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Invitational ONR Lecture

The Two-Faced Microbiologist: Contributions of Pure and Applied Microbiology to Good Research

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INTRODUCTION

"There is no such thing as Pure or Applied science," said a British industrialist recently, "there is only Good or Bad science." The occasion was a meeting of the British Mycological Society dedicated to the proposion that traditionally minded academic mycologists could learn a lot by applying the techniques of submerged culture and the chemostat, and his remark has given me the theme for my own talk. As an academic, it would hardly be proper for me to lecture to the Society for Industrial Microbiology on – say – the manufacture of cephalosporins from activated sludge, and I have never yet been in a position to persuade anyone of the economic importance of my latest discovery. To an audience of fellow-academics it might be worthwhile for me to argue the importance of a proper attitude toward economic, social, and political problems, and to consider how to steer a middle course between selling oneself to the highest bidder and locking oneself in the ivory tower, though I should think that most of us have already been pretty well exposed to those arguments. However, the present occasion gives me a welcome opportunity to look at the reverse process and to acknowledge how much the pursuit of seemingly academic problems owes to the discoveries made in industrial and technological laboratories.

This task of high-lighting the origins of a piece of "pure" science has its dangers. It might make me very unpopular with my peers if I were to argue at length how much their latest and most elegant papers owed to some industrial innovation of which they were in fact quite unaware. I can only expose, therefore, the roots of my own researches, and hope to do so with as little tedium as possible. If the resulting account is too much like a scientific autobiography please excuse me, there must be something I have done which will interest some of you; otherwise, I should never have been honored by your collective invitation!

DISCUSSION

Basidiomycete Polyacetylenes

The polyacetylenes are a large group of natural products which today must be numbered in their hundreds, but 20 yr ago they were still rare curiosities. Many have been produced by basidiomycete fungi, and some of these are antibiotics of no clinical interest; the group as a whole has passed through most of the stages of scientific exploration in a relatively short span of years. Their study was, in fact, the problem to which I was first assigned on arrival at Manchester, and we looked at them, on and off, for about 16 yr.

Polyacetylenes in the plural first emerged as curiosities of the plant kingdom,

particularly from the work of the Sörensens (1953) in Norway, and simultaneously some very apposite new chemistry had been developing in E. R. H. Jones' school at Manchester. The historical roots of that chemical work are themselves of great interest, but I cannot pursue them here; one outcome was that the very distinctive UV absorption spectra of some basidiomycete metabolites described by Anchel (1952) and her co-workers at the New York Botanical Garden (NYBG) had been recognized as polyacetylenic. The New York work came out of a screening program for antibiotic activity, and so it had obvious "applied" origins; moreover, the NYBG had concentrated upon the Basidiomycetes because of their well-established and very practically oriented expertise with these important tree pathogens and timber destroyers.

For similar reasons, since I knew literally nothing about any kind of microbiology when I was assigned the job of growing these organisms, I soon found myself taking a crash course at a comparable British laboratory, that of Forest Products Research at Princes Risborough, where I learned the rudiments of technique and also had my first sight of the chemical problems which a close study of these pathogens and saprophytes might reveal (Cartwright and Findlay 1958). For the remainder of my hasty mycological education I was almost totally indebted to a book by one of the great pioneers of modern mycology, a man to whom "pure" and "applied" science were indistinguishable and who was an intellectual leader both in industry and outside. I refer of course to Jackson W. Foster and his book "Chemical Activities of Fungi" (1949).

