rich media

(Schlenk & DePalma, 1957). The following scheme illustrates the proposed relationships:



* 5-amino-4-imidazole-(*N*-succinylo)-carboxamide.

Moat *et al.* (1956) have also reported the accumulation of diazotizable amine, believed to be 5-aminoimidazole riboside (Friedman & Moat, 1958), by a biotin-dependent strain of *Saccharomyces cerevisiae* when growing in the presence of methionine; as with *S. cerevisiae* yeast 47, adenine and aspartic acid inhibited its accumulation but, unlike yeast 47, 'amine' accumulation was accompanied by that of inosine and not hypoxanthine.

The role of methionine in 'amine' formation is not clear. Methionine greatly enhances 'amine' production but it has no obvious effect on hypoxanthine accumulation, which appears to be solely the result of the imposed biotin deficiency. In liver slices methionine can give rise to formate and serve as a source of the β-carbon of serine (Siekevitz & Greenberg, 1950). In 'amine' synthesis with *Saccharomyces cerevisiae* yeast 47, methionine possibly plays a similar role in generating the one-carbon fragment required for the conversion of glycinamide ribotide to formyl-glycinamide ribotide, a precursor of 5-aminoimidazole ribotide.

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Germination Under Alkaline Conditions and Transmission of Alkali Resistance by Endospores of Certain Strains of *Bacillus cereus* and *Bacillus circulans*

By MARGARET E. CHISLETT*
Microbiology Department, University of Reading, England
and D. J. KUSHNER
Forest Insect Laboratory, Sault Ste. Marie, Ontario, Canada

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SUMMARY

Endospores of *Bacillus cereus* Frankland and Frankland strain R (which is able to grow under highly alkaline conditions) germinated at a higher pH value than spores of an alkali-sensitive strain, Mu-3055. Spores of *B. circulans* Jordan strain Ru 38 (able to grow at pH 11·0) germinated at pH 11·0. Spore germination at any pH value was generally followed by outgrowth to the vegetative rod. Outgrowth of germinated spores of *B. cereus* R at pH 10·0 and of those of *B. circulans* Ru 38 at pH 11·0 took place at 30° but not at 37°. Growth from a vegetative inoculum of *B. cereus* R at pH 10·0 or 10·3 took place more readily at 30° than at 37°. The ability of the vegetative forms of both bacterial species to grow under alkaline conditions was transmitted through the endospore.

INTRODUCTION

Two strains of alkali-resistant *Bacillus* species have recently been described: a strain of *B. cereus* Frankland and Frankland able to grow in media of pH 10·3 (Kushner & Lisson, 1959), and a strain of *B. circulans* Jordan able to grow at pH 11·0 (Chislett & Kushner, 1961). Previous work was largely concerned with the effect of alkaline media on the vegetative growth of these organisms, but gave no information about the effect of such alkaline media on endospore germination. We have now examined the effect of alkaline conditions on the development of the vegetative form from the endospore and have examined how the ability of the vegetative form to grow under alkaline conditions is transmitted through the endospore.

METHODS

Organisms. Bacillus cereus strain Mu-3055 (non-adapted: sensitive to alkali) and *B. cereus* strain R (adapted from Mu-3055 and resistant to alkali); both described by Kushner & Lisson (1959); *B. circulans* strain Ru 38 (maintained routinely on buffered nutrient agar (pH 7·4) but capable of growth at pH 11·0); described by Chislett & Kushner (1961).

* Present address: Division of Applied Biology, Microbiology Section, National Research Council, Ottawa 2, Canada.

Culture media. The media used were: phosphate-buffered nutrient broth (BNB) and phosphate-buffered nutrient agar (BNA) of final pH values 7·4 and higher, prepared as before (Kushner & Lisson, 1959; Chislett & Kushner, 1961).

Preparation of spore suspensions. The three strains were subcultured several times in BNB (pH 7·4), with incubation at 30°, before finally spreading 1 ml. of 6 hr. culture on the surface of 50 ml. BNA (pH 7·4) in a 20 oz. bottle; $MnSO_4.4H_2O$ had been added to the BNA medium to a final concentration of 5 p.p.m. Mn^{++}, and additional agar (Davis, New Zealand) was added to give a final concentration of 3% (w/v) agar in the medium. The *Bacillus cereus* strains were incubated at 30° for 7 days and the *B. circulans* strain for 14 days. Spores were harvested by washing off the growth with sterile distilled water and glass beads. The resulting suspension was washed six times in sterile distilled water at 5° and the deposit, consisting almost entirely of spores, resuspended in 0·01 M-phosphate buffer (pH 7·4). After making viable spore counts on nutrient agar plates, the spore suspensions were diluted with 0·01 M-phosphate buffer (pH 7·4) to contain 10^9 viable spores/ml. and held at 5° until required. Before making viable counts and before use in the experiments described in Table 1, the portion of each spore suspension used was heated at 80° for 15 min., then cooled rapidly in an ice bath and held at 5° until required. In the experiments shown in Table 2 and in those with shaken cultures of *B. circulans*, the spores used had been prepared in a similar manner, with the following variations in technique: BNA medium (pH 7·4) with 5 p.p.m. Mn^{++} added was used; incubation was for 7 days at 37° instead of at 30°. At the end of this time there was a high degree of sporulation and comparatively few vegetative forms remained. The washed suspensions of spores + bacteria were heated for 1 hr. at 60°, adjusted to a concentration of 3×10^8 viable spores/ml. and stored in distilled water at 5°.

