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Bioactive Microbial Products: Search and Discovery

Edited by

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CONTENTS

Chapter 1

INTRODUCTION: NEEDS, WAYS, AND OBSTACLES TO DISCOVERY

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Strategy for Discovery

This book is based on the lectures which were given at a meeting of the Society for General Microbiology's Fermentation Group, held at the Brockham laboratories of Beecham Pharmaceuticals Limited, to whom we are all grateful for financial and organizational help. Both the volume and the meeting have attempted a systematic approach to answering a specific question: "How shall we set about the task of finding new, interesting, and – above all – useful microbial metabolites?". It seems to us that however large the role played in the past by sheer serendipity it is time to ask such a question explicitly, recognizing that successful answers provide an essential foundation, not only for so many wider areas of investigative research but also for the successful and continued existence of important sectors of research-based industry.

In the past, it has been rather easy for most of us to overlook these foundations. Day-to-day, we are mostly concerned with the super-structures: the physiology and biochemistry of producer organisms, the process technology of metabolite production and recovery, the modes of action of microbial products *in vitro* and *in vivo*, the medical (or veterinary or agronomic) technology of their effects and their uses. Equally, year by year, our antibiotics and allied industries are mainly preoccupied with improvements in the production and the marketing of their existing products. Nevertheless, innovation (as distinct from mere improvement) is crucial for the continuation of all these activities. This assembly of studies and reviews is therefore concerned with successive aspects of the search for innovation, starting with the most basic of questions such as "how do we get micro-organisms for study?" and "how do we keep them?", and

proceeding as far as some of the newest approaches to the selection problem and some of the newest results from those approaches.

The study is a timely one for several reasons. First, in a world economic climate which is everywhere adverse, the contribution of innovation to industrial welfare is being sharply argued, and the efficiency of the innovatory process is being critically scrutinized. This is particularly so for companies whose activity has hitherto centred upon a relatively small number of basic and important products whose patent protection is disappearing, or has been lost already. Second, our increased understanding of disease processes as they actually exist, with all the complications of resistance, cross-infection, opportunistic pathogens, and the individuality of patient responses, has positively demanded an increasing sophistication in their chemotherapy, which is the main field of application of the biologically active microbial products. Thirdly, success breeds success. A few years ago the possibility of finding new and interesting microbial metabolites seemed a thing of the past, to be looked at with nostalgia – a state of worldly-wise disillusion which we can now see was entirely in the eye of the beholders. The fact that the collective view has once again changed, that custom has not staled the infinite variety of nature, is almost entirely due to the fact that as soon as new ways of looking for interesting products were introduced, some extremely interesting new products were in fact found.

The starting-point for all of the new ways of search and discovery dealt with in our subsequent chapters has been an increased and wider knowledge of biological phenomena. Selective isolation procedures such as those described by Williams and Wellington are based on a deeper understanding of microbes in their environment; the modern approach to culture conservation discussed by Dietz rests on a profound sympathy in observational microbiology and an up-to-date understanding of the phenomena of instability. After many years of puzzlement and confusion, we are beginning to understand the relationship between phenotype and genotype sufficiently to use the approach considered here by Hütter, designing culture conditions so that we shall be able to see just what our organisms are capable of doing. In the same way, our picture of the whole relationship between the general life-style of microorganisms and those metabolic activities in which we take our specific interest – "secondary metabolism" – has now progressed far enough to provide us with a guide to appropriate discovery strategies, whose effectiveness is illustrated by the results of Zähner and co-workers.

Thus far, the approaches are centred upon the microorganisms themselves. However, we can also add strategies

which are based upon the products, and specifically upon their hoped-for utility. The classic "screen" for antibiotic activity was so devised, and with an increased understanding of its limitations it can be considerably improved, as the work described by Hood shows. Here the increased understanding arose from studies of the mode of action, and more especially of the modes of resistance, of antibiotics, and leads equally directly to screening methods based on individual enzymes involved in the interaction of microbe and antibiotic, as discussed by Fleming and colleagues. Equally, however, the same rationale can be applied in respect of a far wider range of biological phenomena, using our knowledge not only of microbial processes but also of the enzyme and macromolecular biochemistry of cells of all kinds. This has opened up the very wide spectrum of potential uses for microbial metabolites which is covered here in Hamill's account.

Other Directions

In introducing this account, therefore, one is left uncomfortably with the question: "what has been left out?". What other fields of biological understanding might be pressed into our service? Certainly the supporting fields of science already listed can all be expected to continue to advance, and correspondingly we can expect new opportunities along the lines set out, but should we also look elsewhere for new leads?

