FUNGAL SECONDARY METABOLISM RESEARCH: PAST, PRESENT AND FUTURE[1]

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> All the world's a stage,
> And all the men and women merely players:
> They have their exits and their entrances;
> And one man in his time plays many parts,
> His acts being seven ages.
>
> W. Shakespeare, *As You Like It*,
> Act II, scene vii

1. *Introduction*

The objectives of this article are three-fold:

* to overview briefly the past achievements and current status of fungal secondary metabolism research as it relates to the producing organism (this qualification precludes discussion of how secondary metabolites, in the guise of drugs, toxins, etc., interact with other organisms);

* to advocate the more widespread use of solid culture methods in fungal secondary metabolism research, particularly in studies of control and function; and

* to review briefly the principles and practice of radiogas chromatography/ mass spectrometry, a technique that we are using in our work on fungal secondary metabolism but that has, we are convinced, considerable application beyond.

The article will focus exclusively on *fungal* secondary metabolism. This is not simply an arbitrary action; there are good reasons for believing that secondary metabolism, as expressed in bacteria, fungi and plants, is distinct and that failure to accommodate these distinctions clouds rather than clears our overall understanding.

2. *What is a Secondary Metabolite?*

"Secondary metabolite" is a knotty term that several authors would gladly see fall into disuse. It emerged from the fact, first appreciated by plant natural product chemists (1) and later confirmed by their microbial brethern (2), that plant, fungal, bacterial (and mammalian) cells are composed of two distinguishable types of molecules:

a) materials that occur uniquely in a single strain or species, that are found in two or more closely related members of a single genus, or that are found sporadically in a limited number of evolutionarily unrelated species in different genera, families, orders, classes, phyla or kingdoms; and

b) materials that are widely distributed in nature being found at least in all families in an order, often in all orders in a class and in all classes in a phylum, and in some instances, in all phyla in a kingdom and in all five kingdoms.

Cell constituents of type (b) became known as *primary metabolites* (alias intermediary metabolites or "biochemists' compounds"), while cell constituents of

type (a) became known as secondary metabolites (alias shunt metabolites, "special" metabolites, idiolytes, or "chemists' compounds"). [N.B. Enzymes and nucleic acids, species-specific in distribution though they be, are normally excused from the primary/secondary metabolism discussion.]

Over the years, primary and secondary metabolites became endowed with other general characteristics. Thus, it was found that primary metabolites played a well-defined physiological role in the producing organism and were often of relatively simple chemical constitution. By contrast, secondary metabolites appeared to play no crucial role in the growth of the producing organism, were often of complex chemical constitution and, in the microbial sphere, were usually produced in batch-mode, submerged liquid culture following cessation of multiplicative growth. These characteristics are sometimes used as definitions, in preference to the historic criterion of distribution. We deem this unfortunate and advocate retention not only of the terms primary and secondary metabolism but also of their distribution-based definition.

3.   Past Achievements and Present Status of Fungal Secondary Metabolism Research

Fungal secondary metabolism research can be subdivided, as with the Shakespearean ages of man, into seven areas of activity. These areas are reviewed briefly below.

a) ISOLATION/CHEMICAL CHARACTERIZATION/BIOLOGICAL TESTING OF FUNGAL SECONDARY METABOLITES.—Historically this was the first area of research activity. In 1896 Gasio published details of his chemical encounter with what was later to be shown to be the secondary metabolite mycophenolic acid (3). Significant subsequent milestones were: the classic 1912 paper by Alsberg and Black on the extractives of mold-infested maize (4); the discovery of penicillin by Fleming in 1929 (5); and the monumental efforts of the Raistrick group in London in the 1930's that led to the isolation and characterization of nigh on 200 fungal metabolites (see 6, for review). World War II provided a considerable impetus to this area of research in the form of the drive to characterize penicillin and produce it in bulk as a pharmaceutical. In the wake of the euphoria that the penicillin venture produced, the search for antibiotics, then antiviral and antineoplastic agents of fungal origin, began. That search continues today.

