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Secondary Metabolism as an Expression of Microbial Growth and Development

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ABSTRACT. A case is made out for regarding secondary metabolism as part of normal cell growth, related to its interactions with the environment. Secondary metabolism is widespread, especially in fungi and actinomycetes, and is not to be regarded as confined to the production of antibiotics and other special substances. It is part of the normal maturation process. Examples are given of the influence of secondary metabolism in ecological systems. It is also shown that cell productivity can be related to age structure. Secondary metabolism is thus linked with growth, although in many cases this may not be obvious in laboratory work. Initiation of production will arise from the system which regulates growth and differentiation. These processes are little understood at present, but it is clear that the factors involved differ in different instances and that they involve a very great variety of biochemical and physiological processes.

The present discussion of the production of substances by micro-organisms is an interesting opportunity to consider the relation between secondary metabolism and general growth processes. This point has not, on the whole, received much attention, most workers having tended to regard secondary metabolism as a means of producing valuable substances or as an interesting subject for research. Because of the tendency to treat the subject in isolation, it has been seen as irrational, and, a a result of this, explanations of it are often on too narrow a basis. Thus, typically, it is felt that secondary metabolites are produced by relatively few strains, that their production is not associated with the growth phase and they do not appear to be essential for the growth of the organism. It has been asserted that the only advantage they confer upon micro-organisms is the ability to remove excess materials if growth conditions become adverse. In discussing the subject further it is of advantage first to define the nature of secondary metabolism. This is conveniently done by reference to Gaden (1961), who attempted to classify microbial processes as simply as possible, and suggested three main metabolic systems for product formation. (1) Fully growth-associated systems, where the product arises directly from an essential growth process, *e.g.*, alcohol production in the anaerobic growth of yeast. (2) Overproduction of primary metabolic products occurs when one of the enzymes in central metabolism is blocked or eliminated. This effect may be brought about by adjustment of the medium or by mutation and selection. An example of this is the production of citric acid. (3) Secondary metabolic products are evolved by special enzymic reactions, forming substances which are apparently unrelated

to central metabolism, and which may appear to have no value to the organism concerned. It is the contention of the writer that the subject becomes much clearer if it is considered on a wider basis, recognizing that secondary metabolism is not confined to a few substances produced by special microorganisms. It is, in fact, a phenomenon observable in all plants and micro-organisms, in the latter case in the formation of spore colours, odours (especially in the case of actinomycetes), also of toxic or protective substances involved in plant-microbe interaction. Most of the secondary metabolites are complex substances, which must involve many enzymes in their biosynthesis, and it is hardly likely that these are the sort of compounds which would be used simply to absorb surplus material. They must therefore have a much wider significance in the order of nature. It is proposed to discuss the subject under three main headings: I. Ecological aspects of the formation of secondary metabolites; II. maturation and the production of secondary metabolites; III. initiation of secondary metabolism.

I. Ecological aspects of the formation of secondary metabolites

As mentioned above, in many cases, secondary metabolic products seem to have no purpose in the life of the organism which produces them. This has presented a considerable problem in understanding their role in nature. Thus, in the "*Handbuch der Pflanzenphysiologie*" (Ruhland 1968), the volume on Secondary metabolic products shows uncertainty. In the case of the terpenoids (Ruhland 1968) the role is seen in that animals avoid plants containing them, but it is not clear whether that is the reason for their existence. On the other hand (Ruhland 1968) it is recognized that waxes are important in resisting loss of water under hot and dry conditions.

Since then, advances in our understanding of ecology have shown the importance of the part secondary metabolism plays in the interactions between plants, animals and microorganisms. The following brief review is based on Harborne (1972), which provides both information and references. It also gives details of many types of secondary metabolites occurring in plants.

1) Flavour and odour are important in the selection of plants as foods for animals. Relatively few plants are eaten, and it appears that in many cases it is bitterness that is the main deterrent. The effect of alkaloids like strychnine and amygdalin is produced more by their bitterness than by their toxicity.