Germination of spores at pH 7·4 *and at alkaline* pH *values.* Spore suspensions were added to 10 ml. BNB medium in 6 in. × $\frac{5}{8}$ in. tubes at pH values from 7·4 to 11·0, to give a final concentration of 10^7 viable spores/ml. Incubation was at 30° and 37°, and examinations were made at 2, 4, 6, 8, 10, 12 and 24 hr. Spore germination was detected by examining the suspension in a haemocytometer, by using a 4 mm. phase contrast objective (see Berger & Marr, 1960). Under phase contrast, the ungerminated spores appeared refringent, thus allowing a direct spore count. The staining method of Conklin (1934) with malachite green and mercurochrome, whereby ungerminated spores stained green and germinated spores and vegetative forms red, gave a good correlation with the results obtained by the haemocytometer and phase contrast method. For quantitative determinations of the number of germinated and ungerminated spores/ml., and to estimate percentage germination, 0·01 ml. spore suspension was delivered by a dropping pipette on to a marked area of 1 $cm.^2$ on a slide and the suspension stained by Conklin's method. As soon as microscopic observation showed that the spores were germinating, the pH values of the cultures and of control tubes of uninoculated media were measured. The results have been recorded as the relative extent of germination at the earliest time this process was observed (see Table 1). Once germination of even a small proportion of spores had taken place, subsequent vegetative cell growth lowered the pH value of the medium (Kushner & Lisson, 1959; Chislett & Kushner, 1961). For this reason, information about the time-course of germination of a spore population at a given pH value could not readily be obtained in these experiments.

Outgrowth. The successive changes leading to the formation of a vegetative rod from a germinated spore have been termed 'outgrowth' (Campbell, 1957). This process has been recorded here qualitatively simply by noting the first appearance of vegetative rods in the culture medium.

Transmission of alkali resistance. Growing cultures in BNB medium (pH 7·4) resulting from spore germination at this pH value were inoculated into duplicate tubes of BNB medium at different pH values between 7·4 and 11·0. The inocula were two 4 mm. loopfuls of an 18–21 hr. culture at 37°. The tubes were incubated at 30° or 37° and examined at intervals. As soon as growth was visible as turbidity the cultures were examined microscopically, and pH measurements were made of the cultures and of control tubes of uninoculated media.

RESULTS

Spore germination and outgrowth

At pH 7·4, both alkali-sensitive and alkali-resistant strains of *Bacillus cereus* showed considerable germination and outgrowth after 2 hr. at 30° or 37° (Table 1). At pH 9·5, spore germination and outgrowth took place much more rapidly and extensively with the alkali-resistant strain than in the alkali-sensitive strain. Both processes were seen at pH 10·0 with *B. cereus* R, but not with *B. cereus* Mu-3055. Germination did not take place at pH values higher than 10·0 with either strain. At the highest pH values at which germination was possible for each strain, this process and/or outgrowth took place more slowly, if at all, at 37° than at 30°.

Table 1. *Germination and outgrowth of spores of alkali-sensitive and alkali-resistant* Bacillus cereus *and of alkali-resistant* B. circulans

Organism	pH value	Temperature	Spore germination*	Outgrowth†
B. cereus Mu 3055 (alkali-sensitive)	7·4	30°	+ + + at 2 hr.	+ at 2 hr.
		37°	+ + + at 2 hr.	+ at 2 hr.
	9·5	30°	(+) at 4 hr.	+ at 4 hr.
		37°	(+) at 24 hr.	+ at 24 hr.
	10·0, 10·3	30°	− at 24 hr.	− at 24 hr.
		37°	− at 24 hr.	− at 24 hr.
B. cereus R (alkali-resistant)	7·4	30°	+ + + at 2 hr.	+ at 2 hr.
		37°	+ + + at 2 hr.	+ at 2 hr.
	9·5	30°	+ + + at 2 hr.	+ at 4 hr.
		37°	+ + at 2 hr.	+ at 4 hr.
	10·0	30°	(+) at 12 hr.	+ at 24 hr.
		37°	(+) at 12 hr.	− at 24 hr.
	10·3, 10·7	30°	− at 24 hr.	− at 24 hr.
		37°	− at 24 hr.	− at 24 hr.
B. circulans Ru 38 (alkali-resistant)	7·4	30°	+ at 2 hr.	+ at 4 hr.
		37°	+ at 2 hr.	+ at 4 hr.
	9·5, 10·0, 10·3	30°	+ + + at 2 hr.	+ at 2 hr.
		37°	+ + + at 2 hr.	+ at 2 hr.
	10·7	30°	+ at 2 hr.	+ at 4 hr.
		37°	+ at 2 hr.	+ at 4 hr.
	11·0	30°	+ at 4 hr.	+ at 4 hr.
		37°	+ at 4 hr.	− at 24 hr.