In 1960, a new chapter in the fungal secondary metabolite isolation/characterization/testing saga was opened or, more correctly, an old chapter was reopened— 100,000 turkeys died in England and almost simultaneously rainbow trout heading for California were found to have visceral abnormalities (hepatomas). Mycotoxins—the shrouded killers of mediaeval masses—had reappeared. The turkey and trout killing agents were the aflatoxins. Other key mycotoxins subsequently unmasked were the ergot alkaloids, ochratoxin, patulin, zearalenone, gliotoxin and citreoviridin (for review, see 7). Recently another group of mycotoxins, the trichothecenes, has achieved notoriety as alleged agents of chemical warfare ("yellow rain").

As a result of modern chromatographic and instrumental techniques, isolation/characterization/testing is now a straightforward, almost routine, activity.

b) TOTAL SYNTHESIS OF FUNGAL SECONDARY METABOLITES.—Since the very beginnings of organic chemistry, natural products have provided challenges to chemical synthesis. Initially, the objective was to confirm the results of structural elucidation. Subsequently, parallel motives of producing/modifying pharmaceuticals without resort to living systems, or attempting to emulate in the laboratory the constructional elegance, stereochemical control and reagent economy of living organisms, became prominent. The first witting total synthesis of a fungal secondary metabolite was likely that of 6-methylsalicylic acid, reported by Anslow and Raistrick to have been performed for them by Professor A. Robertson in 1931 (8). Notable subsequent achievements have been the syntheses of penicillic

acid (9), lysergic acid (10), griseofulvin (11,12), cephalosporin C (13), trichodermol (14) and gibberellic acid (15,16).

Synthetic work continues, directed towards both applied and academic ends. It is often now tutored by what is known about how organisms themselves manufacture secondary metabolites; organisms themselves are occasionally used as "reagents" in synthesis (see other papers in this Symposium).

c) BIOSYNTHESIS OF FUNGAL SECONDARY METABOLITES.—By the middle 1950's, organic chemists began taking time out from isolation/characterization/synthesis to question how secondary metabolites might be synthesized by the producing organism. The increasing availability of substrates labeled with stable and/or with radioactive isotopes, and of the instruments to assay such isotopes, allowed these questions to be resolved. The first work was done by Birch and his colleagues on 1-^{14}C-acetate incorporation into 6-methylsalicylic acid (17)—a result that confirmed the operation of the polyketide pathway in fungi. Such early work focussed on identifying those primary metabolites that provided carbon, hydrogen, nitrogen, oxygen, etc., to the secondary metabolite in question and how these primary metabolites were disposed physically in the skeleton of the secondary metabolite. The disposition issue called for considerable skill in microchemical degradation and precise isotope assay. Such classical methods established that biosynthetically, fungal secondary metabolites could be divided into three major groups: the polyketides, the terpenes, and the amino acid derivatives.

In parallel with work on secondary metabolite precursor identification and the pattern of precursor disposition in the end product—work that often suggested specific intermediates that might be involved in the biosynthesis—explicit studies of the biosynthetic pathway were undertaken. In the beginning, this involved synthesizing putative intermediates in radiolabeled form (an activity that again relied heavily on the skills of the organic chemist) and feeding them to the producing organism. If a substantial incorporation of isotope into the end product resulted, the intermediary status of the synthesized material was considered established, and visa versa. This is not a totally vigorous argument—aberrant incorporation or impaired cellular permeability of the substrate can cloud the outcome—but most of what we now know about secondary metabolic biosynthetic pathways has relied on use of such methods.