In due course, Anchel's fungi grew for us and their polyacetylenes were characterized (Bu'Lock 1956) more or less simultaneously with the antibiotic mycomycin which emerged from the Pfizer Laboratories (Celmer and Solomons 1952, 1953a,b) with its remarkable structure fully worked out and the parent organism wrongly identified (but that is another story). The Jones research group moved to Oxford; the number of polyacetylene structures grew monthly, with an explosion of new plant polyacetylenes from Bohlmann and more modest additions from our fungi. At Manchester, the accumulating structures threatened to become boring and we started to look for some meaningful pattern in them, the first steps toward elucidating their biosynthesis (Bu'Lock et al. 1957). At the same time, the quite nonchemical job of growing the cultures gave us a real interest in questions of when, and how much, and even why, our metabolites were being produced – the beginnings of an interest in regulation. At the time, the theories of metabolic regulation were in their infancy, even for primary processes, and yet out of the severely practical experience of the people who were busy making antibiotics the ideas about secondary metabolite regulation were already emerging, and indeed their origins are all in the book by Foster (1949).

Of course, with the arrival of Arthur Birch at Manchester our own first steps toward biosynthetic experimentation had a tremendous boost, and we also acquired practical experience as a service laboratory with a much bigger range of fungi. And for this phase there is no doubt in my mind that the biggest generators of interest in natural product biosynthesis were our industrial colleagues, who in effect promoted, by their reception of the results, as much as by direct backing, the establishment of what is now a well-recognized and self-sustaining discipline of chemical biology.

For me, one of the nicest things about the natural polyacetylenes is that by 1966, when the series as a whole was just about to become too big and too miscellaneous to comprehend, I found myself able to rationalize all the known structures – and incidentally, most of those that have since been found – in terms of a relatively small number of biochemical transformations and a unified overall origin; moreover, within a

relatively short period of time we were able to establish the validity of this total scheme (Bu'Lock 1966, Bu'Lock and Smith 1967). My personal taste in science has always been toward the tidying-up of inconvenient facts rather than the generation of new confusions, and so this episode was a very happy one for me. The point in recalling it here is that the key structure had been characterized by lipid chemists in the laboratories of the USDA; I refer to crepenynic acid (octadec-9-en-12-ynoic) which comes from some rather undistinguished Compositae whose seed-oils were being screened in the hope of finding new and commercially valuable fatty acids (Mikolajczak et al. 1964). That program still goes on at the laboratories in Peoria, Illinois, and in the present global situation we should all wish it well, but my own favorite is still crepenynic acid! In effect, this episode ended our polyacetylene work. The regulatory aspects of their production still bothered us, and it would be nice to get back to them one day in the context of their known biosynthetic origin. Meanwhile, other biosynthetic work had exposed us to other fungi, and these had offered us parallel regulatory problems that in the event proved somewhat more accessible.

Biosynthetic Networks

From discussing the "antibiotic" polyacetylenes and the widening field of fungal biosynthesis with the interested applied scientists, I came to attempt some hypothetical frameworks which, in effect, would bring Foster's original views into an up-to-date setting by using the new data on biosynthesis. My first attempt (Bu'Lock 1961) was followed with second thoughts for the Prague antibiotics meeting of 1964 (Bu'Lock 1965). At the end of this period we had arrived at some naive and oversimplified definitions of the phenomena of secondary metabolism which were essentially in terms of regulatory processes (Bu'Lock and Powell 1965). Most of the evidence for our views, over and above our own very minimal contributions, had come from work on commercially important fermentations – for penicillins, gibberellins, actinomycins, etc. Just as the development of biosynthetic studies was needed to bridge the gap between the organic chemistry of natural products which is a diversifying study, and the biochemistry of organisms where the emphasis is on unification, so now the study of regulation phenomena seemed necessary to bridge the gap between the "primary" and "secondary" metabolic processes which our arguments had now emphasized. Both needs had been strongly amplified by the extent and importance of the work done in the antibiotics industry.