* % spores germinated reported as follows: (+) 5 %, + 5–20 %, + + 20–50 %, + + + > 50 %.
† Outgrowth = appearance of rods at time indicated.

The results obtained with spores of the alkali-resistant *Bacillus circulans* strain Ru 38 differed markedly from those obtained with the *B. cereus* strains in that germination was more extensive after 2 hr. at pH values from 9·5 to 10·3 than at pH 7·4. Outgrowth was observed within 2 hr. with the pH range 9·5–10·3, but was not observed until 4 hr. at pH 7·4. Results at pH 10·7 or 11·0 were similar to those found at pH 7·4, except that at pH 11·0, outgrowth took place at 30° but not at 37°.

Transmission of alkali resistance

Following spore germination at pH 7·4, the vegetative form of *Bacillus cereus* strain Mu-3055 in standing tubes showed growth after 4 hr. at pH 7·4 and pH 9·5, at 30° and at 37°; no growth occurred at pH 10·0 or higher. *B. cereus* strain R showed growth after 4 hr. at pH 7·4 and pH 9·5, at 30° and at 37°. At pH 10·0 and pH 10·3, growth was observed within 12 hr. at 30°, but not until 24 hr. at 37°. *B. circulans* strain Ru 38 showed growth after 4 hr. at 37° in the pH range 7·4 to 11·0, but at 30° growth was not observed in this pH range until after 6 hr. incubation.

Table 2. *Effect of* pH *value on the growth of alkali-sensitive and alkali-resistant* Bacillus cereus *following spore germination at* pH 7·4

In Expts. 2 and 3 one drop of spore suspension of strain Mu-3055 or strain R was added to 10 ml. BNB (pH 7·4) medium and incubated 4 hr. with shaking at 37°, to an optical density of 0·7–0·8. Three drops of each culture were added to tubes of BNB medium of different pH values. Shaking and measurements of optical density were as described by Kushner & Lisson (1959).

Data for Expt. 1 from Table 3 of Kushner & Lisson (1959).

Expt.	Inoculum	pH value	Sensitive (strain Mu-3055) $T_{0.4}$ (hr.)*	Resistant (strain R) $T_{0.4}$ (hr.)*
1	Bacteria maintained on plates (mixture of spores and vegetative forms)	9·5	16	7·2
		9·8	> 25	11·0
		10·0	> 25	24·8
		10·1	> 25	> 25
2	Bacteria obtained by spore germination, 2 weeks after preparation of spores	9·4	9	5
		9·8	> 24	10
		9·9	> 24	20
3	Bacteria obtained by spore germination, 16 weeks after preparation of spores	9·4	10	4
		9·8	> 24	21
		9·9	> 24	> 24

* Time at which cultures reached a (net) optical density of 0·4.

An examination of the effects of pH value on the vegetative growth of *Bacillus cereus* derived from spores was also made in shaken cultures at 37° (Table 2). Once growth had begun there was, as before noted (Kushner & Lisson, 1959), little effect of the initial pH value on the growth rate; and the growth rates observed were similar to those reported by Kushner & Lisson (1959). Thus vegetative forms derived from spores 2 weeks after sporulation had retained full alkali resistance. After an additional 14 weeks storage of the spores in distilled water at 5°, however, there was some loss of alkali resistance; the vegetative forms from germinated spores were no longer able to grow at pH 9·9 in 24 hr.

In an experiment with spores of the alkali-resistant *Bacillus circulans*, an inoculum of the vegetative form was prepared by incubating these spores overnight on a BNA medium plate (pH 7·4), and a series of tubes containing BNB medium of different pH values was inoculated and shaken as before (Chislett & Kushner, 1961). The growth curves obtained were virtually identical with those given by the alkali-resistant *B. circulans* which had been maintained at pH 7·4 and transferred as a mixture of spores and vegetative forms (Chislett & Kushner, 1961, Fig. 1*a*).