In more recent years, instrumental methods have replaced, to no small extent, the organic chemists' skills. Nuclear magnetic resonance and mass spectrometry have precluded, in many instances, the need to degrade end products chemically; and, as we shall see later, radiogas chromatography/mass spectrometry and derivative/parallel methods allow *in situ* detection of biosynthetic intermediates.

d) ENZYMOLOGY OF FUNGAL SECONDARY METABOLISM.—This area of fungal secondary metabolism research has been relatively neglected—a fact that, as we will see, is currently impeding progress in other areas. There are several reasons for this neglect; some are rational, some otherwise. A key reason stems from the fact mentioned above that organic chemists, first with a synthetic/degradative bent then with an analytical/instrumentational bent, assumed responsibility for elucidating the pathways of fungal secondary metabolite biosynthesis. Both of these groups were/are by philosophy and training unlikely to resort spontaneously to enzymological methods in the course of their studies Moreover, since these studies proved highly successful without enzymological input, collaborative ventures with biochemists—the group that would spontaneously resort to enzymological methods—were rare. Historically, biochemists have eschewed until very recently most things to do in general with fungi, and with secondary metabolism. A second, less serendipitous, reason for our limited knowledge of fungal enzymology is that filamentous fungi are in principle more difficult to handle from the enzymological point of view than are, for instance, mammalian cells. The cell wall is robust and significant proteolytic activity can

be released when cell wall disruption is eventually achieved.   Whether the enzymes of fungal secondary metabolism are intrinsically more difficult to deal with than are other enzymes, remains to be seen; I personally doubt it.

This said, it must be recognized that some quite elegant work has been done in this area of fungal secondary metabolism research.   The 6-methylsalicylic acid synthase of *Penicillium patulum* has been purified to homogeneity (18) as has the enniatin synthase of *Fusarium oxysporum* (19) and several of the enzymes involved in alkaloid biosynthesis in *P. cyclopium* (20,21).   Both synthases are multienzyme complexes, and their isolation/characterization does not appear to have been unduly difficult.   Additionally, several scores of fungal secondary metabolic enzymes have been dealt with in homogenate, cell-free or partially purified form.   The most integrated set of such enzymes are to be found in the 6-methylsalicylic acid→patulin pathway in *P. patulum* (22,23).   The technique of preparing protoplasts from fungi (biomass preparations free of cell wall) may make the task of releasing fungal enzymes from cells significantly easier (24).

e) FUNGAL SECONDARY METABOLISM AND FUNGAL PHYSIOLOGY.   This is an important question to those interested in the biological role(s) discharged by fungal secondary metabolites in the producing organism and, in more practical vein, to those intent on maximizing yields of a specific fungally-derived pharmaceutical.   It is an area, however, that has only recently been studied in earnest from the biological point of view.   The bulk of earlier work has focussed on gross cytological changes and in medium nutrient compositions as a batch-mode submerged liquid culture ages.   The reason for concentrating on batch-mode liquid culture physiology is that historically this has been the culture mode of industrial choice and, consequently, the most commonly used culture mode in industrial and academic research.

The consensus of the work in batch-mode submerged liquid culture is that secondary metabolite production begins as the growth phase [called "trophophase" by Bu'Lock (25)] of the organism is nearing completion—a consequence of the exhaustion of one or more essential nutrients.   Carbon, nitrogen and phosphorus are the nutrients whose depletion most commonly heralds onset of the production phase [called "idiophase" by Bu'Lock (25)].   For instance, in submerged liquid culture wherein care is taken to ensure biomass homogeneity, 6-methylsalicylic acid production in *Penicillium patulum* is triggered by a nitrogen depletion (22).   There are exceptions to this generality and, as we will see later, some doubts can be raised regarding its true biological interpretability.

The trophophase/idiophase/submerged liquid culture association apart, there are several well-documented instances where fungal secondary metabolites are involved in the physiology of the producing organism.   The best known cases are the fungal sex hormones of the *Achlya* (antheridiol and oogoniol) (26) and of the *Mucorales* (the trisporic acid) (27).