Since microbial metabolites are, in general, the chemical interface between microorganisms and the rest of the universe, our scope is potentially vast. Here I simply wish to draw attention to some aspects of that interface which have not been considered in our main chapters but for which there are, in my view, a sufficient number and variety of "pointers" to suggest we may be able to include some of those wider aspects in our strategic repertoire.

Product-mediated interactions between microorganisms themselves are the basis of the classic screening methods by which most of our present armoury of antibiotics were discovered, but the wider topic of microbial interactions has proved to be an interesting but difficult area. The demonstration of antagonistic interactions *in nature* has remained controversial, and while many cooperative interactions have been studied, particularly by mycologists, and have been shown to be mediated by specific chemical substances, the very nature of these interactions has also implied that the active substances are very specific in their effects. In other words, although such studies give us very important insights into the microbial world, they are unlikely to afford us agents with activities in systems other than the ones for whose purposes they have been evolved.

A more fruitful line for the pursuit of microbial

metabolites with interesting properties over a somewhat wider spectrum is likely to follow from studies of the interactions between microorganism and non-microbial systems – the higher plants, insects, and mammals (including man).

Much of plant pathology is concerned with plant-microorganism interactions, and a significant proportion of these interactions is mediated by special metabolites. Most of this attention has been given to fungal products, and plant pathologists have distinguished between the directly-acting phytotoxins and the indirectly-active "phytoalexins". Both categories already include an interesting variety of molecular structures and perhaps a corresponding variety of physiological activities. A number of true phytotoxins have independently been discovered through antiviral, antifungal, and antibacterial screening of fungal isolates, and this observation has two corollaries – first, that microbial products from other screens might be examined for useful effects in plant systems, and second, that known phytotoxins might similarly be studied for their other activities. In general, neither the biochemical mechanisms of phytotoxin action nor the more general activities of phytotoxins have been very critically examined – but one cardinal exception is, of course, gibberellic acid, the story of which illustrates very well how a study in plant pathology, starting from field observations, can lead to a commercially valuable microbial product.

The phytoalexins have received even less attention outside the field of plant pathology, and even their definition and natural significance remain controversial. As a class, they are "defence" substances produced by more or less unique combinations of plant host and microbial pathogen. However, some appear to be fairly "normal" plant secondary metabolites whose production is enhanced by a variety of traumatic stimuli – including mechanical and inorganic agents on the one hand, and on the other, agents which are identifiable metabolites of the invading pathogen and which would presumably qualify as phytotoxins. Other reputed phytoalexins seem to be microbial products whose formation is similarly enhanced when the microorganism grows on specific hosts; yet others may be the products of truly cooperative metabolism. Unsatisfactory as the whole concept may seem to outsiders, the fact that phytoalexins do exert antimicrobial activities *in vivo* – sometimes quite marked and specific – and that they are produced as a consequence of microbial activity, should recommend them to our notice.

Invertebrate pathologists have likewise developed their special study of microbial effects upon insects and other invertebrates, though in this case their extension into the production of useful microbial agents has been embarked upon with rather more enthusiasm, and the subject

of pest control by microorganisms and microbial products is already well developed. As with all pest-control agents, much of their success or failure depends upon the technology of field application, which is not our concern here. For some of these agents, actual propagation of the infective organism through the field population is required, and these organisms, in line with their high host selectivity, are often difficult to cultivate in large-scale (or even laboratory) systems. Such technical problems are unlikely to resist a determined attack. Closer to our present subject, however, are the microbially-produced toxins, already known in considerable molecular variety and with equally varied activity spectra. Here is a case, however, where the practical inconvenience of direct screening for potential new agents will clearly limit progress – and correspondingly, where progress in our knowledge of invertebrate physiology could in principle lead to the development of far more efficient *in vitro* screens. The potential for really useful agents in this field is, of course, very considerable.

Observations of the interactions between microorganisms and higher animals – specifically, man and the domesticated species – are of course fundamental to medical and veterinary microbiology. The field is too large to review, and even to comment upon it may seem presumptuous. But in all humility I cannot forbear the observation that much of our thinking about chemotherapy is essentially simplistic, concentrating our attention on the simple presence of the pathogen and making our objective its simple eradication. The foundations of this thinking were laid a century and more ago by Lister and Koch; without denying our indebtedness, can we ask: "has nothing more been learnt about the aetiology of disease, that we can ourselves follow-up, at least speculatively and as a small part of our total effort, in directions other than the pursuit of biocides?" The development of microbial products with specific activities in modulating the immune response is thus seen as a particularly significant development.

