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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.

THE PREPARATION OF PAPERS

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

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

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(*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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References. References in the text are cited thus: Brewer & Stewer (1942), (Brewer & Stewer, 1942) Where a paper to be cited has more than two authors, the names of all the authors should be given when reference is first made in the text, e.g. (Brewer, Stewer & Gurney, 1944), and subsequently as (Brewer *et al.* 1944); but papers with more than four authors may be cited, e.g. (Cobley *et al.* 1950) in the first instance. Where more than one paper by the same author(s) has appeared in one year the references should be distinguished in the text and the bibliography by the letters *a*, *b*, etc. following the citation of the year (e.g. 1914*a*, 1914*b*, or 1914*a*, *b*).

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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: degrees Centigrade 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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Nomenclature of Amino Acids and Vitamins. The rules published in the *Biochemical Journal* (1952), **52**, 1–2, should be followed.

Nomenclature and Description of Micro-organisms. Binomial Latin names of micro-organisms, the generic name only with a capital, must be used in accordance with International Rules of Nomenclature; in full at the first mention in each paragraph and in the Summary but in subsequent mention with the generic name abbreviated. Single initial letter abbreviations are used where they are not ambiguous. Binomials should be underlined in the typescript. Scientific epithets or trivial names are not underlined and should be without capitals.

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 Dictionary of the Fungi*, 1961, 5th ed. (Kew: Commonwealth Mycological Institute.)

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

PLANT VIRUSES AND VIRUS DISEASES (1957). *Rev. appl. Mycol.* 35, Suppl. 1–78.

BACTERIA. Author's preferences 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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A Proposal for a Uniform Nomenclature in Bacterial Genetics

By M. DEMEREC, E. A. ADELBERG, A. J. CLARK AND P. E. HARTMAN*

Reprinted from *Genetics*

This proposal was published in *Genetics*, (1966), **54**, 61, but in view of its importance it is reprinted here by permission of the authors and of the Editor and Proprietors of *Genetics*.

A proposal for a descriptive and convenient system of genetic nomenclature for bacteria was drafted by the staff and a number of visitors at Cold Spring Harbor in the summer of 1958 (Demerec, 1958). The proposal had as its basis a system developed by Demerec (1956), which largely adhered to previous genetic conventions yet avoided the complications that have developed in the genetic descriptions of some organisms. At conferences held during the summers of 1962 and 1963 the proposal was critically reviewed and revised in accordance with the increased number of genetic markers available, with usage in other areas (e.g. protein chemistry), with suitability for computer analysis, and with interim developments in bacterial genetics (Demerec, 1963).

The current proposal is an outgrowth of its predecessors, developed by the present authors in consultation with colleagues in other laboratories and in other countries. The basic system has proven convenient to use in the laboratory and has greatly facilitated understanding and communication among many laboratories in the intervening years; increasing use of the system also speaks for its practicality. Thus this proposal does not intend to present a rigid, 'official', frozen system of nomenclature. The system is bound to evolve as knowledge advances in the future. The present communication is aimed at making widely available the proposal as developed to date. Comments, suggestions, and additions are welcome.

The aims of the present proposal are: uniformity; a unique designation for each strain; convenience for typing, editing, printing, record-keeping, and information retrieval; and adaptability, simplicity, clarity, and comprehension by workers in all areas of biology; adaptability to new developments in the foreseeable future. The proposal takes the form of a set of guiding principles for dealing with categories where usage can be clearly defined; application to specific situations is left to each individual worker. The standardized system of genetic symbols is designed to serve the following purposes: (1) To distinguish clearly between symbols representing the *genotype* of a bacterial strain, and abbreviations of words which describe *phenotypic properties*. (2) To provide a uniform set of symbols for genetic loci, mutant alleles and mutation

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sites. These symbols have been designed so as to be readily translatable into computer language, in order that it will be possible to program computers to store the complete genotype of any strain. (3) To provide a system for designating and describing strains that will facilitate both recognition and record-keeping.

The proposed nomenclature is simple and manageable. It avoids the use of unnecessary commas, semicolons, colons, superscripts, subscripts, or Greek letters.

I. GENOTYPE SYMBOLS

Bacterial genetic studies begin with the isolation of a strain from nature or the selection of a prototype strain from an existing culture collection. This strain is arbitrarily designated as wild type; genotype symbols are then devised to designate its genetic determinants, as well as to designate *differences* between the genetic determinants of the wild type and those of derived strains. A set of symbols that describes all the known genetic differences between a derived strain and wild type is used to designate the genotype of the derived strain. A derived strain may differ genetically from wild type in either of two respects: it may carry one or more mutant loci; it may have gained or lost one or more plasmids or episomes. Systems for symbolizing each class of genetic changes will be discussed separately.

A. *Mutant loci*

(1) *General principles*

The terms 'locus' and 'gene' will be used interchangeably to refer to a specific sequence of nucleotides governing the sequence of amino acids in a specific polypeptide (or the sequence of nucleotides in a specific RNA molecule). Nucleotide sequences which themselves may not be transcribed, but which govern the punctuation or regulation of transcription, are also referred to as 'loci'. Minor changes in the nucleotide sequence of a locus (substitutions, small deletions, or insertions) may occur by mutation; the different forms of a locus brought about by such mutations are called *alleles*. The problems involved in recognizing mutation sites and loci will be discussed in section I, D. The following system is proposed for designating loci, alleles, and mutation sites.

Locus symbols in current usage are listed in Appendix A.

Recommendation 1. Each locus of a given wild-type strain is designated by a three-letter, lower-case, italicized symbol.

The existence of a locus is recognized genetically by the occurrence of a mutation within it. In many cases a symbol must be invented long before the polypeptide corresponding to the locus in question has been identified; the investigator may only be aware of a gross phenotypic change produced by the mutation. It is thus a common practice to choose three letters which recall this gross phenotypic change. For example, the symbol *ara* was first coined to refer to the loci in which mutations occur that affect the response of the cell to arabinose as a carbon and energy source.

Recommendation 2. Different loci, any one of which may mutate to produce the same gross phenotypic change, are distinguished from each other by adding an italicized capital letter immediately following the three-letter lower-case symbol.

When, for example, it was recognized that there are three enzymes in the pathway for arabinose utilization, each controlled by a different locus, the three loci in question were designated *araA*, *araB* and *araD* (Englesberg *et al.* 1962). It is useful, but not essential, to assign the capital letters in the same order as the enzymes in the pathway.

(2) *Application to loci concerned with some commonly observed phenotypes*

(*a*) *Loci involved in genetic regulation.* Loci concerned with the regulation of polypeptide formation are designated in accordance with the above convention. For example, the locus which regulates the activity of *araA*, *araB* and *araD* has been designated *araC* (Englesberg *et al.* 1962).

Should an author wish to use a symbol which suggests that the locus in question plays a regulatory function, the capital letter might be chosen accordingly. Thus, a 'regulator' locus, the product of which represses *arg* loci in the *trans* configuration, might be designated *argR*; if more than one such 'regulator' is discovered, they might be symbolized by the sequence *argR*, *argS*, etc. Similarly, an 'operator' locus, which can mutate to derepress an *arg* locus in the *cis* configuration, might be designated *argO*. If more than one operator concerned with arginine biosynthetic enzymes are found, they might be assigned the symbols *argO*, *argP*, etc. It is not necessary, however, to use letters having specific connotations, as long as each locus has a unique designation. *araC*, for example, is a perfectly satisfactory symbol for the locus which regulates *araA*, *araB* and *araD*, and has the advantage of not conveying any preconceived ideas of precise gene function.

(*b*) *Loci governing resistance and sensitivity.* In the absence of knowledge concerning precise mechanisms of resistance or sensitivity, it is customary to choose three letters which recall the deleterious agent. The symbol *str*, for example, was chosen to designate a locus which can mutate to affect sensitivity to streptomycin. When a second locus affecting streptomycin-sensitivity was discovered, the two loci were designated *strA* and *strB* (Sanderson & Demerec, 1965). Similarly, the loci within which mutations affect sensitivity to ultraviolet light have been designated *uvrA*, *uvrB*, and *uvrC* (Howard-Flanders, Simson & Theriot, 1964).

(*c*) *Suppressor loci.* The change in phenotype produced by a mutation in one locus may be partially or fully reversed by a mutation in a second locus. The second locus is then called a 'suppressor locus'.

In many cases it has been demonstrated that genetic suppression involves a change at the translation level of protein synthesis, and it is clear that at least some suppressor loci determine the structures of components of the translation machinery (ribosomes, amino acid activating enzymes, transfer RNA's, etc.). In the absence of any direct information, however, it is necessary to invent symbols which avoid unwarranted connotations. The symbol *sup* has been used, followed by capital letters which distinguish the loci that have been mapped at different places. Thus, one such set of loci have been designated *supH*, *supL*, *supM*, *supN*, *supO*, *supP* and *supT* (Eggertsson & Adelberg, 1965). As more suppressors are mapped, the remaining letters of the alphabet can be used; if these are not sufficient, another set with a symbol such as *spr* may be required.

B. *Mutation sites*

Recommendation 3. A mutation site should be designated by placing a serial isolation number after the locus symbol. If it is not known in which of several loci governing related functions the mutation has occurred, a hyphen is used instead of the capital letter.

For example, Gross & Englesberg (1959) isolated a large number of *E. coli* B mutants unable to utilize arabinose as carbon and energy source. The mutation sites were provisionally designated *ara-1*, *ara-2*, *ara-3*, etc. Later, 17 of these mutations were mapped, and the enzymes which had been altered were identified. It was then possible to complete the designations by substituting capital letters for the hyphen: e.g. *araB1*, *araA2*, *araC3*, *araA4*, *araC5*, *araB6*, etc. Note that the isolation number is not changed at the time that the locus letter is assigned.

According to this system, the symbol *ara-1* originally designated a specific mutation site on the chromosome of *E. coli* B. The symbol *araB1* still refers to the same site, but now conveys additional information about its location. Furthermore, the symbol *araB1* constitutes the designation of a specific allele: thus, the *araB* locus has a given nucleotide sequence as the *araB1* allele, and a different nucleotide sequence as the *araB6* allele.

Although it is possible that two independent mutations may bring about the exact same base-pair change, the probability of this occurring is low, and to prove that it has occurred requires extensive recombination testing. It should thus be assumed, until proved otherwise, that each independent mutation event has occurred at a different site. Even when two mutations are found to occupy identical sites in sensitive recombination tests and to share other properties in common, each mutation still retains its original isolation number.

It is essential that a particular genetic symbol (e.g. *ara-1*) never be utilized on two occasions for two independent mutations. Furthermore, in a useful system of nomenclature, published isolation numbers should not be changed. A few exceptions, however, may occur. For example, a mutation giving rise to valine-resistance in *E. coli* K-12 might receive a particular symbol and isolation number; later, it might be discovered that the mutation actually had occurred in the *ilvB* locus, leading to the formation of an altered, feedback-resistant condensing enzyme elicited by the *ilvB* gene. In this case, the mutation should receive the next available *ilv* isolation number and the change in mutation designation should be noted in the literature.

To avoid duplication of allele numbers, it is urged that geneticists working with the same organism organize a central agency for the assignment of blocks of numbers within each locus. Notices concerning laboratories willing to serve as clearing-houses for this purpose appear regularly in the *Microbial Genetics Bulletin*.

C. *Alleles*

The nature of any particular mutational change is not indicated by the genotypic symbol. For example, *araB1* might be a base-pair substitution or a small deletion. In each case, however, the symbol indicates the presence of a unique nucleotide sequence for the locus in question, and thus constitutes the designation of an *allele*.

In bacterial genetics, the practice of using a plus (+) sign to indicate the wild-type allele of a locus has been borrowed from the genetic nomenclature system used for other organisms. Thus, $araB^+$ is the wild-type allele of the *araB* locus; it stands for the

particular sequence of nucleotides which is found in the *araB* locus of the strain arbitrarily chosen as wild type. Since a locus may have a thousand or more base-pair positions, and since any of four different base-pairs may occupy any one position, the number of possible mutant alleles is very large. It is important that each mutant allele of a particular locus be given a unique designation, e.g. by the use of serial numbers as suffixes. Use of a mutant allele designation is sufficient (e.g. *ara-1*); use of a symbol such as *ara-1*$^-$ is redundant. Superscripts should also be omitted when referring to a particular class of mutants. For example, it is sufficient to speak of all *araB* mutants, or all *ara* mutants, to designate a group of strains, all of which have a mutation in the same locus or in the same set of loci.

A deliberate feature of the system recommended above is that the *allele designation conveys no information concerning phenotype.* For example, all alleles of the *strA* locus can be designated simply by the series *strA1*, *strA2*, *strA3*, etc. Some of these alleles may confer on the cell resistance to low levels of streptomycin, some may confer resistance to high levels of streptomycin, and others may make the cell conditionally or absolutely dependent on streptomycin. None of these facts is relevant to the designation of an allele, however, since an allele is defined as a particular sequence of nucleotide pairs. Furthermore, the phenotype associated with a given allele can often be readily altered by mutations at other loci or by changes in the environment. Thus, according to this proposal, superscripts and suffixes such as 'R' and 'S' for resistance and sensitivity would be rigorously excluded from genotype designations, and should be reserved for use in abbreviations of *phenotype* (see Section II).

D. *Recognition of mutation sites and loci*

When a bacterial strain undergoes a genetic change as a result of a single mutation, the site of that mutation may be assigned a genotype symbol even if its map location is unknown. Proof that the observed change reflects a mutation at a single site requires recombinational analysis; until such an analysis is made, the site description remains tentative.

The existence of a mutation site establishes the existence of a locus whose function has been altered by the mutation. When two or more mutations at different sites have altered the same phenotypic property, however, the assignment of locus designation requires further genetic analysis. For example, genetic mapping of a number of mutants derepressed for alkaline phosphatase synthesis revealed that the mutations had occurred at two widely separated regions of the chromosome. Accordingly, the existence of two loci was inferred, and these were designated *R1pho* and *R2pho* (Garen & Echols, 1962). (To comply with Recommendation 2, these would be changed to *phoR* and *phoS*, respectively.)

When a number of mutation sites all affecting the same phenotypic property are clustered closely together, it is often assumed that the DNA segment within which they are located represents a single functional locus. Without further evidence, however, it is possible that the segment in question includes two or more separate loci having related functions. For example, the early work on mutations affecting the ability to ferment lactose led to the designation of a particular chromosomal region of *E. coli* as the '*lac* locus' (Lederberg, 1947). Later, this region was found to include at least three loci, governing the formation of beta-galactosidase, beta-galactoside permease, and a repressor regulating the other two loci (Jacob & Wollman, 1961).

To prove that two observed mutations are located within the same locus, it is necessary to show that both mutations have affected the amino acid sequence of the same polypeptide. Alternatively, the identity of a locus can be tentatively established by the *cis-trans* test of genetic complementation (Jacob & Wollman, 1961; Garen & Garen, 1963; Helling & Weinberg, 1963; Hayes, 1964; Loper *et al.* 1964).

From the foregoing considerations it is clear that extensive genetic analyses are required before the phenotypic differences between two closely related strains isolated from nature can be ascribed to definable genotypes. For example, a particular phage mutation might be found to be suppressed in *E. coli* K-12 but not in *E. coli* B. Such a difference would reflect an unknown number of genotypic differences between the two strains; without further analysis, no assignment of genotype would be possible.

E. *Plasmids and episomes*

Bacteria are host to a variety of genetic elements capable of independent replication. Such elements include plasmids (remaining autonomous) or episomes (capable of alternating between an autonomous state and a state of attachment to the chromosome).

The known episomes and plasmids include such elements as the sex factor of *E. coli* K-12, the colicinogenic agents, the so-called 'resistance-transfer factors', and a variety of temperate phages such as lambda (λ) and P1. In each case, the element is a DNA structure corresponding to 1–2% of the chromosome in size. As such, it is sufficiently large to contain from 50–100 separate loci of average length.

To include information about plasmids and episomes in the genotype of a bacterial strain, the following are needed: (i) symbols designating the plasmids and episomes which are present; (ii) symbols for the mutant loci and/or mutation sites which they carry.

Recommendation 4. Plasmids and episomes should be designated by symbols which are clearly distinguishable from symbols used for genetic loci.

The first letter of a symbol for a plasmid or for an episome is capitalized, the symbol is *not* italicized, and the symbol is placed in parentheses, e.g. (Col E1).

Recommendation 5. Mutant loci and mutational sites on plasmids and episomes should be designated by symbols of the same kind as those used for loci and sites on the chromosome.

Recommendation 5 is made with the view that loci on plasmids and episomes are not different in kind from loci on the chromosome, and—as part of the total genetic complement of the bacterial cell—should be symbolized according to the unified system of nomenclature.

The complete genotypic description of strains carrying an episome requires a description of the state of the episome: autonomous or integrated. Such information can best be given in words; e.g. 'RTF is integrated between the chromosomal loci *proA* and *proB*'. In the case of strains harbouring the sex factor, F, however, a set of symbols is already in use which requires classification. These symbols are F^-, F^+, F′ (F-prime) and Hfr. Different authors have used these symbols to mean different things; for example, 'Hfr' has sometimes been used to denote the phenotypic property 'high frequency of recombination' (Hayes, 1963) and sometimes to mean the geno-

typic property of integration of sex factor and chromosome (Adelberg & Pittard, 1965). To avoid further confusion, it is recommended that these four symbols be used as follows:

F^- The state of lacking the sex factor, F. The criterion of acting as a genetic recipient in conjugation is not sufficient, since strains carrying F can also act as recipients. The criteria of the F^- state include: activity as a genetic recipient; lack of activity as a genetic donor; failure to transmit F; ability to be infected with F; and resistance to male-specific phages.

F^+ The state of harbouring an autonomous sex factor which does not carry any genetically recognizable chromosomal fragments.

F′ The state of harbouring an autonomous sex factor which carries a genetically recognizable segment of the bacterial chromosome. For example, the strain AB 1206 harbours the sex factor F 14, in which F DNA has become integrated with a chromosomal fragment bearing such loci as *ilvD*, *metE*, and *argA* (Pittard, Loutit & Adelberg, 1963). Such sex factors attached to chromosomal fragments have been called 'substituted sex factors' (Hayes, 1964), 'F-merogenotes' (Clark & Adelberg, 1962), or 'F-genotes' (Ramakrishnan & Adelberg, 1965). The distinction between the F^+ and F′ states is strictly an operational one, since even wild-type sex factors carry regions of homology with the chromosome (Falkow & Citarella, 1965), presumably reflecting incorporated chromosomal fragments (Adelberg & Pittard, 1965).

Hfr The state of harbouring a sex factor which is integrated with the chromosome. Such a state may or may not confer on the cell the phenotype of a high-frequency genetic donor, since this property depends on the functioning of many loci on the sex factor (and possibly on the chromosome as well). Hfr strains in which the sex factor is defective may be extremely low-frequency donors (Cuzin & Jacob, 1965).

Recommendation 6. The description of a strain carrying an episome should include a statement concerning the state and/or location of the episome. The symbols F^-, F^+, F′, and Hfr should be used only to designate the sex factor states as outlined above, and not to convey information concerning the phenotypic properties of mating activity.

F. *Changes in genotype symbols*

Recommendation 7. Genotype symbols which have already been published and which conform to the system recommended above should not be changed. Genotype symbols which do not conform to the above system should be changed accordingly, and the change should be noted when the new symbol is first published.

For example, the set of loci governing the utilization of lactose has been collectively designated as *lac*, but the individual loci have been referred to by the single letters *i*, *o*, *z* and *y* (Jacob, Perrin, Sanchez & Monod, 1960). Mutant alleles have been designated by symbols such as 'i_3^-' 'z_4^-', etc. To conform with the standard system proposed here it would, for example, be necessary to change the latter symbols to *lacI3* and *lacZ4*, respectively.

In exceptional cases the italicized capital letter, designating the gene locus, may have to be changed as subsequent tests define the gene–polypeptide chain relationships more precisely. In these cases, the old and the new gene-locus designations are reported and the reasons for the change are stated in the literature. The change should be referred to in use of the new symbol for an appropriate duration thereafter.

II. PHENOTYPE ABBREVIATIONS

A. *General principles*

The observable properties of a bacterial strain constitute that strain's phenotype. Resistance to a drug such as penicillin, for example, is a phenotypic trait and may reflect any of several diverse genotypes. In publishing a strain description, it is essential that the author make clear whether he is referring to a phenotypic trait or to a genotypic character. In the latter case, a set of symbols such as *penA*, *penB*, *penC*, etc. should be used to designate loci concerned with resistance to penicillin. The phenotype, on the other hand, can best be stated in words: e.g. 'penicillin-resistant'. In practice, however, there is a justifiable tendency to *abbreviate* what might otherwise be a cumbersome description of phenotype. Thus, the abbreviation 'Pen-R10' might be used as an abbreviation of 'resistant to 10 units per millilitre of penicillin', provided that the abbreviation is fully explained the first time that it appears in a given paper.

Care in distinguishing between phenotype abbreviations and genotype symbols is all the more urgent in view of the common practice of inventing genotype symbols which are themselves abbreviations (e.g. '*penA*' for a locus which can mutate to produce resistance to penicillin). The phenotype, on the other hand, can and should be described in words. Abbreviations are needed only for the sake of brevity and of clarity in writing. For example, the sentence 'A cross was performed between a CSD Met$^-$ strain and an SmR100 Met$^+$ strain' is, perhaps, easier to assimilate than the sentence 'A cross was performed between a conditional streptomycin-dependent strain which requires either methionine or streptomycin for growth and a strain which does not require methionine and is resistant to 100 units per millilitre of streptomycin'—*provided that the abbreviations have been clearly defined beforehand.*

To meet these needs, the following recommendation is made:

Recommendation 8. Phenotypic traits should be described in words, or by the use of abbreviations which are defined the first time they appear in a given paper. The abbreviations should be clearly distinguishable from genotype symbols.

To conform with Recommendation 8, three-letter lower-case italicized abbreviations should not be used as phenotype abbreviations. When three letters are used, the distinction between genotype symbol and phenotype abbreviation should be emphasized by capitalizing the first letter of the phenotype abbreviation: e.g. 'The phenotype Met$^-$ is associated with a mutation in the *metA* locus.'

B. *Phenotypic properties involved in mating activity*

The description of mating activity deserves special mention, in view of the problems discussed in connection with the sex factors (Recommendation 6). In many instances, it is necessary to state whether a given strain behaves as a genetic donor or as a genetic recipient, and to indicate the frequency with which it does so. Thus, a strain may be designated as a high-frequency donor, a low-frequency donor, a high-frequency recipient, etc. If such terms require abbreviation, the abbreviations should be carried in accordance with Recommendations 6 and 8; e.g. they should be clearly distinguished from the genotypic symbols F$^-$, F$^+$, F′ and Hfr.

III. DESCRIPTIONS OF STRAINS

A. *Strain designations*

Every strain must have a unique designation. As is the case of symbols for genetic loci, strain designations should be simple (e.g. free of subscripts, superscripts, Greek letters, etc.) and should be compatible with systems for cataloguing and record-keeping. Accordingly, the following recommendation is made:

Recommendation 9. Strains should be designated by simple serial numbers. To avoid duplications, different laboratories should use different letter prefixes. Strain designations should not be italicized.

Phenotypic information should not be included in a strain designation. For example, a designation such as 'C600S^{R}', representing a streptomycin-resistant derivative of strain C600, is undesirable because many different resistant mutants would have the same designation.

Although a strain could be given a unique designation by writing its full or partial genotype (e.g. K-12 (*araB1*, *metE6*, *str-17*)), such designations are cumbersome and greatly complicate the jobs of cataloguing and record-keeping. Instead, such a strain should be given a simple serial number, such as JC1234, and its genotype should be described in a table or footnote, or in the text the first time the strain is mentioned. Some examples of prefixes to strain designations are: CL, for *E. coli* London (Stocker laboratory, retained at Stanford); SB, for Salmonella Baltimore (Hartman laboratory); SW, Salmonella Wisconsin (Lederberg laboratory, retained at Stanford), etc. The exact prefixes are unimportant except that each laboratory must be careful not to duplicate a prefix used elsewhere and thus destroy the uniqueness of the strain designation.

In describing an experiment, it is often helpful to stress a relevant phenotypic or genotypic character of a strain. This can be done by supplementing the strain number, rather than replacing it. For example, 'A cross was carried out between strain AC100 (carrying *araB1*) and AC101 (carrying *araB6*).'

B. *Changes in strain designation*

When a strain is received from another laboratory, it may be necessary to change its designation for the purposes of local record-keeping. To avoid confusion in publication, however, the following recommendation is made.

Recommendation 10. Strain designations which have already been published and which conform to Recommendation 9 should not be changed. Strain designations which do not conform to Recommendation 9 should be changed accordingly, and the change should be noted when the new designation is first published.

C. *Methods for describing strains*

Recommendation 11. When a strain is first mentioned in publication its genotype should be described, and relevant phenotypic information should be given. The genotype includes a list of all mutant loci and/or mutation sites, a list of episomes and/or plasmids, and information concerning the state and location of any episome.

When the genotype includes only one of a few items, it can conveniently be described in the text or in a footnote. When the genotype is long and complex, however, and

when there are many strains, it is extremely helpful to tabulate the information. A sample of such a table, describing some hypothetical strains, is given in Appendix B.

IV. DESCRIPTION OF A CROSS

Once strains are clearly defined, crosses can be described by simple reference to the strain designations, for example, AB712 × AB301.

V. HYBRID STRAINS

The system described above for designating mutant loci and mutation sites presents no problems as long as all strains are derived from a single wild type. As discussed under Recommendation 1, a locus is considered mutant if it differs from the corresponding locus in the arbitrarily chosen wild-type strain.

Thus, a series of mutant loci have been designated within strains derived from *E. coli* K-12, another series within strains derived from *E. coli* B, still another within strains derived from *Salmonella typhimurium*, and so on. But what is the genotype of a hybrid strain, arising from a cross between wild-type *E. coli* K-12 and wild-type *E. coli* B? Some of its loci will be derived from one wild type, and some from the other. If K-12 were considered as the reference strain, the loci inherited from B would be mutant, and vice versa. Furthermore, the genotype of the hybrid could not be written until it was known from which parent each locus was derived.

Should it be possible to determine from which parent a particular wild-type locus was derived, a symbol could be devised to convey this information. Most loci, however, are likely to remain unidentified. In some situations, e.g. when many new strains are to be derived from a particular hybrid, it will be best to designate the hybrid itself as a new prototype strain comparable to a wild type.

SUMMARY

Recommendations are made for a convenient system of nomenclature. These specify the manner of symbolizing or designating loci, mutation sites, plasmids and episomes, sex factors, phenotypic traits, and bacterial strains. Symbols are proposed for known genes in *Escherichia coli* and *Salmonella typhimurium*. The system has been employed in a number of recent papers in *Genetics*, e.g. by Taylor & Thoman (1964) and Sanderson & Demerec (1965).

LITERATURE CITED

ADELBERG, E. A. & PITTARD, J. (1965). Chromosome transfer in bacterial conjugation. *Bact. Rev.* **29**, 161–172.

CLARK, A. J. & ADELBERG, E. A. (1962). Bacterial conjugation. *Ann. Rev. Microbiol.* **16**, 289–319.

CUZIN, F. & JACOB, F. (1965). Analyse génétique fonctionnelle de l'épisome sexuel d'*Escherichia coli* K 12. *C.r. hebd. Séanc. Acad. Sci., Paris* **260**, 2087–2090.

DEMEREC, M. (1956). Terminology and nomenclature. *Publs. Carnegie Instn.* **612**, 1–4.

DEMEREC, M. (1958). Proposal for a uniform nomenclature in bacterial genetics. *Microbial Genet. Bull.* **16**, 38–41.

DEMEREC, M. (1963). Proposal for a uniform nomenclature in bacterial genetics. *Microbial Genet. Bull.* **19**, 30–34.

EGGERTSSON, G. & ADELBERG, E. A. (1965). Map positions and specificities of suppressor mutations in *Escherichia coli* K-12. *Genetics* **52**, 319–340.

ENGLESBERG, E., ANDERSON, R. L., WEINBERG, R., LEE, N., HOFFEE, P., HUTTENHAUER, G. & BOYER, H. (1962). L-arabinose-sensitive, L-ribulose 5-phosphate 4-epimerase-deficient mutants of *Escherichia coli*. *J. Bact.* **84**, 137–146.

FALKOW, S. & CITARELLA, R. V. (1965). Molecular homology of F-merogenote DNA. *J. Molec. Biol.* **12**, 138–151.

GAREN, A. & ECHOLS, H. (1962). Genetic control of induction of alkaline phosphatase synthesis in *E. coli*. *Proc. natn. Acad. Sci. U.S.A.* **48**, 1398–1402.

GAREN, A. & GAREN, S. (1963). Complementation *in vivo* between structural mutants of alkaline phosphatase from *Escherichia coli*. *J. molec. Biol.* **7**, 13–22.

GROSS, J. & ENGELSBERG, E. (1959). Determination of the order of mutational sites governing L-arabinose utilization in *Escherichia coli* B/r by transduction with phage P1bt. *Virology* **9**, 314–331.

HARTMAN, P. E. & SUSKIND, S. R. (1964). Complementation. Chapter 5 of *Gene Action*. Englewood Cliffs, N.J.: Prentice Hall.

HAYES, W. (1963). The mechanism of genetic recombination in *Escherichia coli*. *Cold Spring Harb. Symp. quant. Biol.* **18**, 75–93.

HAYES, W. (1964). *The Genetics of Bacteria and Their Viruses*. New York: Wiley.

HELLING, R. B. & WEINBERG, R. (1963). Complementation studies of arabinose genes in *Escherichia coli*. *Genetics* **48**, 1392–1410.

HOWARD-FLANDERS, P., SIMSON, E. & THERIOT, L. (1964). The excision of thymine dimers from DNA, filament formation and sensitivity to ultraviolet light in *Escherichia coli* K-12. *Mutation Res.* **1**, 219–226.

JACOB, F., PERRIN, D., SANCHEZ, C. & MONOD, J. (1960). L'óperon: groupe de gènes à expression coordonnée par un opérateur. *C. r. hebd. Séanc. Acad. Sci., Paris* **250**, 1727–1729.

JACOB, F. & WOLLMAN, E. L. (1961). *Sexuality and the Genetics of Bacteria*. New York: Academic Press.

LEDERBERG, J. (1947). Gene combination and linked segregations in *Escherichia coli*. *Genetics* **32**, 505–525.

LOPER, J. C., GRABNAR, M., STAHL, R. C., HARTMAN, Z. & HARTMAN, P. E. (1964). Genes and proteins involved in histidine biosynthesis in Salmonella. *Brookhaven Symp. Biol.* **17**, 15–50.

PITTARD, J., LOUTIT, J. S. & ADELBERG, E. A. (1963). Gene transfer by F′ strains of *Escherichia coli* K-12. I. Delay in initiation of chromosome transfer. *J. Bact.* **85**, 1394–1401.

RAMAKRISHNAN, T. & ADELBERG, E. A. (1965). Regulatory mechanisms in the biosynthesis of isoleucine and valine. III. Map order of the structural genes and operator genes. *J. Bact.* **89**, 661–664 **90**, 295.

SANDERSON, K. E. & DEMEREC, M. (1965). The linkage map of *Salmonella typhimurium*. *Genetics* **51**, 897–913.

TAYLOR, A. L. & THOMAN, M. S. (1964). The genetic map of *Escherichia coli* K-12. *Genetics* **50**, 659–677.

APPENDIX A: LIST OF PROPOSED SYMBOLS

(For data on many of these loci in *E. coli* and *S. typhimurium*, see reviews in Taylor & Thoman (1964) and Sanderson & Demerec (1965).)

acr genes determining response to acridine (resistance or sensitivity)
ade see: *pur*
adl genes determining adonitol utilization
ala genes determining and regulating alanine biosynthesis
ank genes determining and regulating K antigen synthesis
ara genes determining and regulating arabinose utilization
arg genes determining and regulating arginine biosynthesis
aro genes determining and regulating biosynthesis of several aromatic amino acids and aromatic vitamins
ars genes determining sensitivity to arginine
asc genes determining and regulating ascorbate biosynthesis
asn genes determining and regulating asparagine biosynthesis
asp genes determining and regulating aspartic acid biosynthesis
att prophage attachment site
azi genes determining response to azide (resistance or sensitivity)

bio genes determining and regulating biotin biosynthesis
chr genes determining response to chromium (sensitivity or resistance)
cit genes determining and regulating citrate utilization
clb genes determining and regulating cellobiose fermentation
clk genes determining response to colicine K (resistance or sensitivity)
(Col K) bacteriocinogenic for colicine K
cys genes determining and regulating cysteine biosynthesis
cyt genes determining and regulating cytosine biosynthesis
dds genes determining D-serine deaminase and regulating its production
dta genes determining and regulating D-tartrate utilization
dul genes determining and regulating dulcitol utilization
fdp gene(s) determining fructose-1,6-diphosphatase
fim genes determining and regulating fimbriation (piliation)
fla genes determining and regulating flagellation (presence of flagella)
gal genes determining and regulating galactose utilization
gas genes determining gas formation from fermentable sugars
gln genes determining and regulating glutamine biosynthesis
glp genes determining and regulating glycerol and glycerolphosphate utilization
glu genes determining and regulating glutamic acid biosynthesis
gly genes determining and regulating glycine biosynthesis
gra genes determining response to gramicidin (resistance or sensitivity)
gua genes determining and regulating guanine biosynthesis
hag genes determining and regulating the synthesis of flagellar antigens
hcr genes determining host cell reactivation
hem genes determining and regulating heme biosynthesis
his genes determining and regulating histidine biosynthesis
hom genes determining and regulating homoserine biosynthesis
ilv genes determining and regulating isoleucine and valine biosynthesis
inl genes determining and regulating inositol utilization
ita genes determining and regulating isotartrate utilization
lac genes determining and regulating lactose utilization
lam genes determining response to phage lambda (resistance or sensitivity)
leu genes determining and regulating leucine biosynthesis
lev genes determining and regulating levulose utilization
lon genes determining cellular growth into filaments
lys genes determining and regulating lysine biosynthesis
mal genes determining and regulating maltose utilization
man genes determining and regulating mannose utilization
mel genes determining and regulating melibiose utilization
met genes determining and regulating methionine biosynthesis
mlz genes determining and regulating melizitose utilization
mot genes determining the functioning of flagella (i.e. flagella present but bacteria nonmotile)
mtl genes determining and regulating mannitol utilization
mut genes determining functions whose aberration leads to heightened spontaneous mutation rates
nfr genes determining response to nitrofuran (resistance or sensitivity)
nic genes determining and regulating nicotinic acid biosynthesis
nit genes determining and regulating nitrate utilization
nml genes determining the presence of *E-N*-methyl-lysine in flagellar protein
nol genes determining response to norleucine (resistance or sensitivity)
nov genes determining response to novobiocin (resistance or sensitivity)
(P1) lysogenicity for phage P1
(P22) lysogenicity for phage P22
pab genes determining and regulating *p*-aminobenzoic acid biosynthesis
pan genes determining and regulating pantothenic acid biosynthesis
pdx genes determining and regulating pyridoxine biosynthesis
pen genes determining response to penicillin (resistance or sensitivity)
pgi gene(s) determining phosphoglucosisomerase
phe genes determining and regulating phenylalanine biosynthesis
pho genes determining alkaline phosphatase and regulating its production
pig genes determining pigment formation (pigment of unknown nature)
pmi gene(s) determining phosphomannosisomerase
pmx genes determining response to polymixin (resistance or sensitivity)

pro	genes determining and regulating proline biosynthesis
pur	genes determining and regulating purine biosynthesis
pyr	genes determining and regulating pyrimidine biosynthesis
raf	genes determining and regulating raffinose utilization
rbs	genes determining and regulating ribose utilization
rec	genes affecting genetic recombination
rha	genes determining and regulating rhamnose utilization
rib	genes determining and regulating riboflavin biosynthesis
(Rtf)	harbouring resistance-transfer-factor
rou	genes determining functions whose aberration leads to rough colony morphology or serotype
scr	genes determining and regulating sucrose utilization
ser	genes determining and regulating serine and glycine biosynthesis
som	genes determining and regulating somatic antigen synthesis
sor	genes determining and regulating sorbose utilization
srl	genes determining and regulating sorbitol utilization
str	genes determining response to streptomycin (resistance, sensitivity, or dependence)
suc	genes determining and regulating succinic acid utilization
sul	genes determining response to sulphonamide (sensitivity or resistance)
tfr	genes determining response to phage T4 (resistance or sensitivity)
thi	genes determining and regulating thiamine biosynthesis
thr	genes determining and regulating threonine biosynthesis
thy	genes determining and regulating thymine biosynthesis
tna	genes determining tryptophanase and regulating its production
ton	genes determining response to phage T1 (sensitivity or resistance)
tre	genes determining and regulating trehalose utilization
trp	genes determining and regulating tryptophan biosynthesis
tsx	genes determining response to phage T6 (resistance or sensitivity)
tur	genes determining and regulating turanose utilization
tyr	genes determining and regulating tyrosine biosynthesis
uvr	genes determining repair of ultraviolet radiation damage to DNA
val	genes whose aberration leads to valine-resistance
vio	genes determining response to viomycin (resistance or sensitivity)
xyl	genes determining and regulating xylose utilization

APPENDIX B: EXAMPLE OF A TABLE ILLUSTRATING DESCRIPTION OF SOME HYPOTHETICAL STRAINS

		Mutant loci and mutation sites								
Strain	Source	*lacZ*	*lacY*	*lacI*	*lacO*	*his*	*trp*	*str*	*proA*	*proB*
AC9991	‡	+	+	3	+	1	6	12	2	+
AC9992	‡	13	+	3	+	+	6	7	+	+
AC9993	‖	+	16	+	4	1	+	+	+	7

		Relevant phenotype*							Episomes†		
Strain	Source	β-gal	Perm	Hist	Tryp	Sm	Prol	M.Act.	λ	P1	F
AC9991	‡	C	C	−	−	R	−	LFD	+	+	1**
AC9992	‡	−	C	+	−	D	+	HFD	+	−	1§
AC9993	‖	C	−	−	+	S	−	Rec	131¶	−	−

* (β-gal) β-galactosidase; (Perm) β-galactoside permease; (Hist) histidine; (Tryp) tryptophan; (Sm) streptomycin; (Prol) proline; (M.Act.) mating activity; (C) constitutive; (−) absent or required; (+) synthesized; (R) resistant; (D) dependent; (S) sensitive; (LFD) low-frequency donor; (HFD) high-frequency donor; (Rec) recipient.

† (+) wild-type episome present; (−) episome absent; numbers are strain numbers of episomes.

‡ This laboratory.

§ F1 is integrated between *proB* and *lacY*; promotes chromosome transfer in the order *proB*, *leu*, *thr*, *metA*...etc.

‖ Obtained from J. C. Sunset. Formerly designated A3-7/*lac*^{c}.

¶ λ 131 carries the mutant alleles *susA*1, *susB*1.

** F1 is autonomous.

APPENDIX C: SUMMARY OF RECOMMENDATIONS

1. Each locus of a given wild-type strain is designated by a three-letter, lower-case italicized symbol.

2. Different loci, any one of which may mutate to produce the same gross phenotypic change, are distinguished from each other by adding an italicized capital letter immediately following the three-letter lower-case symbol.

3. A mutation site should be designated by placing a serial isolation number after the locus symbol. If it is not known in which of several loci governing related functions the mutation has occurred, the capital letter is replaced by a hyphen.

4. Plasmids and episomes should be designated by symbols which are clearly distinguishable from symbols used for genetic loci.

5. Mutant loci and mutational sites on plasmids and episomes should be designated by symbols of the same kind as those used for loci and sites on the chromosomes.

6. The description of a strain carrying an episome should include a statement concerning the state and/or location of the episome. The symbols F^-, F^+, F' and Hfr should be used only to designate the sex factor states as outlined above, and not to convey information concerning the phenotypic properties of mating activity.

7. Genotype symbols which have already been published and which conform to the system recommended above should not be changed. Genotype symbols which do not conform to the above system should be changed accordingly, and the change should be noted when the new symbol is first published.

8. Phenotypic traits should be described in words, or by the use of abbreviations which are defined the first time they appear in a given paper. The abbreviations should be clearly distinguishable from genotype symbols.

9. Strains should be designated by simple serial numbers. To avoid duplications, different laboratories should use different letter prefixes. Strain designations should not be italicized.

10. Strain designations which have already been published and which conform to Recommendation 9 should not be changed. Strain designations which do not conform to Recommendation 9 should be changed accordingly, and the change should be noted when the new designation is first published.

11. When a strain is first mentioned in publication its genotype should be described, and relevant phenotypic information should be given. The genotype includes a list of all mutant loci and/or mutation sites, a list of episomes and/or plasmids, and information concerning the state and location of any episome.

Editors' note: Publication of this 'Proposal for a Uniform Nomenclature in Bacterial Genetics' in the *Journal of General Microbiology* does not imply official endorsement by the Society for General Microbiology. However, it is hoped that the recommendations in the 'Proposal' will serve as a useful guide to authors. The general principles laid down for Genotype Symbols, Phenotype Abbreviations, and Descriptions of Strains should be followed wherever it is practicable to do so.

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Accumulation of Mononucleotides in Washed Suspensions of Myxamoebae of *Dictyostelium discoideum*

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SUMMARY

Washed myxamoebae of *Dictyostelium discoideum* exhibit a transitory accumulation of 2′,3′-mononucleotides in the soluble pool. The accumulation is correlated with the time of transition from the vegetative stage to the initiation of morphogenesis. Materials which stimulate the rate of morphogenesis and prevent the efflux of RNA and protein from washed amoebae, also enhance the accumulation of mononucleotides many fold.

INTRODUCTION

Previous communications have described the ability of a variety of materials to stimulate the rate of morphogenesis in the cellular slime mould *Dictyostelium discoideum* (Krichevsky & Wright, 1963). Further investigation of one class of stimulatory materials (i.e. imidazole-containing compounds) led to the postulate that the site of action of these materials is at the surface of the organisms (Krichevsky & Love, 1964). Stimulants of the rate of development prevented the specific efflux of RNA and protein into the extracellular environment of washed myxamoebae (Krichevsky & Love, 1965). It is well documented (Wright, 1964) that endogenous RNA and protein concentrations decrease in developing cells. Evidence is presented here that the monomeric constituents of one of these macromolecules, RNA, accumulated in washed suspensions of *D. discoideum* myxamoebae under the influence of various stimulants of the rate of morphogenesis.

METHODS

The procedures for growing and harvesting the myxamoebae by using a solid medium were as described previously (Krichevsky & Wright, 1963). The method of Gerisch (1959) was used for growing the myxamoebae in liquid culture. Whether grown on solid or in liquid media, the myxamoebae were washed, suspended and incubated as described by Krichevsky & Love (1965).

Pentose-containing materials were assayed by the orcinol reaction of Mejbaum as modified by Horecker, Smyrniotis & Klenow (1953). The ninhydrin assay for amino acids was performed by the procedure of Rosen (1957). Diphenylamine-reacting materials were determined as described by Dische (1955).

RESULTS

As reported previously, L-histidine stimulated the rate of morphogenesis and prevented the efflux of macromolecules more effectively at higher hydrogen ion concentrations (e.g. pH 5) than at lower H^+ concentrations (pH 7). Therefore, it became of interest to ascertain the ability of L-histidine, at various H^+ concentrations, to promote the accumulation of perchloric acid-soluble pentose-containing materials (2′,3′-mononucleotides, see below). The results are presented in Fig. 1. When the suspending medium was L-histidine at pH 5 and 6 the accumulation was markedly greater than when the medium was either L-histidine at pH 7 or distilled water. Since urocanic acid

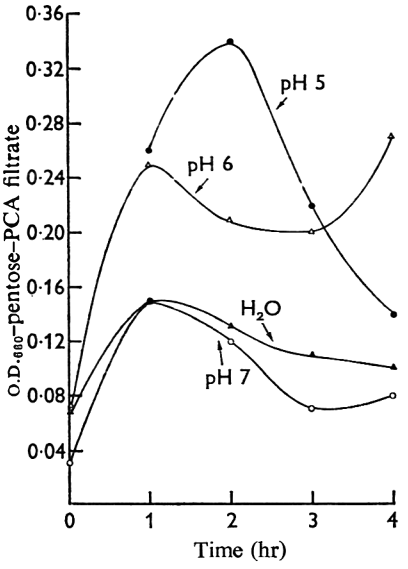
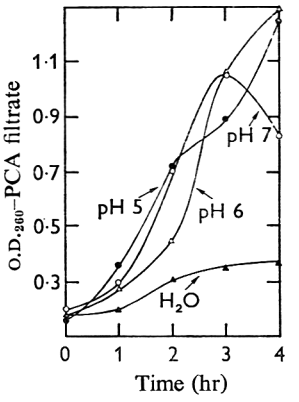


Fig. 1



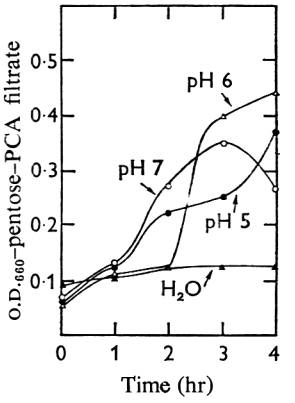


Fig. 2

Fig. 1. Orcinol-reactive material accumulation in the presence of histidine at various hydrogen ion concentrations. Myxamoebae of *Dictyostelium discoideum*, harvested after growth on solid medium, were suspended in 0·04M-L-histidine (pH 5, ●—●; pH 6, △—△; pH 7, ○—○) or distilled water (▲—▲). At the times indicated, 2·0 ml. samples were added to 0·2 ml. *N*-perchloric acid (PCA), chilled in an ice bath, and filtered through 25 mm. diam. Millipore filters (type HA, 0·45 μ pore size). One ml. samples of the filtrate were assayed for their pentose-containing materials.

Fig. 2. Accumulation of orcinol-reactive and ultraviolet absorbing materials in the presence of potassium phosphate buffers. The conditions of incubation of the *Dictyostelium discoideum* myxamoebae were the same as described for Fig. 1. The suspending media were 1·0M potassium phosphate buffers (pH 5, ●—●; pH 6, △—△; pH 7, ○—○) or distilled water (▲—▲). Before the perchloric acid (PCA) filtrates were assayed for pentose, the extinction was read at 260 mμ (1 cm. light path).

is a product of L-histidine metabolism in *Dictyostelium discoideum* (Krichevsky & Love, 1964) and this material has an absorption maximum at 260 mμ in acid, the ultraviolet (u.v.) absorption changes in the samples reflected the urocanate production (ascertained by u.v. absorption at 300 mμ in 0·1 N-NaOH; these are not presented).

The substitution of potassium phosphate buffers for the L-histidine solutions gave the data shown in Fig. 2. Qualitatively similar results were obtained whether the assay

used was u.v. absorption (at 260 mμ) or reactivity with orcinol + $FeCl_3$ reagent (for pentose moeities). In addition, potassium phosphate stimulated the accumulation of the assayed materials irrespective of the pH value; this correlated well with the ability of this material to stimulate morphogenesis at the various pH values.

Other materials which stimulate the rate of morphogenesis and inhibit leakage of macromolecules were tested for their ability to promote increased mononucleotide concentrations (Fig. 3). Of the compounds tested, glucose was by far the most effective; the values obtained having been divided by 3 to bring them within the scale shown. Magnesium ion was somewhat more stimulatory than Na^+ or K^+.

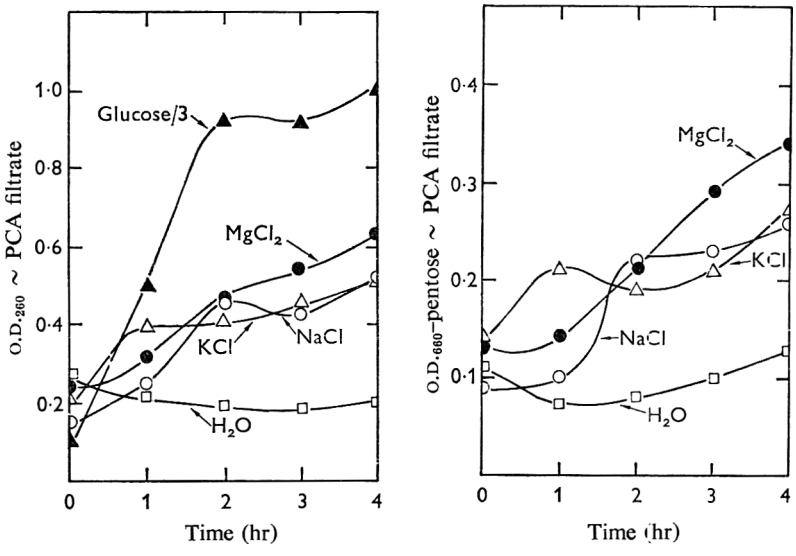


Fig. 3. Accumulation of orcinol-reactive and ultraviolet absorbing materials in the presence of chloride salts or glucose. The conditions for incubation of the *Dictyostelium discoideum* myxamoebae were the same as those described for Fig. 1. The myxamoebae were suspended in the following media: 0·01M-NaCl (○—○); 0·01M-KCl (△—△); 0·005M-$MgCl_2$ (●—●) 0·05M-glucose (▲—▲); and H_2O (□—□). Before the perchloric acid (PCA) filtrates were assayed for pentose, the extinction was read at 260 mμ (1 cm. light path).

Experiments similar to those described above indicated that imidazole in the suspending medium also resulted in increased mononucleotide concentrations. To relate the accumulation phenomenon to the morphogenetic process, the experiment described in Fig. 4 was performed. By both assay procedures it was shown that the stage of development had a marked influence on the results. At the earlier stage, accumulation was observed when either distilled water or imidazole solution was the suspending medium. However, imidazole was more effective in promoting accumulation, and with it maximal accumulation occurred at the early aggregative stage.

Figure 5 shows the effect of developmental stage on the kinetics of amino nitrogen accumulation under the conditions of the experiment just described. Even over the full 4 hr of incubation no particular influence of either morphogenetic stage or suspending medium was evident when compared to the results obtained when mononucleotides were assayed as described for Fig. 4.

Myxamoebae which had been grown in liquid culture in which the only nutrients

were *Escherichia coli* organisms and phosphate buffer exhibited a marked change in accumulating ability with changing developmental stage (Fig. 6). Thus, only the vegetative myxamoebae (the freshly harvested forms) were able to accumulate mononucleotides. As shown by extinction measurements at 260 mμ, the accumulation by the myxamoebae was dependent on the presence of phosphate. The accumulation of orcinol-reactive material in the absence of phosphate (i.e. in the water control sample) was found only in this experiment. The material presumably was not purine or

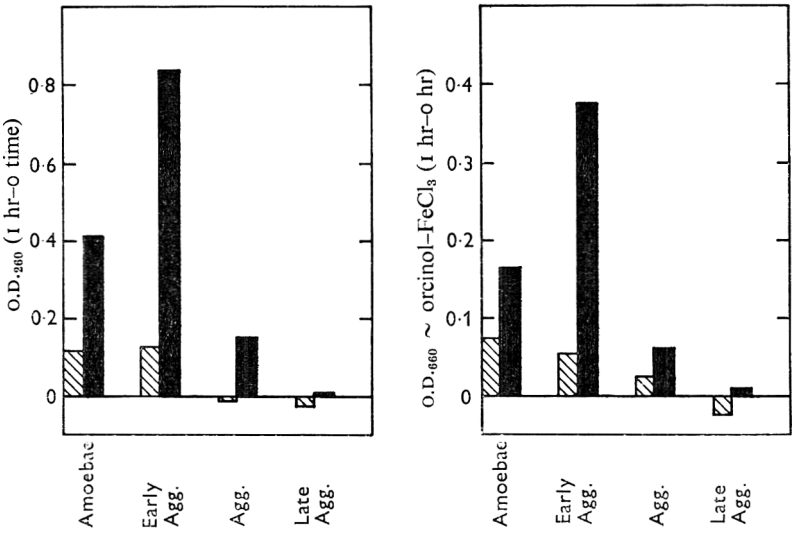


Fig. 4. Effect of developmental stage on the accumulation with imidazole as the stimulant. Myxamoebae of *Dictyostelium discoideum* were grown on solid medium contained in cookie sheets. At intervals through the growth period, groups of cookie sheets were transferred from incubation at 22° to incubation at 19°. This procedure made possible the selection of groups of sheets which, on visual inspection, were determined to be at the developmental stages of vegetative amoebae (amoebae), early aggregation (early agg.), mid-aggregation (agg.), and late aggregation (late agg.). Four suspensions were prepared in the usual manner; one from organisms at each developmental stage. The cell concentrations were adjusted to the same extinctions (1/15 dilution = 0·55 at 660 mμ; 1 cm. light path). The organisms were then further diluted (1/10) in either 0·05M-imidazole, pH 6 (■), or distilled water (▨). The conditions of incubation and assays were the same as described for Fig. 2. However, the data presented here are the changes of the extinction at 260 mμ, or the pentose-moiety contents of the perchlorate-soluble fractions for the first hour only.

pyrimidine in nature since there was no corresponding increase in u.v. absorption. When glucose was used instead of phosphate, the results were like those just described (Fig. 7), indicating that phosphate was not unique in function.

Preliminary determinations of the nature and distribution of the u.v. absorbing orcinol-reactive materials confirmed the observation of Pannbacker (1966) for normal pools as follows. The great majority of the 260 mμ absorption was due to materials which behaved like the 2′- or 3′-mononucleotide constituents of RNA when eluted from Sephadex A-25 ion exchange columns. The isolated materials had u.v. absorption spectra which were identical with adenosine, cytosine, uridine and guanosine-2′,3′-monophosphates. Thin-layer or paper chromatography on polyethylene-imine-cellulose indicated that the isolated materials behaved like the 2′- or 3′-mononucleotides

rather than the 5′-esters. In addition, they were resistant to periodate oxidation under conditions which cleaved the ribose moiety of the 5′-esters. Quantitative assays for diphenylamine reactivity showed that the samples did not contain significant amounts of deoxyribotides.

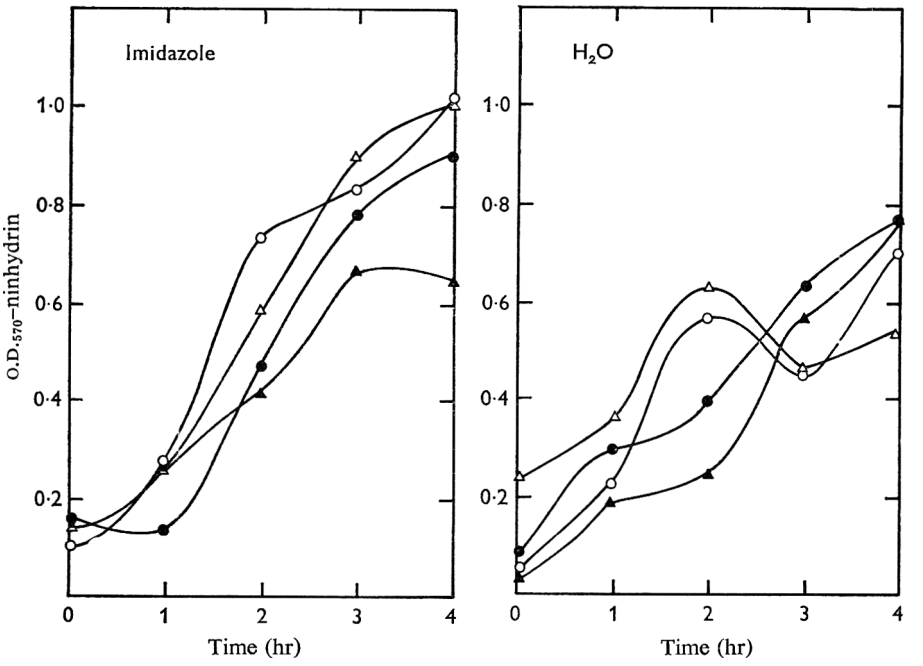


Fig. 5. Amino-nitrogen accumulation, with developmental stage of *Dictyostelium discoideum*, in the presence and absence of imidazole. All conditions were those described for Fig. 4. The samples taken for the ninhydrin assay were 0·05 ml. In this case the data for the full 4hr. incubation period is presented; imidazole on the left and H_2O on the right. The developmental stages are: vegatative amoebae (●—●), early aggregation (○—○), mid-aggregation (△—△), and late aggregation (▲—▲).

DISCUSSION

In those cases tested, materials which have been found to stimulate the rate of sorocarp formation were shown to prevent the efflux of macromolecules from washed *Dictyostelium discoideum* myxamoebae (Krichevsky & Love, 1965). The macromolecules specifically concerned were ribosenucleic acids and proteins. Thus, it was postulated 'that the RNA and protein that leak from the cells are not essential for cellular differentiation but provide stimulatory intermediates through their catabolism to their monomeric constituents, and this only when available inside the cell'. It might be expected that conditions leading to the internal conservation of RNA and protein might lead to an increased pool level of the monomers of these macromolecules. However, this would be true only if the rates of entry of the monomers into the pool exceed the rates of exit from the pool (either through further catabolism or leakage of the monomers themselves or both).

In the case of protein depolymerization, it appears that pool levels of amino acids are not affected markedly by materials which both stimulate the rate of morphogenesis

and prevent the efflux of protein from amoebae under the conditions described. Further, it may be concluded that the pool levels of amino acids are unaffected by the stage of development (at least through aggregation). Thus, the rate of development probably is not controlled by pool levels of amino acids.

The picture is quite different when RNA monomers are considered. At the time when the amoebae are undergoing the transition from the growing stage to the developmental stage of the life cycle, there is a transitory accumulation of mononucleotides in the soluble pool. This accumulation is enhanced many fold by the presence of materials which stimulate the rate of morphogenesis. In addition, these

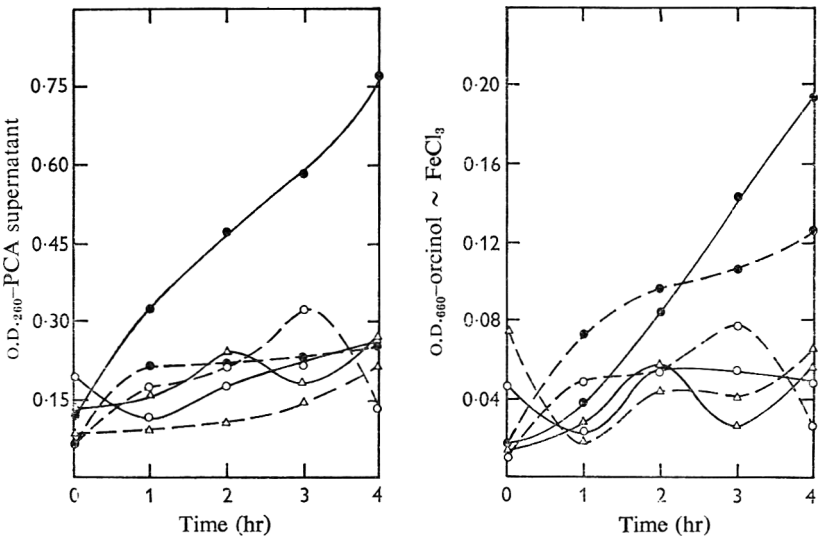


Fig. 6. Accumulation, with developmental stage, of orcinol-reactive and ultraviolet-absorbing materials by *Dictyostelium discoideum* grown in liquid culture. Myxamoebae were grown as described by Gerisch (1959); washed free of residual bacteria and six samples of the crop treated as follows. The first amoeba sample was suspended in a final concentration of 0·1M-potassium phosphate buffer (pH 5) (●—●); the second amoeba sample was suspended in distilled water (● - - - ●); the remaining four samples were spread on cookie sheets containing 2% Difco-Noble agar and allowed to incubate until the desired developmental stage was judged to have been reached by visual inspection. The samples were then washed off the agar surface with distilled water and suspended for incubation, i.e. early aggregation: 0·1M-phosphate (△—△) or distilled water (△ - - - △); mid-aggregation: 0·1M-phosphate (○—○) or distilled water (○ - - - ○). The suspensions were sampled and assayed as described for Fig. 2.

same materials allow the conservation of RNA within the cells. It may be postulated that the rate-limiting step (or steps) in the development of *Dictyostelium discoideum* will be found associated with the further utilization of one or more mononucleotides which, in turn, arise from the deploymerization of endogenous RNA. Further, it would be expected that exogenously supplied mononucleotides would stimulate the rate of morphogenesis. Preliminary experiments indicate that this is true, and at molar concentrations of as little as 10^{-4}M which is at least 100 times as effective as the other stimulants found. However, the form of mononucleotides which is most effective is the 5′-ester.

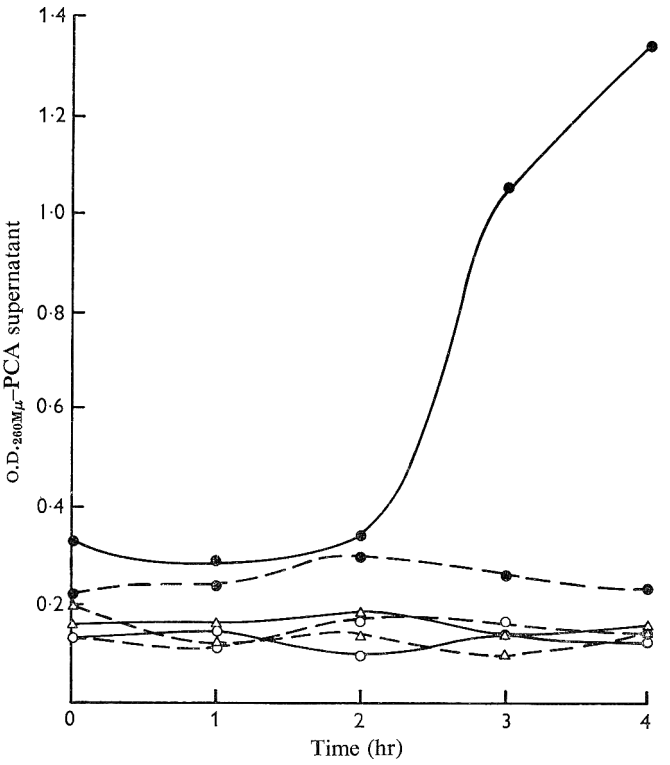


Fig. 7. Accumulation, with developmental stage, of ultraviolet absorbing materials by myxamoebae of *Dictyostelium discoideum* grown in liquid culture. All conditions were identical to those described in Fig. 6 except that 0·05 M-glucose was substituted for the 0·1 M-phosphate.

REFERENCES

DISCHE, Z. (1955). In *The Nucleic Acids*, vol. 1. Ed. by E. Chagaff and J. N. Davidson. New York: Academic Press.

GERISCH, G. (1959). Ein Submerskultuver-fahren für entwicklungsphysiologische untersuchungen an *Dictyostelium discoideum*. *Naturwissenschaften* **46**, 654.

HORECKER, B. L., SMYRNIOTIS, P. Z. & KLENOW, H. (1953). The formation of sedoheptulose phosphate from pentose phosphate. *J. biol. Chem.* **205**, 661.

KRICHEVSKY, M. I. & LOVE, L. L. (1964). The uptake and utilization of histidine by washed amoebae in the course of development in *Dictyostelium discoideum*. *J. gen. Microbiol.* **34**, 483.

KRICHEVSKY, M. I. & LOVE, L. L. (1965). Efflux of macromolecules from washed *Dictyostelium discoideum*. *J. gen. Microbiol.* **47**, 367.

KRICHEVSKY, M. I. & WRIGHT, B. E. (1963). Environmental control of the course of development in *Dictyostelium discoideum*. *J. gen. Microbiol.* **32**, 195.

PANNBACKER, R. G. (1966). RNA metabolism during differentiation in the cellular slime mold. *Biochem. biophys. Res. Comm.* **24**, 340.

ROSEN, H. (1957). A modified ninhydrin colormetric analysis for amino acids. *Archs. Biochem. Biophys.* **67**, 10.

WRIGHT, B. E. (1964). In *Biochemistry and Physiology of Protozoa*, Ed. by S. H. Hutner, vol. 3. New York: Academic Press

J. gen. Microbiol. (1968), **50**, 23–35
Printed in Great Britain

The Adaptation of *Klebsiella aerogenes* to the Inhibitory Action of Triethylene-melamine on Growth and Division

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SUMMARY

The aerobic growth of *Klebsiella aerogenes* adapted to a chemically defined glucose ammonium sulphate medium was studied in the same medium containing triethylene-melamine (TEM). In liquid medium containing TEM up to 205 mg./l. growth and division were only slightly retarded. With concentrations of TEM greater than about 255 mg./l. division was strongly inhibited while growth continued slowly, giving filaments the longest of which later separated from the medium as a white pellicle. Further growth and division occurred in the same culture after a lag. During continued subculture in liquid medium containing a constant concentration of TEM the lag was negligible but growth on return to drug-free liquid or solid medium was impaired. At first filament formation increased to a maximum, the growth rate fluctuated and adaptation was rapidly lost during subculture in drug-free medium. The progressive impairment of division was later opposed by a gradual adaptation which eventually produced bacteria of a more nearly normal size, with a steady growth rate and with resistance to precipitation and to higher concentrations of TEM. TEM was less active in solid medium than in liquid medium. The proportion of filaments and the size of the lenticular areas observed in colonies increased with increasing concentration of TEM but decreased with increasing age and size of the colonies.

INTRODUCTION

Triethylene-melamine (TEM), other biological alkylating agents and ionizing radiations have similar biological effects among which is a tendency to inhibit cell division more than growth (increase in mass). TEM causes bacteria such as *Escherichia coli* to form filaments (Maxwell & Nickel, 1954; Loveless, Spoerl & Weisman, 1954; Katchman, Spoerl & Smith, 1955; Schweisfurth, 1959; Kilgore & Greenberg, 1961). The present work was done as part of a physico-chemical study of the growth of *Klebsiella aerogenes* in chemically defined media containing inhibitory drugs. During repeated subculture of bacteria in media containing a drug, increasing resistance to the drug is reflected in various responses which may include elimination of an initially long lag, increase in growth rate, changes in the enzyme balance and changes in the proportions of other constituents of the organisms. These responses have been interpreted on the basis of physico-chemical principles (Dean & Hinshelwood, 1966). Initial growth of *K. aerogenes* in chemically defined media containing proflavine (Davies, Hinshelwood & Pryce, 1944), *m*-cresol (Spray & Lodge, 1943) or *tert.*-butanol (Hinshelwood & Lodge, 1944) gives rise to abnormally long forms. During repeated

subculture in medium containing one of these drugs, adaptation is marked by a decrease in the proportion of filaments, which indicates that the rate of division is catching up with the rate of growth. The present work shows that the degree of filament formation is a most effective variable for investigating the adaptation of *K. aerogenes* to TEM and, therefore, particular emphasis was placed on a study of the size of the organisms.

METHODS

Organism. The organism used was a strain of *Klebsiella aerogenes* NCTC 418 (*Aerobacter aerogenes*) which was fully adapted to grow in a glucose ammonium sulphate medium by many daily subcultures and which had, so far as could be known, never previously been exposed to TEM or to any other drug.

Triethylene-melamine (2,4,6-*tris*-aziridinyl-1,3,5-triazine; TEM) was a gift from Imperial Chemical Industries Ltd.

Media. Liquid standard and limiting media and solid glucose ammonium sulphate media and the methods employed for growing the bacteria were the same as those used by Drabble & Hinshelwood (1961). Liquid and solid media containing TEM were prepared in the same way as media containing streptomycin described by Drabble & Hinshelwood (1961). The cultures for all measurements were grown in standard medium or on solid glucose ammonium sulphate medium and the inocula were taken from limiting glucose ammonium sulphate medium unless otherwise stated. Subculture of bacteria into fresh media was made daily. One subculture corresponded to about eight generations.

Bacterial mass. The mass of suspended bacteria/ml. culture (M) was estimated turbidimetrically (Drabble & Hinshelwood, 1961; Grant, 1967) by a modification of the method of Monod (1942). M is expressed as the equivalent number of standard-sized cells per 10^{-6} ml. giving the same turbidity. In a suspension for which M was 1 unit the dry weight of bacteria was 0·48 μg./ml.

Growth measurements in liquid media. The lag and mean generation time (T) of cultures were calculated from the growth curves obtained by plotting log M against the time after inoculation. Lags less than about 40 min. were considered to be negligible.

Growth measurements on solid media. The growth of bacterial colonies on solid media was expressed quantitatively in the manner of Dean & Hinshelwood (1955):

$$\alpha_t = \frac{\text{no. colonies of diam. } > 0{\cdot}4 \text{ mm. on drug medium } t \text{ hr after inoculation}}{\text{no. of colonies of diam. } > 0{\cdot}4 \text{ mm. on drug-free medium at the same time}}$$

$= $ fraction of the viable bacteria on the drug medium which formed colonies greater than the standard size in time (t) hr,

$\alpha_\infty =$ fraction of the viable bacteria on the drug medium which ever formed colonies greater than the standard size.

Plots of α_t/α_∞ against t on drug medium or on drug-free medium were sigmoid, rising from zero to unity. The value of t when $\alpha_t/\alpha_\infty = 0{\cdot}5$, is called the 'median delay' (D). $1/D$ is a convenient measure of the rate of growth of the bacteria on solid media.

Bacterial numbers. The total number of bacteria/ml. of culture (N) was determined

directly by counting the bacteria in a Helber counting chamber (Thoma ruling) under a Baker interference microscope, after killing and preserving the bacteria by adding one drop of formalin/ml. suspension. Bacteria in colonies were examined after first cutting out the underlying solid medium, then shaking it with a few ml. of saline (NaCl 8·5 g./l.) to suspend all the bacteria of the colony, and finally killing and preserving the bacteria as described above.

Bacterial sizes. The length (l) of individual organisms was estimated by comparison with the known dimensions of the squares of the counting chamber or by using a calibrated eye-piece graticule. Bacteria which had not separated completely were always counted and measured as single organisms. The relative degree of inhibition of growth and division of the bacteria was studied by plotting histograms for the distributions of bacterial numbers among bacterial lengths and of bacterial mass among bacterial lengths. If n is the number and m is the mass of bacteria in a group of average length l, then number frequency $= n/\Sigma n$ and mass frequency $= m/\Sigma m$. If d is the average density of cellular material, and a is the average cross-section area of the bacteria, $m = dnal$ and mass frequency $= nl/\Sigma nl$. This approximation is justified by the approximate constancy of the observed width of bacteria of different lengths and by the fact that M was found to be proportional to Σnl for a given number of squares of the counting chamber within the limits of experimental error. The average length of the bacteria is equal to $\Sigma nl/\Sigma n$ and a measure of the average size of the bacteria is given by M/N.