These results indicated that growth of the alkali-resistant bacteria took place at a slightly higher pH value in standing than in shaken cultures. However, in standing cultures at 30° and 37° *Bacillus cereus* R showed growth at pH 10·3 (as above) but not at pH 10·7; and *B. circulans* Ru 38 showed growth at pH 11·0 (as above) but not at pH 12·0. The highest pH value at which growth was observed for *B. circulans* Ru 38 was pH 11·4 (pH value of standing culture) after 18 hr. at 37°.

DISCUSSION

In obtaining *Bacillus cereus* strain R the culture method used was designed to select vegetative forms with ability to grow at higher pH values than could the original culture, rather than to select spores that could germinate at higher pH values (Kushner & Lisson, 1959). It was conceivable that such alkali-resistant vegetative forms might produce spores whose germination was as sensitive to highly alkaline conditions as were spores of the original alkali-sensitive cultures. It has now been found that spores of this selected alkali-resistant strain can germinate and develop into vegetative rods at higher pH values than spores of the original strain Mu-3055. The upper pH limit for spore germination of *B. cereus* strain R spores is slightly lower than the upper limit for growth.

Spores of the alkali-resistant *Bacillus circulans* Ru 38 can also germinate in highly alkaline media; when incubation is at 30°, they can germinate in a medium of the highest pH value at which growth is possible. Germination and outgrowth took place more rapidly at pH 9·5–10·3 than at pH 7·4; and it should be noted that vegetative growth of *B. circulans* Ru 38 began after a shorter lag period at pH 9·9 than it did at pH 7·4 (Chislett & Kushner, 1961). The fact that spores of the bacteria studied had the ability to develop into vegetative forms and these to multiply at high pH values suggests that the alkali resistance of these processes may have a common physiological basis.

In so far as it has been studied, the effect of temperature on the processes of growth and spore germination at different pH values seems complex. Although a temperature of 37° could cause growth to take place more rapidly than a temperature of 30°, at the highest pH values at which germination and growth were possible, germination, outgrowth and vegetative growth of *Bacillus cereus* R and outgrowth of *B. circulans* Ru 38 took place more rapidly, or only at 30°. Thus, despite its ability to accelerate growth, the higher temperature also increased the toxic effects of highly alkaline media.

The alkali-resistant *Bacillus circulans* strain Ru 38 has been deposited with the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, and allotted the number NCIB 9218.

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The Diminution of Variation in Bacterial Populations with Special Reference to *Klebsiella pneumoniae* and Drug Resistance

By W. HOWARD HUGHES
Wright-Fleming Institute, St Mary's Hospital Medical School, Paddington, London, W. 2

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SUMMARY

Single organisms of *Klebsiella pneumoniae* were repeatedly selected for resistance on streptomycin agar yielding a population with increased resistance clearly different from that of the parent strain. These two strains were grown together and when retested a uniform population was found to be present with streptomycin resistance intermediate between the initial two.

Two strains of *Klebsiella pneumoniae*, one the type-strain, the other a chloramphenicol-resistant mutant with a different colonial appearance, were grown together and the mixture sampled at intervals. The two populations became progressively more alike until within six hours they were indistinguishable. The resulting uniform population was intermediate in sensitivity to chloramphenicol and the colonies could no longer be assigned easily to either parental type on morphological grounds. When the two strains were separated by a collodion membrane, this diminution of variation did not occur.

The results cannot be explained by overgrowth of one strain by the other. It is suggested that something analogous to inbreeding is taking place.

INTRODUCTION

Previous work has shown that individual bacteria within clones differ from one another (Hughes, 1952, 1953, 1955*a*, *b*). This type of variation is continuous and more common than mutation as ordinarily understood. This work was summarized by Hughes (1957), and the conclusions reached agree in the main with the view advanced by Yudkin (1953) based on his own studies about acquired drug resistance. The subject has received mathematical consideration from Powell (1956, 1958).

The following work was undertaken to find out why, if variation could be found between the individuals, the population as a whole remained stable. In an organism in which sexual reproduction is recognized this is no problem, since any major variation or mutation which is not lethal will be distributed by random crossing. The predominant views on bacterial reproduction predispose against accepting a similar mechanism among cells which multiply by so-called simple fission.

It has now been found that when two populations, derived from the same source but which are different in regard to resistance to streptomycin, chloramphenicol and other characters, are grown together for a short time, a uniform population inter-

mediate in respect to the characters studied emerges very rapidly. This diminution of variation appears to differ in its general properties from the changes associated with established parasexual mechanisms, such as transduction, transformation or conjugation.

METHODS

Organism. A strain of *Klebsiella pneumoniae* (NCTC 7242) first obtained in 1946 for the assay of streptomycin was used. This had been repeatedly subcultured.

Isolation of single organisms. A De Fonbrune micromanipulator and a cheap modification of his oil chamber (made by Hawksley and Sons Ltd., London) were used throughout this series of experiments.