2) It is notable that many brightly coloured birds are distasteful to eat, while cryptically patterned birds are good to eat. It is evident that the conspicuous colouring is a warning to predators.

3) In a similar way, many insects are warningly coloured. It is found that insects of this type feed on plants containing cardiac glycosides which make them distasteful to birds. In addition, these foods may cause them to have odours which are repulsive to birds.

4) Aphids are very sensitive to the flavour of plants, mainly due to secondary metabolites. Aphids arrive on plants at random. They quickly test the flavour and either stay or fly off again in search of the desired plants. There is thus marked host selection. This follows the general rule that any species of plant is attacked by very few parasites, probably only one. This selectivity is at the heart of agricultural entomology and avoids the mass elimination of vegetation by non-specific feeding.

5) Root excretions are of importance in many types of host-parasite relationships. Thus root excretions from host plants cause eel-worms to de-encyst, or in other cases bring about the germination of seeds of Orobanche. Other substances attract

the male gametes to the eggs of fish. Pheromones are of immense importance in the control of insect activity.

6) Phenolic compounds occur in many plants, and protect, for instance, peas from insect attact (Harborne 1964).

7) Luxton (1972) has investigated the feeding habits of orbatid mites in the soil-litter interface in a Danish beech wood. Three species of mites were offered mycelium of an actinomycete and of a number of fungi isolated from the soil (*Candida, Trichoderma, Penicillium, etc.*). It was shown that orbatids may have distinct preferences for consuming certain microbial species, a habit which may be important in terms of their distribution in the soil profile. All the juveniles tested had different preferences from their adults and for foods which are found in different organic layers from those inhabited by their adults. It has also been observed recently (Swift 1978 — *personal communication*) that certain soil inhabitants consume special actinomycetes as a source of cellulase. Secondary metabolites play a clear role in ecological systems concerned with the decomposition of vegetable matter.

8) In fungi there exist an interesting group of substances which are involved in the regulation of germination an early mycelial growth. These have been reviewed by Gottleib (1978).

9) It is known that when *Bacillus subtilis* enters the starvation phase and commences to sporulate, an antibiotic is released, apparently to provide some protection for the cells during the change in growth pattern (Mandelstam 1969).

Another feature worth mentioning is that some secondary metabolic products are formed both by plants and microbes (Turner 1971; Higashide *et al.* 1977). Examples are citrinin (by *Penicillium citrinum* and by *Crotaria crispata*, the gibberellins (by *Gibberella fujikuroi* and by many plants, and ensamytosin (by *Nocardia ansamyces*), which belong to the same chemical type as products of the Maytan tree (Maytansine).

It is the opinion of those working in the fields of ecology and of plant-microbe interactions, that secondary metabolites are factors of major importance in inter-plant competition. Uncertainty as to their precise role is mainly due to the lack of experimental work in this field. They cannot be regarded as resulting from non-essential shunt mechanisms. If this is so, it must be concluded that their formation is part of normal growth processes. These conclusions must also apply to secondary metab. olism in microorganisms, where it can be regarded as part of a maturation process-Fermentation models based on maturation or cell age distribution have been proposed already by several authors (Shu 1961; Brown and Vass 1973; Kobayashi 1963, 1966; Matsumura *et al.* 1973) and will be discussed later. It may be assumed that secondary metabolism will commence at a certain stage of the cell's prowth and will continue till the cells become disorganized by cessation of growth. Even then production may continue for a time if the specific enzymes remain temporarily in existence. Proposals that secondary metabolism arises from maturation are by no means incompatible with arguments that its extent is controlled by growth rate, nor do they necessarily invalidate proposals for process optimization based on that theory.

It is stressed that secondary metabolism is to be interpreted in the widest sense, that is, it refers to all kinds of substances produced by living organisms, which do not form part of the central metabolic pathways. These substances include pigment and similar substances which are observed in ordinary fungi, actinomycetes and bacteria. It is considered that the better known "*secondary metabolites*", antibiotics, alkaloids and so on are simply members of this larger category.