Emerging inexorably from any discussion of fungal physiology and fungal secondary metabolism is the question "why do fungi produce secondary metabolites?"   With the exception of the sex hormones, there is as yet no clear answer to this question.   Elsewhere we have reviewed the options that seem reasonable (28); later we document our reasons for believing that submerged liquid culture is not an appropriate vehicle with which to begin evaluating these various possibilities.   We take great encouragement from the fact that the biological roles of antheridiol, oogoniol and the trisporic acids—three uncontestably legitimate fungal secondary metabolites—became evident when the problem was examined in an appropriate context.

f) THE MOLECULAR BIOLOGY OF FUNGAL SECONDARY METABOLISM.—This area of research is in its infancy but it has great potential in studying how secondary metabolism is controlled, provided progress can be made on the enzymological

front.   Let me explain.   It is now relatively easy to transfer the complete genome of a fungus, in small segments, into plasmids that can be expressed in *Escherichia coli* (see, for instance, reference 29).   Such "gene libraries" contain, *inter alia*, the genes that code for the enzymes of secondary metabolism.   Moreover, these genes are present in an easily studied form, both directly in terms of the primary sequence of the genes and indirectly in terms of their expression into mRNA.

The catch is that genes can only be recognized unambiguously in terms of their gene products, the enzymes for which they bear code.   Since the enzymology of fungal secondary metabolism is so sketchily described, the link between gene and gene product cannot be forged, and the benefits that molecular biology should be providing us are postponed in their realization.

This unfortunate circumstance is compounded by the fact that classical genetics, a methodology that parallels and in some cases duplicates the thrust of molecular biology, is not generally available in the fungal secondary metabolism area.   A majority of the fungi that engage in the phenomenon are imperfect, i.e., they have no sexual cycle.   The parasexual cycle, whereby heterokaryon formation, the formation of diploids and their subsequent mitotic recombination are relied upon, is a viable but less efficient substitute (30).

g)   TOWARDS RATIONAL CONTROL $(+/-)$ OF SECONDARY METABOLITE PRO DUCTION.—I include this last section for completeness since for many individuals it represents the ultimate motive for all the previous ones.   In essence it asks the question "Can we get to a point where we can predict logically and confidently, conditions that will maximize or minimize the production of a given fungal secondary metabolite?"   My conviction is that this will eventually be possible.   The necessary pre-conditions being that we can come to an understanding of how the process is controlled and the function(s) it serves in the producing organism.   In turn this will require increased attention being paid to the enzymology, physiology and molecular biology of the phenomenon.

4.   *A Role for Solid Culture in the Study of Fungal Secondary Metabolism*

a)   THE NEED.—We have already alluded to the central role of batch-mode submerged liquid culture methods in the study and exploitation of fungal secondary metabolism.   Some years ago when we began work in earnest on the control and function of the process—work that was to begin with an exploration of how secondary metabolism correlated with the development cycle of members of the genera *Penicillium* and *Aspergillus*—we became concerned that batch-mode submerged liquid culture might not be optimal for the preliminary stages of this work.   Our concerns were four-fold:

i)   Although the development cycle of the imperfect *Penicillia* and *Aspergilli* is very simple (spore germination, vegetative mycelium formation, aerial mycelium formation, conidial head development, conidiation), that cycle is not executed reproducibly in submerged liquid culture; such cultures, for instance, conidiate sporadically;

ii)   In submerged liquid culture, it is impossible to distinguish aerial from vegetative hyphae; indeed it is not known for sure if both cell types exist in this culture mode or if a third hybrid type is present;

iii)   Even when conidiation occurs in submerged liquid culture [by chance or because of the use of high Ca^{2+} levels in the medium (31)] the environmental conditions encountered by the conidiphore, the conidial head and the conidia, are remote from what surrounds them natively.   A liquid, rather than a gaseous, environment is encountered.   Under ecologically normal conditions, all aerial portions of the culture would be nutritionally dependent on the vegetative mycelium that bears them but have more direct access to O_2 and to a sink for CO_2; in submerged liquid culture, provided care is taken to ensure homogeneity,

all cells have equal access to nutrients (including O_2) and have an equivalent opportunity to rid themselves of CO_2;

iv)   Any physical association that a specific secondary metabolite might have naturally with specific cell types in the aerial portion of a culture will tend to be masked in submerged liquid culture due to the leaching action of the agitated medium on the surface of cells and on their contents.