A topic less central to pharmaceutics, from which we can still learn a little nonetheless, is exemplified by the mycotoxins. These are microbial (predominantly fungal) metabolites with pronounced physiological activities in man and higher animals, which have come to our attention for the rather special reason that they are produced by microbial growth, or "spoilage", on common food and feed products. Like the phytotoxins discussed earlier, the mycotoxins include a number of products which have previously been brought to light as antibiotics; their modes of action and specificities are very diverse (and largely unexplored); their molecular structures are extremely varied, and at least one series of mycotoxins – the lysergic acid derivatives – has

significant therapeutic usefulness. The mycotoxins also resemble the phytotoxins in another important respect: while their production is not unique to particular combinations of microbe and substrate, it is often very materially enhanced in those combinations, to an extent which may be very difficult to duplicate on other laboratory media (again, the ergot alkaloids provide a clear instance of this). If taking notice of the mycotoxins does nothing else, it should caution us yet again that unless a microbial genotype is exposed to an adequate variety of chemical and physical environments its full phenotypic range is unlikely to be displayed for our benefit.

Some Obstacles

A final topic which is very relevant to the development of new bioactive products, and which I venture to introduce because it is not dealt with at all in our ensuing chapters, is the very controversial one of the obstacles to innovation which our society has chosen to impose. For the pharmaceutical industry in particular, innovation is at once its mainspring and its burden. Moreover, the informed opinions to which the general public look for guidance are seen as being very divided as to whether that burden should be eased, or increased.

Apparently by social demand, the costs of all the procedures and tests needed before a really new pharmaceutical can be marketed are now so high, compared with the costs of discovery, as to constitute a very strong disincentive for innovation. This runs directly counter to all the arguments which show that innovation, rather than improvement, is required for progress.

Unfortunately, the resultant debate has been conducted almost entirely in adversarial terms; the pharmaceutical companies have been depicted either as wholly satanic or whiter-than-white, and those of the middle ground have remained silent. Yet for us there is a very real dilemma – how to balance the undoubted, but overt, risks in the introduction of new pharmaceuticals against the equally real, but effectively hidden, risks incurred by delaying, or even preventing, their introduction. Our society is not well equipped even to recognize, let alone quantify, those hidden risks. On the other hand the costs of avoiding the overt risks are now set so high that, given the very high risk that a new product will somewhere fail to meet our requirements, even the largest companies are becoming reluctant to gamble so heavily with their future. The more successful we are in finding new agents of promise, the more frequently industry will be called upon to take that gamble – and the more frequently, in present conditions, industry will decide to play safe; the smaller companies opted out of the game some years ago.

If therefore we are collectively to sustain an industry which is equipped for innovation, and if at the same time we collectively insist on very demanding regulation of new products, we have a responsibility to see that our two requirements are properly balanced, in realistic cost benefit terms, and with due regard for the effect of statistics on the smaller companies. Most of us live and work in free-enterprise economies in which the costs of research failures are intended to be recouped from the profits of successes; perhaps it will be possible to find some acceptable way in which our pharmaceutical companies can collaborate, in a limited but truly mutual manner, so that the statistics of success and failure can be at least partly redistributed. Without it, there is a very real danger that we shall regulate ourselves to a standstill – and all the discoveries we hope for from our searches will have come to nothing in the end.

Chapter 2

PRINCIPLES AND PROBLEMS OF SELECTIVE ISOLATION OF MICROBES

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Introduction

During the past 40 years, the isolation and subsequent screening of microbes from diverse habitats has led to the discovery of many novel and useful secondary metabolites. Nevertheless the approach to the search for potentially useful microbes has been largely empirical. Generally large numbers of isolates are obtained and then screened with the hope that something interesting will turn up. This undirected approach to isolation is to some extent due to our lack of knowledge about the roles, if any, of many secondary metabolites in the microbes' natural habitat. Certain antimicrobial toxins have been detected in natural environments but although most antibiotics are produced by isolates from soil, there is still no conclusive proof that antibiosis occurs in soil [Williams and Khan, 1974; Gottlieb, 1976].

The capacities of different microbial taxa for production of given secondary metabolites vary considerably. This does provide some guidelines for planning selective isolation procedures. The vast majority of antibiotics are produced by actinomycetes and in particular by members of the genus *Streptomyces*. Kurylowicz [1976] estimated that of 1530 antibiotics produced by actinomycetes, 1467 originated from *Streptomyces* species. The biological significance of this unique capacity for antibiotic production remains to be elucidated.

Our aim is to discuss the general principles, problems and relevance of isolation procedures in the search for useful secondary metabolites. Possible future developments will also be considered. Most of the examples will concern actinomycetes, reflecting both their general importance and our own particular interest in this group.

The Basic Stages of Selective Isolation

Five stages may be recognized:
(i) selection of the material containing microbes,
(ii) pre-treatment of the material,
(iii) growth on laboratory media,
(iv) incubation,
(v) colony selection and purification.
Selectivity may be introduced intentionally or unintentionally at any of these stages.