II. Maturation and the production of secondary metabolites

1. Patterns of maturation and productivity

Several authors have proposed models which relate productivity to culture maturity. Shu (1961) used equations in which productivity increased with age and then declined. This gave good models of the citric acid, penicillin and other fermentations. Brown and Vass (1973) used the concept of "maturation time" to designate the productivity of cultures. While this gave good fits, the maturation times were rather artificial, thus, with penicillin, the maturation time was forty hours, much longer than is actually the case. Kobyashi's group (1963, 1966) used an equation which described the age distribution of the cells and applied it to studies of transient states in continuous cultures. Other authors (*e.g.* Meges *et al.* 1970; Smith and Galbraith 1971) have discussed changes in biochemistry and production in relation to mycelial differentiation.

Experiments on penicillin production, using washed mycelium, have been briefly reported (Calam and Davies 1961). The mycelium was shaken in buffer plus lactose and phenylacetic acid, and penicillin measured after two hours. Samples were taken from a freshly inoculated production culture. It was found that production began (at a high rate) after 10—24 h. Penicillin was produced from nitrogen within the cells. It was also found that penicillin production was rapid even when the mycelium was growing at maximum rates, once the initial maturation period had elapsed.

In unpublished work, micro-blocks of agar production medium were inoculated with spores. It was found that penicillin production began when branching started to develop. It is also common experience that if mould colonies develop on agar, production of secondary metabolites can be seen to occur in a zone somewhat behind the advancing edge.

In experiments with washed mycelium (Calam and Davies 1961) it was found that penicillin production could continue, without growth, for as long as 1—2 d. Recent work with stirred cultures (Basil 1977) has shown that when penicillin cultures are in a productive state, cessation of feeding was followed by cessation of production only after 24 h or more. From these results it would appear that the mycelium matures and becomes productive after 10—24 h, and retains its activity for about 2 d after this time.

In order to test this hypothesis, data from a penicillin batch in a 30-litre fermenter (McCann and Calam 1972) were used. From the course of the growth curve it was possible to calculate the weight of cells (per litre) between the ages of 15 and 70 h, which were assumed to be in an active state. It is known that the culture started from about 1 g cells per litre and the cell readings up to 24 h were reduced to allow for the presence of insoluble organic matter in the culture. The calculation of active (*i.e.*, mature) cells was carried out at intervals of five hours. Alongside were calculated the amount of penicillin produced in these periods. The results are given in Fig. 1. The general shapes of the two curves are very similar, supporting the age structure hypothesis. It should be noted, however, that this particular penicillin fermentation has been previously modelled on the basis of growth rate as an indicator of mycelial activity (Calam and Russell 1973). The relationship between the two approaches will be further discussed below.

The cell-age theory of the initiation of secondary metabolism has been successfully applied to continuous culture by previous authors (Shu 1961; Brown and Vass 1973; Kobayashi 1963, 1966). The simple model used above has also been tested in a hypothetical case in which previously grown cells were fed and diluted to give

specific growth rates (SGR) of 0.01, 0.02 and 0.03 g cells per g cells per h, the quantity of active cells being calculated after different periods of continuous culture. It was found that full activity would be retained with SGR's of 0.02 or 0.03, but at 0.01 productivity would fall after 70 h because the original population of cells had not been sufficiently replaced before they lost their activity. On the other hand, an SGR of 0.01 has usually been found adequate to maintain productivity (Ryu and Humphrey 1972). There are two obvious explanations of this difference. (1) The simple model used supposes that activity is suddenly lost after 70 h, in fact the decay of activity is known to be a complicated process, differing in different cultures (Righelato *et al.* 1968) and may go on for a considerable period after new growth has stopped. (2) It has been assumed that the rate of replacement of old cells is given simply by the apparent SGR. In fact, it is known that a considerable amount of re-growth takes place naturally (*e.g.* Duckworth and Harris 1949), so that the actual renewal rate is higher than that indicated by increase in dry weight alone.

