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THE JOURNAL OF GENERAL MICROBIOLOGY

THE PREPARATION OF PAPERS

The more words there are, the more words there are about which doubts may be entertained.'

JEREMY BENTHAM (1748–1832)

What can be said at all can be said clearly; and whereof one cannot speak thereof one must be silent.'

LUDWIG WITTGENSTEIN: *Tractatus Logico-philosophicus* (tr. C. K. Ogden)

The Editors wish to emphasize ways in which contributors can help to avoid delays in publication.

(1) Papers should be written with the utmost conciseness consistent with clarity. The best English for the purpose of the *Journal* is that which gives the sense in the fewest short words, spelt according to the *Shorter Oxford English Dictionary*.

(2) A paper should be written only when a piece of work is rounded off. Authors should not be seduced into writing a series of papers on the same subject as results come to hand. It is better to wait until a comprehensive paper can be written.

(3) Authors should state the objective when the work was undertaken, how they did it and the conclusions shey draw. A section labelled 'Discussion' should be strictly limited to discussing the results if this is necesnary, and not to recapitulation. Typescripts should also carry four key words for index purposes.

(4) Figures and tables should be selected to illustrate the points made if they cannot be described in the text, to summarize, or to record important quantitative results.

(5) Authors should remember that in preparing their typescript they are giving instructions to the printer about layout, etc.) as well as attempting to convey their meaning to their readers.

(6) Editors do not alter typescripts except to increase clarity and conciseness. If an editorial alteration changes an author's meaning one implication is that it was expressed ambiguously. When an editor can rasp the meaning of a sentence unequivocally it may be assumed that anyone can.

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A manuscript should be submitted in double-spaced saping with wide margins, and on paper suitable for ink corrections. The paper must be written in English and should, in general, be divided into (*a*) Summary; (*b*) Introduction; (*c*) Methods; (*d*) Results; (*e*) Discussion (if any) and general conclusions; (*f*) Acknowledgements; (g) References.

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Short Communications. Short Communications will also be published. These will report work of two kinds: (i) results of sufficient moment to merit publication in advance of a more comprehensive paper, and (ii) work which substantially confirms or extends existing knowledge, but which does not justify an extensive paper. Category (i) will be given priority for publication.

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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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Chemical Formulae. These should be written as far as possible on one line. The chemical nomenclature adopted is that followed by the Chemical Society (*Journal of the Chemical Society* 1936, p. 1067). With a few exceptions the symbols and abbreviations are those adopted by a committee of the Chemical, Faraday, and Physical Societies in 1937 (see *Journal of the Chemical Society* 1944, p. 717). Care should be taken to specify exactly whether anhydrous or hydrated compounds were used, i.e. the correct molecular formula should be used, e.g. $CuSO_4$, $CuSO_4.H_2O$ or $CuSO_4.5H_2O$.

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Chemical Nomenclature. Follow the 1969 revision of *Policy of the Journal and Instructions to Authors*, The Biochemical Society, 7 Warwick Court, London, W.C.1.

Nomenclature of Enzymes. The system published in *Report of the Commission on Enzymes on the International Union of Biochemistry*, Oxford: Pergamon Press, 50*s*., is used.

Nomenclature in Bacterial Genetics. The proposal by M. Demerec, E. A. Adelberg, A. J. Clark and P. E. Hartman published in *Genetics* (1966), **54**, 61 has been reprinted in the *Journal* (1968), **50**, 1 as a useful guide to authors. The general principles laid down should be followed wherever practicable.

Nomenclature and Description of Micro-organisms. The correct name of the organisms, conforming with international rules of nomenclature, must be used; if desired, synonyms may be added in brackets when the name is first mentioned. Names of bacteria must conform with the Bacteriological Code and the opinions issued by the International Committee on Bacteriological Nomenclature. Names of algae and fungi must conform with the International Rules of Botanical Nomenclature. Names of protozoa must conform with the International Code of Zoological Nomenclature. Bacteriological Code, Iowa State College Press, Ames, Iowa, U.S.A. (1958); Botanical Code, International Bureau of Plant Taxonomy and Nomenclature, 106 Lange Nieuwstraat, Utrecht, Netherlands (1952); Zoological Code, International Trust for Zoological Nomenclature, London (1961). One or two small changes have been made to these rules at later International Congresses.

The following books may be found useful:

Bergey's Manual of Determinative Bacteriology, 7th edn (1957), edited by R. S. Breed, E. G. D. Murray and A. P. Hitchens. London: Ballière, Tindall and Cox.

V. B. D. Skerman, *A Guide to the Identification of the Genera of Bacteria with Methods and Digest of Genetic Characteristics* (1959). Baltimore, Maryland, U.S.A.: The Williams and Wilkins Company.

S. T. Cowan, *A Dictionary of Microbial Taxonomic Usage* (1968). Edinburgh: Oliver and Boyd.

Ainsworth and Bisby's Dictionary of the Fungi, 5th edn (1961). Kew: Commonwealth Mycological Institute.

Latin Names. The species name is in italics (underlined once in typescript) and is used in full at first mention in each paragraph, but in subsequent mention with the name of the genus abbreviated, single letter abbreviations being used where they are not ambiguous. The genus name is in italic when the whole genus is referred to. When used trivially, genus names are in Roman (not underlined). Anglicized versions are not underlined and are used without capitals. Strain names or numbers are in small capitals (underlined twice in the typescript).

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

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The Discovery of DNA: An Ironic Tale of Chance, Prejudice and Insight

Third Griffith Memorial Lecture

By M. R. POLLOCK
Department of Molecular Biology, University of Edinburgh

(*Delivered at the General Meeting of the Society for General Microbiology on 6 April 1970*)

'Truth emerges more readily from error than from confusion.'
FRANCIS BACON (1620), *Novum Organum*

The Council of our now almost venerable Society must, in inviting me to give the third lecture in this series, have been at their wits' end to have had to scrape the barrel so desperately.

Unlike my two illustrious predecessors who were—in very different ways—natural choices for the job, I cannot claim to have made any significant contribution to scientific knowledge in the areas where Fred Griffith did most of his work. Sincerely honoured though I am, it is obvious to me that I am not really qualified to follow in their footsteps.

So I shall not try to. And I thought that, instead of talking about recent researches of personal appeal to myself, it might be more appropriate to stand aside and view some of the historical aspects of DNA in those early days before, during and shortly after Fred Griffith recorded his all-important discovery of transformation of pneumococcal types.

Hence the rather grandiose title.

Of course I could never attempt to produce anything approaching a complete history—or even a balanced summary—of DNA in the space of one lecture. And I certainly would not wish to compete with Jim Watson's *Double Helix* (1) or try to touch on all the events after 1953 which comprise, in fact, most of modern molecular biology.

It is a tricky business, involving considerable personal risks, touching on contemporary history. Erwin Chargaff, very appropriately, if rather cynically, wrote (2) that such an attempt 'must be very difficult as long as some of the witnesses, with all their quirks, senilities and dubious recollections are still alive'. 'Later it will be easier,' he added, 'no one being left to protest against truth or falsehood.'

I want, if I can, to try to capture the scientific atmosphere of the years between 1928, when Griffith published his now famous paper (3), through 1944 when Avery and his colleagues (4) showed that the transforming agent was DNA, to the great breakthrough in 1953 when Watson & Crick (5) published their structure of DNA and showed how it might explain the chemical mechanism by which cells passed on their characters

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accurately to their daughter cells. And let us have no illusions about this discovery of 1953. Without doubt it represented—that is, it was the culminating point in—one of the most fundamental and important discoveries in biology of all time. This was because it shows not only how living systems replicate *themselves* (and not something different) but has led directly to an understanding of how their functional characters are expressed.

But, having emphasized the excitement of the concluding stages in this development of biology, it is wise to remember that the importance of the three crucial points—Griffith's discovery of transformation(3), Avery's demonstration that the transforming material was DNA(4), and Crick & Watson's solution(5) of its structure—rested on what Maurice Wilkins(6) has emphasized were three essential and much *older* basic concepts: the gene, the macromolecule and the hydrogen bond.

We could paraphrase these concepts respectively as: the 'discreteness' of the inheritance of characters, the huge size of molecules concerned with biological specificity and the great importance of weak inter-atomic bonds (as distinct from the strong covalent links of classical chemistry) in biological systems.

The theme of DNA has now permeated so deeply into the consciousness of most educated people that it is unusual still to find someone—at least in academic circles—who has never heard of it. But it still occurs. Only the other day Bill Hayes and I were showing off our laboratories to a charming and distinguished personality in the Fine Arts Department of Edinburgh University. We stopped proudly before our three-dimensional atomic model of the DNA double helix. 'What is that?' our visitor queried. 'That,' we replied, 'is DNA.' His face was blank. 'You surely know about DNA?' we unwisely reproached him. 'No,' he answered, 'I've never heard of such a thing. What is it?' Now, that's not a particularly interesting story as it stands. What made it especially odd for *us* was that the individual concerned happened to be the perfect double of Francis Crick. The incident had a macabre, almost nightmarish quality that, for an instant, was quite alarming: time passing backwards, visions of all the great scientists denying their discoveries and pleading total ignorance of the field in which they made their names. But at least, after recovery, it reminded us that it takes a very long time for great basic discoveries—as distinct from purely technical advances—to take a real hold on ordinary people.

And we are only really at the very beginning of the sequence of repercussions that the discovery of DNA has initiated. Freud, it is said, believed that there were three really outstanding events in the evolution of human knowledge: the Copernican Theory of the Solar System; the Darwinian Theory of Evolution; and (not surprisingly) the Recognition of Unconscious Mental Processes.

Many will, of course, disagree with such a list, which by its nature must be a gross over-simplification. But the Understanding of the Chemical Basis of Life (which must, of course, include Heredity) might now be a strong candidate for inclusion as a fourth item, though this may be premature and, unlike the others, would not, I think, amount to a new scientific paradigm in the sense used by Kuhn(7). However that may be, it is interesting that all four items share the property of whittling down the cosmic significance of man. The first showed that man is just *part* of the Universe (not the centre), the second that man is just *part* of all living systems (not something fundamentally distinct), the third that man's will and personality—his 'soul'—are just *parts* of mental processes in general, and the fourth (the discovery of DNA) that living

systems themselves are just *parts* of all chemical processes, albeit rather complicated ones and not subject to transcendent vitalistic forces.

Through these discoveries the mystic concept of the stature of man and his cosmic significance has been superseded by a complementary increase in his potential control over the Universe—or what should now be paraphrased as a 'recognition of the increasingly important part the phenomenon of man is playing in the evolution of the Universe'. At all events, he still thinks he is awfully important.

I am rather afraid that Fred Griffith might have been rather shocked, if not horrified, to know that his own work was considered a vital link in the chain of advances in knowledge which could be analysed in such materialistic terms.

But this is the way with history. And I am very conscious that in choosing to tackle this subject of DNA historically I am running many risks—not least of which is that of being a terrible bore—especially to the young.

Peter Medawar, paying tribute to the vital part played by Griffith's discovery, wrote in a review(8) of Jim Watson's *Double Helix* (which makes no mention of Griffith): 'A great many highly creative scientists...take it quite for granted that an interest in the history of science is a sign of failing or unawakened powers...Science in some sense comprehends its history within itself. No Fred, no Jim: that is obvious, at least to scientists, and being obvious, it is understandable that it should be left unsaid ...the history of science does not often interest the scientist as science.'

Whitehead(9)—in a rather different sense—went further when he remarked almost cynically that: 'A science that hesitates to forget its founders is lost.'

Science history *need* not be a bore, but my apology tonight is rather in the nature of hoping that it *can* interest the scientist as science; or at least in close relation to the practice of science; that attempts to follow what in fact has been taking place over the period of an important discovery—the prejudices, the mistakes, the desires and personalities of the participants, the lucky and unlucky chances, the synthesis of ideas, the symbols and analogies, the relationships between entities and the sequence of events—may help in understanding the way human beings behave in their search for knowledge and so give some hints as to how to try to do it better.

The early history of DNA is full of instances of irrational forces that seem both to hinder and to help. But I can really do little more than indicate how the wind was blowing. It is surely premature to try to offer any sort of general theory of discovery. The story of DNA will mean different things to different people; but I believe it carries with it a few clues of how such discoveries are made—or perhaps, rather, of what sorts of things confuse us and delay the rate of progress.

The beginning of the story—of course, there is really *no* true beginning—lies long before 1928. Fred Griffith never hinted in his paper(3) that he contemplated that nucleic acid might have had a part to play in his transformation. But it would have been quite logical and possible for him to have done so, and 'nucleoproteins' were in fact discussed in his laboratory(10) in relation to the phenomenon.

In fact, nucleic acids were first discovered and characterized by Miescher in 1869, i.e. almost exactly 100 years ago(11). Indeed, this remarkable chemist devoted most of his life to studying them. Moreover, as pointed out by Olby & Posner(12), his views on the chemical basis of inheritance were remarkably far-seeing when, in 1892, he postulated heredity being derived from the 'stereo-architecture of only a few very large, complicated molecules'.

It might have been supposed that he was thinking of nucleic acids since he worked for most of his life on little else. Ironically enough, however, it seems he was probably referring to proteins because Mirsky (11) quotes him as saying in an article attacking Weismann (who was in favour of nuclein being the heredity substance) that 'his [Weismann's] speculations are unclear and derived from an outmoded kind of chemistry. When, as is quite possible, a protein molecule has 40 asymmetric C atoms so that there can be a billion isomers...my [stereochemical] theory is better suited than any other to account for the unimaginable diversity required by our knowledge of heredity.'

It was, indeed, at about that time that Staudinger was emphasizing a completely new idea for chemistry—the macromolecular polymer of biological origin. Even earlier than that v. Nägeli was developing the idea of an 'idioplasm'—an intracellular substance that was continuous through cell division and carried with it hereditary determinants (a sort of Weismann substance at the intercellular level), a concept which was well received by people such as de Vries and E. B. Wilson. The possibility came to be considered by several contemporary biologists that it might be identical with chromatin, the material associated with chromosomes.

'In the physiological aspect', wrote Wilson in the first edition of his famous book (13) 'inheritance is the recurrence, in successive generations, of like forms of metabolism; and this is effected through the transmission from generation to generation of a specific substance or idioplasm which we have seen reason to identify with chromatin.... In 1895, in another context (14), he had stated that: '...[this] seems to show that the chromosomal substance, the chromatin, is to be regarded as the physical basis of inheritance'. And he went on: 'Now, chromatin is known to be closely similar to, if not identical with, a substance known as *nuclein*, which analysis shows to be a tolerably definite chemical compound composed of nucleic acid and albumin. And thus we reach the remarkable conclusion that inheritance may perhaps be effected by the physical transmission of a particular chemical compound from parent to offspring.'

I have quoted these passages by Wilson at length because I think they illustrate two interesting points. The first is the remarkable extent—under the existing circumstances—to which the great biologists at the end of the last century had begun to grasp the situation: had got the right end of the stick, so to speak. The second is their failure to recognize the need to explain the basic phenomenon of inheritance: namely, the mechanism by which a character, or the chemical basis of a character, is copied, is multiplied—indefinitely—through successive generations. They talk only of 'transmission' of a character or by a substance—*taking it for granted* that the substance will be faithfully replicated *somehow*. They barely do that, because in fact I do not believe they are completely clear that something must be increased, although—and this is the point—they have arrived at the conclusion that the 'something' is a definable chemical substance. The problem—as it was seen much later on, in the present century—was not then being defined. Nor was it, as I shall emphasize later on, apparently appreciated by Griffith. The 'self-reproducing' molecule was *not* an idea that appeared until much later. At the best, heredity was, in ancient days, the passing on of a minute replica of the parent to the progeny: the homunculus in the head of the sperm, etc. Self-reproduction must have been regarded vitalistically, as an *essential and natural property* of 'life' and 'living systems'—not properly recognized as a phenomenon needing an

explanation, barely recognized as a phenomenon *at all*—as universal gravity before Newton, or the relativity of motion before Einstein.

It was regarded as being as natural and inherent a feature of 'life', as falling bodies were regarded by the Aristotelians as '*naturally*' seeking their true home (the Earth) or the heavenly spheres were regarded by Copernicus and Galileo as moving *naturally* in circles, or by Descartes and Newton *naturally* in straight lines by their own inertia.

This problem of recognizing a problem may, of course, depend upon there being the tools for beginning to solve it. But, above all, it makes one wonder what sorts of problem we may not be capable of recognizing at the moment. Biologists no doubt would like to think they were all a matter for physicists!

With the turn of the century, nucleic acids began to suffer a decline in prestige which persisted until the mid 1930s. For a long time their possible biological significance seems to have been almost completely rejected or forgotten. Even E. B. Wilson(15) in the second edition of his book, published in 1900, seems to have begun to lose the original excited interest he showed in his first edition written only five years previously. He already comes out clearly *against* the 'genetic continuity of chromosomes depending upon a persistence of chromatin'.

In 1909 Strasburger explained the diminishing interest in 'nuclein' when he reasoned that 'chromatin cannot itself be the hereditary substance [because] the amount of it is subject to considerable variation in the nucleus according to its stage of development' (11). (This argument was still operative in the 1940s.)

And in the third edition (1925) Wilson now writes(16): 'It is an interesting fact, which has been emphasized by biochemists, that apart from the characteristic differences between animals and plants referred to above [he means RNA for plants and DNA for animals—the dogma then current], the nucleic acids of the nucleus are on the whole remarkably uniform, showing with present methods of analysis no differences in any degree commensurate with those from the various species of cells from which they are derived. In this respect they show a remarkable contrast to the proteins which, whether simple or compound, seem to be of inexhaustible variety. It has been suggested, accordingly, that the differences between different "chromatins depend upon their basic or protein components and not upon their nucleic acids".'

The cause of this 'eclipse period' was paradoxically, but unquestionably, the rise of biochemistry; in particular, of course, the recognition of the vast variety of proteins (definable as chemical compounds the more strikingly after the crystallization of haemoglobin and egg albumen) with their huge range of distinct biological specificities.

For the chemists who were tearing to pieces the delicate structure of the cell with ever-mounting violence, naturally flushed with the success of isolating the key and relatively robust small molecules of amino acids, vitamins and co-enzymes, the delicate macromolecular 'goo' of nucleic acid may have seemed an amorphous mixture of irrelevancies. It is surprising that the integrity and native configuration of at least some proteins seem to have survived their attack. Nucleic acids were still isolated, but grossly degraded in what was believed to be a tetranucleotide state—containing an emasculated sequence of only four bases.

Even as late as the 1920s arguments were occurring as to whether nucleic acids might possibly range up to a size as could include five or even six bases. And it was not until the 1930s that the older, gentler methods for their extraction began to be reinstated

and it was slowly realized that their molecular weights might be very much larger than the 1500 daltons appropriate for a single tetranucleotide. Even then, most nucleic acid chemists, such as Levene & Bass (whose large book(17) on their properties barely touches on their possible biological significance), regarded them as homopolymers of balanced tetranucleotides containing all four bases in the same order and proportions.

A lot of the trouble, of course, was poor techniques—both for estimating nucleotides and separating what were the grossly degraded hydrolytic products of macromolecular nucleic acids from each other.

It was also misleading that the proportions of bases in the only types of DNA then being studied—those from higher animals—'happened', so to speak, to be roughly equal. We would say now that their proportions of guanine and cytosine (G+C) of total base present being not far off 50%, and since content of guanine must equal that of cytosine, and adenine content must equal that of thymine in all double-stranded DNA, all four bases would inevitably be present in roughly equal amounts. Had the chemists of those days systematically studied bacterial DNA where G+C content can vary between 25% and 75%, the tetranucleotide hypothesis might have died an earlier death.

It is interesting and somewhat ironical to note that in 1931 Levene & Bass(17) do in fact mention the nucleic acid of tubercle bacilli, with a G+C content of DNA now known to be around 66% (Hill(18)), as yielding a material which showed great difficulty in giving a constant composition. But this anxiety was concentrated on the finding that the material contained both thymine *and* uracil. That was either against the great current dogma or showed that bacteria were neither plants nor animals!

The dogma in those days, it will be remembered, was that the nucleic acid of plants ('yeast nucleic acid') was always RNA and that of animals ('thymonucleic acid') DNA. And this further confused the picture.

By then, however, things were perhaps just beginning to look up. The chemical nadir for nucleic acids must surely be represented by some of the opinions expressed in a well-known 'monograph' on *Nucleic Acids*(19) published in 1914, with a preface beginning 'The Nucleic Acids constitute what is possibly the best understood field of physiological chemistry', at the time of the most rapid advances in the Hopkins School of Biochemistry! Its contents include sections such as: 'Both nucleic acids [thymus and yeast] also yield cytosine... but where animal nucleic acids yield thymine, plant nucleic acids yield uracil and this distinction holds in every case. Finally, plant nucleic acids contain a pentose group... On the contrary, all animal nucleic acids give rise to laevulinic acid which is formed from a hexose group in their molecule. These statements are universally granted, and one sufficiently alert to the possible sources of experimental error cannot obtain results which differ from them.' And a little later on: '...It is therefore necessary to discuss only two nucleic acids in order to have an understanding of them all.' It is possible to understand and forgive ignorance and mistakes; but surely not such complacent arrogance as that?

It seems to have been almost in the very nature of biochemistry in its early days—perhaps its need to 'prove itself' as respectable chemistry applied to biological tissues—to operate against ultra-dangerous concepts such as those of huge molecules, weak inter-atomic forces and a type of inter-molecular specificity that might appear ludicrous to the orthodox chemist and mechanistic to biologists.

Even Gowland Hopkins, who pulled no punches in his support for the analytic

attack chemists were making on biological systems at that time, was very much on the defensive when he pointed out in a lecture at Harvard in 1936(20) that 'so long as [the modern biochemist's] analysis involves the isolation of events, and not merely of substances, he is not in danger of so departing from reality that his studies have no longer biological meaning'. Thinking of the structure of DNA and how its solution only 17 years later provided the clue to an understanding of heredity and the biosynthesis of proteins, one is struck by how wide of the mark this observation proved to be. Hopkins, indeed, considered chemical structure could form the basis for explaining morphology, but no more. And two years later he was hard put to it confronting the Cambridge school of cytology under James Gray in defending even that possibility(21).

At the same time, many of the earlier biochemists, like some of the younger and lesser molecular biologists of the present day in their turn, were brash in their claims and interpretations. They tended to shorten and oversimplify the gap between the function and structure of biological systems (on the one hand) and the chemistry of the molecules of which such systems are composed (on the other). As so often happens, the rise of one science set back the development of another.

This, then, was the climate into which Fred Griffith's famous transformation paper was born in 1928(3). And he entered the field laterally, so to speak, without, I believe, realizing it and without appreciating much of what we now, with hindsight, know to have been vitally relevant to the whole history of DNA.

Fred Griffith was a modest and retiring personality who enjoyed working quietly on his own, shunning scientific meetings. According to his colleague V. D. Allison(10), he had to be practically forced into a taxi to attend the London International Microbiology Congress in 1936. And then, I am told(22), he reluctantly and nervously read out his rather boring paper in such an unenthusiastic manner that those not closely concerned with the detailed streptococcal typing techniques he was expounding must hardly have felt it was worth listening to. This was, it seems, the only paper he ever delivered at an open meeting and it had, of course, nothing to do with transformation.

He would not even be persuaded to give an informal paper to the Medical Research Club with a view to becoming a member(22).

He was also very reluctant to write papers; but immensely hard-working, meticulously careful and scrupulously honest in his researches. Before 1928 he had made important contributions to the epidemiology of infectious diseases, first with his brother Stanley in typing tubercle bacilli and later, more or less on his own, in the classification of streptococcal types as well as Salmonella and staphylococci. At that time, Graham Wilson has pointed out(23), he was firmly convinced of the fixity of bacterial types—at least in so far as the mammalian tubercle bacilli were concerned. The vagaries of the pneumococcus in the changes from smooth, virulent (S) to rough, attentuated (R) and back again may well have been a source of irritation as well as interest. His description(3) of his great discovery occupied quite a small proportion of what would nowadays be regarded as an immensely long 46-page paper on 'The significance of pneumococcal types'. In this he showed that heat-killed S pneumococci of (for instance) type I could, when inoculated into mice along with living R cells originally of type II, lead to the appearance of virulent S cells of type I (and vice versa). It must surely have been made almost *despite* his own emotional inclinations, rather than, as is so often the case, *because* of them.

We are used to hearing about how Copernicus, the mystic Sun-worshipper, must have been irrationally impelled towards a theory of the Universe that made the Sun, rather than the Earth, its centre. And, again, about how Pasteur himself, a devout Christian of the old school, launched himself so fiercely into the controversy against the then quite fashionable doctrine of Spontaneous Generation because the idea of the creation of life without God was an impious thought.

With Fred Griffith, it must, if anything, have been quite the opposite. It was his great care, perfectly planned controls and scrupulous honesty that forced him not to hedge and towards the correct conclusion that (to quote) 'there seems to be no alternative to the hypothesis of transformation of type'(3). One almost wonders to what extent he might have wished for an alternative! And he adds that 'this is considered less unacceptable than previously because it is now (since the last few years) widely recognized that R forms occur spontaneously and can spontaneously revert to *their own* S type'. In fact, ironically enough, the S to R change (in the presence of immune serum) was well appreciated even in the previous century, and we now know that it and the reversion of R to S must (presumably) be due usually to point mutations—quite a different process from transformation, which involves substitution of one or more genes (i.e. a long stretch of DNA), through DNA recombination, by their allelic homologues.

The demonstration of specific transformation was an achievement in other respects, as perhaps would only be properly realized if it is appreciated how confusing the situation must have appeared at the time, due to facts such as the following:

(1) Not only could R types revert to their 'own' S types spontaneously, but it had long been known that this reversion could be greatly enhanced by a number of non-specific factors, *including the addition of heat-killed pneumococci of the same or another type*. (This was currently thought to be due to the action, in the culture, of toxic 'aggressins' which operated against leucocytes.)

(2) The temperatures used for killing the S types of cell affected the transforming abilities of different types differentially. Quite apart from that, transforming abilities differed between types, as also did non-specific stimulation of reversions.

However, the key fact elucidated by Griffith was that if *change* of type occurred, it was only in the presence of heat-killed cells of *another* type and towards the same type as that of the added, killed cells.

I hope it will not be taken in any way depreciatory of his achievement if I point out that Griffith seemed to have had little idea of how this transformation came about, nor even of its great and ultimate significance. His discovery was a classic instance of pure serendipity.

After all, he was, in essence, a naturalist. His interpretation was teleogical and looked at from the point of view of the pneumococcus which, being the kind of organism with which he was associated for most of his working life, I suspect he must have loved, with the quiet, but usually unexpressed, *passion* that all true biologists have for the system with which they work.

'The apparent transformation,' he writes, 'is not an abrupt change of one type into another, but a process of evolution through an intermediate stage, the R form, from which the type characters have been obliterated.' In this, of course, he is quite wrong, although, for purely technical reasons, it was a long time before it was found possible to transform S types directly into one another. This view is also rather inconsistent with

the attitude he takes elsewhere in the same paper postulating the idea of 'cycles of states' through which the pneumococcus may pass, and the feeling that, perhaps, in the R state remnants of the previous S state had not been entirely eliminated: 'minor' components of all types could have been present in all types after all, he points out.

'It would appear', he says, 'that the type I antigen no longer serves its *purpose* [my italics] in the presence of the immune substance formed during convalescence, and the pneumococcus consequently develops its type II side.... The idea of a regular sequence of changes in the type of pneumococcus before the development of pneumonia and during recovery... is in harmony with the experimental data.'

Although apparently quite clear that the controlling factor in manifestation of type specificity was the substance responsible for forming the 'specific soluble substance' (SSS) (i.e. the polysaccharide of the capsule) and not the SSS itself, he did not distinguish clearly between it and the factor actually responsible for transformation which, very understandably, he took for granted must be a protein. The actual words he used were: 'When the R form of either type is furnished under suitable experimental conditions with a mass of the S form of the other type, it appears to utilize that antigen as a *pabulum* from which to build up a similar antigen and thus to develop into an S strain of that type.' This seems somewhat inconsistent with his clear conclusion that the heat-lability of the transforming factor indicated that it could not be the heat-stable polysaccharide antigen itself.

Apart from that, and, as with the biologists of the last century to whom I have already referred, there does not appear to be any realization that there is a fundamental problem here. 'Pabulum' simply means a source of food, and there is no indication that what had to be explained was how such a specific pabulum (be it antigen itself or antigen-forming factor) gave rise—indefinitely—to more of the same pabulum. In fact, scientists were only just beginning seriously to attempt to do this at the time, nor indeed was the problem—clear and obvious though it seems to us now—really formulated properly for another eight years, when J. B. S. Haldane wrote a remarkable article to which I will refer in detail later on.

However, H. J. Muller, in a brilliant essay(24) written as early as 1922, had pointed to the difficulty of understanding the 'self-propagation' property of the gene: '... the fact that, within the complicated environment of the cell protoplasm, it reacts in such a way as to convert some of the common surrounding material into an end-product identical in kind with the original gene itself'. But he later (in the same article) admits that 'there is yet no means of deciding whether the chief features of the autocatalytic mechanism [of the gene] reside in the structure of the genes *themselves*, and that the outer protoplasm does little more than provide the building material...', or whether 'gene structure might mean nothing more than the possession by each gene of some very simple character... which enables each gene to enter into combination with certain highly organized materials in the outer protoplasm in such a way as to result in the formulation "by" the protoplasm of more material like this gene which is in combination with it'.

We should now, of course, describe the problem of deciding between these two alternatives as that of determining where the exclusive information for gene replication resides: in the DNA itself or in molecules used for the biosynthesis of DNA, namely the enzymes and building blocks concerned.

Fred Griffith's paper was not ignored (as might almost have been expected). His

findings were confirmed by Neufeld & Levinthal(25) and others only a year or two later. But there was considerable scepticism and even frank disbelief amongst many bacteriologists, none of whom, however, as Graham Wilson emphasized(23), would 'dare express their doubts in public' because of his great reputation for scientific integrity. 'Griffith,' Wilson says, 'they thought might have been misled by some technical error, but he would never have put his name to anything in whose truth he was not profoundly convinced.'

In fact, his firm conclusion on the reality of type transformation must have been all the more convincing to others—and therefore more effective in stimulating their interest—just because he was so well known as a sceptic and as one who demanded the most rigorous proof of a new discovery before accepting it. According to his assistant, W. R. Maxted(26), who knew him well during the last few years of his life, he was only just beginning to accept the Lancefield group classification of streptococci in 1940, several years after it had been generally acknowledged in bacteriological circles.

He never pursued the phenomenon of transformation further himself. S. D. Elliott(22) remembers him explaining that it was now 'up to the chemists'. Nor did he, but rarely, refer to it in conversations with others. Indeed, he turned his own attention increasingly to the new problem of typing haemolytic streptococci—a matter of great importance in epidemiology—and left others to continue the task of pneumococcal typing, using the sera that he had himself prepared.

I do not believe he really could have been very much interested in the phenomenon of transformation which he himself had discovered. But he certainly had started something, and the research developments thus initiated continued—albeit slowly—in a pretty well unbroken series continuously on towards the present time.

So, after 1928, the centre of interest and activity passed to the Rockefeller Institute in New York, under the stimulating guidance of Oswald Avery.

After again confirming type transformation *in vivo* in 1930, Dawson(27) together with Sia(28) demonstrated the phenomenon *in vitro* in 1931, and Alloway's(29) successful transformation with crude cell-free extracts in 1932 and more purified preparations in 1933(30) strengthened the interest and importance that was beginning to be attached to it.

I do not think there is much doubt that Avery had a fair idea of what he was after and certainly of the fundamental importance of transformation. He never actually visited Griffith's laboratory in Endell Street, nor did he and Griffith ever correspond. But, according to one who knew them both well(22), they held each other in high esteem and there is no doubt that Avery was greatly stimulated by Griffith's discovery. He shared his preoccupation with pneumococci and streptococci and in some ways was a rather similar character. Both men were born within a few months of each other and were confirmed bachelors. Both were exceedingly modest, meticulously careful in their experimental work and extremely generous with the time they spent in helping others. Both were almost obsessively cautious in reaching conclusions. Avery is quoted(31) as remarking to his brother Roy, in 1943, that 'it is lots of fun to blow bubbles but it is wiser to prick them yourself before someone else tries to'. And both made their major contributions to knowledge relatively late in life. Griffith was over 50 when he discovered transformation, and Avery 67 when he published his paper on DNA as the transforming agent. Perhaps that will be an encouragement to those of us in our sixth decade, or beyond, to hope that even now all is not quite lost!

In other respects, however, they were rather different: Avery being much more openly friendly and communicative whereas Fred Griffith was very shy and aloof and difficult to get to know.

There were others, besides Avery's group, who had been excited by Fred Griffith's discovery. In 1936 Berry & Dedrick(32) claimed to have transformed the virus of Shope's rabbit fibroma into that of the closely related infectious myxomatosis by the addition of heat-inactivated myxomatosis virus. This effect was confirmed by others and served to strengthen Avery's interest in the whole field.

It is, however, ironic now to know that this virus 'transformation', which at the time emphasized the general significance of the pneumococcal transformation, is no longer considered to be a genetic effect at all, but according to Fenner(33), a rather complex and purely phenotypic 'rescue' of the inactivated myxoma virus due to the induction of a de-coating enzyme in the host cell which liberates, and so reactivates, the myxoma DNA.

DNA itself was still very much in the background in those days. Berry is quoted as believing that 'nucleoprotein' was the operative transforming principle in his effect. And Avery has been mentioned as 'wistfully suggesting' to Hotchkiss(34), as early as 1936, that the transforming agent 'might be a nucleic acid'. But proteins still completely dominated the field of biological specificity; and even if nucleic acids were recognized as respectable chemical entities (still, of course, largely as tetranucleotides!), they were closely linked for biologists with proteins in the 'nucleo-proteins' often referred to and well known as the major constituent of viruses which were then first being studied intensively by biochemists such as the Piries and Barbara Holmes. For instance, as late as 1938 Hopkins(21) could write: 'My colleague, Mr N. W. Pirie, has shown that the active unit in each case [of plant viruses] is, in essential, a complex protein molecule', even though I believe that Bill Pirie(35) was not himself rushing to such a conclusion and already suspected that the RNA was playing a more vital role.

Avery himself, however, may have had good reason not to be so affected by the prevailing fashion regarding the exclusive role of proteins in biological specificity. For he had been chiefly responsible(36) for demonstrating the very high biological specificity shown by the polysaccharides of the pneumococcus capsule. It could have been easier for him, than for many others, to avoid the strong prejudice against considering molecules other than proteins—nucleic acids themselves, for instance, known for half a century or more to be present in, or at least associated with, chromosomes and the nucleus—as possible repositories of powerful specificity.

But despite the remarks quoted above, even Avery seems to have been somewhat surprised at his own finding, because in a guarded letter to his brother Roy in 1943(31), previously referred to, at the time of his great discovery he writes of his conclusion that the transforming agent 'is in all probability DNA: who could have guessed it?'

In fact, however, the possible significance of nucleic acids was beginning to be appreciated by others, and Mirsky(37) had written an important review in 1943 on 'Chromosomes and nucleoproteins' in which he pointed out that 'the specificity of a nucleoprotein may reside in its nucleic acid as well as in its protein moiety'. And, discussing the chemistry of heredity, he also made the important point that 'the kind of experiment that is needed to place the chemistry of the gene on a firm basis is one in which substances extracted from the chromosomes of an organism are administered to a mutant form of the same organism which suffers from a deficiency in its germinal material'. This is a pretty shrewd observation for those days and was, of course,

exactly what Avery was doing at that very time, though neither he nor Mirsky could possibly then have analysed it precisely in that way.

Moreover, during this period, too, there were parallel relevant general advances in biology that served to prepare the way for the final breakthrough.

Caspersson and his school(38) had been developing techniques of ultraviolet spectroscopy applied to intact cells with which it was possible to demonstrate the intense absorption of wavelengths around 260 nm. (characteristic of nucleic acids—and, of course, the single nucleotides and bases of which they were composed) within the nucleus of living cells and by this means demonstrate a rise and fall of nuclear nucleic acid in phase with the mitotic cycle.

During this period, too, the chemists and physicists were at last beginning to realize that molecules of nucleic acid were much larger than the 1500 daltons appropriate for a single tetranucleotide, though they still tended to be regarded as aggregates or simple polymers of 'balanced' tetranucleotides containing all four bases. But the idea of the statistical polytetranucleotide was gaining ground, largely due to the work of Gulland(39).

Their biological role, in so far as it was formulated at all, tended to be considered in the nature of structural support for the gene protein—or (at the best) as what was referred to by Darlington(40) as a 'midwife' molecule to assist non-specifically, in enabling the protein of the gene to replicate itself.

The constant association of nucleic acids with 'self-propagating' systems such as chromosomes and viruses was, however, being stressed by workers such as Caspersson and Astbury, but with reproduction by a direct copying process analogous to crystallization. Astbury(41) in particular was understandably misled by the coincidental fact that the spacing (3·34 Å) of successive nucleotides in a polynucleotide column was almost exactly equal to the spacing of successive side-chains in a fully extended polypeptide.

When Avery(4) mentioned that his transforming DNA might have a molecular weight as large as 500,000, this was not out of keeping with views of chemists and physicists, though we now know that such a size, being barely enough for a single gene, must have been grossly below that actually operative in his transformations, which involve many linked genes. It was difficult to remove DNA-ase completely from the system, and the chain length of the DNA must often have been shortened by enzymic hydrolysis both before and after uptake by the pneumococcus. This, alongside variability of competence factors, must have been largely responsible for the exasperating irreproducibility of the transformation reaction.

During this period and the early years after the identification of DNA as the transforming factor, the lack of consistency in transformation experiments was a continual source of frustration amongst workers on the problem. Indeed, inexplicable 'bad periods' *still* occur, at least with other transformation systems. 'Many were the times', Avery is quoted(31) as saying, 'when we were ready to throw the whole thing out of the window.'

Ideas prevalent 40 to 50 years previously, at a much less sophisticated level, were now beginning to revive. But times were not yet ripe for a concerted attack by biochemists on possible mechanisms for the biological replication of key molecules. The problem had still not been properly broadcast.

But it *had* been defined—defined brilliantly and correctly—if not quite for the first

time, at least for the first time in a manner that made an impact in terms that *could* have been appreciated (even if they were not). This was done by J. B. S. Haldane in an article for Hopkins' 'festschrift'(42) published in 1937. Here, in passages which must be quoted at length, not only is the problem properly defined, but the whole field of molecular biology is outlined, emphasized and characterized—15 years or so before it really became properly recognized.

> Two possibilities are now open. The gene is a catalyst making a particular antigen [referring to blood groups. M.R.P], or the antigen is simply the gene or part of it let loose from its connection with the chromosome. For one essential property of the gene is that it reproduces its like at each nuclear division. . Since a mutated gene is reproduced in its altered form it follows that one gene is reproduced from another. This is an exceptional situation. [This point was originally stressed by Muller(24) in 1922. M.R.P.] A molecule of haemoglobin in a cell is not derived from another similar molecule. If it were, slight changes in the molecule would be perpetuated, and extra-nuclear inheritance would be as common as it is actually rare. But one gene is derived from a like gene. As the gene is of the dimensions of a protein molecule and does not consist of a number of similar parts, we cannot regard its reproduction as a process of growth by accretion ending in division when a limiting size is reached.
>
> It must, on the contrary, be a process of copying. The gene, considered as a molecule, must be spread out in a layer one *Baustein* deep. Otherwise it could not be copied. The most likely method of copying is by a process analogous to crystallization, a second similar layer of Baustein being laid down on the first.
>
> But we could conceive of a process analogous to the copying of a gramophone record by the intermediation of a 'negative' perhaps related to the original as an antibody to an antigen. The process normally stops when one copy has been made, or at least the further copies are not attached to the chromosome.
>
> The whole problem of synthesis is an almost virgin field in biochemistry. And it is absolutely fundamental. If genetics had done no more than pose the question in its sharpest form, it would still be a valuable stimulant to biochemical research. But it has done a great deal more than this. Whereas the classical biochemistry has largely been concerned in analysis of the stages of catabolic processes such as the breakdown of carbohydrates to lactic acid or alcohol and the digestion of proteins, the new brand of biochemistry which will, I believe, arise from genetics, will be concerned largely with the stages of synthesis of such molecules as chlorophyll and cyanin [i.e. macromolecules, such as proteins. M.R.P.] And its final goal will be the explanation and control of the synthesis of genes.

Thus the rise of molecular biology was predicted almost two decades before it began to make its real impact. It was a very remarkable piece of insight—though there was still no mention of nucleic acids.

So I reach the last phase of my historical sketch: the bare decade between Avery and Watson–Crick.

This was very far from a mopping-up period. It was of immense significance that the specificity of the transforming agent had been shown to reside in DNA and not in protein—though there was great reluctance by many, particularly chemists, to accept it.

Even in 1948, and succeeding years, Hotchkiss(43) was still having to argue fiercely to support his exceedingly careful work eliminating the participation of trace amounts of protein in the transformation phenomenon. However, with Hershey & Chase's(44) demonstration in 1952 that bacteriophage left all (that is, experimentally, more than 99 %) of their protein coat and other superficial 'apparatus' behind on infection of the cell, it became very difficult still to maintain that nucleic acid was not the key substance in the maintenance of genetic continuity.

In the period immediately after the Second World War it was still fashionable and perfectly respectable, despite Avery's work, to consider proteins as the key self-

replicating molecules, while in 1946 a chemist(45) could still argue that the specificity of the transforming principle might lie in the *polysaccharide* molecule of the capsule itself, acting as a specific primer (on analogy with the 'directing' action of dextran in biosynthesis of more dextran from sucrose) for further production, by a non-specific enzyme, of the same type of polysaccharide.

It took another 15 years or so for most workers to realize that DNA was not just *another* type of specificity but the origin, the fount, of all or *most* specificities: that all heteropolymeric proteins derive their information from DNA and RNA and not vice versa. Even Avery himself(4) leant over backwards to emphasize that showing the role of DNA in pneumococcal transformation did *not* mean it was the key heredity substance for all living systems! The attitude of most biologists at that very time could be typified by Dubos(46), who, in 1945, giving a full and fair account of Avery's discovery published only a few months previously, wrote: 'Definition of their nature (referring to competence factors) will eventually elucidate the mechanism of the transformation and will, one may hope, provide a pattern for the analysis of the phenomena of bacterial variability.' This was a restrained but perfectly reasonable comment which, however, completely avoided any temptation (if, indeed, any temptation existed) to extrapolate the phenomenon of pneumococcal transformation to the mechanism of heredity in general. Transforming DNA *might* explain some of those curious variations that were continually cropping up in bacterial cultures; that is all.

There were, indeed, a number of misleading clues and confusing facts which delayed progress.

Peter Walker(47) pointed out two of the most important of those red herrings. The first were the ideas of the 1940s stemming mainly from some leading geneticists(48) indicating that nuclear DNA was broken down to nucleotides and resynthesized, possibly through RNA, during each cell division cycle. Despite many other facts supporting Caspersson's theories on the vital role of nucleic acids in cell heredity, these ideas served to implant considerable scepticism regarding genetic continuity of DNA.

The doubts were supported by the second of the red herrings which arose from the apparent decrease in the ultraviolet absorption of the nucleus during telophase—an observation which was in fact due to a big *relative* increase of the u.v. absorption in the cytoplasm, from the synthesis of RNA, only later properly established by Brachet(49), and accumulation of nucleotides therein, and did not represent any absolute loss of nucleic acid from the nucleus.

However, during those days, other workers, in particular Chargaff and his colleagues in New York, and Randall and his group (including, of course, Maurice Wilkins and Rosalind Franklin) at King's College, London, had become interested in nucleic acids (from a chemical and physical standpoint respectively) as large molecules having an important biological activity. Their objectives were to try to discover, with the greatly improved techniques then rapidly becoming available, the basis of the specificity that must surely be present in the molecule. Unquestionably, one of the possibilities envisaged was the sequence of nucleotides in the molecule.

But Chargaff(50) was well aware of the difficulties, pointing out that identity of natural polymers is necessarily difficult because in many cases they will be 'substances that differ from each other in the *sequence*, not in the proportion, of their constituents'.

Up to this time—the late 1940s—the only DNAs properly analysed for base composition were those from higher organisms where the G+C proportion of total base

present in the DNA in different species is now known to range no more than from 40 to 45%, in contrast with that in viruses and bacteria, which spreads from 25 to 75%(51).

Since the ratios A/T and G/C must be unity in double-stranded DNA (and that means practically *all* DNA), in DNAs where the G+C proportion of the total 'happened', so to speak, to be nearly 50%, it follows that the proportions of all bases must be approximately equal, and that, therefore, no variations between DNAs from what were then, of course, considered to be widely differing sources, could possibly emerge. One of the vital clues which led Watson & Crick to the correct solution of the structure of the DNA double helix(1) was therefore lacking until Chargaff and his colleagues(52) started to analyse the DNA of yeast and tubercle bacilli in 1948/9. The G+C content of DNA from these two micro-organisms are 36% and from 62 to 70% respectively of the total. This big difference thus highlighted the constant ratios of near-unity found for A/T of 0·95 and for G/C of 1·0.

Chargaff, though quick to spot a possible clue, was understandably reluctant to push it too far and simply commented as follows at the end of his 1949 paper(52): 'A comparison of the molar proportions reveals certain striking, but perhaps meaningless, regularities.' Further work(53) on DNA of *Serratia marcescens*, *Bacillus schatz* and *Haemophilus influenzae* showed analogous species differences and the same (approximate) relationship of A to T and G to C, despite big, overall differences in G+C%.

But an explanation had not been offered when two years later in 1951 Chargaff(54) wrote: 'As the number of examples of such regularity increases, the question will become pertinent whether it is merely accidental or whether it is an expression of certain structural principles that are shared by many deoxypentose nucleic acids, despite far-reaching differences in their composition and the absence of a recognizable periodicity in their nucleotide sequence.'