We started on the production of patulin by *Penicillium urticae,* for the typically academic reasons that the overall biosynthetic pathways were now known and the organism was already performing adequately (Bu'Lock et al. 1965). In this work, the number of related substances that could be observed in our fermentations, together with others found in different laboratories, was rather large, and this gave us a good example of a phenomenon which is peculiar to secondary biosynthesis, that of the "biosynthetic network." Because the enzymes effecting many of the transformations in secondary metabolism lack the high degree of substrate-specificity which has been evolved for primary biosynthetic processes, the order in which they act on a given molecule may not be absolutely fixed; a priori there may be more than one possible route, and correspondingly more than one set of intermediates between two substances. Today, this ambiguity is well realized in the fields of steroid and carotenoid biosynthesis, and it is becoming accepted that to characterize such a network properly requires rather detailed study, such as has more recently been carried out for the *P. urticae* system itself by Forrester and Gaucher (1972). To be honest, we had been too idle to do such a job

ourselves! However, the concept is an important one. It has turned out to be the key to the problem of sexuality in simple fungi, as I shall show later, as well as illuminating such difficult problems as fungal sterol metabolism and the distinction between genotype and phenotype in chemotaxonomy. In developing the argument for *P. urticae* into its more general form, we were stimulated considerably by the much more complicated case of the tetracyclines, which was being explored so energetically by McCormick (1965) and his co-workers at Lederle. Their determined attack on this difficult system, which is being notably continued by Hostalek et al. (1974) is an excellent example of the contributions of "applied" problems to "academic" science.

Regulation Problems Patulin, Ergot, and Gibberella

Within the metabolic network that represents the genotypic capacity of *P. urticae,* we explored several aspects of its phenotypic regulation. Perhaps the clearest evidence was for the later steps which lead to patulin. These were controlled by two interacting mechanisms acting on a metabolically labile rate-limiting enzyme system. The level of the enzyme was apparently controlled against this turnover by de-repression (by an unidentified substrate) and repression by high nutrient levels (Bu'Lock et al. 1969). This now seems likely to be a rather general pattern in secondary biosynthesis. However, the regulation of 6-methylsalicylate synthetase, which begins the patulin sequence, seemed to be somewhat different, since it was not metabolically labile, and under our conditions it seemed to be formed from the beginning of the fermentation, even though its activity did not become apparent until later. With hindsight I suppose that our nutritional conditions in this fermentation (on nitrate media) were never good enough to reveal the nutrient-repression which others have since found, but the mechanism of the later "activation" remains obscure; it may reflect available substrate levels or there could be an allosteric effect. However, the patulin fermentation turned out to be too recalcitrant for much further exploration; in line with my present theme, we had more success with fermentations that had been improved more effectively by our "applied" colleagues, and with a more wholehearted commitment to some of their fermentation techniques.

After some false starts, we moved on to the ergot alkaloid fermentation with *Claviceps purpurea,* concerning which there was a large log of semi-empirical information as well as some very fine work by Willard Taber, particularly on the strain we were to use, which came from the Northern Regional Research Laboratory, Peoria, Illinois. With this strain we obtained very good evidence for the threefold regulatory mechanism of "ergoline synthase": turnover, substrate induction demonstrably by tryptophan, and repression by nutritionally adequate phosphate levels. Also, we obtained evidence for an interaction between substrate induction and high nutrition which is still somewhat difficult to explain (Bu'Lock and Barr 1968), and for feedback mechanisms of the normal kind in the biosynthesis of the tryptophan substrate. However, this too is a "difficult" and rather slow fermentation, and in particular it was not sufficiently well behaved for us to apply the continuous culture techniques which further study would need.

Historically, both the theory and the practice of continuous culture have developed through a very close interaction of the academic and applied aspects; if at one time the concept was oversold to industrial research, the situation now seems to have adjusted itself, and there is no doubt that for the detailed exploration of fermentation phenomena this is one of the most powerful tools. We were helped both mentally and physically over our own initial barriers by the assistance of Zdenek Hostalek, who came to us on leave

from the very practically oriented Institute of Microbiology at Prague, where continuous culture techniques and theory were highly developed.