2. Variations in responses of penicillin production cultures

When using penicillin cultures it is often forgotten that the mutants used have been put through a very extensive selection process. Thus, the original cultures used in the early forties only worked in surface culture. Successful submerged cultures followed the discovery of the isolate NRRL 1951, from which most of our cultures are descended. In addition, it is often found that mutants which do well in shaken culture fail in stirred fermenters, and that those which succeed in stirred fermenters may fail on the plant scale. On the other hand, with other antibiotics, it may be possible to transfer directly from shake flasks to the full-scale plant. What these results mean is that there is a much wider range of possible relationships between growth and production that is often realised. Our information on culture behaviour and the production of secondary metabolites is nearly always based on cultures or mutants chosen because of their ability to work well under a variety of conditions.

3. Complexity of biosynthesis in secondary metabolism

It is recognized that secondary metabolic products are chemically very complex. For example, it has been found (Hošťálek *et al.* 1974) that the production of tetracyclines involves a large number of enzymes, on the way to a hypothetical tri-cyclic compound from which the final biosynthesis commences. After this a series of twelve steps determines the final product. It is estimated that a total of 45 compounds can be derived from these steps, of which 27 have been identified in cultures, though of course a single main product is usually obtained. Thus, a complex multi-enzyme (and therefore multi-gene) process is involved. Similar consideration hold with other antibiotics. In the case of plants, an enormous range of complex substances are formed, flavonoids, steroids, terpenes, waxes and alkaloids. Each of these represents a multi-gene system quite as complicated as we find in an industrial fermentation. An example illustrates another point. In a class experiment, our students isolate actinomycetes from soil and test them for antibiotic production. Usually about 15 % are active. This suggests that only about 15 % show secondary metabolism, but this is not the case. All show secondary metabolism in some form, if only in colony colours, odour or in some other way. The significance of all these facts is that secondary metabolism cannot be regarded as a chance event. The elaborate systems of enzymes and regulatory mechanisms which are involved must have been evolved to meet some requirement of plant or micro-organism, in the same way that

the other parts of their structures and metabolic systems have been put together, forming a part of the general life system. They do not appear likely to be waste products; these would more likely be carbohydrates or fats.

4. Relation between cell-age and growth-rate models

Evidence has been presented to show that secondary metabolism is part of the normal differentiation process and the degree of productivity is linked to the age structure of the cell population, and it has been shown that this is an adequate basis for modelling (Fig. 1). Fig. 1 shows that an active population was built up which lost production at the predicted age limit. The population became inactive because it was not replaced by growth of new cells, the limitation being oxygen supply which held growth down to a very low level.

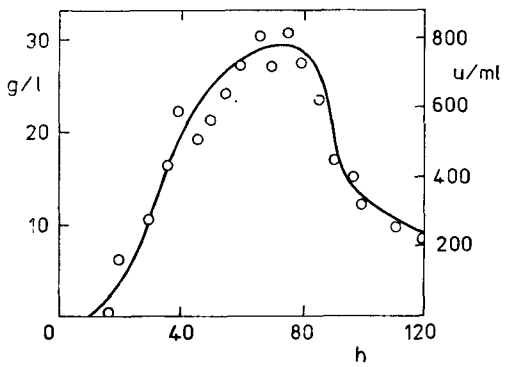

FIG. 1. Concentration of active cells and penicillin; production rate (in g active cells per litre) is shown by the full line; penicillin production increase in 5 h (in units per ml) by the point symbols.

Several points arise from these considerations. It would appear that the fermentation described had been unconsciously designed so as to build up a large quantity of active cells and to use them throughout their expected active life. This was timed to end when the oxygen supply became limiting and the growth-rate declined. The behaviour of the culture could thus be explained strictly in terms of growth rate, concealing the effect of age and the fact that the only way to extend productivity would be to allow the cells to replace themselves by new growth, *e.g.* by removal of culture and dilution, as proposed by Humphrey (1974). In strict "growth rate" terms it would appear possible to keep the cell population in a productive state simply by holding the specific growth rate above the critical level (about 0.01 g per g cells per h). On the basis of age structure this would not, however, be adequate, as it would leave the bulk of the old cells still present and non-productive.