Experimental procedures. Young cultures were obtained by inoculating one loopful of the stock culture stored on the bench into nutrient broth in a screw-capped bottle. This was clipped to a drum rotating at 8 rev./min. in an incubator at 37° and left for 3 hr. One 3·5 mm. loopful of this subculture was spread on a block of agar about 1·0 cm. square mounted on the cover glass of the oil chamber. After air drying the coverslip was reversed and the oil introduced. The specimen was then incubated, usually for 3 hr., and the size of individual colonies recorded. In the presence of streptomycin or chloramphenicol, the colony diameter is an index of the average resistance of the organisms of the colony to the concentration of antibiotic in the agar. Measurements in arbitrary units were made with an eye-piece micrometer (total magnification usually 240 diameters); 100 colonies were measured and the histogram of the population plotted. Any colonies which had fused were discarded from the count.

The planting out of single organisms was impracticable since the time lag between the first and the last isolations would invalidate the investigation. On special occasions the specimens were examined when only one or two divisions had taken place and any colonies found to be too near to one another were decreased in number by removing those likely to come into contact. This was not usually necessary since, with the small implants used less than 5 % of the colonies would have been involved and these could have been recognized and excluded after incubation.

From the most rapidly growing colonies single organisms were picked and grown on to give strains which were again tested for their rates of growth at the same concentration of antibiotic. Those found to be growing better than the previous strains were retained as having a higher resistance. This sequence was repeated until a strain had been selected which grew as well on antibiotic agar as the parent strain on plain nutrient agar. It had already been demonstrated that single streptomycin-resistant cells of *Klebsiella pneumoniae* could be selected and stable strains obtained (Hughes, 1957) as had been done previously with staphylococci with regard to penicillin (Hughes, 1952; Eagle, Fleischman & Levy, 1952).

Having obtained two strains which could be separated by the rates of growth of their colonies in the presence of antibiotic it became possible to reconsider Yudkin's hypothesis on populations of bacteria (Yudkin, 1953). Clearly if differences between organisms could be demonstrated in small populations, then in large populations a great diversity should occur; experience does not support this and there must be some mechanism to counter excessive variation.

RESULTS

Experiment 1 was designed to demonstrate whether or not the two strains grown together would influence one another's resistance to antibiotic. A loopful of a 3 hr. broth culture of the original strain and a similar inoculum of the 17th single cell subculture (each of which had been selected for increased resistance) were separately planted on 1·0 cm. square blocks cut from a poured nutrient agar plate containing 10 μg./ml. of streptomycin. An equal volume of a 17 hr. mixed culture of the two strains diluted to the equivalent concentration was similarly seeded. The three blocks were arranged in the same oil chamber, covered with sterile paraffin oil and incubated at 37° for 3 hr., when the colony diameters were measured.

The resulting histograms are shown in Fig. 1; after incubation together in mixed culture instead of the two peaks characterizing the separate cultures, only a uniform population was present, the average colony diameter falling between the two extremes but, on an arithmetic scale, nearer to the size of the 'sensitive' colony. This strongly suggested that the two populations had, if not 'interbred', at least influenced one another, and further study of the phenomenon was made.

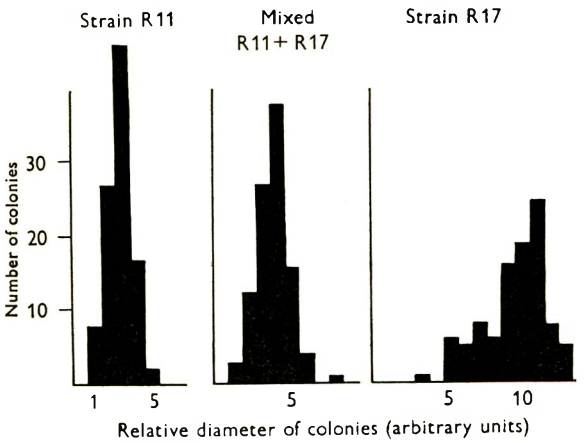

Fig. 1. Histograms showing distribution of size among colonies, growing for 3 hr. on agar containing streptomycin (10 μg./ml.), derived from a streptomycin-sensitive strain of *Klebsiella pneumoniae* NCTC 7242 (R11) and a more resistant strain derived from it (R17). Colony diameter measured in arbitrary units.

Experiment 2. Certain difficulties had been apparent in the earlier experimental arrangements. For example, with streptomycin it was only possible to select for resistance, not for sensitivity, since streptomycin is bactericidal and the most susceptible members of all the populations were lost. Further, streptomycin was unstable under the conditions in which the agar blocks were prepared. In all subsequent experiments freshly prepared chloramphenicol solutions were used.