The above distributions were summarized by placing the bacteria into the following three larger groups and expressing the number in each group as a percentage of the total number of bacteria in all the groups: (*a*) bacteria of length from 0 to 10 μ, corresponding to the vast majority of organisms in a normal culture of the original sensitive strain grown in standard liquid glucose ammonium sulphate medium containing no drug; (*b*) bacteria of length from 10 to 25 μ, corresponding to a tiny minority of organisms in a normal culture described above or to a moderate degree of inhibition of division; (*c*) bacteria longer than 25 μ, which appeared as grotesque snake-like filaments and which were formed only when division was strongly inhibited.

RESULTS

Triethylene-melamine causing 'double growth' in liquid medium

When sensitive bacteria were inoculated into liquid medium containing TEM, from 0 to 205 mg./l., the shape of the growth curves was normal and the end of the logarithmic phase was marked by a decrease in pH value as in drug-free medium. With increasing drug concentration within this range the value of M in the stationary phase decreased and in TEM 205 mg./l. some of the bacteria were actually precipitated from suspension. With TEM 268–2000 mg./l. the growth curves (Fig. 1) were unusual and showed 'double growth'. The initial logarithmic growth ('first phase') ceased while the pH value of the medium was still at its original value and the subsequent decrease in M (of the bacteria in suspension) was accompanied by the formation of a white pellide which adhered to the culture tube at the surface of the medium and which consisted of long snake-like forms. This precipitation did not occur in the absence of TEM, and its degree was insensitive to the rate of aeration and agitation

of the culture. After a lag a further logarithmic increase in *M* occurred ('second phase') which was terminated by a decrease in pH value and a stationary phase (Fig. 1).

Triethylene-melamine in liquid or in solid medium causing inhibition of division

The approximate constancy of *N* over parts of the first phase indicated that growth in this phase was largely an increase in bacterial mass with little division. In accordance with this most of the bacteria grew steadily and continuously up to the maximum value of *M* (Fig. 2). Thereafter, *M* and *N* decreased and the percentage of the smallest organisms increased as a result of the precipitation, and perhaps some division, of

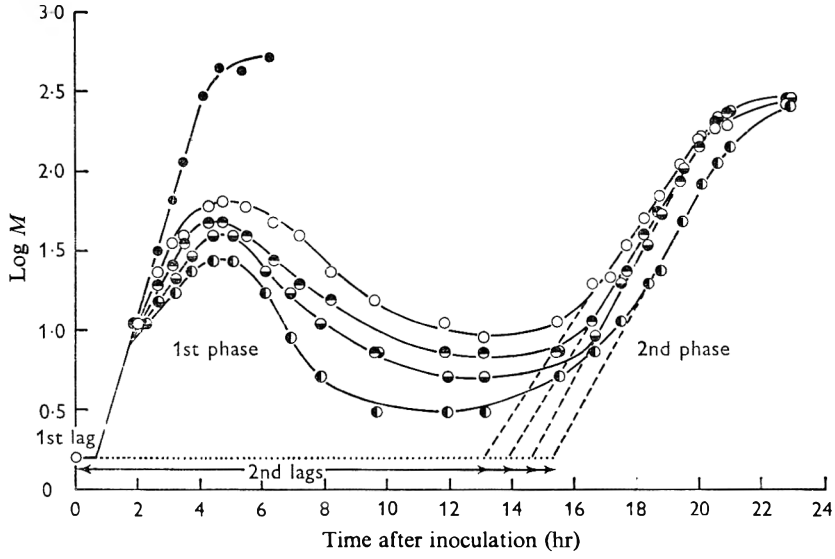

Fig. 1. Growth curves of a sensitive strain of *Klebsiella aerogenes* in glucose ammonium sulphate medium containing the following concentrations of TEM (mg./l.): o (control), ●; 268, ○; 305, ◓; 353, ◒; 399, ◐.

longer forms. Throughout the first phase the distribution of the bacteria with length became increasingly wider; this is shown in Fig. 2 by the continuous increase in the average length of the longest 5% of the bacteria in the culture. This indicated that some of the snake-like forms were still growing in suspension while others were being precipitated.

Throughout the second phase with TEM 353 mg./l. the distribution of the number of bacteria with length merely fluctuated between the following limits: 68·9–79·2% for bacteria of length 0–10 μ; 14·7–22·7% for bacteria of length 10–25 μ; 4·4—10·0% for bacteria longer than 25 μ. Thus, while in the first phase growth occurred largely without division, in the second phase growth and division were more nearly balanced.

The average length of bacteria from colonies grown on solid media, with or without TEM, was much less than that of bacteria from the corresponding liquid media. As division becomes increasingly inhibited relative to growth the longer length groups must become increasingly occupied, whereas the population of the shorter, normal, length groups must decrease. Indeed, this occurred for colonies of the same age (24 hr) and size (0·4 mm.) with increasing concentration of TEM, and for a given

TEM concentration (8000 mg./l.) with decreasing age and size of the colonies. Histograms of the distribution of the number and mass of bacteria with length, for samples taken during the first phase in liquid media containing TEM or taken from colonies growing on solid media containing TEM, had only one mode, like those

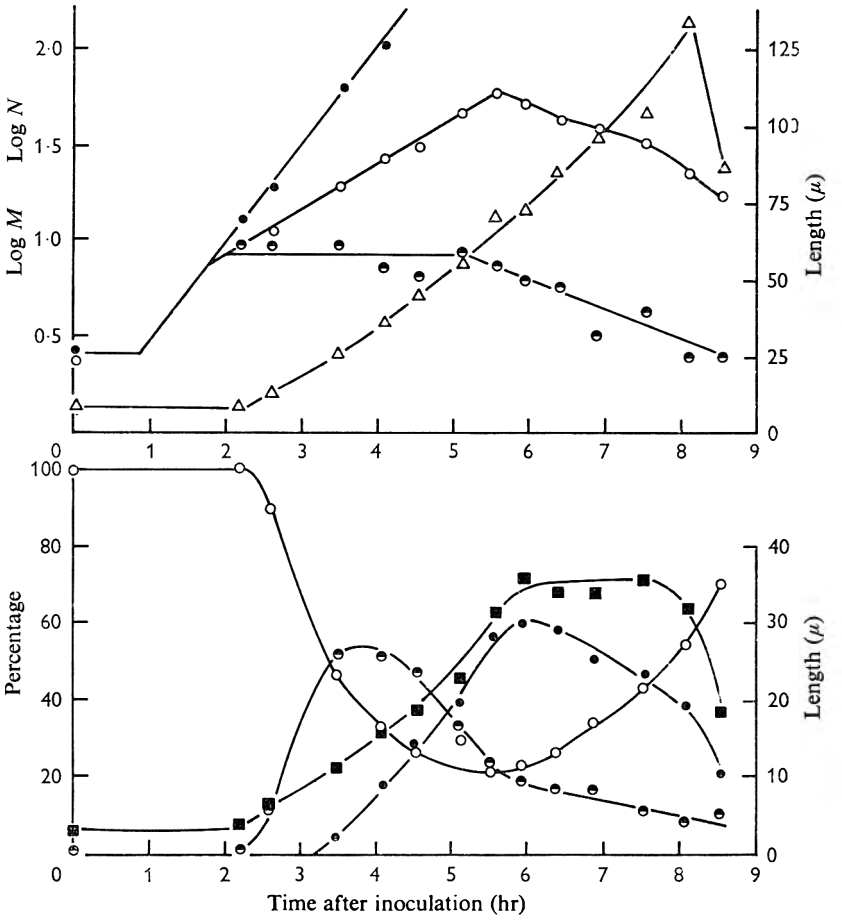

Fig. 2. First phase (initial growth followed by precipitation) for a sensitive strain of *Klebsiella aerogenes* in glucose ammonium sulphate medium containing TEM 353 mg./l. Upper: log M (●) in drug-free medium (control): log M (○), log N (◓) and the average length of the longest 5% of the bacteria (△) in drug medium. Lower: $\Sigma nl/\Sigma n$ = average length of all the bacteria (■) in TEM medium: the percentage of the total number of bacteria in each of the following groups of lengths in drug medium; 0 to 10 μ (○), 10 to 25 μ (◓), longer than 25 μ (●).

for corresponding drug-free cultures. In drug-containing media some of the abnormally long forms consisted of two or more opaque sections connected by translucent membranes as if division were about to occur, and rare, Y-shaped forms with an extra limb joined to the main body were occasionally seen.

Effects of triethylene-melamine on lag, growth rate and colony form

When sensitive bacteria were inoculated into liquid media containing TEM from 0 to 2000 mg./l. the lag was negligible, growth was initially uninhibited (Fig. 1) and

most of the bacteria divided. When M had reached about 8–10 units and N had increased by a factor of about 1·8, division almost stopped (Fig. 2) and growth slowed to an approximately logarithmic rate whose mean generation time (T_c) increased with the drug concentration (c mg./l.) according to a definite relationship (Fig. 3) which may be expressed in either of the following ways:

(*a*) A plot of log $(T_c - T_o)$ against c, where T_o is the value of T_c in the absence of TEM, shows that log $(T_c - T_o) = 0{\cdot}0053\ (c - 58)$ or $T_c - T_o = 0{\cdot}49 \exp(0{\cdot}0121c)$.

(*b*) A plot of T_o/T_c against c, where the symbols have the same meaning as above, leads to a more easily explicable relationship similar to one discussed by Poole & Hinshelwood (1940) and by Dean & Hinshelwood (1966). Since $T_c = \ln 2/k_c$, and $T_o = \ln 2/k_o$, where k_c and k_o are the specific growth rates in the presence and absence of TEM respectively, then $T_o/T_c = k_c/k_o$. When c was about TEM 200–500 mg./l., $k_c/k_o = 1 - (c - 152)/415$. As c increased from 0, k_c/k_o did not start to decrease until c exceeded about TEM 50 mg./l., which shows that the organism tolerated low concentrations of TEM. Furthermore, extrapolation of the linear relationship to $k_c/k_o = 1$ gave a positive value of c, namely, TEM 152 mg./l. With concentrations of TEM exceeding 500 mg./l. T could not be accurately measured but with TEM up to 2000 mg./l. the first phase phenomena were observed, indicating that the initial growth rate was still finite.

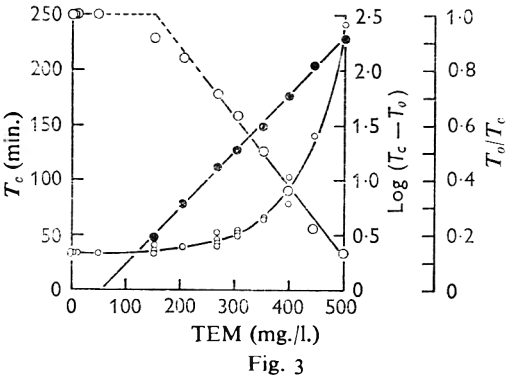

Fig. 3

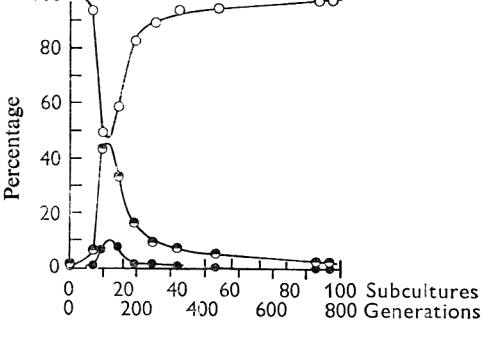

Fig. 4

Fig. 3. T_c (o), log $(T_c - T_o)$ (●) and T_o/T_c (○) for growth of a sensitive strain of *Klebsiella aerogenes* in glucose ammonium sulphate medium containing TEM c mg./l., where T_c is the mean generation time in the presence of TEM c mg./l. and where T_o is the mean generation time of the drug-free control.

Fig. 4. The percentage of the total number of bacteria in each of three groups of organism lengths (0–10 μ, ○; 10–25 μ, ◓; longer than 25 μ, ●) during serial subculture of *Klebsiella aerogenes* in glucose ammonium sulphate medium containing TEM 2000 mg./l.

The time between inoculation and the onset of the second phase of growth (the 'second lag') increased linearly with increasing TEM concentration, being, for example, 13·2 hr at 268 mg./l., 16·5 hr at 500 mg./l., 20·1 hr at 750 mg./l. and 23·9 hr at 1000 mg./l. Above TEM 1000 mg./l. it increased more steeply, being, for example, 39·3 hr at 1500 mg./l. and 109 hr at 2000 mg./l. The values of T during the second phase were not related to the concentration of TEM but were distributed about a mean value of 49 min. in an approximately Gaussian manner, with a standard deviation of 8·2 min. for twenty observations. During the stationary phase M was not related to TEM concentration and more bacteria were precipitated.

On solid media the growth rate decreased with increasing concentration of TEM, D being: 12 hr with 0–353 mg./l., 24 hr with 1000–2000 mg./l., 36 hr with 4000 mg./l.; 57 hr with 8000 mg./l.

On solid media containing TEM from 0 to 4000 mg./l. practically all the viable bacteria inoculated survived to form colonies, indicating that sensitive bacteria could better withstand the action of TEM on solid medium than in liquid medium. With TEM 8000 mg./l. 1 in $1{\cdot}3 \times 10^4$ bacteria survived but with TEM 12,000 and 16,000 mg./l. less than 1 in $2{\cdot}5 \times 10^8$ survived.

In agreement with the observations of Dean & Hinshelwood (1957*a*), lens-shaped insertions called 'lenticular areas' appeared in ageing colonies growing on media containing TEM. The effect (discussed by Dean & Hinshelwood 1957*b*), increased with age and increasing drug concentration and became pronounced in colonies grown on TEM 4000 and 8000 mg./l. from 4 to 7 days after inoculation.

'Training' by repeated subculture in liquid medium containing TEM

At various stages during the first subculture in liquid medium containing TEM 353 mg./l. further subcultures were made into medium of the same type. Subcultures from the first phase grew after considerable lags (8–10 hr), whereas those from the second phase grew after negligible lags although at similar rates (e.g. T = 41–45 min.). The time interval between inoculation of sensitive bacteria into the first medium and the beginning of growth of the second subculture was about 15·9–18·1 hr and was independent of the moment or phase of growth at which the inoculum was transferred from the first to the second sample of medium. A considerable lag after the first phase was therefore a necessary preliminary to further growth, whereas in subsequent subcultures the lags were always negligible and 'double growth' never re-occurred.

During serial subculture in liquid media containing a constant concentration of TEM (353, 1000, 2000 mg./l.) T fluctuated at first but eventually decreased to a steady value after prolonged subculture (e.g. Table 1). The magnitude of the early fluctuations and the final value of T were larger the higher the TEM concentration used. Subculture in liquid medium containing TEM 2000 mg./l. enabled all the bacteria to survive on solid medium containing TEM 8000 mg./l. as compared with only 1 organism in 13,000 for the sensitive strain.

Thus serial subculture in liquid medium containing TEM led to adaptation of the bacteria, but at the expense of slower growth in the absence of TEM (Table 1). Similarly, after 68 or 101 subcultures in liquid medium containing TEM 2000 mg./l. the rate of colony development on solid medium was decreased; D had increased by between 5% and 75% on TEM plates and by 75% and 300% on drug-free plates.

The distribution of the number of bacteria with length changed during subculture in standard glucose ammonium sulphate medium+TEM. When the parent was grown in medium of the same type, the distribution fluctuated markedly throughout the first few subcultures with TEM 353 mg./l., showing that growth and division had not reached a steady balance. Throughout the 37th and 117th subcultures the proportion of longer forms increased to a maximum early in the logarithmic phase and decreased to a constant minimum at the end of that phase. The proportion of filaments at a given point during the 117th subculture was less than that at the corresponding point in the 37th subculture. When the parent was grown in limiting glucose ammonium

sulphate medium + TEM, the proportion of longer forms decreased immediately after inoculation and more gradually, being constant and minimal when M was between 40 and 100 units towards the end of the logarithmic phase. Therefore, in a standard test for the degree of filament formation during serial subculture in TEM-containing medium, the bacteria were grown under these latter conditions and were sampled when M was about 80 units.

Table 1. *Mean generation time (T) during serial subculture of a strain of Klebsiella aerogenes in a chemically defined medium containing a constant concentration of triethylene-melamine (TEM)*

Basal medium: glucose + ammonium sulphate + salts (pH 7·12). Incubation temperature: 40°. Growth: aerobic. Strain: initially adapted to basal medium containing no TEM.

No. of previous subcultures in TEM 353 mg./l. before test	T in TEM 353 mg./l. (min.)	T in absence of TEM (min.)	No. of previous subcultures in TEM 2000 mg./l. before test	T in TEM 2000 mg./l. (min.)	T in absence of TEM (min.)
0	55*	29·5	0	36*	32
1	39·5	—	3	—	33
13	41	39·5	7	69	—
20	41	42	11	108	66
25	45·5	48·5	16	101	—
29	52	46·5	17	62·5	64
36	49	39/54·5	23	54·5	54·5
44	46	45·5	30	91·5	50
52	42	43	39	59·5	60
60	40	38·5	53	142/54†	46·5
74	39	38	90	143/52	45
112	33	30	95	144/53·5	44·5
116	32·5	31	—	—	—

* Second phase of first subculture

† 142/54 indicates that growth with $T = 142$ min. was followed by growth with $T = 54$ min. etc.

Plots of the percentage of the total number of bacteria in various length groups against the numbers of subcultures or generations in medium containing TEM 353, 1000 and 2000 mg./l. (Fig. 4) were very similar in shape. During the early subcultures the degree of filament formation increased to a maximum and then declined as the number of subcultures in presence of TEM increased. The maximum was greater the higher the concentration of TEM and may have resulted from a competition between (*a*) increasing action of TEM in inhibiting the division mechanisms and (*b*) increasing adaptation of the bacteria to resist the action of TEM (see Fig. 7 on p. 57 in Hinshelwood & Lodge, 1944). After many subcultures the distribution of the bacteria with length became narrower, and it and the average bacterial size, as measured by M/N, appeared to approach asymptotically that of the original sensitive strain of *Klebsiella aerogenes* growing in standard drug-free glucose ammonium sulphate medium, showing that growth and division had nearly achieved their original delicate balance. For example, the bacterial number distribution in the standard test during the 105th and 107th subcultures with TEM 353 mg./l. (0–10 μ, 99·2%; 22–25 μ, 0·8%; longer than 25 μ, 0%) was very similar to that for the original sensitive strain in drug-free medium (0–10 μ, 99·3%; 10–25 μ, 0·7%; longer than 25 μ, 0%). Similarly,

bacteria thoroughly adapted to grow with TEM 2000 mg./l. in liquid medium, when grown on solid medium containing either TEM 0 or 8000 mg./l. gave colonies consisting of bacteria with the same distribution among lengths as those of the original sensitive strain growing on drug-free plates; more than 97% of the bacteria were 0–5 μ long. For serial subculture in liquid medium containing TEM histograms of the distribution of the bacteria with length had only one mode and, in general, the degree of filament formation was not related to *T*.

At various stages during 'training' to grow in presence of TEM the organism was

Table 2. *Reversal of adaptation of Klebsiella aerogenes to triethylene-melamine (TEM) by serial subculture in the absence of TEM*

Basal medium: glucose+ammonium sulphate+salts (pH = 7·12). Incubation temperature: 40°. Growth: aerobic. Strains: adapted to basal medium containing TEM 1000 mg./l. *T* = mean generation time.

Growth in absence of TEM		Test of growth in presence of TEM			
No. of previous subcultures before test	*T* (min.) during test	Type of growth curve (see text)	*T* (min.) during 1st phase	Lag (hr)	*T* (min.) during 2nd phase
Reversion after 1 subculture in TEM 1000 mg./l.					
0	32	a	77	.	.
1	33·5	b	69/90·5*	.	.
6	36	c	∞	3	58·5
10	32	d	82·5	1–21	.
11	34·5	d	.	5–17	.
14	35	d	62·5	13·8	52
16	37·5	d	60	7–20	.
20	31·5	d	83·5	6–19	.
Reversion after 18 subcultures in TEM 1000 mg./l.					
0	44·5	a	62	.	.
3	46	b	44/81·5	.	.
4	51	a	56·5	.	.
11	38	d	51·5	27·0	58·5
14	37·5	d	80	5–19	.
18	38	d	54·5	9–17	.
Reversion after 29 subcultures in TEM 1000 mg./l.					
0	42	a	53	.	.
1	41·5	a	43·5	.	.
3	40	a	54	.	.
9	35·5	b	33·5/66	.	.
13	36	a	55	.	.
21	37	a	45	.	.
27	42·5	a	56	.	.
37	30	b	48/90·5	.	.
48	29	b	50·5/134	.	.
62	30	c	∞	5·5–17·5	.
64	37·5	d	65·5	8–18	.
69	32	b	63	.	.
80	.	b	54/102/190	.	.
109	33·5	b	54/142	.	.
140	29·5/52	d	61/125	10–20	.
146	42	c	∞	17·7	34·5

* 69/90·5 indicates that growth with *T* = 69 min. was followed by growth with *T* = 90·5 min. etc.

repeatedly subcultured in TEM-free medium and the degree of adaptation which the strain then possessed was determined by retesting in TEM medium (Table 2). The pattern observed on retesting was as follows: (*a*) Initially the absence of any rapid reversal of adaptation was indicated by a negligible lag and a normal logarithmic phase. During the stationary phase some snake-like forms were precipitated from suspension. (*b*) Later the lag was still negligible but the growth phase was composite: fast growth changed abruptly when M was about 90 units to give what appeared to be slow growth which was wholly or partly a result of the earlier onset of precipitation while growth was still proceeding. Growth and precipitation competed at this early stage of reversion. (*c*) Sometimes more or less normal growth was preceded by a lag. (*d*) The final stage of reversion was marked by 'double growth' in two phases on retesting, as for the initial subculture of sensitive bacteria in TEM medium. Precipitation had at this stage overtaken growth and had brought about the decline in M (of suspended organisms). Continued subculture in TEM-free medium gave a strain indistinguishable from the original sensitive strain. Table 2 shows that in the earlier stages of reversion the order of (*a*) and (*b*) was sometimes reversed, that in the later stages of reversion (*a*), (*b*) and (*c*) did not always occur in this order and that at all stages of reversion T tended to fluctuate. Reversion is clearly a complex process.

The stability of the impressed resistance increased greatly between the 18th and 29th subcultures with TEM 1000 mg./l., reversion being rapid after one subculture or 18 subcultures, but slow after 29 subcultures (Table 2).

As reversion proceeded filamentous forms became progressively rarer in drug-free medium and finally disappeared entirely, leaving bacteria of normal appearance; on retest in TEM medium filamentous forms reappeared and their numbers and average length increased. These changes took place sooner than the changes in the nature of the growth curves on retest in TEM medium; e.g. 44 subcultures in medium containing TEM 353 mg./l. gave a strain which, after 94 or 139 subcultures in drug-free medium, afforded bacteria of normal size in the absence of TEM but gave a high proportion of filaments in TEM-containing medium, whereas the growth curves in TEM medium were still almost normal.

DISCUSSION

The behaviour of *Klebsiella aerogenes* with triethylene-melamine (TEM) is very unusual (Figs. 1, 2, 4). When liquid medium containing TEM was inoculated with the original drug-sensitive strain of *Klebsiella aerogenes*, there was an initial burst of uninhibited growth and division (Figs. 1, 2), which showed that TEM did not act immediately. The effect of a given concentration of TEM on growth and division was less in solid medium than in liquid medium. This is explicable if the reactive ethyleneimine is adsorbed by gel structures in the solid medium or if the bacteria can neutralize the TEM around them more effectively in an immobile medium than in a fluid medium.

During the initial cultivation of sensitive bacteria in liquid medium containing TEM the growth rate in the first phase was not decreased unless the concentration of TEM exceeded about 50 mg./l., which implies that the bacteria have a limited capacity to neutralize the drug. The specific growth rate (k_c) during the first growth phase was given by

$$k_c/k_o = 1-(c-152)/415, \qquad (1)$$

provided that the concentration (c mg./l.) of TEM was between 200 and 500 mg./l. (Fig. 3). This relationship has certain consequences if the following assumptions are made: (*a*) there is an equilibrium between the drug in solution and the drug adsorbed by an enzyme in the bacteria; (*b*) the growth rate of the bacteria is decreased by the inhibition of the enzyme by the drug, such that k_c is proportional to the active surface area of the enzyme free from drug. If S is the fraction of the active surface of the enzyme occupied by the drug, then $k_c \propto (1 - S)$, therefore

$$k_c = k_o (1 - S). \tag{2}$$

From equations (1) and (2)

$$S = (c - 152)/415. \tag{3}$$

If c lies within the limits stated above and if the above assumptions are correct, equation (3) is the adsorption isotherm of the drug on the surface of the key enzyme.

When the concentration of TEM was between about 500 and 2000 mg./l., the growth rate of the first phase still continued to be finite, i.e. $k_c \not\to 0$, contrary to (1). This may be a manifestation of the short initial burst of growth mentioned earlier, or can be explained by postulating that there was still available for metabolism a residue of sites on the enzyme surface immune to or inaccessible to the drug, i.e. if $S \not\to 1$, $k_c \not\to 0$ (cf. equation (2)).

The bacteria which grew during the second phase of the first subculture with TEM were selected organisms which had escaped death or precipitation during the first phase. These selected bacteria could be either favoured organisms of different genotypes (first-stage mutants), since TEM is strongly mutagenic to *Escherichia coli* (Iyer & Szybalski, 1958; Szybalski, 1960); or statistically favoured or physiologically adapted organisms of the same genotype.

During the growth of the bacteria on solid media or in liquid media, with or without TEM, histograms of the distribution of the number or mass of the bacteria with lengths had only one mode. This seems to indicate that, from the point of view of filament formation, only one strain of organisms was present at a given time, with the possible exception of the first phase of the first subculture with TEM.

The susceptibility of the bacteria to form filaments depends on the state of the culture. After repeated subculture in liquid medium containing TEM, the percentage of filaments was greatest early in the growth cycle and then decreased to constant minimum values towards the end of the logarithmic phase. This can be explained by postulating that commencement of division in fresh medium must await the synthesis of a critical amount of a 'division factor' while growth is already in progress (Spray & Lodge, 1943; Hinshelwood & Lodge, 1944). A change in the drug medium from limiting to standard glucose ammonium sulphate medium on inoculation caused a decrease in the percentage of filaments early in the growth cycle, which may be attributed to a change in osmotic pressure accompanying the change in medium (Hinshelwood & Lodge, 1944).

Filaments were formed continuously during serial subculture in drug medium and persisted after many subcultures (Fig. 4), which suggests that all the bacteria were susceptible to inhibition of division and could therefore form filaments. During the early subcultures in drug medium the percentage of filaments at a given point in the growth cycle increased. It seems that a given organism could only form a filament when there occurred at a critical stage in division some special event caused by the

drug. This event must be a rare one since about 100 generations passed before filament formation reached its peak. The susceptibility to filament formation was opposed by a continuing adaptation and the percentage of filaments tended to decrease. The stabilization of adaptation to TEM 1000 mg./l. (Table 2) occurred between the 18th and 29th subculture which was long after the percentage of filaments had reached its peak (at about the 11th subculture).

During subculture in liquid medium containing TEM and afterwards during subculture in drug-free medium the growth rate sometimes changed abruptly during growth and often fluctuated from subculture to subculture (Tables 1, 2). These irregularities in growth partly reflect the complexities of adaptation and partly arise from the disturbance of the measured growth rate by the precipitation of bacteria from suspension. Any relation which might exist between growth rate and filament formation was thus obscured unless adaptation was so far advanced that the complexities and precipitation had disappeared or unless the true growth rates of individual clones (on solid medium) be considered. For example, after prolonged adaptation of *Klebsiella aerogenes* to TEM in liquid medium, both the mean generation time (T) (Table 1) and the percentage of filaments (Fig. 4) had minimum values, and during initial growth on solid medium containing TEM both the median delay and the percentage of filaments increased with increasing concentration of TEM. The minimum value of T and the maximum percentage of filaments increased with increasing concentration of TEM in serial subculture.

The 'lenticular areas' observed in colonies growing on solid medium containing TEM were probably a result of faults arising in the quasi-crystalline structure of the colonies as the size of the bacteria changed during growth (Dean & Hinshelwood, 1957*b*). In agreement with this, the number and extent of the lenticular areas and the tendency of the size of the bacteria to change during growth increased with age and size of the colonies and with increasing TEM concentration.

Although repeated subculture in liquid medium containing TEM (e.g. 2000 mg./l.) usually impaired growth in the absence of drug both in liquid medium (Table 1) or on solid medium, it brought about the following responses: (1) the growth rate in liquid medium containing TEM was optimal (Table 1); (2) nearly all the bacteria survived on solid medium containing TEM 8000 mg./l. which allowed the survival of only about 1 in 13,000 bacteria of the original sensitive strain; (3) the distribution of the bacteria with lengths for growth in liquid medium or on solid medium, with or without TEM, had returned to the distribution for the original sensitive strain growing on the corresponding drug-free medium.

Adaptation of *Klebsiella aerogenes* to TEM seems to be reflected not so much in an increase of the overall rate of growth as in an increase of the rate of division until it equals the rate of growth and in an increase of the ability of the bacteria to resist precipitation and the lethal effects of TEM.

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REFERENCES

DAVIES, D. S., HINSHELWOOD, C. N. & PRYCE, J. M. G. (1944). Studies in the mechanism of bacterial adaptation. *Trans. Faraday Soc.* **40**, 397.

DEAN, A. C. R. & HINSHELWOOD, SIR C. (1955). The rate of development of colonies of *Bacterium lactis aerogenes* on agar plates containing drugs. *Proc. R. Soc.* B **144**, 297.

DEAN, A. C. R. & HINSHELWOOD, SIR C. (1957*a*). The formation of papillae on bacteria. colonies I. *Proc. R. Soc.* B **147**, 1.

DEAN, A. C. R. & HINSHELWOOD, SIR C. (1957*b*). The formation of papillae on bacterial colonies II. *Proc. R. Soc.* B **147**, 10.

DEAN, A. C. R. & HINSHELWOOD, SIR C. (1966). *Growth, Function and Regulation in Bacterial Cells.* Oxford: Clarendon Press.

DRABBLE, W. T. & HINSHELWOOD, SIR C. (1961). Development of resistance to streptomycin by *Bact. lactis aerogenes* (*Aerobacter aerogenes*). I. The role of mutation and of physiological adaptation. *Proc. R. Soc.* B **154**, 449.

GRANT, D. J. W. (1967). Kinetic aspects of the growth of *Aerobacter aerogenes* with some benzenoid carbon sources. *J. gen. Microbiol.* **46**, 213.

HINSHELWOOD, C. N. & LODGE, R. M. (1944). A physico-chemical study of some induced changes in the morphology of *Bacterium lactis aerogenes*. A theory of the balance and adaptive variation of certain enzyme processes in bacteria. *Proc. R. Soc.* B **132**, 47.

IYER, V. N. & SZYEALSKI, W. (1958). Two simple methods for the detection of chemical mutagens. *Appl. Microbiol.* **6**, 23.

KATCHMAN, B. J., SPOERL, E. & SMITH, H. E. (1955). Effects of cell division inhibition on phosphorus metabolism of *Escherichia coli. Science* **121**, 97.

KILGORE, W. W. & GREENBERG, J. (1961). Filament formation and resistance to 1-methyl-3-nitro-1-nitrosoguanidine and other radiometric compounds in *Escherichia coli. J. Bact.* **81**, 258.

LOVELESS, L. E., SPOERL, E. & WEISMAN, T. H. (1954). A survey of effects of chemicals on division and growth of yeast and *Escherichia coli. J. Bact.* **68**, 637.

MAXWELL, R. E. & NICKEL, V. S. (1954). Filament formation in *E. coli* induced by azaserine and other antineoplastic agents. *Science* **120**, 270.

MONOD, J. (1942). *Recherches sur la Croissance des Cultures Bactériennes.* Paris: Hermann et Cie.

POOLE, A. & HINSHELWOOD, C. N. (1940). Physico-chemical aspects of bacterial growth. Part VI. The influence of toxic substances on growth rate, stationary population and fermentation reactions of *Bact. lactis aerogenes. J. chem. Soc.* p. 1565.

SCHWEISFURTH, R. (1959). Einwirkung einiger Gifte auf Zell- und Nucleoid-teilung bei Bakterien. *Zentbl. Bakt. ParasitKde, Abt.* II, **113**, 48.

SPRAY, G. H. & LODGE. R. M. (1943). The effects of resorcinol and *m*-cresol on the growth of *Bact. lactis aerogenes. Trans. Faraday Soc.* **39**, 424.

SZYBALSKI, W. (1960). The mechanism of chemical mutagenesis with special reference to triethylene melamine action. *Developments in Industrial Microbiology*, **1**, 231.

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An Electron Microscope Study of the Mesosomes of a Penicillinase-producing Staphylococcus

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SUMMARY

Thin sections of *Staphylococcus aureus* were examined in the electron microscope, before and after treatments which released part of their cell-bound penicillinase. In all cases, such treatment resulted in alteration of the mesosome structure: the mesosome was replaced by a series of pockets or invaginations in the cytoplasmic membrane. The converse situation was not always true; cocci treated in such a way as to alter the mesosome structure did not necessarily release penicillinase. This leads to the conclusion that structural alteration of the mesosome is only one of a number of steps in the release of penicillinase.

INTRODUCTION

When penicillinase is synthesized by Bacillus and Staphylococcus, only part of the total penicillinase produced is released into the medium as an exo-enzyme, the rest remaining firmly bound to the cell. A mechanism for this synthesis and the subsequent release of penicillinase in Bacillus was proposed by Lampen (1965), based on the assumption that the mesosome—the membranous intracytoplasmic organelle found in many bacteria—is the site of synthesis of cytoplasmic membrane and of penicillinase. Thus it appeared to be worthwhile, in an investigation of the release of penicillinase, to study the ultrastructure of the mesosomes. Suganuma (1963) studied the mesosomes of staphylococci and correlated alteration in mesosome morphology with age of culture; however, he did not state whether the strain he used produced penicillinase. Coles & Gross (1967*a*, *b*) studied some of the factors influencing the release of penicillinase as an exo-enzyme, and described the release of substantial amounts of penicillinase which had previously been bound to the cell. It was decided to examine in the electron microscope thin sections of organisms treated by the methods of Coles & Gross to determine whether the *in vitro* release of penicillinase caused any structural changes, particularly in the morphology of the mesosomes.

METHODS

Staphylococcus aureus strain 8325$\alpha i^{-}p^{+}$ (Novick & Richmond, 1965) was grown overnight in the CY medium used by Novick (1963), and also in a medium modified by using 0·05 M-tris as buffer instead of the β-glycerophosphate specified by Novick; this modified medium will be referred to as tris+CY medium.

Organisms grown in both these media produced penicillinase, but whereas the CY medium resulted in about 45% of the total penicillinase being released to the

growth medium in a soluble form, growth in the tris+CY medium resulted in less than 2% of the total penicillinase being released, the other 98% remaining firmly bound to the organism (Coles & Gross, 1967*a*). Thus the tris+CY grown organisms were the better starting material for the *in vitro* study of penicillinase release, and samples of these organisms were subsequently incubated for 1 hr at 37° in a medium containing 0·15 M-Na_2HPO_4 and 0·01 M-glucose, adjusted to pH 7·6. This treatment resulted in the release in a soluble form of 30% of the previously cell-bound penicillinase. Details of these procedures and the method for determining penicillinase are given in the papers of Coles & Gross (1967*a*, *b*) in which the biochemical aspects of the penicillinase release are reported.

Samples of cocci were fixed by a modification of the method of Kellenberger, Ryter & Séchaud (1958): they were prefixed by the addition of 10% (v/v) of the Kellenberger fixative; after 30 min. they were centrifuged down, and resuspended in the full-strength fixative. After 2 hr, the cocci were again centrifuged down, then suspended in warm 1% (w/v) agar and this suspension spread on a glass slide. After the agar had solidified, it was cut into small cubes which were fixed overnight. A 2 hr treatment with uranyl acetate was used, and after ethanol dehydration the specimens were embedded in Araldite. Sections about 90 mμ thick were cut by using an LKB Ultrotome, stained for 30 min. with a 1% (w/v) solution of uranyl acetate in ethanol, and examined in a Siemens Elmiskop I electron microscope.

RESULTS

Staphylococci grown in CY or tris+CY medium

There was little difference in morphology between cocci grown in CY medium and those grown in the tris+CY medium, apart from the easier visibility of the nuclear material in the CY-grown cocci. Since the cocci grown in tris+CY were used as the starting material in the study of the release of penicillinase, all micrographs of normal cocci shown are of organisms which had been grown in the tris+CY medium.

A conspicuous feature of the morphology of these cocci was the presence of mesosomes in large numbers and of various shapes and sizes. There appeared to be three types of mesosome: a lamellar type composed of concentric whorls of membrane, a vesicular type which was a collection of vesicles bounded by a membrane, and a mesosome which was a mixture of both types.

In Pl. 1, fig. 1, can be seen a coccus containing a mesosome of the lamellar type; the mesosome is bounded by an infolding of the cytoplasmic membrane. In the case of staphylococci prepared for electron microscopy as described above, the cytoplasmic membrane appears as a triple-layered membrane with a dark outer layer, a white gap, and a greyish inner layer. As would be expected, when this cytoplasmic membrane doubles back on itself, the bounding membrane of the mesosome then has a grey outer layer and a black inner layer.

In Pl. 1, fig. 2, is a coccus containing a mesosome of the vesicular type; the vesicles appear to be rolled up portions of the mesosome membrane since they have a dark outer layer, a small gap, and contain greyish material, similar to the grey layer of membrane on the inside. Thus, it would seem that the vesicles result from a further invagination and closing off of the bounding membrane of the mesosome as Fitz-James (1960) suggested.

In Pl. 1, figs. 3, 4, can be seen cocci which contain mesosomes of the mixed type. In the organisms in both these figures, the mesosomes contain vesicles and show some evidence of the lamellar type of structure.

A spatial relationship exists between the mesosome and the developing cross-wall of *Staphylococcus aureus*; this is shown in Pl. 2, figs. 5–7, illustrating adjacent sections of the same coccus. Both mesosomes are seen to be in contact with the developing cross-wall. On the other hand, the set of adjacent sections shown in Pl. 2, figs. 8–10, is of a coccus which contained a completed cross-wall and which also contained two mesosomes in each half of the coccus; three of these mesosomes opened directly into the space between the cell-wall and the cytoplasmic membrane, and so it would seem possible that these mesosomes are responsible for some function other than the synthesis of cell-wall material.

Organisms grown in tris + CY medium and then incubated to release penicillinase

When cocci grown in the tris + CY medium were incubated for 1 hr in glucose + phosphate medium, about 30% of the previously cell-bound penicillinase was released in soluble form. Morphologically, what was seen in these cocci was the almost complete absence of mesosomes and the appearance of a new type of structure, which appeared to be an invagination of the cytoplasmic membrane, probably best described as a pocket.

Plate 3, fig. 11, shows a cell containing several of these pockets. It would appear that there was some relationship between these pockets and the mesosomes which were present in the cocci before enzyme release, since there were frequently vesicles within the pockets, and occasionally in the gap between the cell wall and the cytoplasmic membrane. Thus, a process whereby the mesosome contents were everted into the wall-membrane gap could be postulated, with the pockets resulting from the cytoplasmic membrane surrounding a vesicle which had been everted in this way.

There were large numbers of pockets in these cocci; this is shown in Pl. 3, figs. 12, 13, which were adjacent sections of the one coccus. If eversion of the vesicles occurred as proposed, then the number of pockets observed would be proportional to the total number of vesicles originally present in the coccus. Although it would seem from the micrographs already described that this process of penicillinase release was accompanied by a shrinkage of the cytoplasmic membrane (Pl. 3, figs. 12–13), this seems inconsistent with the proposed mechanism. No membrane shrinkage occurred in the organisms shown in serial section in Pl. 4, figs. 14–16; the shrinkage observed in the other micrographs of the treated cocci might be an artifact, reflecting a greater osmotic sensitivity of these cocci.

One of the rare instances of a mesosome remaining after treatment to release penicillinase is shown in the portion of the coccus on the right of Pl. 4, fig. 15.

Other treatments of normal cocci

Normal cocci were subjected to the following treatments (Coles & Gross, 1967*a*, *b*) which caused penicillinase release and alteration of the mesosome structure: incubation in 0·15 M-phosphate or arsenate for 1 hr at pH 7·6, which caused pocket formation; and incubation with 0·01 M or 0·15 M-sodium citrate for 1 hr, which produced pocket formation and some retention of the original mesosome structure.

Although 0·15 M-phosphate or arsenate at pH 6·0 or 7·6 caused instantaneous pocket formation, at pH 6·0, no penicillinase was liberated by phosphate. On the other hand, treatment with dextran sulphate (Coles & Gross, 1967*a*) which, caused instantaneous liberation of surface-located penicillinase, produced no detectable change in mesosome structure. Thus alteration of the mesosome structure was always observed and might be a prerequisite for the release of penicillinase from within the organism. However, penicillinase release did not always follow such alteration of structure, as shown by the case of phosphate at 0 min. and pH 6·0. Other factors, therefore, appeared to be necessary to release penicillinase; alteration of mesosome structure may be an early step in this sequence.

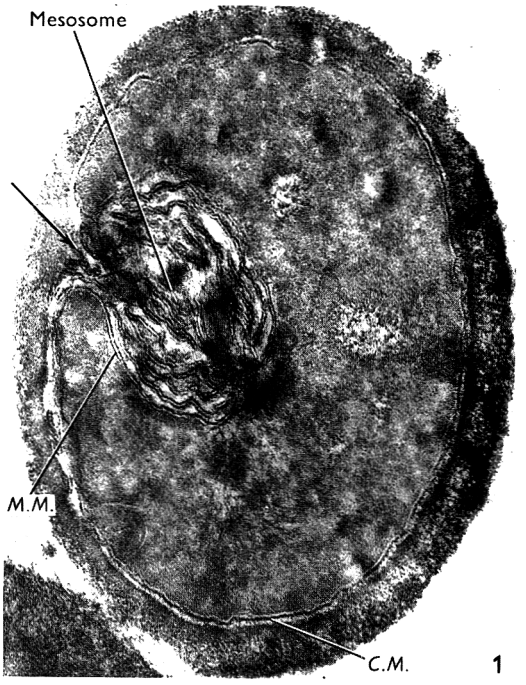
DISCUSSION

There has been much speculation about the function of bacterial mesosomes since they were first discovered. Fitz-James (1960), in his study of spore formation in Bacillus, remarked on the function of the mesosomes and suggested that they were involved in the formation of transverse septa, and the laying down of membrane. Fitz-James also observed vesicles within the mesosomes and he suggested that they were formed by an invagination of the mesosome membrane. It is possible that this is also the case with the Staphylococcus mesosomes (Pl. 1, figs. 3, 4) and that thinner sections would show this relationship more clearly.

Ryter & Jacob (1964) have shown that there is a spatial relationship between the mesosomes and the nuclei and cross-walls of dividing *Bacillus subtilis* organisms. A similar spatial relationship between mesosomes, nuclei and cross-walls is seen in *Staphylococcus aureus* (Pl. 2, figs. 5–7). It is possible that this spatial relationship reflects a functional relationship and that it demonstrates the mesosomes functioning as sites of synthesis of cross-wall material as suggested by Fitz-James (1960). However Pl. 2, figs. 8–10, shows mesosomes with a direct connexion into the wall-membrane gap, in an organism with completed cross-walls. In such a case it would seem that the mesosomes are responsible for some function other than the synthesis of cross-wall material; this function could be the synthesis of other cellular materials such as penicillinase.

Lampen (1965) proposed that the mesosome is an essential element in the secretion of penicillinase by Bacillus. He suggested that penicillinase is inserted into and bound to the cytoplasmic membrane at its point of growth in the mesosome, and that release of this penicillinase is dependent on the membrane growing out of the mesosome. The findings reported here for *Staphylococcus aureus* are consistent with Lampen's hypothesis if it is assumed that vesicles are the sites of growth of new membrane, so that penicillinase is likewise bound to the vesicles; eversion of the vesicles to the wall-membrane space would then make this penicillinase accessible for release.

Eversion of vesicles was demonstrated by Fitz-James (1964) and Weibull (1965) in *Bacillus megaterium*, and by Ryter & Jacob (1964) in *B. subtilis*. These authors used solutions containing sucrose to induce plasmolysis of the organisms; the 'osmotic buffer' used by Fitz-James contained 0·3 M-sucrose, Weibull used sucrose solutions of at least molar concentration for plasmolysis to occur, and Ryter and Jacob used 0·5 M-sucrose. The glucose+phosphate medium used in the present work contained



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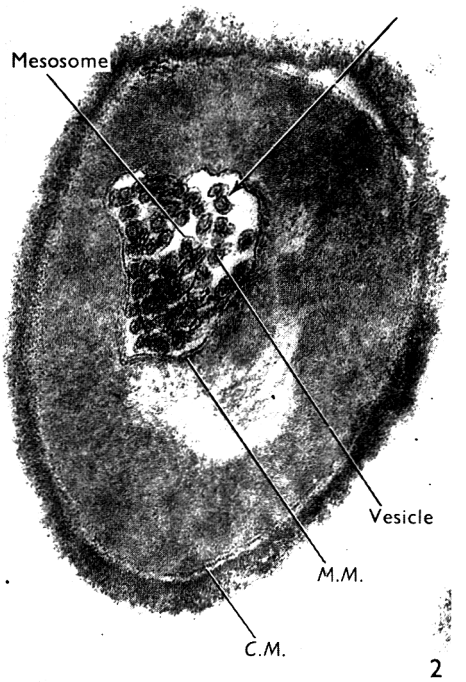

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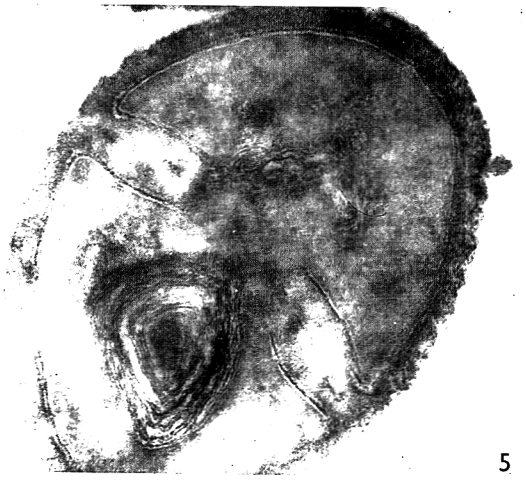

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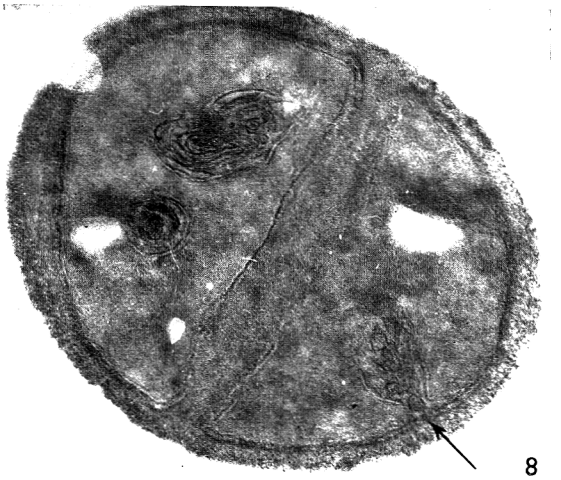

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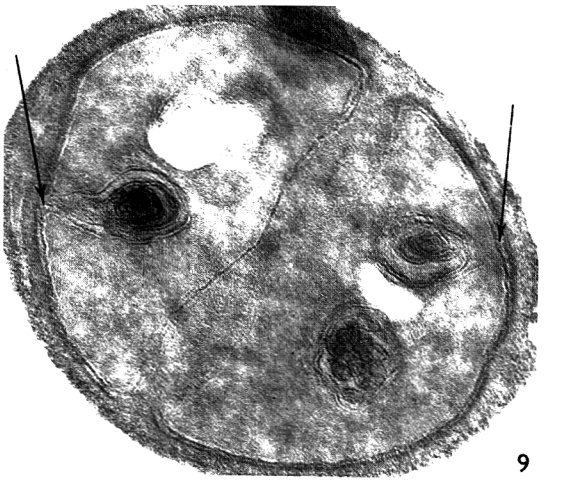

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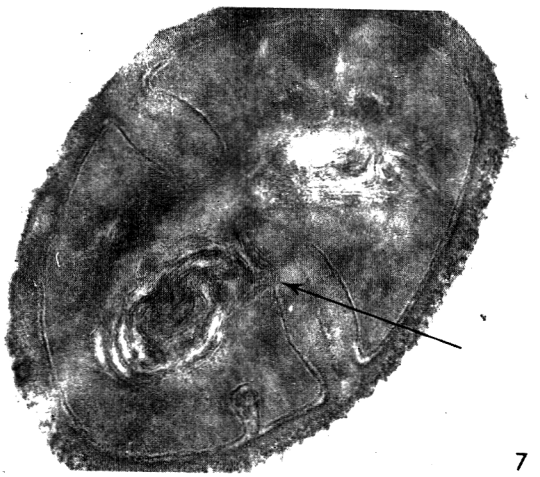

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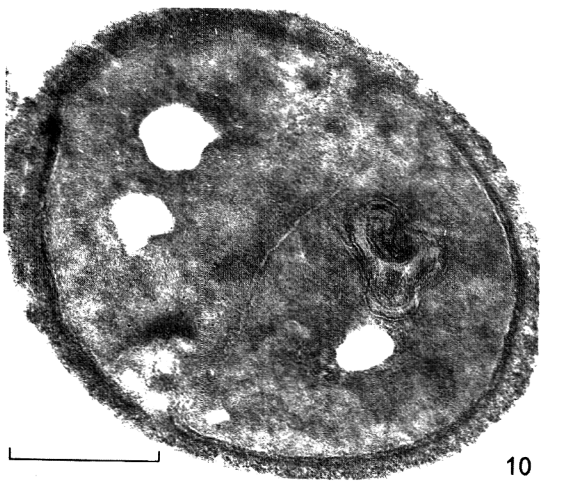

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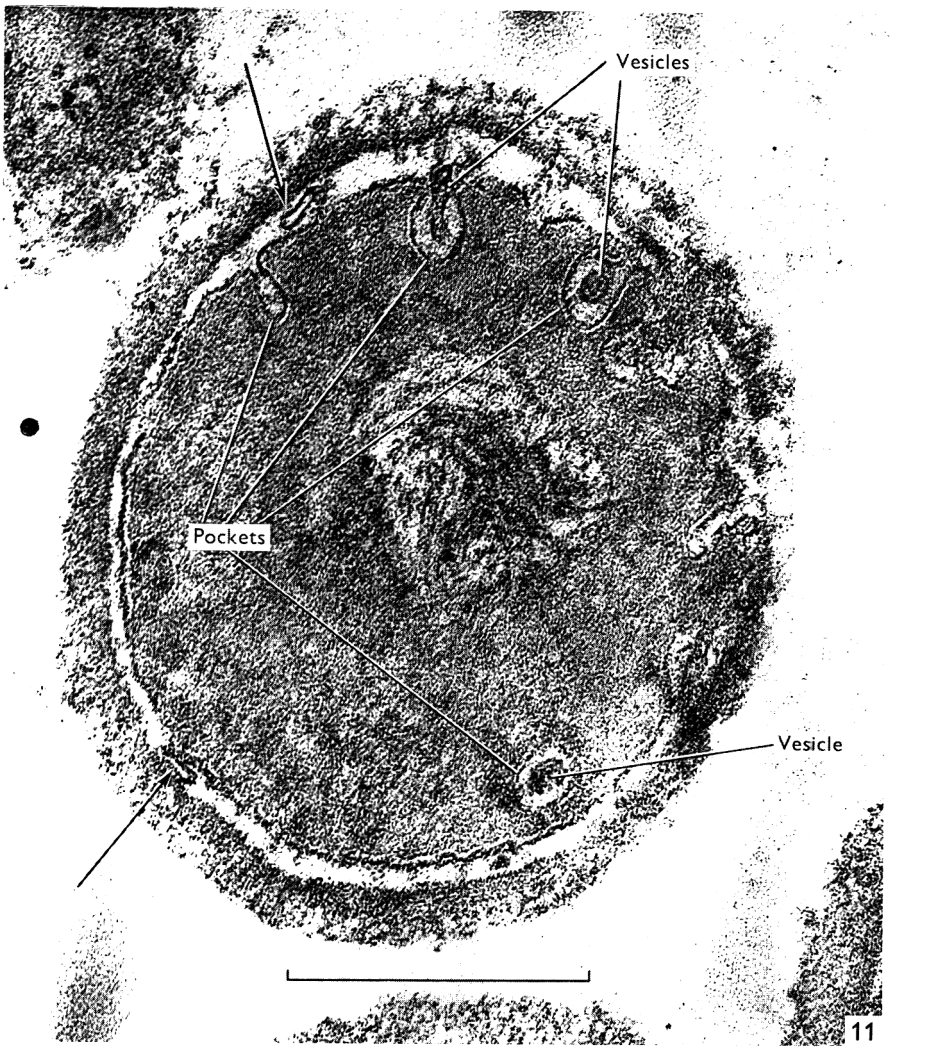

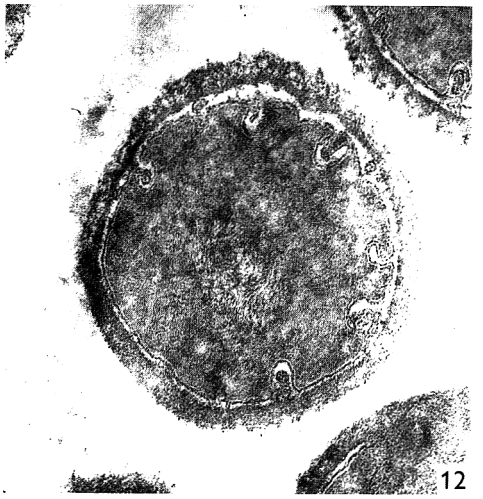

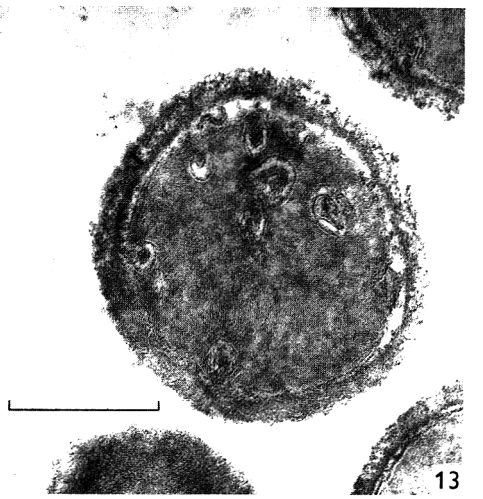

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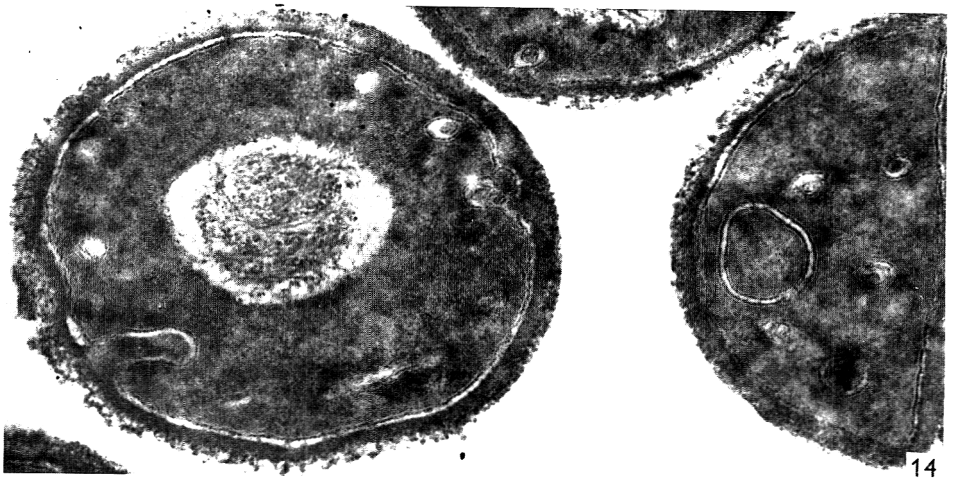
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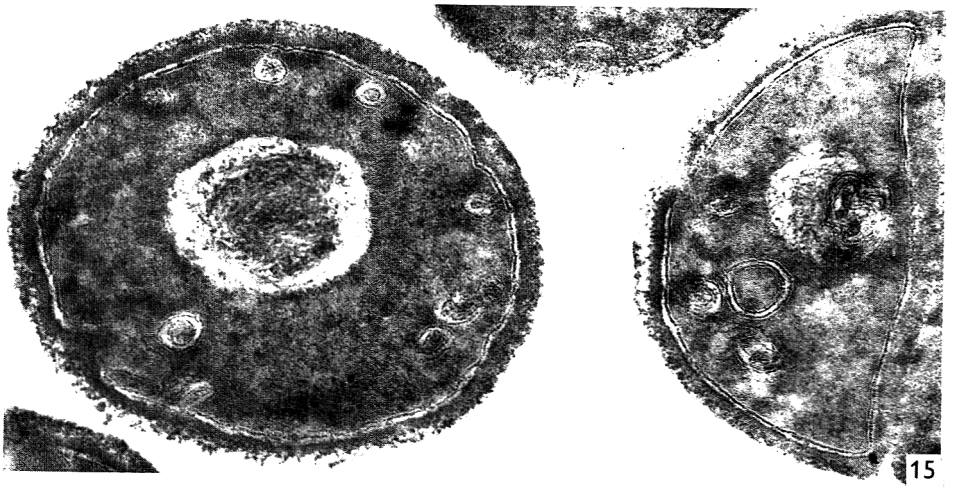
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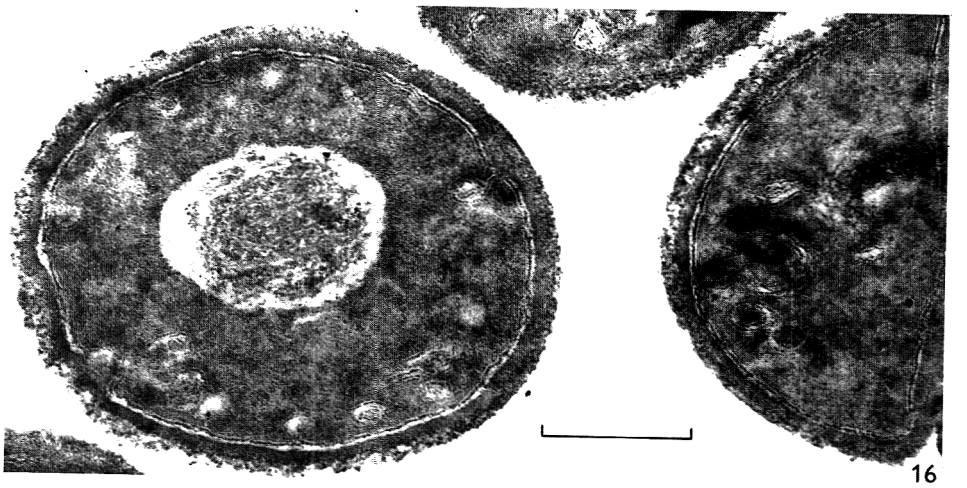
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0·01 M-glucose and 0·15 M-Na_2HPO_4—concentrations too low to cause plasmolysis and thus ruling this out as an explanation for the observed vesicle eversion.

Therefore, although there does not appear to be a direct causal relationship between vesicle eversion and penicillinase release, the results presented here do indicate that the eversion of vesicles may be an early step in the release of penicillinase.

The author thanks Dr R. Gross and Dr N. W. Coles of these Laboratories for supplying the cultures used in this study and for much helpful discussion; he is grateful to Miss F. L. Kirby for technical assistance.

REFERENCES

COLES, N. W. & GROSS, R. (1967*a*). Liberation of surface-located penicillinase from *Staphylococcus aureus*. *Biochem. J.* **102**, 742.

COLES, N. W. & GROSS, R. (1967*b*). Influence of organic anions on the liberation of penicillinase from *Staphylococcus aureus*. *Biochem. J.* **102**, 748.

FITZ-JAMES, P. C. (1960). Participation of the cytoplasmic membrane in the growth and spore formation of bacilli. *J. biophys. biochem. Cytol.* **8**, 507.

FITZ-JAMES, P. C. (1964). Fate of the mesosomes of *Bacillus megaterium* during protoplasting. *J. Bact.* **87**, 1483.

KELLENBERGER, E., RYTER, A. & SÉCHAUD, J. (1958). Electron microscope study of DNA-containing plasms. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. *J. biophys. biochem. Cytol.* **4**, 671.

LAMPEN, J. O. (1965). Secretion of enzymes by micro-organisms. *Symp. Soc. gen. Microbiol.* **15**, 115.

NOVICK, R. P. (1963). Analysis by transduction of mutations affecting penicillinase formation in *Staphylococcus aureus*. *J. gen. Microbiol.* **33**, 121.

NOVICK, R. P. & RICHMOND, M. H. (1965). Nature and interactions of the genetic elements governing penicillinase synthesis in *Staphylococcus aureus*. *J. Bact.* **90**, 467.

RYTER, A. & JACOB, F. (1964). Étude au microscope électronique de la liasion entre noyau et mésosome chez *Bacillus subtilis*. *Ann. Inst. Pasteur, Paris* **107**, 384.

SUGANUMA, A. (1963). Some features of membranous structures in *Staphylococcus aureus*. *J. infect. Dis.* **113**, 179.

WEIBULL, C. (1965). Plasmolysis in *Bacillus megaterium*. *J. Bact.* **89**, 1151.

EXPLANATION OF PLATES

On all micrographs, the bar represents 0·25 μ. The magnification is × 80,000 for all micrographs except fig. 11 for which it is × 160,000.

PLATE 1. Normal cocci

Fig. 1. Section of a coccus illustrating the lamellar type of mesosome; the mesosome is composed of concentric whorls of membrane. The arrow indicates the point of attachment to the cytoplasmic membrane. The mesosome membrane (*M.M.*), and the cytoplasmic membrane (*C.M.*) are marked.

Fig. 2. An organism which illustrates the vesicular type of mesosome. The arrow indicates a vesicle in which the dark outer layer and the greyish inner layer of membrane can be seen.

Figs. 3, 4. Sections showing the mixed type of mesosome structure: the mesosomes show both lamellar and vesicular structure.

PLATE 2. Normal cocci

Figs. 5–7. Adjacent sections of a dividing organism showing the attachment of mesosomes to the developing cross-walls.

Figs. 8–10. Adjacent sections of an organism which contained a completed cross-wall. The points of attachment of the mesosomes to the cytoplasmic membrane are shown.

PLATE 3. Cocci treated to release penicillinase

Fig. 11. Section of an organism showing the pockets which appeared when penicillinase was released. Some of these pockets contain what appear to be vesicles. There is a small amount of membranous material (arrowed) in the space between the cell-wall and the cytoplasmic membrane.

Figs. 12, 13. Adjacent sections of one organism. This pair of micrographs gives some idea of the large number of pockets in each coccus.

PLATE 4. Cocci treated to release penicillinase

Figs. 14–16. Adjacent sections of two cocci, showing pocket formation. There has been negligible shrinkage of the cytoplasmic membrane in these organisms.

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Deoxyribonucleate Binding and Transformation in *Mycoplasma laidlawii*

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SUMMARY

Mycoplasma laidlawii organisms are capable of binding in deoxyribonuclease-resistant form high molecular weight double- or single-stranded homologous or heterologous deoxyribonucleate in amounts of $0{\cdot}1-1{\cdot}0 \times 10^{-15}$ g./organism, in a temperature-dependent mode. The maximum amounts of bound DNA ranged from 25 to 125 molecules of 5×10^6 d. Transformation of streptomycin-sensitive recipients to streptomycin resistance under conditions of maximum DNA uptake was relatively rare, being about 2-4-fold higher than spontaneous mutation. Ultraviolet (u.v.) inactivation studies showed that no caffeine-inhibitable u.v. dark-repair mechanism was present. Photoreactivation was demonstrable; the PR cross-sector was 0·39. From these results it was inferred that *M. laidlawii* is unable to be genetically transformed because of the absence of one or more steps which occur between irreversible DNA uptake and genetic integration. A model system which incorporates the above findings and suggests that donor DNA functions transiently in recipients is presented.

INTRODUCTION

Mycoplasma laidlawii organisms possess a circular single DNA molecule of 800×10^6 d. (H. J. Morowitz, personal communication) and thus are at least three times less complex genetically than *Escherichia coli* (2500×10^6 d.; Cairns, 1963). Conditional lethal mutants of *M. laidlawii* can be isolated with ease (Folsome & Folsome, 1966). In general the organism, by virtue of its genetic simplicity and lack of metabolic versatility, seems to be an ideal subject for a complete genetic analysis ultimately directed towards saturation of the chromosome with conditional lethal mutant cistrons.

The present paper summarizes experiments upon deoxyribonucleate (DNA) binding, transformation, and ultraviolet radiation (u.v.) inactivation in *Mycoplasma laidlawii*. It shows that the organisms bound large amounts of DNA, that genetic transformation frequencies were very low, and that the organisms possessed no demonstrable caffeine-inhibitable dark-repair mechanism. From these results it might be concluded that *Mycoplasma laidlawii* possesses no demonstrable recombination enzyme(s).

METHODS

Organisms. Mycoplasma laidlawii strain A, (ATCC 14089) was obtained from the American Type Culture Collection in 1965. This culture was passaged through 10 subcultures before use: at each stage a typical single isolated colony was used as the inoculum. A spontaneous streptomycin-resistant mutant was isolated (resistant to at least 200 μg. streptomycin/ml.).

Media. Tryptose (T) broth or agar plates containing, g./l. distilled water: 20, Bacto-tryptose; 5, NaCl; 5, Na acetate; 5, tris; 1, glucose; 10, (or 4 for soft agar) Davis New Zealand agar (when required), adjusted to pH 8·0 with 6 N-HCl. Difco PPLO serum fraction was added to all sterile cooled media to a final concentration of 0·5% (v/v). Streptomycin, at a final concentration of 50 or 10 μg./ml., was used as indicated below in soft agar overlays.

Colony-forming unit counts and transformant assays were done by suitably diluting and pipetting 0·01 ml. samples on to the surface of tryptose agar (T) plates. After 4 hr, 1·5 ml. soft agar streptomycin overlays were poured on T agar plates to select for *str-r* organisms. Plates were incubated 2–5 days at 37° and resultant colonies were counted with the aid of a Zeiss-Jena SMXX stereomicroscope at × 40 magnification. The errors for this system of assays were not in excess of 8%, as measured by computation of the standard deviation of many replicate assays.

DNA was isolated from 1 l. *Mycoplasma laidlawii* or 1 l. *Escherichia coli* exponential phase cultures according to the method of Marmur (1961).

[2-^{14}C]*Thymidine labelled DNA* was prepared from *Escherichia coli* 15 T$^-$ cultures grown in 1 l. volumes of thymidine-supplemented minimal medium (after Strelzoff, 1962) which contained 100 to 250 μc of [2-^{14}C]thymidine. [2-^{14}C]Thymidine-labelled DNA was isolated from *Mycoplasma laidlawii* cultures grown in 1 l. volumes of Razin & Cohen (1963) defined medium in which the thymidine concentration was decreased to 10 μg./ml. and to which was added 250 μc of [2-^{14}C]thymidine. The working specific activity of both labelled purified DNA preparations ranged from 15,000 to 25,000 counts/min./extinction unit at 260 mμ (one extinction unit equals 50 μg. native purified DNA.)

[^{14}C]*DNA-binding studies.* Volumes of up to 25 ml. of a reaction mixture of organisms and labelled DNA were filtered through a 0·22 μ average pore size Millipore GS filter, treated as required, and washed with 30 ml. ice cold ultra-violet buffer. Ultra-violet buffer contained, per l. distilled water: tris 5 g., NaCl 5 g., Na acetate 5 g., $MgSO_4 . 7H_2O$ 10^{-3} M, at pH 8·0. The extinction of this buffer was 0·12 at 2537 Å and 10 mm. path length. Organisms were essentially all retained on the surface of the filter. Air-dried filters were fastened to 1·25 in. planchets and the radioactivity measured with an ultra-thin window gas-flow Geiger detector using a Baird-Atomic model 135 scaler. Background counts were 10–11/min.; all count data presented here have been corrected for background.

Genetic transformation conditions were based upon the results of the DNA binding studies. Aerated 37° T broth exponential phase *str-s* recipient organisms were exposed to purified DNA isolated from *str-r* donors. DNAase and MgCl were added after 30 min. to final concentrations of 10–50 μg./ml. and 10^{-3} M respectively. Treated organisms, incubated for various times under non-selective conditions in T broth or on T plates, were then assayed for total colony-forming units on T agar plates, and for *str-r* transformants on streptomycin 10 μg./ml. agar plates or on T agar on top of which was overlaid streptomycin soft agar overlays containing streptomycin 10 or 50 μg./ml. S plates were incubated at 37° for 2–3 days, then at 30° for an additional 4 days. T plates were incubated at 37° for 2–3 days.

Ultra-violet inactivation studies. These were done with Mycoplasma laidlawii or *Escherichia coli* under similar standard conditions: source, single 8 W General Electric germicidal low-pressure mercury lamp (G8T5); distance from material, 40 cm. from

lower edge of lamp tube; temperature, 20°; *Mycoplasma laidlawii* or *Escherichia coli* exponential phase cultures were diluted 1/10 into u.v. buffer and irradiated with various doses in dim yellow light with agitation as 5 ml. volumes contained in the bottom of a 60 × 10 mm. sterile plastic Petri dish, covered until irradiation began with a Pyrex top coated with aluminium foil. Under these conditions a 21 sec. u.v. dose corresponded to one lethal hit for *Escherichia coli* $F^-(\lambda)^s$ exponential phase organisms in u.v. buffer. Irradiated organisms were quickly diluted in T broth or caffeine-supplemented T broth (caffeine 0·75 mg./ml.) and immediately plated on to T or caffeine supplemented (0·75 mg./ml.) T agar plates. Both T and T caffeine (TC) agar plates were incubated at 30° in light-tight boxes; a replicate T agar plate series was immediately exposed to two 15 W daylight fluorescent lamps at a 20 mm distance for 4 hr before incubation to permit photoreactivation (PR).