The penicillin-producing enzyme system will be sensitive to factors which might damage or destroy it. One of these is too low a growth rate. Another factor is too low a concentration of dissolved oxygen. This sensitivity may exist only at certain parts of the growth cycle, perhaps especially when the cells are young. It is known that this is the case with the production of chlortetracycline (Hošťálek 1964) and of citric acid (Lockwood — *personal communication*). Once the productive enzyme system has been destroyed it may recover, but only to a limited extent.

It may be concluded that productivity is primarily associated with the age structure of the cells, provided that other factors do not interfere and destroy the relevant enzyme systems.

III. Initiation of secondary metabolism

The generally accepted view of the mechanism of differentiation is that it depends on a genetically programmed sequence of events, each event being triggered by the completion of the previous stage. Characteristically, the initiation of each stage depends on two factors, one the trigger, the other the state of the cell and its ability to respond. As each stage is triggered, a new series of enzymes appears which bring about the required reactions. This type of behaviour is well shown in the behaviour of *Dictyostelium* (Smith and Galbraith 1971). Other similar systems are known, *e.g.*, the sporulation of *Bacillus subtilis* (Mandelstam 1969). These systems seem to be relatively specific to individual species. Thus the sporulation of *B. subtilis* is induced by starvation. It has been found that with *Clostridium acetobutylicum*, used for solvent production, sporulation begins long before the medium is exhausted and may perhaps be induced by age or by the appearance of the rather toxic butanol in the medium. In mycelial growth, differentiation seems to be regulated by the balance between extension of cell walls and production of cytoplasm. Katz *et al.* (1972) found that branching increased with growth rate. It was concluded that if cytoplasm was being produced faster than cell-wall extension allowed, the accumulation of materials for cytoplasm formation triggered the development of a new branch. Any factor which slowed elongation increased branching. Point mutations changing colonial morphology in *Neurospora* were all concerned with the early stages of glucose metabolism, probably decreasing wall formation more than general metabolism. In other words, each stage of development is preceded by some disturbance of the metabolic pattern; this may arise from a variety of causes, many of which are not yet clear to us. It is considered that as the culture passes through one of these disturbed phases, secondary metabolism will be induced. A few examples may be mentioned. As indicated above (Calam and Davies 1961), penicillin production developed when the culture was very young and still growing rapidly; development occurred as soon as an amino-acid pool appeared in the cells. Chlortetracycline production also appears early (Hošťálek *et al.* 1975), at a time of maximum protein formation. In both cases, there were morphological changes shortly after production began. Melanin production started in *Aspergillus nidulans* as soon as the stationary growth phase began, although already during rapid growth phenolic substances were being formed (Carter and Bull 1969). Bu'Lock, who has given an interesting account of induction stages in gibberellin formation (Bu'Lock *et al.* 1974), has also described the occurrence of a crisis in the metabolism of *P. urticae* immediately before production of patulin commenced (Bu'Lock 1965). We have observed similar behaviour in exponential yeast growth, when the glucose feed rate began to exceed that which the cells could consume efficiently. There was a temporary dramatic fall in respiration rate with recovery after about 20 min. This crisis was followed by less efficient growth — the development of aerobic fermentation. White has shown that this can be triggered by as little as 0.0004 % of glucose (White 1954). In some cases induction of secondary metabolism may be repressed, for instance by glucose. Phosphate prevented production of tetracyclines in some of the early cultures.

It may be concluded that secondary metabolism may be initiated in many different ways, and it may also be limited by a variety of circumstances. Thus, poorly prepared inocula lead to cultures which never perform well and oxygen lack can inhibit formation and colouration of conidia.

DISCUSSION AND CONCLUSIONS

Evidence has been presented to show that (1) secondary metabolism is a natural function of micro-organisms, connected with their survival in their ecological situations; (2) secondary metabolism is part and parcel of normal metabolism, coming into action at the appropriate stage of the life cycle of the cell; it is not confined to antibiotic production or to cases of special research interest; (3) these conclusions indicate that the extent of secondary metabolism depends on the maturity of the cells (or their age distribution), and satisfactory models relating growth to production can be produced, based on this hypothesis.