We had taken as our new system the fermentation of *Gibberella fujikuroi,* the whole story of which is an object-lesson in the origins of scientific advance. The original discovery of the organism and its key metabolite gibberellic acid exemplifies the value of careful observations of uncontrolled field events; it led to major industrial and utilitarian advances and also to a whole new chapter of "academic" plant physiology, as well as a quantity of even more "academic" chemistry. As a starting-point, we had a remarkable body of detailed data for the fermentation, which proved entirely repeatable, from the I.C.I. group (Borrow et al. 1961, 1964). There must be similar data for other fermentations in the research files of many other companies, and from the academic side I can only hold up this example, of putting the data into detailed order and making them public, as something we would like our industrial colleagues to do more often. In the antibiotics industry, the movement of personnel from one company to another seems to have usurped the role of public communication to an extent which is particularly disturbing to academics like myself who might seek to learn more about the general biology of fermentation processes.

In *Gibberella* we could study at least two secondary biosynthetic processes which are regulated quite independently, even though both use acetyl-CoA as precursor. One leads through the diterpene pathway to the gibberellins; it has a very large number of intermediates which recent work by Macmillan at Bristol is at last putting into meaningful relationships, and it is difficult to quantify at the beginning of the production phase, when these intermediates have not reached steady-state levels. The other leads through a multienzyme polyketide synthetase reaction to the bikaverin pigments, which even if they are not very important products, are easy to quantify! To a first approximation and in terms of our 1965 definitions, both are "idiophase" products, formed when either nitrogen or phosphate becomes limiting, but in more detailed study of batch cultures (in which Bob Detroy from the USDA laboratories in Peoria played a key part), we always found that the onset of bikaverin synthesis came measurably before the onset of diterpenoid/gibberellin synthesis. Possibly, then, the regulator for both processes was the same, but the response of one system was quicker; alternatively, they had quite different nutritional regulators (this was soon ruled out) or they responded to different "settings" of a common regulator. It was work with chemostat cultures which demonstrated the correctness of this last hypothesis (Bu'Lock et al. 1974b). We showed that full de-repression of gibberellic acid synthesis occurs only at the very lowest levels of the noncarbon nutrients, whereas bikaverin synthesis is fully de-repressed at levels which are markedly higher, though still well below the optimum for growth. Other mycelial activities, such as carbohydrate accumulation, were progressively de-repressed at all suboptimal levels of the noncarbon nutrients. In this way our naive distinction between "trophophase" and "idiophase" processes could be refined into a graded relationship between individual aspects of "growth" and "nongrowth." We developed now the concept of the regulation by nutritional parameters, not only of the growth-linked or vegetative genome but also of a very diverse "nonvegetative genome." Within the latter could be located a very wide range of phenomena, from simple fat accumulation to complex morphogenetic processes, and of course individual regulatory mechanisms would also operate more specifically within that range, just as they do in vegetative processes.

Demain (1968) introduced fermentation microbiologists to the idea of "catabolite repression," at a time when it was still an ambiguous concept among the bacteriologists

who had introduced the term. I developed the mechanistic possibilities in a lecture to the American Society for Microbiology in Boston in 1970 and Demain (1972) pursued the idea independently. Meanwhile, the molecular biologists' view of the more generalized type of catabolite-repression has been advanced very considerably, and although their data only apply directly to the inevitable few bacteria, they must at least serve as models for wider constructs. Today, the *lac* system of *Escherichia coli* examplifies the idea that a given gene or group of genes can be regulated both by an operon-specific regulator (*lac* repressor protein) and by a regulator which also controls many other genes and acts in a sense determined by the level of growth-linked processes which the nutritional circumstances permit. This latter regulator is the "cyclic-AMP-activated protein" and it acts positively for the transcription of catabolite-repressed genes (there is also less full evidence from other systems of a cyclic-GMP-mediated system operating in the opposite direction).