Others were also working on DNA base compositions, no doubt with the same idea in mind, but they never got so far.

Mirsky's group(55) compared the pneumococcal transforming DNA with thymus DNA and found a rather lower G+C% in the former. Although the A/T and G/C ratios approached unity in both cases, no comment was made on their possible significance. The vital point may here have been obscured by the large number of other base compositions they analysed at the same time, mainly from animals, where approximately equi-molar proportions of the four bases would, of course, have been expected.

Wyatt(56) analysed the DNA of certain insect viruses and found G+C% values varying at least twofold with A/T and G/C ratios all around unity but came to the rather surprising conclusion that 'no direct parallel can exist...between biological relationship and DNA composition, since the groups having equal A+T/G+C ratios include viruses as unlike as any examined'. This argument is difficult to understand if he had conceived that a most likely, or even a *possible*, basis of DNA specificity was in the nucleotide sequence rather than overall base composition. But, of course, it is easy to forget that at that time there could have been no theories about the nature of the DNA/protein code and that most people, in spite of Chargaff's warning, were probably thinking in terms of gross differences both in composition and sequence.

It might be, and often *has* been, asked why Chargaff did not pursue his findings more boldly. But in fact there were not then sufficient data available to go much further. No satisfactory techniques were available for sequencing nucleotides, and it needed a

synthesis with the equally significant findings of Wilkins and Rosalind Franklin for the true solution to emerge.

The base ratio part of the story contains, nevertheless, more than its fair share of the irony in scientific discovery. No one yet knows *why*, so to speak, the DNA of all higher organisms has a G + C content so close to 50 % of the total bases as to have obscured a vital clue for so long and to have been at least partly responsible for persistence of the balanced tetranucleotide hypothesis which long delayed progress in the chemistry of DNA. There is no known molecular reason why this should be so, since bacteria and viruses seem to get on very well with percentages of G + C ranging between 20 and 75. Such a spread is quite consistent with what is known of the degeneracy of the nucleotide/amino acid code. It is, however, difficult to believe that the use of the code, which appears to be universal, and other factors which may contribute to the determination of overall base composition, have no biological significance. At all events a major piece of ill fortune seems here to have been involved.

On the other hand, it is very lucky that Chargaff did not begin his bacterial work with *Escherichia coli* (as most modern molecular biologists would assuredly have done!). This species has a G + C content (50 to 52 %) not differing strikingly from that of animals and plants and such an analysis, at that particular time, would perhaps have so disappointed him and his colleagues that they might have concluded that there was nothing to be found out in DNA by that approach and given it up. An important clue might thus have been missed for even longer.

* * *

This short historical essay is, of course, a lamentably over-simplified account of a fascinating and all-important advance in knowledge. But the story is not, I believe, atypical of the sorts of factors that operate for many scientific discoveries. The eccentricity of human personalities, their prejudices, their personal ambitions, their *un*reason, combine it would seem with the vagaries of fortune to exert more influence on the detailed short-term course of events than the cold force of logic.

Above all, it shows how extraordinarily difficult it is for people to accept new ideas and thus how reluctant they are to *try* to do so—even though the ideas are simple, clarifying and basically quite easy to understand if approached directly, from scratch, so to speak, as a child would do. What a prison we seem to make of our own minds!

A striking instance of the slow permeation of a new concept is illustrated by the inclusion, in a textbook published as recently as 1960(57), of a diagram showing Haurowitz's 1949 (then not unreasonable) hypothesis(58) on the mechanism of protein self-replication by a direct copying process with itself as the template: an inclusion not introduced as an historical curiosity but as an indication of the type of mechanism which must be accepted as a serious contemporary possibility—with very little emphasis on the Watson–Crick hypothesis for the mechanism of DNA replication and all the evidence in support of it, as the fundamental process of biological replication.

'Great discoveries', as André Lwoff puts it(59), 'are dangerous.' They are often hated because they require the immense emotional (and therefore intellectual) effort of abandoning preconceived dogmata. And, because they are hated and feared, they are, as Goethe himself pointed out, nearly always subjected to ridicule. In the present story one particular instance, cited by Hotchkiss(31), occurred as late as 1954 at a symposium on transformation in the United States where an eminent bacteriologist asked

sardonically, but in all seriousness, whether the genetic activity of DNA was 'based on evidence or was merely a voting agreement'.

Even now there are still a few sceptics in the sense of those who suspect that perhaps some (possibly biologically irrelevant) information can be supplied *by* proteins *to* DNA and other polynucleotides—as they are being formed. I suspect they may even be right. But very few would now say that such instances are common or very significant, although some well-known voices (60) are still heard as recently as 1968 with vitalistic and almost meaningless remarks such as:

> Biologists have confronted successively—like a nest of Chinese boxes—levels of complexity ranging from the eco-system to the internal chemistry of the cells. The last box has been opened. According to the Watson–Crick theory, it should have contained the single source of all the inherited specificity of the living organisms—DNA. It is my view that the last box is empty and that the inherited specificity of life is derived from nothing less than life itself.

There are, however, other sceptics of quite a different and much more interesting and valuable type who try to look into the future and consider how our present ideas may develop: those few who feel, like Pirie (himself one of the earliest workers to be concerned in the biology of nucleic acids), that the mechanism of information replication may not be so exclusively centred on nucleic acids as is fashionably supposed at the moment.

We may still be being misled by thinking we know more then we do—in a manner analogous, as Pirie (61) puts it, to the card-player who 'assumes that, although there are 52 types of card, only *he* knows which ones are in his hand because there is only one type of back to a card. He will fare badly when confronted with a card-sharper who has marked the backs so that there are 52 types of back as well. We may be victims of a similar subtlety when we discuss "information" transfer...for we have no means of knowing that we know all the clues that exist. Accumulated biological experience suggests that Nature is as subtle as any card-sharper.'

As a purely fanciful example, suppose we postulate the existence of some highly specific, hitherto unrecognized, slim macromolecule that is in fact necessary for proper replication of DNA (it could even be a type of natural DNA-replicating enzyme). And we suppose that this substance undergoes an extremely slow sequence of chemical changes that result in the 'inaccurate' replication of DNA of a nature such as to lead to a sequential series of modifications in the resultant proteins of a type that is biologically significant to the species concerned—a substance, that is, having the property of a *directing* entity responsible for non-Darwinian evolution. Well, such a hypothesis would, quite reasonably, be rejected with impatience because not only is there no evidence that an entity exists with such properties but (much more important) there is no real *need for it at the moment.*

Some time later on, when molecular biology becomes more complicated again, we can be pretty sure that analogous 'needs' *will* arise (probably in a very different context) and some major reorientation will again be required of us. Just because it is practically impossible to guess what this will be should not absolve us from realizing that it must certainly occur. It should serve to keep our minds as clear as possible from too monolithic and rigid assumptions.

In conclusion, it emerges that in the discovery of DNA there could be few more contrasting pairs of personalities than, on the one hand, Watson and Crick, who were

prepared to stick their necks out and go well beyond the facts in a brilliant thrust of insight, and, on the other hand, Avery and Griffith, who were, if anything, unreasonably careful and almost too modest in their conclusions.

Both types of personality have their drawbacks and virtues, but in this instance what seems to have been of really crucial importance was that they followed each other in the right order: a matter of natural selection, of course, but still worth remembering as an occurrence of critical significance.

Since more is heard of glamour stories and flamboyant personalities in the advance of scientific knowledge than of the quiet workers and those long, tedious and superficially unexciting experiments, with careful controls, that may be just as important, it is well that we should also pay tribute to those who can do great work without noise. And I can do no better than to conclude by quoting Rollin Hotchkiss's tribute(34) to Avery: 'When men and ideas rub against each other, the ideas receive maximal polish if the man is gentle and his principles hard', because I think that applies equally to Fred Griffith.

I am most grateful to Professor E. Chargaff, Mr N. W. Pirie, Professor P. M. B. Walker and Professor M. F. H. Wilkins, who responded generously to my request for information of a personal nature relevant to the more scientific part of this lecture.

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Mutants of *Neurospora crassa*, *Escherichia coli* and *Salmonella typhimurium* Specifically Inhibited by Carbon Dioxide

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SUMMARY

Mutants of *Neurospora crassa*, *Escherichia coli* and *Salmonella typhimurium* are described which are inhibited by CO_2 at concentrations which do not inhibit the parental strains from which the mutants were derived. Sensitivity to inhibition by CO_2 is caused by single gene mutations. The CO_2 inhibitions are reversed by specific substances; for example, the CO_2 inhibition of a methionine-requiring mutant of *N. crassa* is reversed by purines, and the CO_2 inhibition of a prototroph of *E. coli* is reversed by methionine or vitamin B_{12}. The nature of the defects in the CO_2-inhibited mutants is discussed.

INTRODUCTION

Several mutants of *Neurospora crassa* and *Escherichia coli* grow in minimal medium without their specific growth factors when the gas phase is air containing 20 to 30% (v/v) of carbon dioxide (Broadbent & Charles, 1965; Charles & Roberts, 1968). Mutants which are stimulated by CO_2 in this way are called 'CO_2 mutants' (Charles & Broadbent, 1964); they are not usually stimulated by bicarbonate. The mechanism of the stimulation is not known with certainty and may not be the same for all CO_2 mutants. In some CO_2 mutants the mutations affect enzymes which catalyse CO_2-incorporation reactions, and it is probable that high concentrations of CO_2 may compensate for decreased enzymic activity by a mass action effect; this may be the mechanism of the stimulation in some purine-requiring and arginine-requiring mutants which are CO_2 mutants. In other CO_2 mutants, for example certain mutants of *E. coli* which require isoleucine + valine, the mutations apparently affect enzymes which do not catalyse CO_2-incorporation reactions and it is not known how CO_2 stimulates their growth.

CO_2 mutants have also recently been isolated from *Salmonella typhimurium* (H. P. Charles, unpublished).

The present report describes mutants of a new class; these are inhibited rather than stimulated by CO_2. These mutants will be called 'CO_2-inhibited mutants'. They have been found in *Neurospora crassa*, *Escherichia coli* and *Salmonella typhimurium*. They are inhibited by concentrations of CO_2 which stimulate the CO_2-requiring mutants and which do not inhibit the parental strains from which the CO_2-inhibited mutants are derived.

METHODS

Strains. The *Neurospora crassa* wild-type strains used were EMERSON 5256*A* and EMERSON 5297*a*, kindly provided by Professor D. G. Catcheside (Department of Genetics, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T., Australia), and 74-OR23-1*A* and 74-OR8-1*a*, kindly provided by Dr F. J. de Serres (Oak Ridge National Laboratory, U.S.A.). The *N. crassa* methionine-requiring strains *met-1* (35599), *met-2* (K44), *met-3* (36104), *met-4* (39816), *met-5* (9666), *met-6* (35809), *met-7* (4894), *met-8* (P53) and *met-9* (C124), the lysine-requiring strains *lys-2* (37101*a*) and *lys-3* (28815) and the para-aminobenzoic acid-requiring strain *pab-1* (1633*A*) were kindly provided by Mr W. Ogata (Fungal Genetics Stock Center, Dartmouth College, Hanover, New Hampshire 03755, U.S.A.). The methionine-requiring strains *mac* (65108) and *me* (S2706) were kindly provided by Dr Noreen E. Murray (M.R.C. Molecular Genetics Unit, Department of Molecular Biology, King's Buildings, West Mains Road, Edinburgh 9).

The strain of *Escherichia coli* from which the CO_2-inhibited mutants were isolated was strain AB 1621 (F^-; *T6-r, thi, mtl, xyl, str-r, ara, gal, lac, T4-r*), kindly provided by Professor E. A. Adelberg (Department of Microbiology, Yale University, New Haven, Connecticut 06510, U.S.A.).

The CO_2-inhibited mutant of *Salmonella typhimurium* was isolated from strain LT2, kindly provided by Dr P. E. Hartman (Johns Hopkins University, Baltimore, Maryland, U.S.A.).

Media. The minimal medium used for *Neurospora crassa* was that of Vogel (1956) solidified with agar (Difco Bacto, 1·2% w/v) when necessary. Sorbose (5 g./l.) was added to the solid minimal medium to restrict the growth of colonies. The complete medium used for *N. crassa* was the conidiation medium of Horowitz (Ryan, 1950).

The minimal medium used for *Escherichia coli* and *Salmonella typhimurium* was that of Vogel & Bonner (1956). It was supplemented with thiamine (2 mg./l.) for *E. coli*, and was solidified when necessary with Difco Bacto agar (1·2% w/v). The complete medium used contained the following (g./l.): tryptone (Oxoid), 10; yeast extract (Difco), 5; KH_2PO_4, 3; glucose, 5; agar (when required), 15.

Unless otherwise stated, all cultures of *Neurospora crassa* were incubated at 30° and all cultures of *Escherichia coli* and *Salmonella typhimurium* at 37°.

The effect of CO_2 on growth was tested by incubating cultures in Petri dishes in 5 litre vacuum desiccators containing an appropriate mixture of air and CO_2 as described by Charles (1964).

Isolation of mutants. The CO_2-inhibited mutant of *Neurospora crassa*, F4, was obtained from ultraviolet-irradiated conidia of the wild-type strain 74-OR23-1*A* by the filtration enrichment procedure (Catcheside, 1954; Woodward, De Zeeuw & Srb, 1954). The filtration procedure was modified in that the irradiated conidia were incubated throughout the filtration period in a gas phase of air supplemented with CO_2 (about 30%, v/v). This modification was introduced in order to suppress growth of any CO_2-inhibited mutants which might be present in the population and which would otherwise grow and be removed from the suspension by filtration. The duration of the incubation period in the filtration enrichment experiment was 48 h. and the suspension was filtered approximately every 5 h. during this period. Samples of the final filtrate were plated on conidiation medium, and after incubation for 3 days conidia from the

colonies which appeared were tested for ability to grow on minimal medium in air and in air supplemented with 30% (v/v) CO_2. The growth factor requirements of auxotrophs were determined by the auxanographic method (Pontecorvo, 1949). All auxotrophs obtained were tested to determine whether or not the response to their respective growth factor was inhibited by the presence of 30% CO_2 (v/v) in the gas phase.

The CO_2-inhibited mutants of *Escherichia coli* and *Salmonella typhimurium* were isolated as follows. Samples of an ultraviolet-irradiated cell suspension were plated on complete medium and incubated in air. Colonies which grew were then printed by the replica plating technique (Lederberg & Lederberg, 1952) on to each of two plates of minimal medium, one of which was incubated in air and the other in air supplemented with 20% (v/v) CO_2. Plates were observed after incubation for 24 h. and any colonies which failed to appear on the plates incubated in CO_2-supplemented air were picked from the corresponding air-incubated plates and retested to confirm their CO_2-inhibited phenotype.

Crosses. Crosses between strains of *Neurospora crassa* were made on cornmeal agar medium (Smith, 1954) supplemented with the growth factor requirement of the protoperithecial parent. They were incubated at 25° and analysed about 2 weeks after ejection of the ascospores. Germination of the ascospores was induced by placing them at 60° for 45 min. Germinated ascospores were individually transferred to tubes of conidiation medium and the resulting cultures were tested for phenotype after incubation for 3 to 4 days.

RESULTS

One mutant of *Neurospora crassa* which showed inhibition by CO_2 (30%, v/v, in air) was a newly isolated lysine-requiring mutant, F4, derived from wild-type strain 74-OR23-1*A* by the procedure described in Methods. This mutant shows the characteristics of CO_2-inhibited mutants in their simplest form. The sensitivity of the mutant to inhibition by CO_2 was not due to the mutation which causes the lysine requirement, because a cross of the mutant to wild-type (strain 74-OR8-1*a*) yielded some segregants which were inhibited by CO_2 (30%, v/v) but which did not require lysine. These CO_2-inhibited segregants were prototrophic in ordinary air. Crosses of CO_2-inhibited prototrophic segregants to wild-type strains gave only wild-type and CO_2-inhibited prototrophic segregants in equal proportions, showing that CO_2-sensitivity was determined by a single gene mutation. This suggested that an enzyme in the mutant did not function effectively in the presence of 30% (v/v) CO_2. The function of this enzyme might be revealed if the CO_2-inhibition could be reversed by supplying a nutrient in the medium. By auxanographic experiments it was found that cytidine reversed the CO_2-inhibition of mutant F4 and of the prototrophic segregants derived from it and caused them to grow as well as wild-type in the presence of 30% (v/v) CO_2. Other metabolites, including other pyrimidines and pyrimidine derivatives, did not reverse the CO_2 inhibition. Later experiments showed that only one of several samples of cytidine reversed the CO_2 inhibition, indicating that an impurity in the active sample was responsible for reversal of the inhibition. The sample of cytidine was too small to permit identification of its active component and it was not possible to obtain more cytidine of the same batch. The possibility that the impurity was a vitamin was unlikely since all available vitamins were tested on the mutant and failed to reverse the inhibition.

Analysis of crosses between CO_2-inhibited prototrophic segregants derived from mutant F4 and various marker strains indicated that the mutation causing sensitivity to CO_2 was located in the right arm of linkage group V. Crosses with the Group VR markers *lys-2* (37101*a*) and *pab-1* (1633*A*) gave recombination frequencies of 23 and 7% respectively. Crosses of CO_2-inhibited prototrophs with mutants in other linkage groups gave recombination frequencies ranging from 45 to 70% recombination.

The first CO_2 inhibition to be discovered was in *Neurospora crassa* mutant 35809*a* which has a mutation at the *me-6* locus (Beadle & Tatum, 1945; Perkins, 1959). Under ordinary cultural conditions, when the gas phase is air, the mutant requires methionine for growth and does not respond to methionine precursors (Murray, 1960). When the gas phase was 70% (v/v) air + 30% (v/v) CO_2 the mutant did not grow on methionine-supplemented medium. Auxanographic experiments showed that the mutant grew on methionine-supplemented medium, in the presence of 30% (v/v) CO_2, when adenine or other purines were supplied. Purines therefore reversed the inhibitory effect of CO_2. The *me-6* mutant 35809 thus seemed to have two defects, one expressed as a requirement for methionine and the other expressed as a requirement for adenine in the presence of 30% (v/v) CO_2. It is difficult to understand how such defects could be caused by a single gene mutation, and it seemed that the mutant had mutations in two different genes. However, the requirement for methionine and the sensitivity to inhibition by CO_2 did not segregate when the mutant was crossed to wild-type, showing that CO_2 sensitivity was caused either by the same mutation as that which caused the methionine requirement (*me-6*) or by a second mutation closely linked to the *me-6* locus. On the assumption that CO_2 sensitivity was caused by a second mutation closely linked to *me-6*, attempts were made to separate it from *me-6* by crossing the mutant to mutants *lys-3* (28815) and *mac* (65108), which carry mutations close to, and on either side of, the *me-6* locus. Prototrophic segregants arising by recombination in the chromosome interval between *me-6* and *lys-3* or *mac* were selected from the crosses and tested for sensitivity to CO_2 inhibition. None of the prototrophic segregants obtained was CO_2-inhibited. It therefore seems that the CO_2 sensitivity of mutant 35809 was caused by the *me-6* mutation.

Recently, a second methionine-requiring mutant *me* (S2706), kindly given to us by Dr Noreen E. Murray, has been found to resemble mutant *me-6* (35809) in its nutritional characteristics. Mutant *me* (S2706) is leaky but shows a good growth response to methionine; the methionine-dependent growth is inhibited by CO_2, and the CO_2 inhibition is reversed by adenine and other purines. The occurrence of a second mutant resembling mutant *me-6* (35809) provides additional evidence that the methionine requirement and CO_2 sensitivity result from a single gene mutation. Methionine mutants representative of the other known *me* loci, *me-1* (35599), *me-2* (K44), *me-3* (36104), *me-4* (39816), *me-5* (9666), *me-7* (4894), *me-8* (P53) and *me-9* (C124) were not CO_2-inhibited.

When growing in CO_2, mutant *me-6* (35809) partially resembles mutant *mac* (65108), which is reported to require methionine + adenine + cysteine for growth (Dubes, 1953; Perkins, 1959). This similarity in growth requirement together with the apparent close linkage of *mac* to *me-6* (Perkins, 1959) suggested that the mutants might be allelic.

The nutrition of *mac* was investigated. It was found that *mac* grew most quickly with methionine + adenine and less quickly with methionine alone. A requirement for

cysteine was not demonstrable. CO_2 did not inhibit the growth of *mac* under any conditions. Thus *mac* differed from *me-6* (35809) in that it required adenine for growth under all conditions. Experiments by Dr N. E. Murray (personal communication) have shown that *mac* (65108), *me-6* (35809) and *me* (S2706) may be allelic.

Of the CO_2-inhibited mutants isolated from *Escherichia coli* only one has been studied. It grew prototrophically in minimal medium in air but its growth was completely inhibited in a gas phase of air + 20 % (v/v) CO_2. The inhibition by CO_2 was reversed by vitamin B_{12}, L- and D-methionine and was partially reversed by cysteine, cystine, thymidylic acid and DL-serine.

The CO_2-inhibited mutant of *Salmonella typhimurium* similarly grew prototrophically in minimal medium in air and failed to grow in air + 20 % (v/v) CO_2. The CO_2 inhibition of this mutant was specifically reversed by lysine and by the lysine precursor, diaminopimelic acid.

The mutations responsible for the CO_2 sensitivity of the bacterial mutants have not been mapped.

There is evidence that CO_2 does not inhibit growth of the *Neurospora crassa* and bacterial mutants by changing the pH of the medium. Under conditions similar to those of the present experiments, CO_2 caused only trivial changes in the pH of the medium (Broadbent, 1965). Furthermore, the CO_2-inhibited mutants are not more sensitive than the parental strains to changes in this pH. It is therefore unlikely that CO_2 inhibits growth of the mutants by changing the pH of the medium. It is possible that un-ionized CO_2 may penetrate the cells and change the intracellular pH after ionization, but this should cause non-specific inhibition and should not be reversed by single metabolites.

DISCUSSION

Sensitivity to inhibition by CO_2 is caused by single gene mutations at least in *Neurospora crassa*. The CO_2 inhibitions are reversed by single metabolites, and the inhibition of each mutant is reversed by a different metabolite or group of related metabolites. These facts show that the CO_2 inhibitions are specific and affect particular metabolic processes.

Identification of an impurity in one sample of cytidine which reversed the CO_2 inhibition of one of the *Neurospora crassa* mutants (F4) was unfortunately not possible. It may have indicated the nature of the defect in the mutant and have revealed a new growth factor. This is the second unidentified growth factor encountered in the study of mutants showing CO_2 effects; a CO_2-requiring mutant of *Escherichia coli* responds to an unidentified factor in yeast extract (Charles & Roberts, 1968).

The fact that inosine, adenine and other purines reverse the CO_2 inhibition of the methionine-requiring mutant of *Neurospora crassa* (*me-6*, 35809) suggests that CO_2 specifically inhibits an enzyme required for purine biosynthesis. However, the mutation which causes the CO_2 sensitivity does not map at a *purine* locus; both the methionine requirement and the CO_2 sensitivity are caused by mutation at the *me-6* locus. This suggests that the *me-6* locus is concerned with the biosynthesis of a substance required for both methionine and purine biosynthesis. Derivatives of folic acid are required for methionine and purine biosynthesis and it is possible that a reaction in their biosynthesis may be controlled by the *me-6* locus. Mutant *me-6* (35809) may have an incomplete block at this locus, resulting in a requirement for

methionine only, whereas mutant *mac* may have a complete block causing a requirement for both methionine and a purine. CO_2 may cause the purine requirement of *me-6* (35809) by converting the incomplete block into a complete block. Another possible function for the *me-6* locus is control of the biosynthesis of S-adenosyl methionine from its precursors, methionine and adenosine triphosphate. Neither of these suggestions explains why mutation at the *me-6* locus should cause sensitivity to inhibition by CO_2.

The CO_2-inhibited mutant of *Escherichia coli* resembles certain mutants of *Salmonella typhimurium* which also require methionine or vitamin B_{12} for growth. It differs from them in that its requirement is expressed only in the presence of a high CO_2 concentration. The Salmonella mutants have defects in the enzyme N^5-methyltetrahydropteroyl-triglutamate-homocysteine transmethylase (Smith & Childs, 1966) which catalyses the direct methylation of homocysteine in the biosynthesis of methionine (Guest, Friedman & Foster, 1962). Vitamin B_{12} supports the growth of the Salmonella mutants by stimulating the synthesis of methionine from homocysteine by an indirect mechanism (Guest *et al.* 1962; Cauthen & Foster, 1966). It may be that the *E. coli* mutant is unable to effect the direct methylation of homocysteine when the gas phase is air supplemented with CO_2, and thereby becomes dependent upon methionine or vitamin B_{12}.

The CO_2-inhibited mutant of *Escherichia coli* also shows similarities with spontaneously occurring variants of *Mycobacterium tuberculosis* described by Schaefer (1957). Schaefer's variants were inhibited by CO_2 when grown on an oleic acid + albumin medium containing glutamic acid as sole organic nitrogen source. This CO_2 inhibition was reversed by vitamin B_{12}, L- and D-methionine, DL-homoserine, D-serine and D-alanine and 'less regularly' by various other amino acids.

We can propose no explanation of the apparent inhibition of lysine biosynthesis by CO_2 in the CO_2-inhibited mutant of *Salmonella typhimurium*.

The general significance of inhibition by CO_2 is not obvious. Species of micro-organisms differ widely in their relative sensitivity to CO_2. It may be that CO_2 inhibits some micro-organisms by causing non-specific effects such as pH shifts, but it is more difficult to explain large differences in CO_2 sensitivity in the same way. The occurrence of CO_2-sensitive mutants raises the possibility that inhibition of wild-type strains by CO_2 may often be due to effects on single enzymes.

The CO_2 effects shown by the CO_2-inhibited and CO_2-stimulated mutants of *Neurospora crassa*, *Escherichia coli* and *Salmonella typhimurium* suggest that the role of CO_2 in cell physiology is more complex than might be expected from our present knowledge of CO_2 metabolism.

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Effects of Nitrogenous Components of the Medium on the Carbohydrate and Nucleic Acid Content of *Mycobacterium tuberculosis* BCG

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SUMMARY

The effects of varying the concentrations of the nitrogen sources in glycerol+asparagine, glycerol+asparagine+casein hydrolysate and glycerol +ammonium sulphate media on the composition and growth rate of *Mycobacterium tuberculosis* BCG grown in shaken culture for various periods were investigated. The bacteria were fractionated and the soluble, alkali-extractable, hot acid-soluble and residual fractions analysed for carbohydrate, RNA, DNA, free lipid, soluble amino compounds and soluble phosphates.

The results indicate that several forms of growth restriction operated in these experiments: a progressive reduction in growth rate even in conditions when nutrients were not limiting, due to clumping of cells; a reduction in growth rate following any reduction in the amino acid content of the medium; a marked reduction in growth rate when the amino acid content of the medium fell below a critical level, or was replaced by ammonium ions. When the growth rate was reduced because of nutrient limitation, the carbohydrate content of the bacteria increased, but the pattern of accumulation varied with the experimental conditions. The RNA: protein ratio of the cells was little affected by growth rate.

INTRODUCTION

Although much is known about the chemistry of mycobacteria, including *Mycobacterium tuberculosis*, quantitative studies on how their composition varies with environmental conditions have been few. Until very recently, only in the case of *Mycobacterium phlei* had studies been made of the effects of environment on carbohydrate content (Tepper, 1965; Antoine & Tepper, 1969*a*). The studies outlined in this paper were carried out primarily to obtain background information against which to interpret the effects of isoniazid on the carbohydrates of *M. tuberculosis* (Winder, Brennan & McDonnell, 1967; Winder & Rooney, 1970). Since this work was done, Antoine & Tepper (1969*b*) have given a short report on the environmental control of the carbohydrate and lipid content of *M. tuberculosis* RIRv, and their results are essentially in agreement with some of those in this paper.

As far as variations in DNA, RNA and soluble phosphate contents of mycobacteria are concerned, only in the case of *Mycobacterium smegmatis* and *M. phlei* have detailed studies been made (Winder & Denneny, 1956; Winder & O'Hara, 1962; Tepper, 1965; F. G. Winder & M. P. Coughlan, unpublished observations). Consequently, observations on the effects of environment on the amounts of these materials in *M. tuberculosis* are included in this paper.

METHODS

Media. The glycerol + asparagine medium usually had the composition of Sauton medium, as described by Soltys (1952), except that the concentration of glycerol was 75·5 g./l. The glycerol + asparagine + casein hydrolysate medium usually had the same composition, with the addition of 3 g. pancreatic casein hydrolysate/l. Where required, variations in the composition of these two media are noted in the text. All of these media contained ammonium ions (about 400 mg. N/l.) in addition to the organic nitrogen. In the glycerol + ammonium sulphate media, the amino acids were replaced by ammonium sulphate, normally 8 g./l.

Growth of bacteria. Mycobacterium tuberculosis, var. *bovis*, strain BCG (Glaxo Laboratories Ltd) was grown as described previously (Winder *et al.* 1967) under stationary conditions for 4 days and then with shaking for further periods.

Extraction procedures. The 'soluble', 'free lipid' and 'alkali-extractable fractions' were obtained as described by Winder & Rooney (1970), and the remaining insoluble fraction was used to obtain the 'hot acid-soluble' and 'residual' fractions in the manner described by Winder *et al.* (1967).

Assays. Except where otherwise stated, carbohydrates were determined by an anthrone procedure (Winder *et al.* 1967). The phenol method was that of Dubois *et al.* (1956). In both cases the results are given in terms of glucose as the standard. 'Combined phosphate' is the difference between total phosphate and inorganic phosphate, which were determined as described by Winder & O'Hara (1962), as were total nitrogen (determined in the insoluble fraction), RNA and DNA. Amino acids were determined by the method of Moore & Stein (1954), using leucine as the standard.

The organic nitrogen content of the medium at the time of harvesting was estimated by subtracting the insoluble nitrogen content of the bacteria from the initial organic nitrogen content of the medium.

RESULTS

The effects of age on the composition of bacteria from relatively young batch cultures of *Mycobacterium tuberculosis* BCG grown in our normal glycerol + asparagine + casein hydrolysate medium are shown in Tables 1 and 2. The data on yields in the tables showed that, although the cultures were young and far from exhaustion of any component in the medium, growth was not truly exponential: the generation time increased from rather less than 24 h. to rather more than 48 h. over the 6 days of the experiment. These cultures showed a small increase in total carbohydrate content per unit of nitrogen with age over the period studied; this increase was due to an upward tendency in the soluble and alkali-extractable fractions partly balanced by a downwards tendency in the carbohydrate content of the residue. There was no trend in the RNA content per unit of nitrogen of such cultures, but there were marked upward trends in the DNA and lipid contents and perhaps in the content of soluble amino compounds, and a marked downward trend in their soluble combined phosphate content.

When the casein hydrolysate was omitted from the medium, the cultures of *Mycobacterium tuberculosis* BCG grew more slowly (Tables 1 and 2, Expt. *b*). Their carbohydrate content at low yields was higher than in the richer medium, largely due to a much higher content of hot acid-soluble carbohydrate. The carbohydrate content of

Table 1. *Effect of age of culture and medium constituents on the carbohydrate composition of Mycobacterium tuberculosis* BCG

All the media contained glycerol together with (*a*) 4 g. asparagine + 3 g. casein hydrolysate/l., (*b*) 4 g. asparagine/l. and (*c*) reduced asparagine + casein hydrolysate (respectively 1 g. + 0·75 g/l.). Data in (*a*), (*b*) and (*c*) from different experiments. Results are expressed per g. insoluble nitrogen.

Age (days)	Yield of cells (mg. insoluble N/l.)	Organic nitrogen in medium (mg. N/l.)	Carbohydrate fractions (as mmoles 'glucose') Soluble	Alkali-extractable	Hot acid-soluble	Residue	Total carbohydrate
(*a*) Medium with 4 g. asparagine + 3 g. casein hydrolysate/l.							
6	7·9	1310	2·67	1·91	2·39	2·28	9·24
7	17·1	1300	2·74	2·14	2·64	2·45	9·96
8	32·0	1290	3·20	2·01	2·87	2·35	10·42
9	51·3	1270	3·64	2·36	2·31	2·19	10·50
10	72·5	1250	3·43	2·26	2·67	2·13	10·49
12	135·2	1180	3·43	3·03	2·15	1·57	10·17
(*b*) Medium with 4 g. asparagine/l.							
9	4·3	840	2·35	2·62	10·10	2·86	17·93
11	15·4	825	—	—	—	—	16·63
13*	42·2	798	2·48	3·20	8·21	2·37	16·26
15*	55·8	784	3·52	2·56	6·55	1·74	14·37
(*c*) Medium with 1 g. asparagine + 0·75 g. casein hydrolysate/l.							
8*	32·5	297	3·18	2·29	9·65	3·71	18·83
12*	141·5	188	3·29	4·27	11·56	6·73	25·83
17†	318·0	12	8·82	9·03	26·06	5·87	49·53
20†	354·0	—	7·84	11·03	27·52	8·76	55·15

* Mean values from two experiments. † Mean values from four experiments.

Table 2. *Effect of age of culture and medium constituents on the phosphorus components, amino compounds and lipids of Mycobacterium tuberculosis* BCG

Data in (*a*) and (*b*) refer respectively to same experiments as (*a*) and (*b*) in Table 1. Results are expressed per g. insoluble nitrogen.

Age (days)	Soluble fraction: Nucleotides (as μmoles AMP)	Amino compounds (as μmoles leucine)	Combined phosphate (μg. atoms P)	RNA (μg. atoms P)	DNA (μg. atoms P)	Lipids (g.)
(*a*) Medium with 4 g. asparagine + 3 g. casein hydrolysate/l.						
6	220	1140	1650	1350	670	2·40
7	235	1230	986	1440	783	2·78
8	225	1090	900	1290	716	3·08
9	234	1750	976	1460	784	3·28
10	204	1270	728	1310	857	3·40
12	208	2670	483	1450	1460	4·41
(*b*) Medium with 4 g. asparagine/l.						
9	466	1610	1210	1340	935	3·95
11	495	3210	1097	1300	1030	5·23
13*	299	6450	1054	1260	1013	3·85

* Mean values from two experiments.

this fraction and of the residue decreased with age, leading, in spite of an increase in the combined soluble and alkali-extractable fractions, to a smaller total carbohydrate content. The content of soluble amino compounds increased markedly with age. Initially the bacteria had higher DNA, soluble nucleotide and lipid contents per unit of nitrogen than the bacteria from the richer medium.

Cultures were also grown in a medium containing only one-quarter of the normal concentration of asparagine and casein hydrolysate (Table 1, Expt. *c*), so that the initial concentration of amino acid nitrogen in the medium was only 330 mg./l.; this fell to below 200 mg./l. after 12 days and practically to zero after 17 days. Such

Table 3. *Effect of concentration of asparagine + casein hydrolysate in the glycerol + asparagine + casein hydrolysate medium on the carbohydrate composition of Mycobacterium tuberculosis* BCG

Bacteria harvested after (*a*) 9 days growth and (*b*) 14 days growth. (*a*) and (*b*) are different experiments. Results expressed per g. insoluble nitrogen.

Medium composition				Carbohydrate fractions				
Asparagine (g./l.)	Casein hydrolysate (g./l.)	Yield of cells (mg. insoluble N/l.)	Organic N in medium (mg. N/l.)	Soluble	Alkali-extractable	Hot acid-soluble	Residue	Total carbohydrates
				(as mmoles 'glucose')				
				(*a*)				
8	6	43·0	2600	2·37	1·54	2·06	0·922	6·89
4	3	39·0	1280	3·26	2·28	3·36	1·90	10·74
2	1·5	28·0	630	3·06	1·93	3·81	2·38	11·18
1	0·75	25·0	305	3·16	1·83	5·25	3·73	13·97
				(*b*)				
8	6	250	2390	1·55	2·30	2·09	0·593	6·54
4	3	250	1070	1·98	3·07	2·38	0·918	8·34
2	1·5	204	456	3·20	4·07	5·43	3·12	15·82
1	0·75	177	153	4·08	3·69	8·07	5·23	21·07
0·5	0·375	33·8	131	6·45	5·98	10·32	5·03	28·04

Table 4. *Effect of concentration of asparagine + casein hydrolysate in the glycerol + asparagine + casein hydrolysate medium on the phosphorus components, amino compounds and lipids of Mycobacterium tuberculosis* BCG

Expts (*a*) and (*b*) are the same as (*a*) and (*b*) in Table 3. Results are expressed per g. insoluble nitrogen.

Medium composition		Soluble fraction					
Asparagine (g./l.)	Casein hydrolysate (g./l.)	Nucleotides (as μmoles AMP)	Amino compounds (as μmoles leucine)	Combined phosphate (μg. atoms P)	RNA (μg. atoms P)	DNA (μg. atoms P)	Lipids (g.)
			(*a*)				
8	6	210	—	1200	1300	608	—
4	3	259	—	1350	1620	750	—
2	1·5	267	—	1410	1280	663	—
1	0·75	259	—	1470	1070	568	—
			(*b*)				
8	6	139	2340	533	1020	764	4·25
4	3	161	2500	610	1250	681	4·58
2	1·5	216	1120	890	1440	785	4·52
1	0·75	255	890	1130	1150	570	—

cultures grew initially more slowly than normal cultures. This can be seen from the results in Table 3 but is not apparent from those in Table 1 since the picture is confused by the fact that in different experiments (e.g. *a*, *c* in Table 1) the length of the lag period frequently varied. This slower growth was accompanied by a higher content of carbohydrate, even after 8 days, than in the richer cultures, largely due to an increase in hot acid-soluble carbohydrate and, to a smaller extent, in residual carbohydrate. As the growth rate declined after about 12 days, due to the falling amino acid content of the medium, the carbohydrate content of all fractions increased.

In order to confirm the effects on carbohydrate content of changing the concentrations of nitrogen sources, and to avoid complications due to interexperimental variation, cultures containing different initial concentrations of asparagine and casein hydrolysate were incubated together and all were harvested after a fixed time. Tables 3 and 4 show the results of two experiments employing different growth periods. In the early stages of growth of such cultures (e.g. up to 9 days), even though the amino acid nitrogen content of the medium in all the cultures was still more than 300 mg./l., the growth rate diminished slightly but progressively with decreasing concentrations of asparagine and casein hydrolysate. Accompanying this diminished growth rate there was a marked increase in the hot acid-soluble and residual carbohydrate, while the carbohydrate content of the other two fractions remained relatively unaffected. There were no consistent changes in nucleic acid content with the nitrogen content of the medium. When the organic nitrogen content of the medium was reduced to half of the lowest concentration of 0·5 g. asparagine and 0·375 g. casein hydrolysate/l., growth was extremely slow and insufficient material was available for analysis after 9 days growth.

After longer incubation periods there was a tendency for the differences in growth rates to become reduced, probably due to the growth-retarding effect of the formation of cell clumps (see below) which would operate sooner in the more rapidly growing cultures. After 14 days growth the cultures with 2 g. asparagine + 1·5 g. casein/l. showed signs of a general increase of carbohydrate content in all fractions, a decrease in soluble amino compounds and an increase in soluble combined phosphate. All of these results are characteristic of limitation of amino acids in the medium, even though there was still about 450 mg. amino acid nitrogen/l. of medium. These effects were decidedly more marked in the cultures containing 1 g. asparagine + 0·75 g. casein hydrolysate/l., in which the organic nitrogen content of the medium had fallen to 150 mg./l. after 14 days growth. Cultures which initially had a still lower concentration of amino acids in the medium grew very slowly and the accumulation of carbohydrate in all the fractions (except the residue) was more marked.

The results indicate that growth rate and carbohydrate content of the bacteria, particularly in the hot acid-soluble and residual fractions, was affected by the amino acid content of the medium within a wide range of concentrations, but that the effects became marked when the organic nitrogen content of the medium dropped below about 500 mg/l., and particularly when it dropped to 200 mg/l. or below.

Results in general conformity with this were obtained in glycerol + asparagine media (Tables 5, 6). Reduction in the asparagine content of the medium in the range 16 to 4 g./l. reduced the growth rate, though the results for the carbohydrate content of the cells were somewhat erratic. At the lower asparagine concentrations, corresponding to about 400 and 200 mg. amino acid nitrogen/l. much more marked changes occurred. The growth rate was strikingly reduced. The cells had a high carbohydrate content and,

particularly at the lowest nitrogen level (1 g. asparagine/l.), a low content of soluble amino compounds and some increase in soluble combined phosphate. In one respect, however, the effects of reduction in the amino acid content of the glycerol + asparagine media differed from the effects of reduction in nitrogen content of the glycerol +

Table 5. *Effect of concentration of asparagine in glycerol + asparagine medium and of ammonium sulphate in glycerol + ammonium sulphate medium on the carbohydrate composition of Mycobacterium tuberculosis* BCG

Bacteria harvested after 15 days growth. Results are expressed per g. insoluble nitrogen. Data in (*a*) and (*b*) from different experiments.

Nitrogen source (g./l.)	Yield (mg. insoluble N/l.)	Organic N (*a*) or NH_4-N (*b*) in medium (mg. N/l.)	Carboyhydrate fractions: Soluble	Alkali-extractable	Hot acid-soluble	Residue	Total carbohydrate
			(as mmoles 'glucose')				
			(*a*) Asparagine				
16	104·7	3260	3·42	4·70	4·94	1·61	14·67
8	85·7	1590	3·26	4·06	8·56	2·73	18·61
4	58·7	780	3·04	6·40	5·20	1·95	16·59
2	11·3	409	2·74	3·46	15·35	4·10	25·65
1	1·8	208	1·10	7·55	25·80	8·69	43·14
			(*b*) Ammonium sulphate				
8	16·9	1680	9·95	4·55	23·25	4·77	42·52
2	17·8	407	7·43	4·44	23·10	5·58	40·55

Table 6. *Effect of concentration of asparagine in glycerol + asparagine medium and of ammonium sulphate in glycerol + ammonium sulphate medium on the phosphorus components, amino compounds and lipids of Mycobacterium tuberculosis* BCG

Bacteria harvested after 15 days growth. Results are expressed per g. insoluble N. Expts (*a*) and (*b*) are the same as (*a*) and (*b*) in Table. 5.

Nitrogen source (g./l.)	Soluble fractions: Nucleotides (as μmoles AMP)	Amino compounds (as μmoles leucine)	Combined phosphates (μg. atoms P)	RNA (μg. atoms P)	DNA (μg. atoms P)	Lipids (g.)
			(*a*) Asparagine			
16	230	7470	995	1808	1045	3·82
8	227	7500	—	1509	1165	3·84
4	227	6850	1260	1260	1013	3·83
2	258	1750	1323	1528	1044	—
			(*b*) Ammonium sulphate			
8	528	7620	1880	1559	1408	3·67
2	428	2500	1670	1715	1545	—

asparagine + casein hydrolysate media: in the former case the reduction led to a reduced content of soluble carbohydrate in the cells, while in the latter case it led to an increase in this material.

The use of an ammonium salt as the sole nitrogen source led to slow growth and a high carbohydrate content of all fractions (Tables 5, 6). It also led to a high soluble pool of nucleotides and combined phosphates, and to a rather high DNA content.

Reducing the concentration of $(NH_4)_2SO_4$ from 1700 mg. NH_4-N/l. to 400 mg. NH_4-N/l. of medium had little effect on the composition of the cells, apart from the concentration of soluble amino compounds, which showed a substantial decrease (Tables 5, 6). It should be noted, however, that the ninhydrin method, which was used to measure the amino compounds, also responds to ammonium ions, and it seems likely that it was the concentration of these which changed in response to the external ammonium ion concentration.

Table 7. *Changes in the composition of Mycobacterium tuberculosis* BCG *grown in glycerol + asparagine + casein hydrolysate medium and then transferred to other media for 6 h.*

Figures for insoluble nitrogen were obtained by dividing final values by initial values. Other figures were obtained by dividing final values per g. of insoluble nitrogen by initial values per g. of initial insoluble nitrogen. The results are the means from two experiments.

Transfer medium	Insoluble nitrogen	Soluble carbohydrate	Alkali-extractable carbohydrate	Hot acid-soluble carbohydrate	Residual carbohydrate	Total carbohydrate
Glycerol	1·00	1·35	1·27	1·33	1·24	1·32
Glycerol + ammonium sulphate	1·06	1·82	1·15	0·96	0·76	1·18
Glycerol + asparagine + casein hydrolysate	1·18	1·11	1·11	1·25	1·03	1·15

	Soluble fraction				
	Nucleotides	Amino compounds	Combined phosphate	RNA	DNA
Glycerol	1·19	1·01	1·29	1·16	1·05
Glycerol + ammonium sulphate	1·22	1·17	1·05	1·06	1·03
Glycerol + asparagine + casein hydrolysate	1·24	1·60	1·09	1·02	1·02

In order to study the speed with which the composition of *Mycobacterium tuberculosis* BCG responded to altered growth conditions, bacteria which had been grown in glycerol + asparagine + casein hydrolysate medium were washed and resuspended either in the same medium or in glycerol + ammonium sulphate medium, or in glycerol medium with no nitrogen, and incubated for 6 h. (Table 7). As judged by total nitrogen content, organisms resuspended in the original medium resumed growth at something like the previous rate, those in the ammonium sulphate medium grew slightly, while those in the nitrogen-free medium did not change in nitrogen content. Even in the short period of the experiment, the carbohydrate per unit of nitrogen increased markedly in all the fractions obtained from the bacteria incubated in the nitrogen-free medium, while only a slight increase took place in the original medium. In the ammonium sulphate medium there was an unexpected fall in the carbohydrate content of the residue and a marked increase in the soluble fraction. An increase in soluble nucleotide content took place in all media, there was a marked increase in the soluble amino acid content in the original medium and in the soluble phosphate in the nitrogen-free medium. Other

changes in the composition of the bacteria during the experimental period were marginal.