(i) *Selection of Material Containing Microbes*

Although this stage may be simply a matter of trying anything once, some criteria for selection exist. Choice of natural materials, such as soils, may be based on the assumption that samples from widely different locations are more likely to yield novel isolates and therefore, hopefully, novel metabolites. Alternatively, the search may be directed towards habitats in which the microbial populations are adapted to relatively extreme environmental pressures. This approach has met with some success (Table 1). However, more actinomycetes probably still

TABLE 1

Examples of secondary metabolites produced by actinomycetes adapted to environmental extremes

Organisms	Product	Authors
Psychrophilic *Streptomyces* sp.	Antibiotic SP 351	Yoshida *et al.* [1973]
Thermophilic *Streptomyces* sp.	Granaticinic Acid	Maehr *et al.* [1979]
Thermophilic *Thermoactinomyces antibioticus*	Thermorubin	Craveri *et al.* [1964]
Thermotolerant *Saccharopolyspora hirsuta*	Sporaricin	Deushi *et al.* [1979]
Marine *Streptomyces* sp. (salt-tolerant)	Aplasmomycin	Okami *et al.* [1976]
Osmotolerant *Streptomyces* sp.	Unidentified antibiotic	Wong and Griffin [1974]
Basophilic *Streptomyces* sp.	Caerulomycin	Funk and Divekar [1959]
Acidophilic *Streptomyces* spp.	Various antibiotics	Williams and Khan [1974] Nkanga and Hagedorn [1978]

remain to be detected in such habitats. For example, our knowledge of marine microbial populations in general is still limited. The isolation of the halophile, *Actinopolyspora halophila*, from contaminated laboratory media [Gochnauer *et al.*, 1975] and a halophilic *Streptomyces* sp. from a salt farm [Kayamura and Takada, 1970] suggests that actinomycetes may occur in other salt-rich environments.

The actinomycete populations in an acidic soil horizon were shown to be quite different from those in the neutral horizon immediately beneath it [Williams *et al.*, 1971]. Thus it is also possible to increase the variety of isolates by recognizing the heterogeneity in environmental conditions within one habitat.

Populations of natural habitats can be accidentally altered by man's activities. Addition of atrazine to soil resulted in an increase in the numbers of actinomycetes [Percich and Lockwood, 1978] and nocardia-like microbes grew on carboxanilide fungicides [Bachofer *et al.*, 1973]. Introduction of such alien substrates to natural habitats may therefore inadvertently cause rarer microbes to predominate.

More novel habitats still remain to be thoroughly studied, as exemplified by the recent isolation of an actinomycete from nodules of *Comptonia* [Callaham *et al.*, 1978] and the detection of actinomycete-like bacteria in the gut of termites [Bignell *et al.*, 1979]. Screening of anaerobic microbes for secondary metabolites has so far been comparatively neglected [Bull *et al.*, 1979].

(ii) *Pretreatment of Material*

Material may be treated in various ways designed to increase the chances of isolating the desired microbes. Examples of treatments applied in the isolation of actinomycetes are given in Table 2.

Heat treatments of various materials have been frequently used to decrease the numbers of bacteria on actinomycete isolation plates. The basis for this effect is not clearly understood but it appears that many actinomycete propagules, both spores (e.g. *Streptomyces*) and hyphal fragments (e.g. *Rhodococcus*) are more resistant than Gram-negative bacterial cells. Although heating decreases the ratio of bacteria to actinomycetes on plates, numbers of actinomycetes are often also reduced [Williams *et al.*, 1972].

Filtration through a membrane filter (pore size about 0.45 μm) is routinely used to concentrate cells from water. The filter is then placed on the surface of a nutrient medium, where it either remains or is removed after a few hours. The type of filter used can have significant effects on the number of colonies developing [Al-Diwany *et al.*, 1978]. When dealing with sea water containing very low concentrations of actinomycete propagules, Okami and Okazaki [1972] centrifuged samples

TABLE 2

Examples of pre-treatment of material for isolation of actinomycetes

Treatments Physical	Material	Isolates	Authors
Heating:			
55°C for 6 min	Water, soil, dung etc.	*Rhodococcus coprophilus*, *Micromonospora* spp. etc.	Rowbotham and Cross [1977]
40°C for 2 to 16 h	Soil, roots	*Streptomyces* spp.	Williams *et al.* [1972]
100°C for 1 h	Soil	*Actinomadura* spp. *Microbispora* spp. etc.	Nonomura and Ohara (1969, 1971*a*,*b*,*c*,*d*]
Membrane filtration:	Water	*Micromonospora* spp. etc.	Burman *et al.* [1969]
	Water	*Thermoactinomyces endospores*	Al-Diwany *et al.* [1978]
Centrifugation:	Seawater and mud	*Streptomyces* spp.	Okami and Okazaki [1972]