RESULTS

DNA binding

Kinetic studies. Two 60 ml. exponential phase *Mycoplasma laidlawii* cultures were added to equal volumes of warmed T broth supplemented with 0·5% Difco yeast extract and incubated 30 min. at 37°. To one culture was added [^{14}C]*Escherichia coli* DNA (at a final concentration of 12 mg./ml.), to the second [^{14}C]*Mycoplasma laidlawii* DNA (at a final concentration of 15 μg./ml.), and the mixtures incubated. Periodically 10 ml. volumes were withdrawn and filtered. At each time, and for each culture, two filter pads were prepared and washed; one pad was then exposed to and washed further in 10% trichloracetic acid at 4° to precipitate nucleic acids. Figure 1 depicts the kinetics of binding of labelled DNA to intact organisms and to the TCA-precipitable fraction. These results indicate that the *Mycoplasma laidlawii* organisms bound homologous or heterologous DNA equally well. At 37° binding occurred slowly; a plateau was reached by 30–40 min. The amount of activity bound was the same for the intact-organism suspension or for the acid precipitable fraction and suggested that bound DNA was not extensively degraded; however, the label might have been degraded and re-incorporated. The maximum amount of DNA bound/organism at 37° was, for these experiments, about 30% of the organism's total haploid DNA content, or about $0{\cdot}4 \times 10^{-15}$ g. Many experiments of this type, with either homologously or heterologously labelled DNA, demonstrated that the maximum amount of DNA bound ranged from 0·2 to $1{\cdot}0 \times 10^{-15}$ g./colony-forming unit. This range of bound DNA can be expressed as 25 to 125 molecules of size 5×10^3 d., since both the general method of DNA extraction and related experiments made in our laboratory upon DNA-sucrose zone sedimentation indicated the molecular weight range of DNA preparations to be from 5 to 10×10^6 d.

Temperature dependence. Standard volumes of exponential phase culture were mixed with *Escherichia coli* [^{14}C]labelled DNA and incubated for 30 min. at 4°, 30°, 37°, 40°. At the end of this time 4 ml. samples from each mixture were filtered, the filters washed and counted. Results are shown in Fig. 2. These results showed that DNA uptake was a temperature dependent process, possibly enzymic.

Permanent and transient binding of DNA. For this experiment a suspension of exponential phase organisms at 5×10^8/ml. was mixed with *Escherichia coli* labelled DNA (364 c./min./μg., 6 μg./ml. final concentration) and incubated 30 min. at 37°.

Two 4 ml. volumes of culture were filtered. Ten ml. of warmed u.v. buffer were added to the surface of one filter; 10 ml. of u.v. buffer containing DNAase 50 μg./ml. in 10^{-3} M-Mg were added to the second filter. Both filters were incubated at 37° for 20 min. Then both filters were washed with chilled u.v. buffer, dried and counted.

Organisms upon both control and DNAase-treated filters possessed equal radioactivity (725 c./min./2×10^9 colony-forming unit). This binding was equivalent to 8·3% of the total input label; the amount of DNA bound per colony forming unit was $1{\cdot}0 \times 10^{-15}$ g. Apparently no detectable transient binding occurred; bound DNA

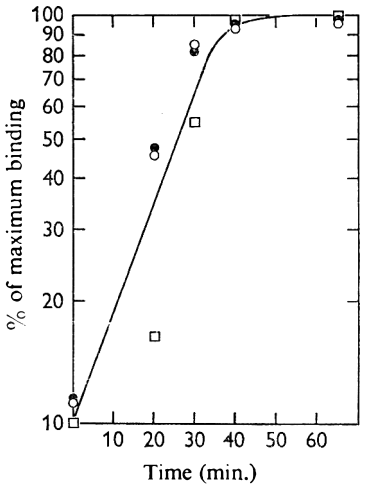

Fig. 1

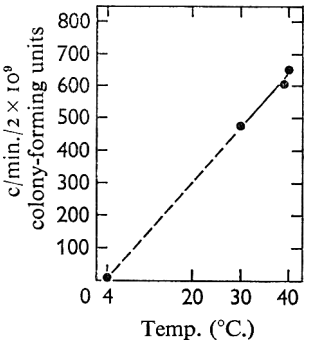

Fig. 2

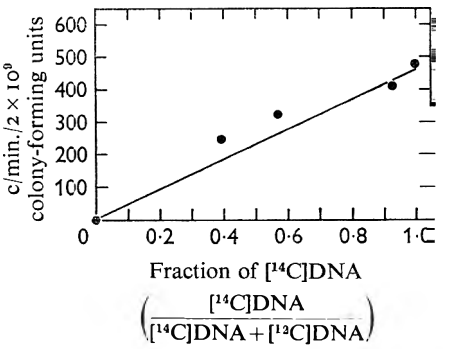

Fig. 3

Fig. 1. The uptake of homologous or heterologous [2−^{14}C]-labelled DNA into intact *Mycoplasma laidlawii* A organisms or into cold acid precipitable fractions. Homologous labelled DNA: intact recipients at $3{\cdot}7 \times 10^8$ colony-forming units/ml. were exposed to labelled DNA 12 μg./ml. of specific activity of 59 c/min./μg. Maximum amount of label bound/organism after 40 min. was $0{\cdot}41 \times 10^{-9}$ μg. (●) intact organisms; (○) acid precipitable fraction. Heterologous labelled DNA: (□) Recipient organisms at $3{\cdot}7 \times 10^8$ colony-forming units/ml. were exposed to *Escherichia coli* labelled DNA at 15 μg./ml. and a specific activity of 375 c/min./μg. Maximum amount of label bound/organism was $0{\cdot}39 \times 10^{-9}$ μg.

Fig. 2. Temperature dependence of DNA binding by *M. laidlawii* A. Recipient organisms at 5×10^8/ml. were exposed to *E. coli* [^{14}C] DNA at 8·3 μg./ml. and a specific activity of 380 c/min. μg. at the temperatures indicated for a time of 30 min. Then 4 ml. volumes were filtered and retained organisms washed, dried and counted.

Fig. 3. Competition between labelled and unlabelled DNA. [^{14}C]-labelled DNA at fixed amount (15·8 μg./ml.), various amounts of unlabelled DNA, and 5 ml. of suspension of 5×10^8/ml. exponential phase *M. laidlawii* organisms were incubated 30 min. at 37°. Then 4 ml. volumes were filtered, and the retained organisms washed, dried and counted.

was present in a form inaccessible to DNAase. However, weak interactions between DNA and organisms would not be observed by the above experiment because of the way in which the organisms were washed. Evidence for weak transient binding is discussed below.

Competition between [^{12}C] *and* [^{14}C]*labelled DNA's.* For this experiment exponential phase organisms 5×10^8/ml. were exposed for a standard 30 min. period at 37° to

mixtures containing a fixed amount of [^{14}C]*Escherichia coli* DNA at 15·8 μg./ml. and 380 c./min./μg., and variable amounts of unlabelled *Escherichia coli* DNA (from 0 to 26 μg./ml.). The amounts of radioactivity retained per 4 ml. sample of filtered organisms were plotted against the fraction of radioactive DNA to give Fig. 3. These results indicated that unlabelled DNA and [^{14}C] DNA competed equally for binding to *Mycoplasma laidlawii* organisms. [^{14}C]DNA binding was decreased by 50% when equal concentrations of unlabelled DNA were present.

Binding of single-stranded DNA. One ml. *Escherichia coli* [^{14}C]DNA at 19 μg./ml. (360 c./min./μg.) was divided into two equal portions. One portion was heated 15 min. in a boiling water bath, then plunged into acetone ice for 5 min.; this treatment, according to Doty, Marmur, Eigner & Schildkraut (1960) thermally denatures DNA from double-stranded to single-stranded molecules. This treatment yielded a 25% hyperchromic shift in u.v. (260 mμ) extinction. Five ml. volumes of exponential phase cultures at 5×10^8 colony-forming units/ml. were added to denatured and to native DNA's at 37° for 30 min. Four-ml. samples of these mixtures were filtered, washed and the radioactivity on the filter measured. Control (native) DNA-treated cultures bound 1·14 μg. (427 c./min./2×10^9 organisms), denatured DNA-treated suspensions bound 1·12 μg. (403 c./min./2×10^9 organisms). One may conclude that *Mycoplasma laidlawii* bound native or denatured DNA equally well under the same conditions, and for 30 min. exposure. Kinetic studies upon single-stranded DNA binding were not done; it is not possible to conclude that the uptake of both molecular species occurred with the same rate.

Dependence on high molecular weight DNA for binding. [^{14}C]*Escherichia coli* DNA at 170 μg./ml. (61, 370 c./min./ml.) was subjected to strong shearing forces in a Waring blendor at 4° under N_2 gas for various lengths of time. One ml. samples of sheared DNA were removed, mixed with 4 ml. recipient culture at 5×10^8 organisms/ml., incubated 30 min. at 37°; 4 ml. were filtered, washed, and radioactivity measured. Figure 4 shows the results, which indicate that maximal DNA binding required high molecular weight DNA. Binding was decreased sixfold with a blending time as short as 1·5 min.

Concentration effect. [^{14}C]*Escherichia coli* DNA (specific activity 370 c./min./μg.; final concentrations from 12 to 0·12 μg./ml.) was added to recipients at 5×10^8 organisms/ml. After 30 min. 37° incubation 4 to 25 ml. samples were taken from each reaction mixture. The results are plotted in Fig. 5 as log fraction of input label bound against log concentration of DNA/ml. reaction mixture. The slope of the line is $-0{\cdot}43$. Hence the relative amount of DNA bound per organism was greater at higher DNA concentrations and lower at lower DNA concentrations. The amount of DNA bound varied exponentially with DNA concentration, but not proportionately. These data suggest that weak transient binding of DNA did occur and that effective contacts between organisms and DNA molecules occurred with exponentially greater probability at higher than at lower DNA concentrations.

Control experiments. Numerous control experiments were used to test the reproducibility and accuracy of the millipore filter method of collecting and washing recipient organisms. [^{14}C]labelled DNA alone at concentrations from 10 μg. to 300 μg./ml. (specific activity 420 c./min./μg.) was passed through a millipore GS filter. After washing with u.v. buffer, no radioactivity exceeding background values was detectable. Recipient organisms at known titre were filtered and the filtrate

assayed directly for colony forming units to determine whether any organisms would pass through the filter; no colonies (fewer than $1/10^7$) were detected. Thus the filtration and washing procedure as used here retained essentially all viable organisms and there was no non-specific retention of DNA to the filter. Samples of thermally denatured DNA were not retained upon the filter.

Transformation

The data obtained from transformation experiments are summarized in Table 1. Clearly, transformation to *str-r* did seem to occur; but only with great rarity, since transformants were only about 2–4 times more frequent than spontaneous mutation to *str-r*. Control experiments in which no *str-r* DNA was present (Expt. I), or in

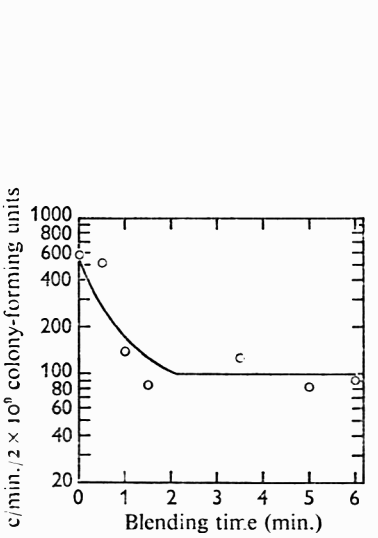

Fig. 4

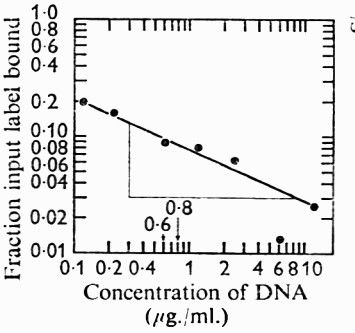

Fig. 5

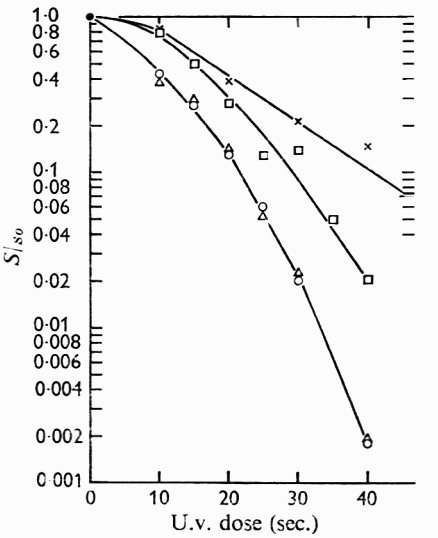

Fig. 6

Fig. 4. DNA binding dependence on high molecular weight. Labelled DNA was blended at 4° under N_2 for various times, removed, mixed with recipient organisms of *M. laidlawii* for 30 min. at 37°, and the organisms then filtered, washed, dried and counted.

Fig. 5. Effect of DNA concentration. Log of input DNA concentration against log of fraction of input label bound.

Fig. 6. Ultraviolet inactivation of *M. laidlawii* A. Log-frequency survivors plotted against u.v.-dose for *M. laidlawii* incubated (○) in dark at 30°, (△) in dark on caffeine T agar at 30°, (□) exposed 4 hr to light for photoreactivation. *E. coli* K-12 F-(λ)$^-$ dark survival is also depicted (×). *M. laidlawii* organisms were filtered through a 0·65 μ Millipore filter to remove clumps before u.v.-irradiation.

which *str-r* DNA was first exposed to DNAase (Expt. IV) indicated that the increased frequency of *str-r* colonies was specifically due to *str-r* donor DNA and not to a less specific effect of DNA upon recipient organisms.

About 120 transformation experiments have been made following protocols similar to those of Table 1. Three of these experiments showed transformation frequencies of 10^{-4} when control *str-r* colony frequencies were as low as 10^{-7}. However, two other experiments of this group showed the converse results; control *str-r* frequencies of

10^{-4} to 10^{-5} and *str-r* DNA recipient frequencies of 10^{-6} to 10^{-7}. Hence, the possibility does exist, although slight, that genetic transformation can occur, provided the correct combinations of elusive experimental parameters are present. A more probable explanation of the data is: (1) that true genetic transformation does not usually occur; (2) that the commonly observed low level of transformants is due to some other process (see below); (3) that the occasionaly high values for 'transformants' observed are due to the random occurrence of 'jackpots' of *str-r* mutants descendant from an early spontaneous *str-r* mutant in either *str-r* DNA-exposed or control cultures.

Table 1. *Genetic transformation of Mycoplasma laidlawii A*

For all experiments reported in this table *str-s* recipient exponential phase organisms at 5×10^8 to 1×10^9 colony-forming units/ml. were exposed to DNA for 30 min. at 37°; DNAase (50 μg./ml.) and $MgSO_4.7H_2O$ (to 10^{-3} M) were added, and the mixture was either (*a*) diluted and pipetted onto T agar, incubated at 37° C for 4 hr, then overlaid with 1 ml. streptomycin soft T agar (Streptomycin 50 μg./ml.) and incubated at 37° for 3–4 days, or (*b*) the broth mixture was incubated a further 100 min. at 37°, and then pipetted on to streptomycin 10 μg./ml. T agar plates and incubated for 3–4 days at 37°.

Experiment	Donor DNA conc. and source	Frequency of *str-r* colonies	Ratio exptl./ control
I. *str-s* recipients exposed under standard conditions. DNAase treated organisms plated on T agar and overlaid with streptomycin (50 μg./ml.) soft agar after 4 hr	Control, no DNA	$4{\cdot}9 \times 10^{-7}$	—
	Control, *str-s* DNA 8 μg./ml.	$5{\cdot}0 \times 10^{-7}$	—
	str-r DNA 10 μg./ml.	$2{\cdot}1 \times 10^{-6}$	4·2
	str-r DNA 5 μg./ml.	$1{\cdot}5 \times 10^{-6}$	3·0
	str-r DNA 0·1 μg./ml.	$6{\cdot}6 \times 10^{-6}$	13·2
II. *str-s* recipients as Expt. I	Control, no DNA	$1{\cdot}7 \times 10^{-7}$	—
	str-r DNA 20 μg./ml.	$3{\cdot}2 \times 10^{-7}$	1·9
	str-r DNA 5 μg./ml.	$3{\cdot}0 \times 10^{-7}$	1·8
III. *str-s* recipients exposed under standard conditions, then incubated in broth 100 min. and plated on to streptomycin (10 μg./ml.) T agar plates	Control, no DNA	$5{\cdot}9 \times 10^{-5}$	—
	str-r DNA 5 μg./ml.	$1{\cdot}1 \times 10^{-4}$	1·9
	str-r DNA 5 μg./ml. (duplicate)	$2{\cdot}0 \times 10^{-4}$	3·4
IV. Repeat of Expt. III, organisms plated on streptomycin (10 μg./ml.) T agar plates	Control, DNAase + *str-r* DNA 6 μg./ml.	$1{\cdot}4 \times 10^{-4}$	—
	Control, no DNA	$1{\cdot}3 \times 10^{-4}$	—
	str-r DNA 6 μg./ml.	$2{\cdot}4 \times 10^{-4}$	1·8
V. Repeat of Expt. III	Control, no DNA	$2{\cdot}2 \times 10^{-4}$	—
	str-r DNA 4 μg./ml.	$3{\cdot}15 \times 10^{-4}$	1·4

None of the experiments reported here has ruled out the distinct possibility that the transformants to *str-r* might have arisen by some means other than recombination between donor *str-r* DNA and recipient *str-r* chromosome. For example, the observed increase in *str-r* transformants might have been due directly to spontaneous mutation occurring at normal frequencies in recipient organisms which were permitted (as a result of incorporation of *str-r* DNA) to undergo additional partial growth on streptomycin agar plates. Such an occurrence could generate data similar to those presented in Table 1. Functional transformation has been observed to occur for pneumococcus by Evans (1964), has been discussed by Folsome (1964), and does appear to exist in *Mycoplasma laidlawii* (T. H. Iha & Folsome, 1966, unpublished experiments).

In addition to the experiments recorded in Table 1, many other experiments have been made in which the effects of: (*a*) culture age from early exponential to early stationary phase, (*b*) pH value from 6·0 to 9·0, (*c*) presence or absence of Mg^{2+} and/or Ca^{2+}, (*d*) temperature from 25° to 40°, (*e*) various defined media, (*f*) aerated or N_2 gassed recipients, (*g*) u.v.-irradiation of recipients to 0·20 survivors have been assessed. In these experiments, without exception, transformation frequencies were not increased. The conditions for optimal transformation thus far are those summarized in Table 1. Concurrently with the above experiments, a search for other modes of genetic exchange was made: a *str-r* strain was cultured with a chloramphenicol resistant (*chl-r*) strain; at various times samples were spread on chloramphenicol streptomycin agar plates in an effort to detect double recombinant, *chl-r str-r*, colonies. The results of over 15 of these experiments were uniformly negative.

Table 2. *Ultraviolet (u.v.) sensitivities of Mycoplasma laidlawii A under various post-u.v.-irradiation conditions, and of Escherichia coli*

Organism	Post-u.v.-condition	Inactivation rate at asymptote ($-dS/dD$)
M. laidlawii A	Dark	−2·27
	Dark plus caffeine	−2·27
	Photoreactivation	−1·39
E. coli K-12$^{-F}(\lambda)^{-}$	Dark	−0·53

PR cross sector (Dulbecco, 1954) = a, where $a+b = 1$, and $b = -dS/dD^{(PR)}/-dS/dD^{(Dark)} = -1{\cdot}39/-2{\cdot}27 = 0{\cdot}61$, $a = 0{\cdot}39$.

Inactivation by ultraviolet irradiation

Survival of *Mycoplasma laidlawii* as a function of u.v. dose under three post-irradiation conditions was measured; the results are shown in Fig. 6. The three post-u.v. conditions were (*a*) dark incubation on T agar plates, (*b*) dark incubation upon TC caffeine 750 μg./ml. agar plates, (*c*) immediate exposure of T agar plates to light for 4 hr for photoreactivation (PR).

There was evidence: (1) for extreme u.v.-sensitivity of *Mycoplasma laidlawii*, (2) that no caffeine-inhibitable dark reactivation appeared to exist (see Metzger, 1964), (3) that PR could be clearly demonstrated (see Table 2). The PR cross-sector (Dulbecco, 1950) was 0·39 (see Table 2 for mode of calculation). This value means that 0·39 of the u.v. lesions at asymptote were capable of photoreactivation. As comparison one might note that coli-phages possess PR cross-sectors ranging from 0·2 for phage T4 to 0·65 for phage T7 (Dulbecoo, 1950). The value for the PR cross-sector reported here for *Mycoplasma laidlawii* (0·39) is a minimal one, since the PR light exposure may or may not have been maximal.

Notable was the extreme u.v.-sensitivity of *Mycoplasma laidlawii* as compared to *Escherichia coli*: the ratio of inactivation rates at asymptote was 4·3 (−2·27/−0·53, see Table 2). If one normalizes the u.v.-sensitivities of these two organisms to a sensitivity per unit DNA, then *Mycoplasma laidlawii* was actually $4{\cdot}3 \times \sim 3) = 13$ times more sensitive. Although this relative sensitivity might be the result of other unknown processes it is consistent with the postulate that *Mycoplasma laidlawii* possesses no u.v. dark reactivation mechanism. Most interesting, however, was the evidence for the lack of caffeine-inhibitable dark repair mechanism. With regard to this charac-

teristic, *Mycoplasma laidlawii* appears to be similar to host-cell-reactivationless (HCR-) mutants of *Escherichia coli* (Sauerbier, 1964) and to u.v.-sensitive mutants of *Escherichia coli* K-12 (Howard-Flanders & Theriot, 1966). Also, transformable *Bacillus subtilis* does show a caffeine-inhibitable DR mechanism (Metzger, 1964).

DISCUSSION

Mycoplasma laidlawii organisms were capable of binding high molecular weight single-stranded or double-stranded DNA to a maximum amount of 0·1 to 0·2 of the organism's genomic DNA content within 30 min. and in a temperature-dependent manner. Bound DNA appeared to remain as high molecular weight material for at least 60 min., as shown by its acid precipitability. One must not discount the possibility that bound DNA was degraded and re-incorporated into newly synthesized recipient DNA.

The comparatively long period required for DNA binding (30 min.) and the apparent DNA exponential concentration-dependence suggested by the results in Fig. 5 can be explained by postulating that concentration-dependent transient binding of DNA occurred within very short time intervals, and that actual DNA entry into the cell required the longer times observed here. The nature of this transient binding must be such that transiently bound DNA is washed off the organisms by the methods used for washing the filter pads. Thus, the experiments on DNA binding were not optimally designed to detect weak transient binding and served mainly to indicate that, once firmly bound, DNA was inaccessible to DNAase and was either within the organism or was held on its surface in a configuration resistant to DNAase attack.

Mycoplasma laidlawii binding of single-stranded DNA also occurred; thus this system, excluding competence, appeared in many ways similar to *Hemophilus influenzae* (Barnhart & Herriott, 1963; Postel & Goodgal, 1966). Although the same amount of ^{14}C label was bound for both native and denatured DNA at 30 min., no kinetic data were available. As a consequence, one cannot state whether similar or different binding rates apply.

One can summarize the stages of DNA binding by listing this most probable sequence of events: (1) transient and quick binding of DNA by weak forces, (2) permanent binding requiring an enzymic mechanism, essentially complete by 30 min. Implicit in the above sequence is the inference that the culture is uniformly competent at least by step 2 and that competence is the normal state of exponential phase cultures, or that transient binding induces competence.

In light of the ability of *Mycoplasma laidlawii* to bind DNA, the experiments on genetic transformation were most surprising since, except for spurious instances, 'transformant' frequencies of only 2–4 times higher than spontaneous mutation were recorded. Two possibilities may be considered: (1) DNA cannot penetrate the organism, but is bound to the surface in a nuclease resistant configuration; (2) DNA can enter the organism, but except for rare instances cannot be genetically recombined into the genophore due to absence of a recombination mechanism. In the event that DNA could not penetrate, one would not expect to observe even the low level of transformants reported. Since the slight transformation which did occur was the specific result of *str-r* DNA, non-penetration could only account for the results if a small subfraction of the population bound DNA internally, and a large fraction

bound DNA in a DNAase resistant form externally. Several experiments on the survival of *str-r* recipients which had bound *str-r* DNA in streptomycin broth have indicated that bound DNA can function (Folsome, unpublished). Thus, for purposes of this discussion, it will be assumed that most bound DNA was internally located.

Were DNA to enter the cell, the question remains as to why transformant frequencies were *str-r* DNA-specific, yet low. The model which best accounts for the result is as follows: (*a*) Within short spans of time, DNA transiently and weakly binds to the surface of recipient organisms. (*b*) Recipients normally possess an enzymic mechanism by which exogenous DNA can enter. (*c*) Once within the organism, the ultimate fate of donor DNA is to be degraded, since *Mycoplasma laidlawii* requires all nucleosides in defined media (Tourtellotte, Morowitz & Kasimer, 1964). Since degradation of DNA does not appear to occur rapidly, it will be assumed that exogenous DNA can function transiently while in recipients. Perhaps exogenous DNA can also become rarely and randomly rescued by the same mechanism through which residual recombination occurs in recombination deficient *Escherichia coli* (Clark & Marguiles, 1965) or ϕx 174 phage which are growing within recombination deficient *Escherichia coli* (Tessman, 1966); (*d*) Recipient organisms are permitted to survive and grow to a greater extent on streptomycin agar plates as a result of the prior functioning of *str-r* DNA; (*e*) The outcome of the sequence of postulated events is that recipients of *str-r* DNA would show a greater frequency of *str-r* spontaneous mutants than control cultures since they undergo greater residual growth under selective conditions.

In this fashion a large part, or all, of the 2–4-fold excess of observed *str-r* colonies resulting from *str-r* DNA treatment of recipients could be due to transitory functional transformation, and not due to genetic integration of *str-r* DNA into *str-s* genophores. It is also possible that a fraction of the *str-r* 'transformants' was the result of the random and rare marker rescue of *str-r* DNA.

Consistent with these possibilities are the results obtained from u.v.-inactivation studies, by which it was shown that *Mycoplasma laidlawii* lacked a caffeine-inhibitable dark reactivation mechanism. From these data one could infer that *M. laidlawii* is similar to recombination-deficient u.v.-sensitive mutants of *Escherichia coli* K-12 which specifically lack a recombination mechanism. The model presented above views *M. laidlawii* as a natural case of a recombination-deficient organism and resolves the results of experiments upon DNA binding, transformation, and u.v. inactivation. Other preliminary experiments have been made in this laboratory in which *M. hominis* H-39, *M. gallisepticum*, and *M. laidlawii* B have been examined for caffeine-inhibitable dark repair mechanisms. To date, these species resemble *M. laidlawii* A and appear not to possess this type of u.v. dark repair system. In an attempt to select for a variant of *M. laidlawii* A which did possess a u.v. dark repair system, survivors of 3 cyc. of 60 sec. u.v.-irradiation were isolated, but showed no difference from the parental strain as regards dark repair.

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REFERENCES

BARNHART, B. J. & HERRIOTT, R. M. (1963). Penetration of DNA into *Hemophilus influenzae*. *Biochim. biophys. Acta* **76**, 25.

CAIRNS, J. (1963). The bacterial chromosome and its manner of replication as seen by autoradiography. *J. molec. Biol.* **6**, 208.

CLARK, A. J. & MARGULIES, A. D. (1965). Isolation and characterization of recombination deficient mutants of *Escherichia coli* K-12. *Proc. natn. Acad. Sci., U.S.A.* **53**, 451.

DOTY, P., MARMUR, J., EIGNER, J. & SCHILDKRAUT, C. (1960). Strand separation and specific recombination in deoxyribonucleic acids: physical chemical studies. *Proc. natn. Acad. Sci., U.S.A.* **46**, 461.

DULBECCO, R. (1950). Experiments on photoreactivation of bacteriophages inactivated with ultraviolet radiation. *J. Bact.* **59**, 329.

EVANS, A. (1964). Introduction of specific drug resistance properties by purified RNA-containing fractions from pneumococcus. *Proc. natn. Acad. Sci., U.S.A.* **52**, 1142.

FOLSOME, C. E. (1964). Functional transformation in mammalian cell culture systems. *Nature, Lond.* **202**, 1023.

FOLSOME, C. E. & FOLSOME, J. (1966). Temperature-sensitive conditional lethal mutants of *Mycoplasma laidlawii* A. *Bact. Proc.* p. 30.

HOWARD-FLANDERS, P. & THERIOT, L. (1966). Mutants of *E. coli* K-12 defective in DNA repair and genetic recombination. *Genetics* **53**, 1137.

MARMUR, J. (1961). A procedure for the isolation of DNA from micro-organisms. *J. molec. Biol.* **3**, 208.

METZGER, K. (1964). On the dark reactivation mechanism in ultraviolet irradiated bacteria. *Biochem. biophys. Res. Commun.* **15**, 101.

POSTEL, E. H. & GOODGAL, S. H. (1956). Uptake of single-stranded DNA in *Hemophilus influenzae* and its ability to transform. *J. molec. Biol.* **16**, 317.

RAZIN, S. & COHEN, A. (1963). Nutritional requirements and metabolism of *Mycoplasma laidlawii*. *J. gen. Microbiol.* **30**, 141.

SAUERBIER, W. (1964). Host cell reactivation of ox 174 at intracellular UV irradiation. *Z. Vererbungslehre* **95**, 145.

STRELZOFF, E. (1962). DNA synthesis and induced mutation in the presence of 5-bromouracil. *Z. Vererbungslehre* **93**, 287.

TESSMAN, I. (1966). Genetic recombination of phage S 13 in a recombination deficient mutant of *E. coli* K-12. *Biochem. biophys. Res. Commun.* **22**, 169.

TOURTELLOTTE, M. E., MOROWITZ, H. J. & KASIMER, P. (1964). Defined medium for *Mycoplasma laidlawii*. *J. Bact.* **88**, 11.

J. gen. Microbiol. (1968), **50**, 55–66
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Mechanism of High-level Resistance to Chloramphenicol in Different *Escherichia coli* Variants

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SUMMARY

Mutants resistant to high levels of chloramphenicol can be obtained in *Escherichia coli* B by one or two mutational events. All of 144 high-level resistant mutant clones examined were powerful inactivators of the drug. Growth of this kind of mutants in nutrient media containing chloramphenicol 100 μg./ml. or more depended on the inoculum size, the composition of the medium, and the concentration of the drug. No growth was observed with lactose as sole energy source unless the organisms had been previously induced for β-galactosidase production.

With a strain of *Escherichia coli* K 12 an entirely different type of resistant mutants occurred. High-level resistant derivatives were obtainable only through several serial mutations. None of 36 high-level resistant mutants was able to inactivate the drug. Growth of these bacteria was extremely slow, even in the absence of drug. This resistance was due to a decreased rate of permeation, which probably was non-specific and concerned many species of micromolecules.

INTRODUCTION

Resistance to chloramphenicol may be due to different mechanisms: (1) resistance of the protein synthesizing machinery (Ramsey, 1958); (2) decreased permeation to the site of antibiotic action (Cavalli & Maccacaro, 1952; Okamoto & Mizuno, 1962, 1964; Vazquez, 1963, 1964); (3) inactivation of the drug (Okamoto & Suzuki, 1965; Rananarayna, Rayaulakshmi & Ehaskaran, 1962; Miyamura, 1964; Chabbert & Debruge, 1956; Dunsmoor, Pin & Sherris, 1963).

Studies with *Escherichia coli* B and *E. coli* K 12 have now shown that high-level resistance to chloramphenicol in *E. coli* B is due to inactivation and in *E. coli* K 12 to impaired permeation. Inactivation of chloramphenicol has been effected also with the susceptible wild-type *E. coli* B, though at a slow rate, but could not be demonstrated with *E. coli* K 12. This is apparently a parallel to the well-known fact that penicillinase-producing mutants are never obtained from initially penicillinase-negative bacterial strains.

METHODS

Bacterial strains. (1) *Escherichia coli* B: the wild strain was inhibited on nutrient agar containing chloramphenicol (cm) 2·5 μg./ml.; for the present work a mutant resistant to dihydrostreptomycin 5000 μg./ml. and sulphathiazole 40 μg./ml. was developed;

Cm-resistant mutants isolated were tested for dihydrostreptomycin and sulphathiazole resistance. (2) *E. coli* K 12–W 945 (lactose-, proline-, thiamine-): this strain was inhibited by Cm 4·0 μg./ml. (3) *E. coli* K 12–W 945: with resistance transfer factor (RTF) mediating resistance to dihydrostreptomycin, sulphathiazole, tetracycline and chloramphenicol. The RTF was transferred from a wild strain of *Shigella flexneri* by using the technique of Watanabe & Fukasawa, 1961. (4) A susceptible wild strain of *Shigella boydii* was used for assay of chloramphenicol concentrations.

Drugs. Chloramphenicol (Cm) was obtained from Abic Ltd., Ramat-Gan, Israel. [^{14}C]Cm (methylene-^{14}C) was purchased from The Radiochemical Centre, Amersham, Buckinghamshire, England; its activity was stated to be 6·81 mC/mM. Tetracycline HCl: Lederle.

Culture media. (1) Bacto blood agar base (BAB). (2) Bacto tryptose phosphate broth (TP): same solidified with 1·5% Bacto agar is designated TPA. (3) Davis's minimal medium (Davis & Mingioli, 1950) and the same solidified with Bacto Noble Agar are designated DB and DA, respectively. The same minimal media were also used with lactose substituted for glucose (lactose DB, lactose DA). For growth of *Escherichia coli* K 12–W 945, the minimal medium was supplemented with L-proline 50 mg. and thiamine HCl 1 mg. per litre.

Mutation rate was estimated by the method of Luria & Delbrück (1943). In each experiment 20–56 independent cultures grown overnight from an inoculum of about 100 bacteria were plated on DA or BAB with Cm. The mutation rate was calculated from the fraction of clones with no mutants.

Bioassay for chloramphenicol. The samples to be tested were sterilized by heating in a boiling water bath during 5 min. This procedure stopped the enzymic activity. Control samples showed no decrease in the activity of chloramphenicol due to the heating. For chloramphenicol (Cm) assay, a susceptible strain of *Shigella boydii* was used as indicator. Samples of the test samples heated to 37° were added to cultures in the exponential phase ($1·7 \times 10^8$–$2·1 \times 10^8$ bacteria/ml.). The amount added was calculated to give a final concentration of Cm 2 μg./ml. if no inactivation of the drug had taken place. Turbidity of the cultures was examined with a Klett Summerson photoelectric colorimeter every 20 min. during 1 hr. Control cultures of *S. boydii* in TP medium with Cm were included in each titration. The following concentrations of Cm (μg./ml.) were used in these controls: 0, 0·5, 1·0, 2·0. From these controls a curve of generation time as a function of Cm concentration was drawn. The Cm concentration in the test samples was then determined from the generation time by interpolation.

Uptake of [^{14}*C*]*Cm.* An exponential culture in TP medium was gathered by centrifugation to a concentration of 4×10^{10} bacteria/ml. and [^{14}C]Cm 2 μg./ml. added. The culture was incubated with slight shaking during 20 min. at 37°, chilled, and the bacteria washed twice with 2 ml. cooled plain TP medium and once with 2 ml. distilled water. After the last deposition of the bacteria by centrifugation, 1 ml. of the supernatant liquid was transferred into a counting vial, and so was the sediment after resuspension. To each sample was added 15 ml. of following scintillator: 6 g. 2,5-diphenyloxazole, 0·3 g. 1,4-bis-2-(5-phenyloxazolyl)-benzene – 100 g. naphthalene in 1 l. *p*-dioxane. The vials were counted in a Packard Tricarb liquid scintillation spectrometer, model 3203. The radioactivity of the intercellular fluid varied between 15% and 25% of the activity of the cell suspension. Uptake of Cm by the bacteria was calculated from the difference between radioactivity of the cell suspension and

the intercellular fluid. Results were compared with radioactivity obtained by arbitrary amounts of [^{14}C]Cm added to vials of bacteria treated as the test samples but without added Cm.

Results were correlated to the wet weight of bacteria, which was determined by centrifugation at 18,000*g* for 10 min., removing the supernatant fluid and draining by tilting the test-tube on adsorbing paper. One g. wet *Escherichia coli* B corresponded to $6{\cdot}8 \times 10^{11}$ bacteria.

RESULTS

Escherichia coli B mutants

Mutation rate was determined in five different experiments using media BAB and DA containing Cm 4·0 μg./ml. The results varied between $5{\cdot}3 \times 10^{-10}$ and $6{\cdot}7 \times 10^{-9}$ mutations/bacterium/division. The degree of resistance was determined for 635 separate mutant colonies. The mutant clones were first purified by several transfers on agar medium containing Cm 4 μg./ml. Heavy suspensions in broth were then streaked on BAB containing different Cm concentrations. Of the clones 578 were inhibited by Cm 8 μg./ml., 21 clones by 16 μg./ml., and 36 clones (5·7%) by 150–300 μg./ml.

One-step high-level resistant mutants were obtained more conveniently by plating the susceptible strain on BAB containing Cm 16 μg./ml. The mutation rate was in this case calculated to be $1{\cdot}2 \times 10^{-10}$ mutations/bacterium/division. Of 100 mutant clones examined 100 grew when streaked on BAB with Cm 100 μg./ml. and 8 on 250 μg./ml. High-level resistant mutants were also isolated from low-level resistant clones (Cm 4 μg./ml.) by plating on BAB with Cm 16 μg./ml. These mutations occurred at a frequency of about 10^{-9}/bacterium/division. It might be concluded from these experiments that *Escherichia coli* B may obtain low-level as well as high-level resistance to chloramphenicol in a single mutational event.

Inactivation of chloramphenicol by Escherichia coli B. The susceptible *Escherichia coli* B inactivates chloramphenicol but at a very slow rate. This was shown in experiments of the following type. A TP culture was grown to the exponential growth phase and the bacteria concentrated by centrifugation to a density of $1{\cdot}6 \times 10^9$ bacteria/ml. Chloramphenicol was added to Cm 100 μg./ml. and the culture incubated for 18 hr at 37°. Assay of residual Cm showed about 80 μg./ml.

On the other hand, 114 one-step and 30 two-step high-level resistant clones all proved to be powerful inactivators of Cm. In the tests with these strains, TP with Cm 100 μg./ml. was inoculated with about $2{\cdot}0 \times 10^8$ bacteria/ml. After 18 hr incubation at 37° and centrifugation the supernatant culture fluid was assayed for Cm; none was found.

The kinetics of Cm inactivation were studied more closely in a few of the one-step high-level resistant clones. Some observations with one of the strains (*Escherichia coli* B/1) will be given. After addition of Cm to 100 μg./ml. to an exponentially growing culture of *E. coli* B/1 in TP, more than 90% of the Cm was inactivated during 30 min. and inactivation was complete after 60 min. (Fig. 1). At this time the growth, which initially was slowed by the Cm, continued at the same rate as in the control culture. With Cm 400 μg./ml., 75% of the Cm was inactivated in 30 min., and after 120 min. inactivation was complete; however, even then growth remained slow (Fig. 2). This problem will be dealt with elsewhere.

Inoculum size and nutrient medium. The minimal inhibitory concentration of Cm

for *Escherichia coli* B/1 depends on the inoculum size and the nutrient medium. This is shown by observations summarized in Table 1. An exponential phase culture in TP was centrifuged, the bacteria washed twice in cold phosphate buffer (pH 7·2; 0·15M) and inoculated on plain TPA as well as on TPA containing various amounts of Cm. With Cm up to 60 μg./ml. the colony counts were as on plain agar. However, with TPA containing Cm 100 μg./ml. growth occurred only when at least 9×10^3 bacteria were seeded, but then growth was confluent as on the control plate with

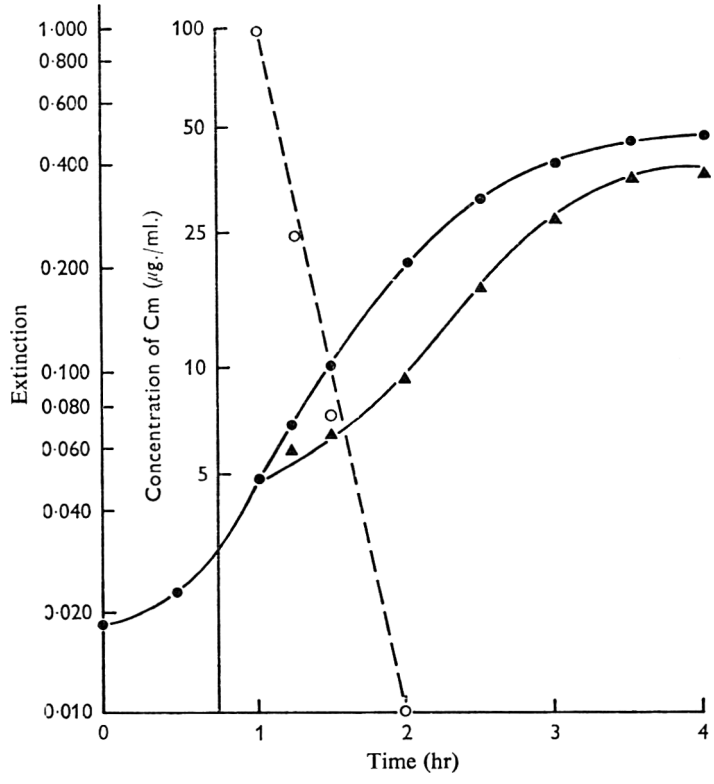

Fig. 1. Inactivation of chloramphenicol by *Escherichia coli* B/1 and growth in tryptose phosphate medium (TP) with and without Cm 100 μg./ml. Extinction and concentration of residual chloramphenicol are plotted on logarithmic scales against a linear time scale. O - - O, Cm concentration; ●—●, growth of strain B/1 without Cm; ▲—▲, growth in presence of Cm 100 μg./ml.

a similar inoculum. To obtain growth on TPA with Cm 200 μg./ml. a 1000-fold increase in inoculum size (to 9×10^6) was required. With this inoculum growth was confluent, though no colonies occurred on the plate inoculated with 9×10^5 bacteria. On minimal medium with glucose (DA) an inoculum size of 9×10^6 was required for growth in Cm 100 μg./ml.

Of special interest was the observation that on lactose DA+Cm 100 μg./ml. no growth was obtained even with an inoculum of 9×10^7 bacteria/ml. However, when the galactosidase-system was induced by incubation with lactose (1 hr), no difference was observed between growth on lactose and glucose media.

Uptake of [^{14}C]*Cm.* No significant difference was noticed in the uptake of labelled

chloramphenicol between *Escherichia coli* B and its high-level resistant derivative *E. coli* B/1 (Table 2). The radioactivity/g wet wt bacteria corresponded very closely to the [^{14}C]Cm 2 μg./ml. initially added to the suspension of the bacteria. Even though a part of the reversibly bound Cm might have been removed by washing, it seems that no marked intracellular concentration of label had taken place, and this in spite of the fact that with the high bacterial concentration used (4×10^{10} bacteria/ml.) virtually all the Cm must have been inactivated (compare Fig. 1). It seems therefore

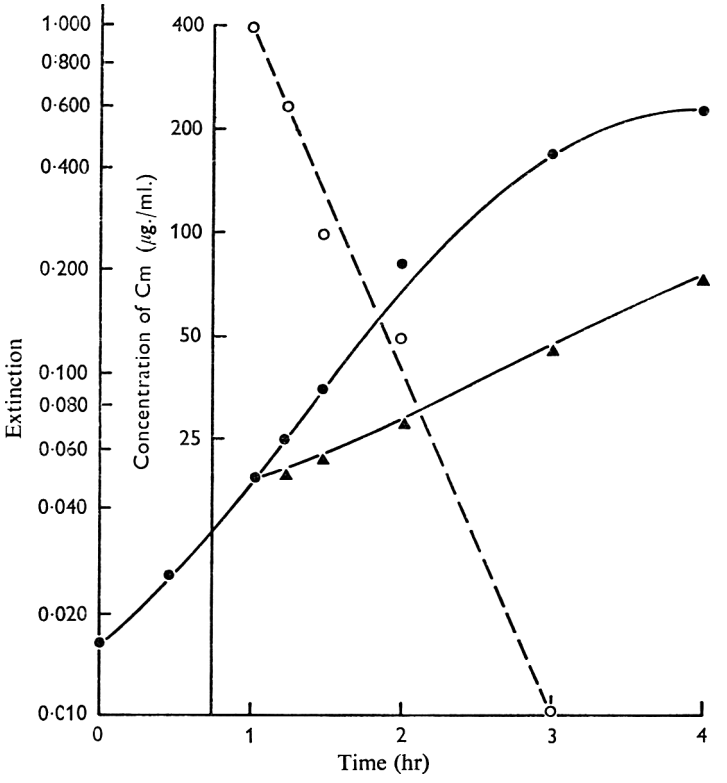

Fig. 2. Inactivation of chloramphenicol by *Escherichia coli* B/1 and growth in tryptose phosphate broth medium with and without Cm 400 μg./ml. O- - -O, Residual Cm ●—●, growth without Cm; ▲—▲, growth in presence of Cm.

that the turnover of Cm molecules was high, so that after 20 min. the same amount of radioactivity was found in the susceptible bacteria as in those which had inactivated about 17 times more Cm than the amount corresponding to the amount of label found.

Cross resistance to tetracycline. No resistance to tetracycline was found in 22 one-step high-level resistant mutants of *Escherichia coli* B. The minimal inhibitory concentration in BAB of tetracycline was 1 μg./ml., both for the mutants and for the susceptible ancestor.

Escherichia coli K 12 mutants

Mutants were isolated by plating on BAB and DA containing chloramphenicol 3–16 μg./ml. Mutation rate varied in different experiments between $4{\cdot}6 \times 10^{-10}$ and

$1{\cdot}3 \times 10^{-9}$ mutations/bacterium/division. High-level resistance was only obtained stepwise. Four mutational events were needed for resistance to Cm 300 μg./ml.

Inactivation of chloramphenicol. Inactivation by the susceptible *Escherichia coli* K 12 was examined on a TP culture concentrated to about $1{\cdot}6 \times 10^9$ bacteria/ml. and

Table 1. *Minimal inhibitory concentration of chloramphenicol for Escherichia coli* B/1*: the effects of inoculum size and growth medium*

Incubation was for 48 hr at 37°.

	Controls				Chloramphenicol (μg./ml.)							
					100		200		100		100	
	In media†											
			Lactose								DB +	DA +
	TP	TPA	DB	DA	TP	TPA	TP	TPA	DB	DA	lactose	lactose
Inoculum size*	Growth response‡											
$9{\cdot}0 \times 10^7$	+	+	+	+	+	+	+	+	+	+	−	−
$9{\cdot}0 \times 10^6$	+	+	+	+	+	+	+	+	−	+	−	−
$9{\cdot}0 \times 10^5$	−	+	+	+	+	+	−	−	−	−	−	−
$9{\cdot}0 \times 10^4$	−	+	+	+	+	+	−	−	−	−	−	−
$9{\cdot}0 \times 10^3$	−	+	+	+	+	+	−	−	−	−	−	−
$9{\cdot}0 \times 10^2$	−	+	+	+	−	−	−	−	−	−	−	−

* No. of viable bacteria inoculated per tube (10 ml. liquid medium) or per plate (10 ml. solid medium) and distributed evenly on the surface by a cotton-wool applicator.

† TP = Tryptose phosphate broth; TPA = same solidified with agar; DB = minimal medium; lactose DB = same with lactose substituted for glucose; DA and lactose DA = same solidified with agar.

‡ Growth response: +, in tube, pronounced turbidity; on Petri dish, confluent growth; −, no growth.

Table 2. *Uptake of* [^{14}C]*chloramphenicol by various strains of Escherichia coli*

	E. coli B		*E. coli* B/1		*E. coli* K 12		*E. coli* K 12/1	
Sample no.	CPM*/ 59 mg.† bacteria	μg. Cm‡/ g. bacteria	CPM/ 59 mg. bacteria	μg. Cm/ g. bacteria	CPM/ 59 mg. bacteria	μg. Cm/ g. bacteria	CPM/ 59 mg. bacteria	μg. Cm/ g. bacteria
1	6030	2·1	5815	2·0	9461	3·2	1701	0·58
2	5297	1·8	4334	1·5	9141	3·1	1426	0·49
3	5144	1·8	.	.	8614	2·9	1320	0·45

* CPM, counts/min.

† All weights are *wet weight* determined for cultures of the same turbidity values as the samples.

‡ Chloramphenicol (Cm) calculated by comparison with CPM obtained with 0·1 μg. [^{14}C]Cm in 59 mg. *E. coli*.

addition of Cm to 100 μg./ml. In 36 high-level resistant derivatives inactivation was examined by growing cultures in TP with Cm 100 μg./ml. After 24–28 hr virtually the entire amount of added Cm was found on assay (95–105%). It was therefore concluded that neither the susceptible ancestor nor its resistant mutants were able to inactivate chloramphenicol.

Properties of high-level resistant mutants. Determination of viable counts of the high-level resistant cultures on plain TPA and on TPA+chloramphenicol gave comparable results. The inoculum size was of no influence on the minimal inhibitory concentration (Cm 350 μg./ml.). The markers L-proline, thiamine and lactose were unaltered in the resistant mutants, but the bacteria were more elongated with 2·7 nuclei/bacterium in an early exponential phase culture instead of 1·4 nuclei/bacterium

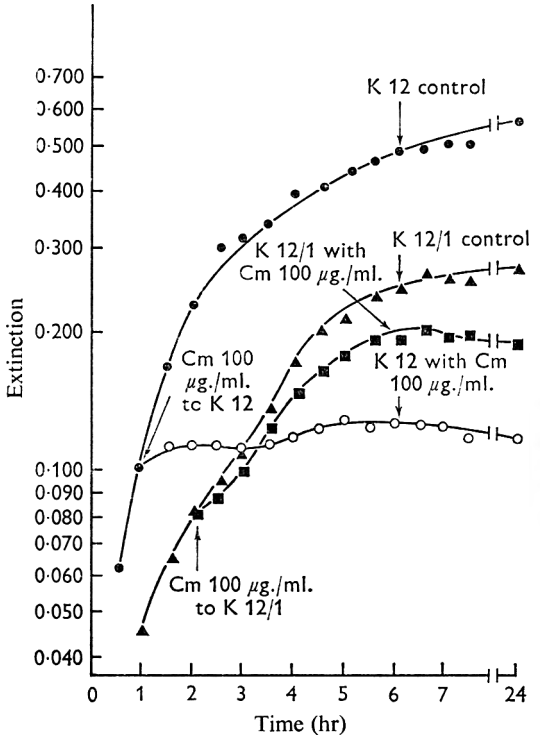

Fig. 3

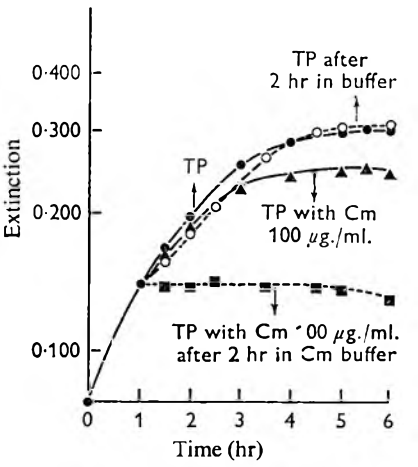

Fig. 4

Fig. 3. Comparison of growth of *Escherichia coli* K 12 and its resistant mutant *E. coli* K 12/1 in tryptose phosphate broth medium, with and without chloramphenicol (Cm).

Fig. 4. Inhibition of growth of *Escherichia coli* K 12/1 after pre-incubation in a buffer solution containing chloramphenicol (Cm) 100 μg./ml., – – –; without pre-incubation ——.

in the susceptible parent strain. All the resistant mutants examined grew very slowly on plain nutrient media as well as on Cm-containing media. In Fig. 3, growth in TP (37°, with shaking) of the susceptible strain is compared with growth of one of the high-level resistant mutants (*Escherichia coli* K 12/1). The shortest generation time was about 30 min. for the susceptible and 100 min. for the resistant strain. Growth ceased at 0·52 extinction for the susceptible strain as compared with only 0·28 for the resistant strain. Addition of Cm 100 μg./ml. to the culture of the resistant strain had almost no effect during the first 90 min.; thereafter the growth slowed down to cease entirely at 0·20 extinction. This might indicate that 'resistance' in *E. coli* K 12/1 was caused by decrease in the permeability of the bacteria to the drug. This assumption was confirmed when the bacteria were suspended in a buffer

solution containing Cm 100 μg./ml. during 120 min. before incubation in Cm+TP medium. Inhibition of growth was in this case immediate and complete (Fig. 4).

Escherichia coli K 12 *infected with resistance transfer factor* (*RTF*). Further evidence for the assumption that permeation by chloramphenicol was delayed in *E. coli* K 12/1 was obtained by infecting this strain as well as its ancestor (*E. coli* K 12) with a resistance transfer factor from a strain of *Shigella flexneri*.

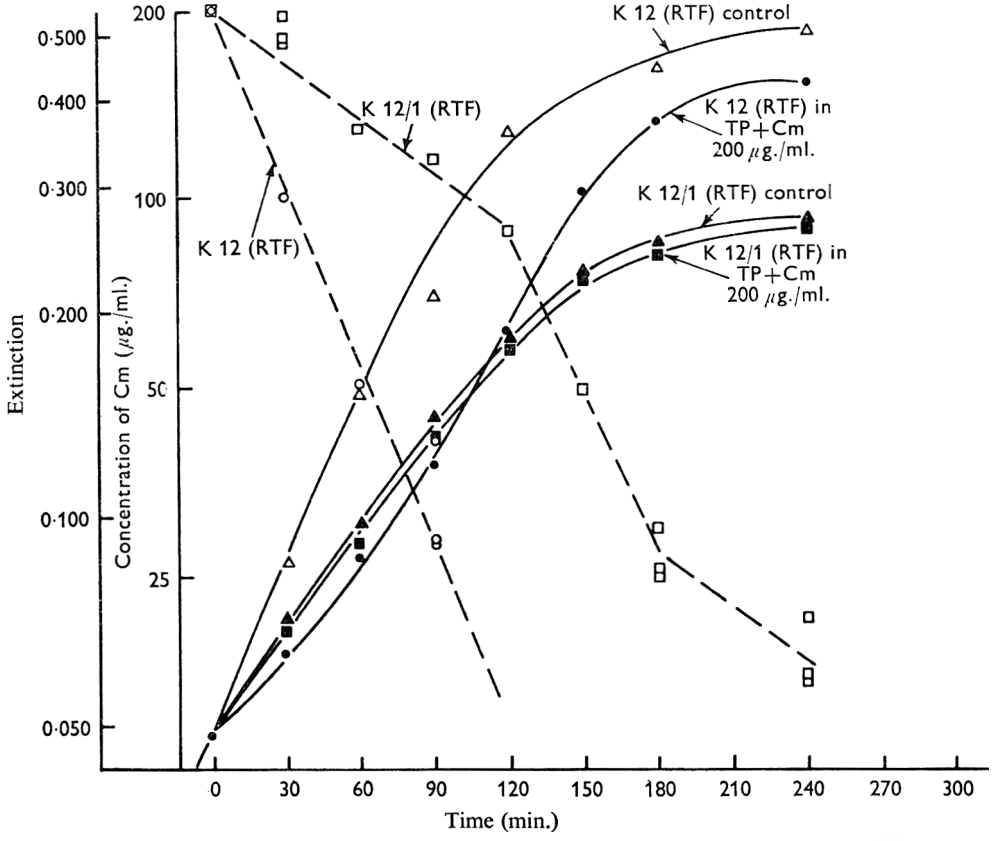

Fig. 5. Inactivation of chloramphenicol and growth in chloramphenicol (Cm) 200 μg./ml. in tryptose phosphate (TP) medium by *E. coli* K 12 with resistance transfer factor (RTF) and the tolerant mutant *E. coli* K 12/1 with the same RTF. Growth of *E. coli* K 12 (RTF) in TP without Cm (△) and with Cm (●). Growth of *E. coli* K 12/1 (RTF) in TP without Cm (▲), and with Cm (■). ○, Cm by assay in culture of *E. coli* K 12 (RTF); □, Cm by assay in culture of *E. coli* K 12/1 (RTF). Cm was assayed in samples of the three parallel cultures separately. When any of the values varied more than ±5% from the mean, all three values are shown; in the other cases only the mean is indicated. The curves for growth in presence of Cm indicate the mean for three parallel cultures. In no case were the deviations in extinction greater than 5%.

The curves on Fig. 5 show the results of three experiments with *Escherichia coli* K 12/RTF and *E. coli* K 12/1/RTF. Both strains were able to grow in TP containing Cm 200 μg./ml. Inactivation of Cm is shown in the figure as a 'semi-log' plot of active Cm against time. During the first 120 min. after addition of the drug, more than 90% was inactivated by *E. coli* K 12/RTF, but only 55% by the tolerant

E. coli K 12/1/RTF, though the concentration of the latter organism was slightly greater than of the former. Similar kinetics of Cm inactivation by these strains were observed in an experiment with Cm 100 μg./ml. As inactivation displays itself intracellularly (Miyamura, 1964; and unpublished observation with *Shigella flexneri* and with *E. coli* B/RTF in our laboratory), it must depend on the rate of uptake.

Uptake of [^{14}C]*chloramphenicol.* Uptake of [^{14}C]Cm was apparently a little higher in *Escherichia coli* K 12 than in *E. coli* B. The resistant mutant *E. coli* K 12/1 showed a significantly lower radioactivity (1/6) than its ancestor (Table 2).

Cross-resistance to tetracycline. The minimal inhibitory concentration of tetracycline in TPA was raised from 1 μg./ml. for the parent strain to 20 μg./ml. for all 22 high-level resistant clones *of Escherichia coli* K 12 which were examined.

Table 3. *Properties of high-level chloramphenicol-resistant mutants of Escherichia coli B and of E. coli K 12*

Bacterial strain	Growth rate	Inactivation of Cm	Uptake of Cm	Effect of inoculum size on resistance	Cross resistance to tetracycline
E. coli B/1	As parent strain	Yes	As parent strain	Yes	No
E. coli K 12/1	Decreased	No	Decreased	No	Yes

DISCUSSION

The main properties of high-level resistant mutants of *Escherichia coli* B and *E. coli* K 12 are summarized in Table 3. All the observations can be explained by the assumption that in *E. coli* B resistance is due to inactivation of chloramphenicol, and in *E. coli* K 12 to impaired permeability. Inactivation of Cm by micro-organisms has been reported by several other authors since the first investigation by Smith & Worrel (1949). Recent publications indicate that an oxidative process requiring acetyl co-enzyme A is responsible for inactivation in *E. coli* (Okamoto & Suzuki, 1965) and in *Staphylococcus aureus* (Suzuki, Okamoto & Kono, 1966). The latter authors as well as Miyamura (1964) have presumed that mutants obtained *in vitro* will never be resistant by virtue of an inactivating ability. Our results with *E. coli* B contradict this. No entirely new enzyme can, of course, be formed by a mutant. Accordingly, this kind of resistance was found only in mutants of *E. coli* B of which the parent strain also proved to inactivate the drug, though to a slight degree. A mutation to an effective degree of inactivation might be one in which the enzyme: (1) is formed in a greater amount; (2) has a higher affinity for the substrate (Cm); (3) is constitutive instead of inducible. It should, however, be pointed out that we have not shown that inactivation of Cm by the resistant *E. coli* B was due to the same enzymic system as functions to a slow degree in the susceptible ancestor.

One-step mutations to high-level resistance to streptomycin are commonly observed. As far as we are aware, such single-step mutation to high-level resistance to chloramphenicol has hitherto only been reported for *Mycobacterium ranae* (Szybalski & Bryson, 1954), but no data are available on the mechanism of resistance in this micro-organism.

In our case it was amply manifested that inactivation was the cause of resistance and not simply a consequence of growth in presence of chloramphenicol, as can be

seen from the following facts. (1) After addition of Cm the growth rate was decreased until inactivation was completed (Fig. 1). (2) The minimal inhibitory concentration of Cm depended on the inoculum size. (3) On Cm-containing media an all-or-none growth response occurred (Table 1). On the plate with Cm 200 μg./ml. TPA, for example, confluent growth was obtained after incubation with $9{\cdot}0 \times 10^6$ bacteria/ml. This was not due to the ability of a small fraction of the population to form colonies at this Cm concentration, since no growth occurred when the inoculum size was decreased by only one-tenth.

The inactivating enzymic system must be constitutive because otherwise the chloramphenicol would prevent its synthesis. Further, confirming the report of Merkel & Steers (1953) that glucose is required for inactivation by non-multiplying bacteria, our results show that inactivation depended on the presence of utilizable carbohydrate. On lactose medium no induction of β-galactosidase was possible, because of the Cm present, and without this enzyme no energy source was available for the inactivation. Pre-incubation with lactose unlocked this metabolic knot.

On media containing Cm 100 μg./ml. or more growth depended on very large inoculum sizes; the smallest inoculum which gave growth was 1000 times greater on minimal medium (DA) than on complex TPA medium. Miyamura *et al.* (1962) reported that small amounts of peptone stimulated inactivation by non-multiplying bacteria. The explanation may be that with an initial high concentration of chloramphenicol virtually all protein synthesis was blocked. Cm-inactivation was therefore limited by the amount of inactivating enzyme present in the sample of bacteria inoculated and co-factors in the bacteria and the nutrient media. On the other hand, with Cm 60 μg./ml. each bacterium of the inoculum grew to a colony, indicating that additional enzyme and/or co-factors might be synthesized *de novo*. This concentration of Cm may not completely prevent protein synthesis (Okamoto & Mizuno, 1962).

Uptake of [^{14}C]chloramphenicol is generally several times lower in resistant bacteria than in sensitive organisms of the same species (Vazquez, 1964; Unowsky & Rachmeler, 1966). Our results with *Escherichia coli* K 12 confirm this; but with *Escherichia coli* B no difference between the susceptible ancestor and the resistant mutant was noticed. The intracellular concentration of Cm, calculated per wet wt bacteria, was near that of the medium, proving that no concentration of Cm occurred. It follows from this that entry to, inactivation, and exit from the cell of Cm molecules must occur at a great rate. From Fig. 2 it can be seen that $1{\cdot}5 \times 10^8$ bacteria (1 ml. at a concentration corresponding to 22 Klett units) inactivated 300 μg. Cm, which is $5{\cdot}6 \times 10^{17}$ molecules Cm/30 min. or $2{\cdot}54 \times 10^6$ molecules/bacterium/sec.

Hitherto we have not isolated mutants of the chloramphenicol-tolerant kind from *Escherichia coli* B, in spite of the fact that many mutant clones have been examined. With *E. coli* K 12, of which 36 mutants were examined, not one Cm inactivator was found. Decreased permeability to Cm was found in mutants of the latter variant of *E. coli*. That resistance was due to this lowered permeability was shown by the complete inhibition of growth after an incubation period in a Cm-containing buffer solution.

The decreased permeability may be non-specific for Cm. Cross-resistance with tetracycline and puromycin (Reeve & Bishop, 1965) should not uncritically be related to common features in the action of these three antibiotics. It might well be a consequence of decreased permeability for a wide range of micromolecular species. The

markedly decreased growth rate in TP medium as well as in minimal medium might simply be a consequence of an unspecific permeability barrier.

The growth curve of the resistant mutant *Escherichia coli* K 12/1 in TP medium (Fig. 3) indicates that there must have been something more than a simple decrease in the rate of permeation, since growth ceased at a much lower population density than for the sensitive strain.

The practical aspect of resistance to chloramphenicol is outside the scope of this communication. It may, however, be mentioned that inactivation is a far more serious form of drug resistance than is the mechanism observed with *E. coli* K 12, which places the micro-organism in absence of the drug at a competitive disadvantage.

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REFERENCES

CAVALLI, L. L. & MACCACARO, G. A. (1952). Polygenic inheritance of drug-resistance in the bacterium, *Escherichia coli*. *Hereditary* **6**, 311.

CHABBERT, Y. & DEBRUGE, J. (1956). La résistance naturelle de staphylocoques au chloramphenicol. *Annls. Inst. Pasteur, Paris* **91**, 225.

DAVIS, B. D. & MINGIOLI, E. S. (1950). Mutants of *Escherichia coli* requiring methionine or B_{12}. *J. Bact.* **60**, 17.

DUNSMOOR, C. L., PIN, K. L. & SHERRIS, I. C. (1963). Observations on the inactivation of chloramphenicol by chloramphenicol resistant staphylococci. *Antimicrob. Ag. Chemother.* p 500.

LURIA, S. E. & DELBRÜCK, M. (1943). Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* **28**, 491.

MERKEL, J. R. & STEERS, E. (1953). Relationship between chloramphenicol reductase activity and chloramphenicol resistance in *E. coli*. *J. Bact.* **66**, 389.

MIYAMURA, S. (1964). Inactivation of chloramphenicol by chloramphenicol resistant bacteria. *J. pharm. Sci.* **53**, 604.

MIYAMURA, S., OKETANT, S., SHIGENO, N. & WATANABE, T. (1962). Chloramphenicol inactivation by microorganisms. III. The action of resistant *Escherichia coli* on chloramphenicol. *Jap. J. Bact.* **17**, 787. After: *Excerpta med.* Sect. 4, 1963, **16**, no. 3212.

OKAMOTO, S. & MIZUNO, D. (1962). Inhibition by chloramphenicol of protein synthesis in the cell free system of a chloramphenicol resistant strain of *E. coli*. *Nature, Lond.* **195**, 1022.

OKAMOTO, S. & MIZUNO, D. (1964). Mechanism of chloramphenicol and tetracycline resistance in *Escherichia coli*. *J. gen. Microbiol.* **35**, 125.

OKAMOTO, S. & SUZUKI, J. (1965). Chloramphenicol, dihydrostreptomycin and kanamycin. Inactivating enzyme from multiple drug resistant *E. coli* carrying episome 'R'. *Nature, Lond.* **208**, 1301.

RAMSEY, H. H. (1958). Protein synthesis as a basis for chloramphenicol resistance in *Staphylococcus aureus*. *Nature, Lond.* **182**, 602.

RANANARAYNA, B., RAYAULAKSHMI, M. R. & EHASKARAN, C. S. (1962). Resistance of *Salmonella typhi* to chloramphenicol. *J. clin. Path.* **15**, 544.

REEVE, E. C. R. & BISHOP, J. O. (1965). Multistep resistance to chloramphenicol in RC-stringent *Escherichia coli* K 12—its effect on the induction of RNA synthesis by antibiotics under amino acid starvation. *Genet. Res.* **6**, 304.

SMITH, G. N. & WORREL, C. S. (1949). Enzymatic reduction of chloramphenicol. *Archs Biochem.* **24**, 216.

SUZUKI, Y., OKAMOTO, S. & KONO, M. (1966). Basis of chloramphenicol resistance in naturally isolated resistant staphylococci. *J. Bact.* **92**, 798.

SZYBALSKI, W. & BRYSON, V. (1954). Genetic studies on microbial cross resistance to toxic agents. III. Cross resistance of *Mycobacterium ranae* to twenty-eight antimycobacterial agents. *Am. Rev. Tuberc.* **69**, 267.

UNOWSKY, J. & RACHMELER, M. (1966). Mechanism of antibiotic resistance determined by resistance-transfer factors. *J. Bact.* **92**, 358.

VAZQUEZ, D. (1963). Antibiotics which affect protein synthesis. The uptake of C^{14} chloramphenicol by bacteria. *Biochem. biophys. Res. Commun.* **12**, 409.

VAZQUEZ, D. (1964). Uptake and binding of chloramphenicol by sensitive and resistant organisms. *Nature, Lond.* **203**, 257.

WATANABE, T. & FUKASAWA, T. (1961). Episome mediated transfer of drug resistance in Enterobacteriaceae. I. Transfer of resistance factor by conjugation. *J. Bact.* **81**, 669.

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Kinetics of the Immune Response of Rabbits to Lower Trypanosomatidae Antigens

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SUMMARY

Experiments tracing the kinetics of the hyperimmune response in rabbits injected with several lower trypanosomatids (*Crithidia fasciculata* (Anopheles strain), *C. fasciculata* (Culex strain, Wallace isolate), *C. fasciculata* (Culex strain, Nöller isolate), *Crithidia* sp. from *Euryophthalmus davisi*, *Blastocrithidia leptocoridis*, *Leptomonas* sp. from *Dysdercus* and *L. mirabilis*) mixed with Freund's complete adjuvant revealed a slow increase in homologous agglutinating titre, followed by a lasting plateau titre. Cross-reactions tested at various homologous titres showed that cross-reacting specificity increased as the titre increased. At peak agglutinating titre, sera were at their maximum specificity and remained like that during the lasting plateau period. Precipitins appeared before agglutinins and faded as the homologous agglutinating titres increased. Precipitins, in contrast to agglutinins, were specific even when the agglutinating titres were low and non-specific. By analogy with work done by other investigators on identification of serum fractions associated with non-protozoan antigens in which immune kinetics, globulin generation, or globulin specificity were traced, we suggest that the early-appearing non-specific agglutinins may be composed of 19S globulins and the late- appearing specific agglutinins and specific precipitins may be composed of 7S globulins.

INTRODUCTION

There have been only four reports in which relationships among the lower Trypanosomatidae have been traced by their immunological affinities (Clark, 1958; McGhee & Hanson, 1963; Noguchi, 1926; Vitetta & Guttman, 1967). Differences in the results of various investigators have led to conflicting statements about group relationships. Some examples are: (1*a*) within the genus *Crithidia*, all organisms isolated from hemipterons are more closely related immunologically to each other than are all organisms originally isolated from dipterons (McGhee & Hanson, 1963), or (1*b*) immunological relationships are independent of host-type (Vitetta & Guttman, 1967); (2*a*) Culex and Anopheles strains of *Crithidia fasciculata* are identical (Noguchi, 1926), (2*b*) Anopheles and only one but not another Culex strain of *C. fasciculata* are identical (Clark, 1958), (2*c*) from immunological and biochemical evidence, the Anopheles and both Culex strains of *C. fasciculata* are distinct (Vitetta & Guttman, 1967).