Since the glycerol concentration in the media employed in this work was high (75·5 g./l.), experiments were carried out to compare the carbohydrate content of *Mycobacterium tuberculosis* BCG grown in such media with its content in media with one-tenth the amount of glycerol. The lower concentration was still sufficient to provide a substantial excess of glycerol, since the concentration in the medium had not been appreciably reduced at the time of harvesting. It was found both with asparagine +

Table 8. *The effect of reducing the glycerol concentration in the medium on the carbohydrate content of Mycobacterium tuberculosis BCG*

Carbohydrate was assayed by the phenol method. Results are the mean values from two experiments.

Medium		Carbohydrate content of fractions			
Nitrogen source (g./l.)	Glycerol (g./l.)	Soluble	Alkali-extractable	Hot acid-soluble plus residue	Total carbohydrate
		(as mmoles 'glucose'/g. insoluble nitrogen)			
Asparagine (4) + casein hydrolysate (3)	75·5	2·88	3·56	8·15	14·59
	7·55	2·78	2·99	5·89	11·66
Ammonium sulphate (8)	75·5	6·87	4·73	15·17	26·80
	7·55	6·61	4·55	9·65	20·82

Table 9. *Carbohydrate compounds of the soluble fraction from Mycobacterium tuberculosis BCG grown with different amounts of glycerol in asparagine + casein hydrolysate medium and in ammonium sulphate medium*

The fraction was concentrated, de-ionized with Amberlite MB-1, chromatographed in ethyl acetate + pyridine + water (10:4:3, by vol.) and the bands eluted and assayed by the phenol method, all as described by Winder & Rooney (1970). The results are given as μmoles 'glucose'/g. insoluble nitrogen.

Medium									
Nitrogen source (g./l.)	Glycerol (g./l.)	Total soluble carbohydrate	Recovery after de-ionization (%)	Trehalose	Polysaccharide	Glucose	Mannose + arabinose	Oligosaccharide	Total recovery from paper (%)
Asparagine (4) + casein hydrolysate (3)	75·5	3140	84	1070	642	65	186	16	75
	7·55	3040	56	850	350	55	69	14	79
Ammonium sulphate (8)	75·5	6415	91	2570	1415	274	174	251	81
	7·55	6645	63	2000	978	94	56	415	85

casein hydrolysate medium and ammonium sulphate medium that this reduction in glycerol concentration slightly reduced the carbohydrate content of the bacteria, the reduction taking place mainly in the insoluble fraction (Table 8). No experiments in glycerol-limited media were carried out.

In one such experiment the carbohydrates of the soluble fraction were subjected to further fractionation by paper chromatography, followed by elution and assay (Table 9). It was found that the change from the asparagine + casein hydrolysate medium

to the ammonium sulphate medium increased the amounts of trehalose and polysaccharide in about the same proportion and also caused a large increase in the oligosaccharide content of the fraction. Reduction in the glycerol content in both media reduced the trehalose and polysaccharide content of the fraction to a greater extent than the total carboyhdrate content. It was noticed that when material from bacteria grown in the low glycerol medium was de-ionized by a mixed-bed resin treatment, the recovery of total carbohydrate was poor, suggesting that the soluble fraction of these bacteria had a high content of ionic derivatives of carbohydrates, presumably phosphorylated compounds.

DISCUSSION

The results obtained suggest that complex combinations of environmental effects operate even in the apparently simple experimental systems employed. Even in young cultures, in which all the known nutrients are far from exhaustion, the growth rate of *Mycobacterium tuberculosis* BCG declines progressively with age of culture This has been observed also in shaken cultures of *M. smegmatis* when dispersing agents are not employed (F. G. Winder & M. P. Coughlan, unpublished observations). The reduction in growth rate is probably due to growth in the form of clumps, which increase in average size during the life of the culture, and within which the ill-defined phenomenon of 'cellular crowding' may take place. Effects of crowding have been demonstrated in *M. smegmatis* (Maruyama, Ono & Sato, 1963), and in *Escherichia coli* crowding appears to operate by leading to a shortage of energy sources which can be utilized under the redox conditions prevailing (Freter & Ozawa, 1963): such conditions may exist within the clumps. Decline in growth rate in *M. tuberculosis* BCG under these conditions is characterized by constant carbohydrate content and an increased DNA:protein ratio.

A second form of growth rate limitation is observed when the concentration or variety of amino acids in the medium is reduced. A progressive impoverishment of the medium in this fashion leads to a progressive decline in growth rate, even though the total pool of soluble amino compounds in the bacteria is relatively constant. This decline in growth rate is accompanied by a relatively small increase in total carbohydrate, confined to the hot acid-soluble and residual fractions. It is suggested that the richness of the medium in amino acids affects the steady state concentration of a limited number of amino acids within the cell, presumably amino acids whose carbon skeleton can only be synthesized slowly from glycerol, and that this affects the rate of protein synthesis. The cellular carbohydrate is largely derived from glycerol (F. G. Winder, P. J. Brennan & S. A. Rooney, unpublished observations) and hence its synthesis tends to outrun that of protein when the synthesis of the latter is thus slowed.

A third form of growth limitation appears when the amino acid concentration in the medium drops below a certain critical value, or when amino acids are replaced by ammonium salts. The critical organic nitrogen concentration in the medium varies with circumstances, but lies in the region of 200 to 500 mg. N/l. Calculations based on the figures of Antoine & Tepper (1969*b*) suggest that a similar range of organic nitrogen concentrations is also critical for *Mycobacterium tuberculosis* RIRv. When the amino acid concentration is below the critical level, the growth rate is very markedly reduced and high levels of all carbohydrate fractions of the bacteria are reached. It is suggested that at concentrations of amino acids below the critical one the organisms have

difficulty in abstracting them from the medium, leading to the observed reduction in the intracellular pool of soluble amino compounds and to a reduction in the rate of protein synthesis with consequent carbohydrate accumulation. These conditions operate even when ammonium ions are present in high concentration, leading to a large intracellular soluble nitrogen pool, as can occur in the glycerol + ammonium sulphate medium; in this case it must be assumed that the provision of at least some of the organism's amino acid requirements from ammonium salts and glycerol is a slow process. Bowles & Segal (1965) have reported that glutamate represses glycerol utilization of *M. tuberculosis* H37Rv, so that de-repression of glycerol utilization might reinforce the tendency of the bacteria to accumulate carbohydrate at low concentrations of amino acids. However, investigations into the uptake of labelled glycerol, asparagine and glutamate in each other's presence do not suggest that this process plays a major role in regulating the carbohydrate:protein ratio (Winder & Rooney, 1970; F. G. Winder, S. A. Rooney, & P. J. Collins, unpublished observations).

Characterization of the carbohydrates present in the various fractions of *Mycobacterium tuberculosis* BCG grown in glycerol + asparagine + casein hydrolysate medium is reported elsewhere (Winder & Rooney, 1970). In the present studies, only in the case of the soluble fraction was an attempt made to identify the individual carbohydrates whose concentration changed in response to growth conditions. In this case, trehalose and a glucan were found to be mainly involved. The carbohydrates which were usually measured in the other fractions contained hexose, since the anthrone method responds only slightly to pentose. Antoine & Tepper (1969*a*, *b*) reported that glycogen is the main carbohydrate which accumulates in *Mycobacterium phlei* and *M. tuberculosis* under their accumulation conditions.

The relative constancy of the RNA:protein ratio in spite of large changes in growth rate is somewhat surprising in view of the fact that in several bacteria it has been found that the amount of RNA (mainly ribosomal RNA) per unit of nitrogen varies approximately linearly with growth rate over a range of conditions (Magasanik, Magasanik & Neidhardt, 1959; Neidhardt, 1963). However, at lower growth rates bacteria appear to approach a constant minimal RNA:protein ratio (Magasanik *et al.* 1959; Maaløe & Kjeldgaard, 1966) and even the maximal growth rate of *Mycobacterium tuberculosis* is far below the range studied with other organisms, so that presumably its whole range of growth rates can be dealt with by a minimal concentration of ribosomes, though the constancy of this value still requires explanation. In the more rapidly growing *M. smegmatis*, variation in this ratio can be observed (Winder & O'Hara, 1962; F. G. Winder & M. P. Coughlan, unpublished observations), even though its maximal growth rate still lies below the rates which have been studied with other bacteria.

On the other hand, mycobacteria appear to have a rather variable DNA:protein ratio. This ratio is generally constant in growing bacteria, although a limited increase can occur during starvation (Neidhardt, 1963; Maaløe & Kjeldgaard, 1966). In *Mycobacterium smegmatis* substantial variations in this ratio can occur (Winder & O'Hara, 1962; F. G. Winder & M. P. Coughlan, unpublished observations), while in *M. tuberculosis* there is clear evidence for an increase in the ratio as the growth rate in a rich medium declines through clump formation.

The maximal RNA:DNA ratio achieved by *Mycobacterium tuberculosis* BCG is very low at 2:1, compared with 4:1 achieved by *M. smegmatis* and the higher values

reached by other micro-organisms. This is presumably related to its low maximal growth rate. The values reported here for this ratio for BCG are similar to those found for a number of other strains of *M. tuberculosis* (Winder & Denneny, 1956). Interference by inorganic polyphosphate in their assay probably contributed to the somewhat high value of 5:1 reported for *M. tuberculosis* BCG by Tsumita & Chargaff (1958), while the very high ratios reported by Youmans & Youmans (1968) for H37Ra were probably due to interference by arabinose-containing polysaccharides in the orcinol method used for measuring RNA.

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Inhibition by Isoniazid of Synthesis of Mycolic Acids in *Mycobacterium tuberculosis*

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SUMMARY

Exposure of growing *Mycobacterium tuberculosis* BCG to 1 μg. isoniazid/ml. inhibited the incorporation of ^{14}C from [U-^{14}C] and [2-^{14}C]glycerol and [1-^{14}C]-glutamate into its walls by about 50 % over 12 h. because ^{14}C incorporation into the mycolic acids of the walls was prevented. Isoniazid, 0·5 μg./ml. with *M. tuberculosis* BCG or 0·1 μg./ml. with *M. tuberculosis* H37Ra, inhibited incorporation of ^{14}C from [U-^{14}C]glycerol into total mycolic acids by about 90 % over 6 h., indicating that inhibition began within 1 h. of the addition of the drug. There was no effect on mycolic acid synthesis in an isoniazid-resistant strain of *M. tuberculosis* BCG. The primary inhibitory action of isoniazid in sensitive mycobacteria is probably on mycolic acid synthesis, and this leads to formation of defective boundary layers of the bacteria.

INTRODUCTION

A detailed examination of the effects of isonicotinic acid hydrazide (isoniazid) on the carbohydrates of *Mycobacterium tuberculosis* suggested that this drug interferes with the formation of the cell envelope of this organism (Winder & Rooney, 1970). Other lines of evidence, discussed by these authors, support this conclusion. This present paper gives an account of subsequent studies into the inhibition of mycolic acid synthesis by isoniazid. Some of these results have appeared in preliminary form (Winder, Collins & Rooney, 1970).

METHODS

Organisms and growth conditions. Mycobacterium tuberculosis, var. *bovis*, strain BCG was obtained from Glaxo Laboratories Ltd, Greenford, Middlesex. The isoniazid-resistant strain was obtained by transfer of a heavy inoculum onto Löwenstein–Jensen medium containing 100 μg. isoniazid/ml., followed by repeated subculture on the same medium. *Mycobacterium tuberculosis*, var. *hominis*, strain H37Ra was obtained from Trudeau Laboratories, Saranac Lake, New York, U.S.A. Both organisms were grown in shaken culture at 37° as previously described (Winder, Brennan & McDonnell, 1967). The medium was based on Sauton medium (Soltys, 1952), but had less glycerol (7·55 g./l.) and asparagine was replaced by 11·2 g. monosodium glutamate/l

Exposure to [^{14}C]glycerol and isoniazid. After about 9 days similar small amounts of [2-^{14}C]glycerol or [U-^{14}C]glycerol of high specific activity was added to each culture. Isoniazid was simultaneously added to half of the cultures, the remainder serving as controls, and both sets of cultures were harvested after 6 or 12 h. by filtration through

Whatman no. 1 paper in a Büchner funnel. The collected material was washed several times on the filter with 0·9 % (w/v) NaCl, the fluid removed by suction and the bacteria weighed (wet weight).

Preparation of bacterial walls. The bacteria were suspended in 67 mM-phosphate buffer, pH 7·8 (10 g. wet wt of bacteria to 50 ml. of buffer), cooled in ice and treated for 20 min. with a 100 W 20 kHz ultrasonic generator (Soniprobe type 1130A, Dawe Instruments Ltd, London W. 3), turned to maximal output. The homogenate was then fractionated as described by Kotani, Kitaura, Hirano & Tanaka (1959) to give a 330*g* sediment (unbroken bacteria and clumps), a 4200*g* sediment (crude-wall fraction) and a 4200*g* supernatant. The crude-wall fraction was treated with trypsin, as described by Kotani *et al.* (1959), after which the residue was freeze-dried. To remove unbound lipids, the walls were then treated for 2 days with chloroform + methanol (2 + 1, v/v) at room temperature, centrifuged after adjustment of the chloroform:methanol ratio to unity, and the residue extracted with ethanol + water (1 + 1, v/v) at 40° for 1 h. The residue of purified cell walls was then dried *in vacuo*. The chloroform + methanol and ethanol + ether extracts were pooled to provide the unbound lipids.

Extraction of mycolic acids from bacterial walls. Purified walls were saponified by refluxing with 2·5 % (w/v) KOH in methanol + benzene (1 + 1, v/v) for 6 h. After removing the insoluble residue by centrifuging, the extract was adjusted to pH 4·0, and mycolic acids (along with other fatty acids and possibly other lipids) were extracted with ether. Mycolic acids were precipitated from the etheral extract by adding two volumes of ethanol, separated by centrifugation and dried *in vacuo*.

Extraction of mycolic acids and other fatty acids from whole bacteria. The bacteria were extracted three times with ethanol + ether (3 + 1, v/v) at 60° for 10 min. to remove unbound lipids. The extracts were pooled, evaporated to dryness, saponified and fatty acids extracted as described above. The bacterial residue was then saponified, fatty acids extracted and mycolic acids precipitated as described above.

Thin-layer chromatography of mycolic acids. Mycolic acids were converted to their methyl esters by the use of diazomethane (Lipsky & Landowne, 1963). The esters were chromatographed on thin-layer plates of silica gel G (E. Merck, A.-G., Darmstadt, West Germany) using light petroleum (b.p. 40 to 60°) + diethyl ether (9 + 1, v/v) as solvent. Samples of authentic mycolic acid esters (gifts from Dr P. J. Brennan of this Department) were used as standards. Mycolic acid esters and other lipids on the plates were detected by exposing plates to I_2 vapour or by spraying with Rhodamine 6G. The chromatogram of each sample was divided into 2 cm. bands, the gel removed from each band and its radioactivity counted.

Measurement of ^{14}C. All samples were counted in Bray's solution (Bray, 1960) containing 2 % Cab-O-Sil (Packard Instrument Company) in a Packard 3380 liquid scintillation counter. The results were converted to d.p.m. using the external standard channels ratio method in conjunction with a quench curve prepared with the same scintillation mixture.

Materials. Isotopically labelled compounds were obtained from The Radiochemical Centre, Amersham, Buckinghamshire.

RESULTS

Effects of isoniazid on bacterial wall synthesis

The incorporation of ^{14}C from [2-^{14}C]glycerol into *Mycobacterium tuberculosis* BCG and fractions derived therefrom is shown in Table 1. Over 90% of the ^{14}C taken up was found in the 4200*g* supernatant and in the 330*g* sediment. Exposure to isoniazid 1 μg./ml. for the 12 h. uptake period had no appreciable effect on the total uptake of ^{14}C. It had a very slight effect on the relative amounts of radioactivity in the 330*g* sediment and the 4200*g* supernatant fluid; this was probably due to a greater fragility of isoniazid-treated bacteria, which was more apparent when shorter periods of disintegration were used (Winder *et al.* 1970). Isoniazid slightly decreased the radioactivity in the crude-wall fraction; when this material was further fractionated, the ^{14}C content of the purified walls was half that of the control while there was little effect on the trypsin extract or the free lipid.

Table 1. *Effect of isoniazid on the incorporation of ^{14}C from [^{14}C]glycerol into Myobacterium tuberculosis BCG and its subcellular fractions*

Fifteen 100 ml. cultures were treated with 1 μg. isoniazid/ml. for 12 h. and the same number used as controls. [2-^{14}C]Glycerol (0·26 μCi/culture) was added to give a final specific activity of approximately 66 d.p.m./μmole. Yields of bacteria (g. wet wt) were: control, 2·6; isoniazid-treated, 2·3.

Fraction	^{14}C content (d.p.m.) Control	Isoniazid-treated
330*g* sediment	127,000	111,000
4200*g* supernatant	189,000	207,000
Wall fractions (4200*g* sediment)		
Trypsin extract	20,000	17,000
Unbound lipid	4,000	3,800
Purified walls	5,900	3,000
Total in bacteria	346,000	342,000
	Yield (mg. dry wt)	
Purified walls	12·6	14·0
	Specific activity (d.p.m./mg. dry wt)	
Purified walls	468	214

Further experiments confirmed this result. In three separate experiments, the incorporation of ^{14}C from [2-^{14}C]glycerol into purified walls over 12 h. in the presence of 1 μg. isoniazid/ml. was 0·51 ± 0·03 (S.E.M.) of the control incorporation, and in two further experiments the incorporation in the presence of 10 μg. isoniazid/ml. was 0·38 ± 0·08 of the control. In neither set of experiments did the total incorporation of ^{14}C into the bacteria in the presence of isoniazid differ appreciably from the control value. Thus isoniazid had a specific inhibitory effect on the incorporation of carbon from glycerol into the cell walls of *Mycobacterium tuberculosis* BCG.

In a similar experiment in which [1-^{14}C]-DL-glutamate, the other carbon source in the medium, was used at 1·25 μCi/l. of medium, the carbon from glutamate was incorporated into walls to a smaller extent than carbon from glycerol. This incorporation was reduced by 20% during a 12 h. exposure to 1 μg. isoniazid/ml. Thus isoniazid inhibited the total incorporation of carbon into the bacterial wall.

To identify the wall component whose synthesis was affected by isoniazid, purified

walls from bacteria which had been exposed to [^{14}C]glycerol in the presence and absence of isoniazid were separately saponified and extracted with ether at pH 4·0. Three fractions were obtained: a mycolic acid fraction; an aqueous fraction which contained part of the cell-wall polysaccharide; and a residue mainly of peptidoglycan. Isoniazid strongly inhibited the incorporation of radioactivity into the mycolic acid fraction, had a much smaller effect on incorporation into the aqueous fraction and no appreciable effect on the residue (Table 2). These results suggested that isoniazid had a primary effect on the synthesis of mycolic acid or its incorporation into the wall and that the effect on the polysaccharide fraction was secondary to this.

Table 2. *Effect of isoniazid on the incorporation of ^{14}C from [^{14}C]glycerol into fractions obtained on saponification of purified walls of Mycobacterium tuberculosis* BCG

Seventeen 100 ml. cultures were treated with 1 μg. isoniazid/ml. for 12 h. and the same number used as controls. [2-^{14}C]Glycerol (0·63 μCi/culture) was added to give a final specific activity of approximately 168 d.p.m./μmole. Yields of bacteria (g. wet wt) were: control, 15·5; isoniazid-treated, 15·4.

Fraction	^{14}C content (d.p.m.)	
	Control	Isoniazid-treated
Purified walls	2,944	1,508
Mycolic acids	768	126
Aqueous fraction (polysaccharide)	1,112	552
Insoluble residue	800	694

Table 3. *Effect of isoniazid on the incorporation of ^{14}C from [^{14}C]glycerol into fatty acids from free and bound lipids of Mycobacterium tuberculosis* BCG

Six 100 ml. cultures were used for each treatment (6 h.). [U-^{14}C]Glycerol (1·25 μCi/culture) was added to give a final specific activity of approximately 333 d.p.m. Yields of bacteria (g. wet wt) were: control, 1·0; with 0·1 μg. isoniazid/ml., 1·2; with 1 μg. isoniazid/ml., 0·9.

Fraction	Control	Isoniazid-treated	
		0·1 μg./ml.	1 μg./ml.
	^{14}C content (d.p.m.)		
Fatty acids from free lipids	53,470	50,440	63,120
Fatty acids from bound lipids			
Mycolic acids	11,840	9,040	1,112
Other fatty acids	19,902	25,050	13,640
	Yield (mg. dry wt)		
Mycolic acids	16·4	18·0	11·8
	Specific activity (d.p.m./mg.)		
Mycolic acids	722	502	94

Effects of isoniazid on mycolic acid synthesis

To determine whether this effect of isoniazid was on the synthesis of mycolic acid or on its incorporation into the wall, experiments were made in which the total mycolic acid was extracted from *Mycobacterium tuberculosis* BCG which had been exposed to [^{14}C]glycerol for 6 h. with and without isoniazid. The results of a typical experiment (Table 3) showed that isoniazid 0·1 μg./ml. slightly inhibited the synthesis of mycolic acid and that it had a strong inhibitory effect at 1 μg./ml. Other experiments showed

that results with 0·5 μg./ml. were similar to those with 1 μg./ml. Effects on the free lipids, and on the non-mycolic fatty acids and related compounds of the bound lipid fraction were relatively small.

Since incorporation of ^{14}C from [^{14}C]glycerol in the medium into the mycolic acid of control bacteria is effectively linear under these experimental conditions for the period involved (F. G. Winder, P. J. Brennan & S. A. Rooney, unpublished observations), the degree of inhibition by 1 μg. isoniazid/ml. of incorporation of ^{14}C into mycolic acid over a 6 h. period indicated that it produced an inhibition of synthesis of mycolic acid between 90% immediately and 100% after a delay of less than 1 h. In view of this degree of inhibition, we conclude that the synthesis of all the mycolic acids present in major amount in this organism was affected.

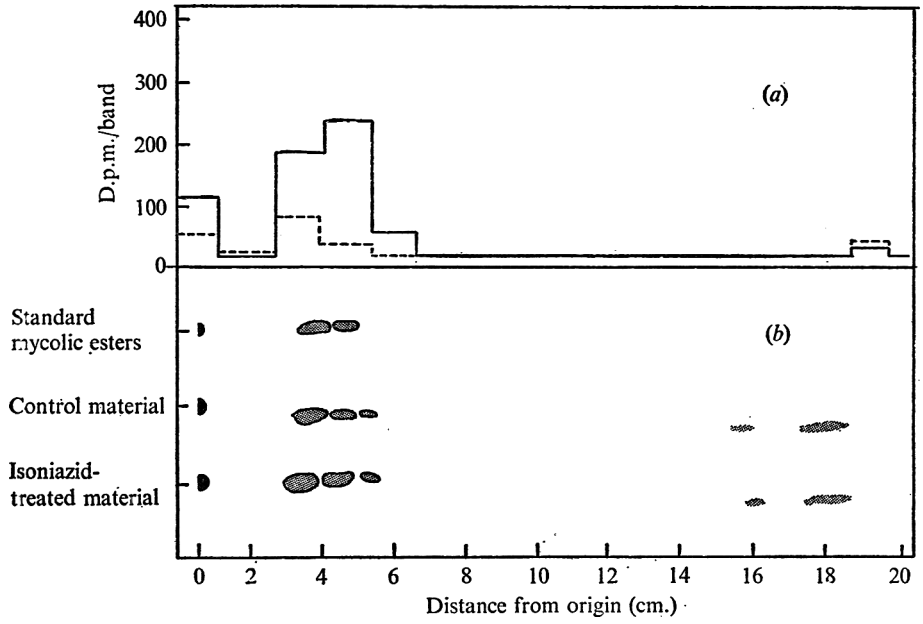

Fig. 1. Thin-layer chromatography of the methylated mycolic acids from the bound lipids of *Mycobacterium tuberculosis* BCG which had been exposed to [2-^{14}C]glycerol with and without 1 μg. isoniazid/ml. for 6 h. (*a*) Distribution of radioactivity in chromatograms of control (—) and isoniazid-treated (---) material. (*b*) Diagram of spots on plate after exposure to iodine vapour.

Thin-layer chromatography of the methyl mycolates showed that they were contaminated with small amounts of fatty acids, but that most of the radioactivity in the case of mycolic acids from the control cells was associated with the mycolic acid area of the plate (Fig. 1). The amount of radioactivity associated with the mycolic acid area from isoniazid-treated cells was much less than with that from control cells. Separation of different components of the mycolic acid fraction was only slight with the chromatographic system used, but the chromatograms confirmed that the major mycolic acid components were all affected by isoniazid, though there was some suggestion that not all were affected equally.

In contrast to the above results with an isoniazid-sensitive strain of BCG, isoniazid

did not inhibit the incorporation of ^{14}C into the mycolic acids of an isoniazid-resistant strain (Table 4).

When similar experiments were made with isoniazid-sensitive *Mycobacterium tuberculosis* H37Ra, it was found that isoniazid at 0·1 μg./ml. led to almost complete inhibition of mycolic acid synthesis over 6 h. (Table 5).

Table 4. *Effect of isoniazid on the incorporation of ^{14}C from [^{14}C]glycerol into mycolic acids of isoniazid-resistant Mycobacterium tuberculosis BCG*

Nine 100 ml. cultures were treated with 1 μg. isoniazid/ml. for 6 h. and the same number used as controls. [U-^{14}C]Glycerol (1·25 μCi/culture) was added to give a final specific activity of approximately 333 d.p.m./μmole. Yields of bacteria (g. wet wt) were: control, 1·2; isoniazid-treated, 1·4.

	Control	Isoniazid-treated
	d.p.m.	
Total ^{14}C mycolic acids	14,108	17,736
	mg. dry wt	
Yield of mycolic acids	29·8	34·2
	d.p.m./mg.	
Specific activity of mycolic acids	474	519

Table 5. *Effect of isoniazid on the incorporation of ^{14}C from [^{14}C]glycerol into mycolic acids of Mycobacterium tuberculosis H37Ra*

Five 100 ml. cultures were treated with 0·1 μg. isoniazid/ml. for 6 h. and the same number used as controls. [U-^{14}C]Glycerol (2·5 μCi/culture) was added to give a final specific activity of approximately 666 d.p.m./μmole. Yields of bacteria (g. wet wt) were: control, 0·68; isoniazid-treated 0·58.

	Control	Isoniazid-treated
	d.p.m.	
Total ^{14}C in mycolic acids	2,660	80
	mg. dry wt	
Yield of mycolic acids	11·5	8·2
	d.p.m./mg.	
Specific activity of mycolic acids	231	9·7

DISCUSSION

These results show that isoniazid at 0·5 μg./ml. and upwards produced complete, or almost complete, inhibition of synthesis of mycolic acids in 1 h. or less in growing *Mycobacterium tuberculosis* BGC, while total incorporation of carbon into the cells as a whole was substantially unaffected for much longer periods. Isoniazid at 0·1 μg./ml. gave even more rapid and complete inhibition of synthesis of mycolic acids in *M. tuberculosis* H37Ra.

Two questions arise. First, is this inhibition related to the bactericidal action of the drug or is it some completely unrelated phenomenon? The fact that this inhibition occurred to a marked extent in both sensitive strains at isoniazid concentrations close to the minimal inhibitory ones under the conditions of the experiment, but did not occur under similar conditions with an isoniazid-resistant strain, is strong evidence that this effect is related to the bactericidal action of the drug. This conclusion is supported further in that isoniazid also inhibits mycolic acid synthesis in *Myco-*

bacterium smegmatis and *M. phlei* but requires higher concentrations, proportional to their minimal growth inhibitory ones (P. J. Brennan, P. B. Collins & F. G. Winder, unpublished observations).

The second question which arises is, accepting that inhibition of mycolic acid synthesis is related to the bactericidal action of isoniazid, where does it fit into the chain of events involved? We suggest that inhibition of mycolic acid synthesis leads to the formation of envelope material low in mycolic acid and hence defective, that this results in loss of further materials from the boundary layers of the bacteria, and that it leads ultimately to loss of acid-fastness and death. We base this hypothesis partly on evidence in the present paper but mainly on evidence discussed elsewhere (Winder & Rooney, 1970). The inhibition of mycolic acid synthesis is established quickly as compared with the time required for most other observed effects of isoniazid (see Winder 1964; Youatt, 1969), while most such effects can be interpreted in terms of it (Winder & Collins, 1969; Winder & Rooney, 1970). The only effects of isoniazid action, other than inhibition of mycolic acid synthesis, which have been shown to occur within 1 h. of exposure are the uptake of the drug, its metabolism and the formation of pigments (Youatt, 1969); these effects may be involved in its action on mycolic acid synthesis. Hence we suggest that the primary site of the inhibitory action of isoniazid lies somewhere in the biosynthetic pathway to the mycolic acids. Since the biosynthetic pathway to mycolic acids is almost unknown (Étémadi, 1967), we do not suggest which enzyme or enzymes may be sensitive to isoniazid nor a mechanism by which isoniazid acts.

The relatively slight inhibitory effect on the incorporation of radioactivity into the polysaccharide fraction derived from the bacterial walls (Table 2) is presumably an indirect effect of inhibition of mycolic acid synthesis. Some of the polysaccharides in this fraction occur, prior to saponification, as mycolic acid esters (Imaeda, Kanetsuna & Galindo, 1968) and failure of mycolic acid synthesis may result either in an indirect inhibition of synthesis of the polysaccharides or in their elution from the bacterial envelope, a process for which there is evidence (Winder & Rooney, 1970).

We thank Dr P. J. Brennan for samples of mycolic acids, and Miss Mairead Kelly and Miss Anne Slevin for technical assistance. This work was supported by grants from the National Science Council, Republic of Ireland, and from the U.S. Public Health Service (AI-07254). P.B.C. was supported by grants from the Department of Education and, subsequently, from The Agricultural Institute, Republic of Ireland.

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Microbial Metabolism of Amino Ketones: D-1-Aminopropan-2-ol and Aminoacetone Metabolism in *Escherichia coli*

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SUMMARY

Aminoacetone was formed from D- or L-1-aminopropan-2-ol, or both, by a variety of micro-organisms. An oxidoreductase capable of oxidizing D-1-aminopropan-2-ol to aminoacetone was purified 38-fold from *Escherichia coli*. It was inactive with L-1-aminopropan-2-ol, L-threonine and DL-glycerol-1-phosphate It was highly active with a variety of diols and hydroxyketones and not narrowly specific as reported by other workers (Decker & Swain, 1968). The effect of growth conditions on activity suggested involvement in mono- or di-hydroxyacetone metabolism. Although D-1-aminopropan-2-ol oxidation was demonstrated in crude extracts of a number of other bacteria, a relationship between L-threonine and D-1-aminopropan-2-ol dehydrogenases and vitamin B_{12} biosynthesis does not appear likely.

INTRODUCTION

A previous study with *Escherichia coli* showed that NAD-dependent oxidoreductase activities toward L-threonine, and both L- and D-1-aminopropan-2-ol were present in extracts and the properties of the first two enzymes were described (Turner, 1967). Aminoacetone was the product in both cases. The present paper describes the properties of a partially purified oxidoreductase acting on D-1-aminopropan-2-ol also to form aminoacetone. Interest in this enzyme arises because of its possible involvement in the reduction of aminoacetone to give D-1-aminopropan-2-ol which then may be incorporated into vitamin B_{12} (Turner, 1966), although it still remains to be demonstrated that it is the D-isomer which is the product of aminoacetone reduction in this organism. Some support for this hypothesis comes from the work of Krasna, Rosenblum & Sprinson (1957), who showed that the incorporation of isotope from [^{15}N]-L-threonine into the D-1-aminopropan-2-ol fragment of vitamin B_{12} occurred in *Streptomyces griseus*.

Some of this work has been summarized previously in a preliminary note (Lowe & Turner, 1968).

METHODS

Micro-organisms and media. Escherichia coli, type I (National Collection of Industrial Bacteria (NCIB) no. 8114) was maintained and cultured as described previously (Turner, 1966). *Pseudomonas* strains 8/IX and DL8A were isolated by enrichment

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culture on DL-1-aminopropan-2-ol as the main carbon and nitrogen source, as described by Higgins, Pickard & Turner (1968). Other micro-organisms were obtained from the NCIB, Aberdeen, Scotland, the National Collection of Plant Pathogenic Bacteria (NCPPB), Hatching Green, Harpenden, Hertfordshire, or were laboratory strains. All micro-organisms were maintained as slope or stab cultures on nutrient agar (2·5 %, w/v). Some vitamin B_{12}-producing micro-organisms (see Table 7) were grown on peptone–yeast extract medium, modified from that described by Hall, Benedict, Wiesen, Smith & Jackson (1953) and consisting of 10 g. glucose, 3 g. yeast extract, 3 g. soya peptone, 1 g. $CaCO_3$, 1·5 g. K_2HPO_4, 1 g. KH_2PO_4 and 10 mg. $CoSO_4.7H_2O$ made up to 1 l. with glass-distilled water. Other micro-organisms were grown on nutrient broth medium (Oxoid no. 2) or simple basal salts media containing 7 g. K_2HPO_4, 3 g. KH_2PO_4, 1·2 g. $Na_2SO_4.10H_2O$, 0·1 g. $MgSO_4.7H_2O$, 1 g. $(NH_4)_2SO_4$ and 5 g. of various carbon sources per litre. Synthetic media were adjusted to pH 7·0 prior to sterilization. Carbohydrates were autoclaved separately.

Preparation of enzyme extracts. Bacteria were harvested in the exponential growth phase and extracts prepared as described by Turner (1966).

Measurement of aminoacetone production and utilization by washed suspensions of bacteria. Aminoacetone production and utilization were measured as described by Higgins *et al.* (1968). The partially resolved amino alcohols were used as substrates for aminoacetone production.

Uptake of radioactive [3-^{14}C]-DL-1-aminopropan-2-ol by growing and resting bacteria. Washed suspensions of *Escherichia coli* were incubated at 4 mg. dry wt/ml. with 0·3 mg. of [3-^{14}C]-DL-1-aminopropan-2-ol (0·085 mCi/mmole) and 0·5 mmole K_2HPO_4 + KH_2PO_4 buffer, pH 7·0, in 5 ml. at 30° with shaking. Samples of culture or bacterial suspensions were then filtered through 0·45 μ Millipore membrane filters (Millipore Filter Corporation, Bedford, Massachusetts, U.S.A.). The bacteria were washed with 0·1 M-phosphate buffer before attaching the filters to planchets and drying. Samples of the combined filtrates and washings were also dried on planchets and examined for radioactivity. Radioactivity was measured using a Nuclear Chicago Model D 47 Gas Flow Detector (Nuclear Chicago, Des Plaines, Illinois, U.S.A.), which had a calculated counting efficiency of 31 %.

Measurement of L-threonine, D- and L-1-aminopropan-2-ol dehydrogenase activities in enzyme extracts. D- and L-1-aminopropan-2-ol dehydrogenase activities were assayed in reaction mixtures containing 50 μmole D- or L-1-aminopropan-2-ol, either as the optically pure bases or partially resolved tartrates, 500 μmole diethanolamine + HCl buffer pH 9·6, 10 μmole NAD^+, and 0·5 mg. protein (dialysed crude extract) or 50 μg. of partially purified enzyme preparation, in a total volume of 1·6 ml. Unless otherwise indicated, 30 μmole $(NH_4)_2SO_4$ was included in the D-1-aminopropan-2-ol dehydrogenase assay mixture. Reactions were started by addition of enzyme to the otherwise complete reaction mixtures equilibrated at 37°. All incubations were for 20 min. at 37°. Reactions were stopped with 0·3 ml. 25 % (w/v) trichloroacetic acid. After centrifuging, 1·0 ml. of supernatant fluid was assayed for aminoacetone. When assaying for L-threonine dehydrogenase, 100 μmole of L-threonine was used as substrate with 500 μmole tris + HCl buffer pH 9·0.

Assay of aminoacetone and protein. Assays were carried out colorimetrically as described previously (Turner, 1966).

Spectrophotometric enzyme assays. D-1-Aminopropan-2-ol and secondary alcohol-

dependent reduction of NAD^+ was measured in reaction mixtures containing 750 μmole diethanolamine + HCl buffer pH 9·6, 10 μmole NAD^+, 50 μmole secondary alcohol substrate, 50 μmole $(NH_4)_2SO_4$ and 0·02 to 0·50 mg. protein in a total volume of 2·5 ml. Aminoacetone and hydroxyketone reductase activities were carried out using 750 μmole sodium acetate + acetic acid (or 3,3'-dimethylglutaric acid + NaOH) buffer pH 6·0, 10 μmole aminoacetone or 2·5 μmole hydroxyketone, 0·75 μmole NADH or NADPH, 50 μmole $(NH_4)_2SO_4$ and 0·02 to 0·50 mg. protein in 2·5 ml. total volume. Substrate or cofactor was omitted from the compensating cells and the reactions were started by the addition of enzyme to otherwise complete reaction mixtures equilibrated at 37°.

Enzymic activities are expressed as mμmoles of NADH produced, or NADH/NADPH oxidized/mg. protein/min. at 37°.

Purification of D-1-*aminopropan-2-ol–aminoacetone oxidoreductase activity*

(*a*) *Heat treatment.* Crude extract was adjusted to 8 mg. protein/ml., heated for 10 min., and precipitated protein was removed by centrifuging at 10,000*g* to give a clear supernatant fluid.

(*b*) *Molecular exclusion chromatography.* Portions of heat-treated enzyme were further purified by gel filtration on Bio-Gel P-300 as described by Turner (1967). Enzyme activity was eluted immediately after the void volume ($V_e/V_o = 1{\cdot}3$). The active fractions were combined for further purification.

(*c*) *Ion-exchange cellulose chromatography.* The enzyme was next adsorbed on 30 g. DEAE cellulose 'Chromedia DE 52' (pre-swollen) previously equilibrated with 0·02 M-phosphate buffer pH 7·0 at 4°. Protein was eluted from the cellulose by a gradient of 0 to 1·0 M-NaCl in 0·02 M-phosphate buffer pH 7·0, at a flow rate of about 30 ml./h. Under these conditions enzyme activity was eluted by 0·35 to 0·40 M-NaCl.

Preparation of optically active 1-*aminopropan-2-ols*

(*a*) *Partially resolved* D-(−)-1-*aminopropan 2-ol* D-(−)-*tartrate.* This was prepared by the procedure of Sullivan (1963) as described by Turner (1967), except that L(+) tartaric acid was replaced by D(−) tartaric acid. The optical rotation of the recrystallized D-(−)-1-aminopropan-2-ol D-(−)-tartrate dihydrate, was $[\alpha]_D^{22\cdot5} = -31{\cdot}5°$ (C. 5 and 10 in water).

(*b*) *Optically pure* D- *and* L-1-*aminopropan-2-ol.* The method used was a modification of that described by Chatelus (1964). For the preparation of D-1-aminopropan-2-ol, 10 g. of L-threonine and 66 g. of freshly distilled acetophenone (mole ratio 3:20) were heated with constant stirring at 130° for 7 h. The resultant deep-red solution (Schiff's base) was hydrolysed by shaking with four 50 ml. volumes of 3 N-HCl at 50° to release the base. The combined aqueous fractions were dried. The hydroscopic residue was dissolved in the minimum of warm dry absolute ethanol. After cooling the solution, D-1-aminopropan-2-ol HCl was precipitated by adding 7 vol. dry diethyl ether and removed by filtration. The crystals were dissolved in ethanol and re-precipitated by ether to yield a flakey white product which was stored under vacuum over a desiccant. The product had a m.p. of 97°, cf. authentic DL-1-aminopropan-2-ol m.p. 96°; and exhibited $[\alpha]_D^{22\cdot5} = -58°$ (C. 2 and 5 in water) and $[\alpha]_D^{22\cdot5} = -33{\cdot}6°$ (C. 1 and 3 in methanol), cf. $[\alpha]_D^{25} = -31{\cdot}5°$ (C. 1 in methanol), Clark,

Jones, Raich & Folkers (1954), and $[\alpha]_D = -58°$ (unknown solvent), Karrer & Klarer (1925), reported for synthesized D-1-aminopropan-2-ol HCl.

Similarly, L-1-aminopropan-2-ol HCl, prepared from D-threonine, exhibited $[\alpha]_D^{22.5} = +38°$ (C. 5 in water). Optical rotation values were determined as described previously (Turner, 1967).

Detection of 1-aminopropan-2-ol formation. Aminoacetone (10 mM) and NADH (0·4 mM) were incubated at 37° with purified enzyme (0·3 mg./ml.) in phosphate buffer, pH 6 (50 mM), in the presence of $(NH_4)_2SO_4$ (20 mM). After 1 to 2 h., reaction mixtures were deproteinized and applied to a column of BioRex 70 cation exchange resin. Basic compounds were eluted with 0·1 N-HCl and freeze-dried. Concentrated solutions were streaked on to thin-layer cellulose plates and chromatographed in butan-1-ol+propionic acid+water (47+22+31, by vol.). Aminopropanol was detected as a distinct ninhydrin-positive band running slightly ahead of aminoacetone (R_F values 0·4 and 0·3 respectively).

Chemicals. The sources of chemicals were those described previously by Turner (1967) and Higgins *et al.* (1968). In addition, D-tartaric acid, *meso*-butan-2,3-diol, DL-propan-1,2-diol, hydroxyacetone and DL-acetoin were obtained from British Drug House Ltd, Poole, Dorset; D-threonine, DL-1-bromopropan-2-ol and DL-1-fluoropropan-2-ol from K and K Laboratories Inc., Plainview, New York, U.S.A.; soya peptone L 44 and yeast extract L 21 from the Oxoid Division of Oxo Ltd, London; dihydroxyacetone from Calbiochem. Inc., Los Angeles, California, U.S.A.; DE 52 Chromedia ion-exchange cellulose from H. Reeve Angel and Co. Ltd, London. [U-^{14}C]-L-Threonine was purchased from The Radiochemical Centre, Amersham, Buckinghamshire. All chemicals were of analytical reagent grade, or of highest quality available commercially.

RESULTS

Aminopropanol and aminoacetone metabolism by intact bacteria

Previous results had shown that *Escherichia coli* was unable to utilize the nitrogen of DL-1-aminopropan-2-ol for growth on a variety of carbon sources (Turner, 1967). The same results were obtained in the present study using aminoacetone. The ability of washed suspensions of *E. coli* to form the amino ketone from DL-1-aminopropanol was shown to be due to the oxidation of only the L-isomer (Table 1). Some micro-organisms among those tested were found, however, to produce aminoacetone from both stereo-isomers (Table 1).

The possible utilization of aminopropanols for biosynthetic rather than catabolic purposes by *Escherichia coli* was studied by adding [3-^{14}C]-DL-1-aminopropan-2-ol (0·66 μCi, 4·2 μmole) to freshly inoculated 10 ml. cultures of *E. coli*, growing either on nutrient broth or on succinate basal salts media. Only about 6 % of the radioactivity was incorporated into bacterial substance within 36 to 60 h. In contrast, micro-organisms capable of growth on DL-1-aminopropan-2-ol, e.g. *Achromobacter* sp. P6 (Gottlieb & Mandel, 1959) and *Pseudomonas* sp. NCIB 8858 (Higgins *et al.* 1968), incorporated about 86 % of the radioactivity under the same conditions. Washed suspensions of *E. coli*, grown on a variety of media, utilized [3-^{14}C]-DL-1-aminopropan-2-ol only after a lag of about 10 h. Virtually all the radioactivity was taken up by the organism within 24 h. and no trace of radioactive products was detectable in suspension media. Suspensions of broth-grown bacteria utilized aminoacetone immediately at

rates of 1 to 2 mμmoles/mg. dry wt/min. at 30° and pH 7. Aminoacetone uptake was decreased 50 % by an equimolar amount of L-1-aminopropan-2-ol whereas the D-isomer lowered uptake by only 5 %. Cultures growing on broth also utilized aminoacetone at slow rates.

Table 1. *Aminoacetone production from D- and L-1-aminopropan-2-ol by washed-cell suspensions of micro-organisms*

Micro-organisms were grown on nutrient broth, or DL-1-aminopropan-2-ol medium (values in parentheses), and aminoacetone production by suspensions was measured as described in the Methods section.

Micro-organism	Strain	Aminoacetone production (mμmoles/mg. dry wt/min. at 30°) from D-Aminopropanol*	Aminoacetone production (mμmoles/mg. dry wt/min. at 30°) from L-Aminopropanol†
Achromobacter anitratum	Laboratory	0	1·2
Aeromonas liquefaciens	Laboratory	0	3·1
Erwinia carotovora	NCPPB 1280	5·0	2·6
Escherichia coli, type I	NCIB 8114	0	3·2
Klebsiella aerogenes, type I	NCIB 418	6·1	5·2
Proteus vulgaris	Laboratory	0	1·2
Pseudomonas aeruginosa	NCIB 8293	6·2	3·2
Pseudomonas sp.	NCIB 8194	0·4	0·7
Pseudomonas sp.	NCIB 8858	0·7 (0·2)	0·9 (8·9)
Pseudomonas sp.	8/IX‡	0·2 (−)	1·2 (13·2)
Pseudomonas sp.	DL 8A‡	7·5 (0·6)	3·2 (10·4)

* Composition 82 % D-1-aminopropan-2-ol, 18 % L-1-aminopropan-2-ol.
† Composition 88 % L-1-aminopropan-2-ol, 12 % D-1-aminopropan-2-ol.
‡ Isolated in this laboratory for ability to grow on DL-1-aminopropan-2-ol.

Table 2. *Effect of activators and inhibitors on D-1-aminopropan-2-ol dehydrogenase activity*

Activity was measured colorimetrically as described in the Methods section, except that $(NH_4)_2SO_4$ was omitted from reaction mixtures in Expt. 1.