If we consider only the three systems discussed here, we can see that mechanisms *of this general kind* – and I must emphasize that the evidence goes no further than such a cautious phrase – will fit them very well (Bu'Lock 1974). We must now take "trophophase" and "idiophase" as being the two ends of what is really a continuum, determined in batch processes by the pattern of nutrient depletion. In such a continuum, we can suppose increasing levels of an activating metabolite, X, whose synthesis reflects the extent to which the actual nutritionally limited growth rate falls below the maximum to which the cellular organization is geared: in terms of fermentation kinetics

$$[X] \propto \mu_{max} - \mu$$

The variation of [X] with time will now reflect nutrient depletion just as μ does, i.e., by a Langmuir-Michaelis-Monod type of relationship. This, of course, is just the picture which emerged from the *Gibberella* work. For the combination of X, or an X-activated protein, with regulatory sites on the genes, we can also write Michaelis-type expressions in [X], but in general for each such site the affinity constants will be different, so that some sites will be "activated" at lower [X] – i.e., higher growth rates – than others. On a time basis, some will be activated sharply and some more gradually.

Carbohydrate assimilation is "activated" gradually over a wide range of [X], formation of the enzymes for bikaverin synthesis is activated at moderate [X] levels, and the process of gibberellin synthesis only at the highest levels, i.e., as $\mu \rightarrow 0$. Similarly in *P. urticae,* 6-methylsalicylate synthetase was formed at the lowest [X] levels obtained under our conditions but the enzyme for patulin formation only at an [X] level within the range of our observations.

In the *lac* system, substrate removal of the repressor protein (de-repression) only leads to galactosidase synthesis when the cAMP-activated protein is bound to its own site on the operon. Similarly in *Claviceps,* direct tryptophan-mediated de-repression can be observed only when nutrient depletion, i.e., the level of [X], is sufficiently high. However, the interaction of the two regulators may not be the same as in the bacterial system, since exposure to tryptophan during nutrient suppression promoted subsequent alkaloid synthesis, and this effect was cumulative. It is possible to construct models which will explain this, for example, by having substrate de-repression operate at transcription and the nutrient depletion effect at translation, or by other exploitations of the greater complexity of gene expression which is possible in eukaryotes, but of course the nature of the actual data makes such detailed hypotheses unwarrantable. The whole process must also be more complicated than our general mechanism suggests because of the

relatively large number of nonlinked genes which all these biosynthetic systems must involve, a point well argued by Vanek et al. (1971). What we see is the resultant of the controlled expression of all these genes plus the interaction of the gene products with substrate pools; on the other hand, this same complexity is itself a strong argument for the kind of control mechanisms we are postulating, which are not restricted to single operons in their scope and which allow for the coordinated regulation of unlinked portions of the genome.

Sex

The last part of our work with which I propose to illustrate my theme is "some studies on heterothallism and homothallism in mucoraceous zygomycetes" – hardly an industrially oriented topic! Currently, indeed, this work seems to have more fundamental significance than most of the things we have done in the past, and yet we came into the field through an applied problem and many of the basic systems we have used were developed in industry.

We had read a review of the β-carotene fermentation and its future potential written by Ciegler (1965), and gone on to learn about the β-factors or trisporic acids which both Upjohn and Farmitalia had shown could promote β-carotene synthesis in single-strain cultures of *Blakeslea trispora,* to a degree found previously only in mixed cultures of the two mating-types. It was therefore as specific regulators of a special metabolite accumulation that the trisporic acids interested us, and since they seemed very probably to be formed from β-carotene itself (as indeed we eventually proved), the implied regulatory mechanism seemed likely to be unusual and perhaps rewarding. We were quite well into the work from this angle when with Graham Gooday, a most able myco-physiologist, we found that the trisporic acids were themselves the hormones responsible for eliciting the actual sexual phenomena in a whole family of fungi, the Mucorales.