Into a dense suspension of *Klebsiella pneumoniae* an equal volume of broth containing chloramphenicol was poured so that the final concentration of chloramphenicol was near the inhibitory concentration for small implants, in this case 8 units/ml. The mixture was incubated overnight and then planted as a wash on a ditch plate containing 30 units chloramphenicol in the ditch of a Petri dish con-

taining 15 ml. agar medium. One single colony was found near the ditch. The strain so obtained (KR) was compared with one of the sensitive strains (K5) chosen for its neat colony form and reliable cultural characteristics. A mixed culture of the two strains in broth at 37°, was sampled at intervals, diluted in broth and planted on agar blocks containing chloramphenicol (8 units/ml.). The filled oil chambers were cooled at 4° until the full series had been collected. They were incubated overnight (17 hr. usually) at 20–22° and then refrigerated again to stop further growth, before being measured.

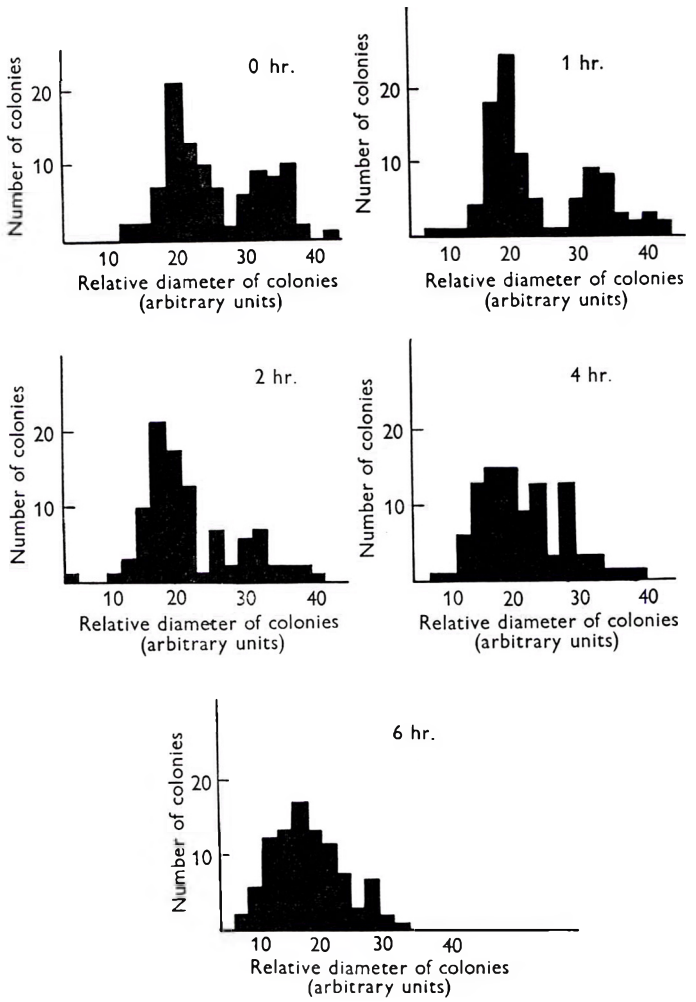

Fig. 2. Histograms showing distribution of size among colonies, growing overnight at 20–22° on agar containing chloramphenicol (8 units/ml), derived from a mixed culture of a sensitive (K5) and a resistant (KR) strain of *Klebsiella pneumoniae*. Samples were plated at 0, 1, 2, 4 and 6 hr. after mixing.

The histograms in Fig. 2 make clear the transition from two independent populations to one which was uniform. The whole cycle with a stationary culture appeared to be complete in only 6 hr. It was fortunate that the resistant strain here used was

distinguishable from the sensitive strain. The resistant strain produced much more slime and capsular material than did the sensitive strain; broth cultures were sticky and tenacious when touched with a wire loop. The individual colonies of the resistant strain at all stages of growth were recognizably different from the sensitive colonies. The resistant colonies were irregular in outline with a coarser surface texture, whereas the sensitive were circular and uniformly smooth.

During incubation with the sensitive strain the size of the colonies of the resistant strain in the samples became progressively smaller and many were morphologically intermediate in type so that while at first it was easily possible to score the two strains separately, later the characters were so mixed as to make this difficult. In Fig. 2, as in other diagrams, not all of the results available are shown; the readings at 3, 5, 7 and 8 hr., however, are in complete accord. A number of controls had to be set up before it could be stated that the change of size, that is the alteration of resistance, was due to the influence of the one strain on the other.

Experiment 3. In order to discover whether the change in average size of colony was due to an overgrowing of one strain by the other in broth culture, the opacity of growing broth cultures of the two strains, separately and together, was measured with a Hilger-Watts Spekker absorptiometer.