Addition	Concentration (mM)	Activity (%)
Experiment 1		
None	—	100
Thioglycollate	5	83
Cysteine	5	81
2-Mercaptoethanol	5	48
$(NH_4)_2SO_4$	10	200
Na_2SO_4	10	140
K_2SO_4	10	103
Experiment 2		
DL-Serine	30	91
DL-1-Dimethylaminopropan-2-ol	30	90
Ethan-1,2-diol	30	86
DL-3-Fluoro-1-aminopropan-2-ol	30	58
Glycerol	30	58
DL-1-Fluoropropan-2-ol	30	57
Propan-1,3-diol	30	52
meso-Butan-2,3-diol	30	43
DL-1-Bromopropan-2-ol	30	17
DL-Propan-1,2-diol	30	9

D-1-Aminopropan-2-ol oxidation by cell-free extracts

The NAD^+-dependent oxidation of D-1-aminopropan-2-ol to aminoacetone was assayed colorimetrically (see Methods section). Under optimum conditions, activities of 12 to 15 mμmoles aminoacetone formed/mg. protein/min. at 37° were found in fresh extracts. Aminoacetone reductase activity could not be measured accurately in crude extracts due to the presence of high NADH oxidase activity.

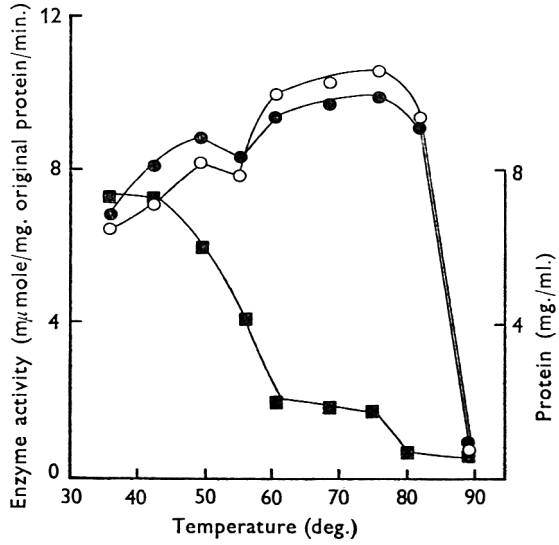

Fig. 1. Heat-treatment of crude cell-free extracts of *Escherichia coli*. Aliquots of crude cell-free extract, adjusted to 8 mg. protein/ml., were held at various temperatures for 10 min. After removing the precipitated protein by centrifuging, supernatants were assayed for D-1-aminopropan-2-ol dehydrogenase activity (○) colorimetrically, DL-propan-1,2-diol dehydrogenase activity (●) (× ⅛ given scale) spectrophotometrically, and protein content (■) as described in Methods. Enzyme activities are expressed in relation to the original protein concentration of untreated extract.

Table 3. *Partial purification of D-1-aminopropan-2-ol dehydrogenase*

Extracts from *Escherichia coli* grown on nutrient broth were fractionated as described in Methods.

Fraction	Volume (ml.)	Protein (mg./ml.)	Enzyme activity (mμmoles/mg. protein/min.)	Total units (IU)	Activity ratio D-1-aminopropan-2-ol DL-propan-1,2-diol
Crude extract	20	19	12	4560	0·15
Heat-treated	20	4·7	46	4300	0·15
BioGel P-300	70	0·45	144	4530	0·14
DE 52 cellulose	25	0·14	178	590	0·14
Peak fraction	3	0·12	450	162	0·14

The enzyme was activated by a number of monovalent cations, but inhibited by sulphydryl compounds and more markedly by substrate analogues (Table 2). Substances with little or no effect at 5 mM included AMP, ADP, ATP, iodoacetate and glutathione.

D-1-Aminopropan-2-ol dehydrogenase was remarkably stable to heat: heating at 70° for 10 min. caused an increase in activity, as well as purification due to protein precipitation (Fig. 1).

Purification and properties of an oxidoreductase acting on D-1-aminopropan-2-ol and aminoacetone

Purification was carried out as a preliminary to studies on substrate specificity and content of aminoacetone reductase activity (Table 3). The relative activities of D-1-aminopropan-2-ol and DL-propan-1,2-diol remained constant during 38-fold purification. During exclusion chromatography on Bio-Gel P-300, the oxidoreductase was completely separated from L-threonine and L-1-aminopropan-2-ol dehydrogenases. The enzyme was eluted immediately after the initial nucleoprotein indicating a mol. wt of about 300,000 (see Turner, 1967).

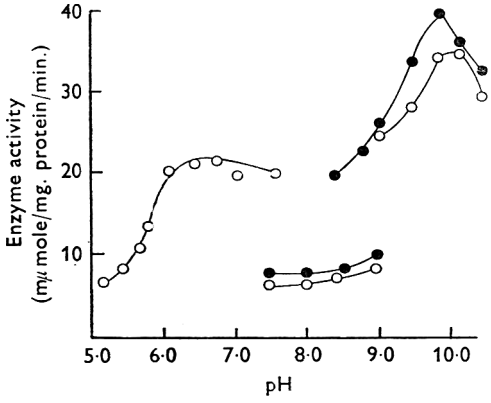

Fig. 2

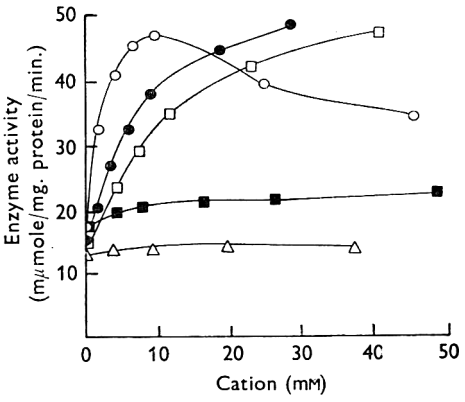

Fig. 3

Fig. 2. Effect of pH and buffers on D-1-aminopropan-2-ol and DL-propan-1,2-diol dehydrogenase activity. Enzyme activities in partially purified preparations were measured spectrophotometrically as described in Methods, although in the absence of $(NH_4)_2SO_4$: D-1-aminopropan-2-ol dehydrogenase activity (○), DL-propan-1,2-diol dehydrogenase activity (●) $\times \frac{1}{2}$ given scale. 0·3 M-Sodium acetate+acetic acid buffers were used at pH 5·1 to 5·9, 0·3 M-K_2HPO_4-KH_2PO_4 buffers at pH 6·0 to 7·5, 0·3 M-tris+HCl buffers at pH 7·4 to 9·0 and 0·3 M-diethanolamine+HCl buffers at pH 8·5 to 10·2.

Fig. 3. D-1-Aminopropan-2-ol dehydrogenase activation by monovalent cations. Activity in partially purified enzyme preparation was assayed colorimetrically as described in Methods, except that $(NH_4)_2SO_4$ in the incubation mixtures was replaced by Li_2SO_4 (●), $(NH_4)_2SO_4$ (○), Na_2SO_4 (□), K_2SO_4 (■) or Rb_2SO_4 (△).

PH optima. Optimum activity for the oxidation of either D-1-aminopropan-2-ol or DL-propan-1,2-diol with NAD^+ occurred at pH 9·8 in diethanolamine+HCl buffer. Dehydrogenase activities were markedly lower in tris+HCl buffer (Fig. 2), particularly in the absence of NH_4^+. For the reduction of aminoacetone and hydroxyacetone with either NADH or NADPH, optimum activity was at pH 6·0.

Cation activation. The activity of the enzyme was stimulated by NH_4^+, Li^+ and Na^+ at about 10, 50 and 100 mM respectively (Fig. 3). These effects, which were not additive, occurred with all substrates tested (see below). Cations were equally effective in both directions of the oxidoreductase.

Substrate and cofactor specificities. Broad substrate specificity was found (Table 4). Inactive as substrates were L-threonine, L-serine, DL-2-hydroxy-2-phenylethylamine and DL-glycerol-1-phosphate. In the alcohol oxidation direction, activity with $NADP^+$ was only 3 % of that with NAD^+. With the reduction of aminoacetone and other ketones, NADPH was at least 60 % as active as NADH at the optimum concentrations of 0·3 to 0·4 mM, but its relative activity was higher at higher (inhibitory) cofactor

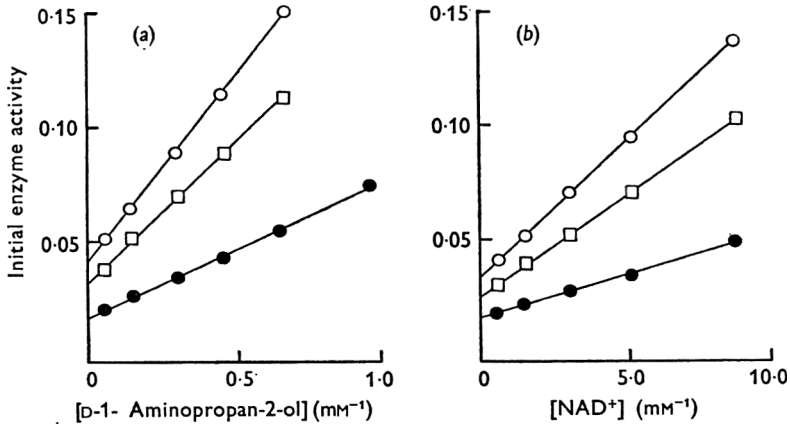

Fig. 4. Double reciprocal plots showing the effect of NH_4^+ on D-1-aminopropan-2-ol dehydrogenase activity. Activity in partially purified enzyme preparation from *Escherichia coli* was assayed colorimetrically as described in Methods except where noted. (*a*) Incubation mixtures contained 2 μmole NAD^+ with D-1-aminopropan-2-ol from 1·0 to 20·0 μmoles in presence of $(NH_4)_2SO_4$ at 1 mM (□) and 20 mM (○). No $(NH_4)_2SO_4$ control (●). (*b*) Incubation mixtures contained 20 μmole D-1-aminopropan-2-ol with NAD^+ from 0·1 to 2·0 μmoles in the presence of $(NH_4)_2SO_4$ at 1 mM (□) and 20 mM (○). No $(NH_4)_2SO_4$ control (●).

Table 4. *Substrate specificity of enzyme acting on D-1-aminopropan-2-ol*

Substrate-dependent reduction of NAD^+ and oxidation of NADH was measured spectrophotometrically at 340 nm. as described in the Methods section. Activities are expressed relative to those with D-1-aminopropan-2-ol, or aminoacetone, measured under appropriate optimum conditions.

Substrate	Activity (%)
Oxidation	
D-1-Aminopropan-2-ol	100
DL-Propan-1,2-diol	680
DL-1-Bromopropan-2-ol	350
meso-Butan-2,3-diol	320
Propan-1,3-diol	320
DL-1-Fluoropropan-2-ol	240
Glycerol	220
DL-1-Aminopropan-2,3-diol	90
L-1-Aminopropan-2-ol*	3
Reduction	
Aminoacetone	100
Dihydroxyacetone	1840
Hydroxyacetone	1200
Methylglyoxal	960
DL-Acetoin	250

* Composition 88 % L-1-aminopropan-2-ol; 12 % D-1-aminopropan-2-ol, used as their L-tartrates.

concentrations. Activities with each cofactor were not additive. D-1-Aminopropan-2-ol formation by reduction of aminoacetone was demonstrated chromatographically (see Methods), but the identity of the stereoisomer was not established.

Table 5. *Michaelis constants for substrates and cofactors of enzyme acting on* D-1-*aminopropan-2-ol and aminoacetone*

K_m values were obtained using method of Lineweaver & Burk (1934). The effects of substrate concentrations were determined in the presence of saturating amounts of cofactors, and vice versa.

Limiting component	Michaelis constant (mM)
D-1-Aminopropan-2-ol	2·0 to 5·0
DL-Propan-1,2-diol	0·3
NAD^-	0·2 to 0·5
Aminoacetone	3·0 to 4·5
Hydroxyacetone	0·1 to 0·2
NADH	0·07
NADPH	0·12

Kinetic behaviour. The effect of D-1-aminopropan-2-ol and NAD^+ on dehydrogenase activity, at various NH_4^+ concentrations, are shown as double reciprocal plots in Fig. 4. Ammonium ions increased V_{max} and decreased K_m values for the substrate. Michaelis constants for various substrates and cofactors are given in Table 5. Activities in the direction of alcohol oxidation were unaffected by high substrate or cofactor concentration and were not influenced by nucleotides. In contrast, ketone reduction was extremely sensitive to high substrate and cofactor concentrations and was inhibited by ATP but not by ADP or AMP. With NADH at the optimum concentration (0·4 mM), excess substrate inhibition with aminoacetone and hydroxyacetone occurred above about 10 and 1·2 mM respectively. Aminoacetone was reduced twice as rapidly as hydroxyacetone when each was tested at 10 mM. With aminoacetone at 10 mM, NADH and NADPH were both inhibitory at above about 0·4 mM

Inhibition by ATP. Whereas ketone reduction with NADPH was inhibited by 84 % by ATP at 0·3 mM, reduction with NADH was inhibited only 10 %. Inhibition by ATP was non-competitive. When the ketone concentration was varied, with NADPH saturating at 1 mM, the K_i for ATP was 0·07 mM. When the cofactor was varied, with ketone constant, the corresponding K_i was 0·60 mM.

Inhibition by substrate analogues and related compounds. Aminoacetone production from D-1-aminopropan-2-ol was inhibited by substrate analogues (Table 2), many of which were more effective substrates (Table 4). DL-Propan-1,2-diol, *meso*-butan-2,3-diol or DL-1-bromopropan-2-ol (ignoring isomeric forms) caused 91, 83 and 57 % inhibition of amino alcohol oxidation when added in equimolar amounts. Inhibition by DL-propan-1,2-diol was found to be competitive (K_i about 0·10 mM). DL-3-Fluoro-1-aminopropan-2-ol also acted as both substrate and inhibitor of D-1-aminopropan-2-ol oxidation. L-1-Aminopropan-2-ol was inactive as inhibitor or substrate.

Effect of growth conditions on dehydrogenase activities

Growth of *Escherichia coli* on simple synthetic media, containing glucose, succinate, fumarate, malate, aspartate, glycerol, lactate, pyruvate or acetate as sole source of carbon and energy, yielded extracts with low D-1-aminopropan-2-ol dehydrogenase activity. Activity towards all substrates tested was about one-fifth that found in

extracts of broth-grown organisms. DL-1-Aminopropan-2-ol added as a supplement to either broth or glucose-mineral salts media had no effect on enzyme activity. Of other alternative substrates tested, only hydroxyacetone induced enzyme formation markedly. Six- to tenfold increases occurred when washed suspensions of bacteria grown on glucose were incubated with hydroxyacetone at 150 mM. Dihydroxyacetone and aminoacetone stimulated 2·5- and 1·6-fold respectively. Similar results were obtained when inducers were added as 0·4% supplements to cultures of *E. coli* growing on glucose. *Escherichia coli* was incapable of growth on the hydroxy and

Table 6. *Dehydrogenase activities in crude extracts of micro-organisms*

Micro-organisms were grown on nutrient broth and dehydrogenase activities were assayed spectrophotometrically as described in the methods section.

Micro-organism	Strain	Dehydrogenase activity (mμmoles/mg. protein/min. at 37°) with substrate shown: D-1-Amino-propan-2-ol	L-1-Amino-propan-2-ol	*meso*-Butan 2,3-diol	DL-Propan-1,2-diol
Achromobacter sp. P6	NCIB 10431	2·8	0·7	32·0	13·0
Arthrobacter globiformis	NCIB 9759	1·5	0·1	n.d.	n.d.
Bacillus licheniformis	NCIB 8061	2·6	0·3	136·0	170·0
B. polymyxa	NCIB 8524	21·0	4·0	850·0	700·0
B. subtilis	Laboratory	1·0	3·0	20·0	31·0
Enterobacter cloacae	NCIB 8529	1·0	0·3	185·0	166·0
Erwinia carotovora	NCPPB 1280	4·1	9·9	n.d.	n.d.
Escherichia coli, type I	NCIB 8114	9·0	10·0	35·0	58·0
E. coli, K 12	NCIB 9481	11·4	n.d.	n.d.	150·0
E. coli, K 12	NCIB 9482	8·5	n.d.	n.d.	125·0
E. coli, K 12	NCIB 9483	7·7	n.d.	n.d.	95·0
Klebsiella aerogenes, type I	NCIB 418	2·0	8·0	n.d.	n.d.
K. aerogenes, type II	NCIB 8017	0·5	0·3	125·0	90·0
Proteus vulgaris	Laboratory	2·0	1·7	n.d.	n.d.
Pseudomonas ovalis	NCIB 9229	2·0	0·3	n.d.	n.d.
Pseudomonas sp.	NCIB 8858	0·3	1·0	n.d.	n.d.
Pseudomonas sp.	DL 8A	8·0	2·0	34·0	64·0

* n.d. = not determined.

Table 7. *L-Threonine and D-aminopropanol dehydrogenases in micro-organisms used for production of vitamin* B_{12}

Micro-organisms were grown on peptone–yeast extract medium and enzymes in extracts assayed colorimetrically as described in the Methods section.

Micro-organism	Strain (NCIB no.)	Growth conditions	Dehydrogenase activity (mμmoles/mg. protein/min. at 37°): D-Aminopropanol	L-Threonine
Flavobacterium devorans	8195	Aerobic	0·4	1·1
Nocardia rugosa	8926	Aerobic	3·1	17·0
Propionibacterium freudenreichii	5959	Anaerobic	0	0
P. shermanii	5964	Aerobic	3·2	19·0
P. shermanii	5964	Anaerobic	0	0
P. shermanii	8099	Anaerobic	0	0
Pseudomonas dentrificans	8376	Aerobic	1·1	3·7
Streptomyces griseus	9004	Aerobic	0·8	14·0
S. olivaceus	8238	Aerobic	0·8	2·0

amino ketones tested, or on the corresponding alcohols. Dehydrogenase activity towards L-1-aminopropan-2-ol was unaffected by hydroxy ketones and analogues, which reduced L-threonine dehydrogenase activity when included in growth media.

Dehydrogenase activities of various micro-organisms

Extracts of micro-organisms grown on broth were examined for dehydrogenase activities towards several secondary alcohols, including D-1-aminopropan-2-ol. Results are shown in Table 6.

A number of micro-organisms known to produce relatively large amounts of vitamin B_{12} were examined for L-threonine and D-1-aminopropan-2-ol dehydrogenase. Two of the propionibacter species would grow only anaerobically, although a third was capable of aerobic growth. The results are shown in Table 7.

DISCUSSION

The small and slow uptake of radioactivity from [3-^{14}C]-DL-1-aminopropan-2-ol by growing cultures of *Escherichia coli* does not allow us to make any unequivocal statement regarding a biosynthetic role for the D-isomer of the amino alcohol. Neither DL-1-aminopropan-2-ol nor aminoacetone act as a growth substrate or nitrogen source for the *E. coli* in synthetic media, although L-1-aminopropan-2-ol can be readily oxidized to aminoacetone by several intact micro-organisms including *E. coli*. However, the enzyme responsible for oxidation of L-aminopropanol in *E. coli* has been studied (Turner, 1967) and its metabolic significance is uncertain.

The enzyme responsible for D-1-aminopropan-2-ol oxidation in cell-free extracts of *Escherichia coli* is concluded to be an oxidoreductase of broad specificity in each direction. Evidence includes the constant activity for D-1-aminopropan-2-ol relative to DL-propan-1,2-diol, throughout purification; the inhibition of amino alcohol oxidation by alternative substrates; and induction of activity by hydroxyacetone or dihydroxyacetone but not aminoacetone or 1-aminopropan-2-ol. In a comparative study, the highest D-1-aminopropan-2-ol dehydrogenase activity was found in *Bacillus polymyxa*, known to possess only the D-specific butan-2,3-diol dehydrogenase (Taylor & Juni, 1960), whereas negligible activity was found in species with the L-specific enzyme, e.g. *Enterobacter cloaca* (Taylor & Juni, 1960).

The metabolic function of the enzyme studied in *Escherichia coli* is not clear, although specificity studies indicate involvement in diol and hydroxyketone metabolism. The acetoin fermentation, however, yielding butan-2,3-diol, is characteristic of *Aerobacter aerogenes* rather than *E. coli*, although the butan-2,3-diol-NAD^+ oxidoreductase of *A. aerogenes* studied by Taylor & Juni (1960) was stereospecific for the D-enantiomorphs of the diol substrates tested. Alternatively, the heat-stability of the enzyme, its activation by monovalent cations, pH optimum and K_m for NAD^+, all suggests close similarity to the glycerol-NAD^+ oxidoreductases studied in *E. coli* (Asnis & Brodie, 1953) as well as *A. aerogenes* (Burton, 1955; Lin & Magasanik, 1960; Strickland & Miller, 1968). It should be noted that glycerol but not glycerol-1-phosphate was a substrate for the enzyme described here (Table 4). The failure of suspensions of *E. coli* to produce aminoacetone when incubated with D-1-aminopropan-2-ol may well be due to competition by endogenously generated substrates for the oxidoreductase found in extracts. The K_m for glycerol found by Asnis &

Brodie (1953) was 10·7 mM, compared with that of 2·0 to 5·0 mM for D-1-aminopropan-2-ol reported here.

The significant activity of NADPH as a cofactor for ketone reduction, in contrast to the inactivity of $NADP^+$ in the oxidation direction, is noteworthy. It is possible that the oxidoreduction between NADPH and hydroxy- or aminoacetone may be catalysed by a separate contaminating enzyme. Work to establish this has not been carried out. Whether aminoacetone reduction, by either NADH or NADPH, yields the D- or L-stereoisomer has not been determined.

The enzyme from *Escherichia coli* K12, claimed by Dekker & Swain (1968) to be specific for the reversible oxidoreduction reaction between aminoacetone and D-1-aminopropan-2-ol, appears to differ from the enzyme described here. The enzyme was optimally active with the D-amino alcohol in tris+HCl buffer between pH 8·0 and 8·6, and inactive with all of a large number of substrate analogues. No activation by monovalent cations or activity with NADPH was reported. The activity with D-1-aminopropan-2-ol in crude extracts was about 14·0 to 14·6 mμmoles/mg. protein/min. at 37° and pH 8·4 (Dekker & Swain, 1968). This activity is about the same as that of the non-specific enzyme described here, and found to be present not only in *E. coli* type I, but also in *E. coli* K12 strains.

No convincing evidence for the joint involvement of L-threonine and D-1-aminopropan-2-ol dehydrogenases in the biosynthesis of the vitamin B_{12} was found, in that Propionbacterium species, which are known to produce good yields of the vitamin during anaerobic growth (Mervyn & Smith, 1964), did not possess either enzymes when grown under such conditions. It now seems clear that in addition to the non-specific nature of the oxidoreductase acting on D-1-aminopropan-2-ol and aminoacetone, L-threonine dehydrogenase may be involved in catabolism yielding glycine and acetyl-CoA (McGilvray & Morris, 1969) as an alternative to aminoacetone.

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Effects of Oxygen on Acetylene Reduction, Cytochrome Content and Respiratory Activity of *Azotobacter chroococcum*

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SUMMARY

The respiratory activities and cytochrome a_2 contents of nitrogen-fixing continuous cultures of *Azotobacter chroococcum* (NCIB 8003) increased with the partial pressure of oxygen encountered during growth. Above 0·6 atm., wash-out of the culture occurred. Acetylene reduction by culture samples of low respiratory activity was far more easily inhibited by oxygenation than was that of samples of high respiratory activity, though their maximum acetylene-reducing activities at their optimal pO_2 values were similar. Inhibition by oxygen was reversible after mild oxygenation: 70 to 100% of the original activity returned immediately when the degree of oxygenation was decreased. Irreversible inhibition occurred after vigorous oxygenation and was associated with a loss of activity in cell-free extracts, which was restored by adding the oxygen-sensitive protein component of Azotobacter nitrogenase. These observations support earlier proposals that augmented respiration can scavenge oxygen from the nitrogen-fixing site and that a conformational change in the state of nitrogenase can prevent damage to the enzyme by oxygen. Vigorous aeration, however, may overcome these protective mechanisms.

INTRODUCTION

Inhibition of growth of nitrogen-fixing Azotobacteriaceae, but not of populations using fixed nitrogen, by oxygen has been known since Meyerhof & Burk's (1928) report. Dalton & Postgate (1969*a*) summarized earlier work and extended the investigation to continuous cultures of *Azotobacter chroococcum* of known nutritional status. Populations whose growth was limited by availability of the phosphate or carbon+energy source were extremely sensitive to oxygen inhibition; populations whose density was limited by their intrinsic rate of nitrogen fixation (called 'N_2-limited' by Dalton & Postgate, 1969*b*) were less oxygen-sensitive, though still much more sensitive than populations assimilating ammonia. Dalton & Postgate (1969*a*) assumed that the oxygen sensitivity of Azotobacteriaceae was related to the known oxygen sensitivity of components of nitrogenase (Bulen & LeComte, 1966; Kelly, 1969*a*, *b*) and proposed the hypotheses that: (i) in resting bacteria, the nitrogenase became 'conformationally protected', i.e. it assumed a conformation in which oxygen-sensitive sites were inaccessible to O_2; (ii) in growing bacteria, nitrogenase assumed a conformation which would be susceptible to damage by oxygen but that the organism adjusted its respiration rate so as to prevent oxygen reaching sensitive sites. They called this control process 'respiratory protection'. Yates (1970) and Drozd & Postgate (1970) observed that growing cultures of *A. chroococcum*, *A. vine-*

landii and *Azomonas macrocytogenes* partly or completely 'switched-off' nitrogenase activity, assayed as rate of acetylene reduction, when vigorously shaken in air. This process was readily reversed on standing and it represented a possible source of error in the practical application of the acetylene reduction test to nitrogen-fixing aerobes in field conditions described by Stewart, Fitzgerald & Burris (1967) and Hardy Holsten, Jackson & Burns (1968).

The concept of an active 'respiratory protection' and a passive 'conformational protection' of nitrogenase in Azotobacter implies that strains of high respiration rate should be more efficient at 'respiratory protection' and less disposed to bring about 'conformational protection'. We report here a study of the oxygen relations of *Azotobacter chroococcum* 'acclimatized' to high and low pO_2 values.

METHODS

Organisms and medium. Azotobacter chroococcum (NCIB 8003) was grown at $30 \pm 0{\cdot}5°$ in a modification of Burk's medium containing mannitol (Dalton & Postgate, 1969*a*, *b*). To avoid transient precipitation in batches of medium which they described, the $CaCl_2$ was autoclaved separately as a 20 % solution and added aseptically to the rest of the autoclaved medium when cool.

Apparatus. For batch cultures, 250 or 500 ml. Erlenmeyer flasks approximately one-quarter full of medium were incubated at 30° on a rotary shaker; we recognize that such cultures became O_2-limited during growth. For continuous culture, apparatus with working volumes of approximately 200 ml. (Baker, 1968) were used with a port in the side for a Clark-type oxygen electrode (Protech Ltd, 21 High Street, Rickmansworth, Hertfordshire). The residual current in the oxygen electrode varied from day to day, so low oxygen tensions were measured by switching off the oxygen supply and stirrer in order to obtain the zero reading; this was then subtracted from the observed value. One port in the lid was closed by a 'Suba-seal' closure for injection of acetylene. Silicone rubber tubing (Esco Rubber Ltd, Seething Lane, London E.C. 3) was used for connexions. The initial pH of the medium was 7·8 and it buffered sufficiently to maintain the pH value constant at 6·7 in the growth vessel with the populations used, so automatic pH control was unnecessary. Partial pressures of gases were regulated using gas flowmeters.

Analytical procedures. An estimate of culture density was obtained from its optical density in an Eel 'Spectra' spectrophotometer. To determine organism concentration more exactly, quadruplicate 10 ml. samples of culture were centrifuged in dry, weighed, glass centrifuge tubes, washed once in 0·85 % NaCl and once in distilled water before drying to constant weight in air at 80°. Respiratory activity was measured directly on culture samples in conventional Warburg manometers containing 0·2 ml. of 40 % KOH in the centre well, shaken at 150 strokes/min., amplitude 2 cm., at 30°. Samples were transferred as rapidly as possible from the culture vessel to the Warburg flask, where they were gassed with argon and oxygen mixtures of the desired pO_2. No extra mannitol had to be added. Cytochrome spectra of whole organisms were measured using a diffuse reflectance unit on a Unicam SP 700 spectrophotometer. To allow for non-specific adsorption, peak heights were measured from an interpolated theoretical scatter curve. Protein in culture samples was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951); protein in crude extracts

was estimated by the method of Gornall, Bardawill & David (1949). The standard was bovine serum albumin (Sigma Chemicals Ltd, London).

Acetylene reduction. Acetylene reduction rates were accepted as measures of nitrogenase activity. Samples of culture (2 ml.) were transferred to 25 ml. conical flasks and gassed with argon/oxygen mixtures of the desired pO_2 value. The flasks were sealed with 'Suba-seal' closures, 1 ml. of acetylene (freshly prepared from CaC_2 and water) was injected and they were shaken at 30°. Gas samples (1 ml.) were taken by syringe for gas chromatography at 5 or 10 min. intervals and replaced by 1 ml. of the appropriate argon and oxygen mixture. This procedure introduced a small systematic error in rate determinations which was unimportant. For tests on samples in Warburg manometer flasks, 1 ml. acetylene was injected through a vaccine cap (Scientific Furnishings, Chichester) on the side-arm of the manometer flask and gas samples (1 ml.) were taken by the same method. With crude extracts (see below) an ATP-generating system was added and acetylene reduction was measured in 8 ml. vials under argon as described by Kelly (1969*a*). The procedures used with continuous cultures are described in the Results section.

Gas chromatography. Ethylene was detected by flame-ionization in a Pye series 104 gas chromatograph with a 5 ft (152 cm.) Porapak R. column, 4 mm. internal diameter, at 45° in a stream of 50 ml. N_2/min. Peak height was taken as being proportional to ethylene concentration.

Preparation of crude cell-free extracts. The culture sample (100 ml.) was centrifuged at 28,000*g* for 10 min. at 4°. The pellet was resuspended in 10 ml. 0·025 M-tris-HCl buffer, pH 7·4, and disrupted under a stream of nitrogen, while cooled in ice, with a Dawe 'Soniprobe' ultrasonic disintegrator at 2 to 5 A for 2 min. This preparation was centrifuged at 38,000*g* for 20 min. to give a clear, light-brown supernatant liquid containing nitrogenase and a pellet containing whole bacteria, membranes and other debris. The supernatant was stored under nitrogen and in ice for up to 2 h. before use, or stored in liquid nitrogen for longer periods. 'Fraction 2', the non-haem iron component of nitrogenase, was prepared from *Azotobacter chroococcum* by Dr M. Kelly in this laboratory.

RESULTS

Effect of growth pO_2 on bacterial density and respiratory rate

A population of *Azotobacter chroococcum* was established growing in continuous culture at a dilution rate of 0·2 $h.^{-1}$ under 0·2 atm. oxygen + 0·8 atm. nitrogen from separate cylinders; the combined gas flow rate was 1 culture vol./min. Cytochrome contents, Q_{O_2} values and dry weights were measured on culture samples. The pO_2 was then altered and comparable analyses made when the culture was considered to be in a new steady state. Population densities of organisms under various partial pressures of oxygen agreed closely with those obtained by Dalton & Postgate (1969*a*) and an almost exact duplicate of their Fig. 1 was obtained. According to population density measurements, oxygen became the growth-limiting nutrient below a pO_2 of 0·09 atm.; above 0·6 atm. O_2, wash-out of the culture occurred. Table 1 shows that the Q_{O_2} values of the organisms increased with increase in growth pO_2.

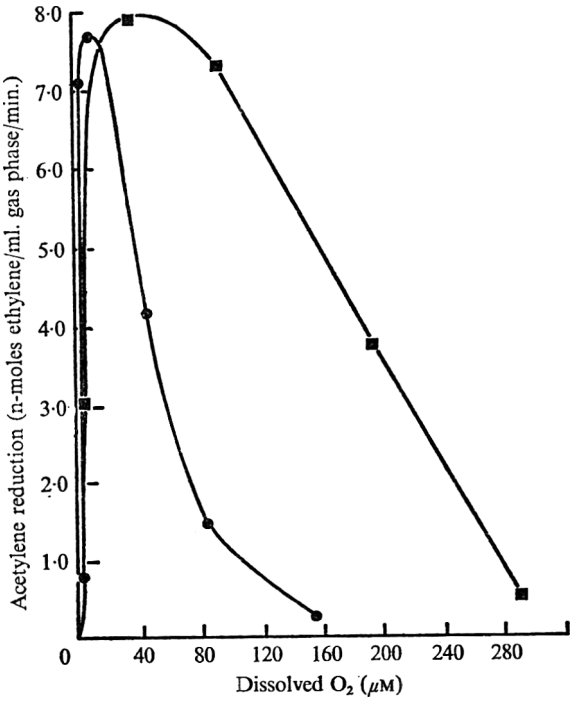

Fig. 1. Rates of acetylene reduction at various molarities of dissolved O_2 by *Azotobacter chroococcum* grown in continuous culture at $D = 0{\cdot}2$ h.$^{-1}$. Tests made on whole contents of growth vessel with medium pump off; O_2 molarities determined from oxygen electrode readings (see text). Populations grown at 0·09 atm. O_2 (●) and at 0·55 atm. O_2 (■).

Table 1. *Relationship between pO_2 of growth, Q_{O_2} and cytochrome content in Azotobacter chroococcum grown in continuous culture under N_2-fixing conditions at $D = 0{\cdot}2$ h.$^{-1}$*

Q_{O_2} values of 1 ml. samples of culture measured in Warburg flasks at 30° under 0·6 atm. Ar+0·4 atm. O_2, with 0·2 ml. 40% KOH in the centre well. Cytochrome spectra were measured by diffuse reflectance spectroscopy in a Pye Unicam SP 700 spectrophotometer in cuvettes with a 1 cm. light path. Cytochrome levels in the bacteria expressed in terms of extinction (E) at reduced α peak. Bacteria from continuous culture at various pO_2 values were centrifuged and resuspended to 11 mg. dry wt/ml. 0·025 M-tris-HCl buffer (pH 7·4). Reduced spectra obtained by gassing the sample with H_2.

Growth pO_2 (atm.)	Q_{O_2} (μl. O_2/mg. dry wt/h.)	Cytochrome levels ($E \times 10^3$/mg. dry wt)	
		a_2	c_4+c_5
0·05	300	0·23	5·25
0·10	1510	0·45	6·83
0·35	2300	1·14	5·60
0·55	2700	1·36	5·50

Effect of dissolved oxygen on acetylene reduction by whole cultures

The rate of acetylene reduction by whole chemostat cultures was assessed by injecting 20 ml. of acetylene into the vessel with the gas inlet and outlet ports clipped off and with the medium pump off (the cultures, of course, ceased to be continuous

during the test). By adjusting the stirrer manually, the dissolved oxygen level could be held at any selected level below saturation. The rate of acetylene reduction over 10 min. was measured at various levels of dissolved oxygen, starting at the highest and then lowering it. Between each determination the culture vessel was flushed out with the gas mixture used for growth. Figure 1 shows that the optimum concentration of dissolved oxygen for acetylene reduction by culture grown at a pO_2 of 0·09 atm. was 12 μM, though reduction could be detected up to 150 μM. The culture grown at a pO_2 of 0·55 atm. showed an optimum at 25 μM-oxygen and acetylene reduction could be detected up to 260 μM.

Effect of ambient pO_2 on respiratory rate and acetylene reduction rate of culture samples

Meyerhof & Burk (1928) reported that respiration in azotobacters is depressed at high pO_2 values. The Q_{O_2} values of samples of cultures grown at pO_2 0·09 atm. and 0·55 atm. were measured under a range of pO_2 values in the manometer flask.

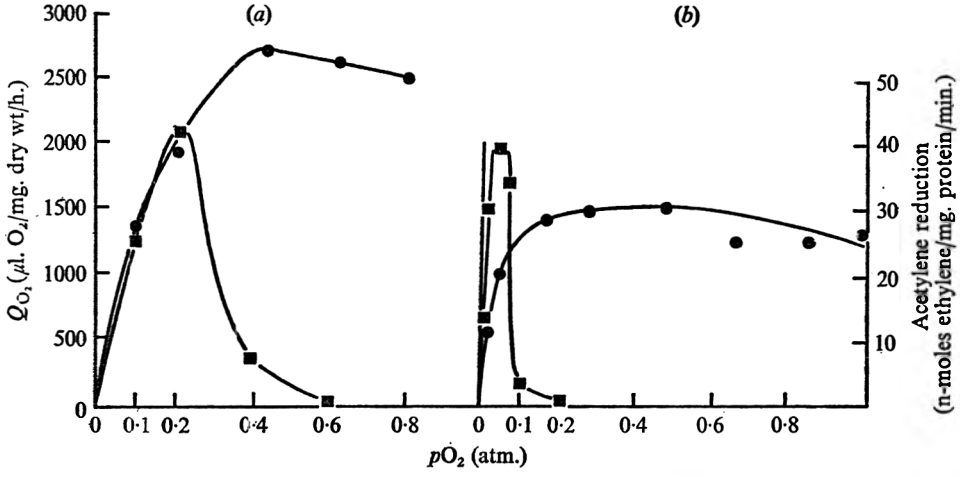

Fig. 2. O_2 uptake (as Q_{O_2}, ●) and acetylene reduction (■) at various pO_2 values by 1 ml. samples of continuous cultures of *Azotobacter chroococcum* (D = 0·2 h.$^{-1}$). In (*a*) organisms grown with 0·55 atm. O_2; in (*b*) grown with 0·09 atm. O_2. (For detailed conditions see text.)

Figures 2(*a*) and (*b*) show that, over 20 min., high pO_2 depressed the respiration rates only slightly. Acetylene reduction measured under similar conditions was much more sensitive to elevated pO_2 and was completely inhibited in populations tested at pO_2 values which gave their maximum respiration rate. The population grown at a pO_2 of 0·09 atm. showed acetylene reduction up to 0·15 atm. with a maximum at 0·05 atm.; the population grown at 0·55 atm. showed activity up to 0·55 atm. with a maximum at 0·2 atm. oxygen. The maximum specific activities at optimal pO_2 values were the same in both cases.

Effect of pO_2 on cytochrome content

Azotobacter vinelandii was reported to possess cytochromes c_4, c_5, b_1, a_1, a_2 and o (Jones & Redfearn, 1967), although cytochrome o was difficult to establish because it was usually only present in traces. Table 1 shows that, with increased pO_2, the cytochrome a_2 content of the organisms increased in parallel with the increase in

Q_{O_2} mentioned earlier. The α peaks of cytochromes c_4+c_5 showed a slight maximum at 0·1 atm. oxygen, the α peaks of cytochrome b_1 appeared as a shoulder on the c_4+c_5 peak and was difficult to measure in these conditions.

Effect of N_2 on cytochromes

Several reports have been published suggesting that the cytochromes of N_2-fixing Azotobacter species interact with N_2 (Wilson, 1958; Ivanov, Sitonite & Belov, 1965; Ivanov, Matkhanov, Belov & Gogoleva, 1967; Ivanov *et al.* 1968). Dalton & Postgate (1969*b*) found no such interaction with carbon-limited populations of *Azotobacter chroococcum*. Since growth pO_2 might influence the response of N_2, we tested populations grown at 0·09 and 0·55 atm. in the following manner. *Azotobacter chroococcum* from the effluent vessel of a continuous culture was resuspended at 11 mg. dry wt/ml. 0·025 M-tris-HCl buffer, pH 7·4. The preparation was gassed successively in a diffuse reflectance cell with high purity H_2, Ar, N_2 and O_2 (Air Products Ltd); the fully reduced spectrum was finally checked by adding dithionite after the O_2 treatment. Hydrogen gave the fully reduced spectrum; Ar did not give any change, nor did N_2. Oxygen gave a 70 % decrease in the β peaks of cytochromes c_4+c_5 and b_1, a 90 % decrease in their α peaks and a shift in their combined γ peaks from 420 to 412 nm.

Effect of NH_4 on cytochromes

Dalton & Postgate (1969*b*) observed no substantial differences between the cytochrome patterns of N_2-grown and NH_4^+-grown continuous cultures in otherwise comparable conditions. Knowles & Redfearn (1968) observed considerable differences in cytochrome contents of subcellular particles from urea- or N_2-grown batch cultures of *Azotobacter vinelandii*, so we repeated Dalton & Postgate's experiment using a different pO_2. *Azotobacter chroococcum* from an NH_4^+-grown (1·5 g. NH_4Cl/l.) continuous culture, $D = 0{\cdot}2$ h.$^{-1}$ under 0·7 atm. argon + 0·3 atm. O_2, had a cytochrome spectrum similar to that of bacteria from the nitrogen-deficient medium grown at the same dilution rate but with 0·7 atm. N_2 + 0·3 atm. O_2.

Michaelis constant for acetylene reduction by whole bacteria

Samples of the cultures grown at low and high pO_2 were tested with argon + O_2 at various partial pressures of acetylene. The K_m values were similar and within the range $0{\cdot}0028 \pm 0{\cdot}0005$ atm. acetylene. Plots of reciprocal velocity against reciprocal substrate concentration showed that nitrogen acted as a competitive inhibitor of acetylene reduction. The K_m values were independent of the pO_2 at which they were determined. A few tests with batch cultures gave similar K_m values.

Reversible inhibition of acetylene reduction by O_2

Samples of *Azotobacter chroococcum* from continuous cultures grown at the high or the low pO_2 value were exposed for 5 to 10 min. in atmospheres of pO_2 just sufficient to inhibit acetylene reduction completely when shaken at 75 strokes/min., amplitude 4 cm. If the pO_2 value was then altered to the optimum for that population, the reduction of acetylene was immediately resumed, usually completely but sometimes up to 30 % below the original specific rate (Fig. 3*a*). This is the 'switch on' and 'switch off' process reported by Drozd & Postgate (1970). The process was too

rapid for destruction and resynthesis of nitrogenase to be involved; this point was nevertheless checked by experiments in which 'switch off' and 'switch on' were induced in the presence of 75 μg. chloramphenicol/ml. to inhibit protein synthesis. The drug had no effect at all on either step in the process. As would be expected, both from our work and that of Yates (1970), culture samples shaken at a fixed pO_2 decreased in activity when shaken more rapidly; on lowering the shaking to

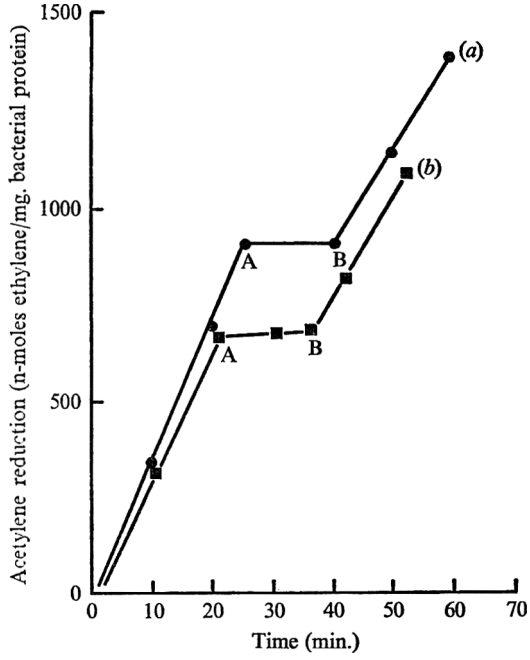

Fig. 3. 'Switch on' and 'switch off' of acetylene reduction by oxygenation in *Azotobacter chroococcum*. In the first experiment oxygenation was altered by increasing the pO_2 (●). A sample (2 ml.) from a continuous culture (D = 0·2 h.$^{-1}$, pO_2 = 0·09 atm.) was shaken in a 25 ml. conical flask at 150 strokes/min., amplitude 4 cm., at 30° under 0·05 atm. O_2+9·95 atm. Ar+0·03 atm. C_2H_2. At A the flask was opened, flushed and resealed within 20 sec. with 0·2 atm. O_2+0·8 atm. Ar+0·03 atm. C_2H_2; at B it was returned to the original atmosphere by a similar procedure. In the second experiment oxygenation was altered by increasing the shaker speed and amplitude (■). A sample (details as for first experiment) was shaken at 75 strokes/min., amplitude 1·5 cm., at 30° under 0·2 atm. O_2+0·8 atm. Ar+0·03 atm. C_2H_2. The shaking was changed to 150 strokes/min., amplitude 4 cm., at A and returned to the original at B.

the original frequency the activity returned immediately, usually completely, but never more than 30 % below the original (Fig. 3*b*). Consistent with the work described earlier, the lower the Q_{O_2} of the population, the more readily was acetylene reduction suppressed by oxygen. These observations show that 70 to 100 % of the inhibitory effect of oxygen was immediately reversible.

Irreversible inhibition of acetylene reduction by O_2

Part of the inhibition by oxygen was sometimes not reversible. To study the question further, a continuous culture of *Azotobacter chroococcum* was set up at D = 0·2 h.$^{-1}$, pN_2 = 0·8, pO_2 = 0·2 atm. and the atmosphere replaced by pure

O_2 for 30 min. or 1 h. Nearly all the organisms in the growth vessel were harvested and the remainder left to grow at 0·2 atm. O_2 again. Extracts of the harvested organisms had no acetylene-reducing activity; populations from the same continuous culture treated similarly, on a different occasion, but with pure argon in place of the O_2, gave extracts showing 80 % of the acetylene-reducing activity of undamaged organisms harvested from the culture. Addition of purified 'fraction 2' to extracts of the oxygen-treated population restored 90 % of the original activity. These findings are illustrated in Table 2, which includes a further control, in which a population which had been grown with sufficient NH_4^+ to repress nitrogenase synthesis was exposed to argon, to show that the acetylene-reducing activity which was recovered was not due to use of impure 'fraction 2'.