In view of these concerns, we opted to work with solid cultures, i.e., with cultures grown on the surface of solid media.   In such cultures, a complete development cycle always takes place unless steps are taken to inhibit that cycle at specific points (see below).   Moreover, aerial and vegetative hyphae are easily distinguished even before the former produces conidial heads, and the natural environmental distinction between aerial and vegetative culture components is preserved.

b)   THE METHODS.—Some technical difficulties had to be overcome before solid culture was used routinely.   Provision had to be made to monitor the morphological development of cultures growing on Petri plates without breaking sterility.   Using 20 ml of growth medium in 100 x 15 mm Petri plates and viewing cultures directly through the plate top with a 4X objective of working distance 21 mm allows this to be achieved.   If the growth medium is any of the standard agars, lateral, epi or diascopic illumination is possible; if the medium is a purée of fruit or a porridge of grain, diascopic illumination is not very effective.   To allow convenient separation of biomass and growth medium, cultures are grown on a circle of dialysis membrane placed on the surface of the growth medium ("over" cultures).   Growing a culture on a dialysis membrane also allows it to be transferred easily from one growth medium to another or to be inverted and returned to the original plate.   The significance of such "inverted over" cultures will become apparent later.   To permit separation of aerial from vegetative hyphae, over cultures can be freed of medium, dried *in vacuo* on the supporting dialysis membrane, and scraped with a coarse brush or metal spatula.   The material scraped off is rich in aerial hyphae; the residue is predominantly vegetative hyphae.   Isotopically labeled substrates can be administered to solid cultures simply by spraying them with an aqueous (or aqueous dimethyl sulphoxide) solution of the substrate.   For this purpose, we use a coarse chromatogram sprayer, the coarseness precluding aerosol formation.   The same technique can be used for administering drugs to cultures.

The solid culture approach used in conjunction with membranes allows a variety of biological manipulations to be made that are not possible in submerged liquid culture.   Firstly, if spores are inoculated onto a dialysis membrane and are then covered with a second dialysis membrane, a "between" culture results.   Such cultures grow between the membrane layers, never forcing them apart nor forming conidial heads.   We believe between culture consist solely of cells of the vegetative hyphal phenotype.   Isotopically labeled substrates and drugs can be sprayed onto between cultures satisfactorily.

Between cultures are valuable in their own right and also as a route to two other culture preparations.   If, following a period of growth, the two membranes in a between culture are separated carefully such that the biomass adheres exclusively to one membrane and this biomass-bearing membrane is returned, biomass uppermost, to the growth medium, a "stripped-between" culture results.   Stripped-between culture develop aerial hyphae rapidly and synchronously across the whole Petri dish—a very valuable attribute where fungal physiology is being examined.   Secondly, if a between culture is constructed from two membranes with the upper membrane being approximately 50% smaller in area than the lower, there comes a time when the growing culture extends to the periphery of the upper membrane.   At the periphery it then forms an aerial mycelium.   Such "ring cultures" allow access to vegetative mycelium (under the upper membrane)

that is associated with, but is not physically supporting, an aerial mycelium. Such preparations are important in studying trigger mechanisms. A third value of between cultures is that in their construction, a Nucleopore filter of defined pore size can be used rather than a dialysis membrane. By use of a range of pore sizes, cultures can be prepared that have no aerials at all or that have aerials threaded through the linear pores of the filter. Preliminary indications are that aerial mycelium can be cleanly shaved off those "Nucleopore-through" cultures.

Reconstructing a between culture after it has been stripped and has been left in the stripped state for a few hours is difficult to do without trapping air bubbles and/or damaging the culture. A reasonable approach to a "reconstituted between" culture is simply to invert a stripped-between (or an over) culture and place it, biomass downwards, onto the growth medium.