In only one of the aforementioned reports (Vitetta & Guttman, 1967) was there used the combination of (*a*) an antibody-generating system said to produce the most physico-chemically homogeneous antibodies (Crowle, 1963; Kabat & Mayer, 1961);

(*b*) high-titre antisera (equal to or greater than 1/2560) for testing all cross-sections; (*c*) routine inactivation of complement. Since Vitetta & Guttman (1967) prepared antibodies by a method which included the use of Freund's complete adjuvant (a mixture which not only enhances production of high-titre antiserum but also lengthens the time required before peak titre is reached), we report here on the kinetic tracking of antibody production during the course of the hyperimmune response in rabbits to antigens mixed the Freund's adjuvant and on cross-reactivity of sera before, at, and after peak homologous titre.

METHODS

The organisms used as antigens in these studies were *Crithidia fasciculata* (Anopheles strain) ATCC 11745; *C. fasciculata* (Culex strain, Wallace isolate) ATCC 12857; *C. fasciculata* (Culex strain, Nöller isolate) ATCC 12858; *C. luciliae* ATCC 14765; *C. oncopelti* (with endosymbiote) ATCC 12982; *Crithidia* sp. from *Euryophthalmus davisi*, ATCC 14766; *Blastocrithidia leptocoridis*; *Leptomonas* sp. from *Dysdercus suturellus*, and *L. mirabilis*.

Methods already reported (Vitetta & Guttman, 1967) were used for preparation of antigens, mixture of antigen with Freund's complete adjuvant for hyper-immunization, generation of antibodies in rabbits and procedures for agglutination tests.

Precipitin tests. All precipitin tests were done in Ouchterlony double diffusion plates (Hyland Laboratories, Los Angeles, California). Disrupted-cell antigen suspensions were placed in the central well and undiluted sera placed in the peripheral wells. The plates were covered with their own plastic lids to prevent drying and were incubated at room temperature (25°) for 24 hr. They were then washed in cold 0·85% (w/v) NaCl solution for 24–48 hr to remove excess antigen and serum and then scanned at × 100 magnification for precipitin lines. The numbers and intensity of precipitin lines were recorded for each antiserum challenged with homologous antigen. Cross-reactions were determined by using sera which showed the most intense and/or greatest number of lines with homologous antigen.

RESULTS

The usual pattern of agglutinating hyperimmune response in rabbits following injection of antigen mixed in Freund's complete adjuvant was an increase in titre beginning 1–20 days after the last antigen injection and continuing to rise for 10–40 more days (Fig. 1). After peak titre was reached, there was a slight decrease in titre and persistence of the agglutinating response at this plateau for at least 140 days (experiments were terminated at that time). Such a pattern of slow rise of titre and maintenance of high titre is typical for adjuvant-injected animals (Uchitel & Khasman, 1965). The one exception to formation of a stable agglutinating titre in our experiments was with one *Crithidia* sp. from *Euryophthalmus* antiserum which reached a rather low peak titre and then decreased rapidly to zero (Figure 2).

For all antisera tested, there was a distinct pattern of relationship between precipitins and agglutinins which was unrelated to either the particular antigen used or to the type of cell preparation (i.e. whole or disrupted cells). Precipitins appeared before agglutinins and usually were most apparent when agglutinating titres were still low (Table 1).

Although all precipitating responses declined as the agglutinating titres increased (Table 1), the precise timing was not constant: precipitins disappeared (*a*) before peak agglutinating titre in two antisera (anti-*C. fasciculata* (Culex strain) serum and anti-*Leptomonas* sp. from *Dysdercus suturellus* serum), (*b*) slightly after peak agglutinating titre in three antisera (two *C. fasciculata* (Culex strain) sera and anti-*B. leptocoridis* serum), and (*c*) persisted in two other antisera (anti-*Leptomonas* sp. from *Dysdercus suturellus* serum and anti-*L. mirabilis* serum).

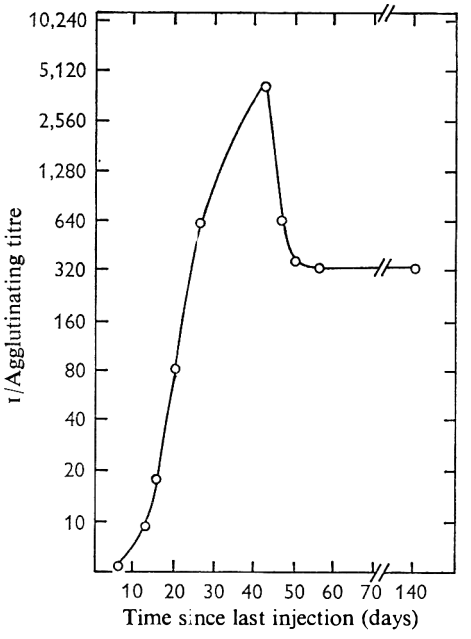

Fig. 1

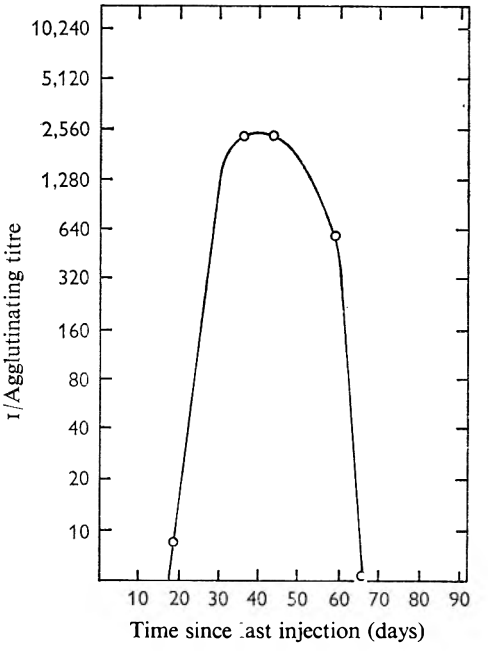

Fig. 2

Fig. 1. Kinetics of antibody generation to whole *Crithidia fasciculata* (Culex strain, Wallace isolate). Similar kinetics were observed in 15 other antisera generated against lower trypanosomatid antigens.

Fig. 2. Kinetics of antibody generation to disrupted organisms of a *Crithidia* sp. (from *Euryophthalmus davisi*).

In our previous study (Vitetta & Guttman, 1967) all agglutinating cross-reactions were determined at peak or plateau homologous titre. We now show (Table 2) the striking decrease in agglutinating cross-reactions concomitant with increase in homologous titre. For antisera to whole or disrupted *C. fasciculata* (Culex strain, Wallace isolate), whole *Leptomonas* sp. from *Dysdercus suturellus*, or whole *B. leptocoridis*, four series in which cross-reactions were followed during the whole sequence of rising, plateau and decreasing titre (Table 2), antibody specificity increased as the homologous titre increased. This specificity was maintained and strengthened at peak and post-peak homologous titres. Although there were insufficient data to follow the development of cross-reacting antibody specificity for other organisms, some generalizations can be made:

(1) At very low agglutinating titres, agglutinating cross-reactions were high and non-specific (Table 3*a*).

(2) Cross-reactivity declined as the homologous agglutinating titre neared a peak, i.e. antibody specificity increased with titre (Table 2).

(3) At peak titre, plateau titre, and decreasing titre after peak, agglutinating cross-reactions remained constantly specific, despite the changes in homologous titre (Table 3*b*).

In contrast to lack of specificity of agglutinating antibodies, precipitating cross-

Table 1. *Kinetics of the production of agglutinating and precipitating antibodies*

Antiserum to	Rabbit no.	Days since last injection	Agglutinating titre	Precipitating lines (no. and intensity)
C. fasciculata (Culex, Wallace)	1	12	1/40	1 faint
		28	1/1280	1 faint
		40	1/5120–1/10240	1 faint
		140	1/5120	0
	2	15	0	0
		20	0	1 faint
		30	1/1280	1 faint
		32	1/5120	1 faint
		40	1/1280	1 faint
		50	1/1280	0
	3	8	1/1	1 sharp
		14	1/10	1 faint
		21	1/80	1 faint
		27	1/640	1 faint
		36	1/2560	0
Leptomonas from *Dysdercus* suturellus	1	10	0	2 sharp
		14	0	2 faint
		21	1/80–1/160	1 sharp
		27	1/320	1 sharp
		30	1/1280	1 sharp
		37	1/2560–1/5120	1 faint
		40	1/5120	1 faint
	2	10	0	2 sharp
		14	0	1 sharp
		21	0	1 faint
		30	1/320	0
B. leptocoridis		5	0	2 very sharp
		10	0	2 sharp
		15	1/640–1/1280	2 faint
		25	1/640–1/1280	1 faint
		30	1/2560	1 faint
		48	1/5120	1 faint
		55	1/10240	1 faint
		110	1/2560	1 faint
		120	1/2560	1 faint
		140	1/1280	0
L. mirabilis		5	0	2 sharp
		10	0	2 sharp
		20	1/640–1/1280	2 faint
		30	1/1280	2 faint
		40	1/10240	1 faint
		56	1/10240	1 faint
		63	1/5120	1 faint
		69	1/2560	1 faint

reacting antibodies are very specific when the homologous agglutinating titre is low and remain specific during the course of the precipitating immune response (Table 4; see also Vitetta & Guttman, 1967).

Table 2. *A comparison of percentage agglutinating cross-reaction at indicated homologous agglutinating titres*

Antiserum to	Whole antigen	Homologous agglutination titre at which cross-reactions were done. Rising titres						Post-peak titre
		1/20	1/640	1/1280	1/2560	1/5120	1/10240	1/320
Whole *C. fasciculata* (Culex strain, Wallace isolate)	*C.* from *Euryophthalmus davisi*	100	100	.	.	25*	.	25
	L. from *Dysdercus suturellus*	100	50	.	.	25*	.	50
	L. mirabilis	100	100	.	.	100*	.	50
	B. leptocoridis	100	100	.	.	100*	.	25
Disrupted *C. fasciculata* (Culex strain, Wallace isolate)	*C.* from *Euryophthalmus davisi*	.	.	100	.	12	12*	.
	L. from *Dysdercus suturellus*	.	.	100	.	25	25*	.
	L. mirabilis	.	.	100	.	50	50*	.
	B. leptocoridis	.	.	50	.	12	12*	.
Whole *Leptomonas* from *Dysdercus suturellus*	*C.* from *Euryophthalmus davisi*	.	100	50	.	12*	.	.
	C. fasciculata (Culex strain, Wallace isolate)	.	100	100	.	25*	.	.
	L. mirabilis	.	100	100	.	25*	.	.
	B. leptocoridis	.	100	100	.	50*	.	.
Whole *Blastocrithidia leptocoridis*	*Crithidia* sp. from *Euryophthalmus davisi*			100	100	25	12*	.
	C. fasciculata (Culex strain, Wallace isolate)			100	100	50	25*	.
	Leptomonas sp. from *Dysdercus suturellus*			100	50	50	50*	.
	L. mirabilis			100	50	50	50*	.

* Peak homologous agglutinating titre.

Table 3*a*. *Percentage agglutinating cross-reaction at low (pre-peak) homologous agglutinating titres*

Antiserum to	Peak titre	Titre when cross-reactions tested	Antigen	% cross-reaction
Whole *C. fasciculata* (Culex strain, Nöller isolate)	1/5120–1/10240	1/1280	*C. fasciculata* (Culex strain, Wallace isolate)	100
			C. fasciculata (Anopheles strain)	100
			C. from *Euryophthalmus davisi*	100
			C. luciliae	100
			C. oncopelti (endosymbiote infected)	100
			L. from *Dysdercus suturellus*	25
Whole *Crithidia* from *Euryophthalmus*	1/2560	1/640	*C. fasciculata* (Anopheles strain)	100
			C. fasciculata (Culex strain, Wallace isolate)	100
			L. from *Dysdercus suturellus*	100
			C. fasciculata (Culex strain, Nöller isolate)	100
			C. luciliae	100
			C. oncopelti (endosymbiote infected)	100
Whole *C. luciliae*	1/5120–1/10240	1/1280	*C. fasciculata* (Anopheles strain)	100
			C. fasciculata (Culex strain, Wallace isolate)	100
			C. from *Euryophthalmus davisi*	50
			C. fasciculata (Culex strain, Nöller isolate)	50
			L. from *Dysdercus suturellus*	100
			C. oncopelti (endosymbiote infected	50

Table 3*b*. *Percent agglutinating cross-reaction at peak and post-peak homologous agglutinating titres*

Antiserum to	Peak titre	Titre when cross-reactions tested	Antigen	% cross-reaction
Whole *C. fasciculata* (Culex strain, Wallace isolate)	1/10240	1/10240	*C. fasciculata* (Anopheles strain)	50
			C. from *Euryophthalmus davisi*	6
			C. fasciculata (Culex strain, Nöller isolate)	50
			L. from *Dysdercus suturellus*	6
			C. oncopelti (endosymbiote infected)	50
			C. luciliae	6
Disrupted *C. fasciculata* (Culex strain, Nöller isolate)	1/5120	1/5120	*C. fasciculata* (Culex strain, Wallace isolate)	50
			C. fasciculata (Anopheles strain)	100
			C. from *Euryophthalmus davisi*	25
			C. luciliae	25
			C. oncopelti (endosymbiote infected)	25
			L. from *Dysdercus suturellus*	25
Whole *C. fasciculata* (Anopheles strain)	1/5120	1/5120	*C. fasciculata* (Culex strain, Wallace isolate)	100
			C. from *Euryophthalmus davisi*	25
			L. from *Dysdercus suturellus*	6
			C. fasciculata (Culex strain, Nöller isolate)	50
			C. luciliae	12
			C. oncopelti (endosymbiote infected)	50
Disrupted *C. fasciculata* (Culex strain, Wallace isolate)	1/5120	1/5120	*C. fasciculata* (Anopheles strain)	100
			C. from *Euryophthalmus davisi*	12
			C. fasciculata (Culex strain, Nöller isolate)	50
			C. luciliae	25
			L. from *Dysdercus suturellus*	25
			C. oncopelti (endosymbiote infected)	50
Disrupted *C.* from *Euryophthalmus davisi*	1/2560	1/320	*L.* from *Dysdercus suturellus*	12
			L. mirabilis	25
			B. leptocoridis	12
			C. fasciculata (Culex strain, Wallace isolate)	25
Disrupted *L.* from *Dysdercus suturellus*	1/2560	1/640	*C. fasciculata* (Anopheles strain)	50
			C. fasciculata (Culex strain, Wallace isolate)	25
			C. from *Euryophthalmus davisi*	100
			C. fasciculata (Culex strain, Nöller isolate)	25
			C. luciliae	25
			C. oncopelti (endosymbiote infected)	25
Disrupted *L. mirabilis*	1/5120–1/10240	1/5120	*C. fasciculata* (Culex strain, Wallace isolate)	50
			L. from *Dysdercus suturellus*	25
			B. leptocoridis	100
			C. from *Euryophthalmus davisi*	25

DISCUSSION

Although it is clear that the specificity of precipitating antibodies was acquired early in the course of the immune response while the specificity of agglutinating antibodies was still changing in the direction of increased specificity during the same period (Tables 2, 3), the biochemical events leading to the changes in specificity are not yet clear. Two possible explanations are: (i) there is an increasing modification of a particular class of antibody molecules as the titre rises; (ii) there is independent synthesis of different classes of antibody proteins each of which differs with respect to specificity and probably to gross molecular structure.

It has now been established by numerous workers that at least two types of globulins are generated after primary inoculation with viral, bacterial, or foreign antigen. The first antibodies generated are 19S globulins (gamma M or macroglobulins) which are later replaced by lighter 7S globulins (gamma G or gamma globulins) (Bauer & Stavitsky, 1961; Daniel, 1965; Benedict, Brown & Ayengar, 1962; Nisonoff & Thorbecke, 1964; Svehag & Mandel, 1964). Normal and immune 7S globulins contain

Table 4. *Precipitating cross-reactions between disrupted antigen and antisera against disrupted cells*

The experiments were done with sera which showed the most intense and/or greatest number of lines with homologous antigen.

	Antigens				
Antiserum to	*Crithidia fasciculata* (Culex-Wallace)	*Crithidia* sp. from *Euryophthalmus davisi*	*Leptomonas* sp. from *Dysdercus suturellus*	*mirabilis*	*Blastocrithidia Leptocoridis*
Crithidia fasciculata (Culex strain, Wallace isolate)	+	–	–	–	–
Crithidia sp. from *Euryophthalmus davisi*	–	+	–	–	–
Leptomonas sp. from *Dysdercus suturellus*	–	–	+	–	–
L. mirabilis	–	–	–	+	–
Blastocrithidia leptocoridis	–	–	–	–	+

two identical light (L) chains linked by disulphide bonds to two identical heavy (H) chains (Edelman & Benacerraf, 1962; Edelman, Benacerraf & Ovary, 1963; Edelman & Gally, 1964; Nossal, Svenberg, Ada & Austin, 1964; Fougereau & Edelman, 1965). There are several alternative classes of H chains which can combine with L chains to make the whole 7S molecule (Potter, Apella & Geisser, 1965). Antibody specificity is dependent upon the amino acid sequence at or near the two H–L interaction sites (Almeida, Cinader & Howatson, 1963; Buckley, Whitney & Tanford, 1963; Koshland & Engelberger, 1963; Roholt, Onoue & Pressman, 1964; Metzger, Wofsy & Singer, 1964; Roholt, Radzimski & Pressman, 1963; Titani & Putnam, 1965; Whitney & Tanford, 1965). The macroglobulin has a molecular weight of one million and contains the same L chains as does the globulin, but different H chains (Edelman & Gally, 1964; Nossal, Svenberg, Ada & Austin, 1964). Since the gamma G globulin and gamma M macroglobulin have different primary structures, the macroglobulins are not merely polymers of smaller 7S globulins (Onoue *et al.* 1965).

Fishman (1961) suggested that the 19S and 7S globulins are each synthesized by different plasma cells which are associated with the same antigen-engulfing macrophage. Nossal *et al.* (1964), on the other hand, postulated that during the course of the immune response, single maturing plasma cells switch from 19S to 7S globulin synthesis. Regardless of whether both 19S and 7S globulins are made by the same or different cells in a competent tissue, the mechanism responsible for the switch-over from production of 19S to 7S globulin in the antibody-synthesizing system as a whole is not yet clear. Some factors which influence this switch-over rate are as follow.

(*a*) The size of the antigen dose. When large doses of antigen are given to the antibody generating system, the time lapse between 19S and 7S globulin synthesis is decreased (Mellors & Korngold, 1963; Uhr & Finkelstein, 1963; Uhr, 1964; Svehag & Mandel, 1964), indicating that the antigen concentration activates the

switch-over mechanism. In cases of these large antigen doses, the 19S globulins are synthesized at a rapid rate, but are short lived while the 7S globulins increase at a slower rate but endure (Mellors & Korngold, 1963; Uhr & Finkelstein, 1963; Uhr, 1964; Svehag & Mandel, 1964).

(*b*) Use of Freund's complete adjuvant. Adjuvant, which has been said to cause non-specific antibody synthesis (Freund *et al.* 1948; John & Gersl, 1959; Uchitel & Khasman, 1965), actually causes an increase in 19S but not in 7S globulin synthesis (Benedict, 1965).

Both the aforementioned factors (*a*) and (*b*) would indicate that the 19S globulin is less specific than is the 7S globulin. Indeed, it has been suggested by Onoue (Onoue *et al.* 1965) that since the macroglobulin has six binding sites per molecule as opposed to two for the 7S globulin, it has greater (and perhaps more random) binding activity (Franklin & Kunkel, 1957; Korngold & Van Leeuwen, 1957; Müller-Eberhard, Kunkel & Franklin, 1956). Work with allergic individuals (Sehon, 1959) also indicates that the 19S Globulin is less specific than is the 7S globulin.

Unlike precipitating antibodies which are exclusively composed of 7S globulin, agglutinating antibodies may be either 19S or 7S globulins (Benedict, 1965; Benedict, Brown & Ayengar, 1962). Thus, it may be inferred from the previous discussion of specificity that precipitating antibodies are always specific, while agglutinating antibodies may be either specific or non-specific depending on whether they are composed of 19S (non-specific) or 7S (specific) globulins.

Since in the experiments reported here we used large doses of antigen in Freund's complete adjuvant, it would be reasonable that, at the time of the first antiserum collection (3 weeks after the first injection), both 19S and 7S globulins were present in the antiserum, the former in high concentration and the latter in low concentration (since it was already 'late' with respect to generation of the first antibodies). Since precipitins are present only in the 7S globulin fraction (Benedict, 1965) and because the 7S globulin is specific (Benedict, 1965), all precipitins present when agglutinating titres are low should be specific, as indeed we have found them to be (Vitetta & Guttman, 1967). Unlike precipitins, agglutinins are present in both the 19S and 7S fractions (Benedict, 1965). At low agglutinating titres, the concentration of 19S globulin should be higher than that of the 7S globulin, thus accounting for presence of non-specific agglutinins (Tables 2 and 3*a*). As the titre increases, the 7S globulin concentration should increase (Benedict, 1965; Daniel, 1965; Mellors & Korngold, 1963), accounting for the more specific agglutinating cross-reactions which were observed (Table 2). By the time peak titre was reached, 19S globulin synthesis should have ceased and 7S globulins exclusively produced (Benedict, 1965; Daniel, 1965; Mellors & Korngold, 1963), thus accounting for the high specificity of the cross-reactions (Table 3*b*). Our preliminary results do indeed show such relationships between 19S and 7S globulins. The fading or complete disappearance of the precipitating reaction at high agglutinating titre (Table 1) might be due to the masking of precipitins by non-precipitating antibody in the same globulin fraction (Gray, 1964). Accordingly, the detection of precipitins would depend on the ratio of precipitating to agglutinating antibody in the 7S globulin fraction at any particular agglutinating titre. At plateau and post-peak titres, despite the changes in homologous titre (slow-down of 7S globulin synthesis) the percentage cross-reaction should remain fairly constant, as indeed was observed (Table 3*b*).

We have thus shown the importance of the use of high-titre serum which is at peak or post-peak homologous agglutinating titre for the determination of specific immunological affinities by means of agglutinating cross-reactions. It is therefore probable that earlier disagreements among different workers (Clark, 1958; McGhee & Hanson, 1963; Noguchi, 1926) about immunological affinities within the genus Trypanosomatidae may have been due to inadvertent failure to select peak or post-peak titre antisera for the most specific cross-reaction determinations.

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REFERENCES

ALMEIDA, J., CINADER, B. & HOWATSON, A. (1963). The structure of antigen-antibody complexes. *J. exp. Med.* **118**, 327.

BAUER, D. C. & STAVITSKY, S. B. (1961). On the different molecular forms of antibody synthesized by rabbits during the early response to a single injection of protein and cellular antigens. *Proc. natn. Acad. Sci., U.S.A.* **47**, 1667.

BENEDICT, A. A. (1965). Sensitivity of passive hemagglutination for assay of 7S and 19S antibodies in primary rabbit anti-bovine serum albumin sera. *Nature Lond.* **206**, 1368.

BENEDICT, A. A., BROWN, R. J. & AYENGAR, R. (1962). Physical properties of antibodies to bovine serum albumin as demonstrated by hemagglutination. *J. exp. Med.* **115**, 195.

BUCKLEY, C. E., WHITNEY, P. L. & TANFORD, C. (1963). The unfolding and renaturation of a specific univalent antibody fragment. *Proc. natn. Acad. Sci., U.S.A.* **50**, 827.

CLARK, JR., T. B. (1958). *A comparative serological and morphological study of the family Trypanosomatidae Doflein, 1901.* Doctoral thesis. University of Minnesota, U.S.A.

CROWLE, A. J. (1963). *Immunodiffusion.* New York: Academic Press.

DANIEL, T. M. (1965). Observations on the antibody response of rabbits to mycobacterial antigens. *J. Immunol.* **95**, 100.

EDELMAN, G. M. & BENACERRAF, B. (1962). On structural and functional relations between antibodies and proteins of the gamma system. *Proc. natn. Acad. Sci., U.S.A.* **48**, 1035.

EDELMAN, G. M., BENACERRAF, B. & OVARY, Z. (1963). Structure and specificity of guinea-pig 7S antibodies. *J. exp. Med.* **118**, 229.

EDELMAN, G. M. & GALLY, J. A. (1964). A model for the 7S antibody molecule. *Proc. natn. Acad. Sci., U.S.A.* **51**, 846.

FISHMAN, M. (1961). Antibody formation in vitro. *J. exp. Med.* **114**, 837.

FOUGEREAU, D. V. M. & EDELMAN, G. M. (1965). Corroboration of recent models of the gamma immunoglobin molecule. *J. exp. Med.* **121**, 373.

FRANKLIN, E. C. & KUNKEL, H. G. (1957). Immunological differences between the 19S and 7S components of normal human gamma globulin. *J. Immunol.* **78**, 11.

FREUND, J., THOMPSON, K. J., HOUGH, H. B., SOMMER, H. E. & PISCINI, T. M. (1948). Antibody formation and sensitization with the aid of adjuvants. *J. Immunol.* **60**, 383.

GRAY, D. F. (1964). *Immunology.* New York: American Elsevier Pub. Co. Inc.

JOHN, C. & GERSL, T. (1959). Parental administration of oil as a factor stimulating antibody production. In *Mechanisms of Antibody Production: Symposium Proceedings.* New York: Academic Press.

KABAT, E. A. & MAYER, M. M. (1961). *Experimental Immunochemistry.* Illinois, U.S.A.: Chas. C. Thomas Publishing Co.

KORNGOLD, L. & VAN LEEUWEN, G. V. (1957). Macroglobulinemia II. Antisera specific for pathological macroglobulins. *J. exp. Med.* **106**, 477.

KOSHLAND, M. E. & ENGELBERGER, F. N. (1963). Differences in the amino acid composition of two purified antibodies from the same rabbit. *Proc. natn. Acad. Sci., U.S.A.* **50**, 61.

MCGHEE, R. B. & HANSON, W. L. (1963). The relationship of various crithidia as determined by serological reactions. *J. Protozool.* **10**, 239.

MELLORS, R. C. & KORNGOLD, L. (1963). The cellular origin of human immunoglobins. *J. exp. Med.* **118**, 387.

METZGER, H., WOFSY, L. & SINGER, S. H. (1964). The participating of A and B polypeptide chains in the active sites of antibody molecules. *Proc. natn. Acad. Sci., U.S.A.* **51**, 612.

MÜLLER-EBERHARD, H. J., KUNKEL, H. G. & FRANKLIN, E. C. (1956). Two types of gamma globulin differing in carbohydrate content. *Proc. Soc. exp. Biol. Med.* **93**, 146.

NISONOFF, A. & THORBECKE, G. J. (1964). Immunochemistry. *Ann. Rev. Biochem.* **33**, 355.

NOGUCHI, H. (1926). Comparative studies of herpetomonads and leishmanias. *J. exp. Med.* **44**, 327.

NOSSAL, G. J. V., SVENBERG, A., ADA, G. L. & AUSTIN, C. M. (1964). Single cell studies on 19S antibody production. *J. exp. Med.* **119**, 485.

ONOUE, K., YAGI, Y., GROSSBERG, A. & PRESSMAN, D. (1965). Number of binding sites of rabbit macroglobin antibody and its subunits. *Immunochemistry* **2**, 401.

POTTER, M., APELLA, E. & GEISSER, S. (1965). Variation in the heavy polypeptide chain structure of gamma myclomaimmunoglobins from an inbred strain of mice and a hypothesis as to their origin. *J. molec. Biol.* **14**, 361.

ROHOLT, O. A., ONOUE, K. & PRESSMAN, D. (1964). Specific combination of H and L chains of rabbit gamma globulins. *Proc. natn. Acad. Sci., U.S.A.* **51**, 173.

ROHOLT, O. A., RADZIMSKI, G. & PRESSMAN, D. (1963). Antibody combining site: the B polypeptide chain. *Science, N.Y.* **141**, 726.

SEHON, A. (1959). Different types of antibodies produced by allergic individuals depending on route of immunization. In *Mechanisms of Antibody Formation: Symposium Proceedings.* New York: Academic Press.

SVEHAG, S. & MANDEL, B. (1964). The formation and properties of poliovirus neutralization antibody. *J. exp. Med.* **119**, 1.

TITANI, K. & PUTNAM, F. W. (1965). Immunoglobin structure: amino and carboxyl terminal peptides of Type I Bence Jones protein. *Science, N.Y.* **147**, 1304.

UCHITEL, Y. & KHASMAN, E. L. (1965). On the mechanism of adjuvant action of nonspecific stimulators of antibody formation. *J. Immunol.* **94**, 492.

UHR, J. W. (1964). The heterogeneity of the immune response. *Science, N.Y.* **147**, 457.

UHR, J. W. & FINKELSTEIN, M. S. (1963). Antibody formation. *J. exp. Med.* **117**, 457.

VITETTA, E. S. & GUTTMAN, H. N. (1967). Immunological relationships among the lower tryponosomatidae. *J. gen. Microbiol.* **48**, 45.

WHITNEY, P. L. & TANFORD, C. (1965). Recovery of specific activity after complete unfolding and reduction of an antibody fragment. *Proc. natn. Acad. Sci., U.S.A.* **53**, 524.

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Activation of Spores of *Bacillus cereus* by γ-Radiation

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SUMMARY

Spores of *Bacillus cereus* strain PX which were exposed to 0·02, 0·06, 0·18, 0·54 and 1·08 Mrad of γ-radiation became progressively more activated, i.e. they germinated more rapidly in the presence of germinants (e.g. L-alanine, inosine, *n*-dodecylamine, calcium dipicolinate) than did unirradiated spores. Heat- and radiation-activation differed in that irradiated spores but not heated spores germinated faster than untreated control spores in *n*-dodecylamine. The two methods of activation were similar in that both sorts of activated spores could be de-activated, and heated and irradiated spores both had increased contents of titratable thiol groups as compared with untreated spores. Germination-like changes still occurred rapidly in spores which had been subjected to doses of γ-radiation sufficient to render them non-viable. Ionizing radiation, heat and other agents which activate spores probably do so by changing the tertiary structure of spore macromolecules and thereby exposing previously masked reactive sites which are important in germination.

INTRODUCTION

Spores of many bacteria only germinate completely and at maximal rate when first activated by some treatment such as: heating at sublethal temperatures (Curran & Evans, 1945); treatment with reducing agents (Keynan, Evenchik, Halvorson & Hastings, 1964) or calcium dipicolinate (Lee & Ordal, 1963); incubation at low or high pH values (Gibbs, 1967) or in the presence of chloral hydrate (Lewis, Snell & Alderton, 1965), dimethylformamide or dimethylsulphoxide (Widdowson, 1967); incubation in solutions of urea (Z. J. Ordal, unpublished results) or incubation of lyophilized spores in air saturated with water vapour (Hyatt, Holmes & Levinson, 1966). These diverse treatments have in common the ability to cause alterations in tertiary structure of macromolecules, and it was postulated by Keynan *et al.* (1964) and Keynan, Issahary-Brand & Evenchik (1965) that activation directly resulted from such alterations.

Ionizing radiation can also cause changes in the tertiary structure of macromolecules, either by direct action or through the agency of reactive free radicals from water, causing rupture of hydrogen bonds, peptide bonds or other covalent linkages (Bacq & Alexander, 1961; see Charlesbury, 1963). Farkas & Kiss (1964, 1965) showed that high lethal doses of X-radiation caused increased permeability and germination-like changes in *Bacillus cereus* spores which might have resulted from large alterations in spore molecular structure; Levinson & Hyatt (1960) described similar changes in spores of *B. megaterium* and *Clostridium sporogenes* when subjected to high lethal doses of radiation with electrons. Roberts & Ingram (1965) and T. A. Roberts

(personal communication) found that the initial parts of dose/survivor curves of spores of several Clostridium species showed an increase in viable count with dose, apparently induced by sublethal doses of γ-radiation. These observations strongly suggested that radiation-activation could occur. It was therefore of interest to study the effect of γ-radiation on the subsequent germination of spores of *B. cereus* in defined media containing known germinants.

METHODS

Spores. Organisms were grown at 30° on the surface of agar containing Oxoid potato extract (0·4%, w/v), yeast extract (0·4%, w/v) and glucose (0·25%, w/v). Sporulation and lysis of sporangia was complete in 3–6 days, after which spores were collected in cold distilled water and washed six times by centrifugation. Suspensions of spores in water (about equiv. 20 mg. dry wt/ml.) were stored at 4°. When used for germination studies suspensions were diluted to about 5×10^8 spores/ml.; for thiol measurements suspensions of spores of *Bacillus cereus* PX were concentrated to equiv. 50–100 mg. dry wt/ml.

Radiation. Suspensions (1·0 ml. amounts in sealed glass ampoules) were irradiated with γ-radiation from ^{60}Co at a dose rate of about 1 Mrad/hr at ambient temperature (about 20°). Following irradiation suspensions were used immediately or stored on ice for not longer than 24 hr.

Heating. Sealed ampoules containing spores for heat activation were immersed in a 70° bath for 30 min., then stored on ice for as short a time as possible before use.

De-activation. Activated spores were partially de-activated by placing suspensions of them in dialysis sacs which were immersed in stirred distilled water for 16 hr at about 20°; control suspensions were stored in sealed ampoules at 4° during this time.

Germination studies. Samples of spores for germination studies were washed out of ampoules into 24 ml. distilled water. One ml. of this dilution was then pipetted into absorptiometer tubes containing 4 ml. medium at 37° to initiate germination. The extinction (which was always initially about 0·3) was immediately read with an absorptiometer with 580 mμ peak transmission filter. Further readings were taken at intervals to follow the decrease in extinction which is characteristic of germination. Germination media used were: (1) L-alanine (10 mM); (2) L-alanine (1 mM) + adenosine (200 μM); (3) inosine (100 μM); (4) *n*-dodecylamine ($\frac{1}{5}$ saturated solution in water); (5) calcium dipicolinate (40 mM-$CaCl_2$ + 40 mM-sodium dipicolinate mixed immediately before adding spores). Germination media nos. 1–4 contained sodium phosphate buffer (100 mM; pH 8·0), and no. 5 contained tris HCl buffer (100 mM; pH 8·0).

Thiol titrations. Spore thiol groups were titrated by using silver nitrate in the back-titration method of Hamm & Hofmann (1965). Samples of spores equiv. 50–100 mg. dry wt suspended in 8 M-urea were used for each estimation.

Colony counts of viable spores. Duplicate samples were decimally diluted in water and poured plates made with yeast glucose agar. Colonies were counted after incubation of plates at 37° for 24 hr.

RESULTS

Viability of γ-irradiated and heated spores

γ-Irradiated spores lost viability as measured by colony counts, the shape of the survivor curve being identical with that reported by Hitchins, King & Gould (1966).

The percentage survivals at the dose values used are shown in Table 1. Spores which are not viable in that they are unable to give rise to colonies may still have their germination capacity unimpaired, or even stimulated (see below). Thus, of the doses of γ-irradiation used later, 0·06 Mrad left more than 75 % of the irradiated spores still viable (i.e. able to grow into colonies), whereas spores irradiated with 0·54 Mrad

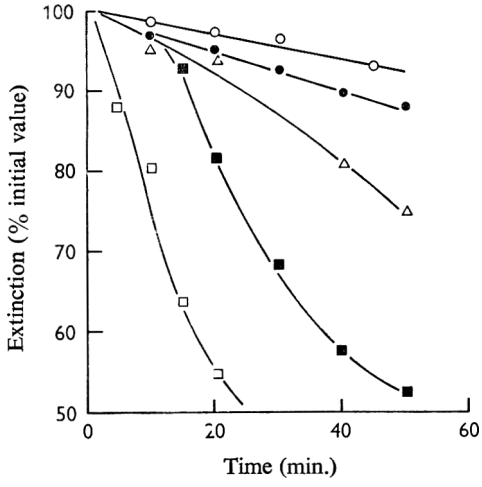

Fig. 1

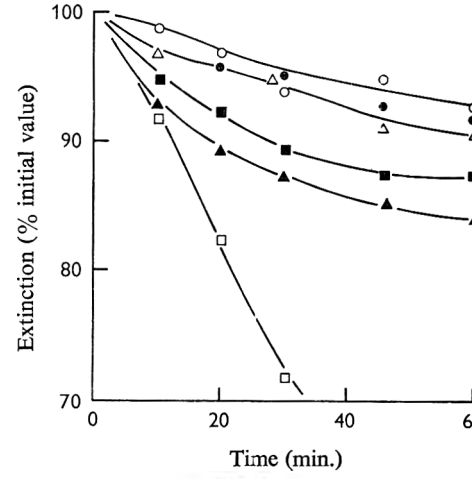

Fig. 2

Fig. 1. Inosine germination of γ-irradiated and heat-activated spores of *Bacillus cereus* strain PX. Spores were incubated at 37° in sodium phosphate buffer (0·1 M; pH 8·0) containing inosine (100 μM). Germination was accompanied by a decrease in extinction of suspensions. Treatment of spores was as follows: ○, unirradiated and unheated control; ●, irradiated with 0·06 Mrad; △, 0·18 Mrad; ■, 0·54 Mrad; □, heated at 70° for 30 min.

Fig. 2. Calcium dipicolinate germination of γ-irradiated and heated spores of *Bacillus cereus* strain PX. Spores were incubated at 37° in Tris HCl buffer (0·1 M; pH 8·0) containing equimolar calcium chloride and sodium dipicolinate (40 mM) Treatment of spores: ○, unirradiated and unheated control; ●, 0·06 Mrad; △, 0·18 Mrad; ■, 0·54 Mrad; ▲, 1·08 Mrad; □, heated at 70° for 30 min The line for 0·06 Mrad has been omitted for clarity.

Table 1. *Viability of γ-irradiated Bacillus cereus spores*

Survivors were estimated by colony counts as described in Methods.

γ-radiation dose(Mrad)	Survivors (%)
0·02	98
0·06	78
0·18	27
0·54	3·4
1·08	0·011

were more than 95 % non-viable (Table 1), although still capable of showing typical germination changes in appropriate media. The heat activation treatment used (70° for 30 min.) did not measurably decrease the viability of *B. cereus* PX spore suspensions.

Activation of spores with γ-radiation and with heat

Spores of *Bacillus cereus* PX which were γ-irradiated, subsequently germinated more rapidly than did unirradiated spores and, over the range studied, the greater

the γ-dose the greater was the degree of activation. Activation was particularly obvious when inosine was used as germinant (Fig. 1). When L-alanine was used as germinant, radiation-activation was detectable but was less marked. With different batches of spores, activation was always clearly seen when inosine was used as germinant, but was variable in extent with L-alanine, sometimes being slight and sometimes more marked. The reasons for these differences are not understood and not investigated further. Activation was always slight when tested with the mixed germinants adenosine + L-alanine. Whichever germinant was used, heat caused greater activation than did the highest radiation dose used (Fig. 1). The degree of radiation-activation of spores of different species varied markedly. For instance, of those tested at the 0·18 Mrad level only spores of strains of *B. cereus* with inosine as germinant were significantly activated (Table 2).

Table 2. *Germination of γ-irradiated spores of different organisms*

Unheated spores were left unirradiated for controls or exposed to 0·18 Mrad of γ-radiation. Subsequent germination was measured as described in Methods. L-Alanine was used at 10 mM and inosine at 1 mM.

	Decrease in extinction (%) after incubation of spore suspension for 20 min. at 37° in the presence of			
	L-Alanine		Inosine	
Organisms	Control spores	Irradiated spores	Control spores	Irradiated spores
B. brevis NCTC 7577	3·4	4·6	1·6	2·6
B. subtilis ATCC 6051	38·3	39·4	0	0
B. coagulans NCTC 3991	41·9	42·4	0	0
B. polymyxa strain M1	43·4	45·4	0	0
B. cereus strain PX	4·8	6·7	18·3	39·5
B. cereus NCTC 945	6·4	9·1	22·2	47·4

Calcium dipicolinate (CaDPA), which is a spore constituent, will germinate spores of most bacteria, probably by de-stabilizing some spore-Ca-DPA structure (Riemann & Ordal, 1961). The long chain primary alkane *n*-dodecylamine (laurylamine) will also germinate many spores, probably by virtue of its surface-active properties, and has been called a 'chemical germinant' to distinguish it from germinants like amino acids and ribosides (Rode & Foster, 1961). Figure 2 shows the effect of radiation and of heat on germination of spores of *Bacillus cereus* PX in CaDPA. Radiation-activation was recognizable with CaDPA or with *n*-dodecylamine as germinant, and therefore differed from heat activation, because heat did not activate spores for germination in *n*-dodecylamine.

Effect of γ-radiation on heat-activated spores

When spores of *Bacillus cereus* PX were activated by heat and subsequently γ-irradiated, the overall effect differed according to the germinant used to assess activation. Spores which had been heated and then γ-irradiated germinated in inosine about as rapidly as when only heated. In L-alanine, however, the germination rate of heated spores was decreased by the additional dose of γ-radiation (Fig. 3). Irradiation appeared to de-activate the heat-activated spores.

Titratable thiol content of activated spores

During heat-activation of *Bacillus cereus* PX spores the titratable thiol content of the spores increased, probably because thiol groups became unmasked by heat-induced tertiary-structure changes in some molecules of the spores. γ-Irradiated spores were found to have a higher content of titratable thiol groups than had unirradiated spores, but not as high as heat-activated spores (Table 3).

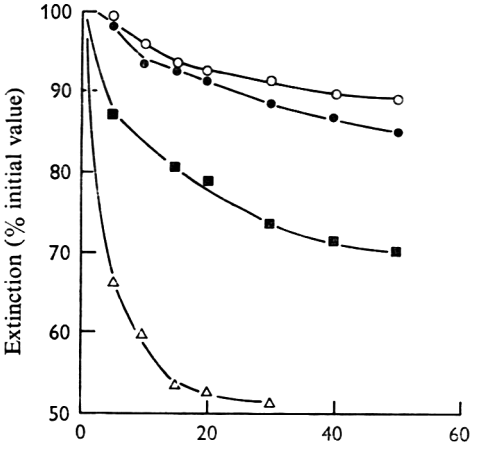

Fig. 3

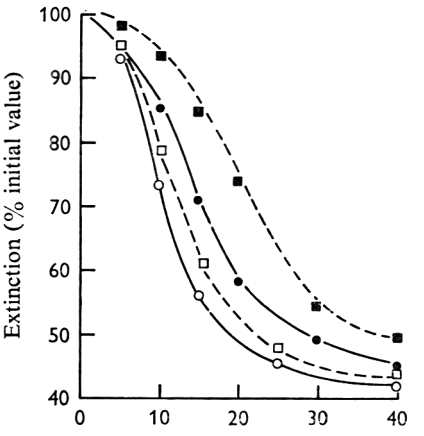

Fig. 4

Fig. 3. Effect of γ-irradiation on germination of heated spores of strain *Bacillus cereus* strain PX. Experimental conditions as defined in Fig. 1, but with L-alanine (10 mM) as germinant in place of inosine. Heating was at 70° for 30 min., radiation dose was 0·18 Mrad. Treatment of spores as follows: ○, unirradiated, unheated control spores; ●, irradiated spores; △, heated spores; ■, spores heated and then irradiated.

Fig. 4. Reversal of heat-activation and γ-radiation-activation of spores of *Bacillus cereus* strain PX. Experimental conditions as in Fig. 1 with inosine (500 μM) as germinant. Treatment of spores: ○ and ●, spores heated 70° for 30 min.; □ and ■, spores irradiated with 0·54 Mrad. Open symbols, before partial deactivation; closed symbols, after partial deactivation. Spores were partially de-activated by storage for 16 hr at 20° in dialysis sacs in water as described in Methods.

De-activation of heat-activated and radiation-activated spores

Heat activation of *Bacillus cereus* spores is reversible in the sense that heat-activated spores become partially de-activated on storage (Keynan *et al.* 1964). γ-Radiation-activated and heat-activated spores of *B. cereus* PX were stored for 16 h in aerated water and then tested for changes in germinability. Figure 4 shows that both heated and γ-irradiated spores germinated less rapidly after such storage, and thus both showed a degree of de-activation.

Radiation activation of Bacillus cereus strain T

In contrast to *Bacillus cereus* strain PX, spores of *B. cereus* strain T were not activated by doses of γ-radiation below about 0·54 Mrad. Doses of 1·08 and 2·16 Mrad were

necessary to cause consistently measurable increase in rate of germination of spores of strain T, at which dose values more than 99·9% kills occurred, as measured by colony count.

Table 3. *Titratable thiol groups in γ-irradiated and heated spores of Bacillus cereus*

Time for 30% decrease in extinction is an approximate measure of germination rate, and therefore of the extent of activation.

Treatment of spores	Thiol titre (μmole SH equiv./ 100 mg. dry weight of spores)	Time for 30% decrease in extinction of spore suspension in inosine medium (min.)
Untreated control	0·4	> 180
Irradiated, 0·18 Mrad	1·4	58
Irradiated, 0·54 Mrad	1·8	28
Heated to 70° for 30 min.	2·7	11·5

DISCUSSION

Ionizing radiation will cause tertiary-structure changes in macromolecules (Bacq & Alexander, 1961) by breaking covalent linkages including peptide bonds, and even by destroying basic amino acids in proteins (Robinson, Weiss & Wheeler, 1966). Destruction of the integrity of spore deoxyribonucleic acid (DNA) by γ-radiation may well cause death of bacterial spores (i.e. destroy their colony-forming ability), but although functional DNA is essential for cell growth it is probably not essential for spore germination and early outgrowth (Steinberg, Halvorson, Keynan & Weinberg, 1965). Indeed, bacterial spores irradiated with 0·54 Mrad during the present work became more than 95% non-viable (not forming colonies) and not only germinated rapidly but also outgrew and shed their coats when incubated on slide cultures of yeast glucose agar. However, growing and dividing forms were never observed. The relative resistance of the germination and outgrowth processes of irradiated spores compared with later vegetative growth has been reported for a number of organisms, e.g. *Clostridium botulinum* (Costilow, 1962), *Bacillus megaterium* (Levinson & Hyatt, 1960) and *B. subtilis* (Hermier, 1963). Clearly the enzymes and cofactors involved in germination must be more resistant to γ-radiation than the sites of damage caused by radiation which lead to loss of colony-forming ability.

From the hypothesis that the various methods known to activate bacterial spores do so by altering tertiary structures (Keynan *et al.* 1964) it was possible to predict that ionizing radiation should cause activation. The efficiency of radiation as an activating agent, however, was (like heat) not the same for all spores. It was more effective for *Bacillus cereus* strains PX and NCTC 945 than for *B. cereus* strain T, and γ-radiation was not an obvious activator for spores of four of the organisms shown in Table 2 or of *B. subtilis* strain SJ2 (Hermier, 1963). Spores of *Clostridium botulinum* suspended in phosphate buffer and irradiated with electrons subsequently germinated at similar rates to un-irradiated spores (Costilow, 1962), but since these spores were heat-activated any radiation-activation may have been masked. Spores of *C. botulinum* irradiated with sublethal doses of γ-radiation in cured ham did become activated (Anellis, Berkowitz, Jarboe & El-Bisi, 1967). Activation of *B. cereus* spores by heat and γ-radiation were similar qualitatively except when *n*-dodecylamine was used as germinant; then

radiation caused activation but heat did not, as noted by Rode & Foster (1961). The mode of action of *n*-dodecylamine as a germinant is not understood, so the different activation responses cannot yet be explained. A further similarity was that heat-activated and radiation-activated spores both became de-activated on storage. De-activation is known to occur with *B. cereus*, but not with all bacterial spores (e.g. *B. subtilis* 5230; Busta & Ordal, 1964), and probably reflects reformation of original tertiary structures (Keynan *et al.* 1964).

Although activation treatments probably alter tertiary structure of some spore macromolecules, the sites of those molecules within the spore are not known. It may be that the structural changes which lead to activation are general and occur to molecules in a variety of spore sites. Evidence supporting this view was obtained when activated spores of *Bacillus cereus* PX were found to have a higher content of titratable thiol groups than control spores, for tertiary-structure changes in proteins containing cysteine residues are known often to result in unmasking or masking of thiol groups. The increased thiol titres measured in radiation-activated spores might be the result of a general loosening of structure and unmasking of groups in a variety of spore molecules. The same loosening may be caused by alkanes, for Rode & Foster (1965) discovered that spores of *B. megaterium* QMB 1551 could be germinated by *n*-alkanes, in particular by butane and pentane, and proposed that the alkanes acted by disrupting hydrophobic bonds in spores. On the other hand, it has been suggested that a major effect of ionizing radiation is disruption of cell membranes (Bacq & Alexander, 1961), so that alterations in membrane permeability might be a factor leading to activation of spores. The observations of Levinson & Hyatt (1960) and Farkas & Kiss (1964, 1965) that electron-irradiated and X-irradiated spores showed changes (which Farkas & Kiss called 'pseudogermination') which were consistent with loss of permeability would support this view. However, the radiation doses necessary to cause pseudogermination were high lethal doses, whereas activation can be caused by low non-lethal doses of γ-radiation. This suggests that activation is due to smaller, more subtle changes than the gross changes in permeability measured by Farkas & Kiss, though probably of the same type. A similar pattern is shown by the effect of heating in that whereas low sublethal heating may activate spores, high sublethal or lethal heating causes increase in spore permeability (Black & Gerhardt, 1962) and leakage of some spore constituents (El-Bisi, Lechowich, Amaha & Ordal, 1962). The observation that ionizing radiation can activate spores is thus compatible with the idea that activation is due either to changes in tertiary structure of spore macromolecules or to an increase in permeability of a spore membrane. In either case the result would be to unmask previously masked reactive sites within the spore and allow more rapid germination.

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REFERENCES

ANELLIS, A., BERKOWITZ, D., JARBOE, C. & EL-BISI, H. M. (1967). Radiation sterilization of prototype military foods II. Cured ham. *Appl. Microbiol.* **15**, 166.

BACQ, Z. N. & ALEXANDER, P. (1961). *Fundamentals of Radiobiology*, 2nd ed. Oxford: Pergamon Press.

BLACK, S. H. & GERHARDT, P. (1962). Permeability of bacterial spores III. Permeation relative to germination. *J. Bact.* **83**, 301.

BUSTA, F. F. & ORDAL, Z. J. (1964). Lack of influence of suspending media on heat activation of *Bacillus subtilis* spores and absence of deactivation. *Appl. Microbiol.* **12**, 111.

CHARLESBY, A. (1963). Radiation chemistry of macromolecules. In *Radiation effects*. Ed. by M. Ebert and A. Howard, p. 72. Amsterdam: North Holland Publishing Co.

COSTILOW, R. N. (1962). Fermentative activities of control and radiation-'killed' spores of *Clostridium botulinum*. *J. Bact.* **84**, 1268.

CURRAN, H. R. & EVANS, F. R. (1945). Heat activation inducing germination in the spores of thermotolerant and thermophilic aerobic bacteria. *J. Bact.* **49**, 335.

EL-BISI, H. M., LECHOWICH, R. V., AMAHA, M. & ORDAL, Z. J. (1962). Chemical events during death of bacterial endospores by moist heat. *J. Food Sci.* **27**, 219.

FARKAS, J. & KISS, I. (1964). Bakteriumsporak sugarkarosodasanak vizsgalata. *Commun. Cent. Food Res. Inst. Budapest*, **1**, 13.

FARKAS, J. & KISS, I. (1965). Observations on biochemical changes in irradiated spores of *Bacillus cereus*. *Acta microbiol. Acad. sci. hung.* **12**, 15.

GIBBS, P. A. (1967). The activation of spores of *Clostridium bifermentans*. *J. gen. Microbiol.* **46**, 285.

HAMM, R. & HOFMANN, K. (1965). Changes in the sulphydryl and disulphide groups in beef muscle proteins during heating. *Nature, Lond.* **207**, 1269.

HERMIER, J. (1963). Les conséquences de l'irradiation gamma de la spore de *Bacillus subtilis* sur la germination de la spore et sur la croissance végétative. *Annls. Nutr. Aliment.* **17**, B439.

HITCHINS, A. D., KING, W. L. & GOULD, G. W. (1966). Role of disulphide bonds in the resistance of *Bacillus cereus* spores to gamma radiation and heat. *J. appl. Bact.* **29**, 505.

HYATT, M. T., HOLMES, P. K. & LEVINSON, H. S. (1966). Activation of *Bacillus megaterium* spore germination by water in the vapour phase. *Biochem. biophys. Res. Commun.* **24**, 701.

KEYNAN, A., EVENCHIK, Z., HALVORSON, H. O. & HASTINGS, J. W. (1964). Activation of bacterial endospores. *J. Bact.* **88**, 313.

KEYNAN, A., ISSAHARY-BRAND, G. & EVENCHIK, Z. (1965). Activation of bacterial spores. In *Spores*, Ed. by L. L. Campbell and H. O. Halvorson, vol. **3**. p. 180. Ann Arbor, Mich.: American Soc. Microbiol.

LEE, W. H. & ORDAL, Z. J. (1963). Reversible activation for germination and subsequent change in bacterial spores. *J. Bact.* **85**, 207.

LEVINSON, H. S. & HYATT, M. T. (1960). Some effects of heat and ionizing radiation on spores of *Bacillus megaterium*. *J. Bact.* **80**, 441.

LEWIS, J. C., SNELL, N. S. & ALDERTON, G. (1965). Dormancy and activation of bacterial spores. In *Spores*, Ed. by L. L. Campbell and H. O. Halvorson, vol. **3**. p. 47. Ann Arbor, Mich.: American Soc. Microbiol.

RIEMANN, H. & ORDAL, Z. J. (1961). Germination of bacterial endospores with calcium and dipicolinic acid. *Science, N.Y.* **133**, 1703.

ROBERTS, T. A. & INGRAM, M. (1965) Radiation resistance of spores of Clostridium species in aqueous suspension. *J. Food Sci.* **30**, 879.

ROBINSON, M. G., WEISS, J. J. & WHEELER, C. M. (1966). Irradiation of deoxyribonucleohistone solutions. I. Amino acid destruction. *Biochim. biophys. Acta.* **124**, 176.

RODE, L. J. & FOSTER, J. W. (1961). Germination of bacterial spores with alkyl primary amines. *J. Bact.* **81**, 768.

RODE, L. J. & FOSTER, J. W. (1965). Gaseous hydrocarbons and the germination of bacterial spores. *Proc. natn. Acad. Sci., U.S.A.* **53**, 31.

STEINBERG, W., HALVORSON, H. O., KEYNAN, A. & WEINBERG, E. (1965). Timing of protein synthesis during germination and outgrowth of spores of *Bacillus cereus* strain T. *Nature, Lond.* **208**, 710.

WIDDOWSON, J. P. (1967). *Bacillus pantothenticus* spores activated by dimethylformamide and dimethylsulphoxide. *Nature, Lond.* **214**, 812.

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A Strain of *Pediococcus cerevisiae* which requires Methicillin for Growth

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SUMMARY

By repeated subcultivation of the parent strain *Pediococcus cerevisiae* ATCC 8081 in the presence of methicillin, a substrain *P. cerevisiae* 8081 CRD was developed which grew only when the partly defined medium was supplemented with methicillin or certain other penicillins. The methicillin-dependent strain was not highly resistant to methicillin and grew only in the presence of a limited range of concentrations of it (about 10–300 μg./ml.). Even with an optimal growth concentration of methicillin (50–100 μg./ml.), the dependent organisms grew less well than did the parent strain without methicillin and showed a longer lag period before growth became visible.

Although other derivatives of 6-aminopenicillanic acid (but not of 7-aminocephalosporanic acid) supported moderate growth of *Pediococcus cerevisiae* 8081 CRD, none was as effective as methicillin, nor was there marked cross-resistance to any of these other derivatives. The more potent the penicillin as inhibitor of growth of the parent strain, the smaller was the optimal concentration needed to support growth of the dependent substrain. Several other antibiotics were ineffective as growth factors.

When methicillin was hydrolysed with acid, alkali or pencillinase, activity as a growth factor was lost. During growth of the parent strain and of the methicillin-dependent strain at pH 6·5 material was produced in the medium which was able to destroy the antibiotic potency of methicillin or other penicillins. The substance was not an enzyme, and the presence of methicillin was not necessary to induce its formation by the parent strain.

The methicillin-dependent strain did not grow, with or without methicillin, when sodium acetate was omitted from the medium. No substance of known chemical structure was found which could replace acetate for growth. *Pediococcus cerevisiae* 8081 CRD grew rapidly in the absence of both acetate and methicillin when the medium was supplemented with yeast extract. However, when acetate was present as well as yeast extract, methicillin again became necessary for growth of the dependent organisms.

INTRODUCTION

The occurrence of strains of bacteria which require streptomycin (or related drugs) for growth is well known, and the mechanisms responsible for dependence have been studied in some detail (Gorini & Kataja, 1964; Anderson, Gorini & Breckenridge, 1965). Such organisms possess a defective structural gene which can be phenotypically corrected by streptomycin owing to a misreading of the genetic code brought about by streptomycin. However, bacteria which are dependent for growth on drugs of other types have seldom been reported (Schnitzer & Grunberg, 1957); cases of

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penicillin-dependence are very rare. An instance of dependence on penicillin was described by Barber (1953*a*, *b*), who isolated colonies of staphylococci which grew only in the presence of benzylpenicillin; the products of penicillin hydrolysis with penicillinase or with acid did not support growth. The dependent strains were rather unstable and tended to revert to the ability to grow in the absence of benzylpenicillin. Jackson (1953) reported further work with the same strains. The penicillin-dependent organisms were much more sensitive to inhibition by oleic acid than were the parent organisms. When traces of oleic acid were removed from the medium (by extraction with ether) the organisms that previously were penicillin-dependent grew in the absence of penicillin.

The present work began with an attempt to develop a substrain of *Pediococcus cerevisiae* that would be resistant to benzylpenicillin. Methicillin (2,6-dimethoxyphenylpenicillin; then called Celbenin) had been introduced (Rolinson *et al.* 1960) and it was of interest to attempt also to produce a methicillin-resistant substrain. *Pediococcus cerevisiae* was used because familiarity with this organism had already been gained during studies of its resistance to 5-fluorouracil (White & Nichol, 1963). In the attempts to develop resistance to benzylpenicillin little success was achieved, but with methicillin a substrain of moderate resistance was obtained which grew only in the presence of some methicillin. This substrain was called *P. cerevisiae* 8081 CRD (Celbenin-resistant-dependent).

The development of this substrain, and some of its properties, are described in this paper. Preliminary reports of part of this work have already been given (White, 1962; Work, 1962).

METHODS

Organisms. Pediococcus cerevisiae (*Leuconostoc citrovorum*) ATCC 8081 was maintained in tryptose glucose agar stab cultures which were incubated overnight at 37°. Stock cultures were transferred monthly and stored at 2°. Development of the methicillin-requiring substrain is described below in Results. Suspensions of organisms of this substrain in sterile horse serum were freeze-dried in ampoules. At about monthly intervals, a fresh ampoule was used to inoculate a tube of liquid partly defined medium (10 ml.) containing methicillin (100 μg./ml.). This culture was left to stand for 1–2 days at 37°; 0·1 ml. of the culture then was transferred to fresh medium (10 ml.) at intervals of 1–2 days. Liquid cultures did not remain viable for much longer than a week, even when stored at 2°. Stab cultures in the partly defined medium containing methicillin (solidified with agar) remained viable for 2–3 weeks, when kept at 2°.

Staphylococcus aureus strain JHM and its cultivation were described by White & Woods (1965).

Media. The partly defined medium (containing leucovorin) for *Pediococcus cerevisiae* was that used by White & Nichol (1963). For growth of the methicillin-requiring organisms, the medium was supplemented (after autoclaving) with a sterile (filtered) solution of methicillin (100 μg./ml. final concentration). Tubes of sterile medium (10 ml.) supplemented with methicillin (for daily transfers of organisms) were kept at 2°. Similar medium solidified with 2% (w/v) agar was used for stab cultures of *P. cerevisiae* 8081 CRD.

The defined medium (containing leucovorin) was that of Sauberlich & Baumann (1948) with uracil omitted.

For counting of viable organisms, Difco CF assay medium (supplemented with leucovorin) was generally used, solidified with 1 % (w/v) agar. This medium was the same as the partly defined medium, except for the presence of uracil in the Difco medium. Sometimes the usual partly defined medium was used for counting.

For plate assays of penicillins the CYLG medium of Marshall & Kelsey (1960) was used without glucose and 2 % (w/v) agar added.

Glucose peptone water contained (g./l.): Oxoid peptone, 20; glucose, 20; sodium chloride, 5; adjusted to pH 7·4.

Conditions for growth experiments. Inocula were fresh overnight cultures in partly defined medium, diluted (sterile water) usually to a reading of 0·002 (in 6 mm. diameter tubes) in the EEL photoelectric colorimeter (Evans Electroselenium Co. Ltd, Halstead, Essex) with neutral density filter (no. 1·0) and with uninocculated medium for the zero setting. Inoculum (0·1 ml.) was added to the medium (4·9 ml.; autoclaved at 120° for 7 min.) in plugged tubes (150 × 19 mm.) which were set up in duplicate and incubated standing upright at 37°, usually in air.

Although cultures in test-tubes grew relatively rapidly and heavily, e.g. the parent strain in 20 hr gave a turbidity reading of 1·5–2·0, such a rate of growth was frequently not achieved when larger volumes of culture were grown in conical flasks. At least two factors seemed to be important, but were not examined in detail: the ratio of volume of medium to the surface area open to the air; and the period of autoclaving of the medium relative to its volume. A good rate of growth was obtained when cultures of 50 ml. or 150–200 ml. were grown in conical flasks of 100 ml. or 250 ml. capacity, respectively, standing at 37°. The medium was autoclaved at 120° for 10 min. and the inoculum was 0·1 ml. of a fresh overnight culture in partly defined medium. When samples for various measurements were removed during incubation of a culture, the medium was only gently stirred, just sufficiently to resuspend the organisms evenly, without causing much aeration of the medium.

For anaerobic growth, tubes were incubated upright in a glass McIntosh and Fildes jar in an atmosphere of hydrogen. The early stages of growth were observed through the sides of the jar, which was not opened until the end of an experiment. As an indicator of anaerobic conditions, a tube of glucose peptone water was used, coloured green by one drop of methylene blue (0·1 % in ethanol). The green colour disappeared completely in about 20 hr when conditions in a jar were anaerobic, and the colour reappeared in a minute or two when the jar was opened to the air.

Assessment of growth. Growth was roughly estimated by inspecting cultures at intervals over several days. When quantitative results were required, the turbidities of cultures were measured in an EEL colorimeter as described above. The relation between colorimeter reading and the dry wt organisms was linear from 0 to 3·0 on the colorimeter scale, and a reading of 1·0 was equivalent to 0·3 mg. dry wt organisms/ml. (for both strains).

Colony counts were made in various modifications of the partly defined medium solidified with 1 % (w/v) agar. A bottom layer of inoculated medium (10 ml.) was poured in a Petri dish at 43°. When this layer had set, an uninoculated covering layer (30 ml.) of the same medium, also at 43°, was poured. Plates were incubated at 37° for 2–3 days until the colonies were large enough to be counted easily. Without a covering layer, colonies did not form evenly throughout the inoculated layer, no colonies being found nearer than about 2 mm. to the surface open to the air.

Pediococcus cerevisiae in liquid cultures in all the media grew as bunches made up of variable numbers of organisms (1 to about 100). Consequently, a colony on a plate might have arisen from one organism or from several. The appearance (with the phase-contrast microscope with oil-immersion lens) of organisms from liquid cultures, and the range of sizes of the bunches were not noticeably altered when the composition of the medium was varied. The parent strain generally seemed to produce larger bunches of organisms than did the methicillin-dependent substrain. The number of bunches in a culture of either strain could readily be determined in a counting-chamber under the phase-contrast microscope with high-power lens, although the number of organisms making up a bunch could not be assessed. A fresh culture of either strain reading 1·0 on the EEL colorimeter scale gave a count of about 2×10^8 bunches of organisms/ml. in the chamber, and when plated in partly defined medium produced about 2×10^8 colonies/ml.

Growth of organisms at a constant pH value. Partly defined medium (350 ml.) containing methicillin or broxil was inoculated with 1·0 ml. of a fresh overnight culture of *Pediococcus cerevisiae* 8081 CRD and incubated at 37°. The medium (in a special vessel) was maintained at pH 6·5 during growth of the culture by the regulated addition of 2N-NaOH through a pH titrator apparatus (Type TTT 1c; Radiometer, 72 Emdrupvej, Copenhagen NV, Denmark). The medium was gently stirred throughout the experiment with a small paddle fixed near the point of entry of the alkali.

Assay of penicillin. Medium CYLG (without glucose) containing 2% (w/v) agar was autoclaved at 120° for 15 min. and then cooled to 43°. The molten medium (50 ml.) was inoculated with 0·5 ml. of a suspension of *Staphylococcus aureus* JHM in sterile water (about 10^8 bunches of organisms/ml., from a fresh overnight slope culture on nutrient agar), and poured into a 15 cm. diameter Petri dish. After the medium had set, holes were cut out with a cork borer (8 mm. diameter). Solutions for assay (25–100 µl.) were pipetted into the holes, and the plates were immediately incubated at 37°. Next day the diameters of the zones of inhibition round the holes were measured, against the general background of growth. A plot of the square of this diameter against the $\log_{10}$ of the concentration of drug gave a straight line. The potencies of solutions under test were found by comparison with the line.

No change in concentration of methicillin occurred during the plate assay. Methicillin (100 µg./ml. final concentration) was added to a culture filtrate of *Pediococcus cerevisiae* 8081 CRD (which was able to decompose methicillin when incubated with it for several hours at 37°) and the mixture (25, 50, 100 µl. samples) was at once plated. A solution of methicillin (100 µg./ml.), in 0·1 M-phosphate buffer (pH 6·5), was similarly plated. After incubation of the plates for 18 hr, equal volumes of the two solutions (with all three volumes of sample) gave identical zones of inhibition. The useful range of the assay was 0·5–20 µg. methicillin per well, or 0·02–1·0 µg. broxil per well. The precision of the estimation was low, but this was unimportant in the experiments for which the assay was used.

Chemicals. Methicillin (2,6-dimethoxyphenylpenicillin) used in the development of the methicillin-dependent substrain was kindly given by Mr J. W. Lightbown, before it was generally available. In most of the subsequent work described in this paper, commercial supplies of methicillin were used. Broxil (6-(α-phenoxypropionamido)-penicillanic acid); phenoxymethylpenicillin (penicillin V) and α-aminobenzylpenicillin (ampicillin) were gifts from Beecham Research Laboratories. Quinacillin (3-carboxy-

2-quinoxalinylpenicillin) was given by Dr D. F. Spooner, and 6-aminopenicillanic acid was given by Dr D. L. Swallow. Stock (sterile filtered) solutions of penicillins were kept at $-15°$.

Cephalosporin C, cephalosporin N and bacitracin were provided by Professor E. P. Abraham. Dr D. L. Swallow gave 7-(phenylacetamido)-cephalosporanic acid. Vancomycin (from Eli Lilley and Co.) was given by Dr P. E. Reynolds.

Mevalonic acid (potassium salt) was provided by Dr G. Popják. Leucovorin (calcium salt of 5-formyl-tetrahydropteroylglumatic acid) was given by the late Professor D. D. Woods. Pencillinase (a sterile filtrate of a broth culture of *Bacillus licheniformis*) was bought from Burroughs Wellcome.

Table 1. *Development of the methicillin-requiring substrain of Pediococcus cerevisiae 8081*

Organisms were grown in partly defined medium (5 ml.) containing increasing concentrations of methicillin. At intervals organisms were transferred from the highest concentration of methicillin in which growth had occurred into a fresh series of tubes. The inoculum for subculture 1 was *P. cerevisiae* 8081 grown in medium without methicillin. After subculture 27 organisms were transferred to stab cultures in partly defined medium containing methicillin (100 μg./ml.). This culture was called 8081 CRD.

Subculture	Highest concn. of methicillin permitting growth (μg./ml.)	Time of incubation before transfer (hr)	Lag period in methicillin-free medium (hr)
1	10	28	< 20
2	40	70	< 20
3	20	48	< 17
4	40	48	< 17
5	40	70	< 17
6	40	72	< 17
7*	40	48	< 18
8	50	72	< 25
9	50	47	< 23
10	60	43	< 22
11	80	123	.
12	100	120	.
13	110	73	< 24
14	140	108	24
15	100	144	45
16	150	95	40
17	200	92	< 24
18	300	147	45
19	400	96	50
20	400	96	60
21	800	145	66
22	600	95	65
23	600	120	100
24	400	144	> 144
25	600	145	> 145
26	600	97	> 97
27	600	112	> 112

* At subculture 7 (and in subsequent subcultures) the inoculum was increased from about 5×10^4 organisms to about 5×10^5 organisms.

RESULTS

Development of the methicillin-dependent substrain

The course of development of this substrain is shown in Table 1. *Pediococcus cerevisiae* 8081 was grown in a series of pairs of test tubes containing the partly defined medium, with methicillin at increasing concentrations. Usually the concentration of methicillin was doubled between each pair of tubes in a series (though sometimes the concentrations were closer than this), and one pair of tubes contained no drug. Organisms from the highest concentration of methicillin at which growth occurred in

Table 2. *Attempt to develop a benzylpenicillin-resistant substrain of Pediococcus cerevisiae 8081*

Organisms were grown in partly defined medium (5 ml.) containing increasing concentrations of benzylpenicillin. At intervals organisms were transferred from the highest concentration of benzylpenicillin in which growth had occurred into a fresh series of tubes. The inoculum for subculture 1 was *P. cerevisiae* 8081 grown in medium without methicillin. For brevity, steps where no resistance was gained have been omitted. After subculture 27 organisms were transferred to stab cultures in partly defined medium. This culture was called 8081 PR (penicillin-resistant).