Table 2. *Restoration of acetylene-reducing activity to extracts of oxygenated Azotobacter chroococcum cultures by addition of 'fraction 2'*

Continuous cultures of N_2-fixing *Azotobacter chroococcum* were interrupted while the contents of the growth vessel were exposed to the gases indicated, harvested and the acetylene-reducing activities of extracts determined. For details see text. Final assay mixture contained 10 μmoles $MgCl_2$, 5 μmoles ATP, 40 μmoles creatine phosphate (Sigma Chemical Co., London, S.W.6), 25 μmoles tris-HCl buffer, pH 7·4, 0·2 mg. creatine phosphokinase (E.C. 2.7.3.2, Sigma, London), 20 μmoles $Na_2S_2O_4$, 0·5 ml. extract (8·5 mg. protein/ml.), 0·025 ml. 'fraction 2' (0·2 mg. protein) when added, H_2O to 1·5 ml. The reaction was started by adding 0·2 ml. acetylene and stopped after 10 min. with 0·1 ml. 30 % trichloroacetic acid.

Treatment	Addition	n-mole ethylene formed/mg. protein/min.
Ar + 0·2 atm. O_2 for 30 min.	None	20
Ar for 30 min.	None	16
O_2 for 30 min.	None	2
	'Fraction 2'	18
Grown with NH_4^+	None	0
	'Fraction 2'	3

DISCUSSION

Respiratory protection of nitrogenase in Azotobacter chroococcum

Evidence for increased efficiency of nitrogen fixation (mg. N fixed/g. carbon source consumed) by azotobacters at low pO_2 values, and concomitant inefficiency at high pO_2 values, has been reported by several workers (Parker, 1954; Parker & Scutt, 1960; Phillips & Johnson, 1961; Dalton & Postgate, 1969*a*). As a rationalization of the effects of nutritional status on the oxygen sensitivity of continuous cultures, Dalton & Postgate (1969*a*) proposed that respiration in *Azotobacter chroococcum*, apart from its normal metabolic function, played a part in protecting functional nitrogenase from damage by oxygen. Our findings logically support their view: culture of *A. chroococcum* at low pO_2 and high pO_2 values led to no differences in the nitrogenase contents of the populations (as expressed in their maximum rates of acetylene reduction) but influenced markedly the pO_2 value at which the maximum rate was obtained. We found a direct relationship between optimal pO_2 for acetylene reduction and Q_{O_2} of the population: the greater the Q_{O_2}, the more environmental oxygen the population could tolerate before inhibition of acetylene reduction appeared. This was true of

samples of cultures grown at high and at low pO_2 values and tested with acetylene over a range of pO_2 values and of samples tested at various shaking frequencies at the partial pressure of O_2 in air; it was also true of the effect of ambient molarities of dissolved oxygen (as registered by oxygen electrodes) on acetylene reduction by the actual 'continuous' cultures (which ceased to be continuous for the duration of the experiment).

Reversible oxygen inhibition and 'conformational protection'

Our observations that O_2 inhibition, in both high and low oxygen populations, is 70 to 100% reversible if exposure to oxygen has not been prolonged is direct evidence for a response too rapid to involve damage to and resynthesis of enzymes or portions of enzymes. Our experiments with chloramphenicol, which inhibits nitrogenase synthesis in *Azotobacter vinelandii* (Strandberg & Wilson, 1968) support this view. Rapidly reversible O_2 inhibition of nitrate reductase activity, also without protein synthesis, occurs in anaerobically grown cultures of *Aerobacter aerogenes* (Pinchinoty, 1963) and the tetrathionate reductase activity of a similarly grown unidentified coliform bacterium (Pinchinoty & Bigliardi-Rouvier, 1963) is also oxygen-sensitive. Pinchinoty attributed these responses to an auto-oxidizable flavin at which O_2 competed with the inorganic acceptors for electrons. Competition by O_2 at the prosthetic site of such enzymes would lead to similar responses. Competition for electrons at the prosthetic site of nitrogenase, or at an auto-oxidizable electron carrier, might equally well account for the 'switch on–switch off' effects described in this paper, but other evidence argues against this view. Since the purified enzyme is oxygen-sensitive yet the crude form is not (Bulen & LeComte, 1966; Kelly, 1969*a*, *b*), this indicates that a 'protected' conformation does exist; in populations from batch culture, 'switch on' is not immediate (Drozd & Postgate, 1970), which a kinetic interpretation of the process would require it to be. Though we accept that there is still no direct evidence that the conformation of the intracellular nitrogenase changes (Dalton & Postgate, 1969*a*, *b*), certainly some event takes place which prevents interaction with oxygen, acetylene and, by implication, nitrogen. Oppenheim & Marcus (1970) and Oppenheim, Fisher, Marcus & Wilson (1970) showed that N_2-fixing *A. vinelandii* is particularly rich in internal membranes and that insoluble oxygen-tolerant enzyme preparations are obtained by a method which yields nitrogenase associated with membrane material; osmotic rupture yielded preparations not associated with membrane which were both soluble and oxygen-sensitive. These two forms of nitrogenase might correspond to the 'switched off' and 'switched on' states of the enzyme. 'Conformational protection', as we shall continue to call it, was sometimes less than perfect in that up to 30% of the original activity was sometimes not restored, and exposure to pure oxygen induced irreversible oxygen damage directly attributable to the oxygen-sensitive component ('fraction 2') of nitrogenase. Kelly (1969*b*) reported that the normally air-stable particulate nitrogenase from *Azotobacter chroococcum*, which we regard as representative of the conformationally protected enzyme complex *in vivo*, lost about 75% activity over 30 min. in pure O_2. Both our work and that of Kelly (1969*a*, *b*) are consistent with the report of Strandberg & Wilson (1968) that prolonged (4 h.) shaking of *A. vinelandii* cultures in air decreased N_2-fixing activity of extracts 28-fold and that synthesis of nitrogenase by NH_4^+-limited *A. vinelandii* was inhibited by hyperbaric (0·4 atm.) oxygen but not by air.

Effect of growth conditions on cytochromes in nitrogen fixation

The cytochrome patterns obtained on altering the growth pO_2 showed that, in O_2-limiting conditions (below pO_2 = 0·09 atm.), the c_4+c_5 level was the same as in N_2-limited populations, but the decrease in a_2 content with decrease in pO_2 continued. This is contrary to the report of Swank & Burris (1969) that the c_4+c_5 content of O_2-limited *Azotobacter vinelandii* was twice that of populations which were presumably N_2-limited. Lisenkova & Khmel (1967) measured the cytochrome content of whole *A. vinelandii* from a continuous culture growing at D = 0·3 h.$^{-1}$, but their maximum Q_{O_2} values are so low (430 μl. O_2/mg. dry wt/h.) that the populations must have been O_2-limited and not N_2-limited in the sense used by Dalton & Postgate (1969*b*). Comparisons of their results and ours are therefore difficult. Like Dalton & Postgate (1969*b*) and consistent with the experiments of Swank & Burris (1969) using bacterial extracts, we could obtain no evidence for direct involvement of cytochromes in nitrogen fixation either from the cytochrome patterns of organisms grown in various conditions or by direct tests for cytochrome oxidation by N_2.

We acknowledge useful critical comments by Dr M. G. Yates during preparation of this manuscript.

ERRATA

Two publications from the laboratory contain mistakes due to oversight in preparing the manuscripts. They are:

Dalton & Postgate (1969*b*), Fig. 2, p. 313:
The rate of ethylene production should be /min., not /h.
Biggins & Postgate (1969), Fig. 1, p. 187:
The figures for μmoles ethylene produced should be divided by 45.

J. R. Postgate and his co-authors apologize for any confusion they may have caused.

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Chemical Composition of Wild-type and Mutant *Aspergillus nidulans* Cell Walls. The Nature of Polysaccharide and Melanin Constituents

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SUMMARY

Chitin and a β-linked glucan were the major chemical components of *Aspergillus nidulans* cell walls. Other monomeric residues identified in enzymic and acid hydrolyses of whole cell walls and cell-wall fractions included galactose, mannose, glucuronic acid and galactosamine. The β-glucan contained (1 → 3) and (1 → 6) linkages and was two-thirds digested by an exo-β-D-1,3 glucanase prepared from a cell-wall lysing Streptomyces species. An α-glucan was identified as a cell-wall component and it also contained (1 → 3) linkages. This latter polysaccharide was distinguishable from nigeran (an α-1,3; α-1,4 glucan present in other Aspergillus species) by infrared spectroscopy and by its low susceptibility to hydrolysis by an endo-α-1,3; α-1,4 glucan glucanohydrolase. Both glucans were alkali-soluble, but the β-glucan was completely solubilized only after acid extraction of the wall. The *N*-acetylglucosamine to galactosamine ratio in the *A. nidulans* cell wall was 1·32 and the two hexosamines were shown to be constituents of distinct polymers. The remaining cell wall was accounted for by protein, lipids, readily extractable and bound, and, in the wild-type, melanin.

The melanin was distributed throughout the cell wall but was associated particularly with the chitin fraction. The pigment has been partially characterized chemically and contains indolic residues; this result does not substantiate earlier views that indolic melanins are peculiar to the animal kingdom. Melanin appears to be a finite heteropolymer both in terms of its molecular size and its chemistry.

INTRODUCTION

A positive correlation has been demonstrated in several species between the presence of melanin, or melanin-like pigments, in the cell walls of fungi and resistance to microbial and enzymatic lysis (Potgieter & Alexander, 1966; Bloomfield & Alexander, 1967). Furthermore, studies on *Aspergillus nidulans* (Kuo & Alexander, 1967) established that the resistance to lysis was affected by the melanin concentration in the cell wall. The mechanism by which melanin may exert its antilytic effect in this fungus has recently been discussed (Bull, 1970*a*).

As an essential prelude to investigations of the melanin effect the cells walls of *Aspergillus nidulans* were examined chemically with reference to the nature of

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melanized and non-melanized walls. Extensive chemical analyses of cell walls of filamentous moulds remain few in comparison with bacteria and, in particular, only two fungal melanins have been studied in any detail (Nicolaus, Piattelli & Fattorusso, 1964).

METHODS

Microbiological techniques. Wild-type strain 13 and an albino mutant 13.1.OL of *Aspergillus nidulans* Eidam (Wint.) have been described previously (Bull & Faulkner, 1964, 1965). The two strains were distinguished by the presence and absence respectively of melanin in their cell walls. Stock cultures were maintained on a glucose–salts agar medium and 10 l. batch cultures for cell-wall preparation were grown in a liquid medium of similar composition (Carter & Bull, 1969; Bull, 1970*a*).

Cell walls of *Aspergillus nidulans* 13.1.OL were degraded extensively by a complex of lytic enzymes synthesized by a Streptomyces species. The lytic organism was isolated from soil enriched with *Phytophthora megasterium* mycelium (Wang, 1964). Cultures of the streptomycete were maintained on a mineral salts agar containing 2 % (w/v) wet wt of blended *Aspergillus oryzae* mycelium (supplied by Wallerstein Co., Division of Travenal Laboratories Inc., Staten Island, New York, U.S.A.) as the sole carbon source. Forty-litre cultures of the streptomycete were grown in a stainless-steel fermenter and the lytic enzymes were precipitated from the culture liquors by 70 % saturation with $(NH_4)_2SO_4$. Full details of these procedures, together with descriptions of β-1,3 glucanase and chitinase purification, have been reported previously (Bull, 1970*a*).

Cell-wall preparation. Two methods of cell-wall preparation were used. (i) Freshly harvested mycelium was washed thrice with Na/K phosphate buffer (0·033 M, pH 6·1), its pH adjusted to 6·8 with 2 N-NaOH and collected by centrifugation. The mycelium was blended for 2 min. in an Omni-Mixer (Ivan Sorvall Inc., Norwalk, Connecticut, U.S.A.), then treated with 1 % (w/v) sodium dodecyl sulphate (10 ml./g. wet wt mycelium) (Mahadevan & Tatum, 1965) and stirred overnight at 4°. Fivefold washing of the hyphal fragments in phosphate buffer was followed by treatment of the residue with increasing concentrations of aqueous ethanol, after which it was suspended in distilled water and freeze-dried. (ii) Phosphate-buffer washed mycelium (1·5 g. amounts in 50 ml. buffer) was macerated in a glass tissue homogenizer (Thomas, Philadelphia, Pennsylvania, U.S.A.) driven by an electric motor at maximum speed and then centrifuged at 800*g* for 10 min. Merthiolate (0·01 %, v/v) (Eli Lilly and Co., Indianapolis, Indiana, U.S.A.) was added to the buffer solution to prevent microbial growth. The supernatant containing cytoplasmic debris was discarded and the combined residues resuspended in buffer (50 ml.). The suspension held below 12° was ultrasonicated in 15 ml. amounts for 2 min. with the sonicator (Biosonik, Bronwill, Rochester, New York, U.S.A.) operating at full power. Cell-wall material was recovered by centrifugation (800*g*, for 10 min.) and the ultrasonication cycle repeated twice more. The residue, collected at 1400*g* for 10 min., was washed twice with chilled buffer and freeze-dried. Wall preparations were examined microscopically during the course of their isolation; the procedures detailed above removed all cytoplasmic material as shown by staining techniques, phase-contrast observation and lack of detectable extinctions at 260 and 280 nm.

Chemical analyses. Chemical fractionation of the cell wall was made according

to the method described by Mahadevan & Tatum (1965). At all times during the fractionation, reaction mixtures were flushed with nitrogen gas to minimize oxidative degradation. The four fractions were characterized as follows: (1) alkali-soluble; (2) acid-soluble; (3) acid-insoluble, alkali-soluble; (4) acid- and alkali-insoluble. Fractions 1, 3 and 4 were suspended in distilled water, neutralized, then dialysed against distilled water for 36 h. and freeze-dried; fraction 2 was neutralized with solid $Ba(OH)_2$ and the supernatant, after centrifugation, was freeze-dried.

In some experiments cell-wall fractions were extracted with Schweizer's cuprammonium reagent, 13·5 g. Cu/l. (Jayme & Lang, 1963). The sample (30 mg.) was twice extracted with the solvent (5 ml.) under N_2 for 3 h. at room temperature. The residue was centrifuged off (14,000*g*, 15 min.), washed exhaustively with distilled water and dried over NaOH pellets.

Carbon, hydrogen and nitrogen determinations were made with a model 185 C-H-N Analyser (Hewlett-Packard, Avondale, Pennsylvania, U.S.A.) using 2, 32 and 8 attenuation respectively for N_2, CO_2 and H_2O. The instrument was calibrated with cyclohexane-2,4-dinitrophenyl hydrazone (N = 20·14 %) and acetanilide (N = 10·36 %). Micro-Kjeldahl determinations of total N were made by the method of Bremner (1960). Phosphorus was determined with the *N*-phenyl-*p*-phenylenediamine hydrochloride chromogen (Dryer, Tammes & Roth, 1957).

Acid hydrolyses of cell walls were made by the procedures described by Bartnicki-Garcia (1966) and were carried out under N_2. After hydrolysis with H_2SO_4, hydrolysates were neutralized with $BaCO_3$ and the clarified supernatants deionized by passage through a bed of Dowex-50W-X8 (hydrogen form). Hydrochloric acid was removed by evaporation *in vacuo* over NaOH pellets. Cell walls were enzymically dissolved with a partially purified lytic complex (Bull, 1970*a*) in 0·05M-citrate–phosphate buffer, pH 5·0, containing 0·01 % (w/v) merthiolate. Reaction mixtures contained 353 units of endo-β-1,3 glucanase (β-1,3(4) glucan glucanohydrolase, E.C. 3.2.1.6), 2·62 units of chitinase (chitin glycanohydrolase, E.C. 3.2.1.14), 93 units of mycodextranase (α-1,3, α-1,4 glucan glucanohydrolase), and unspecified lipolytic and proteolytic activities. Highly purified hydrolases (Bull, 1970*a*) also were used to investigate the nature of various cell-wall polymers.

Cell-wall hydrolysis products were separated by descending paper chromatography (Whatman no. 1) or ascending cellulose thin-layer chromatography (Cellulose F, E. Merck AG, Darmstadt, Germany). Chromatograms were irrigated with either butan-1-ol+pyridine+water (6+4+3, v/v) or propan-2-ol+acetic acid+water (3+0·5+1, v/v) and developed with aniline hydrogen phthalate, ammoniacal $AgNO_3$ (reducing sugars), *p*-dimethylaminobenzaldehyde (amino sugars) or ninhydrin (amino acids). Sugars separated on paper chromatograms were estimated quantitatively by cutting out the sugar spots (located on parallel chromatograms developed with $AgNO_3$), eluting with distilled water and determining the sugar concentration of the clarified eluate with anthrone and *p*-dimethylaminobenzaldehyde.

Total carbohydrate was determined by the anthrone procedure of Loewus (1952) using a glucose standard. Reducing sugars were determined as glucose with a low-alkaline Cu reagent (Somogyi, 1952) and the arsenomolybdate chromagen of Nelson (1944). The method of Reissig, Strominger & Leloir (1955) was used to determine *N*-acetylamino sugars which were expressed as *N*-acetylglucosamine. Glucose and galactose were assayed enzymically with the appropriate sugar oxidases (Glucostat

Special and Galactostat, Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.). Galactosamine identity was confirmed by converting it to the corresponding pentose, i.e. lyxose (Stoffyn & Jeanloz, 1954). Presumed galactosamine spots were cut out from paper chromatograms and eluted with water. The clarified eluate was spotted (20 μl.) on to cellulose TLC plates which, after drying, were sprayed with 2 % (w/v) ninhydrin containing 4 % (v/v) pyridine and heated to 80° for 3 h. in an atmosphere of pyridine and water (1 + 1). The dried plates were irrigated with butan-1-ol + ethanol + water (4 + 1 + 1, v/v) and galactosamine, glucosamine, lyxose and arabinose co-chromatographed for reference. Uronic acids were determined with the Dische carbazole reagent (Gancedo, Gancedo & Asensio, 1968). Passage of hydrolysates through a bed of Amberlite IR-4B removed the mineral acid following which the uronic acids specifically were absorbed on to Dowex-1 (formate form) and recovered by elution with 0·5 N-formic acid. Protein and lipid contents were determined on cell-wall material prepared by method (ii) above. Protein was measured directly in untreated walls using the Folin–Ciocalteu reagent (Lowry, Rosebrough, Farr & Randall, 1951), or in detergent extracts of cell walls (van Soest, 1963). Readily extractable and bound cell-wall lipids were measured by the procedure of Bartnicki-Garcia & Nickerson (1962). Nucleic acid content was estimated by measuring the extinction at 260 nm. of hydrochloric acid digests of cell walls; absorption readings were corrected for background. Ultraviolet and visible spectra were obtained with a Beckman DB-G Spectrophotometer (Beckman Instruments Inc., Fullerton, California, U.S.A.). Samples for infrared spectroscopy were powdered with i.r. quality potassium bromide, pressed under vacuum into discs, and the spectra recorded in a Beckman IR 10 infrared spectrophotometer.

Melanin investigations. Melanin was extracted from wild-type mycelia by the procedures described by Nicolaus *et al.* (1964). The fine amorphous black pigment could be solubilized completely at 60° by 0·5 N-NaOH. Melanin was determined gravimetrically or by measuring the extinction at 540 nm. of NaOH solutions. The molecular-size distribution of melanin samples was investigated by gel filtration on a column (255 × 20 nm.) of Sephadex G-200 (Pharmacia, Uppsala, Sweden). Samples were applied to the column and eluted with 1 M-NaCl. The column was calibrated with the following proteins of known molecular weight: urease ($4{\cdot}8 \times 10^5$), β-glucuronidase ($2{\cdot}8 \times 10^5$), phospholipase ($1{\cdot}0 \times 10^5$) and ribonuclease ($1{\cdot}4 \times 10^4$). Nitrogen was determined by the methods noted above. The preparation of [^{14}C]melanin specimens, specifications of radiochemicals, counting procedures and equipment have been reported previously (Bull, 1968). Attempts to characterize the melanin in terms of its degradation products (Nicolaus *et al.* 1964) were made by (1) fusing in a mixture of sodium hydroxide and sodium dithionate at 300°, and (2) oxidizing with 3 % (w/v) aqueous potassium permanganate. Chemisynthetic DOPA-melanin was prepared by the method described by Bull (1970*b*).

Materials. Insoluble laminaran was obtained from the Seaweed Research Institute, Musselburgh, Midlothian, Scotland; nigeran extracted from *Aspergillus japonicus* QM 332 and *A. luchuensis* QM 873 were gifts from D. E. Eveleigh of the Prairie Regional Laboratory, Saskatoon, Saskatchewan, Canada; chitin was prepared as a colloidal suspension from crustacean chitin (Kylan PC, Moretex Chemical Products, Spartanburg, South Carolina, U.S.A.). Ion exchange resins were purchased from J. T. Baker Chemical Co. (Phillipsburg, New Jersey, U.S.A.) and enzymes from Worthington

Biochemical Corp. A wide range of non-substituted and substituted indoles, catechols, pyrroles and benzoic acids were purchased from Aldrich Chemical Co. Inc., Milwaukee, Wisconsin, U.S.A.; Eastman Organic Chemicals, Rochester, New York, U.S.A.; and K. and K. Laboratories Inc., Plainview, New York, U.S.A.

RESULTS

The growth characteristics of *Aspergillus nidulans* wild-type and albino strains in 10 l. batch cultures were similar to those reported by Carter & Bull (1969). Both strains produced homogeneous, filamentous growth; pellet formation did not occur and only during the late stages of autolysis were abnormal cell morphologies observed. At a growth temperature of 32°, the maximum mycelial dry weight was produced after about 35 h. ($Y_{glucose}$ = 0·40 to 0·44 g. dry wt g.$^{-1}$). Intracellular melanin synthesis became noticeable after about 40 h., by which time the medium was exhausted of glucose. A few hours later the culture began to autolyse and the pH value progressively rose, accompanied by an accumulation of melanin in the medium. Cultures for cell-wall studies were harvested during the maximum population phase (strain 13.1.OL) or, when melanized material was required, after 25 % autolysis had occurred (strain 13). The cell walls of both strains comprised 18 % of the total dry weight of washed mycelia under these conditions.

Chemistry of wild-type and mutant cell walls

The basic chemical features of the cell walls of the wild-type and albino mutants are shown in Table 1. Elemental analyses suggested identical chemical composition of the two cell walls prior to the establishment of secondary metabolism and the subsequent large-scale synthesis of melanin. Control analyses made on cell walls of *Aspergillus oryzae* (N = 3·5; P = 1·0 %) and *Neurospora crassa* (N = 2·9 %; P = 0·7 %) gave results of the same order as the *A. nidulans* data and agreed well with previously reported values (e.g. Mahadevan & Tatum, 1965). Because melanin interfered with the assaying of cell-wall components, analyses of certain components were made on the albino cell walls only.

Neutral sugars. Acid hydrolysates (22·5N-H_2SO_4 for 3 h. at 30°; dilution to 0·85N and 4 h. at 97°) of both cell walls contained three neutral monosaccharides, glucose, galactose and mannose. The amounts in the cell wall of *Aspergillus nidulans* 13.1.OL were determined enzymically and by quantitative paper chromatography of acid hydrolysates (Table 2). The glucose content was considerably underestimated. Paper chromatograms of wall digests contained several reducing sugar spots (equivalent to 18·7 % of the total carbohydrate) which were identified, tentatively, as β-linked (Bull, 1962) and α-linked (see below) glucose oligosaccharides. The two assays of galactose agreed very closely and, because all of this monosaccharide was a substrate for galactose oxidase, a D-configuration was concluded.

Amino sugars. Three amino sugars, having R_{Glc} values of 0·60, 0·72 and 1·25 in the butan-1-ol+pyridine+water solvent, were detected in the acid hydrolysates (6N-HCl for 6 h. at 100°) of the mutant cell walls. The two most rapidly moving sugars had R_{Glc} values identical with those of authentic glucosamine and *N*-acetylglucosamine. The unknown component (R_{Glc} 0·60) had a mobility close to that reported for galactosamine. The identity of the non-acetylated hexosamines was

confirmed by their oxidation to pentoses; only arabinose and lyxose were detected as products from presumed glucosamine and galactosamine respectively. Glucosamine, *N*-acetylglucosamine and galactosamine were present in the cell-wall hydrolysate in the ratio 5·2:1:4·7 (see Table 2).

Uronic Acids. The cell walls of the wild type and the mutant contained significant amounts of a hexuronic acid residue (Table 1) identified on the basis of paper chromatography as glucuronic acid. The most reliable estimates of glucuronic acid were

Table 1. *Chemical composition of the cell walls of Aspergillus nidulans*

Component	Percentage of the cell wall dry weight: Wild type, 13	Mutant, 13.1.OL
C-, H-, N- (C-H-N analyser)	27·6 (45·5)*; 4·8 (7·7)*; 4·1 (3·4)*	41·4; 6·7; 3·3
N, Kjeldahl	3·7	3·0
P (as H_2PO_3)	0·82	0·86
Total carbohydrate	60·5	82·8
Total hexosamine	n.d.	25·1
Uronic acid residues	1·9† 1·0‡	3·5† 2·6‡
Protein	5·0§ 10·0–10·4‖ 8·9–12·7¶	8·0§ 8·6–10·6‖ 8·9–9·4¶
Nucleic acid	0·61	0·60
Lipid		
Free	2·3	2·8
Bound	7·2	6·2
Melanin		
Total	16·3–18·2	0
'Encrusting'	1·0–3·2	0
Sum**	91·5–101·1	101·1–102·7

n.d. = not determined.

* Values refer to pre-melanized cell walls.

† Values derived from paper chromatographically separated material.

‡ Method of Gancedo *et al.* (1968).

§ Method of Lowry *et al.* (1951).

‖ Detergent-extracted cell walls.

¶ Values calculated from total nitrogen data and corrected for total hexosamine and melanin contents of the samples.

** Total carbohydrate, protein, lipid, nucleic acid and melanin.

Table 2. *The neutral and amino sugars present in the cell wall of Aspergillus nidulans 13.1.OL*

Sugar	Percentage of cell wall dry weight
Glucose	28·9; 26·2*
Galactose	3·8; 3·9*
Mannose	2·8
Glucosamine	12·0
N-Acetylglucosamine	2·3 } 14·3
Galactosamine	10·8

Sugars determined by quantitative paper chromatography.

* Sugars determined enzymically.

obtained by using the method of Gancedo *et al.* (1968), which gave values 47 % (wild-type) and 25 % (mutant) less than those obtained by applying the carbazole assay directly to H_2SO_4 hydrolysates. The hexuronic acid contents of *Aspergillus oryzae* and *Neurospora crassa* cell walls were found to be 2·7 % and 3·4 % respectively when estimated by the latter method.

Table 3. *Infrared spectroscopy of purified cell walls: distinctive band assignments*

Strain/wave number ($cm.^{-1}$)		
13	13.1.OL	Interpretation
3270 w sh	3275 m sh	Chitin (N-H stretching)
3110 w sh		Chitin
2920 s p	2930 m p	β-Glucan (CH_2 and CH_3 stretching)
1725 m p	1725 m p	Undissociated carboxyl
1655 s p	1660 s sh	Chitin (amide I band)
1640 w sh	1640 w p	Protein (amide)
1630 w sh	1630 w p	Chitin (C = N)
1550 s p	1540 m sh	Chitin/protein (amide II band)
915 w sh	917 s sh	α-Glucan
889 s sh	890 s sh	β-Glucan
825 s sh		α-Glucan
Major spectral changes following partial enzymolysis*		
889 shoulder lost	890 shoulder lost	
850 appeared	850 appeared	α-Glucan (-1,3)
	820 appeared	α-Glucan (-1,3)
805 appeared	810 appeared	Glucomannan

p, Peak; sh, shoulder; s, strong; m, medium; w, weak.
* Endo-β-D-1,3 glucanase+chitinase (Bull, 1970*a*: carboxymethyl cellulose fractionation, peak I).

Protein. The cell walls of the wild-type and mutant *Aspergillus nidulans* were similar in containing approximately 10 % of protein. Table 1 indicates efficiency of detergent extraction in the determination of cell-wall protein. Cell walls were extracted (×6) with anionic or cationic detergents and the amount of protein recovered agreed well with that calculated on the basis of total nitrogen.

Lipid. The contents of readily extractable and bound lipids in the two cell walls were not significantly different (Table 1).

Nucleic acid. Absorption spectra of cell-wall hydrolysates (6N-HCl for 8 h. at 100°) indicated the presence of nucleic acid bases. Using a molar extinction of 8800 for an equimolar mixture of adenine, cytosine, guanine and uracil (summed molecular weight, 509), the nucleic acid content (as RNA) of the cell walls was calculated to be less than 1 % of the dry weight (Table 1). Exhaustive washing of the cell-wall preparations with phosphate buffer or partial destruction with crude Streptomyces lytic enzymes failed to remove this nucleic acid component.

Melanin. The melanin content of wild-type *Aspergillus nidulans* was found to account for between 16·3 and 18·2 % of the cell wall on a dry weight basis Of this melanin, a small proportion could be released from the cell wall by ultrasonication in the presence of detergents (Table 1) (Bull, 1970*a*).

Infrared spectroscopy. The salient absorption maxima in the i.r. spectra of wild-type and mutant *Aspergillus nidulans* cell walls are summarized in Table 3. Reference spectra were recorded also of representative β-glucans (laminaran, cellulose, pustulan),

α-glucans (nigeran, dextrin) and other polymers of relevance to the chemistry of fungal cell walls (yeast mannan, galactan, native and chemisynthesized melanins). Bands were assigned after comparisons of the cell wall and reference spectra and reference to appropriate literature data (Bacon, Jones, Farmer & Webley, 1968; Barker, Bourne, Stacey & Whiffen, 1954; Pearson, Marchessault & Liang, 1960; Marchessault, 1962; Mitchell & Scurfield, 1967). Infrared analyses indicated the presence of α- (915 and 825 cm.$^{-1}$) and β- (890 cm.$^{-1}$) linked glucans, chitin (3270, 1665, 1630 and 1550 cm.$^{-1}$) and protein (1640 and 1550 cm.$^{-1}$) in the cell walls of both strains. The 1550 cm.$^{-1}$ absorbance can be assigned to amide groups which could have arisen from protein and chitin. Similarly, the undissociated carboxyl absorbance at 1725 cm.$^{-1}$ may have comprised both protein and melanin signals. The presence of melanin in the cell wall of the wild-type strain was not indicated by i.r. spectroscopy. Partial degradation of the cell walls by a mixture of endo-β-D-1,3 glucanase and chitinase caused the 890 cm.$^{-1}$ absorbance to be lost while absorbance maxima characteristic of α-glucans (840 and 820 cm^{-1}) became discernible (Table 3). A shoulder at 805 to 810 cm^{-1} also was indicated after partial hydrolysis, a feature suggestive of a glucomannan component (Marchessault, 1962).

Chemical fractionation of the cell wall

The cell wall was extracted chemically with alkali and mineral acid in order to obtain information on the nature of the polymeric components. Such attempts at fractionation can resolve the polymers only partially, but use of the Mahadevan & Tatum protocol (Mahadevan & Tatum, 1965) with the *Aspergillus nidulans* cell wall enabled at least two polymers to be isolated and identified. Cell walls of the albino mutant or premelanized wild type were used to avoid complications due to melanin which is extracted by the alkali treatments.

Fraction 1. Cell walls (500 mg.) prepared from *Aspergillus nidulans* 13.1.OL were extracted with 2M-NaOH (250 ml.) at room temperature for 16 h. The 24% of the total wall material recovered by this treatment was largely insoluble in cold water, and the only identifiable soluble components were glucose and reducing sugars with R_{Glc} values referable to laminaridextrins (Bull, 1962). Elemental analysis of fraction 1 gave C = 35·8%, H = 6·30%, N = 0·31% and P = 1·11%. The P content represented approximately one-third of the total wall content. Acid hydrolysis produced glucose (62·5%), galactose (4·7%) and galactosamine. The latter was not estimated quantitatively, but paper chromatographic evidence suggested that the majority of the galactosamine was present in this fraction (see fraction 2 below). Fraction 1 material was not very susceptible to β-1,3 glucanase attack and only 7% of the material was solubilized (based on glucose and laminaritetraose recovery).

Infrared spectra of fraction 1 showed a strong shoulder at 917 to 915 cm.$^{-1}$, weak shoulders at 890 cm.$^{-1}$ and 800 cm.$^{-1}$, and a medium peak at 830 to 825 cm^{-1}. These absorbances are indicative of an α-glucan containing −1,3 glucosidic linkages and a small amount of a β-linked glucan. The absorbance at 800 cm.$^{-1}$ could have been due to the α-1,3 glucan or could have arisen from a glucomannan. Treatment of fraction 1 with β-1,3 glucanase produced sharper definition of the 920 to 910 cm.$^{-1}$ shoulder and resolved the 830 to 825 cm.$^{-1}$ absorbance into two small peaks (850 and 835 cm.$^{-1}$), which pointed to the α-glucan being of the α-1,3 type described by Bacon *et al.* (1968) and not of the nigeran (Glcpα1-4Glc; Glcpα1-3Glc) type (see

Fig. 1). Figure 1 shows also that NaOH (3 %, w/v) extractions of the cell wall for 5 h. at high temperature (75°) produced still greater resolution of the α-1,3 glucan absorbance peaks and gave further evidence of mannose-containing polysaccharides (870 cm.$^{-1}$ absorbance). When fraction 1 was incubated with a mycodextranase-containing preparation (Bull, 1970*a*), reducing sugars other than glucose and β-oligoglucosides were released. A preparation having 2·1 units of mycodextranase produced 62·0 %

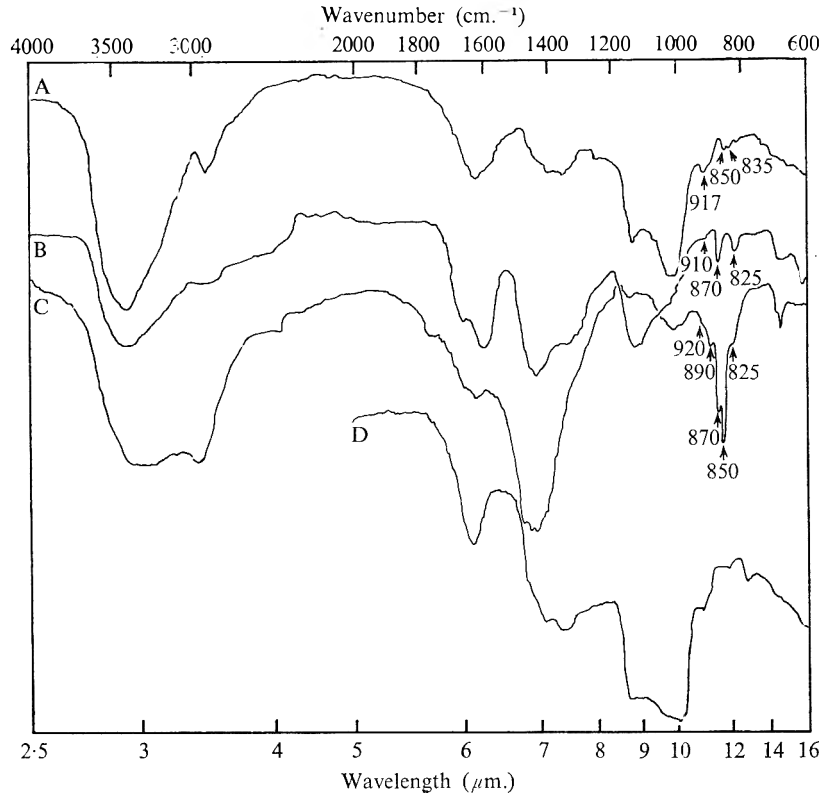

Fig. 1. Infrared spectra of *Aspergillus nidulans* cell walls: α-glucans. A, Cell-wall fraction 1 after treatment with β-D-1,3 glucanase. B, Cell-wall fraction 2. C, Hot alkali extract of the cell wall (see Results section for further experimental details). D, Nigeran (ex *Aspergillus japonicus* QM 332). Tracings displaced vertically to facilitate comparison.

hydrolysis of the nigeran samples and 13·5 % of fraction 1. Two α-linked oligoglucosides detected in nigeran digests were identified as nigerose (Glcpα1-3Glc) and a tetrasaccharide (Glcpα1-3Glcpα1-4Glcpα-3Glc) (chromatographic criteria of Tung & Nordin, 1967). Unequivocal proof of the presence of these sugars in fraction 1 digests was not obtained. Following β-1,3 glucanase digestion of fraction 1, the absorbance characteristic of the β-glucan was lost and consequently the latter contained some -1,3 linked residues; simultaneously, the 800 cm.$^{-1}$ absorbance shifted to 810 to 800 cm.$^{-1}$ which was preliminary evidence of a glucomannan entity in the fraction. The effect of Schweizer's reagent on the i.r. spectrum of fraction 1 was slight; most significant was the retention of the absorbances at 917 and 890 cm.$^{-1}$. Spectroscopic analyses of the corresponding cell-wall fraction from *Aspergillus oryzae*

and *Neurospora crassa* gave evidence of α- and β-glucans and the *N. crassa* spectrum also had a weak absorbance at 770 cm.$^{-1}$, which was suggestive of a galactan.

Fraction 2. The residue from cold alkali treatment was extracted with hot H_2SO_4 (1 N, 96°) for 16 h., then neutralized; the freeze-dried supernatant accounted for 50 % of the total cell wall. The C, H, N and P contents were 23·1 %, 4·02 %, 1·93 % and 0·88 % respectively. The P amounted to 53 % of the total wall content, and when combined with that of fraction 1 gave a recovery of about 84 %. Fraction 2 was completely soluble in cold water, and paper chromatography indicated the presence of glucose, laminaridextrins, galactose, mannose, *N*-acetylglucosamine and glucosamine. The amino sugars were probably derived from chitin (see fraction 4) and, during the partial hydrolysis of the latter, deacetylation occurred. Acid hydrolysis produced glucose (34·0 %), galactose (3·4 %), galactosamine (trace) and mannose (4·1 %) and an uncharacterized mixture of amino acids. The collective data from fractions 1 and 2 were equivalent to galactose and mannose recoveries of about 70 % and 73 % respectively. Assuming the majority of fraction 2 nitrogen to be protein, the protein content was calculated as 6·1 %, a recovery of about 68 % of the total cell-wall protein. β-1,3 Glucanase digestion of fraction 2 caused little depolymerization (about 3 %).

The i.r. spectrum of fraction 2 indicated the presence of an α-1,3 glucan type polysaccharide (910 cm.$^{-1}$, 825 cm.$^{-1}$), a trace of a β-glucan (very weak absorbance at 890 cm^{-1}) and a mannan (870 cm.$^{-1}$) (Fig. 1).

Fraction 3. Fraction 3 comprised material from the second cold 2 N-NaOH extraction and accounted for 13·8 % of the total cell wall. This fraction lacked P, and C, H and N values were respectively 40·60 %, 6·86 %, 0·03 %. Solubility in cold water was slight and the only information derived from paper chromatography of the solubilized products was a reducing sugar reaction at the origin. The trace amount of nitrogen was not further identified. Glucose was released stoichiometrically from fraction 3 by mild acid hydrolysis (3 N-HCl). Digestion by an exo-β-1,3 glucanase released glucose, laminaribiose, laminaritriose, laminaritetraose and two reducing sugars characteristic of gentiobiose, and a trisaccharide containing a β-1,6 linkage (Chesters & Bull, 1964, for method of analysis and identity) (see Fig. 2). The maximum hydrolysis of fraction 3 produced by this enzyme was just over 68 % (reducing sugar: glucose, 1·03) and neither dialysis of the reaction mixture or replenishment of the enzyme induced further degradation. The elemental analysis of this fraction was in accord with its identity as a glucose polymer (theoretical values for a glucan are C, 44·4 %; H, 6·2 %). The collective data from fractions 1, 2 and 3 gave a glucose recovery of 94 %. The only notable feature of the i.r. spectrum was the strong shoulder at 890 to 880 cm^{-1} characteristic of the β-glucan (Fig. 3); this absorbance was not observed after β-1,3 glucanase digestion.

Fraction 4. This final fraction comprised a residue insoluble following the second alkali extraction and was equivalent to 12·2 % of the original wall material. The elemental composition (C, 43·5 %; H, 7·10 %; N, 5·67 %) was very similar to the theoretical analysis of chitin (C, 43·5 %; H, 5·4 %; N, 6·4 %), and when treated with a high purity chitinase from a Streptomyces species (Bull, 1970*a*) chitobiose and *N*-acetylglucosamine were released. Exhaustive incubation with this enzyme produced a maximum 80 % hydrolysis; digestion of the comparable *Neurospora crassa* cell-wall fraction caused 73 % solubilization. The *N*-acetylglucosamine plus glucosamine

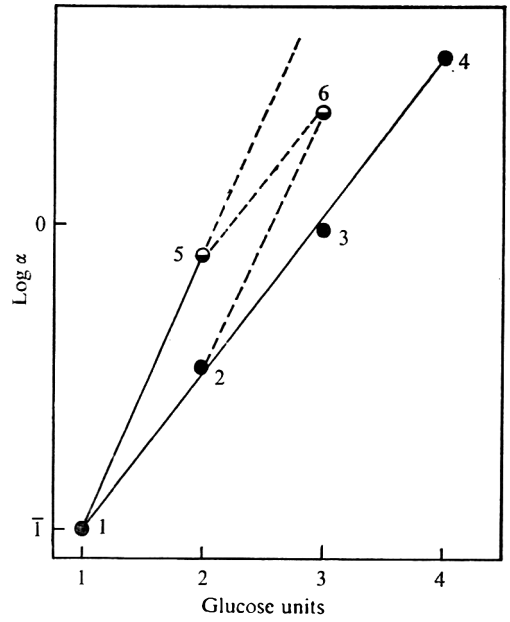

Fig. 2. Products of exo-β-D-1,3 glucanase digestion of cell-wall fraction 3. 1, Glucose; 2, laminaribiose; 3, laminaritriose; 4, laminaritetraose; 5, gentiobiose; 6, triglucoside (Glcpβ1-3Glcpβ1-6Glc). $\alpha = (1 - R_{Glc})R_{Glc}$, see Chesters & Bull (1964). Solvent: butan-1-ol + pyridine + water (6+4+3, by vol.)

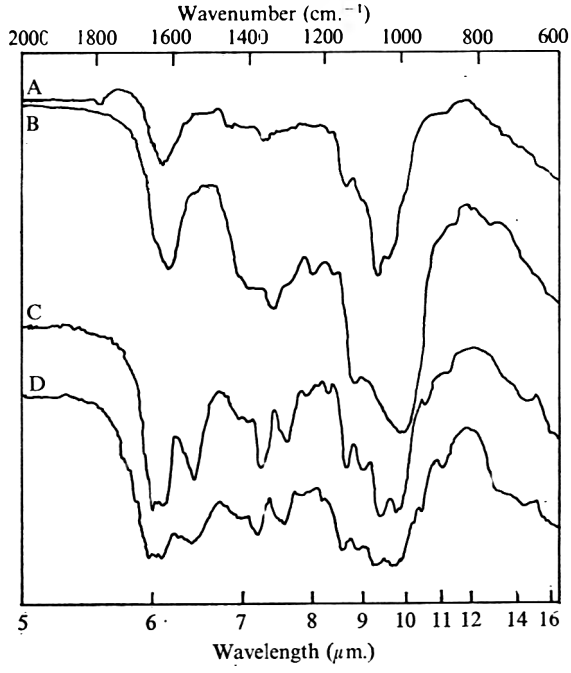

Fig. 3. Infrared spectra of *Aspergillus nidulans* cell walls: β-glycans. A, Laminaran. B, Cell-wall fraction 3. C, Cell-wall fraction 4. D, Purified crustacean chitin. Tracings displaced vertically to facilitate comparison.

content of the cell wall (see Table 2) agreed well with the amount of material recovered as fraction 4. The identity of fraction 4 was confirmed by i.r. spectroscopy (Fig. 2).

Extraction of the wild-type 13 cell wall by the Mahadevan & Tatum procedure revealed that a significant amount of melanin remained associated with the chitin fraction. Conversely, when melanin was being extracted and purified, chitin was the most intractable polysaccharide in the cell wall and was present until the final stages of melanin preparation.

The melanin pigment

The preparation of melanin involved the complete removal of all extraneous cell wall materials by solvent and acid extractions. Nicolaus *et al.* (1964) found that such treatment did not produce major chemical changes in melanins although

Table 4. *Elemental analyses of Aspergillus nidulans melanin: effect of growth conditions on composition*

Strain	Conditions of melanin synthesis	Percentage analysis C	H	N
13	Czapek-Dox medium* (batch culture)	56·40	6·55	3·92
13	Czapek-Dox plus DOPA (0·5 mg. ml.$^{-1}$)†	.	.	5·82
13	Czapek-Dox plus Catechol (0·5 mg. ml.$^{-1}$)†	.	.	1·78
224	DAN‡ (batch culture)	44·80	5·87	4·34
224	DAN (chemostat culture, D = 0·024 h.$^{-1}$)‡	59·52	6·74	3·25
.	Chemosynthetic DOPA-melanin	48·50	4·66	6·25
.	DOPA-melanin, theoretical ($C_9H_8O_4N$)	56·00	3·63	7·26

* Used in earlier investigations by Bull & Faulkner (1965).

† DOPA or catechol added to shake-flask cultures as sterile solutions during the declining growth rate phase.

‡ Defined *Aspergillus nidulans* medium; for medium composition and conditions of continuous culture see Carter & Bull (1969).

partial decarboxylation and deamination probably occurred. The final product accounted for about 16 to 18 % of the *Aspergillus nidulans* wild-type cell wall. The pigment was insoluble in all non-polar solvents tested and in mineral acids; hot NaOH (0·5N, 60°, 24 h.) produced complete solubilization. Precipitation resulted when alkaline solutions were acidified to pH 2 to 3. The *A. nidulans* pigment had properties in common with other melanins: bleached by H_2O_2 (30 %, v/v); colour intensity reduced by glutathione and dithionate and restored on reoxidation; reduced ammoniacal $AgNO_3$; produced characteristic blue-green ferrous sulphate–ferricyanide reaction; formed a flocculent iron-brown precipitate on addition of $FeCl_3$ to an alkaline solution. That the pigment varied in composition with changing growth conditions is indicated by the data in Table 4. The most significant finding was the widely varying N content of the melanin in response to the growth medium, especially when the latter was supplemented with *o*-dihydroxy phenols. Both L-[^{14}C]tyrosine and DL-[carboxy-^{14}C]3(3,4-dihydroxyphenyl)-alanine were incorporated into the cell-wall melanin and the extent of incorporation was dependent on the age of the culture at the time of feeding. Thus, when [^{14}C]tyrosine and [^{14}C]DOPA were supplied to the culture at zero time, 19 % and 28 % of the label was incorporated into cell-wall melanin respectively; the incorporation was 43 % and 69 % when the precursors were fed to the culture after 30 h., that is at the onset of declining growth rate.