The results are shown in Fig. 3; the resistant strain evidently had a longer lag period. When an old culture was used there would therefore be a delay in growth as compared with the sensitive strain, and this would diminish the differences between the two. This difficulty was overcome when cultures already grown in broth for 3 hr. were used.

Experiment 4. To find whether the conditions for growth in the oil chamber favoured one strain more than the other the two were grown both separately and together on plain agar. Under these conditions the sensitive strain formed the larger colonies but both grew well. When equal volumes of young cultures of the two strains were planted together in plain broth and sampled at intervals on to agar, it was found that the ratio of the one to the other did not alter appreciably during incubation. The strains could be differentiated from one another by the colony morphology, at least during the first 6 hr. of incubation. This observation agrees well with the results of the opacity readings in Fig. 3.

Experiment 5. To show whether actual contact is needed between the cells of the two strains in order to bring about the changes seen when they are grown together, the standard experiment was carried out with the two strains separated from each other by a collodion (Gradocol) membrane of average pore size 0·46 μ. The resulting histograms do not suggest that there was any significant change (Fig. 4).

Experiment 6. It was decided to investigate the effect of disturbance on the cultures. The mixture of organisms was put up as before but in tubes which were rocked in a water bath at 37° with about 45 oscillations/min. Samples were taken as before. The preparations were inspected to exclude any possible aggregation into pairs, so that the colonies measured should not be the product of conjoined pairs of organisms. The results are shown in Fig. 5. Again it will be seen that the same trend was present, the shaking merely accelerated the result. A technical difficulty that has not yet been overcome may be mentioned here. Materials for an experiment are prepared and the organisms planted and incubated in broth. Samples withdrawn at predetermined intervals are transferred to the agar medium in oil chambers and

stored in the refrigerator. The results obtained on subsequent culture depend, if they are to be compared, on the starting temperature of each oil chamber being identical and on all the organisms remaining equally in the log phase of growth. When sampling is at hourly intervals, then the rate of equilibration is important; the 7 hr. specimen will be exposed for only 30 min. at 4° while the earlier ones will have had 1·5, 2·5 hr., respectively, and so on. It appears from our results that something of

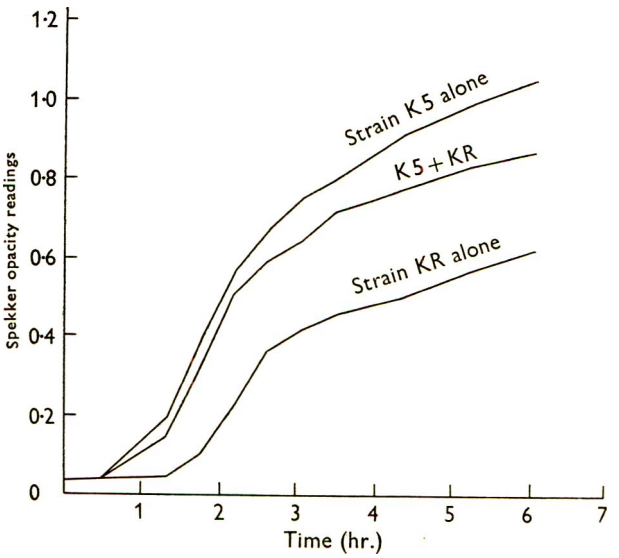

Fig. 3. Growth curves of sensitive strain (K5) and resistant mutant (KR) of *Klebsiella pneumoniae*, in separate and mixed broth culture at 37°. Opacity readings were made with a Spekker absorptiometer.

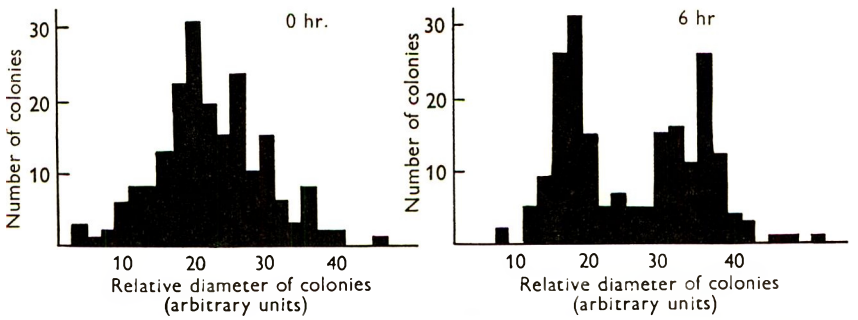

Fig. 4. Histograms showing the distribution of the size of colonies of the sensitive (K5) and resistant (KR) strains of *Klebsiella pneumoniae*, grown in broth and separated from each other by a Gradocol membrane.

the order of cooling for 3 hr. is desirable in the English summer. This conclusion was based on the following observation. A micromanipulator cell, filled with oil containing the agar block was connected to a thermocouple inside a refrigerator, the galvanometer being on a support outside. It was found that while cooling from room temperature began rapidly and evenly, at the lower range temperature fluctuations were marked and a settled low value was not reached until at least 3 hr. By

this time the organisms of the early samples would have passed into the lag phase and all late samples in the series will appear to grow more rapidly than the earlier. This might be overcome by refrigerating for a prolonged period when all samples would show the same lag.