Subculture	Highest concn. of benzylpenicillin permitting growth (μg./ml.)	Time of incubation before transfer (hr)
1	0·8	29
2	1·0	70
3	1·0	48
6	0·5	72
7*	1·0	49
10	1·0	42
11	1·5	123
12	2·5	120
16	3·0	95
20	3·0	96
21	4·0	145
24	5·0	145
26	4·0	97
27	3·0	112

* At subculture 7 (and subsequently) the inoculum was increased from about 5×10^4 organisms to about 5×10^5 organisms.

one series of tubes were used to inoculate a fresh series of tubes. In the earlier subcultures (1–10) transfers were made at intervals of 2–3 days, and only a low degree of resistance developed. The size of the inoculum was increased after subculture 6 and longer periods were allowed between transfers (after subculture 10). During subcultures 11–20 the degree of resistance gradually increased; no large increase in resistance occurred at any one step. There was a gradual increase in the lag period before growth became visible in methicillin-free medium. By passage 20, growth appeared only after a lag of about 72 hr in methicillin-free medium. The lag was shorter in medium containing methicillin, although the difference of the lag in this medium and in methicillin-free medium was not striking at this stage, probably because the lowest concentration of methicillin that was being used in subculture 20 was 200 μg./ml., which was a higher concentration than subsequently proved to optimal for rapid

growth of the dependent organisms. Resistance did not increase further between subcultures 21 and 27, but the failure of the organisms to grow in the absence of methicillin became more and more obvious. By subculture 27 organisms grew rapidly and heavily in the presence of methicillin (100 μg./ml.), but did not show any growth within 5 days in its absence.

Attempt to develop a substrain resistant to benzylpenicillin. Serial transfers of *Pediococcus cerevisiae* 8081 were similarly made in medium containing increasing concentrations of benzylpenicillin (Table 2). As in the development of resistance to methicillin, it was necessary to increase the size of the inoculum and to lengthen the time between subcultures in order to obtain resistant organisms. Even so, the degree of resistance to benzylpenicillin that was achieved after 27 subcultures was only about fourfold. The resistant organisms grew in the absence of benzylpenicillin with a lag period little longer than that of the parent strain; benzylpenicillin did not stimulate growth.

Properties of the methicillin-requiring organisms

Response to methicillin. The methicillin-requiring organisms were not highly resistant to methicillin; growth was inhibited by a concentration of methicillin about 40 times higher than was inhibitory for the parent strain. There was therefore only a limited range of concentration of methicillin within which growth occurred (Fig. 1). About 40–100 μg. methicillin/ml. was optimal for growth of the organisms within 50 hr in the partly defined medium. On longer incubation, organisms were able to grow over a wider range of methicillin concentration and the highest turbidity was eventually reached in relatively low concentrations of it. When an inoculum of less than about 10^4 organisms was used, no growth occurred in methicillin-free medium, but when about 10^5 (or more) organisms were used, then limited growth (about 0·2 on the colorimeter scale) occurred in methicillin-free medium after 2–4 days, depending on the volume of the medium inoculated. This growth never approached the turbidity given by the parent strain in methicillin-free medium.

The response of *Pediococcus cerevisiae* 8081 CRD to methicillin on solid medium is illustrated in Pl. 1, fig. 1. Immediately adjacent to the hole in the medium where methicillin was placed, there was a zone showing no growth, presumably because the methicillin was at an inhibitory concentration. Farther from the hole there was a zone of confluent growth, beyond which there was again no growth, presumably because there the concentration of methicillin had become too low. In this outer region a few single colonies appeared, probably from organisms which had reverted to ability to grow in absence of methicillin.

When incubated in partly defined medium containing methicillin (100 μg./ml.), *Pediococcus cerevisiae* 8081 CRD showed a longer lag phase before turbidity appeared than did the parent strain (growing without methicillin), although the rate of logarithmic growth of the methicillin dependent strain was only slightly lower (Fig. 2). The final turbidity of cultures of *P. cerevisiae* 8081 CRD (with any concentration of methicillin) was lower (at best about 1·5–2·0 on the colorimeter scale) than that of cultures of the parent strain grown without methicillin (about 2·0–2·5). The turbidity of cultures of both strains did not decrease (within 7 days at 37°) after maximum turbidity had been reached.

Colony counts were made during growth of *Pediococcus cerevisiae* 8081 CRD from a small inoculum (Fig. 3). The number of organisms able to form colonies in medium

containing methicillin increased, and eventually reached about 4×10^8/ml., after which there was a decrease in the number that were viable. Initially, no organisms could be detected that were able to form colonies in methicillin-free medium, but such organisms subsequently appeared in the proportion of about 1 among 10^5 methicillin-requiring organisms.

Other properties. A characteristic feature of *Pediococcus cerevisiae* is that 5-formyltetrahydropteroylglutamic acid (leucovorin) is the only form of folic acid that supports growth in a medium which otherwise lacks the vitamin. The parent strain and the methicillin-requiring strain did not grow when leucovorin was omitted from the medium, even when it was supplemented with pteroylglutamic acid (2 mμg./ml.). Both strains grew when leucovorin was added, and the amount of growth (turbidity) was graded over the range of 0–0·1 mμg. leucovorin/ml. The parent strain grew in partly defined medium (after a lag of about 40 hr) when leucovorin was replaced by thymidine (0·1 μg./ml.), although the final turbidity was only about one third of that obtained with leucovorin. The methicillin-requiring strain did not grow (within 120 hr) when leucovorin was replaced by thymidine.

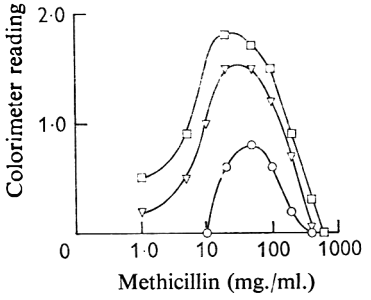

Fig. 1

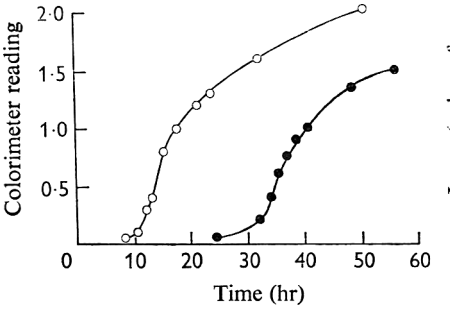

Fig. 2

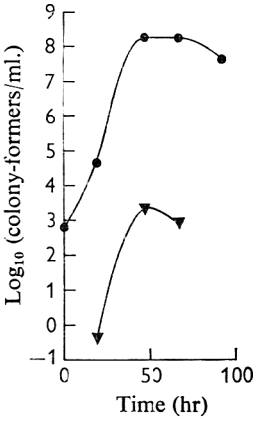

Fig. 3

Fig. 1. Response of *Pediococcus cerevisiae* 8081 CRD to methicillin. A series of 100 ml. conical flasks was prepared, containing partly defined medium (50 ml.) with various concentrations of methicillin. Each flask was inoculated with 0·1 ml. of an overnight culture of *P. cerevisiae* 8081 CRD, and incubated at 37°. At intervals samples were removed and their turbidities were read in the EEL colorimeter as described in the Methods section: ○, after 21 hr; ▽, after 47 hr, □, after 63 hr.

Fig. 2. Growth of *Pediococcus cerevisiae* 8081 and 8081 CRD in partly defined medium. Two 150 ml. flasks were prepared, each containing 100 ml. medium, and to one of which methicillin (100 μg./ml.) was added. Overnight liquid-cultures of *P. cerevisiae* 8081 and 8081 CRD were diluted (sterile water) to give colorimeter readings of 0·02, and 1·0 ml. of each suspension was used as an inoculum: the flask without methicillin was inoculated with *P. cerevisiae* 8081, and that containing methicillin with *P. cerevisiae* 8081 CRD. The flasks were incubated at 37° and samples were removed at intervals, and their turbidities were read in the EEL colorimeter as described in the Methods section. ○, *P. cerevisiae* 8081; ●, *P. cerevisiae* 8081 CRD.

Fig. 3. Colony counts during growth of *Pediococcus cerevisiae* 8081 CRD from a small inoculum. A 100 ml. conical flask containing 50 ml. of partly defined medium plus methicillin (100 μg./ml.) was inoculated with 0·1 ml. of a 1/1000 dilution of a 48 hr culture of *P. cerevisiae* 8081 CRD. The flask was incubated at 37° and samples were removed at intervals for plating in Difco CF assay medium (+leucovorin). ●, Count in agar containing methicillin (100 μg./ml.); ▼, count in agar without methicillin.

Neither *Pediococcus cerevisiae* 8081 nor 8081 CRD grew as rapidly or as heavily at 30° as at 37°, either in defined or in partly defined media. At 30°, methicillin was still required for growth of *P. cerevisiae* 8081 CRD in both media.

Under anaerobic conditions, *Pediococcus cerevisiae* 8081 CRD did not grow in defined medium, with or without methicillin. The parent strain grew to a limited extent under anaerobic conditions in defined medium without methicillin. Addition of yeast extract to defined medium enabled *P. cerevisiae* 8081 CRD to grow anaerobically within 60 hr in the presence and in the absence of methicillin. Anaerobic growth with yeast extract after shorter periods of incubation was not examined.

Some effects of oleic acid on growth of *Pediococcus cerevisiae* 8081 CRD were investigated. In defined medium, oleic acid (adjusted to pH 6·5 before addition prior to autoclaving of the medium) at 0·1 mg./ml. inhibited growth in the presence of methicillin, but did not inhibit at 0·01 mg./ml. Oleic acid (0·01–0·1 mg./ml.) did not replace acetate for growth, with or without methicillin.

When partly defined medium had been extracted three times with ether or chloroform at pH 4·0, or 6·5 (0·5 ml. solvent/ml. medium at each extraction), methicillin was still required for growth of *Pediococcus cerevisiae* 8081 CRD: medium extracted with chloroform at pH 4·0 gave slightly poorer growth than did the unextracted or ether-extracted medium. When testing these extracted media, one from each pair of duplicate tubes was closed with a metal cap instead of a cotton-wool plug. It was thought possible that materials which caused the requirements for methicillin (by *P. cerevisiae* 8081 CRD) might have come from cotton-wool plugs. However, this was not the case, because the growth responses in duplicate tubes, with caps or plugs, were the same.

Growth responses to other derivatives of 6-aminopenicillanic acid, and to other compounds. Several compounds were tested for ability to support growth of *Pediococcus cerevisiae* 8081 CRD in the partly defined medium (Table 3), and determinations were made of their growth-inhibitory concentrations for the parent strain. All the derivatives of 6-aminopenicillanic acid tested had activity as growth factors for *P. cerevisiae* 8081 CRD over limited ranges of concentration. Those which inhibited growth of the parent strain at the lowest concentrations were also effective at the lowest concentrations as growth factors for the substrain. However, none of these compounds gave growth that was as rapid or as heavy as that obtained with optimal concentrations of methicillin. Generally the methicillin-requiring strain was unable to grow in concentrations of penicillins much higher than those which caused inhibition of growth of the parent strain, i.e. *P. cerevisiae* 8081 CRD had appreciable resistance only to methicillin and was not cross-resistant to other penicillins to any marked extent.

The response of *Pediococcus cerevisiae* 8081 CRD to two different amounts of benzylpenicillin in solid medium is illustrated in Pl. 1, figs. 2, 3. The zone of confluent growth was farther from the centre well (containing penicillin) when the larger amount of penicillin was used.

Loss of activity (as a growth factor) on hydrolysis of methicillin. Although methicillin is considerably less sensitive to penicillinase than is benzylpenicillin, it is slowly hydrolysed by the enzyme to a derivative of penicilloic acid. Hydrolysis with alkali gives the same product quite rapidly, while acid-hydrolysis rapidly leads to the formation of a penillic acid derivative. Methicillin (4 mg.) in bicarbonate buffer was incubated with penicillinase (*Bacillus licheniformis* culture filtrate, 0·2 ml.) in Warburg

flasks, as described by Abraham (1955), and a control (methicillin without penicillinase) was also included. After evolution of carbon dioxide had ceased (18 hr) the contents of the flasks were washed out quantitatively and made up to 10 ml.

Methicillin (21 mg.) was also dissolved in 0·1 N-HCl (4·2 ml.), incubated at 37° for 2 hr and then neutralized with 0·1 N-NaOH. Another sample of methicillin (53 mg.) was dissolved in 1·0 N-NaOH (8 ml.), kept at room temperature for 1 hr and then neutralized with 4N-HCl. These two solutions were diluted to the equivalent of 1 mg. (acid-hydrolysate) or 0·5 mg. (alkali-hydrolysate) of original methicillin/ml. Assays with *Staphylococcus aureus* showed that at least 95% of the methicillin in these two

Table 3. *Activities of various compounds as growth factors for Pediococcus cerevisiae 8081CRD and as inhibitors of growth of P. cerevisiae 8081*

Organisms were grown in partly defined medium (for about 4–5 days) in a series of tubes containing the compound under test; generally the concentration of the compound was trebled between each pair of tubes in a series.

Compound	Concn. (μg./ml.) causing: Growth of methicillin-dependent strain 8081 CRD	Inhibition of parent strain 8081
Phenoxymethylpenicillin	0·1–0·3	0·1
Benzylpenicillin	0·1–0·5	0·1–0·5
α-Phenoxyethylpenicillin (Broxil)	0·5	0·3–1·0
α-Aminobenzylpenicillin	1·0	1·0
Quinacillin (3-carboxy-2-quinoxalinylpenicillin)	30	100
Methicillin	30–300	10–20
6-Aminopenicillanic acid	50–100	> 100
Cephalosporin N	100	> 100
Cephalosporin C	100*	> 100
7-(Phenylacetamido)-cephalosporanic acid	10*	.
Bacitracin	Inactive	30–100
Vancomycin	Inactive	.
Cycloserine	Inactive	.
2,6-Dimethoxybenzoic acid	Inactive	.

* Very slight growth after 100 hr.

solutions had been hydrolysed to non-inhibitory products. Control solutions of methicillin (without acid or alkali) were similarly incubated and diluted. When filtered to sterilize and added to partly defined medium (5 ml. final volume) the solutions of penicillinase, acid- or alkali-hydrolysed methicillin (0·25–2·0 ml.) were unable to support growth of *Pediococcus cerevisiae* 8081 CRD, whereas all the controls gave good growth.

Disappearance of methicillin during growth of cultures of Pediococcus cerevesiae 8081CRD

A culture of *Pediococcus cerevisiae* 8081 CRD was grown in partly defined medium (200 ml.) containing methicillin (100 μg./ml.). At intervals during incubation, samples (10 ml.) were removed and their turbidities and pH values measured. Each sample was then adjusted to pH 6·5, organisms removed by centrifugation and the supernatant

fluid Seitz-filtered. The concentration of methicillin in each sample was then found by plate assay; the results are shown in Fig. 4. The initial pH value of the medium (after autoclaving) was 6·2, but as growth increased the pH value decreased to a final value of about 4·5. The concentration of methicillin began to decrease at about the middle of the logarithmic phase of growth, when the medium was about pH 5. The rate at which methicillin subsequently disappeared from the medium was about the same as the rate of disappearance when methicillin (100 μg./ml.) was incubated in 0·1 M-acetate buffer (pH 4·5), at 37°. There was no appreciable disappearance of methicillin or change of pH value when autoclaved uninoculated medium was incubated at 37° for 60 hr.

To establish whether or not the disappearance of methicillin had been due only to the acidity of the medium, a culture of *Pediococcus cerevisiae* 8081 CRD was grown in partly defined medium containing an optimal concentration of broxil (1 μg./ml.), which is a penicillin that is relatively stable to acid. Throughout the growth of this culture the medium was maintained at pH 6·5 by regulated addition of 2N-NaOH. Because of the shape of the culture vessel it was not possible to suspend organisms evenly (for measurement of turbidity) without very considerable agitation of the medium, which tended to delay or even prevent further growth. Consequently the volume of alkali consumed was taken as a measure of growth. The turbidity of the medium and the amount of bacterial growth clinging to the vessel were very roughly estimated by eye. Growth so assessed corresponded in its time of first appearance, and in its increase, with the consumption of alkali, although in the later stages of the experiment the organisms might have been producing acid without further increase in their mass. At intervals samples were withdrawn from the apparatus, organisms removed by centrifugation and the sample assayed for broxil; results are shown in Fig. 5. The concentration of broxil remained unchanged until moderate growth had occurred. As before, the broxil disappeared from the medium during the phase of most rapid growth, and even when all the broxil had disappeared, alkali continued to be consumed, at a slightly slower rate. This rate was not accelerated when more broxil was added (to give 1·0 μg./ml.) and the drug again disappeared from the medium. When the experiment was stopped, 30 ml. of 2N-NaOH had been used. If each molecule of glucose in the medium (350 ml., containing 8·75 g. glucose) had been broken down to two molecules of lactic acid, then 48·6 ml. of 2N-NaOH would have been required to maintain the pH value at 6·5.

A similar experiment was done in which organisms were grown at constant pH value in the presence of methicillin (100 μg./ml.). As before, the methicillin began to disappear from the medium when moderate growth had occurred and consumption of alkali continued after all the methicillin had been destroyed. The organisms grew rather more heavily and quickly with methicillin than they did with broxil, and more alkali (42 ml.) was needed to maintain the pH at 6·5.

In other experiments, organisms were harvested after growing in the ordinary way in partly defined medium containing methicillin (100 μg./ml.) or broxil (1 μg./ml.). The organisms were washed and resuspended in 0·1 M-phosphate buffer (pH 6·5) to about equiv. 1 mg. dry wt/ml. The culture filtrates were Seitz-filtered and adjusted to pH 6·5. Organisms and culture filtrates were seperately incubated overnight at 37° with methicillin (100 μg./ml.), which was assayed at the beginning and end of the incubation. The washed organisms did not destroy the drug. However, the filtrate from broxil-

grown organisms completely destroyed the methicillin, and only about 20% of the drug remained after incubation with the filtrate from methicillin-grown organisms. In another experiment, organisms grown with methicillin were tested manometrically for penicillinase activity, but none was found. Culture filtrates could not be assayed for penicillinase in this way because of the relatively high buffer capacity of the medium.

Three cultures of *Pediococcus cerevisiae* 8081 CRD were grown in partly defined medium (350 ml./500 ml.flask) containing methicillin (100 μg./ml.). The cultures were removed from each flask at a different stage of growth, centrifuged, and the supernatant fluids were adjusted to pH 6·5 and then Seitz-filtered. The three filtrates were incubated overnight with methicillin (100 μg./ml.) which was assayed at the beginning and

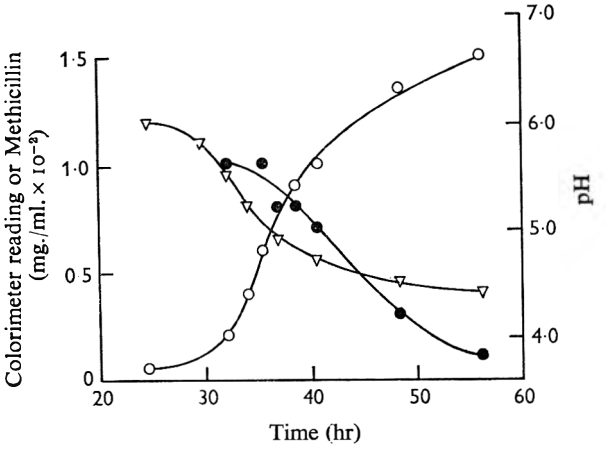

Fig. 4

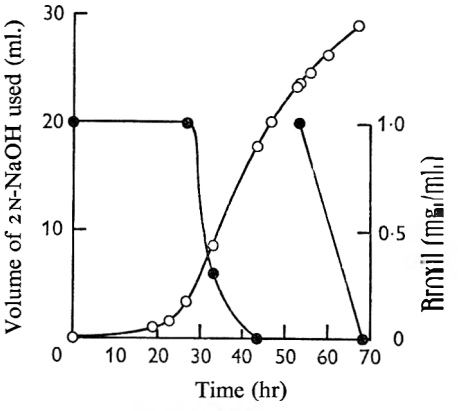

Fig. 5

Fig. 4. Changes in the pH value and the concentration of methicillin in the medium during growth of *Pediococcus cerevisiae* 8081 CRD. Organisms were grown in partly defined medium containing methicillin (100 μg./ml.) and samples were removed at intervals. ○, Colorimeter readings; ●, methicillin concentration; ▽, pH value.

Fig. 5. Changes in the concentration of broxil in the medium during growth of *Pediococcus cerevisiae* 8081 CRD at a constant pH value. Organisms were grown in partly defined medium containing broxil (1 μg./ml.) and samples were removed at intervals and assayed for broxil. The medium was maintained at pH 6·5 by the regulated addition of alkali (see Methods section). After all the broxil had disappeared (52 hr) more broxil was added to give again 1 μg./ml. ○, Volume of 2N-sodium hydroxide used; ●, concentration of broxil.

end of this period, with the following results. The 22 hr filtrate (initially pH 5·4) did not inactivate methicillin; the 47 hr and 67 hr filtrates (initially pH 4·3 and 4·0, respectively) both destroyed about 75% of the methicillin. The 22 hr filtrate itself contained methicillin (about 25 μg./ml.); the other two filtrates did not.

Some properties of the methicillin-destroying factor from Pediococcus cerevisiae 8081 and 8081CRD. At first, production in the culture of material which destroyed the antibiotic potency of methicillin was assumed to be a special property of the methicillin-dependent strain. However, culture filtrates of the parent strain, grown in partly defined medium without methicillin, also inactivated methicillin at a similar rate. Some properties of the methicillin-destroying factor (or factors) were examined, and

no differences were found between the factor from filtrates of the parent or dependent strain.

The factor does not seem to be an enzyme. Its ability to destroy methicillin was not diminished by boiling culture filtrates for 15 min. at pH 3·5 or 6·5, but was considerably decreased by boiling at pH 10·2 for 15 min. On overnight dialysis against water, most of the methicillin-destroying material was diffusible. The factor was stable for at least several months in culture filtrates kept at 2°. Filtrates could be evaporated (under reduced pressure) to one tenth of their original volume, or freeze-dried, without loss of the methicillin-destroying factor.

The inactivation of methicillin became progressively less rapid between pH 6·0 and pH 7·5 (about 50% diminution of the rate of disappearance). At pH 6·5, benzylpenicillin was inactivated at about the same rate as methicillin, i.e. the half life of the compound (initially 100 μg./ml.) in the culture filtrate was about 5 hr. Culture filtrates were also able to inactivate 6-aminopenicillanic acid at a similar rate.

Growth of Pediococcus cerevisiae 8081 CRD in the absence of penicillin derivatives

Addition of Difco yeast extract (1 %,w/v), Oxoid tomato juice (10 %, v/v) or Oxoid liver infusion (0·16 %) to partly defined medium did not enable *Pediococcus cerevisiae* 8081 CRD to grow (within 50 hr) in the absence of methicillin. In tryptose glucose broth, growth occurred only when the medium was supplemented with methicillin (100 μg./ml. was optimal). In the absence of methicillin, addition of Oxoid peptone (1·5 %, w/v) to partly defined medium gave light growth of *P. cerevisiae* 8081 CRD (within 50 hr). Better growth was obtained in peptone water + glucose, and addition of Difco yeast extract (1 %, w/v) gave a further improvement. The organisms which grew in peptone yeast-extract glucose (PYG) medium did not grow when transferred to the partly defined medium, unless this was supplemented with methicillin.

When added together to partly defined medium (without methicillin) peptone and yeast extract were much less effective in promoting growth: it seemed therefore that partly defined medium contained material which inhibited the growth that could occur in PYG medium. The various components of the partly defined medium (at the concentrations used in that medium) were therefore seperately added to PYG medium which was then tested for ability to support growth of *Pediococcus cerevisiae* 8081 CRD in the absence of methicillin. In this way it was found that sodium acetate (2 %, w/v) was the material from the partly defined medium which inhibited growth in PYG medium. The inhibition by sodium acetate was overcome by the addition of methicillin (100 μg./ml.) to PYG medium.

Effects of sodium acetate on growth of Pediococcus cerevisiae 8081 CRD

The growth of liquid cultures of *Pediococcus cerevisiae* 8081 CRD in fully defined medium, with and without sodium acetate, was investigated (Table 4). Medium containing acetate did not support growth unless methicillin was added (about 20 μg./ml. was optimal), but when acetate was omitted there was no growth even when methicillin was present. However, when yeast extract was added to medium without acetate, rapid growth occurred in the absence of methicillin. When acetate was present as well as yeast extract, growth in the absence of methicillin occurred only after a relatively long lag, but this inhibition by acetate could be overcome by adding methicillin.

Several compounds were tested for ability to replace acetate as a growth factor in the defined medium, with and without methicillin (Table 5). Of these compounds, only propionic, butyric and lipoic acids were effective; none gave such rapid or heavy growth as did acetate. Lipoic acid is known to be an 'acetate replacing factor' in lactic acid bacteria (Reed, De Busk, Gunsalus & Hornberger, 1951). However, lipoic acid had only a limited ability to replace acetate for growth of *Pediococcus cerevisiae* 8081 or 8081 CRD. With the parent strain, which also required acetate, lipoic acid (10 μg./ml.) supported moderate growth in absence of acetate, but higher (100 μg./ml.) or lower (1 μg./ml.) concentrations gave very slight growth; lipoic acid at 100 μg./ml. inhibited growth in the presence of acetate (20 mg./ml.). With the methicillin-dependent strain, lipoic acid (5 μg./ml.) gave moderate growth in defined medium without acetate, with or without methicillin, but higher or lower

Table 4. *Growth of Pediococcus cerevisiae 8081 CRD in defined medium with various additions*

Defined medium (5 ml.) in test-tubes was inoculated with *P. cerevisiae* 8081 CRD and incubated at 37°.

	Time of incubation (hr)		
	20	40	60
	Relative growth (turbidity)		
Medium* + Na acetate† (A)	−	−	−
A + methicillin‡	±	+	+
A + yeast extract§	−	−	+ + +
A + methicillin + yeast extract	+	+ + +	+ + +
Medium without Na acetate (B)	−	−	−
B + methicillin	−	−	−
B + yeast extract	+	+ +	+ +
B + methicillin + yeast extract	−	+	+

* Medium: defined, without Na acetate.

† Na acetate $3H_2O$: 20 g./l.; added (before autoclaving) as a solution (20%, w/v) adjusted to pH 6·5.

‡ Methicillin: 100 μg./ml.; added as a sterile (filtered) solution after autoclaving.

§ Yeast extract (Difco): 2% (w/v); added before autoclaving.

concentrations of lipoic acid did not support growth. No improvement in growth of the methicillin dependent strain without acetate resulted when sodium pyruvate (3 mg./ml.) was added with lipoic acid. Mevalonic acid did not replace acetate, at the concentration tested, nor did several 'two carbon' compounds and other substances.

The amounts of sodium acetate or of yeast extract needed to support growth of the methicillin-dependent strain were compared (Table 6). Concentrations of sodium acetate above 2% (w/v) retarded growth even in the presence of methicillin, and concentrations lower than about 0·1% were insufficient to support growth. A concentration of about 0·5% was optimal, giving the most rapid and heavy growth in the presence of methicillin. With this concentration of sodium acetate, a limited amount of growth without methicillin occurred. At least 1–2% (w/v) sodium acetate was needed to cause a delay of growth in the presence of yeast extract (when methicillin was omitted).

Although the organisms which grew in the presence of yeast extract when methicillin and acetate were omitted were not identical with the parent strain (because their growth was inhibited by acetate), it did seem possible that these organisms might be variants distinct from the organisms which required methicillin in medium containing acetate and no yeast extract. To examine this possibility, growth on solid media was investigated (Table 7). A culture of *Pediococcus cerevisiae* 8081 CRD was diluted and

Table 5. *Ability of various compounds to replace sodium acetate for the growth of Pediococcus cerevisiae 8081 CRD*

The compound being tested (in solution adjusted to pH 6·0–6·5 and filtered to sterilize) was added, after autoclaving, to a series of tubes of defined medium (5 ml./tube) without sodium acetate with methicillin 30 μg./ml. Each compound was tested at two or more concentrations. Tubes were inoculated with *P. cerevisiae* 8081 CRD and incubated at 37°.

Compound	Concentration (mg./ml.)	Growth after 40 hr
Formic acid	1–6	−
Propionic acid	2–10	+ (M)
Butyric acid	2·5	± (M)
Valeric acid	3–15	−
Oleic acid	0·01–0·1	−
Lipoic acid	0·01	+
Coenzyme A	0·0001–0·1	−
Acetyl coenzyme A	0·0001–0·1	±
Mevalonic acid (K^+ salt)	0·7	−
Yeast extract (Difco)	10–20	+ + +
Acetaldehyde	1–6	−
Ethanol	1–6	−
Glycollic acid	2–11	−
Glycolaldehyde	0·5–3	−
Ethyleneglycol	2-9	−
Glyoxylic acid	1–3	−
Glyoxal	2–9	−
Oxalic acid	1–4	−
Glycine	2–11	−
Ethanolamine	2–9	−
DL-serine	1–4	−
Na pyruvate	3–16	−
DL-lactic acid	3–14	−
Trisodium citrate.$2H_2O$	43	−
Succinic acid	17	−
Potassium hydrogen phthalate	30	−
N-acetylglucosamine	0·5–2·5	−
Na acetate.$3H_2O$	10–20	+ + (M)
NaCl	1–20	−

M = Growth only in presence of methicillin (20 μg./ml. optimal).

plated in media of various compositions. For comparison, organisms of the parent strain were similarly plated. Because of the variability of the number of organisms occurring in one cluster in liquid cultures, differences in counts of less than tenfold in different media were not considered to be significant. Virtually all the clusters of organisms in the culture of *P. cerevisiae* 8081 CRD gave colonies on medium without acetate but supplemented with yeast extract, with or without methicillin. In medium containing acetate, most of the organisms grew only when methicillin was present, with or without yeast extract.

Table 6. *Quantitative requirements for sodium acetate and yeast extract for growth of Pediococcus cerevisiae 8081 CRD*

Organisms were incubated in a series of tubes containing various concentrations of sodium acetate or yeast extract, with (+M) and without (−M) methicillin. Methicillin 30 μg./ml. in defined medium, 50 μg./ml. in partly defined medium. Sodium acetate $3H_2O$ (adjusted to pH 6·5) Difco yeast extract, added before autoclaving.

Medium (without sodium acetate)	Concn. (% w/v) of:		Growth (after 40 hr)	
	Sodium acetate	Yeast extract	−M	+M
Defined	2·0	.	−	++
	0·4	.	±	++
	0·08	.	−	−
	.	.	−	−
Defined	.	4·0	+++	.
	.	2·0	±++	+
	.	1·0	−++	.
	.	0·5	+	.
	.	0·2	±	.
Partly defined	5·0	.	−	+
	2·0	.	−	++
	1·0	.	−	++
	0·4	.	±	++
	0·1	.	−	+
	.	.	−	±
Partly defined	2·0	4·0	±	+++
	2·0	2·0	±	+++
	2·0	1·0	−	+++
	5·0	2·0	−	.
	1·0	2·0	++	.

Table 7. *Numbers of colonies formed by Pediococcus cerevisiae 8081 and 8081 CRD in the presence of various additions to the medium*

The colonies formed after incubation of plates for 110 hr at 37° were counted as described in methods. The inoculum was a 20 hr culture in partly defined medium (containing methicillin in the case of 8081 CRD), diluted to give about 200 colonies/plate. Medium: partly defined, without Na acetate. Additions as in Table 4.

	Colonies/ml. of undiluted 20 hr culture	
Medium in plates	*P. cerevisiae* 8081	*P. cerevisiae* 8081 CRD †
Medium + Na acetate (A)	$3·4 \times 10^8$	$2·3 \times 10^3$
A + methicillin	0	$4·4 \times 10^8$
A + yeast extract	$3·8 \times 10^8$	$2·0 \times 10^4$
A + methicillin + yeast extract	0	$5·3 \times 10^8$
Medium without Na acetate (B)	*10^9	$4·2 \times 10^2$
B − methicillin	$1·7 \times 10^2$	0
B + yeast extract	$3·1 \times 10^8$	$1·8 \times 10^8$
B + methicillin + yeast extract	0	$5·1 \times 10^8$

* The colonies were very small.

† The culture of *P. cerevisiae* 8081 CRD gave a count of $3·6 \times 10^8$ particles/ml. in the counting chamber, under the phase-contrast microscope.

Colony counts were also made on cultures of *Pediococcus cerevisiae* 8081 CRD growing in liquid media of various compositions (Fig. 6*a–c*). Samples were removed from the cultures and suitable dilutions were plated on solid medium with and without methicillin. For 50 ml. of liquid culture, an inoculum of about 2×10^7 organisms was used, so that some methicillin-independent organisms (about 200/50 ml.) would be present initially. In medium containing sodium acetate (Fig. 6*a*) without methicillin, the number of viable methicillin-dependent organisms decreased fairly rapidly, whereas the number of methicillin-independent organisms increased by a factor of 10^7, although their growth ceased when only slight turbidity was visible in the liquid culture. When methicillin was present, the growth of methicillin-independent organisms was very much diminished, but the methicillin-dependent organisms grew well.

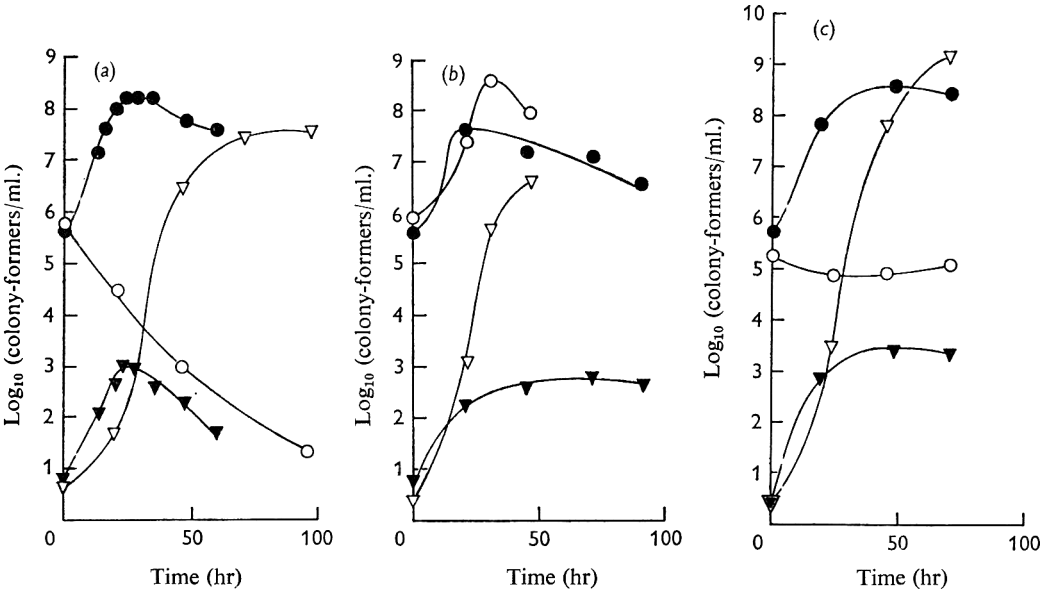

Fig. 6. Colony counts during growth of *Pediococcus cerevisiae* 8081 CRD in liquid media of various compositions. (*a*) Partly defined medium (containing sodium acetate). (*b*) Medium without sodium acetate, containing yeast extract. (c) Medium containing sodium acetate and yeast extract. Medium (50 ml.) in 100 ml. flasks was inoculated with 0·1 ml. of an overnight culture of *P. cerevisiae* 8081 CRD (from medium containing methicillin) and incubated at 37°. At intervals samples were removed for plating in Difco CF assay medium agar (+ leucovorin) with and without methicillin (100 μg./ml.). ●, Methicillin (100 μg./ml.) in liquid medium; viable count in solid medium plus methicillin. ▼, Methicillin in liquid medium; viable count in solid medium without methicillin. ○, No methicillin in liquid medium; viable count in solid medium plus methicillin. ▽, No methicillin in liquid medium; viable count in solid medium without methicillin.

Methicillin-dependent organisms grew in the absence of methicillin in medium without sodium acetate but containing yeast extract (Fig. 6*b*). In this medium, methicillin decreased rather than augmented growth of the methicillin-dependent organisms. Methicillin-independent organisms also grew in this medium, but only to a limited extent when methicillin was present.

In medium containing sodium acetate and yeast extract (Fig. 6*c*) without methicillin, the number of viable methicillin-dependent organisms remained virtually

unaltered during the incubation, while the number of methicillin-independent organisms increased by a factor of 10^9, and the liquid culture (after incubation for about 45 hr) became very turbid. The presence of methicillin in the liquid medium again led to growth of methicillin-dependent organisms, and diminished growth of methicillin-independent organisms.

DISCUSSION

In the present work only one attempt to develop resistant organisms was made with benzylpenicillin and one with methicillin. It is therefore not possible to say whether or not the production of a methicillin-requiring strain of *Pediococcus cerevisiae* was a very rare occurrence, although the fact that dependence became progressively more pronounced in several successive subcultures does suggest that the dependence was not the result of a single infrequent genetic event. Resistance to methicillin also increased by a number of small steps, which is in keeping with the common finding that resistance to penicillins (when not due to penicillinase production) develops only gradually. The failure to develop resistance to benzylpenicillin was not surprising because penicillin resistance does not generally occur with the streptococci (to which *P. cerevisiae* is fairly closely related). Since moderate resistance to methicillin was developed fairly readily and since the organisms were not appreciably cross-resistant to other penicillins, it does seem that methicillin has some special effect of its own, as other studies have suggested already (Rogers & Jeljaszewicz, 1961; Izaki, Matsuhashi & Strominger, 1966).

The methicillin-dependent strain was quite stable, in the sense that the strain was maintained through many subcultures in liquid medium containing methicillin (at a concentration inhibitory to the parent strain). Such a medium would suppress growth of non-resistant revertant organisms (whether or not methicillin-dependent) but would allow growth of resistant strains that had reverted to independence of methicillin. Presumably, if any such revertants did arise, they had no selective advantage over the methicillin-dependent organisms, since these were not overgrown. In spite of this ease of maintenance, about one cluster of organisms in every 10^5 clusters was able to form a colony in the absence of methicillin, and these organisms seemed to be only partly reverted to the parent type, since they grew more slowly and much less heavily than did the parent organisms in a liquid culture.

The sample of methicillin used to develop the methicillin-dependent substrain was from an early batch, and was obtained before the drug was widely available. It seemed possible at the time that growth-promoting effect of the material used might have been due to impurities, but this explanation now seems most unlikely. Every batch of methicillin used has been active, and several other derivatives of 6-aminopenicillanic acid also showed growth-promoting activity. This activity was lost after hydrolysis with penicillinase which would not be expected to degrade an impurity (unless of very similar structure to a penicillin).

If it be accepted that the growth-promoting effect is due to the methicillin molecule itself, its role remains a problem. Methicillin (at 50–100 μg./ml.) gave the most rapid and heavy growth of the dependent organisms; no other penicillin was as effective. Nonetheless, all the derivatives of 6-aminopenicillanic acid tested had some activity as growth factors, often at low concentrations. In every case except methicillin, the concentration at which a penicillin was active as a growth factor was about the same

as the concentration at which the same penicillin was inhibitory to the parent strain (C_i). This relationship rather strongly suggests that there is a close similarity between the way in which various penicillins cause inhibition of the parent strain or support growth of the penicillin-dependent substrain.

The usual inhibitory effect of a penicillin may be the result of acylation (by the β-lactam region of the antibiotic) of a sensitive enzyme (Strominger, Izaki, Matsuhashi & Tipper, 1967): the values of C_i might then be a relative measure of the reactivity of different penicillins in this process. The penicillin-dependent substrain might be supposed to overproduce the sensitive enzyme (with consequent unbalanced growth) and the presence of a penicillin might decrease the activity of the enzyme to a normal value. Alternatively, the dependent strain may produce a toxic metabolite which penicillin may remove by acylation. Either of these possibilities would be consistent with the failure of penicillins to act as growth factors after hydrolysis of the β-lactam ring.

The different values of C_i might instead reflect the ease with which different penicillins were able to penetrate *Pediococcus cerevisiae* 8081 and build up an intracellular inhibitory concentration, which might be about the same for the different penicillins. If this were so, then different penicillins might be broken down intracellularly in *P. cerevisiae* 8081 CRD to yield rather similar amounts of some essential metabolite. That methicillin is broken down intracellularly in *P. cerevisiae* 8081 CRD to provide nutrients seems unlikely; none of the possible fragments that have been tested (except 6-aminopenicillanic acid) supported growth. This might have been because the fragments did not enter the organisms or were not attacked by enzymes, although these possibilities do not seem very likely.

In cases where two penicillins have been shown to cause an inhibitory effect at different concentrations (e.g. Rogers & Mandelstam, 1962) it has been supposed that the two antibiotics differ in their reactivity with a sensitive site rather than in the extent to which they can enter the organisms. The sensitive site, because it is thought to be concerned with formation of the cell wall, would be expected to be near the surface of an organism, and so be accessible to penicillins in the medium.

At an intermediate stage of growth of the parent and the methicillin-dependent strains, material which inactivates methicillin was produced in the medium. This material is not an enzyme; preliminary studies indicated that it had a fairly low molecular weight. Formation of this material by the parent strain is not incompatible with the sensitivity to methicillin of organisms growing from a small inoculum, because the small population would be unable to inactivate the antibiotic before it had produced its toxic effect. Formation of the methicillin-destroying factor might even be disadvantageous to methicillin-dependent organisms by limiting their growth, through loss of methicillin from the medium.

The effects of sodium acetate, yeast extract and methicillin on growth of *Pediococcus cerevisiae* 8081 CRD in partly defined medium, lead to the following conclusions. In yeast extract there is a factor (or factors) able to replace sodium acetate for growth of *P. cerevisiae* 8081 CRD, and when this factor is used in place of acetate, methicillin is no longer needed for growth. Sodium acetate inhibits the response to the yeast-extract factor, but methicillin is able to overcome this inhibition. Preliminary attempts to identify the yeast extract factor were unsuccessful. Several compounds were tested for ability to replace acetate for growth of *P. cerevisiae* 8081 CRD in the presence or

absence of methicillin, but none was found which resembled the factor in yeast extract. Lipoic acid, over a narrow range of concentrations, had a limited ability to replace acetate, but growth with lipoic acid was not heavy or rapid under any conditions.

The reason for the inhibitory effect of acetate on growth of *Pediococcus cerevisiae* 8081 CRD (with yeast extract) in the absence of methicillin is not known. The effect may be indirect and could be the result of an interaction, during autoclaving, between acetate and other compounds in the medium. Acetate may be responsible also for the requirement for methicillin in the absence of yeast extract. Low concentrations of acetate allowed slight to moderate growth without methicillin, but when the usual concentration of acetate was present there was no growth unless methicillin was also added; then good growth could take place. In the present work a penicillin was needed to overcome an inhibitory effect of acetate, while in staphylococci penicillin overcame an inhibition by oleate (Jackson, 1953), and in an L-form of *Proteus mirabilis* high concentrations of penicillin prevented the action of an inhibitor which was tentatively identified as formate (Altenburn, 1961).

The primary inhibitory action of penicillins is believed to be an interference with formation of the bacterial cell wall. It therefore seemed possible that a comparison of the cell walls of parent and methicillin-dependent strains of *Pediococcus cerevisiae* might give some clue to the reasons for methicillin-dependence. Some results of such a study are described in the following paper (White, 1967).

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REFERENCES

ABRAHAM, E. P. (1955). Penicillinase. In *Meth. Enzymol.* **2**, 120.

ALTENBURN, R. A. (1961). Critical factors influencing growth of L-forms of *Proteus mirabilis*. *J. Bact.* **81**, 586.

ANDERSON, W. F., GORINI, L. & BRECKENRIDGE, L. (1965). Role of ribosomes in streptomycin-activated suppression. *Proc. natn. Acad. Sci. U.S.A.* **54**, 1076.

BARBER, M. (1953*a*). Penicillin-resistant and penicillin-dependent staphylococcal variants. *J. gen. Microbiol.* **8**, 111.

BARBER, M. (1953*b*). Antibiotic-resistant staphyloccal variants. *Symp. Soc. gen. Microbiol.* **3**, 235.

GORINI, L. & KATAJA, E. (1964). Phenotypic repair by streptomycin of defective genotypes in *E. coli*. *Proc. natn. Acad. Sci. U.S.A.* **51**, 487.

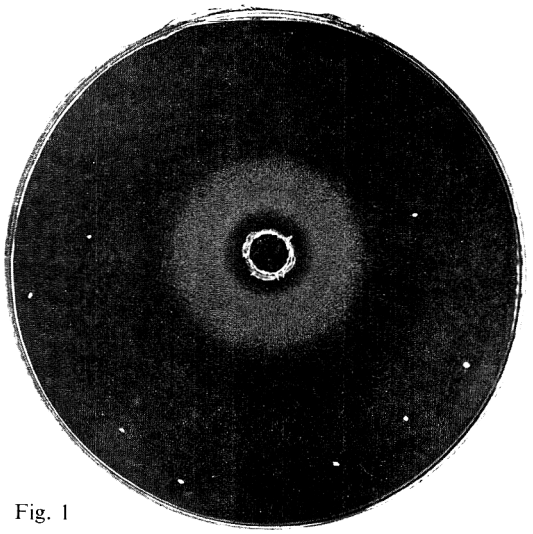

Fig. 1

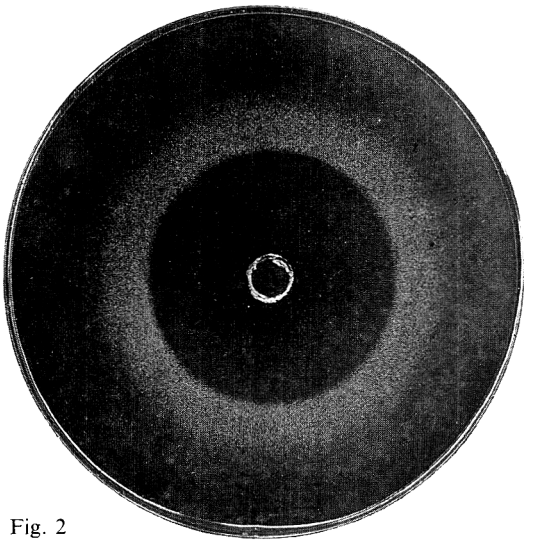

Fig. 2

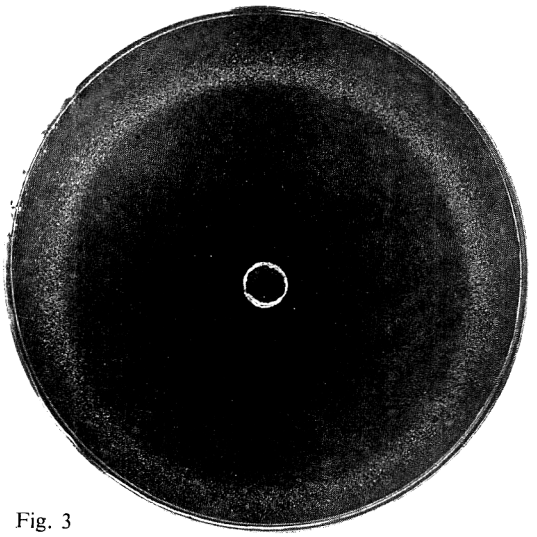

Fig. 3

(*Facing p.* 105)

IZAKI, K., MATSUHASHI, M. & STROMINGER, J. L. (1966). Glycopeptide transpeptidase and D-alanine carboxypeptidase: penicillin-sensitive enzymatic reactions. *Proc. natn. Acad. Sci. U.S.A.* **55**, 656.

JACKSON, F. L. (1953). Discussion. *Symp. Soc. gen. Microbiol.* **3**, 249.

MARSHALL, J. H. & KELSEY, J. C. (1960). A standard culture medium for general bacteriology. *J. Hyg. Camb.* **58**, 367.

REED, J. L., DE BUSK, B. G., GUNSALUS, I. C. & HORNBERGER, C. S. (Jr.) (1951). Crystalline α-lipoic acid: a catalytic agent associated with pyruvate dehydrogenase. *Science, N.Y.* **114**, 93

ROGERS, H. J. & JELJASZEWICZ, J. (1961). Inhibition of biosynthesis of cell-wall mucopeptides by penicillins. Penicillin-sensitive and penicillin-resistant strains of *Staphylococcus aureus*. *Biochem. J.* **81**, 576.

ROGERS, H. J. & MANDELSTAM, J. (1962). Inhibition of cell-wall-mucopeptide formation in *Escherichia coli by* benzyl penicillin and 6-[D(−)-α-aminophenylacetamido]penicillanic acid (ampicillin). *Biochem. J.* **84**, 299.

ROLINSON, G. N., STEVENS, S., BATCHELOR, F. R., CAMERON-WOOD, J. & CHAIN, E. B. (1960) Bacteriological studies on a new penicillin-BRL. 1241. *Lancet*, ii, 564.

SAUBERLICH, H. E. & BAUMANN, C. A. (1948). A factor required for the growth of *Leuconostoc citrovorum. J. biol. Chem.* **176**, 165.

SCHNITZER, R. J. & GRUNBERG, E. (1957). *Drug Resistance of Microorganisms.* New York: Academic Press Inc.

STROMINGER, J. L., IZAKI, K., MATSUHASHI, M. & TIPPER, D. J. (1967). Peptidoglycan transpeptidase and D-alanine carboxypeptidase: penicillin-sensitive enzymatic reactions. *Fedn. Proc.* **26**, 9.

WHITE, P. J. (1962). A penicillin-dependent substrain of *Pediococcus cerevisiae Abstr. VIII Congr. int. Microbiol. Montreal, p.* 75.

WHITE, P. J. (1967). A comparison of the cell walls of *Pediococcus cerevisiae* and of a substrain that requires methicillin for growth. *J. gen. Microbiol.* **50**, 107.

WHITE, P. J. & NICHOL, C. A. (1963). Effects of uracil and thymidine on the development of resistance to 5-fluorouracil in *Pediococcus cerevisiae. J. Bact.* **85**, 97.

WHITE, P. J. & WOODS, D. D. (1965). The synthesis of *p*-aminobenzoic acid and folic acid by staphylococci sensitive and resistant to sulphonamides. *J. gen. Microbiol.* **40**, 243.

WORK, E. (1962). Discussion in *Resistance of Bacteria to the Penicillins.* Ed. by A. V. S. de Reuck and M. P. Cameron, CIBA Foundation Study Group no. 13. p. 50. London: J. and A. Churchill Ltd.

EXPLANATION OF PLATE

Stimulation by methicillin or benzylpenicillin of growth of *Pediococcus cerevisiae* 8081 CRD in solid medium. Bottom layers (10 ml.) were poured in Petri dishes from partly defined medium (at 43°) seeded with 0·1 ml. of a 24 hr culture of *P. cerevisiae* 8081 CRD. A hole (8 mm. diam.) was cut out and was filled with 0·1 ml. of a solution of methicillin or benzylpenicillin in medium containing agar. Cover layers (30 ml. uninoculated medium) then were poured, and the plates were incubated at 37°. At the end of the incubation the holes were again cut out, so that their positions would show more clearly on photographs.

Fig. 1. Methicillin (500 μg.) in centre well. Plate incubated for six days. The zone of confluent growth appeared after two days. Eight colonies of methicillin-independent organisms can be seen These first appeared on the fourth day of incubation.

Fig. 2. Benzylpenicillin (25 μg.) in centre well. Plate incubated for 3 days.

Fig. 3. Benzylpenicillin (125 μg.) in centre well. Plate incubated for 3 days.

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A Comparison of the Cell Walls of *Pediococcus cerevisiae* and of a Substrain that Requires Methicillin for Growth

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SUMMARY

Cell walls were prepared from: (1) *Pediococcus cerevisiae* 8081 grown (in partly defined medium) without methicillin; (2) *P. cerevisiae* 8081 grown with a sub-inhibitory concentration of methicillin (10 μg./ml.); (3) *P. cerevisiae* 8081 CRD (methicillin-dependent substrain) grown with an optimal concentration of methicillin (100 μg./ml.). All three preparations contained glucosamine, muramic acid, *N*-acetyl groups (no *O*-acetyl groups), alanine, glutamic acid, lysine and aspartic acid, in proportions which suggested that they were components of a mucopeptide polymer; small amounts of serine, glycine and threonine were also present. The mucopeptide components made up about 50% by weight of walls (1) and (2) but about 80% in walls (3). Glucose and phosphorus were present in the walls, in greater amounts in walls (1) and (2) than in walls (3). No free amino groups were detected in any of the walls, and most of the lysine was released as ϵ-(aminosuccinyl)-lysine when mucopeptide from the parent strain was hydrolysed for a short period. About 35% of walls (1) and (2) was removed by extraction at 2° with trichloroacetic acid; only 16% was removed from walls (3). Nearly all the glucose and most of the phosphorus was removed from all the walls by such extraction; the residues after extraction all contained about 90% of mucopeptide components. Teichoic acids were isolated from each trichloroacetic acid extract: phosphorus, glucose, alanine and glycerol were found in all. Teichoic acid from walls (3) contained, in addition, about 7% of mucopeptide components. Walls and trichoroacetic acid insoluble residues from the methicillin-dependent substrain were hydrolysed much more rapidly by lysozyme than were walls or residues of the parent strain, whether grown in absence or presence of methicillin.

INTRODUCTION

A substrain of *Pediococcus cerevisiae* has been obtained which requires methicillin (or some other penicillins) for growth, and some properties of the substrain have now been examined (White, 1967). It seemed possible that changes in the chemical composition of the cell wall might accompany the development of this growth requirement for a penicillin, since interference with the biosynthesis of the cell wall is thought to be a primary effect of penicillins on susceptible bacteria (see review by Reynolds, 1966). The composition of cell walls of *P. cerevisiae* has already been investigated by Ikawa & Snell (1960). In the present study, walls of this organism, grown in the absence and in the presence of methicillin, were re-examined, and were compared with walls of the methicillin-dependent substrain. A preliminary report of this work has already been given (White, 1966).

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METHODS

Organisms. Pediococcus cerevisiae ATCC 8081 and the methicillin-dependent substrain (*P. cerevisiae* 8081 CRD) were maintained as described previously (White, 1967).

Medium and growth of organisms. The partly defined medium (containing leucovorin) for *Pediococcus cerevisiae* was that used by White & Nichol (1963). The parent strain was grown in the absence of methicillin or in the presence of a sub-inhibitory concentration of it (10 μg./ml.). *Pediococcus cerevisiae* 8081 CRD was grown in the presence of methicillin (100 μg./ml.). In each case, ten 2 l. flasks, each containing 1 l. medium, were autoclaved at 120° for 15 min., cooled, and sterile methicillin was added when required. Each flask was inoculated with 1 ml. of an overnight culture, then was left to stand at 37°. Organisms were harvested by centrifugation after 2 days (parent strain) or 4 days (methicillin-dependent substrain), by which time fairly heavy growth had been reached. The organisms were washed once in 0·9% sodium chloride and once in water, then were resuspended in water. The dry weight of this suspension was found (by heating a sample to constant weight at 105°); water was added to the suspension as required to give about equiv. 20 mg. dry wt organisms/ml.

Isolation of cell walls. The organisms were broken in a shaker head (Shockman, Kolb & Toennies, 1957) fitted on a PR 2 centrifuge (International Equipment Co., Boston, Mass., U.S.A.) as described by Allsop & Work (1963). Immediately after breaking, the containers were heated for 10 min. at 60° (to inactivate lytic enzymes) before the glass beads were removed by filtration through a coarse sintered glass filter. The filtrate was centrifuged for 5 min. at 400 *g*. The supernatant liquid (containing cell walls) was centrifuged at 20,000 *g* for 20 min., and the pad was washed twice with water. The pad was resuspended in 0·05 M-tris HCl buffer (pH 7·8; 200 ml.) containing crude trypsin (1 g.) and one drop of chloroform. The suspension was shaken gently overnight at 37°, and then was centrifuged at 400 *g* for 5 min. The supernatant liquid (containing cell walls) was centrifuged at 20,000 *g* for 20 min. The walls were washed twice with 1·0 M-sodium chloride, twice with 0·05 M-tris HCl buffer (pH 7·8) and six times with water, with centrifugation at 20,000 *g* for 20 min. between each wash. The walls were dried on a grid, and examined without further preparation in the electron microscope (by Dr Hilary Rose). Very many empty cell-evelopes were seen, while no whole organisms were found. The walls were freeze-dried and finally dried over phosphorus pentoxide in vacuum and weighed.

Isolation of teichoic acids. This was based on the procedure of Shaw & Baddiley (1964). About 250 mg. dry walls were suspended in 10% (w/v) trichloroacetic acid (20 ml.) at 2° and stirred continuously for 1 week at 2°. The suspension was centrifuged at 20,000 *g* for 10 min. at 2° and the solid residue extracted as before with a further 20 ml. 10% trichloroacetic acid. The solid residue after this extraction was washed twice in water, then resuspended in water, freeze-dried and weighed. The combined extracts were shaken three times with ether (15 ml.) to remove trichloroacetic acid. Nitrogen was bubbled through the aqueous layer to remove ether, and then the solution was freeze-dried. The solid was redissolved in 10% (w/v) trichloroacetic acid (5 ml.) at 2° and ethanol (15 ml.) was added. The precipitate was left to settle at 2° overnight. The supernatant liquid was decanted, more ethanol (25 ml.) was added to it and the precipitate was left to settle at 2° overnight. The combined precipitates were dissolved (in a weighed tube) in 10% (w/v) trichloroacetic acid (3 ml.) at 2°, ethanol

(15 ml.) was then added and the precipitate allowed to settle at 2° overnight. The precipitate was washed with acetone and then with ether and was dried in vacuum over phosphorus pentoxide.

Amino acid analysis. Cell walls or teichoic acids (about 5 mg.) were heated at 105° in sealed tubes for 18 hr with 6 N-HCl (1 ml.). The acid was removed by drying in vacuum over phosphorus pentoxide and sodium hydroxide, then adding water and drying twice more. The dried residue was taken up in 0·1 N-HCl (10 ml.) and the amino acids in a sample determined by use of a Technicon AutoAnalyser (Technicon Instruments Co. Ltd, Hanworth Lane, Chertsey, Surrey).

Amino sugars. Cell walls or teichoic acids (about 5 mg.) were heated at 100° in sealed tubes for 4 hr with 4 N-HCl (1 ml.). The acid was removed as above, and the residues taken up in water (1 ml.). Total hexosamine (glucosamine and muramic acid) in the hydrolysate was estimated by the borate method of Strominger, Park & Thompson (1959). Glucosamine was estimated by the method of Cessi & Piliego (1960) in which muramic acid does not react. Attempts to estimate muramic acid by the method of Ghuysen & Strominger (1963) were unsuccessful, as were attempts to separate glucosamine and muramic acid on charcoal+Celite columns (Perkins & Rogers, 1959). Consequently the amount of glucosamine was subtracted from the total hexosamine to give a value for muramic acid.

Glucose. Cell walls or teichoic acids (about 5 mg.) were heated at 100° in sealed tubes for 1 hr with 0·5 N-HCl (1 ml.). The acid was removed by drying in vacuum as above, and the residues taken up in water (1 ml.). Glucose in these solutions was estimated with a Glucostat kit (Worthington Biochemical Corporation, Freehold, N.J., U.S.A.).

Phosphorus. The method of Fiske & SubbaRow (1929) was used to estimate total phosphorus. Inorganic phosphorus was estimated by the method of Lipman & Tuttle (1944).

Acetyl groups. The method of Ludowieg & Dorfman (1960) was used to estimate total acetyl groups (*O*- and *N*-). To estimate *N*-acetyl groups, cell walls (about 25 mg.) were stirred overnight in 0·01 N-NaOH (2 ml.) at room temperature to remove *O*-acetyl groups, and then washed three times with water. The washed walls were dried and weighed before acetyl groups (i.e. *N*-acetyl) were estimated as before. No difference between total acetyl groups and *N*-acetyl groups was found, so that no *O*-acetyl groups were present in the walls.

Determination of free amino groups. (H. R. Perkins, personal communication, based on the method of Ingram & Salton, 1957.). Cell walls (5 mg.) were suspended in 0·1 M-ammonium acetate, pH 6·5 (5 ml.). The walls of *Pediococcus cerevisiae* 8081 (grown in the absence or presence of methicillin) readily formed an even suspension when they were rubbed with a glass rod in the buffer. Walls of *P. cerevisiae* 8081 CRD, however, were not dispersed so easily; instead small flakes were formed which settled from suspension very quickly. A more even suspension of these walls was made by ultrasonic treatment for a minute or two in an MSE 60 W ultrasonic disintegrator (Measuring and Scientific Instruments Ltd, 25–28 Buckingham Gate, London, S.W. 1).

The suspension of walls (0·3 ml.), 1 % (w/v) $Na_2B_4O_7.10H_2O$ (borax) (1·2 ml.) and 0·1 M-1-fluoro-2,4-dinitrobenzene (0·15 ml. in ethanol) were rapidly mixed in a test-tube (150 × 15 mm.), and the mixture was heated at 60° for 30 min. After cooling, unreacted fluorodinitrobenzene was removed by extraction three times with ether (2 ml.) and traces of ether removed from the aqueous layer with a stream of nitrogen. The walls were

centrifuged (20,000 *g* for 10 min.) and washed once in water to remove borax, then re-suspended in 4 N-HCl (2·25 ml.) and heated in a sealed tube at 100° for 4 hr. After cooling, the contents of the tube were diluted with water (2·25 ml.). The extinction of this solution was measured at 420 mμ in a 2 cm. cuvette against a zero of water, in order to assess roughly the amount of DNP-amino acids that had been formed. A calibration curve was established with DNP-alanine for which ϵ was found to be $6{\cdot}5 \times 10^3$. The solution was extracted as before with ether to remove any DNP-derivatives of acidic and neutral amino acids. Both this extract and the aqueous residue (which should contain DNP-derivatives of basic amino acids) were evaporated to dryness and taken up in 50% (v/v) ethanol in water (100 μl.). The solutions (20 μl.) were spotted onto a thin-layer chromatography plate (silica gel), which was developed with chloroform + methanol + acetic acid (95 + 5 + 1 by vol.) for about 1 hr at room temperature. The solutions in dilute ethanol (20 μl.) were also examined by electrophoresis on Whatman no. 3 paper in 0·1 M-sodium carbonate at 500 V for 3 hr.

Digestion with lysozyme. Cell walls (5 mg.) were suspended evenly (as above) in 0·1 M-ammonium acetate (pH 6·5; 4·75 ml.) at 21°, in a colorimeter tube (15 mm. diameter). One drop of 0·9% sodium chloride was added. At time zero, 0·25 ml. of a solution of lysozyme in 0·1 M-ammonium acetate (1 mg./ml.) was added at 21°. The turbidity of the suspension was measured at intervals in an EEL colorimeter (Evans Electroselenium Co. Ltd, Halstead, Essex) against a zero of water, using a neutral density filter (no. 1·0).

Chromatography. For preliminary investigation of the amino acids present in hydrolysates of cell walls, two-dimensional chromatograms (on Whatman no. 4 paper) were run, using as solvents: (1) phenol saturated with water, with a few drops of concentrated ammonia solution and a few crystals of potassium cyanide added to the tank; (2) butan-1-ol + acetic acid + water (67 + 10 + 23, by vol.). After development, the chromatograms were treated with ninhydrin (0·1%) in acetone.

To detect hexosamines, chromatograms (on Whatman no. 1 paper) were irrigated with ethyl acetate + pyridine + acetic acid + water (5 + 5 + 1 + 3, by vol.; Fischer & Nebel, 1955). After development, chromatograms were treated with ninhydrin (as above) or with the reagents for hexosamines described by Partridge (1948), which however gave only weak spots.

For detection of carbohydrates, chromatograms (on Whatman no. 1 paper) were developed with butan-1-ol + pyridine + water (6 + 4 + 3, by vol.) or propan-1-ol + aq. ammonia solution (sp.gr. 0·88) + water (6 + 3 + 1, by vol.). Chromatograms were treated with alkaline silver nitrate (Smith, 1960), or, to detect glycols, with periodate-Schiff's reagents (Baddiley, Buchanan, Handschumacher & Prescott, 1956).

Electrophoresis. Phosphate esters were separated by electrophoresis (on Whatman no. 3 paper) for about 3 hr at 500 V and 20 mA, in collidine acetate buffer (pH 7·0; 0·08 M in respect of γ-collidine). The method of Hanes & Isherwood (1949) was used to detect phosphate esters.

Chemicals and enzymes. Bacitracin was a gift from Professor E. P. Abraham, and muramic acid (isolated from cell walls of *Micrococcus lysodeikticus*) was a gift from Dr H. R. Perkins. Trypsin ('from pancreas') and lysozyme ('crystalline, from egg white') were purchased from British Drug Houses Ltd (Poole, Dorset). Alkaline phosphatase ('from *Escherichia coli*, salt-fractionated') was purchased from Worthington Biochemical Corporation (Freehold, N.J., U.S.A.).

RESULTS

Cell walls

Batches of cell walls were isolated from the parent strain grown without methicillin, and from the methicillin-dependent substrain grown in the presence of the drug (100 μg./ml.); one batch of walls was also examined from the parent strain grown in the presence of a sub-inhibitory concentration of methicillin (10 μg./ml.) in order to establish whether such conditions led to changes in the composition of the cell wall. Chromatography showed the presence in each batch of alanine, aspartic acid, glutamic acid, lysine, glucosamine and traces of glycine. Glucosamine and muramic acid were the only hexosamines, and glucose was the only reducing sugar found on chromatography of hydrolysates of the walls. No component was seen in one batch of walls that was not present in the others.

Table 1. *Composition of the cell walls of Pediococcus cerevisiae 8081 and 8081 CRD*

Analyses for the various components of the walls were performed (in duplicate) as described in the Methods section. Values are m-moles/10 g. of wall. Ammonia was present in hydrolysates from all of the walls.

Component	8081 grown without methicillin	8081 grown with methicillin (10 μg./ml.)	8081 CRD grown with methicillin (100 μg./ml.)
Glucosamine	5·7	5·8	9·7
Muramic acid	4·6	4·9	8·5
N-acetyl	11·3	11·0	17·3
O-acetyl	0·0	0·0	0·0
Alanine	9·9	10·0	12·4
Glutamic acid	4·7	5·4	7·9
Lysine	3·9	4·6	6·4
Aspartic acid	5·7	6·3	9·1
Serine	1·1	0·4	0·5
Glycine	0·6	trace	trace
Threonine	0·5	trace	trace
Glucose	4·3	2·7	3·0
Phosphorus	12·4	11·0	4·9
Yield of walls*	26	30	31

* Percentage of dry weight washed organisms obtained as dry cell walls.

Quantitative analyses for amino acids, hexosamines, acetyl groups, glucose and phosphorus were made, the results of which are shown in Table 1. Glucosamine and muramic acid occurred in walls of the parent strain in roughly equimolar amounts, and approximately 2 moles of *N*-acetyl were present for each 2 moles of hexosamine. No *O*-acetyl groups were detected. Glutamic acid, lysine and aspartic acid were found in amounts roughly equimolar with the muramic acid, and there was a larger amount of alanine. These quantitative relationships strongly suggested that these substances were all components of a mucopeptide polymer (Perkins & Rogers, 1959). Serine, glycine and threonine were found in small amounts: there was considerably less of these compounds in walls of organisms grown in the presence of methicillin. The presence of glucose and phosphorus suggested that the walls contained a teichoic acid (Baddiley & Davison, 1961). This possibility was confirmed

when it was found that all the glucose and most of the phosphorus could be removed by trichloroacetic acid, and that teichoic acid could be precipitated from the acid extract.

No marked changes in the composition of the wall were found when the parent strain was grown in the presence of methicillin. However, in walls of the methicillin-dependent substrain 8081 CRD the mucopeptide components made up a larger proportion of the weight than they did in walls of the parent strain (77% and 51% in the two strains respectively, allowing for the loss of weight on condensation between components). The walls of the parent strain on the other hand contained more than twice as much phosphorus as did walls of the substrain and a greater weight of material was extracted by trichloroacetic acid from walls of the parent strain.

Ammonia could not be determined in the amino acid analyser, though it was found in hydrolysates of every batch of walls. If the value for ammonia (3·3%, w/w) obtained by Ikawa & Snell (1960) is taken for walls of the parent strain, and if an amount of glycerol equimolar with the phosphorus extracted by trichloroacetic acid is assumed to be present (see later) and phosphorus is assumed to be present as orthophosphate, then 78% of the dry wt of the walls of the parent strain (grown without methicillin) can be accounted for as known compounds. If similar assumptions regarding phosphorus and glycerol are made for the walls of the methicillin-dependent substrain (but ammonia be neglected) then account can be made for 90% of the dry wt of these walls.

In case the observed differences between parent and methicillin-dependent strains were no more than chance variations between different batches of walls, further samples of walls of the parent strain (grown without methicillin) and of the substrain were isolated. Hydrolysates of these walls were found chromatographically to be closely similar to those from the earlier batches. Analyses for hexosamines, glucose and phosphorus showed that in the new samples there was again a higher proportion of mucopeptide and less teichoic acid in the walls of the substrain than in walls of the parent strain.

Analysis of residues after extraction of cell walls with trichloroacetic acid

After extraction, the residues were made up very largely of the mucopeptide components, and the difference between the residues from the parent strain and the substrain were slight (Table 2). If acetyl groups equimolar with the total hexosamines were assumed to be present in the residues, it was possible to account for 92% of the dry weight of the residue from the parent strain (grown without methicillin) and for 91% of the dry weight of the residue from the substrain, as mucopeptide components. Almost all the glucose was extracted, but an appreciable amount of phosphorus remained. The way in which this phosphorus is combined is not known.

When hydrolysates of the residues were examined in the amino acid analyser a peak was found on the recorder trace near the position where tryptophan or 3-methylhistidine would be expected. It seemed possible that this material might be ϵ-(aminosuccinyl)-lysine, which has been shown to be formed by cyclization of aspartic acid and lysine when bacitracin or cell walls of lactobacilli are heated with hydrochloric acid (Swallow & Abraham, 1958). After hydrolysis of a sample of bacitracin (5 mg.) at 105° for 6 hr with 6 N-HCl (1 ml.), a peak was found in the same position on the recorder trace as the unknown compound; the other peaks were all identifiable as

amino acids that are present in bacitracin: aspartic acid, lysine, glutamic acid, isoleucine, leucine, phenylalanine, ornithine, histidine.