Attempts to characterize the *Aspergillus nidulans* melanin on the basis of its degradation products were only partially successful. The pigment (100 mg.) was oxidized by 37·8 ml. $KMnO_4$ (3 %, w/v) but ether extraction of the solution did not reveal any identifiable products. In contrast, alkali fusion of the melanin (100 mg.) with NaOH (300 mg.) and sodium dithionite (50 mg.) at 300° for 10 min. produced

Table 5. *Degradation products from the alkali fusion of Aspergillus nidulans melanin*

Tentative identity	Chromatography R_F (BAW)*	Chromatography R_F (PAW)†	Co-electrophoresis (PyA)‡
5,6-Dihydroxyindole-2-COOH	0·57	.	.
Indole-2-COOH§	0·93	.	+
5-Hydroxyindole§	0·90	.	.
Pyrrole-2-COOH	0·87	0·76	+
Pyrrole-3-COOH§	0·84	0·49	.
Pyrrole-2,4-COOH, or pyrrole-2,5-COOH	.	0·27	.
3,4-Dihydroxybenzoic acid	.	.	+

\+ Present.
* Butan-1-ol+acetic acid+water (60+15+25, v/v).
† Propan-1-ol+ammonia+water (60+30+10, v/v).
‡ Pyridine+acetate, pH 6·1.
§ Spots of strongest intensity indicated.

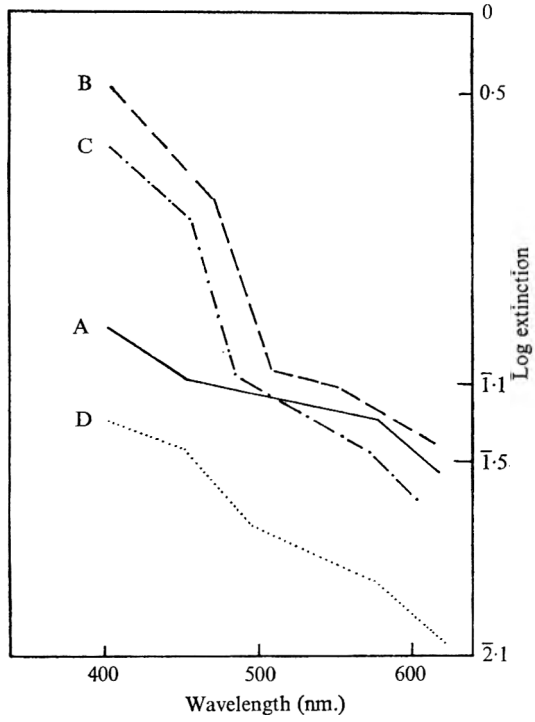

Fig. 4. Extinction of wild-type and mutant *Aspergillus nidulans* melanins. A, Wild-type 13 melanin. B, Purple mutant 13y.ma alkali-extracted pigment. C, Purple mutant 13y.ma×pink mutant 13y.pi alkali-extracted hybrid pigment. D, Chemisynthesized DOPA-melanin. Mutants 13y.ma and 13y.pi are described by Bull & Faulkner (1965)

23·8 % degradation; degradation of the chemisynthesized DOPA-melanin by similar treatment was 28·6 %. Of the 18 ether-extractable degradation products from the fungal melanin, half were identified tentatively (see Table 5) on the basis of co-chromatography and co-electrophoresis, colour reactions with ethanolic 3 % $FeCl_3$, diazotized sulphanilic acid and Ehrlich's reagent, and response to u.v. light. The chromatographic mobilities of authentic samples and published values (Nicolaus *et al.* 1964) often differed by as much as one or more R_F units.

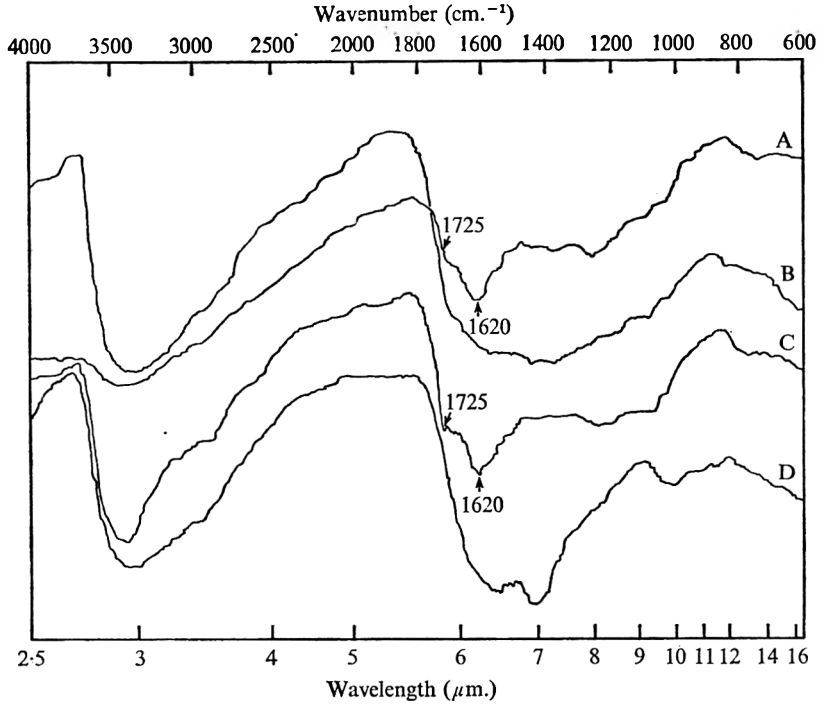

Fig. 5. Infrared spectra of native and synthetic melanins. A, Wild-type *Aspergillus nidulans* 13 melanin. B, As A but extracted with 0·5 N-NaOH. C, Chemisynthesized DOPA-melanin. D, As C but extracted with 0·5 N-NaOH. Tracings displaced vertically to facilitate comparison.

Logarithmic plots of absorbance in the visible range for the wild-type melanin and melanoid pigments from previously described mutants (Bull & Faulkner, 1964; 1965) produced a series of straight lines as shown in Fig. 4. The i.r. spectrum of the wild-type melanin was very similar to that of DOPA-melanin. Neither spectrum had features of significant diagnostic value but the absorbances at 1620 and 1725 $cm.^{-1}$ gave some indication of carboxyl groups. The latter absorbances disappeared after brief treatment of the pigments with dilute NaOH (Fig. 5).

Finally, the homogeneity of various samples of the *Aspergillus nidulans* melanin was investigated by gel filtration on a Sephadex G-200 column previously calibrated for molecular size determination. All samples were separable into a series of fractions having different molecular sizes equal to or greater than 2×10^6 (Fig. 6). Recovery of the melanin from this column was high in all experiments when based upon the extinction at 540 nm. The elution pattern of extracted melanins was composed of three commonly positioned peaks (P1, P2*b*, P4) and other minor peaks (P2, P3*a*, P3*b*)

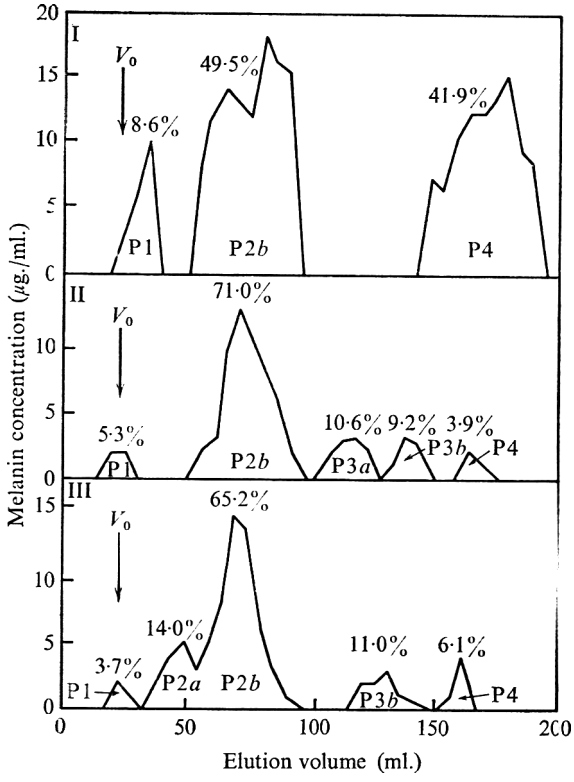

Fig. 6. Fractionation of *Aspergillus nidulans* melanins on Sephadex G-200 gel. I, Cell-wall melanin produced in unsupplemented cultures. II, Cell-wall melanin produced in L-[^{14}C]-tyrosine cultures. III, Cell-wall melanin produced in DL-[carboxy-^{14}C]DOPA cultures. Column size: 255 × 20 mm.; V_0 (void volume) measured with Dextran Blue (M.W. = 2·0 × 10^6). Elution with 1 M-NaCl at 10° in the dark. Fractions (10 ml.) collected automatically and melanin assayed spectroscopically. Recovery of melanin applied to the column: I (82·1 %); II (88·0 %); III (100·0 %).

Table 6. *Molecular size and specific activity of melanin fractions*

	Melanin sample†				
	I: Unsupplemented	II: [^{14}C]tyrosine		III: [^{14}C]DOPA	
Peak*	(mol. size‡)	Mol. size	Sp.act.§	Mol. size	Sp.act.
P1	⩾ 2·0 × 10^6	⩾ 2·0 × 10^6	161	⩾ 2·0 × 10^6	240
P2*a*	.	.	.	6·6 × 10^5	53
P2*b*	3·3 × 10^5	3·2 × 10^5	494	3·8 × 10^5	1278
P3*a*	.	4·7 × 10^4	80	.	.
P3*b*	.	1·0 × 10^4	39	1·3 × 10^4	104
P4	2·7 × 10^3	3·1 × 10^3	191	2·7 × 10^3	56

* See Fig. 5.

† Melanins extracted from wild-type *Aspergillus nidulans* 13. Growth in: I, defined medium (DAN); II and III, DAN supplemented with L-[^{14}C]tyrosine and DL-[^{14}C]DOPA during the declining growth rate phase.

‡ Molecular size estimated by gel filtration on Sephadex G-200.

§ mμCi mg.$^{-1}$

characterized the tyrosine- and DOPA-labelled melanins. Also, the data in Table 6 indicate that the specific activity of the ^{14}C-labelled melanin fractions varied markedly and was not related in a simple way to molecular size.

DISCUSSION

The hyphal walls of *Aspergillus nidulans* comprised largely polysaccharide with minor amounts of lipid and protein and, in the case of the pigmented wild-type strain, melanin. The high glucan plus chitin content of the *A. nidulans* cell wall (60 to 64 %) is typical of Ascomycetes, as is also the presence of galactose and galactosamine. Mannose also is a usual component of Asomycete cell walls (see Bartnicki-Garcia, 1968, for a critical review). Until recently, the evidence for uronic acids in fungal cell walls has been disputed, but the mild hydrolysis and separation procedures recommended by Gancedo *et al.* (1968) have enabled this question to be resolved. Application of these methods to *A. nidulans* revealed the presence of approximately 1 to 3 % of glucuronic acid in its cell wall, a value of the same order as reported for Alternaria, Fusarium and Penicillium (Gancedo *et al.* 1968). It must be stressed that the percentage composition data presented in this paper are not absolute and can be expected to vary significantly with the conditions of culture of the fungus. Moreover, the data on the recovery of various components are subject to the limitations of the extraction techniques used. For example, β-1,3 glucans are susceptible to degradation by β-alkoxyl elimination and hence the value of 13·8 % obtained for the β-glucan (fraction 3) was probably a serious underestimate; this interpretation seems warranted by the detection of laminaridextrins in cell-wall fractions 1 and 2. The low recovery (about 68 %) of protein from fractionated cell walls was because it was partially extracted with lauryl sulphate during the initial stage of the Mahadaven & Tatum (1965) fractionation procedure. Indeed, the present investigation has demonstrated the efficacy of detergent extraction for measuring cell-wall protein, especially when the conventional, direct methods such as biuret are subject to interference by materials such as melanin.

Chemical fractionation studies of *Aspergillus nidulans* cell walls allowed a number of conclusions to be made on their polymer composition. There appeared to be at least two alkali-soluble glucans, one of which, the β-glucan, was released only after the acid extraction, whilst the α-glucan was solubilized by the initial alkali treatment. The infrared spectral analyses of the α-glucan indicated its close relation to the α-1,3 glucan of Cryptococcus cell walls reported by Bacon *et al.* (1968) and distinguished it clearly from the mixed linkage α-glucan nigeran. Furthermore, the release of reducing sugars from fraction 1 and attributable to mycodextranase action amounted to only a very small portion of the total glucose present. The *A. nidulans* α-glucan resembled other strongly dextrorotatory fungal glucans in not giving a colour reaction with iodine (Gorin & Spencer, 1968); moreover, it was not attacked by amylases. The β-glucan, which was partially destroyed during acid and alkali extraction, was of a non-cellulosic type as demonstrated by its susceptibility to exo- and endo-β-1,3 glucanase hydrolysis and the lack of complex formation with Schweizer's reagent. On the basis of its insolubility in cuprammonium hydroxide and paper chromatography of enzyme hydrolysates, it was concluded that the β-glucan contained a preponderance of β-1,3 glucosidic linkages and a smaller

proportion of β-1,6 linkages. The 68 % limit to hydrolysis by exo-β-1,3 glucanase suggested that the β-glucan might have a 'block-type' structure in which a number of say -1,6 linkages are surrounded by -1,3 linkages.

Aspergillus nidulans cell walls contained two hexosamine polymers: chitin and one composed of, or containing, galactosamine. The galactosamine polymer was largely extracted as fraction 1 by 2N-NaOH whereas the chitin remained resistant to alkali and acid treatments; the two hexosamines are therefore components of different polymers. The *N*-acetylglucosamine to galactosamine ratio in the wall of the albino mutant was 1·32, a value similar to that found in the walls of *Helminthosporium sativum* (Applegarth & Bozoian, 1969). Much smaller galactosamine contents have been reported for other fungal cell walls, including a different strain of *A. nidulans* (Mahadevan & Tatum, 1965; Cohen, Katz & Rosenberger, 1969).

The polymers containing galactose, mannose, amino and glucuronic acids were not characterized. Most of the cell-wall mannose was found in fraction 2, and i.r. spectroscopy suggested the presence of a glucommanan. Galactose was extracted in almost equal proportions by cold alkali and acid (fractions 1 and 2). Protein was associated with fraction 2. Emiliani & Ucha de Davie (1962) claimed that such components were found as glucomannoprotein and galactomannoprotein complexes in *Aspergillus phoenicis*, while an alkali insoluble glucomannogalactan has been proposed as an entity in an unidentified *Aspergillus* species (Ruiz-Herrera, 1967).

The presence of melanin in the wild-type cell wall is clearly responsible for its resistance to lytic enzymes (Bull, 1970*a*). However, the respective limit hydrolyses of the β-glucan (68 %) and chitin (80 %), which represented 6 to 7 % of the cell wall not degraded, suggest that factors other than melanin may be involved in the prevention of total cell wall dissolution. Previously I have found (Bull, 1970*a*) that a resistant 'core' of chitin and α-glucan persisted after enzymic lysis of *Aspergillus nidulans* cell walls.

Histochemical data and evidence from enzymolysis experiments indicated that the melanin was located throughout the thickness of the wall and, probably, formed an especially intractable complex with chitin (Bull, 1970*a*). In gel filtration experiments of *Aspergillus nidulans* melanin preparations, all melanin samples analysed possessed three common peaks, corresponding to molecular weights of approximately 2,000,000, 350,000 and 2900. These data suggest that the melanin is a finite heteropolymer whose component molecules differ in molecular size, in contrast to an infinite homopolymer whose size is limited merely by the amount of available precursors. This size distribution of melanin may reflect a location of pigment molecules of specific molecular dimensions in different parts of the cell wall. Brown, Falkehag & Cowling (1967) favoured a similar explanation for the macromolecular structure of lignin in sweetgum wood.

On the basis of the degradation studies, the *Aspergillus nidulans* melanin appears to be, at least in part, indolic in nature. A number of degradation products have been tentatively identified but further interpretation of these results must be cautioned on the grounds of artefact production during melanin extraction: (1) strong acid treatment of the cell wall may have caused acid-catalysed rearrangements in the quinonoid structure; (2) the degradation products (note their low yields) may have been differentially susceptible to further decomposition by alkali fusion. Hence the relative intensities of products noted in Table 5 are not necessarily significant in any

definition of the melanin structure. Visible spectra of the pink and purple mutants' pigments invariably had broad extinction maxima at 520 to 550 nm. and, less frequently, a sharper extinction at 480 nm. These extinctions closely correspond to those of melanochromes and dopachrome (Bu'Lock, 1960) and add further support to the characterization of the *A. nidulans* melanin as indolic.

Few detailed analyses of fungal melanins are available with which to compare those from *Aspergillus nidulans*. Potgieter (1965) quotes elemental analyses of melanins extracted from *Rhizoctonia solani* (C, 62·0; H, 4·8; N, 3·3 %) and *Cladosporium herbarum* (C, 60·7; H, 4·0; N, 2·2 %) which are very similar to those of *A. nidulans*. Further information on the Rhizoctonia and Cladosporium pigments is not available, consequently reference of the nitrogen content to indolic residues cannot be made. Nicolaus *et al.* (1964), however, concluded that the melanins of *Ustilago maydis* spores and *Capnodium nerii* hyphae are non-indolic, the melanin from the former species being catecholic in composition.

During the present investigations, carboxyl groups were indicated in the *Aspergillus nidulans* melanin by i.r. spectroscopy and by the incorporation of [carboxy-^{14}C]DOPA into the pigment. Carboxyl groups in the fungal pigment were titrated against 0·2 N-Na_2CO_3 by the method of Piattelli & Nicolaus (1961) (A. T. Bull, unpublished experiments). A titration difference (0·2 N-HCl) of 133·3 ml. g.$^{-1}$ was observed for melanin synthesized in unsupplemented media (Table 4, first entry), while a chemisynthetic DOPA-melanin had a corresponding value of 99·2 ml. g.$^{-1}$. The origin of these carboxyl groups is open to doubt; they may reflect incorporation of DOPA itself into the melanin molecule, or may be derived from oxidative decomposition of quinones during the synthesis of melanin. The question of whether melanin is a *chemically* homogeneous polymer (single type of subunit and linkage group) or heterogeneous polymer (multiple types of subunit and bondings) also has been debated (see Mason, 1966). The data reported in this paper on the elemental composition of the pigment synthesized under different growth conditions and on the specific activities of [^{14}C]melanin fractions offer some support for the heterogeneous view of melanins.

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Dominance of the Wild-type Alleles of Methionine Regulatory Genes in *Salmonella typhimurium*

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SUMMARY

F′ elements carrying wild-type alleles of the methionine regulatory genes *metJ* and *K* have been transferred from *Escherichia coli* to *metJ* and *K* mutants of *Salmonella typhimurium*, and the resulting heterogenotes tested for resistance to methionine analogues and for repressibility of cystathionase (one of the methionine biosynthetic enzymes). The wild-type alleles of both genes were dominant in both tests. Increased *metK* gene dosage was found to have no effect on the rate of uptake of [^{14}C]methionine by cell suspensions. Possible roles for the *metJ* and *K* gene products in the regulation of methionine synthesis are considered.

INTRODUCTION

The six structural genes controlling methionine biosynthesis in *Salmonella typhimurium* are not contiguous (Fig. 1; Smith, 1961; Sanderson & Demerec, 1965; Childs & Smith, 1969), and the repression of their expression by exogenous methionine appears not to be co-ordinate (Lawrence, Smith & Rowbury, 1968). Two kinds of mutants in which expression of these genes was constitutive were found among mutants resistant to methionine analogues (Lawrence *et al.* 1968). *metJ* mutants were resistant to DL-ethionine, but not to α-methyl-DL-methionine or DL-norleucine, while *metK* mutants were resistant to all three analogues. Some *metK* mutants were also isolated in which regulation was normal, and, unlike constitutive *metK* mutants, these did not show a reduced growth rate in minimal medium compared with the wild-type. *metJ* mutations were about 95% cotransduced with *metB*, and *metK* mutations about 1% with *serA* (Fig. 1).

In the present work F′ episomes carrying, respectively, the *metB* and *serA* regions of the *Escherichia coli* genome (K. Brooks Low, personal communication) have been transferred into *Salmonella typhimurium* strains, and the dominance of various *metJ* and *K* alleles tested in the resulting hybrid heterogenotes. The possibility that *metK* may specify a component required for methionine uptake has also been tested, by examining the effect of increasing the *metK* gene dosage on the rate of uptake of radioactive methionine.

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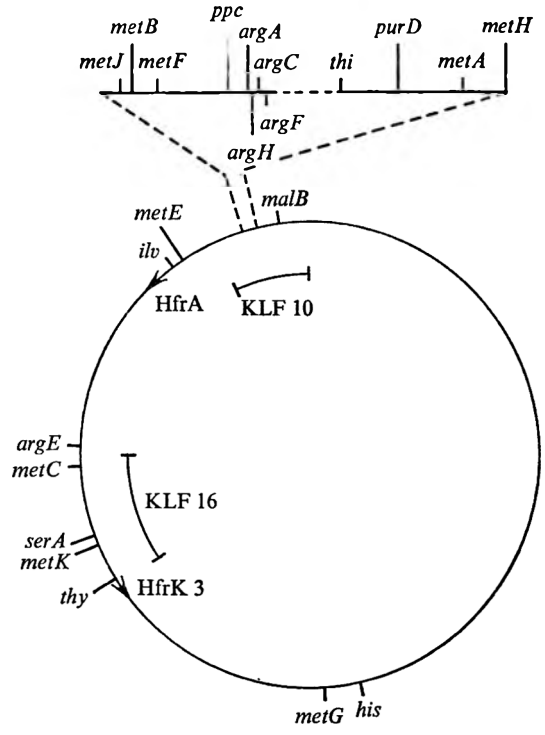

Fig. 1. Linkage map of *Salmonella typhimurium*. The map and nomenclature are based on those of Sanderson (1967). Bracketed markers are cotransduced. Arrows represent the sites of insertion of sex factors in high frequency donors. KLF 10 and KLF 16 represent F′ episomes derived from *Escherichia coli* by K. Brooks Low, and carrying regions of the *E. coli* genome homologous with the regions of the *S. typhimurium* genome covered by the arcs.

METHODS

Media. Nutrient agar (NA) and nutrient broth (NB) were supplied by Oxoid Ltd. The minimal medium (MM) was as described by Smith (1961). Minimal agar (MA) was obtained by adding 1·5% (w/v) Oxoid agar no. 1 to MM. MM was supplemented as required with the following: L-arginine, 20 μg./ml.; DL-ethionine, 1000 μg./ml.; α-methyl-DL-methionine, 1000 μg./ml.; L-methionine, 20 μg./ml.; L-serine, 200 μg./ml.; uracil, 20 μg./ml.

Organisms. The nomenclature is that used by Sanderson (1967). Two *Escherichia coli* F′ donor strains were kindly provided by K. Brooks Low: a JC1553 (*argEhisleu metBlacmalxylstr-rrecA1*) donor of KLF 10, an episome carrying the *malB* and *metB* genes (Fig. 1), and a JC1553 *thy* donor of KLF 16, an episome carrying the *serA* and *thy* genes. All other strains were derived from *Salmonella typhimurium* strain LT2. A list of the regulatory mutants is given in Table 1, and Table 2 shows the derivation of multiple mutants.

Techniques. Cultures were grown at 37° unless otherwise stated. The maintenance of stock cultures, and the propagation, assay and maintenance of transducing phage P 22 or its non-lysogenizing variant L 4 (Smith & Levine, 1967; supplied by H. O. Smith) were as described by Smith (1961). Growth experiments in MM were essentially

Table 1. *Methionine regulatory mutants of Salmonella typhimurium*

All strains were derived from *Salmonella typhimurium* LT2. Tests of resistance to the methionine analogues α-methyl-DL-methionine (AMM), DL-ethionine (ETH) and DL-norleucine (NOL), and detection of methionine excretion by cross-feeding of a methionine auxotroph, were as described by Lawrence *et al.* (1968). The nomenclature is that of Sanderson (1967).

Structural gene mutations	Methionine regulatory gene mutations	Resistance (R) or sensitivity (S) to AMM	ETH	NOL	Excretion of methionine
argFI11	–	S	S	S	–
argFI11	*metJ713*	S	R	S	+
argFI11	*metJ743*	S	R	S	+
argFI11	*metK747*	R	R	R	+
serA11	–	S	S	S	–
serA11	*metK747*	R	R	R	+
serA11	*metK751*	R	R	R	–
serA11	*metK752*	R	R	R	+
serA13	*metJ744*	S	R	S	+

Table 2. *The derivation of Salmonella typhimurium multiple mutants*

Spontaneous (spont) methionine regulatory mutants were obtained as colonies growing on MA containing ethionine (Lawrence *et al.* 1968). *N*-methyl *N'*-nitro *N*-nitrosoguanidine treatment (NG), conjugation (conj) and transduction (trans) were as described by Ayling & Chater (1968). Temporary strain numbers are for use only in the context of this table, recipient numbers preceding those of donors in descriptions of crosses. Bracketed strains were used only in deriving strains and are not referred to in the text. A linkage map including the markers used is given in Fig. 1.

Temporary strain number	Genotype	Mode of origin, or source
1	*argFI11*	J. D. Childs
2	(*argFI11ilv*)	From 1 by NG
3	(*argFI11metB406*)	2 × 18 (conj)
4	*argFI11metJ713*	3 × 10 (trans)
5	*argFI11metJ743*	From 1 by spont. mutation
6	*argFI11metK747*	2 × 21 (conj)
7	(*argFI11rec-301*)*	From 1 by NG
8	*argFI11rec-301pyr**	From 7 by NG
9	(*argFI11serA13*)	2 × 20 (conj)
10	(*metJ713*)	D. A. Lawrence
11	*serA11*	D. A. Lawrence
12	*serA11metK747*	From 11 by spont. mutation
13	(*serA13*)	K. E. Sanderson
14	*serA13metJ744*	9 × 18 (conj)
15	(*serA13rec-302*)*	From 13 by NG
16	*serA13rec-302met-754**	From 15 by NG
17	(*metK747*)	12 × wild-type LT2 (trans)
18	(*HfrAhisD23metB406*)	J. D. Childs
19	(*HfrK3serA13*)	K. E. Sanderson
20	(*HfrK3serA13his*)	From 19 by NG
21	(*HfrK3metK747his*)	20 × 17 (trans)

* Recombinant formation in conjugation and transduction was greatly reduced with *rec-301* or *-302* recipients, though F' merodiploid formation was normal. The *rec* mutations also resulted in hypersensitivity to ultraviolet irradiation and, in the case of *rec-301*, to NG (Chater, 1969).

as described by Lawrence *et al.* (1968). Episome transfer was carried out by the spot method of Fink & Roth (1968). F′ heterogenotes were purified by subculturing the crude isolates in selective MM to stationary phase, and spreading a 10^{-6} dilution on selective MA. After incubation of such plates for 48 h., discrete colonies were obtained. Haploid segregants from heterogenote cultures were detected, after subculture in NB to stationary phase (sometimes followed by subculture in NB + acridine orange (20 μg./ml.; Hirota, 1960)), by spreading a suitable dilution on NA, incubating for 24 h., and replicating to appropriately supplemented MA, so that auxotrophic segregants and prototrophic heterogenotes could be distinguished.

Assay of cystathionase activity. Late logarithmic phase MM cultures grown at 25° in randomized positions in a shaking water-bath were harvested by centrifugation, washed in culture volumes of 10 mM potassium phosphate buffer (pH 7·4), and finally resuspended in the same buffer. The optical density of each suspension was measured at 650 nm., and usually adjusted to 1·5 units (1 unit = 0·375 mg. dry wt/ml.), though less dense suspensions were sometimes used. Toluene was added (to 1% of the total volume), and the suspension emulsified using a Whirlimixer, prior to incubation at 30° for 20 min. Such suspensions were then used immediately, or after storage at 0° for up to 2 h. The method for assaying cystathionase activity was adapted from that of Rowbury & Woods (1964) with cell-free extracts. Incubation was at 30°. The reaction mixture contained, in a final volume of 1 ml. (μmoles): potassium phosphate buffer, pH 7·4, 100; pyridoxal phosphate, 0·0025; DL-allocystathionine, 10; and toluene-treated cells, 0·04 to 0·3 mg. dry wt. Cystathionine was added to the pre-incubated mixture to start the reaction, which was stopped after measured time intervals of up to 40 min. by the addition of 0·33 ml. of a 1% solution of 2,4-dinitrophenylhydrazine in 2 M-HCl. After a further 15 min., 2 ml. of 2 M-KOH was added. The extinction at 445 nm. was determined after 10 min. had elapsed, against a blank from which both cystathionine and toluene-treated cells had been omitted. Corrections to this reading were made by subtraction of readings obtained with controls lacking, respectively, cystathionine and toluene-treated cells. Under the conditions of the assay 1 μmole of pyruvate 2,4-dinitrophenylhydrazone gave an extinction rate at 445 nm. of 5·0. The formation of pyruvate from cystathionine by toluene-treated cells proceeded at a constant rate for at least 40 min. This rate was shown to be proportional to the quantity of cells added to the reaction mixture.

Assay of methionine permease activity. Cultures were grown overnight (with shaking) in 10 ml. MM + 0·4% glucose, then added to 40 ml. fresh medium and incubated for a further 75 min. Chloramphenicol (200 μg./ml.) was added, and incubation continued for 30 min. The cells were then harvested by centrifugation, washed in 50 ml. ice-cold MM + glucose + chloramphenicol, and finally resuspended in 5 ml. of the same medium. The extinction at 650 nm. of each suspension was determined, and the volume required to give a final concentration of 50 μg. dry wt/ml. was added to a 25 ml. beaker containing a suitable quantity of MM + glucose + chloramphenicol, in a shaking water-bath at 25°. After 1 min. 50 sec., shaking was stopped and aeration continued through the rest of the experiment by the operation of a spring-loaded syringe with its tip in the reaction mixture. Methyl-labelled [^{14}C]methionine (Radiochemical Co., Amersham, Bucks.) (10·7 μCi/μmole, final concentration 5 μM) was added 10 sec. later, and 1 ml. samples removed by means of the syringe to Millipore filtration units so that filtration took place at 10, 25, 40, 55 and 70 sec. Filtration was

immediately followed by washing twice with 5 ml. MM + glucose + chloramphenicol at room temperature. In all experiments, a final sample was taken into 1 ml. 10% trichloroacetic acid at 85 sec. The insoluble material was filtered and washed twice with 5 ml. trichloroacetic acid. All filters were then dried under a lamp and added to 5 ml. of scintillation fluid (xylene Pop-op Ppo) prior to assaying in a liquid scintillation counter.

The method is based on that of Piperno & Oxender (1968) as modified by P. D. Ayling & E. S. Bridgeland (manuscript in preparation). I am indebted to Dr Ayling for his collaboration in the performance of these experiments.

RESULTS

Dominance tests with metJ mutants

The properties of KLF 10 in Salmonella typhimurium. The episome to be used in the *metJ* dominance tests, KLF 10, was first transferred from its *Escherichia coli* JC1553 host into *Salmonella typhimurium argF111rec-301pyr*. As the episome carried the *argH* gene (i.e. the *E. coli* homologue of *argF* in *S. typhimurium*) but not the *pyr*$^+$ allele, the required heterogenotes were selected on MA + uracil. The episome could then be freely transferred to further *S. typhimurium argF111* recipients, selecting for prototrophic growth, without loss of efficiency due to restriction (Okada, Watanabe & Miyake, 1968). KLF 10 heterogenotes segregated frequent arginine-requiring organisms (10 to 50%) when subcultured into NB. Of more than 300 such segregants from cultures of *F111metJ*/*arg*KLF10 heterogenotes examined during this investigation none was ethionine-sensitive, showing that recombination between the episome and the host's chromosome was rare.

Ethionine sensitivity of metJ heterogenotes. Ethionine sensitivity was tested by streak tests on MA + ethionine and by growth experiments in MM + ethionine. Typical results of the latter experiments (each of which was carried out at least twice) are shown in Fig. 2; essentially similar results were obtained from the streak tests. Possession of either of two mutations *metJ713* and *J743* resulted in resistance of the haploid host cell to ethionine (Fig. 2*b*,*c*). When such cells were made heterozygous for *metJ* by the introduction of KLF 10, they became sensitive to the analogue (Fig. 2*f*,*g*). The degree of sensitivity was not the same in the two heterogenotes: *metJ713*/KLF10 was as sensitive as a wild-type (*metJ*$^+$/*metJ*$^+$) homogenote (Fig. 2*e*), whereas *metJ743*/KLF10 was slightly more resistant.

The sensitivity of the two *metJ*/*metJ*$^+$ strains could have reflected either dominance of *metJ*$^+$ over *metJ*, or some other, independent, effect on ethionine resistance of the presence of KLF 10. The latter possibility was unlikely, because the presence of KLF 10 in the ethionine-resistant strain *metK747*, in which the lesion resulting in resistance was located outside the region covered by the episome, did not result in ethionine sensitivity (Fig. 2*d*, *h*).

Repressibility of the methionine enzymes in metJ heterogenotes. Lawrence *et al.* (1968) showed that the ability of *metJ* and some *metK* mutants to cross-feed a methionine-requiring strain in streak tests on MA resulted from constitutive synthesis of the methionine biosynthetic enzymes. Haploid strains possessing *metJ713* or *J743* mutations gave such cross-feeding (Table 1). However, the introduction into these strains of *metJ*$^+$, on the episome KLF 10, resulted in loss of the ability

to cross-feed, while in control experiments the wild-type (*metJ*$^+$) failed to cross-feed, and a *metK747* (*metJ*$^+$*metK*) strain succeeded in doing so, both with and without the episome. Thus repressibility of the methionine biosynthetic enzymes appeared to be dominant to their constitutive synthesis in *metJ*/*metJ*$^-$ heterogenotes. To confirm and measure this effect, one of the enzymes was assayed in the appropriate strains grown in the presence and absence of methionine. Cystathionase, the enzyme specified by *metC* (Fig. 1), was selected because of the convenience of the assay method, but it had

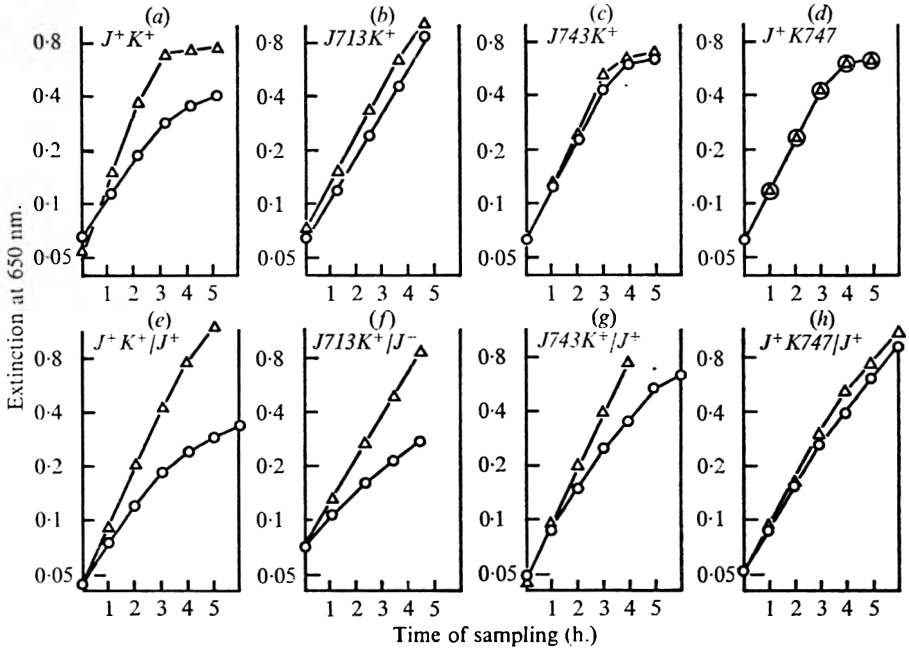

Fig. 2. Growth of *metJ* heterogenotes and control strains in the presence of ethionine. All the parental haploid strains had the *argF111* mutation (see Table 1). Heterogenotes were obtained by transferring the *Escherichia coli* episome KLF 10 (Fig. 1) into these parental strains. Inocula were in the exponential phase of growth. Cultures were grown in MM (△) and MM + ethionine, 1000 μg./ml. (○). The relevant genotypes (i.e. with respect to *metJ* and *K*) are indicated on the graphs: where merodiploids were used, relevant episomal markers are given after a diagonal stroke.

the disadvantage of relatively low sensitivity to regulation. For this reason, the experiments were carried out with cultures grown in groupings that permitted appropriate analyses of variance to be made on results obtained in essentially identical conditions. The results of these experiments are given in Table 3. Before analysis, all results were transformed into logarithms to ensure that, for example, a twofold difference between untransformed readings was given equal weighting in the analysis whatever the absolute value of the readings.

In Expts 1 and 2 the activity of cystathionase in the *metJ*/*metJ*$^+$ heterogenotes was compared with the mean of the values for the wild-type and mutant haploids. In the absence of dominance of either *metJ*$^-$ or *metJ*$^+$, no significant difference would have been expected. However, *metJ713*/KLF 10 (*J*/*J*$^+$) closely resembled the haploid wild-type (*J*$^+$), and differed significantly (P = 1 to 0·1 %) from the mean of the two haploids

(Expt 1). This suggested that *metJ713* was completely recessive to the episomal *metJ*$^+$. In contrast, the second *J*/*J*$^+$ strain (*metJ743*/KLF 10; Expt 2) differed only marginally (P = 10 to 5%) from the mean of the two haploids, and thus *metJ743* appeared not to be fully recessive to *metJ*$^+$. In confirmation of this, the *metJ743*/KLF 10 heterogenote was found to differ significantly (P = 5 to 1%) from a *J*$^+$/*J*$^+$ strain (Expt 3). Finally, the effect of KLF 10 on the regulation of cystathionase synthesis was tested in a wild-type (*metJ*$^+$*metK*$^+$) strain and the constitutive mutant *metK747* (*J*$^+$*K*) in which the regulatory mutation was situated outside the region duplicated by the episome (Expt 4). No significant effect of the episome was detected in these strains, showing that the effect of KLF 10 on regulation observed in *metJ* mutants was due to a true dominance effect, and not to some other, nonspecific, regulatory effect of the episome.

Table 3. *Cystathionase activity in metJ heterogenotes and control strains*

Each value is the mean of two or more replicate experiments in which cystathionase activity was determined by the toluene-treated cell procedure described in Methods. The activity is given as μmoles pyruvate produced from cystathionine in 1 h. by 1 mg. (dry wt) of cells, at 30°. All strains possessed a chromosomal *argF111* mutation which was expressed only in haploid strains. Arginine (20 μg./ml.) was therefore added to haploid, but not to partially diploid cultures. In the column 'Pertinent genotype', chromosomal methionine regulatory gene mutations precede the diagonal stroke, and regulatory genes carried by the episome KLF 10 follow it. Arginine-requiring segregants were obtained from all heterogenote cultures used, following subculture into nutrient broth, proving that the parental cultures had been heterogenotic.

The results of statistical analysis of these experiments are given in the text.

Experiment	Regulatory mutations	Possession of KLF 10	Pertinent genotype	Cystathionase activity: Methionine absent from growth medium	Cystathionase activity: 10 mM-methionine present in growth medium	Number of replicates
1	None	−	*J*$^+$	0·83	0·45	2
	metJ713	−	*J*	1·92	1·79	
	metJ713	+	*J*/*J*$^+$	0·85	0·45	
2	None	−	*J*$^+$	0·91	0·52	3
	metJ743	−	*J*	1·41	1·49	
	metJ743	+	*J*/*J*$^+$	0·91	0·69	
3	None	+	*J*$^+$/*J*$^+$	0·64	0·37	2
	metJ743	+	*J*/*J*$^+$	0·85	0·61	
4	None	−	*J*$^+$*K*$^+$	0·61	0·28	2
	None	+	*J*$^+$*K*$^+$/*J*$^+$	0·53	0·21	
	metK747	−	*J*$^+$*K*	1·36	1·09	
	metK747	+	*J*$^+$*K*/*J*$^+$	1·01	1·01	

Dominance tests with metK mutants

The properties of KLF 16 in Salmonella typhimurium. As the episome KLF 16 extended from *thy* on one side of *serA* to a point well to the other side (Fig. 1; K. Brooks Low, personal communication), it was considered that it might carry the *metK* gene (*metK* and *serA* being cotransduced; Lawrence *et al.* 1968), and therefore be suitable for use in *metK* dominance tests. The episome was transferred from its *Escherichia coli* JC 1553 *thy* host into *Salmonella typhimurium serA13rec-302met-754*, with selection of the required heterogenotes on MA + methionine. One of these was purified and used for transfer of KLF 16 to further *S. typhimurium serA* recipients, the resulting heterogenotes being selected as prototrophs. KLF 16 was found to be more stable than

KLF 10 in *S. typhimurium* hosts: fewer than 1% serine-requiring segregants were usually obtained after growth of KLF 16 heterogenotes in NB. However, acridine orange treatment (Hirota, 1960) increased the proportion of segregants. No ethionine-sensitive recombinants were detected among more than 100 serine-requiring segregants from various *metK*/KLF 16 cultures, so that, as with KLF 10 heterogenotes, recombination between the episome and the host's chromosome was rare.

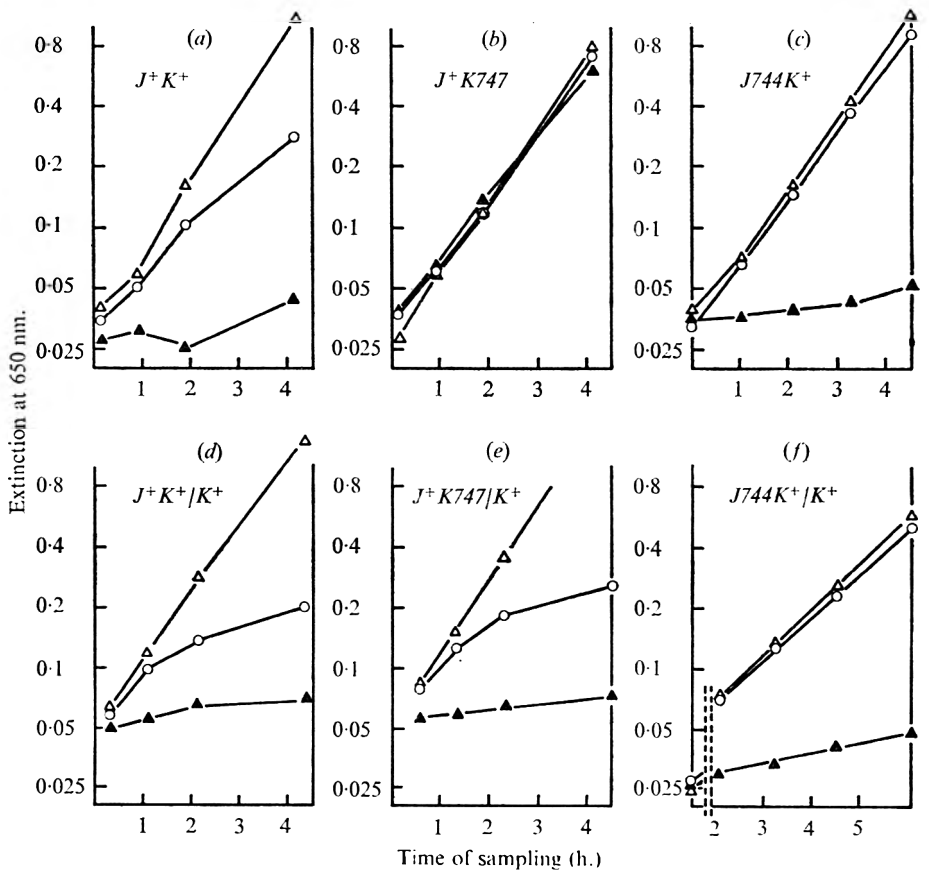

Fig. 3. Growth of *metK* heterogenote and control strains in the presence of methionine analogues. All the parental haploid strains possessed *ser.A* mutations (see Table 1). Heterogenotes were obtained by transferring the *Escherichia coli* episome KLF 16 (Fig. 1) into these parental strains. Inocula were in the exponential phase of growth. Cultures were grown in MM (△), MM+ethionine, 1000 μg./ml. (○), and MM+α-methylmethionine, 1000 μg./ml. (▲). The relevant genotypes (i.e. with respect to *metJ* and *K*) are indicated on the graphs: where merodiploids were used, relevant episomal markers are given after a diagonal stroke.

Methionine analogue sensitivity of metK heterogenotes. As with *metJ* heterogenotes, the results of streak tests of the analogue resistance of *metK* heterogenotes were confirmed by growth experiments in liquid media. Examples of the latter results are shown in Fig. 3. Possession of any of the three mutations *metK747*, *K751* and *K752* by a haploid strain resulted in its resistance to α-methylmethionine and ethionine (Fig. 3*b*). The introduction into these strains of *metK*+, on KLF 16 (Fig. 3*e*), made them as

sensitive to both analogues as the wild-type (*metK*$^+$) haploid and homogenote (Fig. 3*a*,*d*). The absence of a nonspecific effect of KLF 16 on analogue resistance was confirmed (Fig. 3*c*,*f*) by the observation that ethionine resistance in a *metJ* mutant was independent of the presence or absence of the episome, which did not cover the *metJ* region (Fig. 1). Thus the analogue sensitivity observed in *metK*/*metK*$^+$ heterogenotes reflected a true dominance of the wild-type over the mutant alleles.