The analytical chemistry associated with solid culture is not particularly difficult. Indeed, the scale on which extractions, etc., need to be done, is conveniently small. Standard thin layer, gas and high pressure liquid chromatographies are appropriate, as are standrd gel electrophoresis and immunological methods. As the latter section of this article will describe, radiogas chromatography (rgc) and rgc/mass spectrometry (rgc/ms) have proved to be invaluable comprehensive analytical techniques; radioliquid chromatography, possibly also used in conjunction with mass spectrometry, may also have a significant future role to play. Microscopy (light, fluorescent and electron), including cytochemical localization, can be expected to become an important analytical method in the context of solid culturing.

c) SOME REPRESENTATIVE RESULTS.—Resort to a new culturing format is only appropriate if it delivers experimental insights that other culturing formats cannot provide. Listed below are findings which we believe could not have been obtained from the submerged liquid culture format because: the complete development cycle, through conidiation, could not have been guaranteed to occur; vegetative and aerial hyphae could not have been distinguished; and the leaching action of the medium would have masked specific associations of specific secondary metabolites with specific culture locations.

i) In *P. brevicompactum*, mycophenolic acid (I), asperphenamate (II) and brevianamides A and B (III, A and B are diastereomeric at position *) are not produced by between cultures. Mycophenolic acid and asperphenamate appear in over or in stripped between cultures just after an aerial mycelium begins forming; brevianamides A and B appears after the aerial hyphae have formed conidial heads and conidiation has begun (32–34).

ii) In *P. brevicompactum* ring cultures, the three secondary metabolites are found only in association with the biomass of the ring.

iii) In *P. brevicompactum*, mycophenolic acid is excreted into the growth medium; asperphenamate and the brevianamides are found almost exclusively in the aerial mycelium. Epi-fluorescence microscopy locates the brevianamides on the upper reaches of the conidiophore, on the penicillus and on the immature conidia close to the sterigmata (33,34). [Conidial heads containing the brevianamides spin in their long axis on illumination with near ultraviolet light, spinning back to their original position when the light is extinguished (35). This observation could certainly never have been made in submerged liquid culture.]

iv) In *P. brevicompactum*, nutrient depletion in the growth medium (C, N or P) is not a prerequisite for initiation of production of any of the three secondary metabolites (32,33).

v) In *P. patulum*, 6-methylsalicyclic acid (IV), production does not occur in between culture, but does occur in over or stripped-between cultures that are forming aerial hyphae. Conidial head formation is not required for 6-methylsalicylic acid production (36).

I

II

III

IV
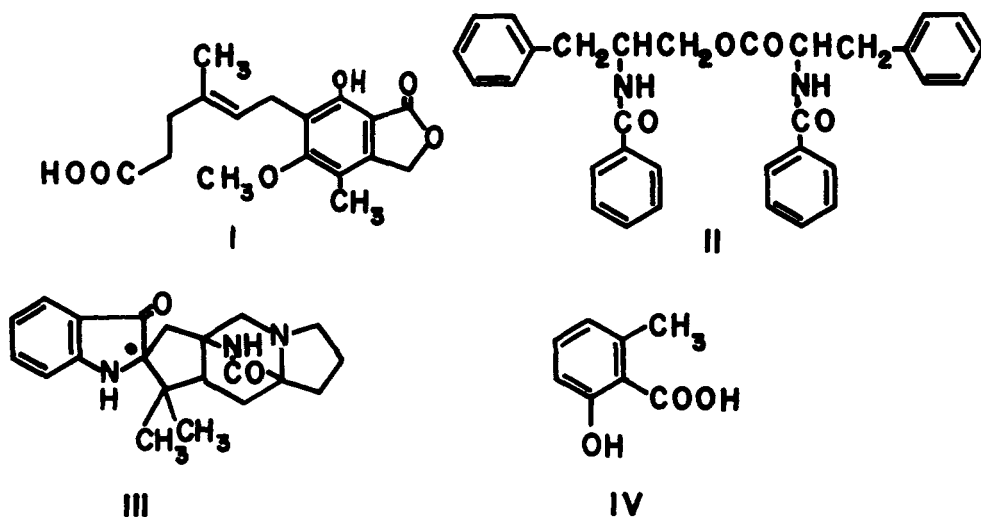
vi)   In *P. patulum*, 6-methylsalicylic acid is excreted into the growth medium (28).

vii)   In *P. patulum*, nutrient depletion in the growth medium (C, N, or P) is not a prerequisite for 6-methylsalicylic acid production or for aerial mycelium formation in over or stripped between culture. Moreover, nutrient depletion (natural) or manipulated by culture transfer) in a between culture does not trigger 6-methylsalicylic acid production (36). This behavior is in total contrast to what is found in submerged liquid culture (see 22).