Cell-wall residues hydrolysed in 6 N-HCl for various times at 105° were analysed for aspartic acid and lysine and for the unknown compound (Table 3). As the times

Table 2. *Composition of residues after extraction of cell walls of Pediococcus cerevisiae 8081 and 8081 CRD with trichloroacetic acid*

Analyses for the various components of the residues were performed (in duplicate) as described in the Methods section. Acetyl groups were not estimated. Values are m-moles/10 g. of residue. Ammonia was present in hydrolysates of all the residues.

Component	8081 grown without methicillin	8081 grown with methicillin (10 μg./ml.)	8081 CRD grown with methicillin (100 μg./ml.)
Glucosamine	7·9	8·0	11·4
Muramic acid	9·4	9·2	8·0
Alanine	16·8	18·7	15·0
Glutamic acid	10·6	9·8	9·2
Lysine*	9·6	9·3	10·1
Aspartic acid*	10·2	7·8	10·0
Serine	1·0	0·4	0·5
Glycine	0·8	Trace	Trace
Threonine	0·8	Trace	Trace
Glucose	0·2	0·1	0·2
Phosphorus	2·4	2·2	1·6
Loss of wt. on extraction†	34	38	16

* After hydrolysis in 6 N-HCl for 30 hr at 105°.
† Percentage of dry weight of walls removed by extraction with trichloroacetic acid.

Table 3. *Release of aspartic acid and lysine from ε-(aminosuccinyl)-lysine after prolonged acid hydrolysis of mucopeptide from Pediococcus cerevisiae 8081*

Cell wall residue (after extraction with trichloroacetic acid) was hydrolysed in 6 N-HCl at 105° for various times, and the hydrolysates were examined in the Technicon AutoAnalyser. Values are m-moles/10 g. of residue.

Period of hydrolysis (hr)	Aspartic acid	Lysine	ε-(aminosuccinyl)-lysine*	Glutamic acid	Alanine
6	3·5	4·7	4·5	9·0	15·9
18	8·2	7·2	1·4	10·6	16·8
30	10·2	9·6	Trace	9·4	17·0

* Calculated on the assumption that ε-(aminosuccinyl)-lysine gave the same colour yield with ninhydrin as was given by norleucine, which was used as a standard.

of hydrolysis were increased, so the yield of the two amino acids went up, while the peak due to the unknown compound decreased. The unknown compound therefore appeared to be ε-(aminosuccinyl)-lysine. Its formation on hydrolysis implied that in the wall residues, some molecules of lysine were joined by their ε-amino groups to the α- or β-carboxylic acid groups of aspartic acid. After hydrolysis for 6 hr, only about 40% of the lysine and aspartic acid had been set free, whereas all the alanine and glutamic acid had been liberated. Thus, not less than 60% of the lysine in the residues was joined by its ε-amino group to a carboxylic acid group of aspartic acid.

No ε-(aminosuccinyl)-lysine was found in the hydrolysates of unextracted walls for which analytical data are given in Table 1. However, this compound was detected in the amino acid analyser after unextracted cell walls had been hydrolysed for about 12 hr in 6 N-HCl. On two-dimensional chromatograms treated with ninhydrin ε-(aminosuccinyl)-lysine gave an extended spot close to lysine.

Teichoic acid

Results of analyses are shown in Table 4. Glucose, phosphorus (calculated as orthophosphate) and alanine made up 60% of the dry weight of the teichoic acid from the parent strain, and 74% of that of the methicillin-dependent substrain; glucose was the major component by weight. Teichoic acid from the parent strain grown in the presence of methicillin was only examined chromatographically.

Table 4. *Composition of the teichoic acid from Pediococcus cerevisiae 8081 and 8081 CRD*

Analyses for the various components of the teichoic acids were performed as described in the Methods and Results sections. Values are mmoles/10 g. teichoic acid.

Component	8081 grown without methicillin	8081 CRD grown with methicillin (100 μg./ml.)
Glucose	18·9	28·9
Phosphorus	28·0	27·0
Alanine	3·6	2·0
Glycerol	Present	Present
Ammonia	Present	Present
Glucosamine	0·2	1·0
Muramic acid	0·2	1·2
Glutamic acid	< 0·1	0·6
Lysine	0·1	0·6
Aspartic acid	0·1	0·8
Serine	0·2	0·1
Glycine	0·1	0·1
Threonine	Trace	Trace
Yield of teichoic acid*	55	37

* Percentage (by weight) of material extracted from cell walls by trichloroacetic acid recovered as teichoic acid.

Chromatograms of acid hydrolysates of the teichoic acid from the methicillin-dependent *Pediococcus cerevisiae* strain 8081 CRD, developed in ethyl acetate + pyridine + acetic acid + water and treated with ninhydrin, showed the presence of hexosamines and other mucopeptide components. These compounds (estimated in the AutoAnalyser) made up 7% of the dry weight of this teichoic acid (Table 4), but were present only in traces in the teichoic acid from the parent *P. cerevisiae* 8081 (grown with or without methicillin). The mucopeptide components were not removed from the teichoic acid of *P. cerevisiae* 8081 CRD when a solution was passed through a column of Sephadex G-25. Fractions from the column were assayed for phosphorus, and the fractions from the peak of phosphorus content were combined and evaporated. After hydrolysis, the mucopeptide components were still present in the same proportions relative to the phosphorus. This suggested that the mucopeptide was covalently bound to the teichoic acid.

Glucose was the principal sugar found in the teichoic acids. Faint unidentified spots, situated between glucose (R_F about 0·4) and the origin line, were seen on chromato-

grams developed in butan-1-ol + pyridine + water and treated with alkaline silver nitrate.

With all the teichoic acids an unexpected difficulty was that the presence of glycerol or ribitol (or anhydroribitol) was not shown chromatographically in acid hydrolysates (2–4 hr at 105° in 2 N- or 4 N-HCl). Markers of these compounds on chromatograms were detected with periodate-Schiff reagents, or equally well with alkaline silver nitrate. To release polyols, teichoic acids from *Pediococcus cerevisiae* 8081 (grown without methicillin) and from *P. cerevisiae* 8081 CRD (methicillin-dependent) were first hydrolysed for 4 hr in 4 N-HCl at 105°. After neutralization part of each hydrolysate (corresponding to about 500 μg. teichoic acid) was incubated with alkaline phosphatase (10 μl.) in 0·1 M-tris HCl buffer (pH 8·0; 200 μl.) overnight at 20°. The hydrolysates, before and after treatment with the enzyme, were examined by chromatography and paper electrophoresis. The acid hydrolysates contained two principal phosphate esters, one of which corresponded in its position to α-glycerophosphoric acid (which moved 10·5 cm. towards the anode after electrophoresis for 3 hr at pH 7·0) while the other moved 14·5 cm. in the same direction. Two other phosphate esters (which moved 5·5 cm. and 6·8 cm. towards the anode) were present in traces, and inorganic phosphate (which moved 12·5 cm. towards the anode) was also present. After enzymic treatment of the hydrolysates the phosphate esters had disappeared, and all the phosphorus was in the inorganic form; free glycerol was detected but no ribitol or anhydroribitol. Estimations of glucose in the hydrolysates, before and after treatment with alkaline phosphatase, showed that no liberation of free glucose accompanied the release of inorganic phosphate. It was concluded that a glycerol-teichoic acid was the only type present in the cell walls, and it has been assumed for quantitative purposes that the glycerol in the teichoic acid is equimolar with the phosphorus. When this assumption was made, 78 % (parent strain) and 96 % (methicillin-dependent substrain) of the dry weight of the teichoic acids could be accounted for as identified compounds.

Determination of free amino groups in cell walls

No evidence for the presence of free amino groups was obtained with any of the walls since the hydrolysates of walls treated with fluorodinitrobenzene contained no detectable DNP-amino acids. It was thought possible that the walls which had been examined might have lacked free amino groups because the organisms from which the walls were obtained had reached the stationary phase of growth when there was extensive cross-linking of the mucopeptide side-chains. A batch of walls was therefore prepared from *Pediococcus cerevisiae* 8081 CRD harvested in the logarithmic phase of growth. The yield of these walls was only 9 % of the dry weight of the intact organisms, which suggested that the walls were less thick than those of stationary-phase organisms; however, these walls were also devoid of free amino groups.

Digestion by lysozyme of cell walls

The course of digestion by lysozyme of walls and of residues (after extraction with trichloroacetic acid) is illustrated in Figs. 1 and 2. The walls of the substrain were rapidly dissolved by lysozyme at 21°, whereas those of the parent strain were more resistant, being lysed at only one tenth of the rate. Walls from the parent strain grown in the presence of methicillin were digested by lysozyme about one and a half

times more rapidly than those of parent-strain organisms grown in the absence of the drug. Extraction of teichoic acid from the walls did not increase their susceptibilities to attack by lysozyme; in fact the residue from the parent strain (grown without methicillin) was digested only slowly and incompletely at room temperature, although at 37° overnight digestion almost completely dissolved the residue.

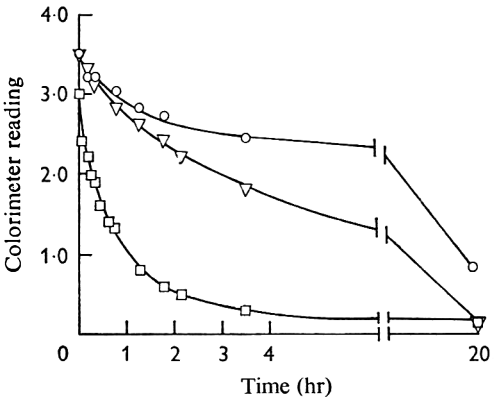

Fig. 1

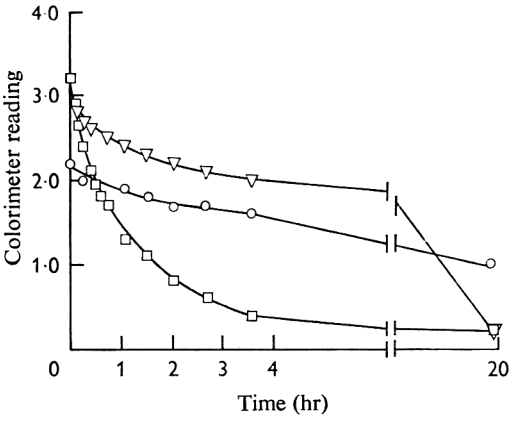

Fig. 2

Fig. 1. Digestion by lysozyme (100 μg./ml. at 21°) of walls of *Pediococcus cerevisiae*. ○, *P. cerevisiae* 8081 grown without methicillin; ▽, *P. cerevisiae* 8081 grown with methicillin (10 μg./ml.); □ *P. cerevisiae* 8081 CRD grown with methicillin (100 μg./ml.).

Fig. 2. Digestion by lysozyme (100 μg./ml. at 21°) of walls of *Pediococcus cerevisiae* after extraction with trichloroacetic acid. ○, *P. cerevisiae* 8081 grown without methicillin; ▽, *P. cerevisiae* 8081 grown with methicillin (10 μg./ml.); □, *P. cerevisiae* 8081 CRD grown with methicillin (100 μg./ml.)

DISCUSSION

In the present study the results of Ikawa & Snell (1960) were confirmed in respect of the qualitative composition of the cell walls of *Pediococcus cerevisiae* 8081. Apart from traces of glycine and threonine no substances were found in these walls other than those reported by Ikawa & Snell. In the present work, the cell wall made up a higher percentage (26%) of the dry weight of the organisms than previously had been found (9%). This might be because in the present work the walls were prepared from organisms at a later stage of growth, when the wall had become thickened, or it may be only the result of a difference of efficiency in the method of isolating the walls. Quantitatively, a few differences were found, though on the whole the compositions were similar: Ikawa & Snell (1960) found more glucose (10% of dry wt wall) and less phosphorus (1·4%) than were found in the present study (glucose 7·7%; phosphorus 3·8%).

Growth of *Pediococcus cerevisiae* 8081 in presence of a sub-inhibitory concentration of methicillin did not lead to any marked change in the composition of its cell wall. The walls of the methicillin-dependent *P. cerevisiae* 8081 CRD contained the same components as the walls of the parent strain but had relatively more mucopeptide and less teichoic acid. The interpretation of this finding is uncertain. Penicillins are generally considered to inhibit the biosynthesis of mucopeptide, and it may be that in the methicillin-dependent substrain a mechanism of mucopeptide biosynthesis

operates which is relatively resistant to methicillin; this mechanism might lead to an increase in the proportion of mucopeptide in the wall. Alternatively (or simultaneously) the formation of teichoic acid might be suppressed by methicillin, as some studies might suggest (Saukkonen, 1961; Roberts & Johnson, 1962; Rogers & Garrett, 1963).

When teichoic acid had been extracted from the walls, the residues from the parent and methicillin-dependent strains were similar in chemical composition, being made up almost entirely of mucopeptide components. However, there must be differences between the mucopeptides from the two strains since the mucopeptide from *Pediococcus cerevisiae* 8081 CRD was much more susceptible to hydrolysis by lysozyme than was that from the parent strain. Teichoic acid extracted from walls of *P. cerevisiae* 8081 CRD contained a fragment of mucopeptide. Similar complexes have been isolated from walls of *Staphylococcus aureus* after partial enzymic degradation (Ghuysen, Tipper & Strominger, 1965), but these complexes were broken by trichloroacetic acid. Thus, in *P. cerevisiae* 8081 CRD some part of the mucopeptide is bound unusually firmly to some of the teichoic acid. Possibly the mucopeptide fragment is separate in the cell wall from the main part of the mucopeptide, which is not soluble in trichloroacetic acid. The data are insufficient to decide whether all the molecules of teichoic acid from *P. cerevisiae* 8081 CRD carry a small fragment of mucopeptide or whether a proportion of the teichoic acid molecules carry a relatively large fragment.

Cross-linking in mucopeptide polymers is thought to occur by the formation of a bond between an amino group of a basic amino acid in one peptide chain, and a carboxylic acid group (probably on the terminal alanine) in a neighbouring chain (see review by Rogers, 1965). This bond might be formed directly between amino acids that are each in separate peptide chains or through an amino-acid bridge, perhaps aspartic acid in the present case. In either event, the number of free amino groups in a cell wall might be expected to give some measure of the degree of cross-linking of the mucopeptide.

Lysine was the only basic amino acid found in the cell walls of *Pediococcus cerevisiae* and it was expected that cross-linking of peptide chains might take place through the ϵ-amino group of lysine. Since no amino acid of the mucopeptide was present in considerable molar excess over muramic acid or lysine it seemed unlikely that cross-linking was achieved by a polypeptide bridge, as in *Staphylococcus aureus* (Petit, Muñoz & Ghuysen, 1966). No free amino groups (α- or ϵ-) of lysine or other amino acids were detected in walls of *P. cerevisiae*, and in the parent strain at least 60% (and possibly much more) of the ϵ-amino groups of lysine were combined with a carboxylic acid group (α- or β-) of aspartic acid. These findings suggest that the mucopeptide of the parent strain is cross-linked by an aspartic acid link between lysine and perhaps alanine. However, this point is not established, since the terminal alanine carboxyl groups have not been shown to be bound to aspartic acid in adjacent chains.

Free amino groups would be expected to occur on the alanine residues in the teichoic acid. The alkaline conditions used during the treatment of cell walls with fluorodinitrobenzene would probably liberate this ester-linked alanine into solution, so that the DNP-alanine would be lost when the treated walls were washed with water before hydrolysis.

The absence of free amino groups from the walls does not seem to be a result of

the walls being isolated from organisms harvested at a late stage of growth, when the wall might have become thickened (Shockman, Kolb & Toennies, 1958) and extensively cross-linked. Walls from *Pediococcus cerevisiae* 8081 CRD harvested in the logarithmic phase of growth also had no free amino groups, but there was a lower yield of walls from these younger organisms. This suggests that only fully cross-linked walls were isolated by the methods used, and the less cross-linked, more soluble, material from younger organisms might have been lost during the isolation procedure.

If the teichoic acid were concerned in cross-linking of mucopeptide chains, it might be expected (as was found) that walls of the parent strain would be less readily digested by lysozyme, since these walls contain more teichoic acid than do walls of the methicillin-dependent substrain. However, after removal of teichoic acid, the residues from either strain would be expected to show increased, and probably similar, susceptibility to hydrolysis by lysozyme; this was not found to be the case.

Wise & Park (1965) and Izaki, Matsuhashi & Strominger (1966) showed that benzylpenicillin inhibited a cross-linking reaction between mucopeptide chains, in *Staphylococcus aureus* and *Escherichia coli*, respectively. Professor J. T. Park (personal communication) has suggested that *Pediococcus cerevisiae* 8081 CRD, in the absence of a penicillin, might form a cell wall that was too completely cross-linked, so that the organisms became encased and unable to multiply further. The presence of methicillin (or other penicillins) at a suitable concentration might partly inhibit the formation of cross-links and allow growth to proceed. As this hypothesis would predict, *P. cerevisiae* 8081 CRD did die when the organisms were incubated in the partly defined medium without methicillin. However, the hypothesis does not explain why the organisms did not die in the absence of methicillin when yeast extract was added to the medium; in fact the organisms multiplied in drug-free medium containing yeast extract, provided that sodium acetate was omitted (White, 1967). Another obstacle to the acceptance of this hypothesis is that the cell walls of the parent strain, grown in the presence or absence of methicillin, appear (so far as they have been examined) to be as highly cross-linked as those of the substrain, and yet the organisms were viable when transferred to fresh medium.

Thus the results of the present study, while they reveal some interesting differences between the walls of *Pediococcus cerevisiae* 8081 and its methicillin-dependent substrain 8081 CRD, do not lead to an explanation of the requirement for methicillin by *P. cerevisiae* 8081 CRD. Because of the known effect of methicillin on formation of cell walls, it seems reasonable to suppose that the altered features of the walls of *P. cerevisiae* 8081 CRD are related to the metabolic changes which have caused the nutritional requirement for methicillin. However, this might not be the case, since the methicillin-dependent substrain seemed to arise through a number of genetic steps, rather than from a single mutational event (White, 1967).

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REFERENCES

ALLSOP, J. & WORK, E. (1963). Cell walls of *Propionibacterium* species: fractionation and composition. *Biochem. J.* **87**, 512.

BADDILEY, J., BUCHANAN, J. G., HANDSCHUMACHER, R. E. & PRESCOTT, J. F. (1956). Biosynthesis of purine nucleotides. I. Preparation of *N*-glycylglycosylamines. *J. chem. Soc.* p. 2818.

BADDILEY, J. & DAVISON, A. L. (1961). The occurrence and location of teichoic acids in lactobacilli. *J. gen. Microbiol.* **24**, 295.

CESSI, C. & PILIEGO, F. (1960). Determination of amino sugars in presence of amino acids and glucose. *Biochem. J.* **77**, 508.

FISCHER, F. G. & NEBEL, H. J. (1955). Nachweis und Bestimmung von Glucosamin und Galaktosamin auf Papierchromatogrammen. *Hoppe-Seyler's Z. physiol. Chem.* **302**, 10.

FISKE, C. H. & SUBBAROW, Y. (1929). Phosphocreatine. *J. biol. Chem.* **81**, 629.

GHUYSEN, J.-M. & STROMINGER, J. L. (1963). Structure of the cell wall of *Staphylococcus aureus* strain COPENHAGEN. I. Preparation of fragments by enzymatic hydrolysis. *Biochemistry* **2**, 1110.

GHUYSEN, J.-M., TIPPER, D. J. & STROMINGER, J. L. (1965). Structure of the cell wall of *Staphylococcus aureus* strain COPENHAGEN. IV. The teichoic acid-glycopeptide complex. *Biochemistry* **4**, 474.

HANES, C. S. & ISHERWOOD, F. A. (1949). Separation of the phosphoric esters on the filter paper chromatogram. *Nature, Lond.* **164**, 1107.

IKAWA, M. & SNELL, E. E. (1960). Cell wall composition of lactic acid bacteria. *J. biol. Chem.* **235**, 1376.

INGRAM, V. M. & SALTON, M. R. J. (1957). The action of fluorodinitrobenzene on bacterial cell walls. *Biochim. biophys. Acta* **24**, 9.

IZAKI, K., MATSUHASHI, M. & STROMINGER, J. L. (1966). Glycopeptide transpeptidase and D-alanine carboxypeptidase: penicillin-sensitive enzymic reactions. *Proc. natn. Acad. Sci. U.S.A.* **55**, 656.

LIPMANN, F. & TUTTLE, L. C. (1944). Acetyl phosphate: chemistry, determination, and synthesis. *J. biol. Chem.* **153**, 571.

LUDOWIEG, J. & DORFMAN, A. (1960). A micromethod for the colorimetric determination of *N*-acetyl groups in acid mucopolysaccharides. *Biochim. biophys. Acta* **38**, 212.

PARTRIDGE, S. M. (1948). Filter-paper partition chromatography of sugars. I. General description and application to the qualitative analysis of sugars in apple juice, egg white and foetal blood of sheep. *Biochem. J.* **42**, 238.

PERKINS, H. R. & ROGERS, H. J. (1959). The products of the partial acid hydrolysis of the mucopeptide from cell walls of *Micrococcus lysodeikticus*. *Biochem. J.* **72**, 647.

PETIT, J.-F., MUÑOZ, E. & GHUYSEN, J.-M. (1966). Peptide cross-links in bacterial cell wall peptidoglycans studied with specific endopeptidases from *Streptomyces albus* G. *Biochemistry* **5**, 2764.

REYNOLDS, P. E. (1966). Antibiotics affecting cell-wall synthesis. *Symp. Soc. gen. Microbiol.* **16**, 47.

ROBERTS, J. & JOHNSON, M. J. (1962). Effect of penicillin on the cell wall of *Bacillus subtilis*. *Biochim. biophys. Acta* **59**, 458.

ROGERS, H. J. (1965). The outer layers of bacteria: the biosynthesis of structure. *Symp. Soc. gen. Microbiol.* **15**, 186.

ROGERS, H. J. & GARRETT, A. J. (1963). The action of benzylpenicillin and cycloserine upon ribitol teichoic acid formation by *Staphylococcus aureus*. *Biochem. J.* **88**, 6*P*.

SAUKKONEN, J. J. (1961). Acid-soluble nucleotides of *Staphylococcus aureus*: massive accumulation of a derivative of cytidine diphosphate in the presence of penicillin. *Nature, Lond.* **192**, 816.

SHAW, N. & BADDILEY, J. (1964). The teichoic acid from the walls of *Lactobacillus buchneri* NCIB 8007. *Biochem. J.* **93**, 317.

SHOCKMAN, G. D., KOLB, J. J. & TOENNIES, G. (1957). A high speed shaker for the disruption of cells at low temperatures. *Biochim. biophys. Acta* **24**, 203.

SHOCKMAN, G. D., KOLB, J. J. & TOENNIES, G. (1958). Relations between bacterial cell wall synthesis, growth phase and autolysis. *J. biol. Chem.* **230**, 961.

SMITH, I. (1960). Silver nitrate reagent. In *Chromatographic and Electrophoretic Techniques*, 2nd ed. Ed. by I. Smith, vol. I, p. 252. London: William Heinemann.

STROMINGER, J. L., PARK, J. T. & THOMPSON, R. E. (1959). Composition of the cell wall of *Staphylococcus aureus*: its relation to the mechanism of action of penicillin. *J. biol. Chem.* **234**, 3263.

SWALLOW, D. L. & ABRAHAM, E. P. (1958). Formation of ε-(aminosuccinyl)-lysine from ε-aspartyl-lysine from bacitracin A and from the cell walls of lactobacilli. *Biochem. J.* **70**, 364.

WHITE, P. J. (1956). The chemical composition of cell walls from *Pediococcus cerevisiae* and from a substrain that requires methicillin for growth. *J. gen. Microbiol.* **44**, i.

WHITE, P. J. (1967). A strain of *Pediococcus cerevisiae* which requires methicillin for growth. *J. gen. Microbiol.*

WHITE, P. J. & NICHOL, C. A. (1963). Effects of uracil and thymidine on the development of resistance to 5-fluorouracil in *Pediococcus cerevisiae*. *J. Bact.* **85**, 97.

WISE, E. M. Jr. & PARK, J. T. (1965). Penicillin: its basic site of action as an inhibitor of a peptide cross-linking reaction in cell wall mucopeptide synthesis. *Proc. natn. Acad. Sci., U.S.A.* **54**, 75.

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The Activity and Specificity of the Proline Permease in Wild-type and Analogue-resistant Strains of *Escherichia coli*

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SUMMARY

The main characteristics of the previously described proline-specific transport mechanism (permease) of *Escherichia coli* were confirmed in strain C4. The same permease was responsible for entry of a number of proline analogues, including 3,4-dehydroproline, 4-methyleneproline, *cis*- and *trans*-4-chloroprolines, thiazolidine-4-carboxylic acid (thioproline) and the lower homologue, azetidine-2-carboxylic acid. These analogues also entered the cells by an exchange reaction between extracellular analogue and previously accumulated intracellular proline. Growth of the parent (C4) strain was inhibited by 3,4-dehydroproline and azetidine-2-carboxylic acid, both of which were incorporated into cellular protein. Several classes of mutants, selected for resistance to either dehydroproline or azetidine, failed to incorporate one or both analogues into protein. Some of these mutants owed their resistance to failure to produce a functional proline permease. At least one strain, resistant to azetidine but not to dehydroproline, possessed an altered permease with little affinity for azetidine-2-carboxylic acid but still capable of transporting proline and 3,4-dehydroproline; the permease of this strain could no longer promote exchange between intracellular proline and extracellular proline or proline analogues.

INTRODUCTION

Exposure of bacterial cells to a wide variety of structural analogues of 'protein' amino acids results in inhibition of growth, frequently accompanied by incorporation of the analogue into the proteins of the organism (Cohen & Munier, 1959; Munier & Cohen, 1959; Richmond, 1962; Fowden, Lewis & Tristram, 1967). Recently, two analogues of proline have been investigated, namely, azetidine-2-carboxylic acid (a 'non-protein' amino acid occurring naturally in certain higher plants) and 3,4-dehydroproline. These compounds are strongly growth-inhibitory to *Escherichia coli* and some higher plants, inhibition being specifically annulled by proline. Both analogues are incorporated into the proteins of these organisms, stoichiometrically replacing proline residues (Smith, Ravel, Skinner & Shive, 1962; Fowden & Richmond, 1963; Fowden, Neale & Tristram, 1963; Peterson & Fowden, 1965). Incorporation of dehydroproline into the alkaline phosphatase of *E. coli* has been shown to result in a marked increase in thermolability of the enzyme (Neale & Tristram, 1964). Thiazolidine-4-carboxylic acid (thioproline) also inhibits growth and is incorporated into the proteins of *E. coli* (Beerstecher, 1950; Unger & DeMoss, 1966). The biological activi-

ties of other proline analogues have recently been reviewed (Mauger & Witkop, 1966; Fowden *et al.* 1967).

Numerous examples of the development of resistance to growth-inhibitory amino acid analogues have been described (Moyed, 1964; Richmond, 1965; Fowden *et al.* 1967). The mechanisms whereby resistance is acquired vary; one type of resistance involves mutation leading to failure of the analogue to penetrate into the cell, thereby excluding the analogue from the site (or sites) at which toxicity is exerted. Several investigators have described the isolation of analogue-resistant bacterial strains which failed to accumulate either the analogue or the corresponding natural amino acid (Schwartz, Maas & Simon, 1959; Ferroluzzi-Ames, 1964; Shifrin, Ames & Ferroluzzi-Ames, 1966; Lester, 1966). These observations not only provide an explanation of the mechanism of resistance, but also demonstrate that the analogues enter the cell by the specific transport mechanisms normally responsible for entry of the corresponding natural amino acids. The latter conclusion was also reached in a study of the uptake of phenylalanine and its analogue, *p*-fluorophenylalanine by *Escherichia coli* (Kempner & Cowie, 1960).

A specific energy-dependent transport mechanism (permease) permitting the accumulation of proline by *Escherichia coli* has been described (Britten & McClure, 1962; Britten, 1965; Kessel & Lubin, 1962). Strains deficient in proline permease activity have been obtained (Lubin, Kessel, Budreau & Gross, 1960; Kessel & Lubin, 1962). Evidence will be presented that 3,4-dehydroproline, azetidine-2-carboxylic acid and some other proline analogues are accumulated in the cell by the proline permease and that, compared with the parent strain, some of the mutants resistant to the two named analogues have an impaired ability to concentrate proline. A preliminary account of some of these experiments has already been presented (Neale & Tristram, 1965).

METHODS

Chemicals. L-Proline, *N*-acetyl-DL-proline and glycyl-L-proline were obtained from the Sigma Chemical Company; D-proline, L- and D-azetidine-2-carboxylic acid from Calbiochem; L-thiazolidine-4-carboxylic acid, DL-pipecolic acid, 4,5-dehydro-DL-pipecolic acid, 4-methylene-DL-proline, *cis*- and *trans*-4-chloro-L-proline were the gift of Professor L. Fowden, F.R.S.; 3,4-dehydro-DL-proline was a gift from Dr B. Witkop and Professor A. V. Robertson. Labelled amino acids were purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England. All amino acids and analogues were checked for purity by chromatography. Where possible, all other chemicals were 'Analar' grade, obtained from British Drug Houses Ltd.

Media. The basal glucose mineral salts medium (designated 232G) described by Tristram (1960) was used throughout the investigation. For selection of analogue-resistant mutants, the medium was supplemented with 25 μg. 3,4-dehydro-DL-proline/ml. (232G/DHP), or 25 μg. L-azetidine-2-carboxylic acid/ml. (232G/Az). Solid media were prepared by addition of 2% (w/v) Bacto agar. The effect of analogues on growth was measured in shaken cultures at 37° as described by Neale & Tristram (1963).

Organisms. Escherichia coli C4 (a strain derived from K-10), isolated and described by Echols, Garen, Garen & Torriani (1961), was used as the source of analogue-resistant strains described below. Strains resistant to 3,4-dehydroproline were isolated by plating between 10^8 and 10^9 bacteria of strain C4 (without exposure to mutagens) on

232G/DHP agar. Similar attempts to isolate azetidine-resistant strains by plating on 232G/Az resulted, after a lag, in a sparse 'lawn' of growth which covered the plates. Accordingly, strains resistant to this analogue were obtained by repeated passage of *E. coli* C4 through 232G/Az liquid medium, followed by plating on 232G/Az agar to obtain individual colonies. Resistant colonies were picked from 232G/DHP and 232G/Az plates, purified by streaking on the same media and maintained on slopes of 232G/DHP or 232G/Az agar in which the glucose concentration was reduced to 0·1 % (w/v) to prevent development of highly acid conditions. Mutants were allocated numbers prefixed by 'DHP' or 'AZ', denoting strains selected for resistance to 3,4-dehydroproline or azetidine-2-carboxylic acid respectively.

Excretion of proline by analogue-resistant mutants was detected on double-layer plates consisting of a lower layer of 232G agar covered by a thin layer of the same medium in which was suspended about 10^{10} organisms of *Escherichia coli* strain H208 (a proline-dependent derivative of K12, kindly provided by Dr K Fisher). Mutants were streaked on such plates and proline excretion detected by cross-feeding of the auxotrophic strain suspended in the agar.

Accumulation of amino acids; measurement of exchange between intra- and extracellular amino acids. The procedure for measurement of the uptake of amino acids was essentially that described by Britten & McClure (1962). Unless otherwise stated, uptake was followed in continuously aerated suspensions containing 125 μg. dry wt. organism/ml. and maintained at 10°. In those experiments involving uptake in the presence of chloramphenicol, exponential cultures growing in 232G at 37° were cooled to 10° and, when necessary, diluted with 232G medium, followed by addition of chloramphenicol (in 232G medium) to give a final concentration of 150 μg./ml. The suspensions were distributed in suitable amounts in Pyrex tubes, aerated with a stream of water-saturated air and used within 15 min. of addition of chloramphenicol. For experiments not involving addition of chloramphenicol, cultures growing exponentially at 37° were transferred to 10° and aeration continued for 2 hr before dilution and distribution as already described. Additions of amino acids and/or analogues were made in volumes not exceeding 1 % (v/v) of the total volume of suspensions. Total intracellular radioactivity was measured by filtration of 1 ml. samples through Millipore filters (type HA; 23 mm. diameter) held in stainless steel holders (Tracerlab) and covered, immediately prior to receiving the sample, with 2 ml. ice-cold 232G medium. After filtration, which occupied about 5 sec., filters were transferred to aluminium or stainless steel planchets, held in position by a slip-on retaining ring (Tracerlab), dried over calcium chloride and counted by use of a thin end-window G-M tube (Isotope Developments Ltd., England). In all instances at least 1000 counts over background were obtained and, where necessary, counts were converted into μmoles amino acid by calculation from specific radioactivities and the known efficiency of the counting equipment. For kinetic studies, the amount of radioactivity taken up by suspensions could be determined at 20 sec. intervals and the initial rates of uptake were calculated by the method of Algranati (1963).

In earlier experiments involving exchange between accumulated radioactive proline and extracellular unlabelled proline or analogue, the bacteria were 'loaded' by exposure to $1{\cdot}22 \times 10^{-7}$ M- or 2×10^{-6} M-L-[U-^{14}C]proline for 20 min. or 40 min., respectively, at 10° in aerated 232G medium containing chloramphenicol (150 μg./ml.). Later exchange experiments involved exposing bacteria, suspended in 232G

medium containing chloramphenicol, to $1{\cdot}22 \times 10^{-7}$ M-L-[U-^{14}C]proline for 10 min. at 25°. The suspensions were then cooled to 0° with continuous aeration for 15 min., followed by centrifugation at 0° (10,000 *g*; 10 min.) or rapid filtration through HA Millipore membrane filters (47 mm. diameter) and resuspension of the cells in fresh 232G medium, previously cooled to 0° containing chloramphenicol (150 μg./ml.). Aeration at 0° was continued for at least a further 30 min. before exchange commenced. In both procedures the exchange reaction was initiated by rapid addition of a suitable sample of preloaded bacteria to Pyrex tubes already containing a solution of the compound to be tested for exchange activity. Samples were filtered and intracellular radioactivity measured as described above.

Incorporation of amino acids and analogues into protein; extraction of free amino acid pool. Incorporation of analogues into protein was determined after acid hydrolysis of the 'principal protein fraction' (Roberts *et al.* 1955) of dehydroproline-containing protein or, for azetidine-containing protein, by alkaline hydrolysis, as described previously (Neale & Tristram, 1963). Proline in hydrolysates was assayed after electrophoresis on Whatman 3MM paper (6000 V.; 30 mA.; formic+acetic acid buffer, pH 2, containing 61·2 ml. formic acid and 97·2 ml. acetic acid per litre of solution), followed by spraying with isatin and comparison of spot intensities with those of standard proline solutions, using a 'Chromoscan' (Joyce-Loebl and Co. Ltd., Newcastle-on-Tyne, England) fitted with filter 595 (A. Hussain, personal communication). 3,4-Dehydroproline was assayed on the same electrophoretogram by over-spraying with Ehrlich's reagent (Fowden, Neale & Tristram, 1963) and scanning as described above. Azetidine-2-carboxylic acid in hydrolysates was determined after two-dimensional chromatography, as described by Fowden & Richmond (1963).

Before extraction of the free amino acid 'pool', bacterial suspensions were centrifuged (20,000 *g*; 15 min.) at the temperature at which amino acid accumulation occurred (to avoid changes in 'pool' size due to temperature shock). The pellet of bacteria was resuspended in 70% (v/v) ethanol in water to extract the amino acid 'pool' and, after overnight storage at 4°, cell debris was removed by centrifugation and the constituents of the ethanol-soluble fraction separated and detected by conventional chromatography or by electrophoresis as already described. Radioautography was carried out on 'Kodirex' X-ray film.

Incorporation of radioactivity into trichloroacetic acid-insoluble material was determined as described by Neale & Tristram (1963).

RESULTS

Growth in the presence of analogues

In common with many amino acid analogues (see reviews by Richmond, 1962; Fowden *et al.* 1967), addition of 3,4-dehydro-DL-proline (25 μg./ml.) or L-azetidine-2-carboxylic acid (20 μg./ml.) to cultures of *Escherichia coli* strain C4 growing exponentially in 232G medium resulted in 'linear' growth, i.e. whereas the extinction of control cultures increased exponentially, in the presence of the analogue increase in extinction was arithmetic. In cultures containing azetidine, onset of linear growth was virtually immediate. However, in cultures receiving dehydroproline there was little effect on growth for the first 40 min. of exposure, after which linear increase in optical density was established (Fig. 1*a*, *b*; see also Fowden *et al.* 1963).

Addition of analogues to exponentially growing cultures of suitable bacterial concentration was advocated by Cohen & Munier (1959) and allows a sufficient increase in dry weight of organism to facilitate isolation and study of incorporation of analogues into cell protein. When either 3,4-dehydro-DL-proline (25 μg./ml.) or L-azetidine-2-carboxylic acid (20 μg./ml.) were added to 232G medium at the same time as inoculation with a small inoculum (about 5×10^5 bacteria/ml.), then detectable growth was completely inhibited for at least 12 hr. Addition of much higher (400 μg./ml.) concentrations of L-thiazolidine-4-carboxylic acid yielded a curve similar to that

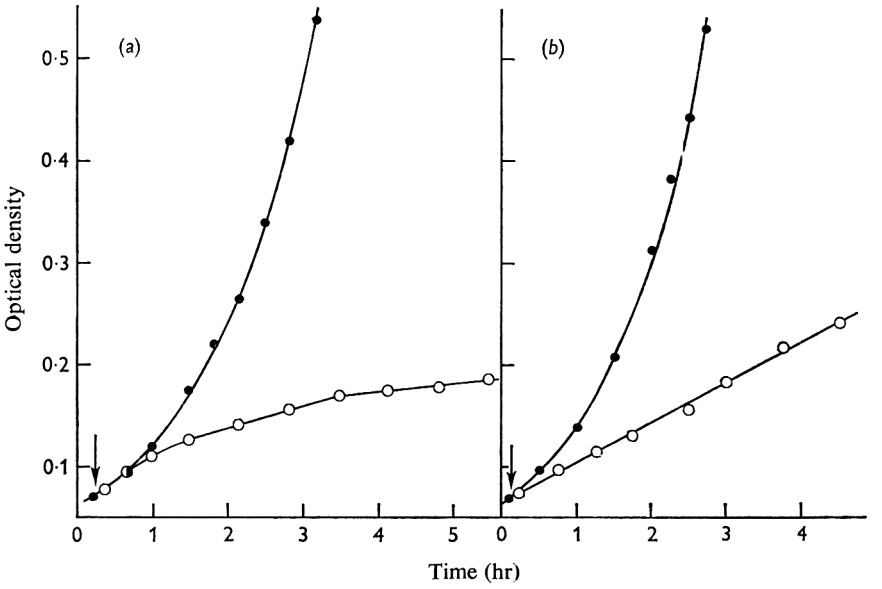

Fig. 1. The effect of addition of (*a*) L-azetidine-2-carboxylic acid (20 μg./ml.) and (*b*) 3,4-dehydro-DL-proline (25 μg./ml.) to exponential cultures of *Escherichia coli* C4 growing in 232G medium at 37°. Analogues were added at points indicated by arrows. ●—●, Control (no analogue); ○—○, plus analogue.

obtained on addition of 3,4-dehydroproline, thus confirming the observations of Unger & DeMoss (1966). *Cis*- or *trans*-4-chloro-L-prolines did not inhibit growth of *Escherichia coli* C4 at concentrations up to 200 μg. analogue/ml., nor was growth inhibited by DL-pipecolic acid or 4,5-dehydro-DL-pipecolic acid, in agreement with earlier observations (Fowden & Richmond, 1963).

In studying growth of mutants resistant to 3,4-dehydroproline or azetidine, 232G, 232G/DHP and 232G/Az media were inoculated with bacteria growing exponentially in 232G medium to give an initial bacterial concentration equiv. to about 10 μg. dry wt bacteria/ml. Dehydroproline-resistant strains grew in the presence of 3,4-dehydroproline or azetidine at rates comparable with that displayed by the parent (C4) strain in the absence of analogue. All dehydroproline-resistant strains tested displayed cross-resistance to both analogues. However, although fewer resistant strains were isolated when using azetidine as selective agent, all those tested (fourteen in all) were completely resistant to azetidine (judged by their capacity to grow in 232G/Az medium at rates comparable with that of the parent strain in 232G medium), but were still

sensitive to growth inhibition by dehydroproline when inoculated into 232G/DHP medium.

Incorporation of analogues into proteins of parent and mutant strains

The 'principal protein fraction' (Roberts *et al.* 1955) isolated from strains grown in 232G, 232G/DHP and 232G/Az was examined for the presence of incorporated analogues (see Methods). As previously reported (Fowden & Richmond, 1963; Fowden *et al.* 1963), both 3,4-dehydroproline and azetidine were incorporated into proteins of wild-type *Escherichia coli*, with a corresponding decrease in the proline content (Table 1). Although not all the analogue-resistant strains isolated were tested,

Table 1. *Incorporation of 3,4-dehydroproline and azetidine-2-carboxylic acid into the 'principal protein fraction' of Escherichia coli C4 and analogue-resistant derivatives*

Organisms were grown at 37° in 232G, 232G/DHP and 232G/Az media. The 'principal protein fraction' was isolated, hydrolysed and the content of proline and, where appropriate, 3,4-dehydroproline and azetidine-2-carboxylic acid determined (see Methods). Cultures grown in the presence of analogues were harvested after a 2·5- to 3-fold increase in extinction. Figures reported are not corrected for the proline content of analogue-free protein present in cells before addition of analogues (see Fowden & Richmond, 1963; Fowden *et al.* 1963). The proline content of all strains harvested during exponential growth in 232G medium was about 24 μg. proline/mg. 'principal protein fraction'.

	Growth in 232G/DHP		Grown in 232G/Az	
Strain	3,4-Dehydroproline (μg./mg. protein)	Proline (μg./mg. protein)	Azetidine-2-carboxylic acid (μg./mg. protein)	Proline (μg/mg. protein)
C4	13·1	10·8	5·4	18·1
DHP15	0	22·8	0	23·2
DHP27	0	23·9	0	24·1
DHP29	0	24·4	0	23·8
AZ3	12·9	11·0	0	22·7
AZ642	13·3	10·6	0	24·2
AZ643	14·6	9·4	0	24·0

the results presented in Table 1 show that dehydroproline-resistant strains (also cross-resistant to azetidine) did not incorporate either dehydroproline or azetidine into protein, whereas strains which had been selected for resistance to azetidine did not incorporate this analogue but still incorporated dehydroproline into cell proteins. Moreover, the degree of incorporation of dehydroproline by these azetidine-resistant strains was comparable with that observed in strain C4 (Table 1).

Excretion of proline by analogue-resistant mutants

Excretion of proline was detected by ability to cross-feed proline auxotrophs (see Methods). Whereas many strains selected for resistance to dehydroproline excreted proline, most azetidine-resistant strains studied were unable to cross-feed proline-dependent organisms. The significance of proline excretion will be discussed below (see Discussion).

Proline uptake by the parent strain

Addition of 10^{-6} M-L-[U-^{14}C]proline to a suspension of *Escherichia coli* C4 maintained at 10° in the absence of chloramphenicol resulted in rapid accumulation of radioactivity in the cells (Fig. 2). Incorporation into TCA-insoluble material was linear for about 40 min., the rate of incorporation subsequently decreasing with approaching exhaustion of added proline. The proline 'pool' (obtained as the difference between

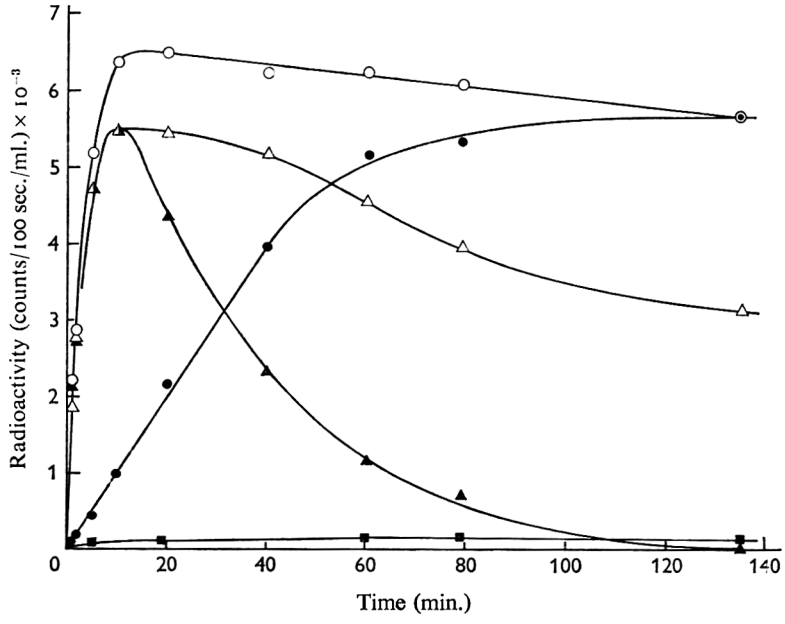

Fig. 2. Total uptake and incorporation of radioactivity into trichloroacetic acid-insoluble material by *Escherichia coli* C4 in the presence of 10^{-6} M-L-[U-^{14}C]proline (31·25 μc./μmole). Bacteria (equiv. 125 μg. dry wt/ml.) were suspended in 232G medium, with or without addition of chloramphenicol (150 μg./ml.) and aerated at 10°. In the absence of chloramphenicol: ○—○, total uptake; ●—●, incorporation into the trichloroacetic acid-insoluble fraction; ▲—▲, calculated proline 'pool' (total uptake minus trichloroacetic acid-insoluble fraction). In the presence of chloramphenicol: △—△, total uptake (= 'pool'); ■—■, incorporation into the trichloroacetic acid-insoluble fraction.

total uptake and TCA-insoluble incorporated material) rose to a maximum after 10 min. and then decreased as the limited amount of proline supplied passed into protein. In the presence of chloramphenicol, incorporation into protein was suppressed, and the total uptake may be taken as representing incorporation into the 'pool'. Although under these conditions some radioactivity was lost from the bacteria, the initial rate of accumulation in the 'pool' was identical in the presence or absence of chloramphenicol (Fig. 2). This overall picture of proline uptake in strain C4 is similar to that described for *E. coli* B by Britten & McClure (1962).

The addition of relatively high concentrations of labelled proline to *E. coli* strain C4 at 10° in the presence of chloramphenicol resulted in rapid uptake of radioactivity which reached a maximum and subsequently decreased due to leakage of accumulated proline. This effect was less pronounced at lower initial proline concentrations. For

example, in the presence of 2×10^{-6} M-L-proline about 50% of the accumulated proline was lost in 150 min. In the same time-period leakage accounted for 38%, 32%, 25% and 17% of the maximum 'pool' size after addition of 10^{-6} M, $2{\cdot}6 \times 10^{-7}$ M, 10^{7} M and $0{\cdot}33 \times 10^{-7}$ M-L-[U-^{14}C]proline, respectively. This 'overshoot' phenomenon has been noticed by W. Maas (personal communication) during investigation of the arginine permease of *E. coli*, and has been studied and discussed in detail with respect to sulphate transport in *Salmonella typhimurium* (Dreyfuss & Pardee, 1966).

Following uptake of L-[U-^{14}C]proline in the presence of chloramphenicol, extraction of the 'pool' followed by chromatography and radioautography (see Methods) revealed that virtually all the accumulated radioactivity could be accounted for as proline. Similarly, after uptake of the labelled amino acid in the absence of chloramphenicol followed by isolation, and hydrolysis and electrophoresis of the 'principal protein fraction', only the proline spot was labelled.

Inhibition of proline accumulation by analogues

Since radioactively labelled analogues were not available, their uptake could not be measured directly. A measure of the affinity of the proline permease for proline analogues was obtained by investigating inhibition of L-[U-^{14}C]proline uptake by the

Table 2. *Inhibition of uptake of* L-[*U*-^{14}C]*proline by proline analogues in Escherichia coli strain C4*

Bacterial suspensions (equiv. 125 μg. dry wt bacteria/ml.) in 232G medium containing chloramphenicol (150 μg./ml.) at 10° were simultaneously exposed to $1{\cdot}22 \times 10^{-7}$ M-L-[U-^{14}C]proline and $1{\cdot}1 \times 10^{-4}$ M-analogue (or unlabelled proline), as indicated below. Analogue concentrations were calculated on the basis of the content of L-isomer. Results are expressed as percentage inhibition of the initial rate of uptake in the presence of $1{\cdot}22 \times 10^{-7}$ M-L-[U-^{14}C]proline only.

Addition	Inhibition (%)
L-Proline (unlabelled)	95·7
3,4-Dehydro-DL-proline	94·8
L-Thiazolidine-4-carboxylic acid	86·7
L-Azeticine-2-carboxylic acid	77·5
4-Methylene-DL-proline	75·9
cis-4-Chloro-L-proline	76·1
trans-4-Chloro-L-proline	56·9
Glycyl-L-proline	13·9
D-Proline	0
D-Azeticine-2-carboxylic acid	0
N-Acetyl-DL-proline	0
DL-Pipecolic acid	0
4,5-Dehydro-DL-pipecolic acid	0

analogues. Table 2 (line 1) reveals that the presence of excess unlabelled proline inhibited accumulation of radioactive proline. Many analogues, including 3,4-dehydro-DL-proline, L-thiazolidine-4-carboxylic acid and a number of 4-substituted prolines had a similar effect. The lower homologue of proline, L-azetidine-2-carboxylic acid also inhibited the uptake of labelled proline, though to a lesser degree; on the other hand, the higher homologue of proline, DL-pipecolic acid, together with 4,5-dehydro-DL-pipecolic acid and *N*-acetyl-DL-proline were without effect (Table 2).

When studied as a function of initial proline concentration, the initial rates of uptake in the presence of chloramphenicol obeyed Michaelis–Menten kinetics (Fig. 3), with

a K_m of $6{\cdot}4 \times 10^{-7}$ M. Inhibition of proline uptake into the amino acid pool by 3,4-dehydroproline and azetidine was studied in detail. Inhibition by both analogues was competitive (Fig. 3). Calculation of K_i (see Dixon & Webb, 1964) yielded a value of $2{\cdot}6 \times 10^{-6}$ M and $2{\cdot}4 \times 10^{-5}$ M for 3,4-dehydroproline and azetidine, respectively. It should be noted that, since only racemic (DL) 3,4-dehydroproline was available, the calculation of K_i for this analogue was based on the assumption that only the L-isomer was active in inhibiting uptake of proline. Thus, in the parent strain *Escherichia coli* C4, the affinity of the proline transport system for 3,4-dehydroproline was comparable with that for proline itself, but affinity of the system for azetidine was considerably lower (see also Table 2).

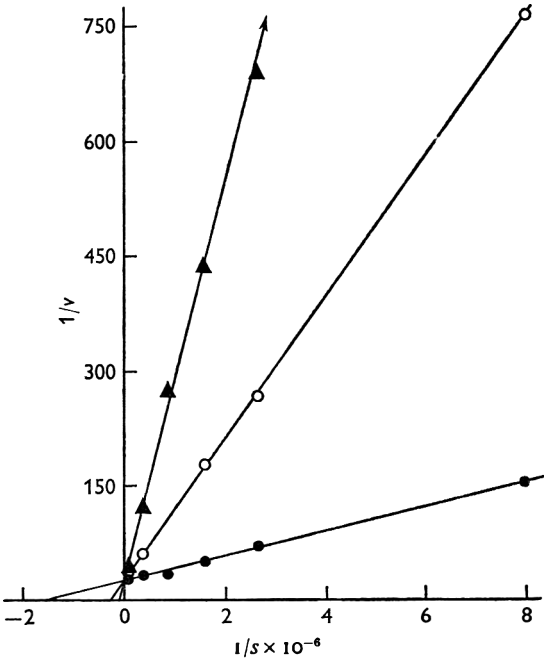

Fig. 3. Lineweaver-Burk plot of the initial rate of uptake of radioactivity as a function of L-[U-^{14}C]proline concentration in the presence and absence of 5×10^{-5} M-3,4-dehydro-DL-proline or 10^{-4} M-L-azetidine-2-carboxylic acid. *Escherichia coli* C4, suspended in 232G medium containing chloramphenicol (150 μg./ml.), were aerated at 10°. Various concentrations of substrate were obtained by addition of suitable amounts of carrier L-proline to L-[U-^{14}C]proline (130 μc./μmole). ●—●, L-proline only; ○—○, L-proline + L-azetidine-2-carboxylic acid; ▲—▲, L-proline + 3,4-dehydro-DL-proline.

Accumulation of proline by analogue-resistant mutants

A wide range of strains selected for resistance to either 3,4-dehydroproline or azetidine were tested for their ability to accumulate labelled proline. Some resistant strains appeared to take up labelled proline at rates comparable to that displayed by the parent strain *Escherichia coli* C4. However, in many other mutants the capacity to accumulate proline was impaired. A representative group of results are shown in Fig. 4. The activity of the impaired permease varied from virtually complete absence to values with which the initial rate of proline uptake was about 25% of that measured in the parent strain C4. In many of the strains selected on 3,4-dehydroproline, the

kinetics of proline uptake were abnormal (see strain DHP 21, Fig. 4) in that radioactivity entered the bacteria at a high rate, but was quickly lost. These strains also excreted proline in considerable amounts. The significance of this behaviour will be considered in a later publication.

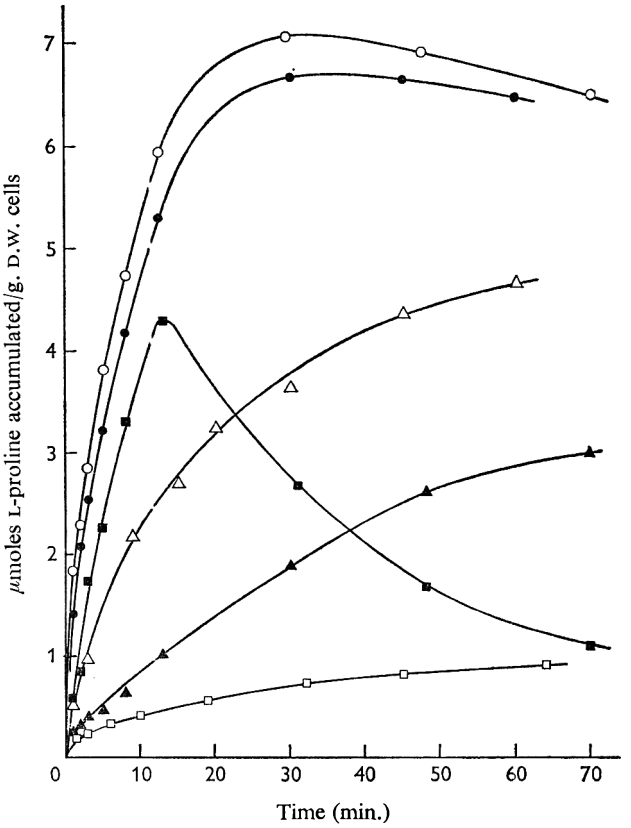

Fig. 4. Uptake of radioactivity by *Escherichia coli* wild-type C4 and analogue-resistant strains in the presence of 2×10^{-6} M-L-[U-^{14}C]proline (31·25 μc/μmole). Bacteria (equiv. 125 μg. dry wt/ml.) were suspended in 232G medium containing chloramphenicol (150 μg./ml.). Azetidine-resistant strains: ▲—▲, AZ3; △—△, AZ642; ●—●, AZ643. 3,4-Dehydroproline-resistant strains: ■—■, DHP21; □—□, DHP27. ○—○, strain C4.

The presence of 10^{-3} M-2,4 dinitrophenol almost completely suppressed the uptake of 2×10^{-6} M-L-[U-^{14}C]proline by the parent strain and by those mutants having somewhat impaired permease activity. However, the inhibitor was without significant effect on the very low intracellular levels of those strains which were virtually devoid of permease activity (see also Britten & McClure, 1962; Kessel & Lubin, 1962).

The uptake of $1{\cdot}65 \times 10^{-6}$ M-L-[U-^{14}C]arginine (12·5 mμc/ml. suspension) by the parent and all mutant strains tested was identical, suggesting that the impaired permease activity of the mutants was specific for the proline permease.

The mutants selected for azetidine-resistance were of particular interest since the majority of these strains were not cross-resistant to 3,4-dehydroproline, and the latter compound was still incorporated into protein (see Table 1). The affinity of the per-

mease of one of these strains (AZ642) for proline ($K_m = 9{\cdot}0 \times 10^{-7}$ M) and 3,4-dehydroproline ($K_i = 3{\cdot}6 \times 10^{-6}$ M) differed only slightly from that found for the parent strain. However, in strain AZ 642, uptake of proline was much less sensitive to inhibition by L-azetidine-2-carboxylic acid compared with the parent (C4) strain (Fig. 5). The K_i for azetidine determined for AZ 642 was $3{\cdot}0 \times 10^{-4}$ M, an approximately tenfold increase over the corresponding value for strain C4. As in the parent strain, both analogues competitively inhibited uptake of proline in strain AZ 642.

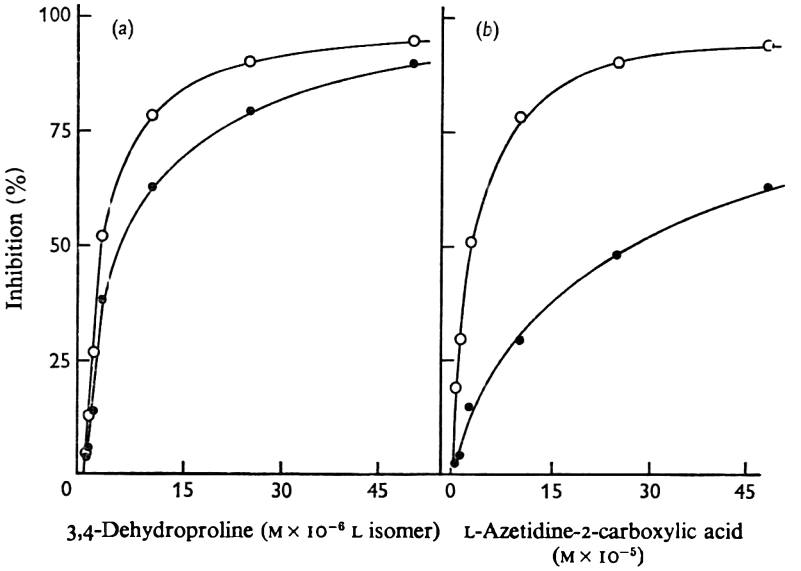

Fig. 5. Inhibition of uptake of labelled proline by varying concentrations of (*a*) 3,4-dehydroproline and (*b*) azetidine-2-carboxylic acid by *Escherichia coli* C4 and the azetidine-resistant strain AZ642. Bacteria (equiv. 125 μg. dry wt/ml.) were aerated at 10° in 232G medium containing chloramphenicol (150 μg./ml.), to which was added $1{\cdot}22 \times 10^{-7}$ M-L-[U-^{14}C]proline (130 μc/μmole) and, where necessary, either 3,4-dehydro-DL-proline or L-azetidine-2-carboxylic acid at concentrations indicated. Uptake of radioactivity was measured at 20 sec. intervals over the first 80 sec. and results are expressed as % inhibition of the initial rate of proline uptake in the absence of inhibitor. ○—○, Strain C4; ●—●, azetidine-resistant strain AZ642.

Exchange between intracellular proline and extracellular proline or analogues

Several investigators have shown that a variety of metabolites, once accumulated in the intracellular 'pool', can exchange with extracellular metabolite (Cohen & Kepes, 1962; Kessel & Lubin, 1962; Britten & McClure, 1962; Britten, 1965). Experiments with *Escherichia coli* strain C4 confirmed the results obtained with *E. coli* B by Britten & McClure (1962). Proline accumulation did not occur at 0°, though proline previously accumulated at higher temperatures could still exchange with extracellular proline at 0°. At 0° proline pools of moderate size were stable for at least 3 hr. When a stable pool at 0° was established by exposure to $1{\cdot}22 \times 10^{-7}$ M-L-[U-^{14}C]proline (see Methods), the rate of exchange between intracellular and unlabelled extracellular proline was independent of the initial external proline concentration in the range 10^{-7} M to $7{\cdot}5 \times 10^{-4}$ M. At 10° the rate of exchange was not markedly dependent on external proline concentration, but the final value of intracellular radioactivity

observed was concentration-dependent, due to the establishment of a new equilibrium between internal and external proline. Further, when the logarithm of the residual intracellular radioactivity was expressed as a function of time, the exchange process was revealed as an initial rapid phase followed by a period during which exchange was slower (see Britten & McClure, 1962; Kessel & Lubin, 1962). These workers interpreted this as indicating the presence of at least two pool components, one capable of rapid exchange with extracellular proline, the other a slowly exchanging component.

Several proline analogues also exchanged with intracellular radioactive proline. A direct correlation was demonstrated between ability of an analogue to inhibit proline uptake in the presence of chloramphenicol (see Table 2) and ability to effect exchange. When tested at 5×10^{-5} M- (based on the concentration of L-isomer), 3,4-dehydro-DL-proline, L-azetidine-2-carboxylic acid, L-thiazolidine-4-carboxylic acid and 4-methylene-DL-proline exchanged readily at 10° with the pool established by prior exposure

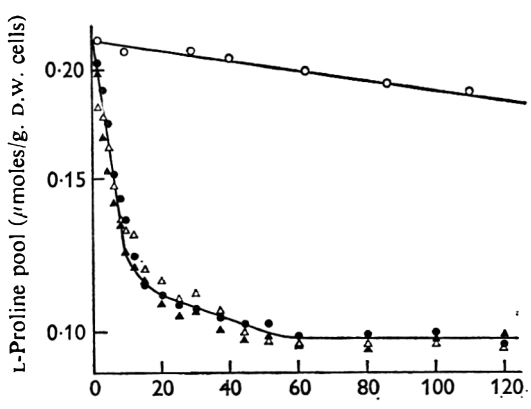

Fig. 6

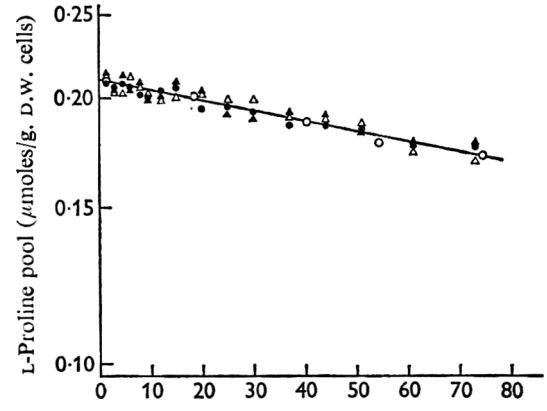

Fig. 7

Fig. 6. Exchange between labelled L-proline in the amino acid pool of *Escherichia coli* C4 with extracellular 3,4-dehydro-DL-proline, L-azetidine-2-carboxylic acid or (unlabelled) L-proline at 0°. The amino acid pool of bacteria maintained at 25° in 232G medium containing chloramphenicol (150 μg./ml.) was 'loaded' by exposure to $1{\cdot}22 \times 10^{-7}$ M-L-[U-^{14}C] proline (130 μc/μmole) for 10 min., followed by cooling the bacterial suspension to 0° and resuspension in fresh 232G medium containing 150 μg. chloramphenicol/ml. Exchange was initiated by adding samples of this bacterial suspension to tubes containing either L-proline, 3,4-dehydro-DL-proline or L-azetidine-2-carboxylic acid solutions to yield final concentrations of 5×10^{-5} M (based on the content of L-isomer). ○—○, Control; △—△, L-proline; ▲—▲, 3,4-dehydro-DL-proline; ●—●, L-azetidine-2-carboxylic acid.

Fig. 7. Exchange between labelled L-proline in the amino acid pool of azetidine-resistant *Escherichia coli* strain AZ642 with extracellular 3,4-dehydro-DL-proline, L-azetidine-2-carboxylic acid and unlabelled L-proline at 0°. Details and symbols as described for Fig. 6, except that, owing to the diminished permease activity of *E. coli* strain AZ642, time of 'loading' of the pool at 25° was extended to 30 min.

to $1{\cdot}22 \times 10^{-7}$ M-L-[U-^{14}C]proline. Under the same conditions glycyl-L-proline supported very little exchange, while DL-pipecolic acid, 4,5-dehydro-DL-pipecolic acid, D-proline and D-azetidine-2-carboxylic acid were unable to exchange with intracellular L-proline.

The exchange reaction between accumulated labelled proline and extracellular 3,4-

dehydroproline or azetidine-2-carboxylic acid was studied in detail at 0° and 10°. At 0° not only the rates of exchange but also the final steady state pool values after exchange between a labelled pool (established by exposure to $1{\cdot}22 \times 10^{-7}$M-L-[U-^{14}C] proline) and external L-proline, 3,4-dehydro-DL-proline or L-azetidine-2-carboxylic acid (5×10^{-5} M, based on the content of L-isomer) were virtually identical (Fig. 6). At 10° both analogues exchanged with intracellular labelled proline, but in the presence of L-azetidine-2-carboxylic acid loss of radioactivity from the pool ceased after the first (rapid) phase of exchange. Other experiments, in which the steady-state pool values were measured following simultaneous addition of $1{\cdot}22 \times 10^{-7}$ M-L-[U-^{14}C] proline and L-azetidine-2-carboxylic acid at concentrations ranging from 5×10^{-5} M to $2{\cdot}5 \times 10^{-3}$ M, established that this behaviour was due to the preferential re-accumulation of labelled proline molecules which had left the pool by exchange with azetidine. The final pool values found in these experiments were dependent on the

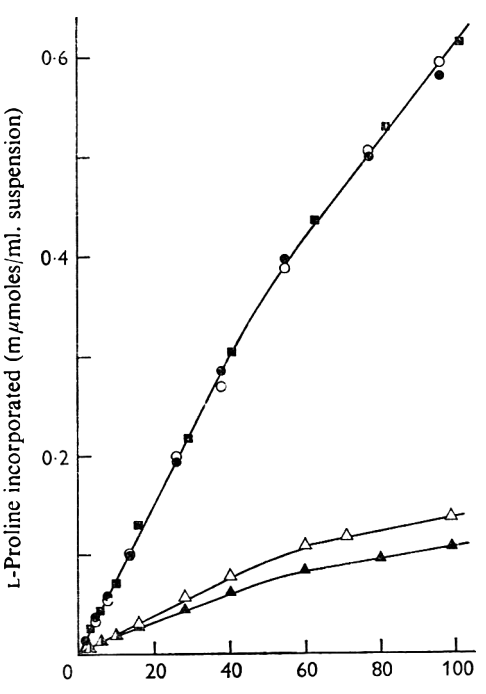

Fig. 8

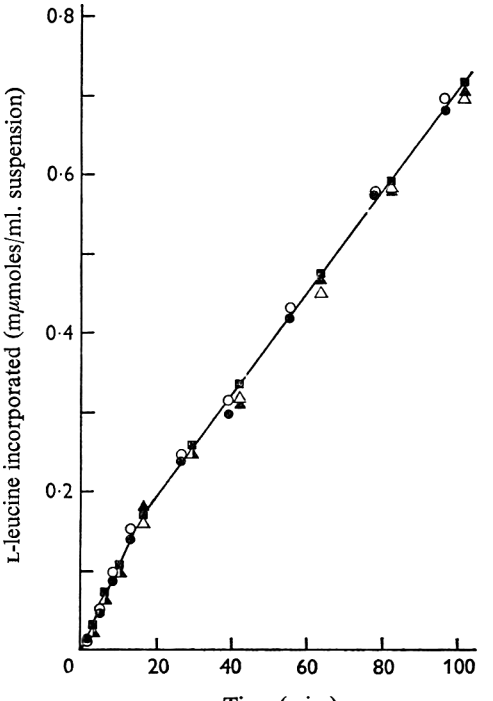

Fig. 9

Fig. 8. Incorporation of labelled L-proline into the trichloroacetic acid-insoluble fraction of *Escherichia coli* wild-type C4 and analogue-resistant strains. Cultures growing exponentially at 37° in 232G medium were cooled and maintained at 10° for 2 hr. L-[U-^{14}C]Proline (31·25 μC/μmole) and L-leucine were added to give final concentrations of 2×10^{-6} M. Samples were withdrawn at intervals for determination of total uptake of labelled proline and radioactivity incorporated into trichloroacetic acid-insoluble material (see Methods). Azetidine-resistant strains: ●—●, AZ3; ○—○, AZ642. 3,4-Dehydroproline-resistant strains: ▲—▲, DHP27; △—△, DHP29. ■—■, strain C4.

Fig. 9. Incorporation of labelled L-leucine into the trichloroacetic acid-insoluble fraction of *Escherichia coli* wild-type C4 and analogue-resistant strains. Details and symbols as described for Fig. 8, except that L-[U-^{14}C]leucine (50 μC/μmole) and L-proline replaced L-leucine and L-[U-^{14}C]proline.

azetidine-2-carboxylic acid concentration and were identical with those observed when the same amounts of azetidine were added to comparable bacterial suspensions in which a pool had previously been established by exposure to $1{\cdot}22 \times 10^{-7}$ M-L-[U-^{14}C] proline.

The exchange reaction could not be conveniently studied in many analogue-resistant mutants possessing very low permease activities. An azetidine-resistant strain (AZ 642) displaying a diminished permease activity with much reduced affinity for azetidine (see Fig. 5) completely lacked the ability to promote exchange between intracellular radioactive proline and external (unlabelled) proline, 3,4-dehydroproline or azetidine (Fig. 7).

Incorporation of proline and leucine into TCA-insoluble material of analogue-resistant strains

Compared with the parent strain *Escherichia coli* C 4, the capacity to incorporate exogenous L-[U-^{14}C]proline into TCA-insoluble material was markedly decreased in mutants selected for 3,4-dehydroproline resistance (Fig. 8). This diminution in proline incorporation was not due to a decrease in the rate of overall protein synthesis in the mutants, since the rate of incorporation of L-[U-^{14}C]leucine into protein was identical in both the parent strain and in all the mutants studied (Fig. 9). This suggests that those mutants with a decreased capacity for the uptake of exogenous proline continue to synthesize (unlabelled) proline and preferentially use this endogenous source for synthesis of protein. Although the rate of uptake of proline from the medium was decreased in most of the azetidine-resistant mutants tested, the residual permease activity was still capable of ensuring a sufficient supply of proline for protein synthesis since the rate of incorporation of exogenous labelled proline into protein was identical in the parent strain and in at least two azetidine-resistant mutants (Fig. 8).