Repressibility of the methionine enzymes in metK heterogenotes. Cross-feeding tests showed that the possession of KLF 16 by *metK747* and *K752* mutants prevented the excretion of methionine observed in the parental haploid strains, while excretion by strain *metJ744* was unaffected by possession of the episome. Thus repressibility of the methionine biosynthetic enzymes was apparently dominant to their constitutive synthesis in *metK*/*metK*$^+$ heterogenotes. As in the previous experiments with *metJ*

Table 4. *Cystathionase activity in metK heterogenotes and control strains*

Each value is the mean of two replicate experiments in which cystathionase activity was determined by the toluene-treated cell procedure described in Methods. The unit of cystathionase activity is given in Table 3. All strains possessed a chromosomal *serA* mutation which was expressed only in haploid strains. Serine (200 μg./ml.) was therefore added to haploid, but not to partially diploid, cultures. In the column headed 'Pertinent genotype', chromosomal methionine regulatory gene mutations precede the diagonal stroke, and regulatory genes carried by the episome KLF 16 follow it. Serine-requiring segregants were obtained from all heterogenote cultures used, following subculture into nutrient broth containing acridine orange (Hirota, 1960). This proved that the parental cultures had been heterogenotic.

The results of statistical analysis of these experiments are given in the text.

Experiment	Regulatory mutations	Possession of KLF 16	Pertinent genotype	Cystathionase activity: Methionine absent from culture medium	Cystathionase activity: 10 mM-methionine in culture medium
1	None	−	*K*$^+$	0·75	0·37
	metK747	−	*K*	1·36	1·15
	metK747	+	*K*/*K*$^+$	2·56	1·25
2	None	−	*K*$^+$	0·32	0·16
	metK751	−	*K*	0·96	0·35
	metK751	+	*K*/*K*$^+$	2·03	0·72
3	None	−	*K*$^+$	0·56	0·16
	metK752	−	*K*	1·47	1·28
	metK752	+	*K*/*K*$^+$	2·18	1·04
4	None	+	*K*$^+$/*K*$^+$	2·35	0·93
	metK747	+	*K*/*K*$^+$	2·18	0·75
5	None	−	*J*$^+$*K*$^+$	0·53	0·24
	None	+	*J*$^+$*K*$^+$/*K*$^+$	2·42	1·01
	metJ744	−	*JK*$^+$	2·24	1·60
	metJ744	+	*JK*$^+$/*K*$^+$	12·05	9·30

heterogenotes, this effect was examined quantitatively by assaying cystathionase activity in the appropriate strains grown in the presence and absence of methionine (Table 4). The experiments were again designed to permit statistical analysis of the results.

In Expts 1 to 3 three *metK*/*metK*$^+$ heterogenotes were compared with the wild-type (*metK*$^+$) and the appropriate *metK* haploid strain. One of the strains (*metK751*) was

of the nonexcreting type (Table 1), and this was the only one of the three *metK* mutants to show repressibility in the haploid state. However, all three K/K^+ heterogenotes showed repressibility, but in each case the total activity was several times greater than in the haploid wild type ($metK^+$) and was indeed significantly greater in two cases than in the *metK* haploid strain ($P = 5\%$ with *metK747*, $<0{\cdot}1\%$ with *K751*, in non-orthogonal comparisons).

A simple hypothesis to explain these results was that KLF 16 carried the *metC* gene, leading to a gene dosage effect on cystathionase activity in the heterogenotes. To test this, strain *serA13rec-302met-754*/KLF16 was crossed with the mutant *metC104* (Smith & Childs, 1966) on MA. Confluent growth was obtained. A purified subculture of this prototrophic growth was treated with acridine orange, and methionine-requiring segregants were obtained, confirming that KLF 16 carried the *metC* gene. In addition, the cystathionase activities of *metC104*/KLF16 (C/C^+) and the wild-type homogenote (C^+/C^+) were compared. The values obtained for cells grown in the absence and presence, respectively, of 10 mM methionine were: 1·23 and 0·51 units for C/C^+, and 2·05 and 0·80 units for C^+/C^+. The two strains differed significantly ($P = 1$ to $0{\cdot}1\%$), and by about the amount which would be predicted if the C/C^+ strain possessed one copy fewer of the *metC* gene than did the C^+/C^+ strain. Clearly, then, a gene dosage effect was responsible for the high activities found in KLF 16 heterogenotes. (It was not possible to estimate the number of copies of the episome per chromosome from the increase in cystathionase activity when KLF 16 was present, because the enzymes from *Escherichia coli* and *Salmonella typhimurium* may differ in their repressibility and catalytic activity.)

The activity of cystathionase observed in the latter experiment with the wild-type homogenote ($metC^+K^+/C^+K^+$) was very similar to that of the K/K^+ strains (Table 4, Expts 1 to 3). A further experiment confirmed this: no significant difference was detected between *metK747*/KLF16 (K/K^+) and the K^+/K^+ strain (Table 4, Expt 4). Thus the *metK* phenotype was not expressed in a strain possessing KLF 16. Finally, the effect of KLF 16 was examined in a normally regulated (J^+K^+) strain, and a *metJ* (JK^+) mutant in which constitutivity resulted from a mutation outside the diploid region (Table 4, Expt 5). The effect of the *metJ* mutation was highly significant ($P<0{\cdot}1\%$), regardless of the presence of the episome; similarly, the effect of the episome was also highly significant ($P<0{\cdot}1\%$), regardless of which *metJ* allele was present. However, no significant difference could be detected between the effect of the episome on the two strains (J^+K^+ and JK^+): in each case it caused four or five times more cystathionase activity than was found in the corresponding haploid, due to the *metC* gene dosage effect. Thus KLF 16 affected the *regulation* of cystathionase synthesis only in constitutive *metK* mutants. This specific effect of the episome could only be interpreted to mean that $metK^+$ was dominant to *metK*.

The absence of effects of metK mutation and metK gene dosage on the rate of methionine uptake by cell suspensions

Lawrence *et al.* (1968) found that growing cultures of *metK* mutants incorporated less radioactivity from ^{14}C-labelled ethionine or methionine, measured over 30 min. intervals, than did *metJ* or wild-type strains. This observation, coupled with the cross-resistance of *metK* mutants to three methionine analogues that all inhibit the growth of *Salmonella typhimurium* in different ways, indicated that a methionine uptake

system (permease) might be defective in *metK* mutants. A permease specific for methionine has been detected in *Escherichia coli* (K_m (methionine), $2{\cdot}27 \pm 0{\cdot}39$ μM; Piperno & Oxender, 1968), and preliminary results (P. D. Ayling & E. S. Bridgeland, personal communication) indicate that a transport system having a rather similar K_m (methionine) also exists in *S. typhimurium*. Therefore any gross differences in the rate of methionine uptake between *S. typhimurium* strains should have been revealed by measuring the initial rate at which cells incorporated radioactivity from ^{14}C-labelled methionine at an extracellular concentration of 5 μM, i.e. above the K_m value. This method was used not only to detect differences between wild-type and *metK* strains, but also to test the effect of *metK* gene dosage on the rate of uptake. This was undertaken following the observation of Ames & Roth (1968) that the presence of an episome

Table 5. *The effects of metK mutation and gene dosage on the rate of uptake of [^{14}C]-L-methionine by cell suspensions*

Uptake of radioactivity by cells was measured as described in Methods, at 15 sec. intervals during the first 75 sec. after addition of [^{14}C]-L-methionine to cell suspensions maintained at 25°. The rates of uptake were calculated from these data by the method of least squares. In each experiment, determinations were made with all four strains, so that valid comparisons could be made between strains.

Strain	Pertinent genotype	Rate of uptake of [^{14}C]-L-methionine (nmoles/mg. dry wt cells/min.)			
		Expt 1	Expt 2	Expt 3	Mean
serA11	*metK*$^+$	0·74	1·31	0·83	0·96
serA11metK752	*metK*	0·52	1·36	0·85	0·91
serA11/KLF16	*metK*$^+$/*K*$^+$	0·30	1·22	0·82	0·78
serA11metK752/KLF16	*metK*/*K*$^+$	0·26	1·09	0·81	0·72

carrying the histidine permease gene resulted in a two- to threefold increase in the rate of histidine uptake by *S. typhimurium*. Thus if *metK* specified methionine permease, the presence of KLF 16 (which carried *metK*$^+$) in a cell should have resulted in a two- to threefold increase in the rate of methionine uptake. (It is assumed that the V_{max} of the *E. coli* and *S. typhimurium* permeases is the same; this appears to be so for histidine permease (Ames & Roth, 1968), and unpublished preliminary results obtained by P. D. Ayling and E. S. Bridgeland suggest that it is also true for methionine permease.) Triplicate experiments were therefore carried out with *metK*$^+$, *K752*, *K*$^+$/*K*$^+$ and *K752*/*K*$^+$ strains (in collaboration with Dr P. D. Ayling). The results are given in Table 5. Although the absolute rates of uptake varied between experiments, it was clear that the rate of uptake was neither decreased by *metK* mutations nor increased when several copies of the *metK* gene were present (i.e. in KLF 16 merodiploids): indeed, the presence of KLF 16 appeared slightly to reduce the rate of uptake, though this effect was not statistically significant. It was concluded that the *metK* gene did not specify a high affinity methionine permease, though the possibility that it could specify a low affinity one having a K_m (methionine) greater than 5 μM was not excluded by these results.

DISCUSSION

To what was previously known about *metJ* and *K* mutants from the work of Lawrence *et al.* (1968) (see Introduction) it can now be added that representative mutant alleles of both loci were recessive to the respective wild-type alleles derived from *Escherichia coli* (Fig. 2, 3; Tables 3, 4).

The complete dominance of *metJ*$^+$ to the *metJ713* allele indicated that the *metJ*$^-$ product was active, and the *metJ713* product inactive. That the gene product is a rather large molecule is suggested by the similarity in the lengths of the *metB* and *metJ* genes, deduced from the finding that the variation in cotransduction frequencies of various mutations in the two genes with particular *metF* mutations was similar (*metB-metF31*, 21·6 to 34·1%; Smith, 1961: *metJ-metF96*, 31 to 52%; Lawrence *et al.* 1968): since the molecular weight of the *metB* polypeptide (the subunit of cystathionine-γ-synthetase) is about 40,000 daltons (Kaplan & Flavin, 1966), the *metJ* gene product is likely to be comparable in size. More direct evidence that the *metJ* gene product is indeed a protein has been provided by the recent isolation and identification of a *metJ* mutant suppressible by nonsense suppressors (A. J. Minson, personal communication).

At this point it is suggested that the *metJ* protein may be an apo-repressor of methionine synthesis. If so, the observation that the *metJ743* allele, unlike *metJ713*, was not completely recessive to *metJ*$^+$ (Fig. 2; Table 3), could be explained if the *metJ* product normally forms an oligomeric protein, in which case inactive mixed protein molecules containing mutant and wild-type subunits might have formed in the *metJ743*/KLF10 heterogenote. Thus the *metJ* gene product, if an apo-repressor, may resemble the *lac* apo-repressor of *Escherichia coli* in being an oligomer of high molecular weight (Muller-Hill, Crapo & Gilbert, 1968).

Recently, evidence that *metJ* specifies an apo-repressor has been obtained with a strain carrying a *metJ* mutation isolated by D. A. Lawrence (unpublished) as a suppressor of a methionine auxotroph. In four determinations, this strain consistently gave very similar, *partially* derepressed cystathionase activity whether grown in the presence or absence of methionine (10 mM) (mean values, 0·56 and 0·63 units, respectively: Chater, 1969). It is difficult to envisage any explanation for this observation other than that in this strain the *metJ* product is a methionine apo-repressor whose conformation is unaffected by the presence of co-repressor, but which has some regulatory activity.

Ethionine inhibits the growth of bacteria by replacing methionine in protein synthesis (Spizek & Janecek, 1969): it has no effect on *S*-adenosylmethionine synthesis (Cox & Smith, 1969; Gross, 1969) and little effect on the activity of the methionine first enzyme (D. A. Lawrence, personal communication). Thus the ethione resistance of *metJ* mutants is probably the result of reduced incorporation of the analogue into proteins, attributable to the high intracellular methionine level.

The complete dominance of *metK*$^+$ to the three *metK* mutations tested indicated that the *metK*$^+$ allele produced active, and the *metK* mutant alleles inactive, product, with respect both to methionine analogue resistance and (where relevant) to regulation of methionine synthesis. This eliminated a positive role for the *metK* product in the regulation of methionine synthesis. We may also be certain that the *metK* mutations did not result in the acquisition of novel properties by the mutant gene product such

that the methionine analogues were actively prevented from reaching their sites of inhibition. The resistance of *metK* mutants to α-methylmethionine, which acts specifically by inhibiting the activity of the methionine first enzyme (Schlesinger, 1967; Rowbury, 1968), could not have been the result of an altered component of the first enzyme, because α-methylmethionine resistance would then have been dominant to sensitivity; moreover, this enzyme was sensitive to feedback inhibition by methionine in a *metK* strain (Chater & Rowbury, 1970).

The possibility that the *metK* phenotype might result from an altered specificity or activity of methionine permease was eliminated by the finding that the rate of uptake of radioactive methionine was not influenced by *metK* mutation or by changes in *metK* gene dosage. This conclusion has recently been confirmed by the identification of a gene specifying methionine permease, which is located far from *metK* on the linkage map (P. D. Ayling & E. S. Bridgeland, personal communication).

Lawrence *et al.* (1968) were able to reject the possibility that the *metK* gene product was an apo-repressor; and Gross & Rowbury (1969) showed that methionyl-tRNA was unlikely to have any effect on regulation, while methionyl-tRNA synthetase was specified by the *metG* gene. Thus conventional explanations for the *metK* phenotype all appear inadequate, and it is necessary to look for a feature of methionine biosynthesis that is not shared by other well-established biosynthetic systems. The most obvious such feature is the role of methionine as the immediate precursor of *S*-adenosylmethionine, which is the universal donor of methyl groups in transmethylation reactions (Cantoni, 1965). *S*-Adenosylmethionine is known to be involved in feedback inhibition of the first enzyme of methionine biosynthesis in *Escherichia coli* (Lee, Ravel & Shive, 1966) and *Salmonella typhimurium* (D. A. Lawrence, unpublished data); it is reasonable to suppose that it might also be involved in repression. *S*-Adenosylmethionine deficiency would then be expected to cause release from both feedback inhibition and repression, which might in turn result in the resistance to both α-methylmethionine and ethionine which is one of the features of the *metK* phenotype. The release from control of methionine biosynthesis would be most marked in strains with the most serious *S*-adenosylmethionine deficiencies, and such strains would also be expected to be somewhat slow-growing. Methionine-excreting *metK* strains may illustrate this situation.

Some difficulties arise in the application of this hypothesis to *metK* strains. Chater & Rowbury (1970) have demonstrated that feedback inhibition by methionine occurred in a *metK* strain, though it could be argued that sufficient methionine was accumulated by the cells in the conditions of the experiment to overcome a K_m barrier to *S*-adenosylmethionine synthesis. Secondly, A. J. Minson (personal communication) has obtained viable *metK* mutants suppressible by nonsense suppressors: although this is evidence that the *metK* gene product is a protein, it suggests that the function of the protein is not indispensable, as it would be expected to be if it were required to maintain a pool of *S*-adenosylmethionine.

Thus our present working hypothesis may be summarized as follows: the central factor in regulating methionine biosynthesis is the intracellular level of *S*-adenosylmethionine, which is dependent upon the activity of the *metK* gene product (on the simplest model, this would be the enzyme *S*-adenosylmethionine synthetase). *S*-Adenosylmethionine participates both in feedback inhibition, by interaction with the enzyme homoserine-*O*-transsuccinylase, and in repression, by activating a (possibly oligomeric)

protein apo-repressor coded for by the *metJ* gene. The active repressor acts in an unspecified way to reduce further synthesis of the enzymes of methionine synthesis.

A recent report by Green, Su & Holloway (1970) describes *S*-adenosylmethionine synthetase deficient mutants of *Escherichia coli* which were ethionine-resistant, and possessed high levels both of the methionine biosynthetic enzymes and of methionine. No further data were presented that would permit comparison of the *E. coli* mutants with the known kinds of ethionine-resistant mutants of *Salmonella typhimurium* (Lawrence *et al.* 1968).

Some interesting incidental points arise from the use of F′ factors derived from *Escherichia coli* in this study. The rarity of recombination observed between the episomes and the chromosomes of the *Salmonella typhimurium* host cells suggested the absence of fine structure homology of the two types of DNA. Despite this, the *E. coli* regulatory elements efficiently controlled the expression of *S. typhimurium* genes. This is therefore another example of the evolutionary preservation of those parts of proteins concerned with quaternary interactions (Balbinder, 1964; Ito, 1969). Similar conclusions may be drawn from the work of Fink & Roth (1968). Finally, the regulation of methionine synthesis in *E. coli* appears to be similar to that in *S. typhimurium*.

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A Genetical Study of the Feedback-sensitive Enzyme of Methionine Synthesis in *Salmonella typhimurium*

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SUMMARY

The homoserine-*O*-transsuccinylase activity of three kinds of methionine-excreting mutants of *Salmonella typhimurium* was examined. In a *metI* mutant the enzyme was resistant to inhibition by methionine or its analogue α-methylmethionine. Feedback inhibition in a *metJ* and a *metK* strain was normal. *metI* was dominant to *metI*$^+$ only when coupled in the *cis* position with the wild-type allele of the closely-linked *metA* (homoserine-*O*-transsuccinylase) gene, and a deletion analysis of nine *metI* mutations showed that they were all located within the *metA* gene. Thus both the regulatory and catalytic sites of homoserine-*O*-transsuccinylase are specified by a single polypeptide species. An estimate was made of the length of the *metA* gene, based on recombination data.

INTRODUCTION

The first step specific to methionine biosynthesis in *Salmonella typhimurium* and *Escherichia coli* is the *O*-succinylation of homoserine (Rowbury, 1964; Rowbury & Woods, 1964). Mutations in *S. typhimurium* resulting in the loss of homoserine-*O*-transsuccinylase activity are located in the *metA* gene, which is cotransduced with a second methionine structural gene (*metH*) (Childs & Smith, 1969) and with genes controlling the biosynthesis of purines (*purD,H*) and thiamine (*thi*) (Sanderson, 1967). Sensitive regulation of methionine synthesis is achieved by the feedback inhibition of homoserine-*O*-transsuccinylase activity by methionine and its important derivative *S*-adenosylmethionine, either singly or (more effectively) in combination (Rowbury, 1964; Lee, Ravel & Shive, 1966). False feedback inhibition by the methionine analogue α-methylmethionine has been demonstrated, and since this substance is unable to replace methionine in protein synthesis, its addition to growing cultures rapidly inhibits further cell division (Schlesinger, 1967; Rowbury, 1968; Smith, 1968).

Mutants of *Salmonella typhimurium* that were resistant to α-methylmethionine and overproduced methionine were described by Lawrence, Smith & Rowbury (1968). Genetical analysis of these mutants revealed two groups, one of which was defective in *metK*, a locus situated far from any of the known methionine genes in the linkage map, the other group being designated *metI*. *metI* mutations were all more than 95% cotransduced with *metA*, and it was considered probable that they possessed a homoserine-*O*-transsuccinylase that was not subject to inhibition by methionine or its

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analogue. Preliminary deletion mapping experiments led Lawrence *et al.* (1968) to speculate that *metA* and *metI* might be separate cistrons, *metA* specifying a catalytically active subunit of the enzyme and *metI* a regulatory subunit. This idea was reinforced by the data of Ayling & Chater (1968), who found that three independent pairs of *metA* and *I* mutations had the same orientation relative to outside markers.

The present account includes biochemical evidence that *metI* mutations do indeed give rise to feedback inhibition resistance, and genetical evidence, from complementation and deletion mapping experiments, that they occur *within* the *metA* cistron. A preliminary account of some of the genetical experiments has appeared elsewhere (Chater, 1970*b*).

METHODS

Media. The media and most of the supplements used are given in an accompanying paper (Chater, 1970*a*). Additional supplements to minimal media were adenine HCl and L-tryptophan (20 μg./ml.). Abbreviations used are: NA, nutrient agar; NB, nutrient broth; MA, minimal agar; MM, liquid minimal medium.

Organisms. The nomenclature is that of Sanderson (1967). *Escherichia coli* 1553/KLF10 was provided by K. Brooks Low, and is described in an accompanying paper (Chater, 1970*a*). All other strains were derived from *Salmonella typhimurium* strain LT2. Those previously described were: *metA15*, *A54*, *A229* and *A309*, *metA22tryB2*, and *metA43purE11* (Smith & Childs, 1966); *argF111*, *argF111metI708*, *metA746metI706*, *metB23metI708* and *metB23metJ744* (Ayling & Chater, 1968); *metI701*, *I702*, *I703*, *I704*, *I705*, *I706*, *I708*, and *I712* (Lawrence *et al.* 1968); *argF111rec-301pyr* (Chater, 1970*a*), and *metB23metK747* (Chater, 1969). Mutant *metI749* was provided by P. D. Ayling, and strain *metA7* was obtained by transduction of the double mutant *metA7cysA21* (Smith & Childs, 1966) to cysteine independence. The maintenance of stock cultures, and the propagation, assay and maintenance of transducing phage P22, were as described by Smith (1961). Cultures were grown at 37° unless otherwise stated. Growth experiments in MM were essentially as described by Lawrence *et al.* (1968).

Assay of homoserine-O-transsuccinylase activity. The incubation mixture and assay system for estimating the [^{14}C]homoserine-dependent accumulation of [^{14}C]-*O*-succinylhomoserine by a cell suspension are described in the legend to Table 1.

Episome transfer and the detection of haploid segregants. The methods used are described in an accompanying paper (Chater, 1970*a*).

Transduction. Transduction and recombinant analysis were as described by Ayling & Chater (1968), except in the deletion analysis of *metI* mutants, when the normal method would have involved the use of too much expensive α-methylmethionine. Instead, use was made of the methionine excretion of *metI* mutants, by analogy with a similar study of histidine feedback inhibition-resistant mutants of *Salmonella typhimurium* (Sheppard, 1964). Eighteen h. NB cultures of recipients were harvested by centrifugation and resuspended in culture volumes of T2 buffer (adsorption medium: Hershey & Chase, 1952), and donor phage added at a multiplicity of infection of 5. The transduction mixture was incubated at 37° for 5 min. before the addition of up to 1 ml. per tube to tubes containing 10 ml. of molten minimal soft agar maintained at 45°. The contents of each tube were then poured into a Petri dish containing a thin solidified layer (10 ml.) of MA. Plates were incubated at 37° for 48 h., and at room temperature (20 to 25°) for a further 24 h. Upon examination with a binocular micro-

scope at low magnification it was possible to distinguish methionine-excreting from non-excreting colonies: excretion resulted in growth of the background of methionine-requiring recipient cells, visible as a fuzzy edge to the excreting colony, while the boundaries of non-excreting colonies were smooth and clearly defined. Up to 300 colonies per plate could be scored. The reliability of the technique was established in reconstruction experiments with artificial mixtures of *metI* and I^+ strains in the presence of a large excess of a *metA* deletion mutant. In addition, as further confirmation of the scoring in all experiments, all apparently non-excreting colonies were restreaked on MA, and their resistance to α-methylmethionine tested by replica plating. To

Table 1. *The effect of methionine and α-methylmethionine on the synthesis of O-succinylhomoserine by strains metB23 and metB23metI708*

Organisms (0·25 mg dry wt/ml.) were incubated at 37° in a mixture consisting of glucose minimal medium (0·75 ml.), [^{12}C]homoserine (800 nmoles), and [^{14}C]homoserine (0·2 μCi), with additions as stated in the table, and water to 1·5 ml. After 15 min., tubes were heated in a boiling water-bath, and cell debris was removed by centrifugation. [^{14}C]-*O*-Succinylhomoserine in the supernatant fluids was estimated as follows: 0·5 ml. aliquots were treated with 0·1 ml. M-KOH, and heated on a water-bath at 100° for 1 min. to convert *O*-succinylhomoserine to *N*-succinylhomoserine. Duplicate aliquots were left untreated. Treated and untreated samples were added to 5 cm. columns of Dowex-50 resin (H^+ form), and eluted with water. *N*-Succinylhomoserine passes through the column, while unreacted [^{14}C]homoserine is retained. 3·5 ml. of each eluate was collected, of which 1 ml. was added to 15 ml. of scintillation fluid (0·5% butyl PBD in toluene-triton X 100; 3:2, v/v) and counted for 5 min. in a Packard Tri-Carb Liquid Scintillation Spectrometer 3320. [^{14}C]-*O*-Succinylhomoserine was given by the difference between treated and untreated samples.

	O-Succinylhomoserine formed (% of control)	
Additions	*metB23*	*metB23metI708*
None (control)	100	100
L-Methionine		
0·02 mM	31	108
0·05 mM	8	86
0·25 mM	0	92
α-Methyl-DL-methionine		
0·001 mM	52	105
0·005 mM	5·5	101
0·02 mM	4	97
0·05 mM	3·5	90
0·25 mM	0	105
1·0 mM	0	103

confirm that any rare *metI*$^+$ recombinants were not contaminants, any second auxotrophic marker present in the recipient was checked in the recombinants by replica plating. Where such markers were not available, the colonial morphology of the *metI* and I^+ recombinants was examined, as this quality was sufficient to distinguish between the different recipients used, and was therefore unlikely to be shared with any chance contaminant.

RESULTS

Tests of the sensitivity of methionine analogue-resistant mutants to feedback inhibition by methionine. The assay of homoserine-*O*-transsuccinylase depending on the homoserine-dependent accumulation of [^{14}C]-*O*-succinylhomoserine can be used only with

metB mutants, since the ester is further metabolized in other strains. The isolation of strains *metB23metI708*, *metB23metJ744* (Ayling & Chater, 1968) and *metB23metK747* (Chater, 1969) permitted the study of feedback inhibition in representatives of three classes of methionine analogue-resistant mutants. The effects of added methionine and α-methylmethionine on *O*-succinylhomoserine synthesis in the *metI* mutant and a *metI*$^+$ strain are given in Table 1 and Fig. 1. External concentrations of methionine or α-methylmethionine giving effectively total inhibition of *O*-succinylhomoserine synthesis in *metI*$^+$ cells had no effect at all on this activity in the *metI* mutant. Since *metB23metI708* cultures grew only in the presence of added methionine, one must assume that methionine could enter and be utilized by the cells, so that the only explanation for the observations in Table 1 and Fig. 1 is that the homoserine-*O*-transsuccinylase of *metB23metI708* was altered in such a way that it was no longer inhibited by methionine or α-methylmethionine.

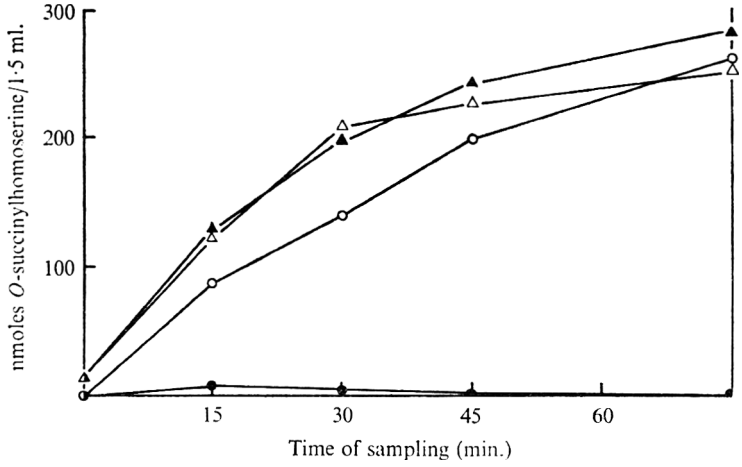


Fig. 1. Time course for the synthesis of *O*-succinylhomoserine by strains *metB23* and *metB23metI708* in the presence and absence of methionine. Organisms (0·25 mg. dry wt/ml.) were incubated at 37° in the incubation mixture described in the legend to Table 1. At the stated intervals, samples were removed and assayed for [^{14}C]-*O*-succinylhomoserine (see Table 1). ○, *metB23* in absence of methionine; ●, *metB23* in presence of 0·1 mM-methionine; △, *metB23metI708* in absence of methionine; ▲, *metB23metI708* in presence of 0·1 mM-methionine.

The rate of *O*-succinylhomoserine synthesis in the *metJ* and *metK* mutants was reduced in the presence of L-methionine (0·05 mM) to 7% and 5%, respectively, of that obtained in controls lacking methionine. Thus neither *metJ* nor *metK* mutations result in altered feedback sensitivity of homoserine-*O*-transsuccinylase.

Absence of complementation between metA and metI. Lawrence *et al.* (1968) suggested that *metA* and *I* might be separate genes. If they were, it should be possible to demonstrate complementation between them in a suitable strain. The genotype of the merodiploid required for such a test is *metA*$^-$*I*$^-$/*A*$^+$*I*$^+$. α-Methylmethionine-resistant growth of a strain of this genotype would indicate that the products of the *metA*$^+$ and *I*$^-$ alleles were complementing each other, i.e. *metA* and *I* were separate cistrons. The required merodiploid was obtained by transferring the episome KLF 10 (which carried the *metA* region of the *Escherichia coli* genome: Chater, 1970*a*), from

its *E. coli* 1553 host, via *Salmonella typhimurium argFIIIrec-301pyr*, into strain *metA746metI706*, with selection on MA for prototrophic heterogenotes. In addition, for control purposes, KLF 10 was transferred into strain *argFIII* to give the merodiploid $metA^+I^+/A^+I^+$, and into strain *argFIIImetI708* to give the merodiploid A^+I^-/A^+I^+.

The growth of the heterogenotes and their haploid parents was followed in MM with and without the addition of α-methylmethionine (Fig. 2). As expected, the haploid A^+I^+ strain (Fig. 2*a*) was sensitive to the analogue. The presence of KLF 10 in this strain (A^+I^+/A^+I^+; Fig. 2*d*) increased its resistance a little (probably due to a

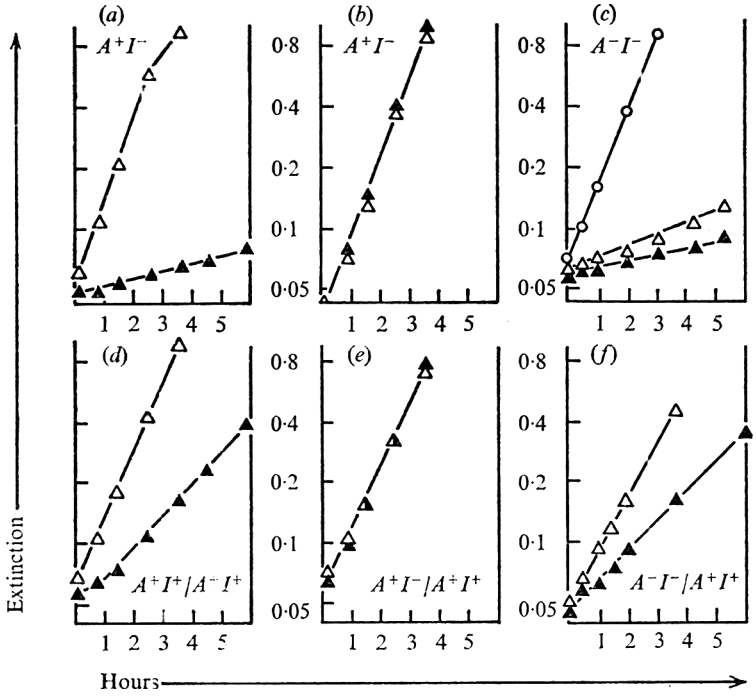


Fig. 2. A test of complementation between *metA* and *metI*. The *Escherichia coli* episome KLF 10 (*argF*$^+$ *metA*$^+$ *metI*$^+$) was transferred into various *Salmonella typhimurium* strains and the growth of the haploid parent strains and their heterogenote derivatives was followed, by extinction measurements, in minimal medium containing: △, no addition; ▲, DL-α-methylmethionine (1000 μg./ml.); ○, L-methionine (20 μg./ml.). The relevant genotypes (i.e. with respect to *metA* and *I*) are indicated on the graphs: where merodiploids were used, relevant episomal markers are given after a diagonal stroke.

metA gene dosage effect), though not to a level comparable with that of the A^+I^- strain (Fig. 2*b*), which, moreover, was unaffected by possession of the episome (A^+I^-/A^+I^+; Fig. 2*e*). Thus *metI* was dominant to *metI*$^+$ (as had been anticipated in the design of the complementation test). Figure 2*f* shows that the presence of the episome in the A^-I^- double mutant (giving the critical genotype A^-I^-/A^+I^+) permitted its growth in unsupplemented MM, while the haploid strain (Fig. 2*c*) gave only slow, leaky growth in MM unless methionine was added. However, the growth of the A^-I^-/A^+I^+ strain was inhibited by α-methylmethionine to about the same extent as

was that of the A^+I^+/A^+I^+ strain (Fig. 2*d*). Thus the dominance of *metI*$^-$ to *metI*$^+$ suggested by Fig. 2*e* was apparently dependent upon its coupling in the *cis* position with *metA*$^+$. It was therefore concluded that the *metA* and *I* mutations used in the complementation test were probably located in the same cistron.

This conclusion is based on the assumption that the *Escherichia coli* and *Salmonella typhimurium* homoserine-*O*-transsuccinylases have not undergone enough changes during evolution from a presumed common ancestral form to prevent the efficient formation of mixed enzyme in hybrids. Two lines of evidence suggest that this assumption is justified. First, *E. coli–S. typhimurium* hybrid tryptophan biosynthetic enzyme complexes are easily obtained when extracts of the two organisms are mixed (Balbinder, 1964; Ito, 1969); and secondly, the expression of the histidine and methionine structural genes of *S. typhimurium* is efficiently controlled by regulatory elements derived from *E. coli* (Fink & Roth, 1968; Chater, 1970*a*). It seems that those sites of homologous enzymes from the two organisms that are concerned with quaternary interactions have undergone relatively little evolutionary divergence, so that the absence of complementation observed between *metA* and *I* is unlikely to be due to failure of the required quaternary interactions to take place.

Deletion mapping of metI mutations. The absence of complementation between *metA746* and *metI706* described above cast doubt on the suggestion of Lawrence *et al.* (1968) that *metA* and *I* were separate genes. To clarify the relationship between mutations causing *metA* and *metI* phenotypes, the locations of *metI* mutations in the *metA* deletion map were investigated. The original deletion map of Smith & Childs (1966) has been modified by the properties of a new deletion mutant, *metA596*, which gave prototrophic recombinants with *metA43* but not with *metA15* (D. A. Smith, personal communication). The resulting improvement in the map (Fig. 3) received support from the occurrence of prototrophic recombinants in the crosses *metA43* × *A54* or *A746*, and their absence in the crosses *metA15* × *A54* or *A746* (Chater, 1969). The criterion established by Smith & Childs (1966) for absence of recombination between two *metA* mutations was failure to produce prototrophic recombinants in three replicate reciprocal transduction crosses. Their crosses were performed by a spot technique in which crosses between non-allelic methionine mutants gave 40 to 100 recombinant colonies per spot. In order that the resolution of the mapping of *metI* mutations should be comparable with that obtained by Smith & Childs, at least 500 recombinant colonies were examined before absence of recombination between the donor and recipient mutant sites was concluded.

The results of crosses involving 9 *metI* mutants are presented in Table 2. Mutant *I702* gave no *I*$^+$ colonies among 506 recombinants obtained from a cross with *metA229*, a mutation located at one extremity of the deletion map (Smith & Childs, 1966). *A229* and *I702* were therefore placed in the same deletion group (x in Fig. 3). In only one case, in which *I705* was the donor, was an *I*$^+$ recombinant obtained in crosses with *metA7*, but so few prototrophic recombinants occurred with this recipient that no significance could be attached to the negative results, which are therefore not included in Table 2. The positive result with *I705* is, however, taken into account in the assignment of this mutation to deletion group x. The result for *I706* did not agree with that given by Lawrence (1967), who obtained one *metI*$^+$ colony among 90 recombinants from an *A43* × *I706* cross. No explanation can be offered for this disparity.

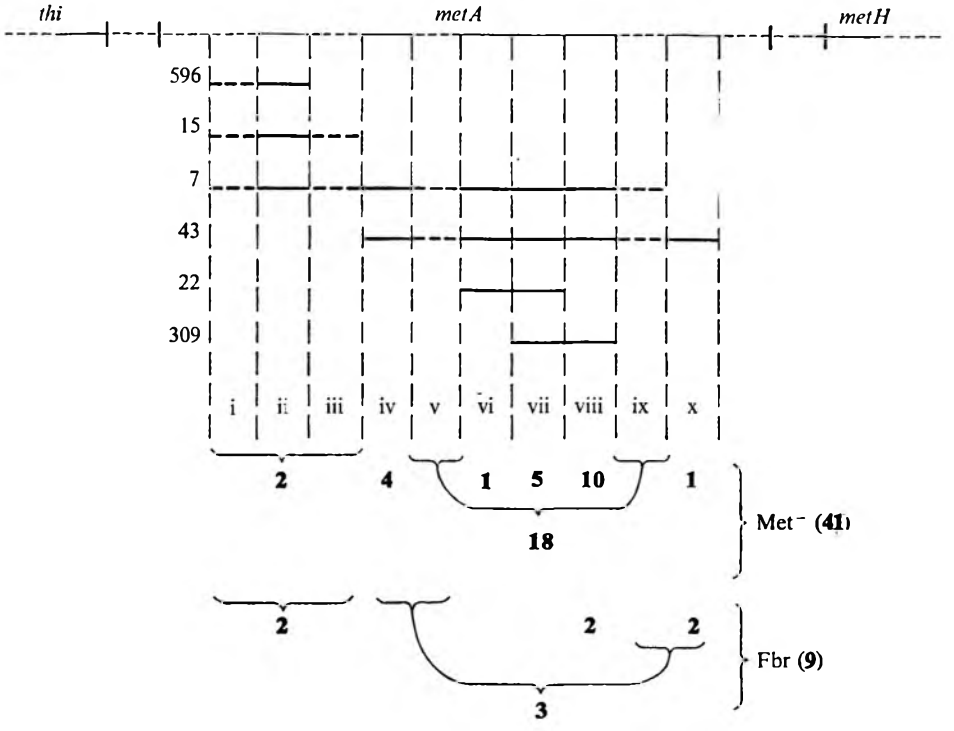


Fig. 3. A revised deletion map of the *metA* gene. The map is based on that of Smith & Childs (1966, and unpublished) and on data given in Results. Heavy unbroken lines represent regions deleted in *metA* deletion mutants (isolation numbers given at left end of each line). Heavy broken lines indicate regions whose existence is not certain. Deletion regions are not drawn to scale. Bold-face figures give the numbers of methionine auxotrophic (Met⁻) and feedback resistance (Fbr) mutations so far mapped in the various deletion regions (i to x). The total numbers mapped are also given (far right). The orientation of regions vi, vii and viii relative to the rest of the map has not been established.

Table 2. *Deletion mapping of metI mutations*

Phage P22 propagated on *metI* mutants was used to transduce *metA* deletion mutants to prototrophy. Recombinants were scored for inheritance of the *metI* phenotype (see Methods). In the table, the number of colonies with *metI*⁺ phenotype is followed in each case by the total number of colonies scored. Assignment to deletion groups refers to Fig. 3, and for *metI702* and *705* utilizes additional information given in the Results. Parentheses indicate assignments based on negative results with fewer than 500 recombinant colonies.

	No. of colonies with *metI*⁺ phenotype among recombinants from cross with recipient					
Donor	*metA43*	*metA15*	*metA596*	*metA22*	*metA309*	Deletion group
metI701	4/724	0/578	1/372	n.t.	n.t.	i or iii
metI702	0/1162	1/103	n.t.	n.t.	n.t.	x
metI703	13/1775	0/660	2/886	n.t.	n.t.	i or iii
metI704	0/894	4/150	3/67	1/56	c/181	(vii)
metI705	0/1934	0/287	3/217	14/82	11/577	x
metI706	0/1830	4/439	1/144	9/45	23/648	iv, v, ix or x
metI708	0/737	0/193	2/126	11/48	5/162	iv, v, ix or x
metI712	0/660	0/236	2/243	10/69	26/579	iv, v, ix or x
metI749	0/526	9/318	5/200	2/149	c/1065	viii

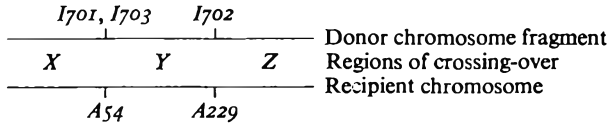
n.t. = Not tested.

These results confirmed the conclusion drawn from the *metA-I* complementation test, namely that mutations leading to the *metA* and *I* phenotypes are located in the same gene. Moreover, mutations resulting in the *metI* phenotype did not seem to be clustered in any particular region(s) of the *metA* gene.

The frequency of recombination within the metA gene. Using *metA* and *I* mutations situated at the ends of the deletion map (*A54*, *I701* and *I703* in deletion groups i, ii or iii, and *A229* and *I702* in deletion group x; see Results, preceding section, and Fig. 3), crosses were made to estimate the frequency of recombination along the *metA* gene (more precisely, that part of *metA* containing deletion regions iv to ix, within which 86% of all *metA* and *I* point mutations tested have been located: Fig. 3) during P22-mediated transduction (Table 3). In crosses 1 & 3 the generation of *metA*$^+$ recombinants required

Table 3. *Estimation of recombination frequency within the metA gene during transduction*

Phage P22 propagated on *metI* mutants was used to transduce *metA* auxotrophs to prototrophy. Recombinants were scored for inheritance of *metI* phenotype by replica plating to MA+α-methylmethionine. Controls indicated that *metI*$^+$ colonies were unlikely to have resulted from recipient reversion.



Cross	No. of recombinants with crossing-over in regions: *X*, *Y*	*Y*, *Z*	*X*, *Z*	Estimated recombination frequency in region *Y**
†1. *metA229* × *metI701*	—	13 (*I*$^+$)	708 (*I*$^-$)	0·026
2. *metA54* × *metI702*	18 (*I*$^+$)	—	508 (*I*$^-$)	mean 0·033
†3. *metA229* × *metI703*	—	29 (*I*$^+$)	567 (*I*$^-$)	0·040

* Calculated as follows: $\dfrac{(X, Y)+(Y, Z)}{2[(X, Y)+(Y, Z)+1]}$,

where (*X*, *Y*) and (*Y*, *Z*) are the frequencies of *X*, *Y* and *Y*, *Z* recombinants per *X*, *Z* recombinant (represented by the figure '1' in the denominator) obtained from crosses with reciprocal marker arrangements. The denominator is doubled to take account of the requirement for two recombination events in the generation of a viable recombinant. Mutations *metA54*, *I701* and *I703* on the one hand, and *A229* and *I702* on the other, are taken to define the ends of region *Y* of the diagram.

† Data of P. D. Ayling.

a recombination event to the right, and in cross 2 to the left, of the *metA* gene. Combining the data from crosses 1 and 2 or crosses 2 and 3, estimates of the recombination frequency along the *metA* gene could therefore be made that took into account differences between the numbers of recombination events occurring on either side of the gene. The mean of the two estimates obtained was 0·033, i.e. 1 in 30 of all the recombination events occurring along the whole transduction fragment took place within the *metA* gene.

DISCUSSION

The failure of methionine or α-methylmethionine to inhibit *O*-succinylhomoserine synthesis in a *metI* mutant (Table 1; Fig. 1) was attributable either to failure of these substances to penetrate the cells, or to an alteration of homoserine-*O*-transsuccinylase such that it was not sensitive to feedback inhibition. Two observations precluded the former interpretation. First, strain *metB23metI708* grew at the same rate as strain *metB23* on methionine at concentrations down to 0·02 mM (i.e. the lowest concentration used in these experiments: R. J. Rowbury, unpublished observations); and secondly, α-methylmethionine resistance was dominant to sensitivity in a *metI*$^-$/*metI*$^+$ heterogenote (Fig. 2*e*), which contrasts with the observation of Ames & Roth (1968) that histidine analogue resistance resulting from mutation of the histidine permease gene was recessive to sensitivity. Thus the *metI* gene product cannot be a component of the methionine transport system, so must be a component of homoserine-*O*-transsuccinylase. This conclusion has also recently been reached by D. A. Lawrence (personal communication), who has tested the feedback sensitivity of homoserine-*O*-transsuccinylase activity in cell-free extracts of four other *metI* mutants: in no case was the activity affected by methionine or α-methylmethionine.

Smith & Childs (1966) observed no abortive transduction, indicating no complementation, between strains bearing any of 37 *metA* point mutations, all of which were located within the *metA* deletion map; hence the map involves a single cistron only. As *metI* mutations were scattered within this map, and one of them failed to show complementation with a *metA* mutation, *metA* and *I* mutations must both result in alterations of the same polypeptide, which must specify both the substrate and the inhibitor binding sites of homoserine-*O*-transsuccinylase. Thus aspartate transcarbamylase in *Escherichia coli* remains the only case yet described in which the catalytic and regulatory sites of an enzyme are carried by distinct polypeptide species (Gerhart & Schachman, 1965). Deletion mapping of histidine feedback-resistant mutants of the *hisG* gene of *Salmonella typhimurium* (Sheppard, 1964) gave similar results to those described in the present work, though the clustering of most of the *hisG* feedback resistance mutations in a single region of the *hisG* deletion map is in contrast to the lack of gross clustering of methionine feedback resistance mutations in the *metA* map (Fig. 3). As *metI* mutations are now known to be situated within the *metA* gene, such mutations will in future publications be designated *metA* (e.g. *metI701* will become *metA701*). This accords with the convention suggested by Demerec, Adelberg, Clark & Hartman (1966).