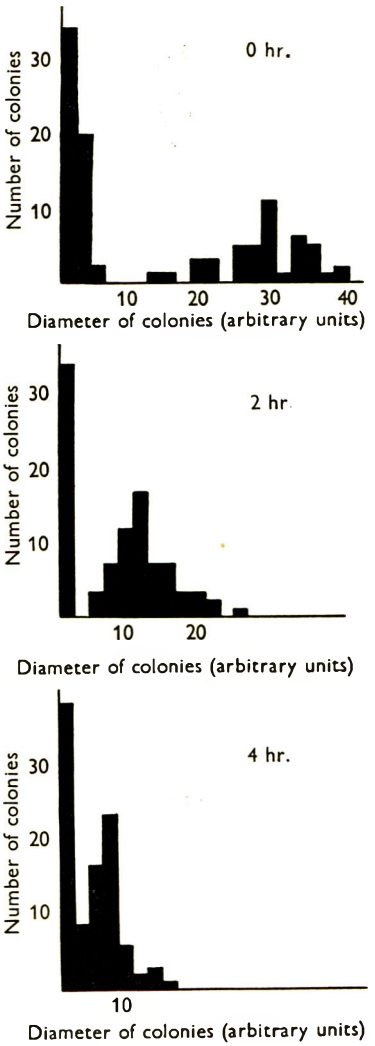

Fig. 5. Histograms showing the distribution of colony size as before. Sensitive and resistant strains of *Klebsiella pneumoniae* grown together in a broth culture, rocked at about 45 oscillations/min. in a water bath at 37°, and sampled on the 8 units chloramphenicol/ml. agar.

DISCUSSION

It has long been recognized that many drug-resistant 'mutants' selected by whatever method tend to alter on subculture, becoming rather more sensitive than when first isolated. This can be explained easily enough if it be supposed that the colony taken contains a mixture of organisms which, when in subculture, influence one

another towards a mean. In the present experiments two strains both derived from the same pure culture of *Klebsiella pneumoniae* and grown repeatedly from single organisms, when incubated together merged progressively into a uniform population. This disappearance of the mutant strain or, in the first series of experiments where streptomycin was used, of the clone selected for streptomycin-resistance, was not due to overgrowth by the sensitive strain, for, when the growth rates were balanced by using only actively growing cultures, the ratio of the numbers of one strain to the other remained constant throughout the experiment.

That the strains did not influence one another when separated by a coarse grade of collodion membrane through which any large molecule would pass easily suggests that actual contact between the organisms may be necessary. Relatively gentle agitation only accelerated the process and did not at all inhibit it. A suitable technique for demonstrating the results of violent agitation such as is needed to separate recombining *Escherichia coli* organisms (Wollman, Jacob & Hayes, 1956) has not been devised for the present work.

A demonstration of the kind given here seems necessary as a corollary to Yudkin's work and in particular to his theoretical considerations about bacterial populations and the emergence of resistant strains. Yudkin pointed out (1953; Fig. 2) that if cells divided 'unequally' then a population would be obtained which would be progressively variable when grown in the absence of a drug (or of other selecting mechanism) until, after many subcultures, the permanent form of the distribution curve would be reached. For one character, e.g. drug resistance, there is obviously a limit to variation; finality is reached when the population contains some completely resistant individuals. For all the factors that make up the complex genetic pattern of the population, almost infinite variation is possible. Nevertheless, since various microbial diseases described by ancient writers are still recognizable today, their causal organisms cannot have greatly changed. Similarly, when the Oxford staphylococcus was collected from a number of centres and re-examined after about 10 years' use as a standard, variation was within the limits of the assay methods in use. This is no longer true, however; it is probable that substitution of other strains has now taken place (Oeding & Ostervold, 1959).

It seems probable that the relative uniformity of members of microbial populations depends on regular interchange of hereditary material. Under the highly artificial conditions of laboratory culture on solid media, or where deliberate selection with chemicals or otherwise takes place, pressures will be brought to bear on the population which will disclose its latent heterogeneity. Usually variations can be detected only in early divisions of the organisms on solid medium; once they have been separated as isolated colonies, no further variation takes place. This was noticed in the earlier experiments with spontaneously occurring long forms of *Escherichia coli* (Hughes, 1953). Progressive homogenization of a mixture of strains has been found repeatedly, irrespective of the time of incubation or the concentration of antibiotic used for the detection of strain differences.

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