I will not go through the ensuing work in detail, save to note that our contributions have been equalled by those of van den Ende in Holland and Sutter in the United States, but merely summarize the situation as we now see it (Bu'Lock et al. 1973, 1974). The picture draws not only from direct work on the problem but also from some of the conceptual frameworks whose origins I have already discussed, and so it forms a useful conclusion.

The mating-type in heterothallic Mucorales, plus or minus, is determined by two alleles of a single gene, and since the manifestations of the mating-type difference are multifarious, we have to deal with the amplification of that gene rather than its direct expression. The constitutive gene product is, *inter alia,* a regulator for the synthesis of several of the enzymes required to convert β-carotene into trisporic acids. The genetic equipment for doing this exists in both mating-types. Synthesis of these enzymes is also controlled by the nutritional regulator mechanism previously considered (which equally regulates genes involved in asexual reproduction). Even when this regulator is released, syntheses of the enzymes making trisporic acid are repressed in single mating-types, apparently by the specific gene product from the mating-type locus. However, this repression is not complete, and there is a low level of gratuitous enzyme synthesis; since the repressor is not the same in the two mating-types, the selection of enzymes synthesized in the two mating-types at this gratuitous level is also different. Moreover, parts of the reaction sequence from β-carotene to trisporic-acids constitute a "metabolic

grid," in which enzymes which are not wholly substrate-specific can act in more than one sequence.

Consequently, in the single strain, while no trisporic acids are formed, small amounts of precursors accumulate and these are different in the two mating-types; moreover, the reactions still needed to convert the plus accumulations into trisporic acids are precisely those which the minus can carry out, and vice versa. Thus when both mating-types are grown together, and once the nutritional regulator has been lifted, trisporic acids are produced. These de-repress the synthesis of the enzymes by which they have been formed, in both mating-types, and so the hormones are now produced autocatalytically.

The trisporic acid-mediated de-repression also affects, directly or indirectly, a whole battery of other enzyme syntheses (which are also under the control of the nutritional regulator and part of the "nonvegetative" genome). These together bring about a sequence of morphological development leading to sexual union between the two mycelia, in which still further regulatory effects are, of course, involved.

This is, therefore, a dramatic working demonstration of the way in which a cascade of regulatory events can amplify the action of one or two genes, and one in which at least some of the steps are relatively well worked-out. It is also a valuable illustration of the very complex manner in which two sets of nuclei can interact. In the mating process these two sets occur in the two mycelia and can interact only through metabolites which diffuse between them. But given this picture, we should surely suspect even more complex intracellular interactions in, say heterokaryotic or diploid cells, even though they might well prove very difficult to disentangle. As if in confirmation of this suspicion, we have found that an apparent sex diploid of *Mucor hiemalis* autonomously produced what are clearly "sexual" spores on single suspensors – i.e. parthenogenetically (Gauger 1966). The homothallic Mucorales are a further instructive variant; in these, different parts of the same hyphae differentiate into plus and minus structures which then reunite "sexually." Here there is evidence that the genetic equipment for trisporic acid is again present in its entirety, but the differential regulation which gives rise to the localized plus and minus characters seems wholly due to hyphal gradients of the nutritional regulator, locally sharpened by septum formation. Chemically, the plus and minus regions correspond completely with the equivalent heterothallic types. Thus, where in the heterothallic species a group of genes is regulated in one of two patterns because of the genotype, in the homothallic species the two corresponding patterns are selected phenotypically from a common genome. We have evidence that cyclic AMP is at least one of the mediators in this phenotypic regulation, which I suppose makes our ideas very up-to-date.

Postscript

I set out to illustrate the role of industrial microbiology as a generator of fundamental problems, using examples from my own research because we can only guess at the sources of inspiration in other laboratories. I hope that I have illustrated the theme adequately enough to persuade my academic colleagues that the ideas of industry are intellectually rewarding, and perhaps my industrial colleagues that their work often deserves rather wider publicity than it is sometimes allotted.

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