Here is not the place to attempt to interpret those results; suffice that it be realized that they represent findings which could only have been obtained in solid culture.

One final word in this section: by advocating the more widespread use of solid culture, we do not intend to abandon or discourage the use of liquid culture methods. We foresee a time when, armed with a reasonable, solid culture-derived understanding of how fungal secondary metabolism is integrated into the development cycle of the organism, definitive research and production runs will be made in the liquid culture format.

5.   *Radiogas Chromatography/Mass Spectrometry*

Radiogas chromatography/mass spectrometry (rgc/ms) permits separation of mixtures of organic molecules and simultaneous identification and assay of each mixture component for amount and isotope content. Both radioactive and stable isotopes can be handled. Component separation is achieved by gas chromatography (gc); component identification is based on gc retention times and mass spectral fragmentation patterns; component quantitation involves integration of peak areas of total ion current (tic) and/or selected ion current (sic) profiles and relation of such areas to areas produced by standard amounts of the component; component radioactivity is assayed directly in a flow counter of the proportional or Geiger type; and stable isotope enrichments are assayed by analysis of the mass spectral parent molecular ion cluster ratios. The only limits bounding the use of rgc/ms are the natural or inducible volatilities of mixture components, the resolving power of the gc column, the sensitivity of the various rgc/ms unit detectors, and the capability of the user to attribute structures to components based solely on gc retention and mass spectral data.

Rgc/ms is an ideal technique for studying primary or secondary metabolite biosynthesis, for monitoring biodegradation, and for elucidation of how drugs,

etc., affect the flow of mass through metabolic pathways and grids. It is comprehensive in coverage of nonpolymeric cell constituents, is possessed of high separation resolution—particularly if capillary gc columns are used in the gc module, is capable of detecting mixture components at both high sensitivity (1–100 molecule/cell) and with high selectivity, and has kinship with rgc, radioliquid chromatography (rlc) and rlc/ms.
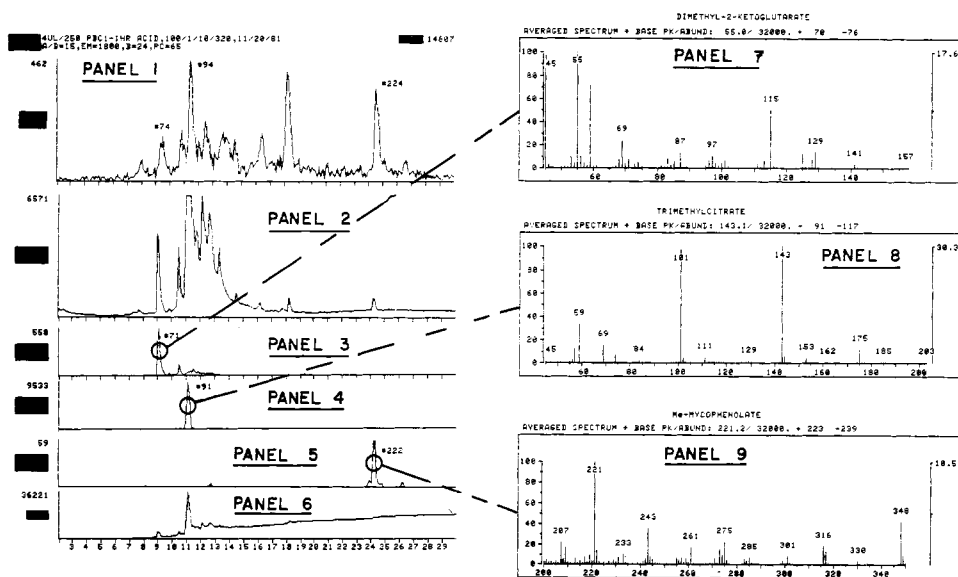