DISCUSSION

Earlier experiments on the proline permease of *Escherichia coli* strains B and W (Britten & McClure, 1962; Kessel & Lubin, 1962) have been confirmed with *E. coli* strain C 4, a derivative of *E. coli* K-10. The observations were extended to include a study of the specificity of the permease towards proline analogues. Inhibition of proline accumulation by 3,4-dehydroproline and azetidine-2-carboxylic acid, the two analogues studied in detail, was competitive, suggesting that both analogues enter the cell by the mechanism responsible for transport of proline. That 3,4-dehydro-proline and azetidine actually enter the cell is indicated by two types of observation: (i) both analogues are incorporated into the proteins of *E. coli* (Fowden & Richmond, 1963; Fowden *et al.* 1963); (ii) both analogues exchanged with previously accumulated proline (Fig. 6).

Other analogues capable of inhibiting proline uptake (Table 2) also compete with proline for sites leading to accumulation within the cell. The proline permease of the parent culture displayed affinity for a number of 4-substituted proline derivatives. The analogues *cis*- and *trans*-4-chloroprolines are of particular interest since the former inhibited the uptake of proline to about the same extent as azetidine, but the latter possessed far less inhibitory action (Table 2). This is in contrast to the observations of Gottlieb, Fujita, Udenfriend & Witkop (1965), who showed that incor-

poration of *cis*-4-fluoroproline into TCA-insoluble material exceeded that of the *trans* isomer in a cell-free system from *Escherichia coli*. Whereas azetidine, the lower homologue of proline, markedly inhibited uptake of proline, the higher homologue, pipecolic acid, was devoid of inhibitory action, an effect presumably attributable to the larger ring structure of the latter compound.

A large number of strains were obtained from *Escherichia coli* C4 following selection for resistance to the growth-inhibitory analogues 3,4-dehydroproline or azetidine-2-carboxylic acid. In general, strains selected for resistance to 3,4-dehydroproline were also resistant to azetidine and failed to incorporate either analogue into protein. Some of these strains displayed impaired ability to take up exogenous proline; others excreted proline, but possessed normal permease activity. In many of the proline-excreting mutants the accumulation of proline showed anomolous behaviour in that labelled proline passed into the cells, but uptake was followed by rapid loss of radioactivity (see, for example, strain DHP 21, Fig. 4). Mutants of this type will be discussed fully in a later publication.

Several workers have shown changes in permeability in mutants selected for resistance to amino acid analogues. Failure of a toxic analogue to enter the cell serves to exclude it from the site (or sites) at which toxicity is exerted and accounts fully for resistance to the analogue. It is commonly found that failure to transport an analogue is accompanied by failure of the corresponding natural amino acid to enter the cell (Schwartz *et al.* 1959; Ferroluzzi-Ames, 1964; Lester, 1966; Shifrin *et al.* 1966; see also reviews by Moyed, 1964; Fowden *et al.* 1967). In the present investigation the impairment of the proline permease in dehydroproline-resistant strains was substantiated by decreased incorporation of exogenous proline into protein (Fig. 8). Presumably 3,4-dehydroproline and azetidine-2-carboxylic acid were excluded from protein by the same mechanism.

Strains selected for resistance to azetidine did not show cross-resistance to 3,4-dehydroproline. In some strains the proline permease was normal; proline excretion, if it occurred at all, was weak. The mechanism of resistance in these strains is under investigation. The majority of the azetidine-resistant strains isolated possessed an impaired capacity for uptake of proline (Fig. 4), though the residual permease activity was sufficient to meet the needs of protein synthesis, at least at 10° (Fig. 8). The behaviour of these mutants is of interest since growth was inhibited by 3,4-dehydroproline yet they still incorporated this analogue into protein. It was shown that the affinity of the proline permease of one of these strains (AZ 642) for proline and 3,4-dehydroproline was comparable with that of the parent strain, but affinity for azetidine was considerably reduced. Thus, strain AZ642 displayed a highly selective effect towards proline analogues, owing its resistance to azetidine to the capacity to exclude this compound from the cell. Other isolates behaved similarly, but it should be noted that the method of selection of azetidine-resistant strains (see Methods) makes it possible that most of these cultures were derived from a single clone and hence genetically identical.

Although in the parent organism affinity of the proline permease for 3,4-dehydroproline was comparable with that for proline itself, its affinity for azetidine was somewhat lower. This difference, however, was not apparent in the exchange of intracellular labelled proline with extracellular (unlabelled) proline or proline analogues. The rates of exchange of proline, 3,4-dehydroproline and azetidine were virtually

identical (Fig. 6). The azetidine-resistant strain AZ 642 failed to promote the exchange reaction between intracellular proline and extracellular proline, 3,4-dehydroproline or azetidine (Fig. 7). Kessel & Lubin (1962) described an *Escherichia coli* mutant lacking proline permease activity which also lacked the capacity to exchange internal and external proline at 0°. They concluded that uptake and exchange are closely related phenomena. The isolation of AZ 642, a strain unable to promote exchange yet possessing a permease which, though impaired, was still capable of proline uptake, suggests that the two processes may not be closely interrelated. Other experiments (Britten & McClure, 1962; Britten, 1965) lead to the same conclusion. For example, uptake could not occur at 0° or in the absence of an energy source, though exchange was rapid under these conditions.

Excretion of proline implies failure of the metabolic control of proline biosynthesis. Proline-excreting dehydroproline-resistant derivatives of *Escherichia coli* K-12 and azetidine-resistant strains of *Salmonella typhimurium* SW1061 which excrete proline have also been obtained, but not yet studied in detail (Tristram, unpublished observations). In *E. coli* proline is formed from glutamate, via glutamic γ-semialdehyde and its spontaneous cyclization product, Δ^1-pyrroline-5-carboxylic acid. The overall conversion of glutamate to Δ^1-pyrroline-5-carboxylic acid is inhibited by proline, 3,4-dehydroproline and, to a lesser extent, by azetidine (Strecker, 1957; Baich & Pierson, 1965; Tristram & Thurston, 1966). Formation of Δ^1-pyrroline-5-carboxylic acid by a proline-excreting 3,4-dehydroproline-resistant mutant had lost its sensitivity to proline (Baich & Pierson, 1965). At least the earlier enzymes of proline biosynthesis are subject to enzyme repression by the final product of the pathway (Tristram & Thurston, 1966). Preliminary experiments have shown that some of the analogue-resistant strains which excrete proline are no longer subject to repression; in others the formation of Δ^1-pyrroline-5-carboxylic acid is insensitive to end-product inhibition (Neale & Tristram, unpublished observations). The genetic study of dehydroproline- and azetidine-resistant strains has commenced and should lead to recognition of proline regulatory genes and also the gene (or genes) determining the proline permease.

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REFERENCES

ALGRANATI, I. D. (1963). Determination of initial rates in enzymic non-linear progress reactions. *Biochim. biophys. Acta* **73**, 152.

BAICH, A. & PIERSON, D. J. (1965). Control of proline synthesis in *Escherichia coli*. *Biochim. biophys. Acta* **104**, 397.

BEERSTECHER, E. (1950). Bacterial inhibition by a penicillin moiety. *Proc. Soc. exp. Biol. Med.* **73**, 200.

BRITTEN, R. J. (1965). The concentration of small molecules within the microbial cell. *Symp. Soc. gen. Microbiol.* **15**, 57.

BRITTEN, R. J. & MCCLURE, F. T. (1962). The amino acid pool in *Escherichia coli*. *Bact. Rev.* **26**, 292.

COHEN, G. N. & KEPES, A. (1962). Permeation. In *The Bacteria; a Treatise on Structure and Function*, Ed. by I. C. Gunsalus and R. Y. Stanier, vol. 4, p. 179. New York: Academic Press.

COHEN, G. N. & MUNIER, R. (1959). Effets des analogues structuraux d'amino acides sur la croissance, la synthèse de protéines et la synthèse d'enzymes chez *Escherichia coli*. *Biochim. biophys. Acta* **31**, 347.

DIXON, M. & WEBB, E. C. (1964). *Enzymes*, 2nd ed. London: Longmans.

DREYFUSS, J. & PARDEE, A. B. (1966). Regulation of sulphate transport in *Salmonella typhimurium*. *J. Bact.* **91**, 2275.

ECHOLS, H., GAREN, A., GAREN, S. & TORRIANI, A. M. (1961). Genetic control of repression of alkaline phosphatase in *E. coli*. *J. molec. Biol.* **3**, 425.

FERROLUZZI-AMES, G. (1964). Uptake of amino acids by *Salmonella typhimurium*. *Archs Biochem. Biophys.* **104**, 1.

FOWDEN, L., LEWIS, D. & TRISTRAM, H. (1967). Toxic amino acids: their action as antimetabolites. *Advauc. Enzymol.* **29**, 89.

FOWDEN, L., NEALE, S. & TRISTRAM, H. (1963). Effect of 3,4-dehydro-DL-proline on growth and protein synthesis. *Nature, Lond.* **199**, 35.

FOWDEN, L. & RICHMOND, M. H. (1963). Replacement of proline by azetidine-2-carboxylic acid during biosynthesis of protein. *Biochem. biophys. Acta* **71**, 459.

GOTTLIEB, A. A., FUJITA, Y., UDENFRIEND, S. & WITKOP, B. (1965). Incorporation of *cis*- and *trans*-4-fluoro-L-prolines into proteins and hydroxylation of the *trans* isomer during collagen biosynthesis. *Biochemistry* **4**, 2507.

KEMPNER, E. S. & COWIE, D. B. (1960). Metabolic pools and the utilization of amino acid analogues for protein synthesis. *Biochim. biophys. Acta* **42**, 401.

KESSEL, D. & LUBIN, M. (1962). Transport of proline in *Escherichia coli*. *Biochim. biophys. Acta* **57**, 32.

LESTER, G. (1966). Genetic control of amino acid permeability in *Neurospora crassa*. *J. Bact.* **91**, 677.

LUBIN, M., KESSEL, D. H., BUDREAU, A. & GROSS, J. D. (1960). The isolation of bacterial mutants defective in amino acid transport. *Biochim. biophys. Acta* **42**, 535.

MAUGER, A. B. & WITKOP, B. (1966). Analogues and homologues of proline and hydroxyproline. *Chem. Rev.* **66**, 47.

MOYED, H. S. (1964). Biochemical mechanisms of drug resistance. *A. Rev. Microbiol.* **18**, 347.

MUNIER, R. & COHEN, G. N. (1959). Incorporation d'analogues structuraux d'aminoacides dans les protéines bactériennes au cours de leur synthèse *in vivo*. *Biochim. biophys. Acta* **31**, 378.

NEALE, S. & TRISTRAM, H. (1963). Effect of *O*-methyl-DL-threonine and *O*-methyl-DL-serine on growth and protein synthesis in *Escherichia coli*. *J. Bact.* **86**, 1241.

NEALE, S. & TRISTRAM, H. (1964). Incorporation of 3,4-dehydroproline into alkaline phosphatase of *E. coli*. *6th Int. Cong. Biochem.* Abstracts **4**, 121 (p. 325).

NEALE, S. & TRISTRAM, H. (1965). Loss of ability to concentrate proline in *Escherichia coli*: basis of resistance to proline analogues. *J. gen. Microbiol.* **39**, vii.

PETERSON, P. J. & FOWDEN, L. (1965). Purification, properties and comparative specificities of the enzyme prolyl-transfer ribonucleic acid synthetase from *Phaseolus aureus* and *Polygonatum multiflorum*. *Biochem. J.* **97**, 112.

RICHMOND, M. H. (1962). The effect of amino acid analogues on growth and protein synthesis in microorganisms. *Bact. Rev.* **26**, 398.

RICHMOND, M. H. (1965). The enzymic basis of specific antibacterial action by structural analogues. *Biol. Rev.* **40**, 93.

ROBERTS, R. B., ABELSON, P. H., COWIE, D. B., BOLTON, E. T. & BRITTEN, R. J. (1955). Studies of biosynthesis in *Escherichia coli*. *Publs Carnegie Instn*, no. 607, p. 1.

SCHWARTZ, J. H., MAAS, W. K. & SIMON, E. J. (1959). An impaired concentrating mechanism for amino acids in mutants of *Escherichia coli* resistant to L-canavanine and D-serine. *Biochim. biophys. Acta* **32**, 582.

SHIFRIN, S., AMES, B. N. & FERROLUZZI-AMES, G. (1966). Effect of the α-hydrazino analogue of histidine on histidine transport and arginine synthesis. *J. biol. Chem.* **241**, 3424.

SMITH, L. C., RAVEL, J. M., SKINNER, C. G. & SHIVE, W. (1962). 3-4-Dehydroproline, a proline antagonist. *Archs Biochem. Biophys.* **99**, 60.

STRECKER, H. J. (1957). The interconversion of glutamic acid and proline. I. The formation of Δ^1-pyrroline-5-carboxylic acid from glutamic acid in *Escherichia coli*. *J. biol. Chem.* **225**, 825.

TRISTRAM, H. (1960). The adaptive degradation of L-histidine by *Paracolobactrum aerogenoides*. *J. gen. Microbiol.* **23**, 425.

TRISTRAM, H. & THURSTON, C. F. (1966). Control of proline biosynthesis by proline and proline analogues. *Nature, Lond.* **212**, 74.

UNGER, L. & DEMOSS, R. D. (1966). Action of a proline analogue, L-thiazolidine-4-carboxylic acid, in *Escherichia coli*. *J. Bact.* **91**, 1556.

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The Aerosol Survival of *Escherichia coli* B in Nitrogen, Argon and Helium Atmospheres and the Influence of Relative Humidity

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SUMMARY

The survival of *Escherichia coli* strain B sprayed from distilled water into atmospheres of nitrogen, argon and helium, as a function of relative humidity (RH) at an aerosol age of 30 min. was good at low RH, while at high RH values regions of marked instability occurred. At high RH differences in survival were observed, indicating that the gas atmospheres were not completely biologically inert. The results indicated that the initial evaporation rates of the aerosol droplets did not influence the long-term survival of *E. coli* B in the aerosol. An alternative reason for the importance of RH is discussed, together with considerations of death mechanisms.

INTRODUCTION

The aerosol survival of *Escherichia coli* depends upon several variables (Cox, 1965, 1966*a*, *b*, 1967; Cox & Baldwin, 1966, 1967). These include an air stress at low relative humidity (RH) caused by the toxic action of oxygen, possibly modified by trace contaminants (Cox & Baldwin, 1967). Hess (1965) found that *Serratia marcescens* and *E. coli* B were also killed by oxygen at low RH. At high RH, regions were found where *E. coli* survival was particularly sensitive to RH, in a manner that was dependent upon the spray fluid and collecting fluid (Cox, 1966*a*, *b*, 1967; Cox & Baldwin, 1966). Relative humidity changes before collection also influenced survival, either beneficially or detrimentally, depending upon the strain of *E. coli* used and the nature of the spray and collecting fluids (Cox, 1966*b*, 1967). These results obtained with different collecting fluids and RH changes showed that the death mechanisms of *E. coli* in nitrogen atmospheres must be influenced by the manner in which water re-enters the bacteria after collection. In certain instances all the deaths can be attributed to the manner in which water re-enters the bacterium, e.g. *E. coli* B and COMMUNE sprayed from raffinose into nitrogen at high RH (Cox, 1966*a*, *b*). Microbial survival in aerosols was reviewed in general terms by Anderson & Cox (1967).

The present paper reports the survival of *Escherichia coli* B sprayed from distilled water into atmospheres of nitrogen, argon and helium. The object of this work was to determine whether these atmospheres were really inert and to examine the possibility that the initial evaporation rates of the aerosol droplet influence the long-term survival of *E. coli* B in the aerosol. A further possibility, namely that protective agents operate by a modification of this rate, was also considered.

METHODS

The techniques used were as previously reported (Cox, 1966*a*), except that only *Escherichia coli* B sprayed from distilled water was studied. Since wet- and dry-bulb thermometer readings were used as a measure of the relative humidity (RH), it was necessary to determine the psychrometric constant for argon and helium so that wet-bulb depression could be converted into RH. As far as is known psychrometric tables exist only for air. The technique was to determine the dew-point as a function of wet-bulb depression in various atmospheres. From these data the psychrometric constants were determined and psychrometric tables calculated. The dew-point hygrometer was very kindly lent by Mr W. R. Sparks of the Meteorological Office, Bracknell.

Anderson (1966) and Cox (1966*a*) provided evidence that *Bacillus subtilis* var. *niger* spores were not altogether satisfactory as a tracer, since under certain conditions they lost viability, especially at high RH. Anderson 1966 developed a tracer technique based on labelling *Echerichia coli* B with ^{14}C. This method was examined as an alternative to the use of *B. subtilis* var. *niger* spores, but the results of 28 experiments showed the technique to be unsuitable for the present work. The reasons for reverting to *B. subtilis* var. *niger* as a tracer are given later in the Results.

RESULTS

The tracer technique

The use of a radioactive tracer necessitates the collection of a much larger number of bacteria than does the spore-tracer technique, so that high aerosol densities and long sampling times are needed to give radioactive counts of ten times (or greater) the background count. As a consequence the ^{14}C technique required a 3-jet Collison spray (Green & Lane, 1957) in place of the 1-jet spray as used previously (e.g. Cox, 1966*a*). During aerosol formation considerably greater cooling occurred in the 3-jet than in the 1-jet spray so that the control of temperature and RH was much inferior to that when using a 1-jet spray. As had been the practice previously (e.g. Cox, 1966*a*), samples were removed from the spray pot both before and after spraying, with the intention of checking for any spray damage to *Escherichia coli*. In all of the 28 experiments it was observed that the radioactive count increased after spraying, often by as much as 30%, without a concomitant increase in the count of *E. coli* B and *Bacillus subtilis* var. *niger* which was also added as a second tracer. These results tended to exclude the possibility that concentration of the spray fluid occurred through evaporation during spraying. Ultrasonic treatment, or extraction with trichloroacetic acid, of *E. coli* B did not produce a similar increase in radioactive count. The phenomenon thus remains unexplained; it threw considerable doubt on the radioactive count per bacterium. An investigation of the effect of adding sucrose to the phosphate-buffer collecting fluid could not be made because sucrose caused quenching during the scintillation processes involved in the radioactive assay. The extent of quenching was variable and time-dependent and was also related to the degree of radioactivity in the sample to be assayed. For these three reasons the results on aerosol survival presented later were obtained by using a 1-jet spray and *B. subtilis* var. *niger* spores as tracer.

Psychrometric constants

The % relative humidity (RH) is obtained from the relationship,

$$RH = 100 \times (e/e_a)\%,$$

where e_a is the saturation vapour pressure at the ambient temperature, *Ta*, and e is given by the expression $e = e_w - A(Ta - T_w)$, where e_w is the saturation vapour pressure at the wet-bulb temperature T_w, and A is the psychrometric constant. According to the theory of August & Apjohn (Penman, 1958) the value of A may be calculated from $A = (p.Cp)/(E.L)$ where p = total pressure, Cp = specific heat of gas at constant pressure, E = ratio of densities of water vapour and dry gas at the same temperature (*Ta*), and L = latent heat of vaporization of water at the temperature *Ta*. For air, the value of A calculated as above gives excellent agreement with that used for the derivation of the psychrometric tables of, for example, Marvin (1941) and of the *Handbook of Chemistry and Physics* (1948). The values of A calculated and determined experimentally for the particular conditions used (e.g. a well-ventilated wet bulb) are given in Table 1. The units of pressure were mm. of mercury and of temperature were degrees centigrade. The agreement between the calculated and experimental values of A for air and nitrogen was excellent, while argon gave reasonable agreement; however, the agreement for helium was very poor and suggests that the simple formula of August & Apjohn does not always apply.

Table 1. *Psychrometric constants of the gases used*

Measurements were made at 26·5° over a relative humidity range of 50–16%

Gas	A (calc.)	A (expt.)	No. of determinations	S.D.
Air	0·499	0·506	13	0·020
Nitrogen	0·496	0·500	8	0·013
Argon	0·343	0·358	16	0·010
Helium	0·354	0·532	16	0·035

The aerosol survival in nitrogen

Cox (1966*b*) showed that *Escherichia coli* strain COMMUNE gave variants which could have slightly differing survival characteristics. To minimize the possibility that the use of different variants of *E. coli* B might influence the comparisons of the survival in various atmospheres (even though the same stock culture was used throughout) the survival in nitrogen was determined again, both before and after the experiments with argon and helium. In all, 62 experiments in nitrogen were made; Fig. 1 gives the results for the survival at 26·5° and at an aerosol age of 30 min., for collection into phosphate buffer, with and without M-sucrose. For clarity not all the experimental results are given in Fig. 1. The previous data (Cox, 1966*a*; Cox & Baldwin, 1966) showed minima in the survival versus RH curves at 97%, 85% and 50% RH and also poor survival at 100% RH. The present data are very similar, except that the minimum at 50% was not detected and that the minimum at 85% split into a double minimum (or else reproducibility in this region was poor). Since the survival in argon (see later) showed a similar pattern and also the reproducibility of the results in nitrogen at RH values to one side of the minima was good, the former explanation seems more likely.

The aerosol survival in argon

In general terms the survival in argon at an aerosol age of 30 min. for collection in phosphate buffer with and without M-sucrose was very like that in nitrogen, in that instability occurred between 80 % and 90 % RH and between 97 % and 100 % RH, as shown in Fig. 2. Closer examination suggested that the actual position of the minima might be slightly different. A sharp peak (mean of 6 determinations) occurred at 86 % RH, which coincided with a maximum for survival in nitrogen, within the limits of RH measurements. The effect of the collecting fluid was somewhat similar for argon and nitrogen, except in the region of 85 % RH.

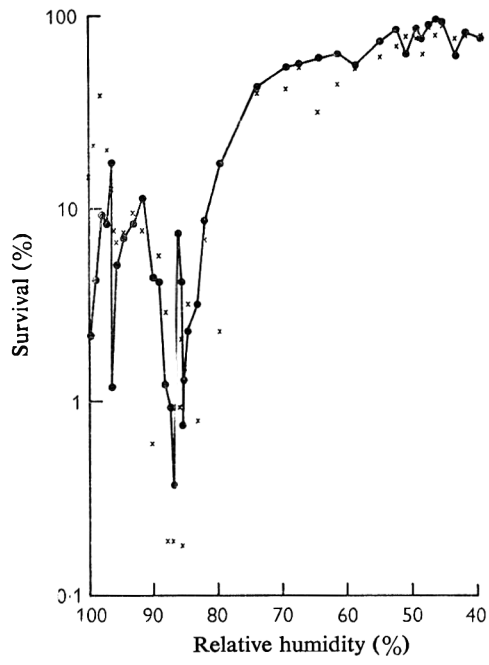

Fig. 1

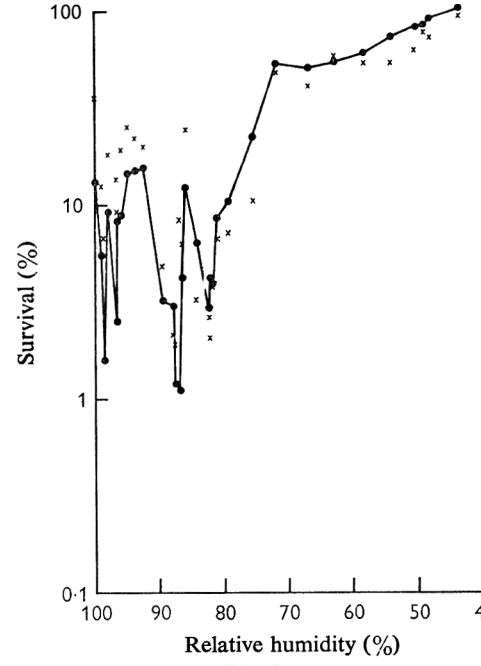

Fig. 2

Fig. 1. The aerosol survival of *Escherichia coli* B sprayed from distilled water into nitrogen as a function of RH, at an aerosol age of 30 min. and at 26·5° ●, Collection into phosphate buffer; ×, collection into phosphate buffer + M-sucrose

Fig. 2. The aerosol survival of *Escherichia coli* B sprayed from distilled water into argon as a function of RH, at an aerosol age of 30 min. and at 26·5° ●. Collection into phosphate buffer; ×, collection into phosphate buffer + M-sucrose

The aerosol survival in helium

Because of the very high cost of helium only limited experiments could be made with it. However, the data (Fig. 3) at an aerosol age of 30 min. for collection in phosphate buffer with and without M-sucrose are sufficient to show great similarity to the survival in nitrogen and argon. Instability occurred between 80 % and 90 % RH, at 97 % RH and at 100 % RH. However, the collection difference between phosphate buffer with and without M-sucrose was probably more marked than with the other

gases in the unstable regions. Like argon and nitrogen, there was a slight peak at 86% RH, within the limits of RH measurements. For all three gases the survivals at low RH were similar.

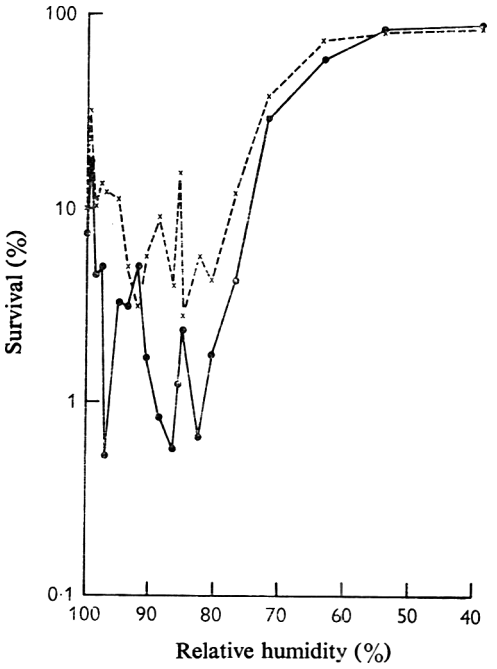

Fig. 3. The aerosol survival of *Escherichia coli* B sprayed from distilled water into helium as a function of RH, at an aerosol age of 30 min. and at 26·5° ●, Collection into phosphate buffer; ×, collection into phosphate buffer + M-sucrose

DISCUSSION

The results show that the overall survival patterns for *Escherichia coli* B sprayed from distilled water into nitrogen, argon and helium atmospheres were similar. These data are like those previously reported for the survival of this organism in nitrogen (Cox, 1966*a*, *b*; Cox & Baldwin, 1966), with the exception that the minimum at 50% RH was not detected in the present work. The effect was probably due to the use of variants; *E. coli* strain COMMUNE also gives rise to variants (Cox, 1966*b*). Susceptibility tests to phages T1–T7 inclusive did not show any difference between the present *E. coli* B and that previously used. Anderson (1966) found minima in the survival of *E. coli* B at different RH values, which approximately correspond with those previously reported (Cox, 1966*a*, *b*; Cox & Baldwin, 1966). A further possible difference between the latter results and those of the present study was that the minimum at approximately 85% RH was in fact a double minimum. Owing to the uncertainty in measuring RH to an accuracy of greater than ±1·0% it is difficult to be sure in the case of nitrogen whether the double minimum existed or whether reproducibility in this region was poor. It is suggested that a double minimum does in fact exist, since the results in argon and helium also indicated a double minimum, and because reproducibility of results at RH values to one side of the minima was good. Results such

as these emphasize the need for an instrument that accurately measures RH to an order of $\pm 0{\cdot}2$ RH % or better.

Biological inertness of the three gases

A possible difference between the survival in the three gases was that the corresponding RH values for one part of the double minima were 85·7 %, 82·5 % and 82·5 %, respectively, for nitrogen, argon and helium, whereas the other part was $86{\cdot}8 \pm 0{\cdot}3$ % RH for all three gases. The difference between nitrogen and argon is outside the experimental error, while the value of 82·5 % for helium agrees very well with that of argon. Hence there would seem to be a slight difference between nitrogen and argon or helium. Also differences occurred at high RH in terms of the method for collection. As a consequence of these findings it is suggested that slight differences in behaviour do exist for aerosols of *Escherichia coli* B sprayed from distilled water into nitrogen, argon and helium, and that these atmospheres are not truly inert. This indicates that the gaseous atmosphere is involved in the death processes which occur at high RH, and which are independent of the presence of oxygen (Cox & Baldwin, 1967). It is possible that the actual water structure is involved in these death processes, and in which case the gases may operate through modification of this structure. It is known that nitrogen and argon, but not helium, form gas hydrates (clathrates) with water and modify the water lattice (van der Waals & Platteeuw, 1959). Also all three gases can help stabilize clathrates of a more polarizable solute which is also present (van der Waals & Platteeuw, 1959). It is also possible that the gases and water compete for the same sorption sites.

Influence of initial evaporation rate on survival.

Cox (1965; 1966*a*) discussed the possibility that aerosol survival is influenced by the initial evaporation rate of the aerosol droplet, and Poon (1966) concluded from his investigation of the aerosol survival of *Escherichia coli* that the rate of death and the initial rate of water evaporation followed a similar relationship with respect to RH and temperature. Webb (1959) also tried to correlate rate of death with initial rate of water evaporation.

The data presented in the present paper are suited for a critical examination of the relationship between death and evaporation rate. As shown, at high RH, zones exist where *Escherichia coli* B sprayed from distilled water is particularly susceptible to death in nitrogen, argon and helium. Hence if the degree of killing is related to the initial evaporation rate, then the position of the zones in the survival versus RH curves in helium would have been very different to those in nitrogen and argon, for the reasons outlined below. The approach of Eisner, Quince & Slack (1960) was used to calculate the initial evaporation rate of water droplets, namely the equations (1) and (2) below.

$$\frac{dm}{dt} = \frac{4\pi MD}{RT}(f.p_t - p_\theta)\frac{a}{(D/aV\alpha + 1)}, \tag{1}$$

where

dm/dt = rate of evaporation of a droplet radius, a,

M = molecular weight of water,

D = diffusion coefficient for water vapour into the gas,

f = relative humidity as a fraction,

p_t = vapour pressure of water at the ambient absolute temperature, T,
p_θ = vapour pressure of water at the droplet temperature, θ,
R = gas constant,
$V = (RT/2\pi M)^{\frac{1}{2}}$
α = coefficient of evaporation,

and

$$\theta = \frac{LMD(f.p_t - p_\theta)}{KRT(D/aV\alpha + 1)} \qquad (2)$$

where L is the latent heat of vaporization of water, and K is the thermal conductivity of the gas.

It is necessary to calculate the equilibrium temperature θ of an evaporating water droplet, because water is comparatively volatile and cooling of the droplet occurs during evaporation, and hence affects the vapour pressure of the water. At 85% RH and 26·5° the rate of evaporation in helium is approximately twice that in nitrogen. At this temperature the evaporation rate in nitrogen at 85% RH would be achieved in helium at 93% RH. Hence if the initial evaporation rate of an aerosol droplet influenced the degree of killing of *Escherichia coli* B, then the position of the minimum in helium would have been at 93% RH and not at 82·5%. Such a change would be very readily detected; in fact it clearly did not occur (Fig. 3). Hence it is possible to conclude that viability does not depend upon the initial rate of evaporation. Further support for this statement comes from a study of equation (1), which indicates that the evaporation rate increases as the RH decreases. Yet the present work (Figs. 1–3) and previous work (Cox 1966*a*, *b*, 1967; Cox & Baldwin, 1966) show that in inert atmospheres the survival is greater at low rather than at high RH values. For *E. coli* such studies and comparisons must be made in inert atmospheres because at low RH, oxygen is toxic (Hess, 1965; Cox & Baldwin, 1967) and therefore such comparisons of aerosol survival in air would be invalid.

The rate of evaporation in helium is greater than that in nitrogen at a given temperature and humidity for two reasons: (i) the diffusion coefficient of water vapour into helium is much greater than into nitrogen (Schwertz & Brow, 1951); (ii) the thermal conductivity of helium is very much greater than that of nitrogen (*Handbook of Chemistry and Physics*, 1965).

The influence of relative humidity on survival.

Because the degree of killing does not depend upon the initial rate of evaporation of the aerosol droplet it seems very likely, as suggested by Cox (1966*a*), that protective agents do not operate through a modification of initial evaporation rate. Since the initial evaporation rate has been excluded as being related to the degree of killing, it is of interest to consider in what other way RH can influence survival. Scott (1958), Webb (1960) and Bateman, Stevens, Mercer & Carstensen (1962) have shown that the water content of bacteria is related to RH. The nature of the relationship is fairly typical of the water sorption isotherms of other biological materials (e.g. Bull, 1951). A plot of water sorbed versus RH yields an S-shaped curve, with the slope being greatest between 70 and 100% RH, i.e. a small change in RH in this range produces a comparatively large change in water content. It is suggested that RH operates by controlling the water content of the bacterium which in turn is related to the

mechanism of death. Such an explanation also provides a reason why the survival of *Escherichia coli* is so dependent upon RH, since the region of critical RH zones is in the 70–100% range where water content changes rapidly with RH. When survival is plotted as a function of water content of the bacterium, rather than RH, the regions where *E. coli* is unstable are much broadened. Cox (1966*a*) suggested that DNA might be affected by aerosolization owing to the formation of hydrates which occurs in a semi-reversible manner and produces a biologically inactive moiety. Benbough (1967) has shown that aerosolization does not impair DNA synthesis in *E. coli*, although RNA synthesis may be slightly affected. The reason that *E. coli* B when collected from the aerosol at high RH and subjected to phage T7 does not reproduce this phage (Cox & Baldwin, 1966) would therefore not seem to be caused by inability to synthesize DNA. The impairment of RNA synthesis would not seem to be sufficiently great to suppress drastically colony formation and phage T7 reproduction. The cause of these phenomena for *E. coli* B collected from the aerosol at high RH remains unexplained. However, the manner in which water re-enters the bacterium after collection from an aerosol can play a very important role in the death mechanisms which occur for aerosolized *E. coli* stored at high RH (Cox, 1966*a*, *b*).

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REFERENCES

ANDERSON, J. D. (1966). Biochemical studies of lethal processes in aerosols of *Escherichia coli*. *J. gen. Microbiol.* **45**, 303.

ANDERSON, J.D . & COX, C. S. (1967). Microbial survival. *Symp. Soc. gen. Microbiol.* **17**, 203.

BATEMAN, J. B , STEVENS, C. L., MERCER, W. B. & CARSTENSEN, E. L. (1962). Relative humidity and the killing of bacteria: the variation of cellular water content with external relative humidity or osmolality. *J. gen. Microbiol.* **29**, 207.

BENBOUGH, J. E. (1967). Death mechanisms in airborne *Escherichia coli*. *J. gen. Microbiol.* **47**, 325.

BULL, B. B. (1951). *Physical biochemistry*. New York: John Wiley and Sons Inc. London: Chapman and Hall Ltd.

COX, C. S. (1965). The mode of action of protecting agents. *1st Int. Symp. on Aerobiol.*, p. 345. Published by Naval Biological Laboratory, Naval Supply Center, Oakland, California.

COX, C. S. (1966*a*). The survival of *Escherichia coli* atomized into air and into nitrogen from distilled water and from solutions of protecting agents, as a function of relative humidity. *J. gen. Microbiol.* **43**, 383.

COX, C. S. (1966*b*). The survival of *Escherichia coli* in nitrogen under changing conditions of relative humidity. *J. gen. Microbiol.* **45**, 283.

COX, C. S. (1967). The aerosol survival of *Escherichia coli* Jepp sprayed from protecting agents into nitrogen atmospheres under shifting conditions of relative humidity. *J. gen. Microbiol.* **49**, 109.

COX, C. S. & BALDWIN, F. (1966). The use of phage to study causes of loss of viability of *Escherichia coli* in aerosols. *J. gen. Microbiol.* **44**, 15.

COX, C. S. & BALDWIN, F. (1967). The toxic effect of oxygen on the aerosol survival of *Escherichia coli* B. *J. gen. Microbiol.* **49**, 115.

EISNER, H. S., QUINCE, B. W. & SLACK, C. (1960). The stabilization of water mists by insoluble monolayers. *Discuss. Faraday Soc.* **30**, 86.

GREEN, H. L. & LANE, W. R. (1957). *Particulate Clouds: Dusts, Smokes and Mists*. London: E. and F. N. Spon Ltd.

Handbook of Chemistry and Physics (1948). Relative humidity from wet and dry bulb thermometers, p. 1922. 30th ed. Cleveland, Ohio, U.S.A.: Chemical Rubber Publishing Co.

Handbook of Chemistry and Physis (1965). Thermal conductivity of gases, p. E-2. 46th ed. Cleveland, Ohio, U.S.A.: Chemical Rubber Publishing Co.

HESS, G. E. (1965). Effects of oxygen on aerosolized *Serratia marcescens*. *Appl. Microbiol.* **13**, 781.

MARVIN, C. F. (1941). Psychrometric tables. *W.B.* no. 235. Washington: United States Government Printing Office.

PENMAN, H. L. (1958). *Humidity*. The Institute of Physics; Monographs for Students. London: Chapman and Hall Ltd.

POON, C. P. C. (1966). Studies on the instantaneous death of airborne *Escherichia coli*. *Am. J. Epidem.* **84**, 1.

SCHWERTZ, F. A. & BROW, J. E. (1951). Diffusivity of water vapour in some common gases. *J. chem. Phys.* **19**, 640.

SCOTT, W. J. (1958). The effect of residual water on the survival of dried bacteria during storage. *J. gen. Microbiol.* **19**, 624.

VAN DER WAALS, J. H. & PLATTEEUW, J. C. (1959). Clathrate solutions. *Advanc. chem. Phys.* **2**, 1.

WEBB, S. J. (1959). Factors affecting the viability of airborne bacteria. I. Bacteria aerosolized from distilled water. *Can. J. Microbiol.* **5**, 649.

WEBB, S. J. (1960). Factors affecting the viability of airborne bacteria. III. The role of bonded water and protein structure in the death of airborne cells. *Can. J. Microbiol.* 6, 89.

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The Application of Computers to the Classification of Streptococci

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SUMMARY

Computers were used for numerical studies as an aid to the classification of streptococci. The results obtained with 75 biochemical and physiological tests of 216 strains were examined by three different computer programmes. Two of these programmes formed clusters in general agreement with the well-established species and also formed three clusters among the viridans-like streptococci. These 3 clusters seem to provide a basis for the recognition of 3 divisions among the streptococci which are at present usually described as lacking adequate distinguishing characters.

INTRODUCTION

The streptococci form a group of bacteria of considerable medical and veterinary importance yet, with the exception of one or two species, their classification is very unsatisfactory. There has been a tendency to attribute over-riding importance to a single character, namely the so-called group antigen, but various workers have shown that this may lead to the allocation to one taxon of quite disparate organisms as, for example, among strains of serological group K (Williams, 1956), group M (Skadhauge & Perch, 1959), and group D (Deibel, 1964). It seems that a satisfactory classification will be achieved only by taking into account several different characters, and attempting to define taxa on the basis of overall similarity of the constituent strains. In an attempt to unravel some of the taxonomic problems of this genus, a collection of some 350 strains of streptococci was assembled and subjected to a large number of tests.

Numerical taxonomy appeared to offer a good method for examining the collection of strains, even if, with Mayr (1965), one does not necessarily accept the thesis that one is bound to analyse the occurrences of large numbers of attributes chosen at random, or to accept machine-formed clusters as the final outlines of taxa. The first analysis of 216 strains, by using a programme devised by Rogers & Tanimoto (1960), yielded clusters which often conflicted seriously with those recognized by tradition or intuition. The same material was subsequently examined with two other programmes, which yielded clusters that are reasonably conformable one with the other, and are intuitively more 'satisfactory'. This paper reports an outline of the classification of streptococci as derived from this numerical analysis and a comparison of the classifications indicated by the use of the three different computer programmes

METHODS

Strains examined. The strains in the collection do not represent any natural population, but were selected to give as wide a representation as possible of strains which have in the past been given more or less specific names, and to include a large number of strains that cannot at present be adequately classified. Some of the strains were from culture collections but many were freshly isolated; most of the strains were of human origin.

A total of 216 strains is included in this study: 203 streptococci, 10 aerococci, 2 leuconostocs, and 1 pediococcus. The attributes of the aerococci included have been given in detail elsewhere (Colman, 1967).

Reference numbers for each strain are given in Table 2. The numbers on the extreme left of the table are the catalogue numbers of the strains. The letters NCTC show the strains supplied by the National Collection of Type Cultures, Colindale, the letters FW indicate that the strains were, with the exception of those numbered 1, 5, 6, 7, 9, 13, 15, 16, 32, 34, 35, 36, 37, 49, and 250, isolated at the Wright Fleming Institute. The prefix RB is given to strains isolated by Dr K. B. Rogers, Birmingham. S, with the exception of S 1230 and S 1545, indicates strains from the collection of Professor M. Seelemann formerly of Kiel, and BU indicates strains from the collection of the late Dr Coralie Baldovin-Agapi of Bucharest. Numerous other workers generously sent strains for examination and as far as possible their numbers are used to denote the strains they sent; these strains make up the rest of the table.

Bacteriological tests. This study is based on the results from a total of 75 tests on each strain as listed in Table 1.

One of the computer programmes, the single-linkage method, allows tests to be considered as 'qualitatives' or 'quantitatives' as well as 'dichotomies'. With this programme the tests scored as qualitatives are: arrangement in glucose broth; changes in horse blood agar; colonial form on sucrose agar. Tests scored as quantitatives are: ability to grow on bile agar; growth in NaCl broth; growth in tellurite broth; presence of trace or readily detectable amounts of rhamnose in cell-wall hydrolysates. All these tests are scored as dichotomies in the other programmes.

The programmes used

Single-linkage method. The programme for this method was prepared by G. J. S. Ross of The Rothamsted Experimental Station and is a version in I.C.T. Orion machine language of J. C. Gower's programme originally prepared for the Elliott 401 digital computer. All procedures with this programme were carried out by Mr Gower and other members of the staff of the Rothamsted Experimental Station. The tests with this programme are of three kinds and are called dichotomies, qualitatives and quantitatives. Dichotomies are tests in which the response is binary, for example, the response of the indicator in the test for the production of acid from lactose is recorded as either + or −. Qualitatives are tests with mutually exclusive attributes such as the changes produced in the blood agar beneath the colonies. These are recorded as one of the following: haemolytic, greening, indifferent. Quantitative tests are those which form a series such as the ability to grow in 4 % and 6·5 % NaCl broth.

These different sorts of tests affect the coefficient of similarity in different ways.

Dichotomies are scored as follows. When lactose is fermented by two strains, *i* and *j*, in calculating the similarity coefficient of these two strains there is a count of 1 in both numerator and denominator. When lactose is fermented by only one strain there is a count of 1 only in the denominator. When lactose is not fermented by either strain there is no score in either numerator or denominator. This method of scoring does not take a negative match as a similarity; the arguments for and against this procedure were discussed by Sneath (1962). In the scoring of qualitative tests, when

Table 1. *The bacteriological tests used in this work*

Arrangement in glucose broth: pairs; chains
Changes in horse blood agar: haemolysis; greening; indifference (= no change)
Serological grouping procedures (23 sera): Lancefield groups A, B, C, D, E, F, G, H, K, L, M, N, O, P, Q, R, S, and T; Ottens and Winkler types I, II, III, IV, and V (Williams, 1958)
Bile solubility (Anderson & Hart, 1934)
Optochin sensitivity (5 μg. disc)
Bacitracin sensitivity (0·1 U. disc)
Nitrofurazone sensitivity (10 μg. disc)
Growth on bile agar: 10% bile; 40% bile (Williams, 1958)
Growth on MacConkey agar
Growth in salt broth: 4% NaCl; 6·5% NaCl
Survival after heating at 60° for 30 min.
Growth at 45°
Ammonia from arginine
Colonial forms on 5% sucrose agar: colonies without special features; fleshy colonies; adherent colonies; watery colonies
Hydrolysis of hippurate (Ayers & Rupp, 1922; sulphuric acid reagent)
CAMP test (Munch-Petersen & Christie, 1947)
Production of hyaluronidase (Henriksen & Joner, 1962)
Production of β-glucuronidase (Williams, 1954)
Acetoin produced from glucose (α-naphthol reagent)
Hydrolysis of starch (Andrewes, 1930)
Splitting of aesculin (ferric citrate as indicator)
Production of acid from fermentable substances (phenol red as indicator): glucose; maltose, glycerol (aerobically); glycerol (anaerobically); mannitol; sorbitol; arabinose; lactose; sucrose; trehalose; raffinose; salicin; inulin
Detection of cell-wall residues: glycerol; anhydroribitol; rhamnose readily detectable; rhamnose present in trace amounts; glucose; galactose; galactosamine; fucosamine (Colman & Williams, 1965)
Growth in tellurite broth: 0·1% potassium tellurite; 0·025% potassium tellurite; 0·01% potassium tellurite

the strains are both haemolytic there is a score of 1 in both numerator and denominator, but there is no score in either numerator or denominator when one strain is greening and the other haemolytic. In the scoring of quantitative tests there is a range of 2 when measuring the ability to grow in NaCl broth, and there is a count of 1 in both numerator and denominator when both strains grow in 6·5% NaCl broth, and a count of ½ in the numerator and 1 in the denominator when one strain grows only in 4% and the other in both 4 and 6·5% NaCl broth.

The 'similarity coefficient' of two strains is obtained by dividing the sum of the scores of similarity by the number of relevant tests. The similarity coefficients are sorted by the machine to produce the clusters. The strains are first examined for any that are identical; there were no completely identical pairs of strains in our collection. The collection of similarity coefficients is then examined for strains similar

at the 97·5 % or a higher level. In our collection 3 pairs of strains were linked at this level of similarity: the strains in the array in Table 2 numbered 9 and 10, 95 and 96, and 159 and 160. Restricting our attention to the first pair, no further strains were added at the 95 % level, but at the 92·5 % level a third strain, numbered 8, was added. At the 90 % level of similarity, 3 more strains were added to this cluster, making at this level of similarity a cluster of 6 strains. By this method each strain will eventually join a cluster and the clusters will eventually join together until all the strains in the collection form one cluster.

The Harrison method. This programme was prepared by P. J. Harrison of Imperial Chemical Industries, Wilmslow, originally for the classification of chemicals. (Harrison 1968). Our data were given to Mr Harrison, and he and other members of the staff of I.C.I. did all the computer procedures.

In this programme all tests are treated as dichotomies and the probabilities of the attributes not being randomly distributed are calculated. In the calculation of the similarity of a pair of strains, a weighting of 2 was given to positive matches and a weighting of 1 to the negative matches and mismatches. The 'expected' distribution of attributes is calculated from the matrix of similarities and this serves as a reference for calculating the odds that a particular combination of attributes could arise by chance. For each probability level in turn the largest cluster is found, and then the next largest cluster with the following conditions: that a strain previously clustered is not to be the centre of a new cluster, at least one-half of the strains in the new cluster are to be strains not previously clustered, and all clusters must contain at least five strains. The programme is repeated while clusters can be formed that fulfil these conditions.

Table 2 contains the clusters formed at the 99 and 80 % significance levels. As would be expected, not all strains in the collection were clustered, and some strains appeared in more than one cluster.

The Rogers & Tanimoto method. Rogers & Tanimoto (1960) described a method for the classification of plants and International Business Machines, London, made available to us a version of this programme (I.B.M. 7090/94 Taxonomy Application) which was run on the I.B.M. 7090 digital computer in the Centre for Computing and Automation at Imperial College. All tests were examined as dichotomies.

The computer first determines the similarity coefficients of all pairs of strains by dividing the number of attributes possessed in common by a pair of strains by the number of attributes possessed by either of them. The computer then makes a count for each strain of the number of strains with at least one attribute in common. The results of this count are used to provide the primary ranking of the strains. A strain with something in common with 100 other strains ranks above a strain with something in common with 99 other strains. As more than one strain may have something in common with 100 other strains the computer then performs a secondary ranking of the strains. This is done by multiplying together all the non-zero values of the similarity coefficients, using the negative of the binary logarithm. The logarithms of numbers less than 1 but greater than 0 are negative in sign, and the logarithm of the number increases in magnitude as the number becomes smaller. The strain that is ranked first among those having something in common with 100 other strains is the strain with the lowest sum. A geometrical analogy was used by Rogers & Tanimoto (1960) and they likened strains to points in space and similarity to distance, like strains

being separated by short distances. Multiplying together similarities and choosing the strain with the lowest sum should lead to the selection of a strain in the centre of a cluster as the strain at the head of the list, or the prime strain.

The cluster is formed from the results of the primary and secondary ranking of the strains. The strain at the head of the list is the centre or prime strain of the cluster and the second strain in the list provides the boundary. The cluster is formed from those strains which have a similarity coefficient, relative to those ranked first, greater than or equal to those ranked second. The strains forming the cluster are not further considered and the process of clustering is repeated while there are two or more strains remaining unclustered.

The size of the cluster is thus determined by the similarity of the second strain in the ranking to the prime strain. When the strain ranked second is very similar to the strain ranked first the cluster will be of two specimens only, or the second strain may provide the boundary for a homogeneous cluster. When the second strain is dissimilar the cluster formed will contain many specimens with a wide range of similarity to the prime strain. All of these possibilities occurred.

The whole process was repeated three times with deletions of the most common attributes and the large clusters were also separately studied as discrete populations. The results of these additional runs did not appear to add anything to the results of the first examination of our strains, using all of the attributes. It is the result of the latter study that is given in Table 2.

RESULTS

The programmes

The table. The results of the computer studies are summarized in Table 2. Reading from the left the first column gives the catalogue number of the strain. The second column lists the serological group, if any, and also the specific name when this is well established; otherwise the appearance of the colonies on blood agar plates is listed. Changes in blood agar do not form an adequate basis for classification but they are useful descriptive characters frequently used in medical microbiological laboratories. The diagram is a summary of the results with the single linkage method and it shows the similarity level (at 75% or more) at which the strains are clustered. The first two strains in Table 2 are linked at the 90% level, which means that the coefficient of similarity of these two strains is equal to or greater than 90% but less than 92·5%. A third strain is added at 77·5% and this similarity could be with one or both of the first two strains; this strain appears third on the table because it was by chance further down the list of strains fed into the computer.

The series of numbers, 1 to 216, to the right of the diagram is the order in which the strains were printed out from the computer in the single-linkage method. It is these numbers which are referred to throughout this paper. Further to the right of Table 2 are the results obtained with the two other programmes. The strains in the clusters formed by these programmes have been re-arranged, within the clusters, to agree as closely as possible with the order of the strains in the single linkage method. An asterisk is used when a strain can be placed in the same relative position as it has with the single-linkage method. When strains cannot be aligned in this way the numbers of the strains in the single-linkage method are used to indicate the composition of the clusters.

Single-linkage method. In this method all strains were brought into a single cluster at 45% overall similarity, and 208 of the 216 strains were formed into one cluster at the 65% level. The limits of the smaller clusters formed at higher similarities and discussed below were not solely machine-determined but were also influenced by subsequent inspection. For instance, one could choose any one of the four strains, 105, 108, 112, or 116, as the lower limit of the cluster that began with strain 88.

The admission of a new strain to a cluster, at perhaps the 90% level will occur when its similarity to any strain in the cluster is 90% or higher, as is indicated by the name—single-linkage method. Sokal & Sneath (1963) suggested that this method could lead to the linkage of two clusters through the similarity of two strains, one in each cluster, the other members of the cluster being more dissimilar. Something of this sort appears to have occurred with our material. Strains 41, 42, and 43 (indifferent streptococci) are seen in Table 2 to come between strains 37 to 40 and 44 to 48, all of these nine being salivarius strains. The two separate clusters of salivarius are joined at the 80% level by the linkage of strains 39 and 45, and they are joined to the cluster bounded by strains 4 and 28 by the similarity at the 80% level of strains 17 and 46. The strains 41, 42, and 43 separate the salivarius strains because at the 80% level these too are linked to the large cluster and these three strains were listed in the data after the salivarius strains 37 to 40 and before the salivarius strains 44 to 48.

The Harrison method. At the 99% probability level, 87 strains were formed into 7 clusters, and at the 80% level 130 strains were formed into 9 clusters.

The Rogers & Tanimoto method. When all attributes were used in the analysis 17 clusters were formed, but as can be seen from Table 2 the composition of the clusters appears to be unrelated to the conventional classification of the streptococci. Therefore, the presentation of the results with this programme is restricted to a listing, in Table 2, of the composition of these clusters.

The strains

The well-established species. Our collection of strains includes representatives of species established by conventional methods. It is useful to present the results of numerical studies with these strains before considering the results with other strains.

Streptococcus agalactiae (Lancefield group B) has a number of distinctive properties, including ability to grow on 40% bile agar, growth in 4% but not 6·5% NaCl broth, ability to hydrolyse hippurate and give the CAMP reaction (Munch-Petersen & Christie, 1947). It is heat sensitive and does not split aesculin (Munch-Petersen, 1954). With the single-linkage method all 7 strains included formed one cluster as strains 168 to 174. With Harrison's method these same 7 strains are clustered at the 99% significance level, with the addition of one strain of Lancefield's group G which was the only strain clustered in Harrison's method in a way that caused surprise. Harrison's method was used with a double weighting on positive matches, and this strain of group G does have some features in common with the group B strains, such as ability to grow on 40% bile, production of acid from glycerol aerobically, production of hyaluronidase and β-glucuronidase and the splitting of arginine, but it does not grow in salt broth, split hippurate, or give the CAMP reaction. In the single-linkage method the overall similarity of this strain to the 7 agalactiae strains was in all cases less than 70%.

Streptococcus salivarius is also a distinctive species and if we wish to classify strains on the basis of a single distinctive character, levan production characterizes

a reasonably homogeneous species (Williams, 1956). Ten salivarius strains are included and in the single-linkage method they are found as strains 37 to 40, 44 to 48, and strain 118. Strain 118 has a similarity coefficient of greater than 70% with 6 of the other 9 strains. Nine of the 10 salivarius strains are included in a cluster with other strains, and 5 of these form a discrete cluster at the 82·5% level. At the 99% probability level Harrison's method clusters 7 of these 10 strains and includes in the cluster strain 80, a strain which does not produce levan but which is remarkably like the salivarius strains.

Streptococcus faecalis and other enteric streptococci have been studied previously by numerical methods. Colobert & Blondeau (1962) examined a collection of enterococci which fulfilled Sherman's (1938) criteria. They examined 173 strains for the occurrence of 38 characters, and since their study was restricted to enterococci they included more tests of value for distinguishing these strains than was done in the present work. They found two major divisions, one containing varieties of *S. faecalis*, var. *faecalis* and var. *liquefaciens*, the other division almost forming a linear series with centres of population formed by *S. faecium*, *S. durans*, and some strains for which Colobert & Blondeau propose the name *S. innominatus*. Raj & Colwell (1966) examined 40 enterococci and 8 other streptococci and found all the enterococci to form a homogeneous cluster (they included *S. bovis* but excluded *S. equinus* from the enterococci). This finding could have been determined by their choice of tests which was heavily weighted by appearance in culture.

In the present work the single-linkage method formed, at the 85% level, a homogeneous cluster of *Streptococcus faecalis* and *S. faecium* strains, numbered 133 to 144. Harrison's method at the 99% probability level formed the same cluster with the addition of five strains, one strain each of *S. faecium*, serological group Q and *S. durans*, and two strains of aerococci which occurred in another cluster. There are more *S. faecalis* and *S. faecium* strains in this collection than *S. durans*, *S. bovis* and *S. equinus* strains and this is a possible reason why the faecalis and faecium strains were clustered together. However, they are very similar.

The aerococci are similar to the enterococci in a number of properties such as salt tolerance and bile tolerance. They can be mistaken for streptococci (Colman, 1967). The aerococci formed a discrete cluster by the single-linkage method, strains 200 to 207, and by Harrison's method, strains 200 to 209. As mentioned above, some strains by Harrison's method occurred in both the cluster of the aerococci and the enterococci.

There are within the present collection other strains that would generally be accepted as distinctive populations at a level equivalent to that of species. They appear only in the clusters formed with the single-linkage method because of the limit imposed on cluster size in Harrison's method. Three strains of *Streptococcus pyogenes* (Lancefield group A) are found clustered 1 to 3, five strains of Lancefield group G (haemolytic and large-colony variety) are clustered 83 to 87, three haemolytic strains of Lancefield group C are clustered at 130 to 132, and three strains of pneumococcus are clustered at 164 to 166.

Some proposed specific groupings. In describing the residues found in hydrolysates of whole cell walls it was noted by Colman & Williams (1965) that some indifferent (non-haemolytic) streptococci of Lancefield groups A, C, and G differed from the more common haemolytic members of these serological groups and that their cell-wall residues were similar to those from indifferent streptococci of Lancefield's group F

and from the strains called *S. milleri* (Guthof, 1956). All these strains show a number of similar physiological and biochemical reactions, such as resistance to bacitracin and nitrofurazone, ability to split arginine, production of acetoin from glucose, and ability to grow in 0·01 % potassium tellurite. It is therefore not surprising that these strains should be classified together in both the single-linkage method and in Harrison's method. At the 85% similarity level in the single-linkage method the cluster was formed of the strains numbered 5 to 23, and 17 of these strains occurred in the 19 strains clustered at the 99% probability level in Harrison's method. One of the strains added at the 82·5% level to the cluster in the single-linkage method was a representative of the 'minute' group G streptococci (Bliss, 1937).

Another cluster, the strains numbered 55 to 69, was made up of strains that either belonged to serological group H or produced a dextran-like polysaccharide from sucrose (the diagnostic character of *S. sanguis*; Hehre & Neill, 1946) or had both these characters. The strains in this cluster all contained readily detectable amounts of rhamnose in the cell wall and split arginine yielding ammonia, most produced acid from the sugars lactose, sucrose, trehalose and salicin, and most grew in the presence of 10% bile.

A third large cluster, strains 88 to 112, contained strains that either gave a precipitate with sera of the groups O, K, or M, or else lack recognized 'Group' or 'Ottens' antigens. These strains had no readily detectable amounts of rhamnose in the cell wall and usually yielded the residue anhydroribitol in the cell-wall hydrolysates; most of the strains gave zones of greening on blood agar, few gave acid from trehalose and salicin, and none produced ammonia from arginine. These strains would usually be called *Streptococcus viridans* or *S. mitis*. The same set of characters was also possessed by strains 113 to 115, which would usually be classified as *S. sanguis*; these three strains belong to the second serological type established among the *S. sanguis* strains by Washburn, White & Niven (1946). If we classify solely on ability to produce a polysaccharide from sucrose these three strains would be called *S. sanguis*, but on overall similarity they belong with the strains numbered 88 to 112.

Thus the results of the numerical studies suggest that the viridans-like streptococci of man contain three recognizable divisions in addition to the well-established species *Streptococcus salivarius*.

DISCUSSION

In so far as there is a formal classification of streptococci, it is based on a combination of physiological, biochemical and serological reactions. Some bacteriologists, in their enthusiasm for a diagnostic scheme that is excellent for the haemolytic streptococci originally included, have gone so far as to make serological grouping the sole basis of classification. I cannot accept this view and I shall later present evidence, some of which is apparent here, that a classification based solely on serological group will yield physiologically heterogeneous taxa in at least all the groups from A to N. I believe that the definition of species should be based on the possession of a number of distinctive, independent and highly correlated attributes. Among the well-documented species isolated from man I suggest, on the basis of past experience, that *Streptococcus agalactiae*, *S. pneumoniae*, *S. pyogenes*, *S. salivarius* and *S. faecalis* and its varieties, fulfil these criteria.

There are many streptococci outside these well-defined species and it is among

these that taxonomic confusion has reigned. While it is possible that they form a series with a common gene pool, it seemed worth applying the methods of numerical taxonomy to seek clusters of strains that possess characters highly correlated within the cluster, but which rarely occur together outside the cluster. Two of the three programmes used in this work produced clusters that conformed generally to the well-established taxa mentioned above, and they both produced approximately the same three divisions among the less well-known strains. The third clustering method, that devised by Rogers & Tanimoto, produced clusters that seemed to be unrelated to the conventional classification of streptococci. This last method was originally used in a study of higher plants, for which its originators clearly found it to give satisfactory results; a possible reason for its failure in the present work may lie in the nature of the data; ours is largely binary in form; theirs was multi-state qualitative.

The three population nodes found among the viridans-like streptococci can usefully serve as the centres of aggregated species, which can be subdivided, if so desired for special purposes, into sub-species. The central node of one of these aggregated species contains representatives of the indifferent streptococci of Lancefield groups A, C, F and G, some strains that contain antigens of the Ottens or Ottens & Winkler series, and some strains that lack recognized antigens but which were called *Streptococcus milleri* by Guthof (1956). The central node of the second aggregated species contains representatives of the species *S. sanguis* and members of serological group H. The third central node contains representatives of the serological groups O, K, and M, and this node can readily be distinguished from the other two because of the combination of unusual cell-wall composition and unusual biochemical reactions.

The data presented in this paper indicates strongly that if a bacterial population contains clusters of strains with attributes distributed in the non-random manner that is characteristic of a species, then numerical methods can demonstrate these clusters. However, evidence is also presented that these methods will only do this when the right programme is chosen.

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REFERENCES

ANDERSON, A. B. & HART, P. D. (1934). The lysis of pneumococci by sodium desoxycholate. *Lancet* ii, 359.

ANDREWES, F. W. (1930). Note on the fermentation of starch by certain haemolytic streptococci. *J. Path. Bact.* **33**, 145.

AYERS, S. H. & RUPP, P. (1922). Differentiation of hemolytic streptococci from human and bovine sources by hydrolysis of sodium hippurate. *J. infect. Dis.* **30**, 388.

BLISS, E. A. (1937). Studies upon minute streptococci. III. Serological differentiation. *J. Bact.* **33**, 625.

COLMAN, G. (1967). Aerococcus-like organisms isolated from human infections. *J. clin. Path.* **20**, 294.

COLMAN, G. & WILLIAMS, R. E. O. (1965). The cell walls of streptococci. *J. gen. Microbiol.* **41**, 375.

COLOBERT, L. & BLONDEAU, H. (1962). L'espèce *Streptococcus faecalis*. I. Étude de l'homogénéité par la méthode adansonnienne. *Annls Inst. Pasteur, Paris* **103**, 345.

DEIBEL, R. H. (1964). The group D streptococci. *Bact. Rev.* **28**, 330.

GUTHOF, O. (1956). Über pathogene 'vergrünende Streptokokken'. Streptokokken-Befunde bei dentogenen Abszessen und Infiltraten im Bereich der Mundhöhle. *Zentbl. Bakt.* (*Abt. I. Orig.*) **166**, 553.

HARRISON, P. J. (1968). Cluster Analysis. *Appl. Statist.* (in the Press).

HEHRE, E. J. & NEILL, J. M. (1946). Formation of serologically reactive dextrans by streptococci from subacute bacterial endocarditis. *J. exp. Med.* **83**, 147.

HENRIKSEN, S. D. & JONER, P. E. (1962). Production of hyaluronic acid by a strain of *Streptococcus zymogenes*. *Acta path. microbiol. scand.* **56**, 70.

MAYR, E. (1965). Numerical phenetics and taxonomic theory. *Syst. Zool.* **14**, 73.

MUNCH-PETERSEN, E. (1954). Note on *Streptococcus agalactiae*. *Int. Bull. bact. Nomencl. Taxon.* **4**, 129.

MUNCH-PETERSEN, E. & CHRISTIE, R. (1947). On the effect of the interaction of staphylococcal β-toxin and group-B streptococcal substance on red blood corpuscles and its use as a test for the identification of *Streptococcus agalactiae*. *J. Path. Bact.* **59**, 367.

RAJ, H. & COLWELL, R. R. (1966). Taxonomy of enterococci by computer analysis. *Can. J. Microbiol.* **12**, 353.

ROGERS, D. J. & TANIMOTO, T. (1960). A computer program for classifying plants. *Science, N.Y.* **132**, 1115.

SHERMAN, J. M. (1938). The enterococci and related streptococci. *J. Bact.* **35**, 81.

SKADHAUGE, K. & PERCH, B. (1959). Studies on the relationship of some alpha-haemolytic streptococci of human origin to the Lancefield group M. *Acta path. microbiol. scand.* **46**, 239.

SNEATH, P. H. A. (1962). The construction of taxonomic groups. *Symp. Soc. gen. Microbiol.* **12**, 289.

SOKAL, R. R. & SNEATH, P. H. A. (1963). *Principles of Numerical Taxonomy*, p. 180. San Francisco and London: W. H. Freeman and Company.

WASHBURN, M. R., WHITE, J. C. & NIVEN, C. F., Jr. (1946). Streptococcus s.b.e.: immunological characteristics. *J. Bact.* **51**, 723.

WILLIAMS, R. E. O. (1954). Glucuronidase production by serotypes of *Streptococcus pyogenes*. *J. gen. Microbiol.* **10**, 337.

WILLIAMS, R. E. O. (1956). *Streptococcus salivarius* (vel *hominis*) and its relation to Lancefield's group K. *J. Path. Bact.* **72**, 15.

WILLIAMS, R. E. O. (1958). Laboratory diagnosis of streptococcal infections. *Bull. Wld Hlth Org.* **19**, 153.

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Specific Chromosomal Affinity of a Resistance Factor

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SUMMARY

R factors, responsible for transmissible drug resistance in Enterobacteriaceae, convert their hosts into donors of genetic material, but fewer recombinants for chromosomal genes are produced than with F. For one R factor, at least, this was merely a consequence of less frequent conjugation. With a mutant where conjugation was not repressed in contrast to the wild type, this R factor, R 1, was shown to behave like an F′ factor and to transfer the bacterial chromosome at high frequency from a fixed origin near the *try* genes.

INTRODUCTION

The factors responsible for transmissible drug resistance in Enterobacteriaceae resemble the F factor of *Escherichia coli* K 12 in conferring on their hosts the ability to donate the factor by conjugation to other bacteria (Watanabe, 1963). The existence of F was originally detected by the ability of an F^+ bacterium to produce genetic recombinants with an F^- recipient (Lederberg, Cavalli & Lederberg, 1952; Hayes, 1953), but the presence of an R factor may be detected directly by transfer of the drug-resistance determinants as a part of the factor itself. The recipient is thus converted to a drug-resistant bacterium at the same time as it in turn becomes able to conjugate and to pass on its resistance. R factors occasionally bring about transfer of chromosomal genes and the formation of genetic recombinants between bacteria both of which are F^- (Sugino & Hirota, 1962). In contrast to the situation with F, however, chromosomal recombinants appear at very low frequency, corresponding ordinarily only to about 10^{-7} to 10^{-8} of the R^+ population instead of 10^{-4} to 10^{-5} of an F^+ population.

Several different R^+ strains of *E. coli* K 12 58–161/*sp met*$^-$ were tested for their ability to give genetic recombinants with an F^- recipient, J 6-2 *pro*$^-$ *his*$^-$ *try*$^-$, and one of the factors, R 1, was remarkable for the relatively large number of *try*$^+$ recombinants produced. While *pro*$^+$ and *his*$^+$ recombinants occurred at a frequency of 10^{-7} to 10^{-8} per R^+ donor cell, there were more than 10^{-5} *try*$^+$ colonies. Tryptophan independence was not conferred, like drug resistance, by the R factor itself; crosses between R 1$^+$ F^- bacteria and a recipient strain mutated in the genes *pyrF*, *try* and *purB* (Signer, Beckwith & Brenner, 1965; see Fig. 1) showed that it was indeed this region of the bacterial chromosome that was transferred.

Almost every F^+ bacterium can conjugate, but with other factors conjugation is

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repressed and happens with only a minority of the cells (Ozeki, Stocker & Smith, 1962; Meynell & Datta, 1966*a*; Meynell & Lawn, 1967). Since, when conjugation occurs it only occasionally leads to chromosome transfer, when it is infrequent the numbers of genetic recombinants must be correspondingly small. Recombination with R 1 was first studied with the wild-type R factor, but the nature of the preferential transfer of the *try* region could be investigated only after the isolation of a derepressed mutant, R 1 *drd* (Meynell & Datta, 1967), with which conjugation occurred about as often as with F, and which therefore allowed the rates of production of different classes of chromosomal recombinants with the two sex factors to be directly compared.

METHODS

Culture media. Defined medium was the mineral salts medium described by Tatum & Lederberg (1947), with the omission of asparagine and solidified with 1·5% (w/v) Davis N.Z. agar. In a few experiments, M9 medium (Adams, 1959) was used. These were appropriately supplemented with amino acids, purines and pyrimidines at 20 μg./ml., thiamine at 1 μg./ml. and sugars used as carbon source at 0·2% (w/v).

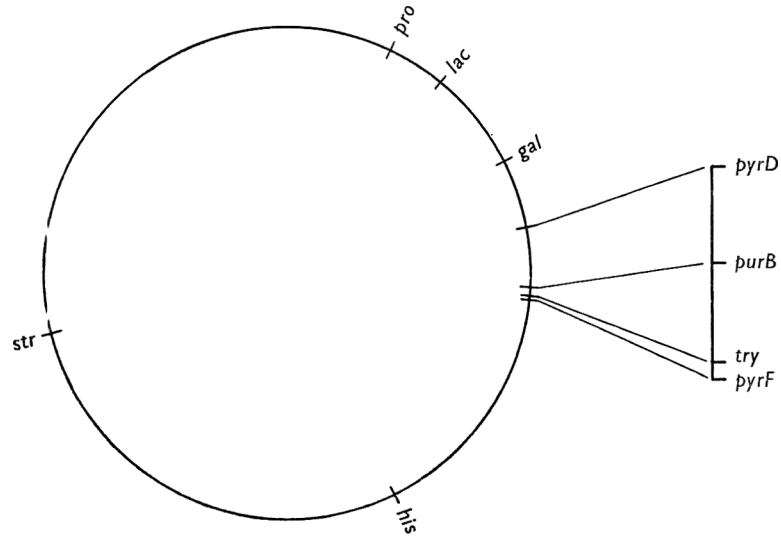

Fig. 1. The genetic map of *Escherichia coli* showing the loci which are referred to.

Broth was either Oxoid broth No. 2, or L-broth (Lennox, 1955) adjusted to pH 7·4; for nutrient agar, these were solidified with 1·35% (w/v) Davis N.Z. agar.

Bacteria. These were all derivatives of *Escherichia coli* K12 and are described in Table 1.

R factor. R 1 (Meynell & Datta, 1966*a*) confers resistance to chloramphenicol, kanamycin, ampicillin, streptomycin and sulphonamide. Mutants of R 1 whose conjugation function is no longer repressed have been isolated by Meynell & Datta (1967), and one of these, R 1 *drd-19*, was used in addition to the wild-type R factor. The R factor was introduced into a strain by conjugation, selecting for resistance to chloramphenicol (25 μg./ml.) added to defined medium supplemented to allow growth of the recipient, but not the donor, strain.