Finally, by combining the results of the deletion mapping with those of three-point transduction crosses described by Ayling & Chater (1968) (which placed *metI706* between *metA746* and *metH*, and *A15* between *I749* and *thi*), the *metA* deletion map can be orientated unambiguously with respect to *metH* and *thi*. This orientation is indicated in Fig. 3. The estimated recombination frequency of 0·033 along the major part of the *metA* gene, i.e. that containing deletion regions iv to ix (Fig. 3), indicates that it occupies about 1/30th part of the average transduction fragment carrying it (assuming that recombination events occurred at random in the crosses analysed in Table 3).

We are grateful to Dr D. A. Smith, Dr D. A. Lawrence and Dr P. D. Ayling for communicating their unpublished results to us, and to Mr R. J. Harold for useful

discussion. Part of this work was carried out by one of us (K.F.C.) during tenure of a Science Research Council Research Studentship, and formed part of a Ph.D. thesis (Chater, 1969) submitted to the University of Birmingham.

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Suppression of Methionyl Transfer RNA Synthetase Mutants of *Salmonella typhimurium* by Methionine Regulatory Mutations

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SUMMARY

Prototrophic 'revertants' of methionine-requiring methionyl-tRNA synthetase mutants (*metG*) of *Salmonella typhimurium* were examined following an observation that some of them excreted methionine. A number were found to be phenotypically indistinguishable from a class (*metK*) of methionine regulatory mutants. The mutations causing reversion, like *metK* mutations, were located between a serine (*serA*) and a methionine (*metC*) structural gene on the linkage map, and it was concluded that the 'suppressors' were *metK* mutations. The ability of independently isolated *metK* mutations to cause suppression of *metG* mutants was tested and was apparently related to their ability to cause methionine overproduction. A second kind of methionine regulatory mutation (*metJ*) also caused suppression, while a *metA* (homoserine-*O*-transsuccinylase) mutation leading to feedback insensitivity failed to do so. Spontaneous *metG* revertants due to secondary *metJ* mutations were rare, and none due to secondary *metA* mutations was detected.

INTRODUCTION

Gross & Rowbury (1969) have recently shown that methionine-requiring *metG* mutants of *Salmonella typhimurium* (Smith & Childs, 1966) have a methionyl-tRNA synthetase with reduced affinity for methionine. Lawrence (1967) observed that some of the frequent prototrophic 'revertant' colonies that grew when a *metG* culture was spread on unsupplemented minimal agar medium were surrounded by haloes of background growth. The present report describes an investigation of the nature of these 'revertants' in the context of wider studies of the regulation of methionine biosynthesis in *S. typhimurium*. The properties of the three known groups of methionine regulatory mutants, *metA*, *metJ* and *metK*, in this organism were first described by Lawrence, Smith & Rowbury (1968), and are summarized in two accompanying papers (Chater, 1970; Chater & Rowbury, 1970).

We have found that the *metG* 'revertants' tested had secondary *metK* or, more rarely, *metJ* mutations that suppressed the *metG* phenotype, and we have investigated the specificity of the suppression effect. In the course of the work, new *metK* linkage data were obtained.

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METHODS

Media. The media and most supplements have been described in an accompanying paper (Chater, 1970). Additional supplements to minimal media were: L-cysteine and L-histidine (20 μg./ml.): DL-norleucine (1000 μg./ml.); $Na_2S.9H_2O$ (750 μg./ml.); Na_2SO_3 (400 μg./ml.). Abbreviations used are: NA, nutrient agar; NB, nutrient broth, MA, minimal agar; MM, liquid minimal medium.

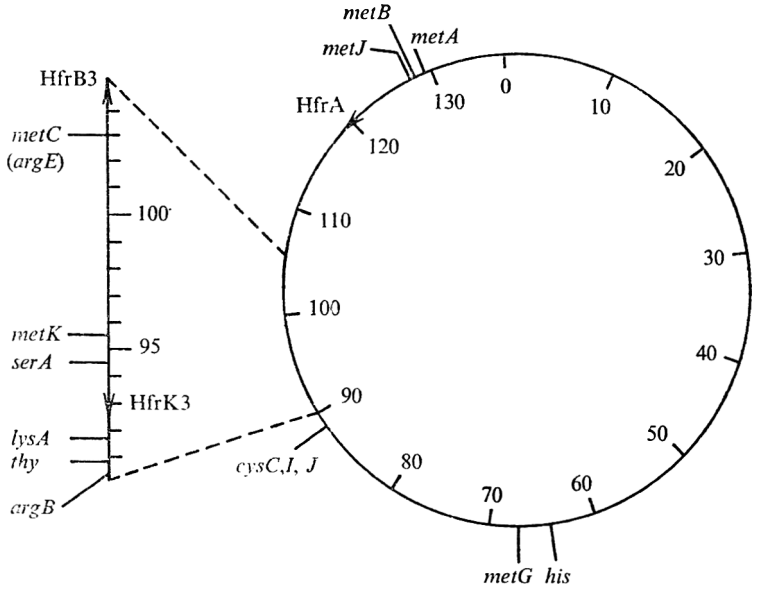

Fig. 1. An abbreviated linkage map of *Salmonella typhimurium*. The map and nomenclature are based on those of Sanderson (1967). Arrows represent the sites of sex factor insertion and direction of chromosome transfer of high frequency donor strains. Figures are time intervals obtained from interrupted mating experiments (Sanderson, 1967). The location of *argE* is only known approximately.

Organisms. The nomenclature is that of Sanderson (1967). All strains were derived from *Salmonella typhimurium* strain LT2, of which a linkage map appears in Fig. 1. Those strains previously described were: *metA94*, *B23*, *G319* and *G419* (Smith & Childs, 1966); HfrA*hisD23metA708* (feedback-resistant), *metK721* and *K731* (Lawrence *et al.* 1968); and *serA11metK747*, HfrK3*metK747his*, and HfrK3*serA13his* (Chater, 1970). The wild-type strain LT2 and mutants *argB69*, *argE8*, *serA11* and *serA13* were provided by J. D. Childs and K. E. Sanderson. HfrA*hisD23metJ744* was obtained as an ethionine-resistant histidine-requiring recombinant after transduction with phage grown on mutant *metJ744* (Ayling & Chater, 1968). New HfrK3*metKhis* strains were obtained by transduction of HfrK3*serA13his* with phage propagated on the relevant *metK* strain. HfrB3*hisD23cysC200* was obtained from HfrB3*hisD23* (provided by D. A. Smith) in two steps. First, the parent strain was treated with *N*-methyl *N'*-nitro *N*-nitrosoguanidine, and a derivative isolated which responded to cysteine or sulphide, but not to sulphite, +histidine. This strain was possibly defective in the *cysI* or *J* genes, both of which are cotransducible with *cysC*, and it

was transduced with phage grown on strain *cysC200* (provided by M. A. Qureshi) on MA + histidine + sulphite. Of the recombinants, 17 % were histidine + sulphite-requiring, and one of these was purified as HfrB3*hisD23cysC200*. HfrB3*hisD23serA11metK747* was obtained as one of the 3 % of serine + histidine requiring colonies among the recombinants that grew when HfrB3*hisD23* was transduced with phage grown on strain *serA11metK747*, on MA + histidine + serine + ethionine. Strain *metC378serA11* was obtained as a methionine + serine requiring recombinant from the cross *metC378cysCD519* (Smith & Childs, 1966) × HfrB3*hisD23serA11metK747*. Since ethionine resistance could not be tested in a methionine auxotroph, to confirm that the new double mutant did not possess *metK747* it was crossed with HfrK3*his* and HfrK3*metK747his* on MA, and the prototrophic recombinants were scored for ethionine resistance. With the former donor, none of 359 recombinants was resistant, while with the latter donor 5 % of the recombinants were sensitive. The latter result in particular confirmed the presence of *metK*$^+$ in the recipient.

Techniques. All incubation was at 37° unless stated otherwise. The maintenance of stock cultures, and the propagation, assay and maintenance of transducing phage P22, were as described by Smith (1961). *N*-Methyl *N'*-nitro *N*-nitrosoguanidine mutagenesis, and conjugation and transduction experiments, were performed as described by Ayling & Chater (1968). Detection of methionine excretion and analogue resistance by streak testing, and growth experiments in liquid media, were essentially as described by Lawrence *et al.* (1968). Enzyme assays followed the procedures of Rowbury & Woods (1966) and Gross & Rowbury (1969).

RESULTS

The isolation and phenotypes of revertants of metG mutants. In the initial experiments, about 2×10^8 bacteria from saturated NB cultures of mutants *metG319* and *G419* were spread on MA. After 48 h. incubation, between 10 and 100 revertant colonies grew on each plate. A number of these colonies were picked off and taken through two single colony isolations on NA. The resulting strains were then tested for their ability to cross-feed a methionine auxotroph; their growth rates in MM in the presence and absence of the methionine analogues α-methylmethionine, ethionine and norleucine were measured; and the activity and repressibility of two of their methionine biosynthetic enzymes, cystathionine synthetase (*metB*) and cystathionase (*metC*), were assayed. It was also desirable to test whether the phenotypes of the revertants indicated by these tests were retained in the absence of the *metG* mutations, and genetic manipulation (described in the next section of the Results) permitted the isolation of strains retaining the analogue resistance characteristic of the original revertants, despite possession of the *metG*$^+$ allele.

The results of such tests with a representative revertant of *metG319* and one of *G419* (retrospectively designated *metG319metK755* and *metG419metK756*) and their *metG*$^+$ derivatives are given in Table 1. For comparison, representative methionine-excreting and nonexcreting *metK* strains (*K721* and *K731*, respectively) and the wild-type were also tested. The phenotypes conferred by possession of mutations that suppressed *metG* mutants were the same in *metG*$^+$ strains as in the original revertants; the revertants, like known *metK* mutants, were resistant to all three analogues, and fell into methionine-excreting and nonexcreting categories; and the enzyme levels of

the nonexcreting revertant (*metG419metK756*) were normally regulated, like those of wild-type and *metK731* strains, while those of the excreting revertant (*metG319 metK755*) were as high as those of strain *metK721* in the absence of methionine, but were repressed during growth in the presence of methionine. Analogue resistance and methionine excretion tests carried out with 14 other revertants of *metG319* and 6 of *G419* were in agreement with these results, all the *G319* revertants excreting, while the *G419* revertants failed to do so. Later tests have shown that some excreting *G419* revertants also occur.

Table 1. *The phenotypes of metG319 revertants and other strains*

Abbreviations: MM = minimal medium; AMM = MM + α-methylmethionine (1000 μg./ml.); ETH = MM + ethionine (1000 μg./ml.); NOL = MM + norleucine (1000 μg./ml.); −MET = cells grown in the absence of methionine; +MET = cells grown in the presence of 10 mM methionine; S = exponential growth not obtained. Enzyme activities expressed as μmoles product/mg. protein/h.

	Excretion of methionine		Generation time (min.)				Enzyme activities: Cystathionine synthetase		Enzyme activities: Cystathionase	
Strain	25°	37°	MM	AMM	ETH	NOL	−MET	+MET	−MET	+MET
*metG319metK755**	+	−	65	70	70	75	2·00	0·16	1·27	0·83
*metG419metK756**	−	−	63	76	63	70	0·14	0·01	0·65	0·34
metK755(metG⁺)†	+	−	66	66	66	70	1·25	0·15	0·88	0·30
metK756(metG⁺)†	−	−	58	59	59	68	0·34	0·02	0·70	0·32
metK721(metG⁺)	+	+	72	69	69	75	2·00	2·40	1·30	1·34
metK731(metG⁺)	−	−	55	69	58	59	0·20	0·05	0·63	0·41
Wild type	−	−	55	S	S	S	0·18	0·03	0·65	0·38

* Retrospective designation of *metG* revertants.

† For derivation, see Results ('Location of the suppressor mutations on the *Salmonella typhimurium* linkage map').

The location of the suppressor mutations on the Salmonella typhimurium linkage map

The accompaniment of suppression of the *metG* phenotype in the revertants by the *metK* phenotype suggested that the suppressors might be *metK* mutations. These are 1 to 3 % cotransduced with the *serA* gene (Lawrence *et al.* 1968). To test the linkage of the suppressors with *serA*, phage propagated on the revertant strains *metG319metK755* and *G419K756* was used to transduce strain *serA11* to prototrophy, and 1350 and 781 recombinants, respectively, were tested by replication for ethionine resistance. In both cases cotransduction of resistance with *serA*⁺ occurred, the frequency being 1·7 % with *metK755* and 3·2 % with *K756*. The strains *metK755(metG⁺)* and *metK756(metG⁺)* in Table 1 were originally obtained as resistant recombinants in these crosses.

Thus the phenotypic and genetic similarities between the suppressor mutations and *metK* mutations were such as to leave little doubt of the identity of the two types of mutation. Nonetheless, the possibility still existed that the suppressors might be situated on one side of *serA*, and *metK* on the other: indeed, it was possible that the two types of *metK* mutation, differing with respect to methionine excretion, originally described by Lawrence *et al.* (1968) might be separated by *serA*. Experiments were therefore designed which simultaneously eliminated these possibilities and revealed

the orientation of the *serA–metK* region with respect to the whole linkage map (Fig. 1). The experiments utilized a new donor strain, HfrK3, isolated in mutant *serA13* by K. E. Sanderson (personal communication). He found that the site of insertion of the HfrK3 sex factor was close to *serA*, *argB* being transferred very late in HfrK3-mediated conjugation. It was first necessary to locate the sex factor more accurately. For this purpose, the strain HfrK3*metK747his* was crossed with mutants *argB69*, *argE8* and *serA13* on MA, and the prototrophic recombinants tested for ethionine resistance by replication. The frequency of recombinants was about 100-fold greater with *serA13* and *argE8* recipients than with *argB69*, indicating that *serA* and *argE* were transferred early, and *argB* late, by HfrK3. As 57 % of the recombinants obtained with *serA13* were ethionine-resistant, *metK* was also an early marker.

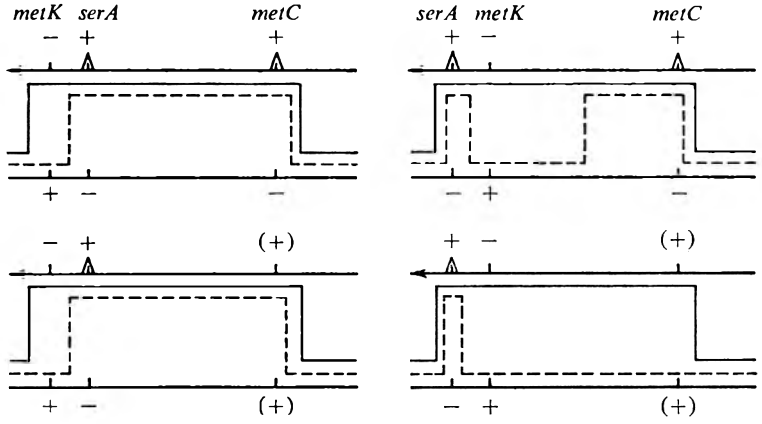

Fig. 2. The generation of prototrophic recombinants in crosses between *serA* or *serAmetC* recipients and an HfrK3*metKhis* donor. Heavy unbroken lines represent donor and recipient chromosomes and an arrow the leading point in transfer of the donor chromosome. Light unbroken lines indicate the recombination events generating *metK* recombinants, and broken lines those generating *metK*$^+$ recombinants. The situations for the alternative sequences are given to the left and right, and for the crosses with *serA* and *serAmetC* recipients at the bottom and top, respectively, of the figure. Triangles indicate selected alleles.

The ease with which *metK* derivatives could be obtained from HfrK3*serA13his* made it useful for comparative studies of different presumptive *metK* alleles. In all, five such derivatives were used, carrying *metK721*, *K731*, *K747*, *K755* and *K756*. These mutations differed in their modes of origin and methionine excretion phenotypes, as listed in Table 2. Each strain was crossed with the two recipients *serA11* and *serA11metC378*, with selection for prototrophic recombinants, which were tested for ethionine sensitivity by replication. Figure 2 shows that if a *metK* mutation were situated between *serA* and the leading point in chromosome transfer, the ratio of sensitive to resistant recombinants should have been independent of selection for the donor's *metC*$^+$ allele, i.e. the same proportion of sensitive colonies should have occurred with both recipients. However, if the *metK* mutation were situated between *serA* and *metC*, the proportion of sensitive recombinants should have been smaller when simultaneous selection was made for the donor's *serA*$^+$ and *metC*$^+$ alleles than when the former was selected separately. The results of those crosses are given in Table 2. Plainly, the proportion of sensitive recombinants was greatly decreased

when *serA11metC378* was the recipient. We concluded that all the *metK* mutations tested were situated between *serA* and *metC* on the linkage map. (It should be pointed out that a certain amount of difficulty was experienced in distinguishing between resistant and sensitive recombinants in conjugational crosses involving *metK755*, which may account for the relatively lower number of resistant recombinants detected in those crosses).

Table 2. *The location of metG suppressor and metK mutations relative to metC and serA*

Mating mixtures of the two recipients and various *metK* and suppressor derivatives of HfrK3*his* were diluted in NB, and 0·1 ml. amounts spread on MA. After incubation for 48 h., plates with fewer than 200 recombinant colonies were replicated to MA + ethionine to identify ethionine sensitive (ETH-S) colonies. Controls showed that none of the markers reverted significantly.

	Recipient			
	serA11metC378		*serA11*	
Relevant donor marker	Number scored	ETH-S (%)	Number scored	ETH-S (%)
*metK721**	263	4	254	38
metK731†	270	3	417	12
metK747‡	261	5	247	32
metK755§	181	19	201	47
metK756§	264	2	282	10

* Mutations resulting in strong methionine overproduction.
† Mutations not resulting in methionine overproduction.
‡ Mutations resulting in weak methionine overproduction.
§ Mutations obtained as suppressors of *metG* mutants.

Further support for this gene sequence was obtained when strain *serA11metC378* was crossed with four of the *metK* donor strains, but with selection for *metC*$^+$ only, serine being present in the selective medium. The recombinants were tested by replication for ethionine resistance and ability to grow without serine. About 10% of the recombinants from each cross were serine-requiring, and among these the numbers that were ethionine-resistant, together with the relevant donor marker and the number of colonies scored, were: *metK721*, 3/22; *K731*, 1/9; *K747*, 3/10; and *K755*, 9/40. If the clockwise sequence *–metK–serA–metC–* were correct, resistant, serine-requiring recombinants should have constituted a rare four-crossover class, whereas on the alternative sequence *–serA–metK–metC–* the ratio of the distance between *serA* and *metK* to that between *serA* and *metC* (Fig. 2) should have been reflected in the frequency of resistance among serine-requiring colonies. The former interval (*serA* to *metK*) is probably about 1 min., as these markers are about 1% cotransduced, and the latter interval was shown by Sanderson & Demerec (1965) to be 5 to 8 min. (see also Fig. 1). In each case the frequencies obtained were compatible with the ratio of these intervals, favouring the sequence *–serA–metK–metC–*.

For independent confirmation of the map location of *metK*, we determined the proportion of ethionine-resistant colonies among prototrophic recombinants from the crosses *cysC200* × HfrB3*hisD23serA11metK747* and *serA11metK747* × HfrB3*his-D23cysC200*. If the correct clockwise sequence were *–cysC–metK–serA–*, the same proportion of recombinants should have been resistant in both crosses, as this

proportion would have been determined by the radio of the intervals between *metK* and *serA*, and *cysC* and *serA*. In contrast, if the correct clockwise sequence were *–cysC–serA–metK–*, the reciprocal crosses should have given widely different frequencies of resistant recombinants since, when *cysC200* was present in the recipient, resistant recombinants would have required four crossovers for formation. We found that when *cysC200* was the recipient 5 % of 891 recombinants scored were resistant. When *metK747serA11* was the recipient, however, 16 % of 407 recombinants were resistant. The relative rarity of resistant recombinants in the former cross favoured the clockwise sequence *–cysC–serA–metK–*, which was compatible with the clockwise sequence *–serA–metK–metC–* previously determined.

As *metK* and *serA* are only weakly cotransduced, it is possible that they might also be carried by overlapping neighbouring transduction fragments. In this context, none of more than 500 recombinants scored in each of the crosses *serA11* × *lysA8*, *lysA8* × *serA11*, *argE8* × *metK721*, or *metC50* × *metK721*, gave any evidence of co-transduction of the donor phenotype, though it is not at all certain that the transduction fragments involved were contiguous.

An examination of the allele specificity of the metG–metK relationship

As the suppressors of *metG319* and *G419* had been clearly identified as *metK* mutations, it was of interest to examine the allele specificity of the *metG-metK* relationship. Attempts to transduce *metG* recipients to prototrophy with phage propagated on various *metK* mutants failed to yield any clear results because the

Table 3. *Suppression of metG mutants by various metK mutations*

Overnight NB cultures of recipients and donors were mixed in the ratio 9:1, diluted 10^{-4} in NB, and 0·1 ml. amounts spread on MA. The numbers of colonies growing after 48 h. incubation were recorded.

	Number of colonies					
	With HfrK3*his* donors possessing:					Control of recipient alone
Recipient	*metK721**	*metK731*†	*metK747*‡	*metK755*§	*metK756*§	
serA11	234	189	379	312	329	0
metG319	156	2	11	219	7	1
metG419	107	53	222	242	523	22
Control of donor alone	0	0	0	0	0	

* Mutations resulting in strong methionine overproduction.
† Mutations not resulting in methionine overproduction.
§ Mutations resulting in weak methionine overproduction.
‡ Mutations obtained as suppressors of *metG* mutants.

frequency of transduction was lower than the reversion frequency of the recipients. An alternative method, using HfrK3 mediated conjugation, was therefore devised.

Conjugational crosses between *metG* recipients and HfrK3 donors of *metK*, with selection on MA for prototrophic recombinants, could give rise only rarely to true *metG*$^-$ recombinants, because the donor's *metG*$^+$ marker was transferred late (i.e. after about 110 min.) and was closely linked to the counter-selected donor *his* marker (Fig. 1). In contrast, the donor's *metK* marker was transferred very early, and was therefore very frequently inherited (see preceding section). If the incoming *metK* allele caused suppression of the recipient's *metG* phenotype, this should have resulted

in large numbers of prototrophic recombinant colonies. The high fertility of HfrK3 in plate crosses permitted the use of highly diluted (10^{-4}) mating mixtures, which made it improbable that colonies due to reversion of the recipient could interfere significantly with the clarity of this test. To permit estimation of the frequency of entry of the donor *metK* allele into the *metG* recipient, parallel crosses were carried out with a *serA* recipient, as *metK* had been shown (Table 2) to be more than 50 % linked to *serA* in HfrK3-mediated conjugation. The results are given in Table 3. Of the five donor strains tested, two (carrying *metK721* and *K755*) gave high numbers of recombinants with all three recipients; two more (carrying *metK747* and *K756*) did so with *serA11* and *metG419* but not with *metG319*; and the donor of *metK731* did so only with *serA11*. In all crosses with *metG319* and *G419* giving a high frequency of recombinants, more than 90 % of the colonies proved to be ethionine resistant, confirming that the mutations causing suppression and resistance were closely linked. (In the crosses with *metG319* that failed to give frequent recombinants, and on the *G319* control plate, all the colonies were ethionine-sensitive, while most of the colonies on the *G419* control plate were resistant.)

We concluded from this experiment that suppression of *metG* mutations was rather non-specific, although certain *metG-metK* combinations failed to give suppression. A pattern was apparent in which mutations suppressing *metG319* also suppressed *G419* (though the reverse did not always happen) and were of the methionine strongly excreting class, while those suppressing only *metG419* were of the weakly or non-excreting classes.

The K_m (methionine) of methionyl-tRNA synthetase in suppressed metG mutants

As suppression of *metG* mutants by *metK* mutations lacked specificity, it was probably not due to missense or nonsense suppression (Yanofsky, Helinski & Maling, 1961; Garen, 1968). In confirmation of this, the K_m (methionine) values of the *metG* enzyme, methionyl-tRNA synthetase, in *metG319metK755* and *G419K756* were found to be $3{\cdot}3 \times 10^{-3}$M and $1{\cdot}0 \times 10^{-2}$M, respectively. The values reported previously (Gross & Rowbury, 1969) for the enzymes from the unsuppressed *metG319* and *G419* parent strains were $1{\cdot}0 \times 10^{-2}$M and $1{\cdot}0 \times 10^{-3}$M, respectively. Thus the presence of the suppressor mutations did not significantly decrease the K_m (methionine) values of the mutant synthetases, which were several orders of magnitude higher than the wild-type value of $1{\cdot}8 \times 10^{-5}$M (Gross & Rowbury, 1969).

Tests of the ability of other methionine regulatory mutations to suppress metG mutants

If methionine overproduction is the cause of suppression of *metG* mutants by *metK* mutations, then *metA* (feedback-resistant) and *metJ* mutations resulting in methionine overproduction should also suppress *metG* mutants. To test this, *metG319* and *G419* recipients were crossed with HfrA*hisD23* donor strains possessing the mutations *metA708* (feedback resistance) or *metJ447*, with selection on MA. As in similar experiments with HfrK3 donors of *metK* alleles, *metG*$^+$ was transferred late and was closely linked to the donor's counter-selected *his* marker (Fig. 1), while the *metA* and *J* mutations were transferred early, so that if prototrophic colonies arose very much more frequently in these crosses than on recipient control plates, it could be concluded that effective suppression had occurred. The results of these experiments are given in Table 4. Crosses with *metA94* (auxotrophic) and *B23* recipients showed

the frequency with which *metA* and *metJ* respectively were transferred. Only *metJ744* seemed able to suppress the *metG* mutants, in that crosses involving it gave fairly similar high numbers of colonies with *metB23*, *G319* or *G419* recipients, while the numbers on control plates were relatively low. In contrast, crosses between the *metG* recipients and HfrA*hisD23metA708* gave relatively few colonies, there being no significant excess over the controls. Considerably more colonies grew in the cross with the recipient *metA94*, and they all proved on replication to be resistant to α-methylmethionine, confirming that *metA708* was present and behaving normally in the donor.

Table 4. *Suppression of metG mutants by a metA (feedback resistance) and a metJ mutation*

Overnight NB cultures of recipients and donors were mixed in the ratio 9:1, diluted 10^{-3} in NB and 0·1 ml. amounts spread on MA. The numbers of colonies growing after 48 h. were recorded. Where two figures are given they indicate the numbers of colonies on duplicate plates.

	Number of colonies			
	With HfrA*hisD23* donors possessing:			Control of recipient a one
Recipient	*metA708*	*metJ744*	*metA⁺metJ⁺*	
metA94	264	.	144	1
metB23	.	*ca.* 1,500	453	0
metG319	128;208	817;664	116;95	110;146
metG419	27;41	235;259	39;35	22;27
Control of donor alone	1	1	1	.

As a further proof that the prototrophic colonies growing in the crosses between *metG* recipients and the *metJ* donor possessed *metJ* but not *metK* mutations, two colonies from each cross were found to be resistant to ethionine, but not to α-methylmethionine or norleucine, in streak tests. Phage propagated on these strains gave 96% cotransduction of ethionine resistance with *metB*, but none with *serA*.

We concluded that *metJ* mutations could suppress *metG* mutants, but at least one *metA* (feedback resistance) mutation was unable to do so. It should therefore have been possible to identify spontaneous revertants of *metG* mutants resulting from *metJ* mutations. E. S. Bridgeland (personal communication) screened *metG319* revertants by replication for resistance to ethionine but sensitivity to α-methylmethionine. Colonies apparently possessing this phenotype were often found, but most of them proved after purification to be of *metK* phenotype. However, one revertant which retained its *metJ* phenotype after purification was obtained. Its growth in MM was unaffected by ethionine, but drastically inhibited by α-methylmethionine, and phage propagated on it gave 97% cotransduction of ethionine resistance with *metB*. The suspected presence of a *metJ* mutation was therefore confirmed.

In the course of these experiments, no revertants with the *metA* (feedback resistance) phenotype of α-methylmethionine resistance but ethionine sensitivity were obtained among several hundreds of *metG319* revertants screened. If such revertants ever occur, they must be considerably rarer than the *metK* type, and possibly also rarer than the *metJ* type of revertant.

DISCUSSION

The *metG* mutants used in this study possessed methionyl-tRNA synthetases with relatively low affinities for methionine (Gross & Rowbury, 1969). The mutants' methionine requirement was suppressible by various *metJ* or *K* mutations, either occurring spontaneously or being introduced by recombination. We feel that suppression probably occurs because the increase in the methionine pool resulting from the regulatory mutation is sufficient to compensate for the reduced affinity of the mutant tRNA synthetase for methionine. It is not understood why *metA* feedback resistance mutations apparently fail to cause suppression. In an analogous situation described by Roth & Ames (1966) a *hisS* (histidyl-tRNA synthetase) mutant was suppressible by a *hisG* (histidine feedback resistance) mutation. A second anomaly was the suppression of *metG419* (but not *G319*) by a *metK* mutation that did not give rise to detectable methionine excretion: our hypothesis demands that, despite their phenotype, in such mutants a higher methionine pool must be available for protein synthesis than in the wild-type.

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SHORT COMMUNICATIONS

Genetic Recombination in a Thermophilic Actinomycete, *Thermoactinomyces vulgaris*

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Thermoactinomyces vulgaris is strongly thermophilic, growing rapidly above 50° (Tsilinsky, 1899). Its spores have a multilayered outer integument (Cross, Walker & Gould, 1968; Dorokhova, Agre, Kalakoutskii & Krassilnikov, 1968), possess dipicolinic acid (Cross *et al.* 1968) and withstand boiling in aqueous suspension for considerable periods (Tsilinsky, 1899; Erikson, 1952; Cross, 1968). In all these features they resemble the spores of the eubacterial bacilli and Clostridia and differ from those of the mesophilic Streptomycetes (Glauert & Hopwood, 1961; Rancourt & Lechevalier, 1964; Bradley & Ritzi, 1968; Wildermuth & Hopwood, 1970).

Thermoactinomyces vulgaris is one of two organisms causally implicated in the disease known as Farmer's Lung, a condition in which hypersensitivity to antigens of the organisms occurs as a result of inhaling vast quantities of their spores from mouldy hay (Pepys *et al.* 1963; Cross, 1968).

We had several motives for attempting to develop a useful system of genetic analysis in *Thermoactinomyces vulgaris*. One was to provide a genetic comparison with the mesophilic *Streptomyces coelicolor* from the point of view of spore morphogenesis (Hopwood, Wildermuth & Palmer, 1970), sexual biology (Hopwood, Harold, Vivian & Ferguson, 1969) and genome organization (Hopwood, 1967). Another was to open the way for a genetic approach to the biochemical basis of thermophily; we are not aware of any previous report of genetic recombination in a thermophilic bacterium. An obvious attraction of the organism for genetic studies is its very rapid growth rate.

The strains of *Thermoactinomyces vulgaris* used in our work had a nutritional requirement satisfied by hydrolysed casein in Czapek–Dox medium. Our 'minimal medium' (MM) therefore consisted of Czapek–Dox medium (Oxoid), containing 0·6% (w/v) Bacto vitamin-free Casamino-acids (Difco); the 'complete medium' (CM) consisted of MM supplemented with L-tryptophan (50 μg./ml.), adenine, guanine, thymine and uracil (10 μg./ml. each), biotin, nicotinamide, *p*-aminobenzoic acid, pantothenic acid, pyridoxin, riboflavin and thiamine (1 μg./ml. each). Auxotrophic mutants were isolated after exposure of spore suspensions in water to approximately 2000 ergs/mm.^{2} ultraviolet light, plating on CM to yield 100 to 300 colonies per plate, and replicating by means of velvet to MM. Streptomycin-resistant mutants were isolated by spreading dense spore suspensions on CM containing 25 μg./ml. streptomycin sulphate. All cultures were incubated at approximately 52°.

We started with strain CUB76 of *Thermoactinomyces vulgaris* kindly supplied by Dr T. Cross, University of Bradford. Preliminary attempts to demonstrate genetic

recombination in mixed cultures of two mutant derivatives of CUB 76 were not successful; a few colonies appeared on the selective plates, but the yield was no greater from the mixed culture than from the parental strains grown separately.

Since recombination was not demonstrated in CUB 76, other wild-type strains of *Thermoactinomyces vulgaris* were isolated from soil samples, using the selective isolation conditions described by Cross (1968). Samples of soil from various localities were shaken in water and drops of the liquid were spread on plates of MM containing 25 μg./ml. sodium Novobiocin and 50 μg./ml. Actidione. The plates were incubated for 24 h at 52°, when colonies of *T. vulgaris* appeared on all plates. A single colony was chosen from each of several soil samples, and the resulting strains were used as the starting cultures for the isolation of auxotrophic and streptomycin-resistant mutants. Attempts were then made to select prototrophic streptomycin-resistant recombinants from mixed cultures of an auxotrophic streptomycin-resistant mutant with its wild-type progenitor. One wild-type, stock number 1227 (isolated from garden soil kindly collected by Mr G. R. Davies at Wymondham, Norfolk), gave clear evidence of recombination with its nicotinamide-requiring, streptomycin-resistant derivative, and attention was then confined to this strain and its mutant and recombinant descendants. Further auxotrophic mutants were isolated, including strains requiring adenine, riboflavin, thiamine, thymine, tryptophan or uracil. Mixed cultures of these strains yielded recombinants on selective media with frequencies in the region of one per 10^3 to 10^5 spores of parental phenotype; these frequencies were at least 100 times higher than could be explained by mutation of the markers concerned as judged by mutation frequencies in cultures of the parents grown separately.

A first attempt to characterize the process of genetic recombination in strain 1227 involved four-point crosses of the kind used by Hopwood (1959) to analyse recombination in *Streptomyces coelicolor*. The procedure was to plate samples of the same spore suspension, derived from a mixed culture of the two parents, on four selective media, on each of which two different markers were selected, leaving two markers unselected. A sample of recombinants was picked from each medium to plates of the same composition and classified into the four theoretically possible classes by replica plating to test media. Considering all four media together, nine out of the 16 possible combinations of the parental markers were theoretically recoverable, comprising both members of two complementary pairs and one member of five others. Neither member of the parental pair of genotypes could, of course, be selected.

The following four markers were used in a series of crosses: requirements for nicotinamide (*nic*), thiamine (*thi*) or uracil (*ura*) and resistance to streptomycin (*str−r*). Table 1 gives the results of the first cross, involving two mutant strains, *nic thi ura*$^+$ *str−r* × *nic*$^+$ *thi*$^+$ *ura str−s*. Recombinants obtained by crossing these strains were used to make two further crosses with the same markers in different coupling arrangements: *nic*$^+$ *thi ura str−r* × *nic thi*$^+$ *ura*$^+$ *str−s* and *nic thi*$^+$ *ura str−r* × *nic*$^+$ *thi ura*$^+$ *str−s*.

The first conclusion from these crosses is that we are dealing with a true recombination process in which substitution of homologous segments of genetic material occurs, rather than with some kind of replication (Clark, 1967) in which genetic material from one strain is merely added to the genetic complement of the other. The most direct evidence for this conclusion is the isolation of certain classes of progeny (*nic*$^+$ *thi ura str−r*; *nic thi*$^+$ *ura str−r*; Table 1) expressing auxotrophic markers, which are unlikely to be dominant, inherited from both parents. Confirmation of this

conclusion came from the results of the crosses of pairs of recombinants. These pairs did not give rise to a majority of colonies displaying the phenotype of one of their own parents.

Table 1. *Results of crossing strains nic-1 thi-3 ura-1$^+$ str-1 and nic-1$^+$ thi-3$^+$ ura-1 str-s*

	Numbers of recombinants of each genotype on selective media supplemented as indicated†			
Genotypes of progeny*	Nicotinamide uracil streptomycin	Nicotinamide	Thiamine uracil streptomycin	Thiamine
nic thi + *str-r*	—	—	—	—
+ + *ura str-s*	—	—	—	—
nic + + *str-r*	20	4	—	—
+ *thi ura str-s*	—	—	—	—
+ + *ura str-r*	57	—	73	—
nic thi + *str-s*	—	—	—	—
+ *thi* + *str-r*	—	—	23	12
nic + *ura str-s*	—	—	—	—
+ + + *str-s*	—	94	—	169
nic thi ura str-r	—	—	—	—
+ + + *str-r*	1	0	0	1
nic thi ura str-s	—	—	—	—
nic + *ura str-r*	1	—	—	—
+ *thi* + *str-s*	—	—	—	0
+ *thi ura str-r*	—	—	1	—
nic + + *str-s*	—	0	—	—
Total recombinants classified	79	98	97	182

* Arranged in complementary pairs; the top pair is parental; the bottom three pairs differ from both parents by two markers; the remainder differ from one parent by only one marker.

† Spores from the mixed culture were plated on the four selective media and samples of the resulting recombinant colonies were classified in respect of the non-selected markers (giving four possible genotypes on each medium). Total colony counts on the four media were approximately equal. A dash indicates that a particular genotype could not grow on the medium in question.

The second conclusion is that *Thermoactinomyces vulgaris* resembles all other protokaryotes so far studied in having partially diploid zygotes. The evidence for this is the finding that the great majority of the recombinants differed from one or other parent by only a single marker; these were the classes *nic thi$^+$ ura$^+$ str−r*, *nic$^+$ thi$^+$ ura str−r*, *nic$^-$ thi ura$^+$ str−r* and *nic$^+$ thi$^+$ ura$^+$ str−s*, irrespective of the coupling of the markers in the three crosses. Had both parents contributed a complete genome to the zygotes, the observed result is incompatible with any of the three possible sequences of four markers on a single circular linkage group, any of the 12 sequences on a linear linkage group, or an arrangement involving more than one linkage group.

A final conclusion is that the process of genetic transfer involves fragments representing only a small fraction of the total genome, otherwise recombinants inheriting two markers from each parent should have been frequent, as they are in *Streptomyces coelicolor*. An alternative possibility is that the markers so far studied are carried on separate chromosomes, genetic transfer normally involving only one. In any event, the results preclude the assessment of linkage at this stage; in this they differ from the results of comparable crosses in *S. coelicolor* in which linkage was successfully demon-

strated by such crosses because the linkage map is comparatively short, and genetic transfer involves large portions of the genome (Hopwood, 1959, 1965, 1966, 1967); thus in *S. coelicolor* the situation did not arise in which all four markers in a cross could fail to show linkage.

We conclude that our strain of *Thermoactinomyces vulgaris* has a process of true recombination that should qualify it as a suitable organism for some of the genetic studies alluded to at the beginning of this note.

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Nitrogen Fixation by Sporulating Sulphate-reducing Bacteria Including Rumen Strains

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The acetylene test for nitrogen fixation has been an important tool in reassessing the ability of various groups of micro-organisms to fix nitrogen (Parejko & Wilson, 1958; Millbank, 1969; Hill & Postgate, 1969). As a result of this reassessment, it has been found that several aerobic genera such as *Azotomonas*, *Pseudomonas*, *Nocardia*, *Pullularia* and yeasts probably do not fix N_2; on the other hand, nitrogen fixation has proved to be far more widespread among the sulphate-reducing bacteria of the genus *Desulfovibrio* than was earlier thought (Reiderer-Henderson & Wilson, 1970). This communication reports evidence for fixation by type strains of mesophilic, spore-forming, sulphate-reducing bacteria, genus *Desulfotomaculum* (Campbell & Postgate, 1965), including strains originating from the rumens of hay-fed sheep. Some data with type strains of Desulfovibrio are included to supplement the findings of Reiderer-Henderson & Wilson (1970).

Desulfotomaculum ruminis and *Dm. orientis*, as well as the Desulfovibrio species, were incubated at 30° and *Dm. nigrificans* at 55° in medium B (Postgate, 1966). Growth and acetylene reduction in N-deficient medium was tested for in Pankhurst (1967) tubes as described by Campbell & Evans (1969), except that gassing with N_2 was omitted and instead, about 2 h. after setting up, 10 ml. N_2 were injected through the side arm to replace oxygen absorbed by the pyrogallol plug. The N-deficient medium was a variant of medium B: ammonium chloride was omitted and the trace-element mixture specified by Postgate (1966) was included. In all tests a tube containing 100 μg. yeast extract/ml. (cf. Reiderer-Henderson & Wilson, 1970) was included and also one with 2 mg. NH_4Cl/ml. to repress nitrogenase synthesis. When growth was obvious because of blackening of the culture (the 1 % to 10 % inoculum carried over sufficient fixed N for marginal growth), 2·5 ml. of N-free or NH_4-containing medium was injected aseptically into the culture and, 24 to 48 h. later, 1 ml. of C_2H_2, freshly prepared from $Ca_2C_2 + H_2O$, was injected via the side arm. Three to 5 ml. N_2 were then injected to allow an excess of gas for sampling; gas samples were removed at intervals up to 3 days and analysed for ethylene by vapour-phase chromatography as reported elsewhere (Hill & Postgate, 1969). Progressive formation of ethylene, which did not take place in cultures containing NH_4Cl, was taken as presumptive evidence for the presence of nitrogenase; negative cultures were tested again after 5 days, before discarding.

Though cultural tests had earlier failed, this procedure confirmed the presence of nitrogenase in the marine strain of *Desulfovibrio desulfuricans*, NORWAY 4, in *D. vulgaris*, strain HILDENBOROUGH, and in *D. gigas*, as reported by Reiderer-Henderson & Wilson,

(1970). *D. desulfuricans*, strains BERRE SOL (NCIB8388) and BERRE EAU (NCIB8387) grew readily in N-free media as claimed by Le Gall, Senez & Pichinoty (1959) and reduced acetylene readily; the holotype strain of *D. desulfuricans*, strain ESSEX6 (NCIB8307), reduced acetylene and so did a strain of unusual semilunar morphology provided by Dr H. Veldkamp; type strains of *D. africanus* (strain BENGHAZI; NCIB8401) and *D. salexigens* (strain BRITISH GUIANA; NCIB8403) did not, nor did a second halotolerant strain of *D. desulfuricans* (strain EL AGHEILA A; NCIB8309). Acetylene reduction, when observed, was inhibited by NH_4Cl, usually completely, but in one test (see below), only partially. It was not consistently affected by the small amount of yeast extract: sometimes yeast extract accelerated this reaction, sometimes the reaction was slowed.

Table 1. *Acetylene reduction and nitrogen fixation by Desulfotomaculum species*

For procedures see text. 'Y' signifies 100 μg. yeast extract/ml.

Organism	nmoles C_2H_4 produced/7 ml. culture: After 1 day −Y	After 1 day +Y	After 3 days −Y	After 3 days +Y	μg. N/ml. after 12 days in medium+Y, control acidified with H_2SO_4: Control	Culture	Atom % ^{15}N excess after 19 days under Ar+0·1 atm. 99 % $^{15}N_2$ in medium+Y
Dm. ruminis							
(NCIB 10,149)	64	85	483	484	22·4	27·6	0·009
(NCIB8542)	13·9	9·8	143	67	24·6	27·6	0·010
Dm. orientis							
(NCIB8382)	3·4	13·2	3·3	83	23·8	28·0	0·027
Dm. nigrificans							
(NCIB8395)	0	0	0	0	—	—	—

Desulfovibrio desulfuricans BERRE SOL cultures produced 12 to 330 nmole C_2H_4 in 1 day in 12 comparable tests; the amount of fixed N in a culture increased from 12·6 to 15·86 μg. N/ml. over 11 days; another reached 0·395 atom % excess ^{15}N in 19 days.

Table 1 lists the results of tests on Desulfotomaculum species. Like Reiderer-Henderson & Wilson (1970), I obtained no evidence of fixation by the thermophile *Dm. nigrificans*. The two strains of *Dm. ruminis* and the one strain of *Dm. orientis* showed unequivocal activity, completely repressed by NH_4Cl, except in one out of three tests with *Dm. orientis* where repression was only partial. The values for ethylene produced given in Table 1 cannot be taken as measures of the relative activities of the strains because the time at which to inject acetylene was judged subjectively and the population densities were unlikely to have been similar. From several experiments Desulfotomaculum species appeared to reduce acetylene at about 10 % of the rate usually found with BERRE strains of Desulfovibrio. Assuming N_2 is reduced one-third as rapidly as C_2H_2, one can calculate approximate rates of N_2 fixation to which the figures for acetylene reduction correspond: for Desulfotomaculum species they would be in the region of 2 to 5 μg. N fixed/ml. culture. Table 1 includes analytical data and tests with $^{15}N_2$ which, though not impressive on their own, support the presumptive evidence of the acetylene test and establish nitrogen fixation among the mesophilic members of the genus *Desulfotomaculum*.

Reiderer-Henderson & Wilson (1970) deduced from their experiments that N_2 fixation is more widespread than hitherto thought in the genus *Desulfovibrio*. My

experiments support this view, and the relatively small fixations obtained by analytical or isotopic tests on cultures taken direct from ammonia-containing media may offer a partial reason for earlier difficulties in detecting nitrogen fixation among members of this genus. The ability of the two strains of *Desulfotomaculum ruminis* to fix N_2 is of ecological interest in that both strains were isolated from the rumens of sheep (Coleman, 1960), though whether they represent normal rumen inhabitants or itinerants introduced with food is uncertain. Bergersen & Hipsley (1970) have evidence that facultatively anaerobic bacteria in the intestines of men and guinea pigs may, in certain circumstances, be actively fixing nitrogen. The rumen of a ruminant mammal might, in conditions in which the dietary nitrogen was low, be a logical environment in which commensal N_2-fixation by anaerobes could take place and such fixation might be of benefit to the host animal.

Miss K. Williams assisted with part of this work, and Mr E. Kavanagh performed the nitrogen analyses. $^{15}N_2$ was estimated by mass spectrometry by Dr C. W. Crane (Queen Elizabeth Hospital, Birmingham).

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