FIGURE LEGEND: An overview of the information that can be obtained from a single rgc/ms run. The sample was obtained by treating an over culture of *Penicillium brevicompactum*, approximately 500 mg of biomass, with [1-¹⁴C]acetate for an hour. Thereafter, an ethyl acetate extract was made of the biomass and that extract was methylated with ethereal diazomethane. An aliquot of this methylated extract was used in this analysis. Panel 6 shows the raw total ion current profile. Panel 2 shows a processed form of that profile in which only ions between m/z 45 and 450 were used in the profile generation. This procedure excludes much of the gc column background and allows for more meaningful scaling of the profile. Panel 1 is the radioactivity profile (derivative form), called as the m/z 5 selected ion current profile (see 42). The numbers above three of the peaks are scan number indices. Panels 3, 4 and 5 contain the selected ion current profiles for m/z values 115, 143 and 221 respectively. These three ions are key ions in the mass spectra of the methylated forms of 2-ketoglutarate, citrate and mycophenolate, respectively. Panels 7, 8 and 9 confirm that the ion maxima in Panels 3, 4 and 5 are indeed the dimethyl ester of 2-ketoglutarate, the trimethyl ester of citrate and the methyl ester, methyl ether of mycophenolate, respectively. The flow characteristics of the rgc/ms unit is such that a peak arrives at the mass spectrometer a few seconds before it arrives at the radioactivity detector; hence, ion maxima are 2–3 scan numbers lower than are the corresponding radioactivity maxima.

The simplest way to construct an rgc/ms unit is to couple a commercial gc/ms to a commercial gas flow radioactivity counter. In all constructions reported to date (37–43), the counter operates in parallel with the spectrometer. This coupling mode is accomplished by inserting a splitter into the gas stream emerging from the gc column. The majority of the gas flow is directed to the counter with the balance entering the mass spectrometer. Because of their fast scanning capabilities, low resolution spectrometers are most commonly found in commercial gc/ms units. Of the two frequently encountered forms of low resolution mass filter, the quadruple is preferred over the magnetic sector in rgc/ms work since it copes more easily with multiple selected ion monitoring (sim).

In assembling the rgc/ms unit, make-up gas flows may be needed at one or more points. The design and mode of operation of the rgc/ms dictates when and where such flows are required and what specific gas(es) should be used. An

additional aspect of rgc/ms unit construction can be the writing/modification of a data system (42,43).

Several reports have already appeared of how we have used rgc/ms in pursuit of both fungal secondary metabolic and of more general ends (39,44–47). Space does not permit inclusion of any significant amount of new material. Suffice to note that from an rgc/ms run (the figure shows part of the output from a methylated total extract of a *P. brevicompactum* 1-^{14}C-acetate-fed over culture) one derives estimates of radioactivity and mass for duly identified extract components. Assays of stable isotope enrichment could also have been obtained had 1-^{13}C-acetate, etc., been fed. From data sets like this we can determine, for example:

- when acetate is being converted to mycophenolic acid;
- what other cell constituents are being made from the precursor;
- if we increase sensitivity, what are the intermediates in the mycophenolic acid biosynthetic pathway;
- if we carry out the above analysis on culture subcomponents, e.g., aerial hyphae, which cells are making mycophenolic acid, etc.;
- if we sample the biological system (whole or in part) as a function of time following tracer administration, what are the rates of acetate transfer to the various acetogenic end products;
- if we conduct the tracer administration as a function of the development of the culture, when mycophenolic acid production begins and how that onset relates to status of other acetogenic product;
- if we manipulate the culture genetically or through use of drugs prior to tracer administration, how these manipulations affect mycophenolic acid production, etc.

Like fungal secondary metabolism research, and man himself, rgc/ms can "play many parts".

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ERRATUM

Vol. 45, No. 4, Pages 462–465—Podraneoside instead of Pondraneoside, *Podranea* instead of *Pondranea*.