Bacterial crosses and measurements of frequency of R factor transfer. Donor and recipient strains were grown overnight in broth without shaking. Next day, the culture of the donor strain was diluted 1/10 in fresh broth and incubated for 2 hr without aeration until the bacterial concentration reached $2–5 \times 10^8$/ml. The temperature of incubation greatly affected the donor ability of the culture; at 35° or less very little chromosome or R transfer occurred. Mating mixtures contained 0·5 ml. of the donor

Table 1. *Bacterial strains: all derivatives of Escherichia coli K12*

Strains used as donors	Characters*
58–161 F^+	*met-1* (Lederberg, 1947)
58–161/*sp*	Spontaneous F-defective mutant of 58–161 F^+ (Hayes, 1953; Meynell & Datta, 1966*b*)
58–161 F^-	58–161 F^+ cured of F by acridine orange
J5–3 F^-	*pro-1 met-2* (Clowes & Rowley, 1954), cured of F by acridine orange
J5–3 F^+	J5–3 carrying F transferred from 58–161 F^+
K12F	Wild-type strain K12 cured of F by acridine orange
W1655 F^-	*met-1*(λ)$^-$ λ-*res* (Lederberg & Lederberg, 1953), cured of F by acridine orange
C600	*thr*$^-$ *leu*$^-$ *thi*$^-$ *lac*$^-$(λ)$^-$ F^- (Appleyard, 1954)
W1177	*thr*$^-$ *leu*$^-$ *thi*$^-$ *lac*$^-$ *str-r* F^- (Lederberg, 1951)
W677	*thr*$^-$ *leu*$^-$ *thi*$^-$ *lac*$^-$ F^- (Lederberg, 1950)
HfrC	Hfr Cavalli derived from 58–161 F^+ by integration of F giving *pro* as a leading marker (Cavalli-Sforza, 1950)
HfrB10	Derived from W1655 F^+ by integration of F giving *try* as a leading marker (Broda, 1967)
Strains used as recipients	
J6–2	*pro-2 his-1 try-1 lac*$^-$ (Clowes & Rowley, 1954), cured of F by acridine orange
J6–2 *str-r*	streptomycin-resistant mutant of J6–2
PA309	*thr*$^-$ *leu*$^-$ *thi*$^-$ *try*$^-$ *his*$^-$ *arg*$^-$ *lac*$^-$ *gal*$^-$ *mal*$^-$ *xyl*$^-$ *str-r*(λ)$^-$ λ-*res* F^- (kindly provided by Dr W. Hayes)
X195	*pro*$^-$ *his*$^-$ *try*$^-$ *met*$^-$ *purB*$^-$ *pyrF*$^-$ *tyr*$^-$ *lac*$^-$ *str-r* F^- (from the collection of the M.R.C. Laboratory of Molecular Biology, Cambridge, by courtesy of Dr J. Shapiro)
MS0	*pyrD*$^-$ *his*$^-$ *str-r* F^- (Shapiro, 1967)

* Location of genetic markers shown in Fig. 1.

culture and 4·5 ml. of the overnight recipient culture, and were rotated at 37° in a ½ oz. screw-cap bottle on an inclined turntable revolving at 33 rev./min. With wild-type R1 and with F^+ donors, the mixture was incubated for 60 min. and dilutions in buffer were then plated on appropriate media to isolate recombinants. In all except the preliminary experiments with R1 *drd-19*, the mating mixture was incubated for only 5 min. to allow cell-to-cell contacts to form, and was then gently diluted 1/100 into fresh warm broth (de Haan & Gross, 1962). This was held at 37° either for 60 min. or for the time stated in the experiments where mating was interrupted. Before plating, the mating pairs were separated by agitating the sample with a 'Whirlimixer' (Fisons Scientific Apparatus Ltd., Loughborough, Leicestershire). Samples were plated in a layer of soft agar, using 2·5 ml. volumes of 0·6% (w/v) agar in water; control experiments showed that as many recombinant colonies were produced by this method as by spreading the sample on the surface of the medium. Ability to synthesize a given nutrient was tested by omitting it from an otherwise complete defined medium; ability to utilize a particular sugar was tested by providing it as sole energy source.

Contraselection against the R^+ donor strain with streptomycin. The bacteria used as recipients were one-step mutants to high level streptomycin resistance and, although

streptomycin is one of the antibiotics to which the R factor R 1 confers resistance, the maximum concentration which allows normal growth of R 1$^+$ bacteria on nutrient agar is about 20 μg./ml. The concentration of streptomycin initially used to select against the donor strain was 250 μg./ml., but when this was shown to be insufficient to abolish growth of J 5-3(R 1) on defined medium (Pearce & Meynell, 1968), the concentration was increased to 5000 μg./ml., which was the least concentration needed to prevent completely the growth of the R 1$^+$ donor, but which still allowed normal growth of the highly streptomycin-resistant recipient strains.

RESULTS

Selected cases of recombinants

In preliminary tests of R$^+$ donors with a recipient strain J 6-2 *pro*$^-$ *try*$^-$ *his*$^-$ *str-r* the factor R 1 gave 10^{-7} to 10^{-8} *pro*$^+$ and *his*$^+$ recombinants per donor cell, while *try*$^+$ colonies appeared much more frequently, at a rate of 10^{-4} to 10^{-5}. The original donor strain, 58–161/*sp met*$^-$ carries a defective F factor (Meynell & Datta, 1966*b*) which might have been responsible for the observed recombination, so R 1 was re-tested in strain J 5-3 *pro*$^-$ *met*$^-$, an F$^-$ strain cured of F by acridine orange. Since donor and recipient were both *pro*$^-$ here as a result of mutations in closely linked genes, only the numbers of *his*$^+$ and *try*$^+$ recombinants could be compared; however, in crosses between J 5-3 (R 1) and J 6-2 *str-r* large numbers of *try*$^+$ colonies were again obtained (Table 2). Their *try*$^+$ gene was not an integral part of the R factor itself because about 200 times more recipients became drug-resistant than become tryptophan-independent, while any *try*$^+$ strain was converted into a high-frequency donor of the character when R 1$^+$ derivatives were selected for drug-resistance alone. While most *try*$^+$ recombinants were drug-resistant, 17 out of 423 were R$^-$; in addition, R$^-$ segregants of R$^+$*try*$^+$ recombinants remained *try*$^+$.

In the first crosses with strain J 6-2 *str-r pro*$^-$ *his*$^-$ *try*$^-$ as recipient, only 250 μg./ml. streptomycin was added to the medium to inhibit the donor (Pearce & Meynell, 1967) and it was observed that the plates without tryptophan used for scoring *try*$^+$ recombinants were covered with non-recombinant bacterial growth in contrast to those in which either proline or histidine had been omitted. This indicated considerable cross-feeding for tryptophan and suggested that the high incidence of *try*$^+$ recombinants might have resulted from mating on the plates. However, when strain J 6-2 *str-r* was replaced as recipient by strain X 195 carrying *pyrF*$^-$ and *purB*$^-$, loci closely linked to *try*$^-$, and the concentration of streptomycin was increased to 5000 μg./ml., the numbers of *pyrF*$^+$ and *purB*$^+$ recombinants were as high as those of *try*$^+$ recombinants, though when adenine or uracil was omitted there was no background growth, and presumably no cross-feeding and continued mating on the plates. Moreover, when the derepressed mutant of R 1, which gives a much higher rate of conjugation, was substituted for wild-type R 1, *try*$^+$ recombinant colonies could be counted after plating dilutions of the mating mixture which did not give any background growth.

Table 2 shows the frequencies of the different recombinant classes obtained with the various conjugation factors. With F, the frequency of recombinants for all markers was 1×10^{-5} to 2×10^{-4} and, although it was not unusual to find more recombinants for *purF*, *try* or *purB* than for other loci, the difference was seldom more than twofold. The small numbers of *pro*$^+$ recombinants using strain J 5-3 *met*$^-$ *pro*$^-$ as donor was

Table 2. *Escherichia coli* K12: *frequencies of different classes of selected recombinants expressed as number of recombinants per donor*

Factor	Cross: Donor	Cross: Recipient	*thr leu*	*pro*	*lac*	*gal*	*pyrD*	*purB*	*try*	*pyrF*	*his*	*tyr*	*arg*
R 1	J5-3	J6-2 *str-r*	—	$<1\times10^{-8}$	—	—	—	—	10^{-4}–10^{-5}	—	1×10^{-8}	—	—
R 1	J5-3	PA 309	1×10^{-7}	—	—	—	—	—	10^{-4}–10^{-5}	—	10^{-7}–10^{-8}	—	5×10^{-11}
R 1	J5-3	X 195	—	—	$<1\times10^{-7}$	—	—	1×10^{-5}	$1{\cdot}5\times10^{-5}$	$2{\cdot}5\times10^{-5}$	1×10^{-7}	1×10^{-7}	—
R 1	K 12F$^-$	PA 309	$2{\cdot}5\times10^{-7}$	—	—	—	—	—	$1{\cdot}5\times10^{-5}$	—	—	—	—
R 1 *drd-19*	J5-3	X 195	—	—	2×10^{-5}	—	—	$2{\cdot}8\times10^{-3}$	$3{\cdot}8\times10^{-3}$	6×10^{-3}	—	1×10^{-5}	—
R 1 *drd-19*	K 12F$^-$	PA 309	5×10^{-4}	—	—	—	—	—	8×10^{-3}	—	$2{\cdot}5\times10^{-5}$	—	—
R 1 *drd-19*	58-161/*sp*	J6-2 *str-r*	—	7×10^{-4}	—	—	—	—	2×10^{-2}	—	3×10^{-5}	—	—
R 1 *drd-19*	58-161 F$^-$	J6-2 *str-r*	—	2×10^{-4}	—	—	—	—	6×10^{-3}	—	2×10^{-5}	—	—
R 1 *drd-19*	W 1655 F$^-$	J6-2 *str-r*	—	2×10^{-4}	—	—	—	—	7×10^{-3}	—	5×10^{-6}	—	—
R 1 *drd-19*	C 600	J6-2 *str-r*	—	8×10^{-5}	—	—	—	—	4×10^{-3}	—	3×10^{-6}	—	—
R 1 *drd-19*	W 1177	X 195	—	—	—	3×10^{-5}	—	9×10^{-4}	1×10^{-3}	$1{\cdot}2\times10^{-3}$	—	3×10^{-5}	—
R 1 *drd-19*	W 677	X 195	—	—	—	—	—	4×10^{-4}	—	—	—	1×10^{-5}	—
R 1 *drd-19*	J5-3	MSO	—	—	—	—	2×10^{-4}	—	—	—	1×10^{-5}	—	—
F	J5-3	PA 309	1×10^{-5}	—	—	—	—	—	$2{\cdot}5\times10^{-5}$	—	6×10^{-6}	—	—
F	J5-3	X 195	—	6×10^{-6}	5×10^{-5}	—	—	7×10^{-5}	1×10^{-4}	$1{\cdot}5\times10^{-4}$	—	2×10^{-5}	—
F	58-161 F$^+$	J6-2 *str-r*	—	2×10^{-4}	—	—	—	—	2×10^{-4}	—	1×10^{-4}	—	—
F	W 1177	X 195	$3{\cdot}5\times10^{-5}$	—	—	—	—	—	1×10^{-5}	—	—	—	—
F	W 677	X 195	1×10^{-5}	—	—	—	—	2×10^{-5}	2×10^{-5}	—	—	7×10^{-6}	—
F	Hfr C	J6-2 *str-r*	—	$1{\cdot}5\times10^{-1}$	—	—	—	—	4×10^{-5}	—	5×10^{-5}	—	—
R 1 *drd-19* and F	Hfr C	J6-2 *str-r*	—	$1{\cdot}3\times10^{-1}$	—	—	—	—	1×10^{-2}	—	5×10^{-5}	—	—

due to close linkage of *pro*⁻ in donor and recipient. With R 1, recombinants for *pyrF*⁺, *try*⁺ and *purB*⁺ occurred at a frequency of 10^{-4} to 10^{-5}, about 100 times higher than for other markers.

R 1 *drd-19* is a mutant of the wild type R 1 in which conjugation is no longer repressed (Meynell & Datta. 1967). The frequency of recombinants for chromosomal genes with R 1 *drd-19* was increased to the same extent as the frequency of conjugation

Table 3. *Escherichia coli K12: comparison of recombination between strain J5-3 as donor and strain X195 as recipient mediated by R1 drd-19, wild-type R1 and F*

Factor	Ratio of frequencies of R transfer	Ratios of frequencies of selected recombinants				
		lac	*purB*	*try*	*pyrF*	*tyr*
R 1 *drd-19* compared with F	—	1	42	29	46	1
R 1 *drd-19* compared with R 1	250	—	343	280	325	—
F compared with R 1	—	—	8	9	7	—

Table 4. *Escherichia coli K12: patterns of segregation of unselected markers in crosses between J5-3 and X195*

Pattern of segregation of markers					J5-3 F⁺		Recombinants with J5-3 (R 1 *drd-19*)		J5-3 (R 1)	
lac	*purB*	*try*	*pyrF*	*tyr*	No.	%	No.	%	No.	%
S	o	o	o	o	39	67·2	48	55·75	—	—
S	1	1	[illegible]	o	14	24·0	29	33·7	—	—
S	1	o	o	o	3	5·0	3	3·5	—	—
S	1	1	o	o	2	3·4	1	1·15	—	—
S	o	o	1	o	o	o	2	2·3	—	—
S	o	1	1	o	o	o	2	2·3	—	—
S	1	o	1	o	o	o	1	1·15	—	—
o	S	o	o	o	21	34·4	11	12·6	6	9·5
o	S	1	o	o	2	3·3	3	3·45	2	3·2
o	S	1	1	o	18	29·5	69	79·3	53	84·0
o	S	o	1	o	1	1·6	1	1·15	1	1·6
1	S	1	1	o	9	14·7	2	2·3	1	1·6
1	S	1	c	o	4	6·5	1	1·15	o	o
1	S	o	c	o	4	6·5	o	o	o	o
o	S	o	c	1	2	3·3	o	o	o	o
o	1	S	1	o	30	49·2	29	33·7	22	40·7
o	o	S	1	o	23	37·7	49	57·0	28	51·8
o	1	S	c	o	1	1·6	3	3·5	o	o
1	1	S	1	o	6	9·8	o	o	o	o
o	o	S	o	o	1	1·6	5	5·8	4	7·4
o	1	1	S	o	25	43·0	23	26·1	21	30·0
o	o	1	S	o	14	24·0	50	56·8	28	40·0
o	o	o	S	o	7	12·0	13	14·7	20	28·6
o	1	o	S	o	o	o	1	1·15	o	o
1	1	1	S	o	12	20·6	1	1·15	1	1·4
o	o	o	o	S	52	88·0	24	43·7	5	100·0
o	1	1	1	S	5	8·4	25	45·45	o	c
o	o	1	1	S	2	3·4	3	5·4	o	o
o	1	1	o	S	o	o	2	3·6	o	o
o	1	o	o	S	o	o	1	1·3	o	o

1 = allele inherited from donor; o = allele inherited from recipient (Lederberg, 1951); S = donor allele selected.

measured by transfer of the R factor (Table 3), indicating that the numbers of genetic recombinants obtained with wild-type R 1 was limited principally because conjugation was infrequent. The mutant R 1 *drd-19* behaved exactly like the wild-type R 1 in giving about 100 times more recombinants for the chromosomal region *pyrF...try...purB* than for other genes, and the relative frequencies of three classes of recombinants for genes *pyrF*, *try* and *purB* were the same with R 1 *drd-19* as with R 1 (Table 2). Chromosomal recombinations brought about by the R factor could therefore be compared directly with that due to the sex-factor F, provided that the mutant R 1 *drd-19* was used, for then the frequencies of recombinant production were then no longer complicated by repression of mating in the wild type. In Table 3, R 1, R 1 *drd-19* and F are compared in crosses between strain J 5-3 as donor and strain X 195 as recipient. R 1 *drd-19* gave nearly as many recombinants as F for genes outside the *try* region, but about 30 times more for *pyrF*, *try* and *purB*. The same relationship between the numbers of recombinants for the markers *pyrF*, *try* and *purB*, and for other markers was observed with all lines of *E. coli* K 12 tested as donors (Table 2).

Unselected markers

The patterns of segregation of unselected markers are shown in Tables 4 and 5. Most of the alleles were those of the R^- parent indicating that, as with the F factor,

Table 5. *Pattern of segregation of unselected markers in crosses between F^+ and $R1^+$ donor strains and strain PA 309*

Pattern of segregation of markers										Recombinants with 58-161 (F)		Recombinants with J 5-3 (F)		Recombinants with J 5-3 (R 1)	
str	*mal*	*xyl*	*man*	*met*	*arg*	*thr leu*	*lac*	*try*	*his*	No.	%	No.	%	No.	%
C	o	o	o	C	S	o	o	o	o	—	—	—	—	8	57·15
C	o	o	o	C	S	1	o	o	o	—	—	—	—	3	21·45
C	o	o	o	C	S	1	1	1	1	—	—	—	—	2	14·3
C	o	o	o	C	S	o	o	o	1	—	—	—	—	1	7·15
C	o	o	o	C	o	S	o	o	o	27	96·4	14	100·0	5	50·0
C	o	o	o	C	o	S	o	1	o	1	3·6	o	o	3	30·0
C	o	o	o	C	1	S	o	1	o	o	o	o	o	1	10·0
C	o	o	o	C	1	S	o	o	o	o	o	o	o	1	10·0
C	o	o	o	C	o	o	o	S	o	75	77·4	37	90·0	233	92·0
C	1	o	o	C	o	o	o	S	o	o	o	o	o	7	2·7
C	o	o	1	C	o	o	o	S	o	o	o	o	o	4	1·58
C	o	o	o	C	o	o	o	S	1	4	4·1	4	10·0	o	o
C	o	o	o	C	o	1	o	S	1	o	o	o	o	1	0·39
C	o	o	o	C	o	1	o	S	o	18	18·55	o	o	8	3·15
C	o	o	o	C	o	o	o	o	S	6	75·0	—	—	4	57·0
C	o	o	o	C	o	o	o	1	S	2	25·0	—	—	1	14·3
C	o	o	o	C	1	o	o	1	S	o	—	—	—	1	14·3
C	o	o	o	C	o	1	o	o	S	o	o	—	—	1	14·3

1 = allele inherited from donor; o = allele inherited from recipient (Lederberg, 1951); S = donor allele selected; C = donor allele contraselected.

transfer occurred from the R^+ to the R^- cell. In the initial experiments, not shown in Tables 4 and 5, with wild-type R 1, using strain J 6-2 *str-r pro⁻ his⁻ try⁻ lac⁻* as recipient, only 6 out of 196 recombinants selected for *try*$^+$ also received *lac*$^+$, and when *pyrF*,

try or *purB* were selected, the chromosome fragment transferred did not often extend beyond this region. The relative numbers of recombinants for *pyrF*, *try* and *purB* (Table 2) suggest that these three genes were transferred in ordered fashion o...*pyrF*... *try*...*purB*, starting from a fixed origin as in an Hfr strain; further support for a fixed gradient of transfer, in which *pyrF* regularly preceded *purB*, was provided by the observation that amongst recombinants selected for *purB*$^+$ (the distal marker), 80–90% were also *pyrF*$^+$, whereas only 30% of the recombinants selected for *pyrF*$^+$ received *purB*$^+$. A strain, MSO, mutated in *pyrD*, situated about the same distance as *pyrF* to the other side of *purB* (Signer *et al.* 1965), was kindly provided by Dr J. Shapiro, and when crossed with strain J5-3 (R1 *drd-19*), *pyrD*$^+$ recombinants were produced at intermediate frequency, as expected (Table 2).

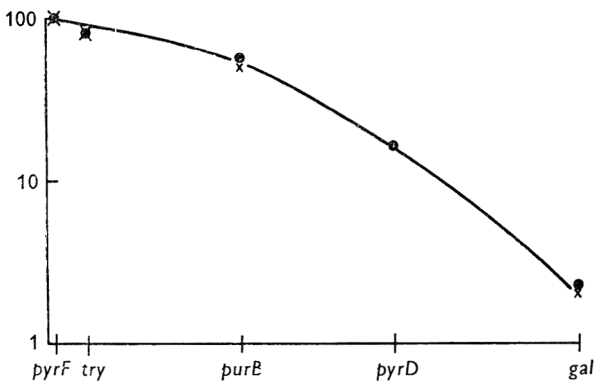

Fig. 2

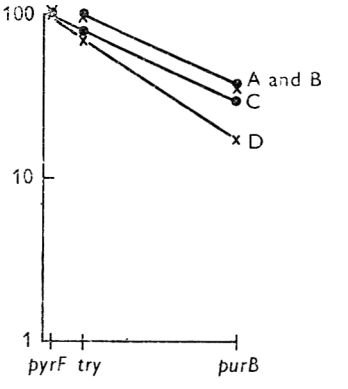

Fig. 3

Fig. 2. Comparison of the relative frequencies of recombinants for *pyrF*, *try*, *purB*, *pyrD* and *gal* with J5–3 (R1 *drd-19*) and HfrB10 as donors. The crosses, J5–3 (R1 *drd-19*) × X195 and HfrB10 × X195, were performed as described in Methods, the mating mixtures being diluted 1/100 after 5 min., and then allowed to stand for 1 hr before blending and plating. Map distances on the abscissa are as proposed by Signer *et al.* (1965), and the percentage of recombinants obtained with J5–3 (R1 *drd-19*) (●) and HfrB10 (×), relative to the frequency of *pyrF* recombinants in each case, are plotted on a logarithmic scale.

Fig. 3. Comparison of the frequencies of inheritance of the donor *try* and *purB* genes as unselected markers in recombinants selected for *pyrF*, and of the frequency of *purB* in recombinants selected for *try*, with J5–3 (R1 *drd-19*) and HfrB10 as donors. The percentage of recombinants with the marker in the cross J5–3 (R1 *drd-19*) × X195 (●) and the cross HfrB10 × X195 (×) are plotted on a logarithmic scale, the map distances on the abscissa being those proposed by Signer *et al.* (1965). Curves A and B selected marker *try*; curves C and D selected marker *pyrF*.

Comparison of strain J5-3 (R1 drd-19) with an Hfr strain

The donor behaviour of strain J5-3 (R1 *drd-19*) was compared with that of an Hfr strain by using HfrB10, which transfers in the order o...*try*...*lac*...*pro*...(Broda, 1967) and which is therefore likely to initiate chromosome transfer near the presumptive origin of strains carrying R1 *drd-19*.

Crosses of the donors HfrB10 and J5-3 (R1 *drd-19*) with the recipient X195 are compared in Figs. 2 and 3. In Fig. 2 the relative frequencies of recombinant classes selected for *pyrF*, *try*, *purB*, *pyrD* and *gal* are plotted logarithmically against the corresponding map distances as determined by Signer *et al.* (1965), and it can be seen that although the gradient of transfer was steeper than the simple exponential function

of distance from the origin (de Haan & Gross, 1962), precisely the same gradient was observed with J5-3 (R1 *drd-19*) as with HfrB10. Figure 3 shows the proportion of recombinants selected for the proximal gene, *pyrF*, that also received *try* and *purB*, and the proportions selected for *try* that received *purB*. Once again, essentially the same result is given by the two donor strains.

Times of entry of pyrF and purB genes

The production of $pyrF^+$ and $purB^+$ recombinants was measured in a cross between J5-3 (R1 *drd-19*) and X195 with interrupted mating. Figure 4 shows the curves obtained when successive samples were plated for recombinants inheriting $pyrF^+$ and $purB^+$. Extrapolating the ascending parts of the curves back to the abscissa to measure the time taken for transfer of a particular segment of chromosome (Wollman & Jacob,

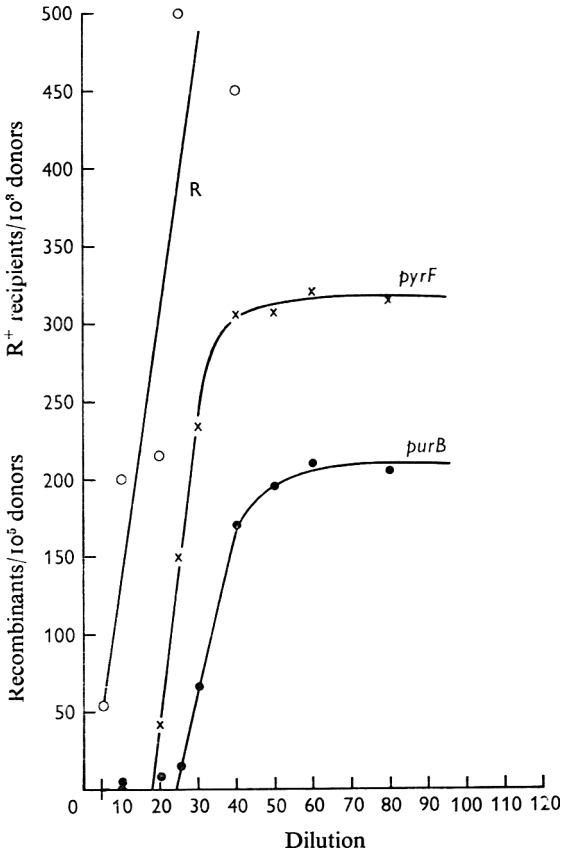

Fig. 4. Times of entry of *pyrF* and *purB* genes. The mating mixture consisted of J5-3 (R1 *drd-19*) 5×10^7/ml. and X195 5×10^8/ml. and was diluted 1/100 into fresh broth after 5 min. Samples taken at the times given on the abscissa were blended and then plated for recombinants inheriting $pyrF^+$ (×) and $purB^+$ (●), which are plotted as number of recombinants per 10^5 donor cells. The number of $purB^+$ recombinants, 350 per 10^5 donors, produced by a sample of the mating mixture which had been incubated without dilution for 120 min., showed that at least half of the total possible number of cell pairs had formed within 5 min. The curve for R factor transfer (○) plotted as number of R^+ recipients per 10^3 donors was obtained by plating samples on defined medium fully supplemented for growth of strain X195 and containing chloramphenicol 20 μg./ml.

1958; Wollman, Jacob & Hayes, 1956), the times of entry for *pyrF*$^+$ and *purB*$^+$ recombinants were shown to be separated by 6 min. The rare recombinant colonies appearing in the earliest samples presumably arose from mating pairs which had failed to separate. The maximum number of recombinants for *pyrF* was reached by about 40 min., and for *purB* by about 50 min., and by that time, there were about 1·6 times as many for *pyrF* as for *purB*, which agreed more or less with the relative numbers obtained when mating was uninterrupted for 60 min.

Figure 3 also shows the curve for R transfer. The actual numbers of drug-resistant colonies differed somewhat between experiments, depending largely on the time allowed for the R$^+$ recipient cells to express their resistance before plating on medium containing the drug. However, the shapes did not differ significantly.

Behaviour of an Hfr strain also carrying R 1 drd-19

The orientation of chromosomal transfer by Hfr donors is determined by the position of the sex-factor F. In strain HfrC, where F is between *pro* and *gal*, *pro* is one of the first genes to be transferred and *try* one of the last. Thus, many recombinants inherit the *pro* gene of the donor, whereas very few acquire *try*. Crosses were made between HfrC, both R$^-$ and carrying R 1 *drd-19*, and strain J62 *str-r* allowing 5 min. for cell contacts to form and then incubating a 1/100 dilution of the mixture for 60 min. before blending and plating to select *pro*$^+$, *his*$^+$ and *try*$^+$ recombinants. The presence of R 1 *drd-19* in HfrC did not appreciably alter the frequency of *pro*$^+$ recombinants (Table 2); however, the number of *try*$^+$ recombinants increased about 250-fold, and thus the R factor evidently directed the transfer of this particular chromosomal segment in HfrC just as it did in an F$^-$ strain. When wild-type R 1 was present in HfrC, the results were altogether comparable to those with R 1 *drd-19*, although the effect was masked to a large extent by the repression which R 1 exerts on conjugation, which greatly lowered the numbers of all classes of recombinants.

DISCUSSION

Although genetic recombinants for chromosomal markers are known to be produced during conjugation brought about by R factors (Sugino & Hirota, 1962), a distinction is drawn between conjugation factors like R factors and *col* I (Ozeki, Howarth & Clowes, 1961) on the one hand, and F on the other, in that recombination is more frequent with F. The number of recombinants, however, necessarily depends on the number of bacteria that conjugate, and both *col* I and R factors also differ from F in producing a repressor which prevents synthesis of the specific pilus, and thus conjugation, in all but a small minority of bacteria (Stocker, Smith & Ozeki, 1963; Monk & Clowes, 1964; Meynell & Datta, 1966*a*; Meynell & Lawn, 1967). In the case of R 1 at least, the lower rate of recombination observed with the wild-type R factor was largely due to less frequent mating, as was shown by using a mutant, R 1 *drd-19*, which does not produce repressor (Meynell & Datta, 1967).

The striking feature of recombination with R 1 is the relatively high frequency with which the *try* region of the donor chromosome appears among recombinants. This peculiarity of R 1 was difficult to analyse with the wild-type R factor, since it gave few recombinants of any kind, but using the de-repressed mutant R 1 *drd-19* it became clear that a genetic donor carrying R 1 transferred the bacterial chromosome as though

it were an Hfr strain with the F factor integrated between the *try* region and *his*, not far from *pyrF*. This phenomenon is distinct from the high frequency of recombinants for *met*, reported by Sugino & Hirota (1962), when R factors bring about conjugation in the Richter ♀3 strains. The ♀3 strain, though losing ability to conjugate, nevertheless retained the chromosomal origin near *met* characteristic of its parent Hfr strain (Richter, 1961); thus, when conjugation factors are introduced which restore its donor ability, recombinants for *met* are produced at high frequency. The same thing is observed when 'F-refractory' Hfr strains (Lederberg & Lederberg, 1956) are infected with R factors (Hirota, Fujii & Nishimura, 1966). With R 1, on the other hand, the oriented transfer of the *try* region is a characteristic feature of the R factor and is independent of the particular bacterial strain used as donor.

Using as recipients with R 1$^+$ donors, strain x 195 *pyrF*$^-$ *try*$^-$ *purB*$^-$ which was also *gal*$^-$ and the uracil-requiring strain MSO *pyrD*$^-$, it was possible to show that the relative numbers of recombinants inheriting the various donor markers between *pyrF* and *gal* coincided with their order on the genetic map as determined by Signer *et al.* (1965). That the order of transfer was *pyrF...try...purB...gal*, starting from a fixed origin, was supported by the patterns of unselected markers. Most recombinants selected for the more distal *purB*, also acquired the proximal *pyrF*; but selected for *pyrF*, fewer recombinants had *purB*. Finally, by using the de-repressed mutant, R 1 *drd-19*, an R1$^+$ donor could be directly compared with a known Hfr strain, and also examined in an interrupted mating experiment for the relative times of entry of *pyrF* and *purB*. These tests showed that an R1$^+$ donor transferred its chromosome in the way associated with the Hfr state. There were, however, two differences. First, recombinants were rather fewer; this may be misleading, as pointed out above, since, in comparing recombination rates with different conjugation factors, one may in reality be comparing the frequencies at which conjugation takes place, rather than chromosome transfer itself. Second, and more important, the recombinants had nearly always received the R factor also. These are precisely the two features which distinguish recombination brought about by F′ factors such as F2 (Adelberg & Burns, 1960) which alternate rapidly between the autonomous state and integration at a particular chromosomal site, from recombination with a typical Hfr donor where the only F factor present is integrated and seldom transferred. The relative frequencies of sex-factor transfer and production of the most frequent class of recombinant with R1 are altogether comparable to those reported for F 2 by Adelberg & Burns (1960). R1 thus appears as another genetic element with a specific affinity for a particular chromosomal site, which it probably acquired in the same way as an F′ factor; that is, by recombination with the chromosome of a previous host. The ultimate source of the resistance determinants carried by R factors remains unknown, but the region of R 1 homologous with the chromosome did not lie in its resistance genes: one segregant without kanamycin-resistance, as well as another which lost all except resistance to kanamycin, continued to give large numbers of recombinants for the *try* region as before. If the homologous region of R 1 is indeed associated with the conjugation factor proper, it is worth noting that its site of integration is one of those where F integrates (to give the Hfr strain HfrB 10 described by Broda, 1967).

Integrated and autonomous F′ factors can co-exist in the same cell because of the instability of chromosomal attachment, which does not last long enough to cause the autonomous F to disappear (Adelberg & Burns, 1960). Presumably the same explana-

tion applies to R 1, which must therefore alternate frequently between the integrated and the autonomous state. Transient integration, if it lasted for a few generations, might lead to detectable fluctuation in the numbers of recombinants produced by different donor cultures, provided that young enough cultures were examined. In fact, a significant degree of variation appeared in an experiment where replicate 1 ml. broth cultures of J 5-3 (R 1 *drd-19*) grown from an inoculum of 200 bacteria/ml. to a concentration of 2×10^5/ml. were tested for the production of *pyrF*$^+$ recombinants after mixing with strain X 195 at 5×10^8/ml. for 1 hr. When 19 individual 0·02 ml. samples from each of two cultures were plated, the average numbers of recombinants were 15·3 and 16·5 with variances of 14·1 and 19·5 equal to 0·92 and 1·2 times the mean respectively. However, with single samples from each of 130 further cultures, the average number of recombinants was 12·47 and the variance 40·7; that is, considerably greater than the mean, as expected for a non-random distribution.

The frequency with which different Hfr types arise, as well as their stability, depends directly on the length of the homologous region (Broda, 1967) and, on this argument, the region in R 1 homologous with the chromosome of *Escherichia coli* K 12 should be of considerable length. The members of that class of R factors comprising R 1 so resemble each other and the F factor in the conjugation pilus and the genetic control of its synthesis that all are likely to have evolved from a common ancestor (Meynell & Datta, 1966*a*). Nevertheless, the conjugation factor of R 1 is not F itself, for, apart from several perhaps only insignificant differences such as in susceptibility to acridines and in restriction of different phages (see Watanabe, 1966) they manifest no superinfection immunity towards each other. In contrast to the mutual exclusion between two F factors (Scaife & Gross, 1962), or between F and *col* V (Macfarren, 1966), bacteria carrying either F or R 1 readily accept the other, and both factors are able to co-exist stably in the same cell.

The characteristic behaviour of R 1 in an F$^-$ strain could also be observed with Hfr bacteria, although here again it was necessary to use the de-repressed mutant to avoid repression of conjugation mediated by F as well as by R. When R 1 *drd-19* was introduced into HfrC, two kinds of recombinant were produced at high frequency, the usual *pro* recombinants resulting from the oriented transfer of the Hfr chromosome and, superimposed on these, the *try* recombinants due to a second point of origin determined by the R factor. Strain HfrC (R 1 *drd-19*) thus resembled the 'double male' strain of Clark (1963), which carries two F factors integrated at different chromosomal sites. Watanabe & Ogata (1966) also refer to the similar behaviour of an Hfr strain carrying, as well as the integrated F factor, a particular hybrid of an R factor with F.

It has been questioned whether the term 'episome' (Jacob & Wollman, 1958) should be applied to conjugation factors like *col* factors and R factors which have never been shown with certainty to integrate with the bacterial chromosome in the same way as the F factor (Clowes & Moody, 1966). However, from the way in which R 1 brings about the transfer of the chromosome from the donor cell, it now seems that this is one R factor which may be described as an episome within the original definition.

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REFERENCES

ADAMS, M. H. (1959). *Bacteriophages*. New York: Interscience Publishers Inc.

ADELBERG, E. A. & BURNS, S. N. (1960). Genetic variation in the sex factor of *Escherichia coli*. *J. Bact.* **79**, 321.

APPLEYARD, R. K. (1954). Segregation of new lysogenic types during growth of a doubly lysogenic strain derived from *Escherichia coli* K 12. *Genetics* **39**, 440.

BRODA, P. (1967). The formation of Hfr strains in *Escherichia coli* K 12. *Genet. Res., Camb.* **9**, 35.

CAVALLI-SFORZA, L. L. (1950). La sessualita nei batteri. *Boll. Ist. sieroter. Milan* **29**, 281.

CLARK, A. J. (1963). Genetic analysis of a 'double male' strain of *Escherichia coli* K 12. *Genetics* **48**, 105.

CLOWES, R. C. & MOODY, E. D. M. (1966). Chromosomal transfer from 'recombination-deficient' strains of *Escherichia coli* K 12. *Genetics* **53**, 717.

CLOWES, R. C. & ROWLEY, D. (1954). Some observations on linkage effects in genetic recombination in *Escherichia coli* K 12. *J. gen. Microbiol.* **11**, 250.

HAAN, P. G. DE & GROSS, J. D. (1962). Transfer delay and chromosome withdrawal during conjugation in *Escherichia coli*. *Genet. Res. Camb.* **3**, 251.

HAYES, W. (1953). Observations on a transmissible agent determining sexual differentiation in *Bact. coli*. *J. gen. Microbiol.* **8**, 72.

HIROTA, Y., FUJII, T. & NISHIMURA, Y. (1966). Loss and repair of conjugal fertility and infectivity of the resistance factor and sex factor in *Escherichia coli*. *J. Bact.* **91**, 1298.

JACOB, F. & WOLLMAN, E. L. (1958). Les épisomes, éléments génétiques ajoutés. *C. r. hebd. Séanc. Acad. Sci., Paris* **247**, 154.

LEDERBERG, E. M. & LEDERBERG, J. (1953). Genetic studies of lysogenicity in *Escherichia coli*. *Genetics* **38**, 51.

LEDERBERG, J. (1947). Gene recombination and linked segregations in *Escherichia coli*. *Genetics* **32**, 505.

LEDERBERG, J. (1950). The selection of genetic recombinations with bacterial growth inhibitors. *J. Bact.* **59**, 211.

LEDERBERG, J. (1951). Streptomycin resistance: a genetically recessive mutation. *J. Bact.* **51**, 549.

LEDERBERG, J., CAVALLI, L. L. & LEDERBERG, E. M. (1952). Sex compatibility in *Escherichia coli*. *Genetics* **37**, 720.

LEDERBERG, J. & LEDERBERG, E. M. (1956). Infection and heredity. In *Cellular Mechanisms in Differentiation and Growth*. Ed. by D. Rudrick, p. 101. Princeton, N. J.: Princeton University Press.

LENNOX, E. S. (1955). Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* **1**, 190.

MACFARREN, A. C. (1966). *A study of three related colicin factors and their products*. Ph D. thesis; University of London.

MEYNELL, E. & DATTA, N. (1966*a*). The relation of resistance transfer factors to the F factor of *Escherichia coli* K 12. *Genet. Res., Camb.* **7**, 134.

MEYNELL, E. & DATTA, N. (1966*b*). The nature and incidence of conjugation factors in *Escherichia coli*. *Genet. Res., Camb.* **7**, 141.

MEYNELL, E. & DATTA, N. (1967). Mutant drug resistance factors of high transmissibility. *Nature, Lond.* **214**, 885.

MEYNELL, G. G. & LAWN, A. M. (1967). Sex pili and common pili in the conjugational transfer of colicin factor Ib by *Salmonella typhimurium*. *Genet. Res., Camb.* (in the Press).

MONK, M. & CLOWES, R. C. (1964). The regulation of colicin synthesis and colicin factor transfer in *Escherichia coli* K 12. *J. gen. Microbiol.* **36**, 385.

OZEKI, H., HOWARTH, S. & CLOWES, R. C. (1961). Colicine factors as fertility factors in bacteria. *Nature, Lond.* **190**, 986.

OZEKI, H., STOCKER, B. A. D. & SMITH, S. (1962). Transmission of colicinogeny between strains of *Salmonella typhimurium* grown together. *J. gen. Microbiol.* **28**, 671.

PEARCE, L. E. & MEYNELL, E. (1968). Mutation to high level streptomycin-resistance in R^+ bacteria. *J. gen. Microbiol.* **50**, 173.

RICHTER, A. (1961). Attachment of wild-type F factor to a specific chromosomal region in a variant strain of *Escherichia coli* K 12: the phenomenon of episomic alternation. *Genet. Res., Camb.* **2**, 333.

SCAIFE, J. & GROSS, J. D. (1962). Inhibition of multiplication of an *F-lac* factor in Hfr cells of *Escherichia coli* K 12. *Biochem. biophys. Res. Comm.* **7**, 403.

SHAPIRO, J. (1967). *The structure of the galactose operon in Escherichia coli* K 12. Ph.D. thesis; University of Cambridge.

SIGNER, E. R., BECKWITH, J. R. & BRENNER, S. (1965). Mapping of suppressor loci in *Escherichia coli*. *J. molec. Biol.* **14**, 153.

STOCKER, B. A. D., SMITH, S. M. & OZEKI, H. (1963). High infectivity of *Salmonella typhimurium* newly infected by the *col* I factor. *J. gen. Microbiol.* **30**, 201.

SUGINO, Y. & HIROTA, Y. (1962). Conjugal fertility associated with resistance factor R in *Escherichia coli*. *J. Bact.* **84**, 902.

TATUM, E. L. & LEDERBERG, J. (1947). Gene recombination in the bacterium. *J. Bact.* **53**, 673.

WATANABE, T. (1963). Infective heredity of multiple drug resistance in bacteria. *Bact. Rev.* **27**, 87.

WATANABE, T. (1966). Evolutionary relationships of R factors with other episomes and plasmids. *Fedn. Proc.* **26**, 23.

WATANABE, T. & OGATA, C. (1966). Episome mediated transfer of drug resistance in *Enterobacteriaceae*. IX. Recombination of an R factor with F. *J. Bact.* **91**, 43.

WOLLMAN, E. L. & JACOB, F. (1958). Sur le processus de conjugaison et de recombinaison chez *Escherichia coli*. V. Le méchanisme du transfert de matériel génétique. *Ann. Inst. Pasteur* **95**, 641.

WOLLMAN, E. L., JACOB, F. & HAYES, W. (1956). Conjugation and genetic recombination in *Escherichia coli* K 12. *Cold Spr. Harb. Symp. quant. Biol.* **21**, 141.

J. gen. Microbiol. (1968), **50**, 173–176
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Mutation to High-level Streptomycin-resistance in R^+ Bacteria

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SUMMARY

Bacteria carrying an R factor conferring resistance to low concentrations of streptomycin frequently give highly resistant variants. Resistance to high concentrations of streptomycin can arise in a sensitive bacterium either from a single mutation or as the result of successive mutations in different genes (Demerec, 1948; Hsu & Herriott, 1961). The resistance genes may be either in the chromosome or in an R factor.

METHODS

R 1 (Meynell & Datta, 1966) confers resistance to sulphonamide, streptomycin, chloramphenicol, kanamycin and ampicillin. The bacteria in which it was tested were *Escherichia coli*, K 12 strains J 5-3 *pro*$^-$ *met*$^-$ and J 6-2 *pro*$^-$ *his*$^-$ *try*$^-$ (Clowes & Rowley, 1954), and the defined media and methods used for R factor transfer were as described in Pearce & Meynell (1968).

Isolation and detection of R^- *segregants.* Unlike the F factor (Hirota, 1960), R 1 is not eliminated by growth in acridine broth, and since it carries an ampicillin-resistance gene, which determines the production of penicillinase (Datta & Kontomichalou, 1965), R^- bacteria cannot be isolated by the penicillin-screening method in the ordinary way (Watanabe & Fukasawa, 1961). Nevertheless, the screening technique could be successfully applied when cephalosporin, a penicillin which is relatively insensitive to penicillinase, was substituted for ampicillin. An overnight broth culture of $R1^+$ bacteria was diluted in fresh broth to a concentration of 10^4 bacteria/ml. and incubated for 2 hr until the bacteria were growing exponentially; chloramphenicol was then added to 25 μg./ml. and the culture re-incubated for 1 hr. At this time, cephalosporin (Cephaloridine, Glaxo) was added to 20 μg./ml. and the culture further incubated for 4 hr, after which the number of viable bacteria was found to be 0·3–0·05% of that present when the cephalosporin was added. Dilutions were spread on nutrient agar plates which were incubated overnight; next day R^- clones were detected as penicillinase-negative colonies by the starch–iodine method (Foley & Perret, 1962, modified by Hennessey; T. Hennessey, personal communication). For this method, the plates were overlaid with 3 ml. of 0·6% water agar containing 0·3 ml. of a 2% solution of starch; and when the layer had set, 3 ml. of a mixture of 3·2 M-potassium iodide and

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0·08 M-iodine solutions containing 100 mg./ml. benzylpenicillin was poured on the surface. In 2–3 min., all the R^+ colonies were surrounded by a white halo, and it was possible very quickly to subculture R^- clones before all the bacteria were killed. When strain J5-3 (R1) and three streptomycin-resistant mutants, H53, H54 and H55 were treated in this way, penicillinase-negative colonies amounted to 22, 13, 23 and 85%, respectively.

RESULTS AND DISCUSSION

R1 conferred only low-level resistance to streptomycin and growth was inhibited on nutrient agar by concentrations exceeding streptomycin 20 μg./ml. It was therefore surprising to find in crosses with $R1^+$ donor strains (Pearce & Meynell, 1968) that on defined medium containing streptomycin 250 μg./ml., a 0·1 ml. inoculum of a broth culture of *Escherichia coli* K12 strain J5-3 (R1) gave about 500 medium-sized colonies as well as many more smaller ones. The presence of the R factor was responsible for the appearance of these colonies, since a similar inoculum of either strain J5-3 before infection or an R^- segregant of strain J5-3 (R1) gave no growth. The colonies produced by strain J5-3 (R1) contained bacteria with various degrees of increased streptomycin-resistance. Thus the limited streptomycin-resistance conferred by R1 was evidently enough to allow the inoculated bacteria to multiply in the presence of streptomycin at 250 μg./ml. to a population density where resistant mutants appeared.

The degree of streptomycin resistance of the variants was determined by streaking one loopful of a fully grown broth culture across a series of plates of defined medium containing graded concentrations of streptomycin increasing by steps of $\sqrt{2}$ from 1 μg./ml. to 16,384 μg./ml., the degree of resistance being recorded as the highest concentration of streptomycin which permitted confluent growth (Reeve, 1966). This method had the advantage of giving a sharp end-point, although the estimated degree of resistance was higher than that obtained by assessing the numbers and appearance of individual colonies. As others have noted (Tzagaloff & Umbreit, 1963; Gundersen, 1965*b*) on defined medium the bacteria grew in the presence of higher concentrations of streptomycin than on nutrient agar.

Mutation to a low degree of streptomycin resistance occurs comparatively often (Demerec, 1948) and, if the mutated chromosomal gene acted synergistically with the resistance determinant of the R factor, a highly resistant organism would result. Several variants of greater resistance were isolated as individual colonies on plates where growth was no longer confluent. Table 1 shows that R^- segregants of H53 grew only on streptomycin 22·4 μg./ml. and, when the R factor was transferred to the sensitive strain J6-2, all of 5 individual R^+ recipient colonies showed only the usual degree of resistance, i.e. to 128 μg./ml. Thus the variant, H53, derived from strain J5-3 (R1) and resistant to streptomycin 2048 μg./ml. was the product of a sensitive bacterium mutating to resistance to only 22·4 μg./ml. while carrying an R factor conferring no more than its original resistance to 128 μg./ml. Again, when the R factor was introduced into H56, a mutant of J5-3 resistant to streptomycin 128 μg./ml., the result was a culture resistant to as much as 11,500 μg./ml. Thus, the resistance genes on the chromosome and the R factor clearly co-operated to produce a bacterium of disproportionately high overall resistance. To see whether the conditions for mutation to high-level streptomycin-resistance could be reproduced equally well when the determinant for low-level resistance was in the chromosome instead of in the R factor,

a mutant of J5-3 whose resistance level was as nearly as possible the same as that conferred by R1 was isolated on streptomycin agar. This mutant, H56, gave confluent growth, like J5-3 (R1), on concentrations of streptomycin up to 128 μg./ml. and when 0·1 ml. volumes of overnight broth cultures of the two strains were plated in parallel on defined medium containing streptomycin 250 μg./ml., both gave about 70 full-sized colonies of bacteria later shown to be resistant to streptomycin 16,000 μg./ml. The similar behaviour of the mutant and the R+ strain in increasing the rate at which highly resistant variants arose strongly suggested that the function of the R factor was simply to confer an initial degree of resistance. In each case, a further mutation of the kind that occurs relatively frequently and confers only a small degree of resistance on

Table 1. *Escherichia coli K12. Mutation to streptomycin-resistance in R1+ bacteria*

Strain or variant	Obtained		Resistance to strepto-mycin (μg./ml.)	Further procedure	Resulting derivative	Resistance to strepto-mycin resistance (μg./ml.)
	From	By				
J5-3			4	—	—	—
H56	J5-3	Selection on streptomycin	128	Infection with R1	H56 (R1)	11,500
J5-3 (R1)	J5-3	Infection with R1	128	—	—	—
H55	J5-3 (R1)	Selection on streptomycin	180	Curing of R1	H69	22·4
H53	J5-3 (R1)	Selection on streptomycin	2048	Curing of R1	H67	22·4
				Transfer of R1 to J6-2	J6-2 (R1) (5 isolates)	128
H54	J5-3 (R1)	Selection on streptomycin	2867	Curing of R1	H68	22·4

a sensitive bacterium then results in a highly resistant organism. There still remained some slight difference between J5-3 (R1) and the mutant H56, which may perhaps only have reflected a difference in the mechanism of resistance (Okamoto & Suzuki, 1965), for while the plates inoculated with the R+ strain had by 48 hr developed a large number of tiny colonies, no such colonies appeared on the plates spread with the mutant.

R1 confers resistance to streptomycin 128 μg./ml., but, at the same time, it can be seen greatly to increase the rate of appearance of highly streptomycin-resistant variants. Gundersen (1963, 1965*a*) and Ginoza & Painter (1964) observed what may be the same phenomenon, which was attributed to '*genetic instability*' resulting from a '*mutator gene*' present on an episome. The particular episome studied by Gundersen itself gave resistance to low concentrations of streptomycin (Gundersen, 1965*a*), and Ginoza & Painter (1964) noted that the apparently mutagenic effect of R factors was limited to drugs where the R factor already conferred some degree of resistance. The chromosomal mutation was not in the gene where high-level resistance is ordinarily acquired by a single mutational step (Gundersen, 1963); and in the bacterial variants examined by Ginoza & Painter (1964) two genes, one present in the R factor and the

other in the chromosome, and each of which singly confer resistance to streptomycin 25 μg./ml., co-operated to give an organism resistant to 1000 μg./ml. Ginoza & Painter concluded from this result that the chromosomal gene was acquired as a direct result of genetic recombination between chromosome and R factor. On the other hand, the similar rates of mutation to high-level resistance in bacteria carrying R 1 or a chromosomal mutation giving the same degree of resistance implies rather that the R factor acts simply by providing an initial degree of resistance sufficient for the bacterial population to reach a concentration at which one of the comparatively frequent mutations which ordinarily gives low degrees of resistance can occur. When the bacterium already carries one resistance determinant, either an R factor or chromosome, the synergistic action of the two resistance genes produces an organism which is highly resistant.

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REFERENCES

CLOWES, R. C. & ROWLEY, D. (1954). Some observations on linkage effects in genetic recombination in *Escherichia coli* K 12. *J. gen. Microbiol.* **11**, 250.

DATTA, N. & KONTOMICHALOU, P. (1965). Penicillinase synthesis controlled by infectious R factors in Enterobacteriaceae. *Nature, Lond.* **208**, 239.

DEMEREC, M. (1948). Origin of bacterial resistance to antibiotics. *J. Bact.* **56**, 63.

FOLEY, J. M. & PERRET, C. J. (1962). Screening bacterial colonies for penicillinase production. *Nature, Lond.* **195**, 287.

GINOZA, H. S. & PAINTER, R. B. (1964). Genetic recombination between the resistance transfer factor and the chromosome of *Escherichia coli*. *J. Bact.* **87**, 1339.

GUNDERSEN, W. B. (1963). New type of streptomycin resistance resulting from action of the episome-like mutator factor in *Escherichia coli*. *J. Bact.* **86**, 510.

GUNDERSEN, W. B. (1965*a*). Transduction of the mu-factor in *Escherichia coli*. *Acta path. microbiol. scand.* **65**, 621.

GUNDERSEN, W. B. (1965*b*). Reduced streptomycin killing in *E. coli* carrying the mu-factor in its extrachromosomal state. *Acta path. microbiol. scand.* **65**, 627.

HIROTA, Y. (1960). The effect of acridine dyes on mating type in *Escherichia coli*. *Proc. natn. Acad. Sci. U.S.A.* **46**, 57.

HSU, Y-C. & HERRIOTT, R. M. (1961). Studies on transformations of *Haemophilus influenzae*. III. The genotypes and phenotypic patterns of three streptomycin-resistant mutants. *J. gen Physiol.* **45**, 197.

MEYNELL, E. & DATTA, N. (1966). The relation of resistance transfer factors to the F factor of *Escherichia coli* K 12. *Genet. Res., Camb.* **7**, 134.

OKAMOTO, S. & SUZUKI, Y. (1965). Chloramphenicol-, dihydrostreptomycin- and kanamycin-inactivating enzymes from multiple drug-resistant *Escherichia coli* carrying episome 'R'. *Nature, Lond.* **208**, 1301.

PEARCE, L. E. & MEYNELL, E. (1968). Specific chromosomal affinity of a resistance factor. *J. gen. Microbiol.* **50**, 159.

REEVE, E. C. R. (1966). Characteristics of some single-step mutants to chloramphenicol resistance in *Escherichia coli* K 12 and their interactions with R-factor genes. *Genet. Res., Camb.* **7**, 281.

TZAGALOFF, H. & UMBREIT, W. W. (1963). Influence of streptomycin on nucleotide excretion in *Escherichia coli*. *J. Bact.* **85**, 49.

WATANABE, T. & FUKASAWA, T. (1961). Episome-mediated transfer of drug resistance in Enterobacteriaceae. II. Elimination of resistance factors with acridine dyes. *J. Bact.* **81**, 679.

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Apical Organization in the Somatic Hyphae of Fungi

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SUMMARY

By using phase-contrast microscopy a 'Spitzenkörper' was seen at the hyphal apex of all the 'septate' fungi examined, but none was seen at the apex of any Phycomycete. The spitzenkörper accepts staining with cationic dyes. A method is described for consistently obtaining ultra-thin sections longitudinally through hyphal apices. Electron micrographs indicate that the spitzenkörper is an aggregation of vesicles just beyond the apex, namely at the region where wall-formation occurs during extension growth. It is suggested that the vesicles are formed posteriorly and migrate to the apex where they fuse with the plasma membrane liberating their contents as part of the process of wall growth.

INTRODUCTION

During an investigation of nuclear behaviour and mating strains in the genus *Coprinus* Brunswik (1924) examined iron-haemotoxylin stained preparations of two species (*C. sterquilinus*, *C. narcoticus*) and observed their hyphal tips to contain a strongly staining area at the growing point. This area he described as a 'nucleus-like' body, but because of its variable size and because he never observed it in the process of division, he concluded that it was not a nucleus but a special organelle, connected in some way with apical growth of the hypha; from its position he called it the 'Spitzenkörper'. Girbardt (1955) showed that this structure might be observed in living material by phase-contrast microscopy, and he detected its presence in the tips of several species of Basidiomycetes and in species of *Penicillium* and *Aspergillus*. He also noted that it disappeared when extension growth ceased and formed again just before extension growth restarted in a checked tip. Girbardt later (1957) used 14–16% (w/v) gelatin in the growth medium to match the refractive index of the immersion medium with that of the fungal cytoplasm and was able to make a more detailed study of the relationship between apical growth and the behaviour of the spitzenkörper. The position of the structure in the apical dome was related to subsequent direction of extension, an eccentric position preceding a turning of the tip. No information was obtained about the source or the nature of the spitzenkörper.

Little is known of the mechanism of wall extension at the hyphal apex during growth of fungi, yet the nature of the wall here and the mechanism of its extension have considerable significance for fungal morphogenesis. The present report arises out of a study of fungal fine structure in its relation to morphogenesis.

METHODS

The fungi examined most frequently in this work were *Aspergillus niger* v. Tiegh., *Fusarium oxysporum* Sny. et Hans., and *F. solani* Sny. et Hans. The nutrient medium used for growth was: glucose, 0·7 g.; KH_2PO_4, 0·2 g.; $MgSO_4.7H_2O$, 0·5 g.; NH_4NO_3, 0·1 g.; distilled water to 1 l.; agar media contained 10 g. Oxoid no. 3 agar/l. Cultures were incubated for at least 2 days at 25° to allow apices to attain a mature condition.

For material to be fixed and stained for light microscopy and electron microscopy sterile cover glasses were prepared with a thin (0·5 mm.) layer of agar medium. These were point-inoculated with spores of the fungus, and incubated on moist sterile filter papers in a humid chamber. Fixation for light microscopy was by freeze-substitution in absolute ethanol at −70°, the fixed material being allowed slowly to reach 4° in the ethanol.

For phase-contrast observations of growing hyphae the nutrient medium was made up in 25% (w/v) acacia gum (Barer & Joseph, 1958) and the fungus mounted for microscopy in the same medium, the refractive index of which is close to that of the cytoplasm (Park & Robinson, 1957). The thin-layer cultures to be used for electron microscopy were made on coverslips which had previously been sprayed lightly with PTFE (polytetrafluoroethylene). The cultures were incubated for 3 days at 25° before treatment. The coverslips with colony and medium attached were then immersed for 1 hr in 2·5% (w/v) glutaraldehyde at 0°, followed by washing for three 30 min. periods in 0·1 M-phosphate buffer (pH 7·0). After post-fixation for 2 hr with 2% osmium tetroxide in 0·1 M-phosphate buffer (pH 7·0), the tissue was dehydrated by passing it through a graded series of acetone solutions kept at 0° at 30 min. intervals, finally transferring to anhydrous acetone for not less than 2 hr. A 1:1 Araldite:anhydrous acetone mixture was added over a period of 1 hr until the mixture immersing the tissue became near 1:1 resin:acetone. The tissue was allowed to remain in this mixture for 3 hr and then the acetone was allowed to evaporate for 24 hr, residual acetone being removed in a vacuum oven at 60° and a pressure of 5×10^{-3} mm. Hg.

The preparation was embedded in Araldite by using a modification of the clamped holder first described by Sheffield (1965) with slight modification. BEEM capsules (supplied by LKB Instruments Ltd.) trimmed to remove the frustrum of the hexagonal pyramid were put into the holders, and the coverslips placed on top so that the margin of the growing colony lay across the diameter of the capsule. A glass slide was then placed on top of the coverslips, the compression plate put on top of the glass slide and the whole assembly carefully bolted into position. The holder was then inverted and the capsules filled with resin. In this way the thin agar layer containing the hyphal tips was embedded and also initially orientated so that the hyphae were parallel with the flat lower exposed face of the polymerized cylinder. After the normal polymerization process the compression plate and the glass slide were removed and the coverslips gently pulled away from the capsules. The PTFE coating made this possible. The capsules were then removed from the holder.

To determine the orientation of the hyphae the blocks were examined under a binocular microscope. The block was then trimmed so that when placed in the microtome the hyphae lay with their long axes parallel to the knife edge. Sections cut with a diamond knife and a Porter–Blum microtome were placed on platinized grids,

stained with uranyl acetate and Reynold's lead citrate, and stabilised with a thin layer of carbon.

Sections were examined in an A.E.I. EM6B making use of 250 μ condenser lens aperture, 50 μ objective lens aperture and cold finger to prevent specimen contamination. The beam acceleration voltage was 60 kV and the beam current was 200 μ. The recording emulsion was Ilford N 40.

RESULTS

Phase-contrast observations of living hyphae

The method used gave very good optical resolution of cytological detail by eliminating the lens effect of the narrow cylindrical hyphae. Nuclei appeared bright, each delimited by a dense boundary. Each nucleus had an opaque nucleolus situated to one side. Vacuoles (not present at the tip) were transparent and occasionally contained dense granules showing random erratic movement. The boundaries of the vacuoles were not rigidly fixed but constantly undulated, and not infrequently the vacuolar membrane invaginated into the vacuole or evaginated into the cytoplasmic matrix. These observations suggest that there was no marked pressure difference between the vacuole and the cytoplasmic matrix. The mitochondria were optically dense, filamentous, and in the tip region of the hyphae densely aggregated into a tuft. They moved actively with a serpentine motion. Small, highly refractive spherical organelles, in some cases actively moving, in other cases static, were present. These corresponded closely with the Woronin bodies described by Buller (1933). All these structures appeared clearly and with a sharp outline. The 'Spitzenkörper', on the other hand, while clearly evident could not be focused sharply even though it showed no obvious rapid movement (Pl. 1, fig. 1). Its boundaries were vague. Commonly associated with, and just posterior to, the spitzenkörper were two or three of the Woronin bodies moving fairly rapidly with an apparent random motion but keeping more or less the same station, not frequently traversing as did some of the other refractive bodies elsewhere. At intervals a refractive spherical body from a region posterior to the tip was seen to move forwards and take up a position behind the spitzenkörper, or from a position at the apex move steadily posteriorly.

Our observations support those of Girbardt in that the spitzenkörper is only visible during active extension growth of the hyphal tip. When growth was stopped by any of a number of agencies (poor aeration, hypertonic solution, vacuolation factor) the spitzenkörper faded out gradually. It was not seen to move away. Some of the agents used, e.g. hypertonic solutions, had only a temporary inhibitory effect and the spitzenkörper re-appeared before growth became evident once more.

We have examined several species of fungi other than the three named earlier. The list in Table 1 shows that a spitzenkörper was seen in every imperfect fungus examined, but in none of the Zygomycetes or Oomycetes. In *Rhizopus nigricans* some especially favourable preparations showed an area just behind the apex and slightly larger than the usual spitzenkörper, with small, just resolvable, particles in highly active movement. We do not consider the range of fungi examined large enough to warrant any generalization.

In material fixed by freeze-substitution in ethanol at $-70°$ the spitzenkörper stained with methyl green + pyronin (Brachet, 1953) and with other cationic dyes (Pl. 1,

fig. 2). However, it gave this reaction even after prolonged incubation in RNAase solution so that it seems unlikely that the reaction is one for RNA. The stains used did not give a DNA reaction; the nucleus remained uncoloured whereas the nucleolus stained densely.

Table 1. *Fungi examined for presence of Spitzenkörper*

	Presence of Spitzenkörper
Pythium mamillatum Meurs.	−
Pythium ultimum Trow.	−
Mucor hiemalis Wehm.	−
Zygorhynchus moelleri Vuill.	−
Rhizopus stolonifer (Ehrenb. ex. Fr.) Lind.	−
Aspergillus niger v. Tiegh.	+
A. oryzae (Ahlb.) Cohn	+
Botrytis cinerea Pers. ex Fr.	+
Fusarium culmorum (W. G. Smith) Sacc.	+
F. oxysporum Schlect. sensu Sny. & Hans.	+
F. solani (Mart.) App. & Wollenw. sensu Sny. & Hans.	+
Neurospora crassa Shear & Dodge	+
Penicillium citrinum Thom	+
P. expansum Link. emend Thom	+
Trichoderma viride Pers. ex Fr.	+

Electron microscopy

The primary interest of this work is the special organization near the hyphal apex and the following description is restricted to those points of interest especially relevant to this region. The cell wall over the apex showed little structural difference from that seen in other regions of the hyphae, being largely electron transparent, and continuous over the whole apical dome in all sections examined. The wall over the dome was in some sections thicker (about 50–60 mμ) than the wall along the tubular part of the hyphae (about 30 mμ) (Pl. 2, fig. 4). This may, at least in part, have been due to some of the sections being truly longitudinal but not truly median, and therefore not cutting the wall normal to the surface.

Mitochondria in this region were filamentous, having a diameter as little as 120 mμ but usually 220–250 mμ. Their length was difficult to determine accurately because of their sinuous form, but lengths of 3·5 μ were common, and lengths of up to 4·5 μ were seen in individual sections near the tip. Mitochondria 5–7·5 μ long are common in phase-contrast photomicrographs. Mitochondria near the hyphal tip characteristically had a high content of cristae, which were tubular and orientated parallel to the long axis. A significant characteristic of the mitochondrial tuft at the tip was that it invariably stopped short (about 1–2 μ) of the apex. Mitochondria were thus excluded from the area immediately behind the apical dome.

Similarly, ribosome groups, whose distribution was dense in the tip region, were infrequent in the region immediately behind the extending apex proper (Pl. 3, fig. 5). Occasional ribosomes were found at the apex but were much less common and less commonly associated into groups. Nuclei were never found closer to the apex than 400 μ.

The unit membrane-bound vesicles immediately behind the apical dome are of interest. They appeared in all longitudinal sections of apices in extension growth at

the time of fixation. The vesicles varied in size from 70 mμ to 120 mμ and electron-dense strands or particles were included in them. Similar vesicles were occasionally present in regions of hyphae proximal to the tip (Pl. 4, fig. 6), but not in the same frequency as at the apex. The region where these vesicles were most numerous corresponds with that occupied by the spitzenkörper, and was the region of exclusion of mitochondria and of ribosome groups.

The apices of lateral branches show exactly the same features as the leader hyphae from the colony margin (Pl. 1, fig. 3). This figure shows the presence of vesicles like those in the apical aggregate near the junction of the branch with the main axis. Zalokar (1959) has demonstrated that the normal rate of extension of an apex of a filamentous fungus is dependent on the presence of a substantial amount of subtending hyphae, and Butler (1961) has shown a correlative situation between the extension rates of leader hyphae and its branches. Competition between apices for a common supply from posterior hyphal regions may be responsible for this correlative growth pattern.

A fusion of vesicles with the unit-membrane of the plasmalemma was clearly evident (Pl. 4, fig. 6; Pl. 3, fig. 5). It is always difficult to interpret the direction of any dynamic movements in living systems from fixed material. It seems probable that the spitzenkörper is a presentation of the mass of aggregated vesicles. Supporting this is the fact that sections from apices, the extension growth of which had been inhibited before fixation, showed no similar aggregation of vesicles at the apex. The staining with cationic dyes might indicate that the contents included acid carbohydrates. We suggest that the vesicles arise proximal to the apex and move forward, eventually to fuse with the plasmalemma, their contents being concerned with cell-wall synthesis. We have no evidence as to the source of the vesicles or to the site of their formation. No Golgi apparatus was seen in any of our sections of the three species examined by this technique. The vesicles may be produced in the region of dense mitochondria and ribosomal groups, but in the absence of information on their possible rates of migration their distribution does not exclude other origins.

Other structures regularly found in the region just behind the apical dome were the larger spherical unit membrane-bound structures (140–310 mμ) with electron-dense contents (Pl. 3, fig. 5). These appeared very similar to the Woronin bodies which we found near septa in more mature regions; there were often two to four near the apex.

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REFERENCES

BARER, R. & JOSEPH, S. (1958). Concentration and mass measurements in microbiology. *J. appl. Bact.* **21**, 146.

BRACHET, J. (1935). The use of basic dyes and ribonuclease for the cytochemical detection of ribonucleic acid. *Q. J. microscop. Sci.* **94**, 1.

BRUNSWIK, H. (1924). Untersuchungen über die Geschlechts- und Kernverhältnisse bei den Hymenomyceten gattung *Coprinus*. *Bot. Abh. K. Geobel.* Heft. 5.

BULLER, A. H. R. (1933). *Researches on Fungi*, Vol. 5. London: Longmans, Green and Co.

BUTLER, G. M. (1961). Growth of hyphal branching systems in *Coprinus disseminatus*. *Ann. Bot.* **25**, 341.

GIRBARDT, M. (1955). Lebendbeobachtungen an *Polystictus versicolor* (L). *Flora, Jena* **142**, 540.
GIRBARDT, M. (1957). Der Spitzenkörper von *Polystictus versicolor* (L). *Planta* **50**, 47.
PARK, D. & ROBINSON, P. M. (1967). A fungal hormone controlling internal water distribution normally associated with 'cell' ageing in fungi. *Symp. Soc. exp. Biol.* **21**, 323.
SHEFFIELD, H. G. (1965). A device for flat-face, epoxy resin embedding of tissues and coverslip cultures. *Stain Technl.* **40**, 143.
ZALOKAR, M. (1959). Growth and differentiation of *Neurospora* hyphae. *Am. J. Bot.* **46**, 602.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. *Fusarium oxysporum*. Hyphal tip growing in 25% (w/v) gum acacia solution. Filamentous mitochondria, refractive spherical bodies (Woronin bodies?) and the spitzenkörper are visible. Note the tuft of mitochondria just back of the apex. No nuclei are present this near the apex. Phase contrast. × 2000 (625 × 3·2).

Fig. 2. *Aspergillus niger*. Fixed hyphal tip stained methyl green + pyronin. Phase contrast. × 2000 (625 × 3·2).

Fig. 3. *Aspergillus niger*. Longitudinal section of lateral branch and main axis. The apex is organized in the same way as a leader apex. Electron micrograph, glutaraldehyde-OsO_4. × 10,875 (7500 × 1·45)

PLATE 2

Fig. 4. *Aspergillus niger*. Longitudinal section of hyphal apex. The unit membrane-bound vesicles (Vs) constituting the spitzenkörper contain an electron-transparent cortical region surrounding an electron-dense core. M, mitochondrion; R, ribosome groups; P, plasma membrane, CW, cell wall. Electron micrograph, glutaraldehyde-OsO_4. × 72,000 (20,000 × 3·6).

PLATE 3

Fig. 5. *Aspergillus niger*. Longitudinal section of hyphal tip. Two Woronin bodies (W) appear in this section. The configuration of the plasma membrane (P) at the apex suggests fusion of the vesicles (Vs) with the membrane. M, mitochondria; R, ribosome groups. Electron micrograph, glutaraldehyde-OsO_4. × 32,000 (10,000 × 3·2).

PLATE 4

Fig. 6. *Aspergillus niger*. Longitudinal section of hyphal tip. Filamentous mitochondria (M) and groups of ribosomes (R) stop short of the spitzenkörper region, which contains many vesicles (Vs). Similar vesicles are present at lower frequency more than 12 μ from the apex. The plasma membrane (P) presents a papillate outline more markedly at the apex than in non-extending regions. CW, cell wall. Electron micrograph, glutaraldehyde-OsO_4. × 19,000 (10,000 × 1·9).