On this basis, secondary metabolism can be expected to be initiated at any culture age, depending on the requirements of the cells in their particular environment, that is to say, at the appropriate moment in the life cycle of the cells. Each organism will be provided with its appropriate form of induction, no general system will be found to operate among all microbes. Microbes will differ considerably in their responses to induction. However, because of the widely based tests used in mutant selection, culture behaviour often seems more simple and flexible than is the case with wild strains.

It has been noted that micro-organisms of a given species vary widely in antibiotic production. This probably reflects genetic run-down which has taken place over a long period of time. It is probable also that the ecological factor which led to the production of particular secondary metabolites will, in many cases, have long since passed away and now be unidentifiable. The switch to production of secondary metabolites may well show up as a metabolic crisis (cf. Bu'Lock 1965). Such crises are probably common in metabolic systems, representing the lag involved in enzyme replacement after a genetic regulatory change. Such changes can be observed in many metabolic systems overloaded with one of the input components.

It is considered that the type of maturation model. connecting growth with secondary metabolism, is more satisfactory that one depending wholely on growth rate. Secondary metabolism is a universal phenomenon, and it is possible, in this way, to understand it in a fuller and more useful way.

There are two areas where our ignorance is very great. The first of these is that the processes of differentiation and its induction are very little understood, so that it is difficult to be certain how they operate, except in a limited number of cases. The second is that a very great part of cell activity (apart from growth) is devoted to maintenance activity. In particular, at low growth rates most of the energy produced is used in this way. Estimates of the energy required for cell biosynthesis are very much less than actual consumption (Forrest and Walker 1971). What becomes of this energy is unclear, but it is thought to be used in turn-over and structural reactions. It has been estimated that in *Penicillium chrysogenum* energy used for penicillin production is only 8 % of that used for maintenance (Righelato *et al.* 1968). Thus, in considering the initiation of secondary metabolism, it can be seen that this is mixed up with a great number of other reactions which are proceeding at the same time. In the case of a particular product we know a great deal about the biosynthetic operations involved in its production, but very little about other reactions proceeding at the same time. As was found in considering the significance of secondary metabolism in ecology, it is our general lack of knowledge of the factors govering cell metabolism and differentiation which prevents us from recognizing the true significance of secondary metabolism in micro-organisms.

Because of the way in which cells are usually grown, and, consequently the age-distribution develops, it happens that for a considerable time productivity

appears to be independent of growth, provided that growth rate does not fall below a critical level. However, at the end of the main production phase, when the main bulk of the cells are becoming over-aged, there will be a fall in productivity in any case. This is partly offset by feeding and dilution, but it would be difficult to replace the whole of the old population, as it would be necessary to restore full productivity. This matter is very complicated, in any case, because of the various ways is which cells mature, die, autolyse and re-grow, especially with mycelial cultures. Genetic run down may also occur. It is known that in some cases fermentation processes can be prolonged, efficiently, by feeding, dilution and culture withdrawal. With penicillin this seems difficult, especially because the cells present continuously demand respiration for maintenance energy, which puts a limit on what is available for new growth, and it seems that it would be difficult to set up a stable and highly productive system. Calculation of the optimal environment would be difficult, but an excpert might find a way by experience. More data and thought are needed.

It is not easy to foresee the way in which modelling will develop in future. Growth--rate models are evidently convenient, age-structure models more realistic. It would seem inevitable that both systems will be combined, based on the hypothesis that cells are active over a certain period of time, provided that other factors, such as oxygen supply and growth rate, do not fall below critical levels. This limitation will obviously apply during growth in fermenters, rather than under field conditions. The main problem is our lack of information about the role of secondary metabolism in the general field of biology, as well as its function in the general metabolism of micro-organisms. It is possible that out best leads will come from ecological work where matters can be viewed from a broader position than is usually possible in laboratory microbiology.

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