Review Article

SCREENING MICROBIAL METABOLITES FOR NEW DRUGS — THEORETICAL AND PRACTICAL ISSUES

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Introduction

The discovery of novel, non-antibiotic, small molecule pharmaceuticals or product candidates through screening microbial secondary metabolites is an expanding activity throughout the pharmaceutical industry and one that is becoming increasingly fruitful. There is widespread acceptance that microorganisms are a virtually unlimited source of novel structures with many potential therapeutic applications.

The process of producing, identifying and isolating microbial metabolites of interest involves a number of unique organizational and technical issues. Despite the expanding success of this approach to drug discovery, a concise operational review of these issues is not available. This is likely a reflection of the fact that the vast bulk of this work is conducted in the proprietary R&D environs of pharmaceutical companies. The present essay is, therefore, an attempt to fill that void and is intended to benefit researchers not previously exposed to this rather complex endeavor. Thus, a synopsis is provided of some of the critical issues that need to be considered in producing and screening these molecules for new leads of pharmacological interest. Both practical and theoretical issues are discussed.

Why and How Companies Screen Microbial Metabolites

The Return on Investment

There exists a 50 year history of screening microbial secondary metabolites for antimicrobial activity which has led to the discovery and subsequent development of many important antibiotics. In the middle 1960's, the late H. UMEZAWA of the Institute for Microbial Chemistry in Tokyo began screening for molecules from microbial sources with activities as selective enzyme inhibitors. This approach, which continues today, has resulted in numerous therapeutic products[1]. UMEZAWA's successes provided impetus for the expansion of microbial metabolite screening as a path to non-antibiotic drug discovery. As elegantly reasoned more than a decade ago by WOODRUFF[2], the structural novelty and diversity of microbial secondary metabolites make them a logical source for exploitation not only for molecules that exhibit antibiosis, but also molecules that interact with mammalian receptors, signal transduction or biosynthetic pathways which represent potentially important drug targets.

Continuing successes vindicate and support this view[3~5]. From an economic perspective, it is difficult, if not impossible, to anticipate and quantify the specific investments required for the discovery and development of individual drugs. However, as judged by the large number of major pharmaceutical companies presently engaged in screening microbial metabolites, the return on investment from this activity is widely perceived as having a high benefit to risk ratio. The revenues from such products as the avermectins (discovered in a collaboration between Merck & Co. and the Kitasato Institute, Tokyo), lovastatin (Merck & Co., Inc), pravastatin (Sankyo Co., Ltd.) and cyclosporin A (Sandoz, Ltd.), along with anticipated revenues from FK-506 (Fujisawa Co., Ltd.) and numerous other candidates under development, would clearly seem to justify the costs and efforts required to find and develop these products. One perspective of costs and rewards is provided by the recent commentary on the discovery of the HMG-CoA reductase inhibitor, lovastatin, which, remarkably, was found after only three weeks of screening soil microorganisms[6].

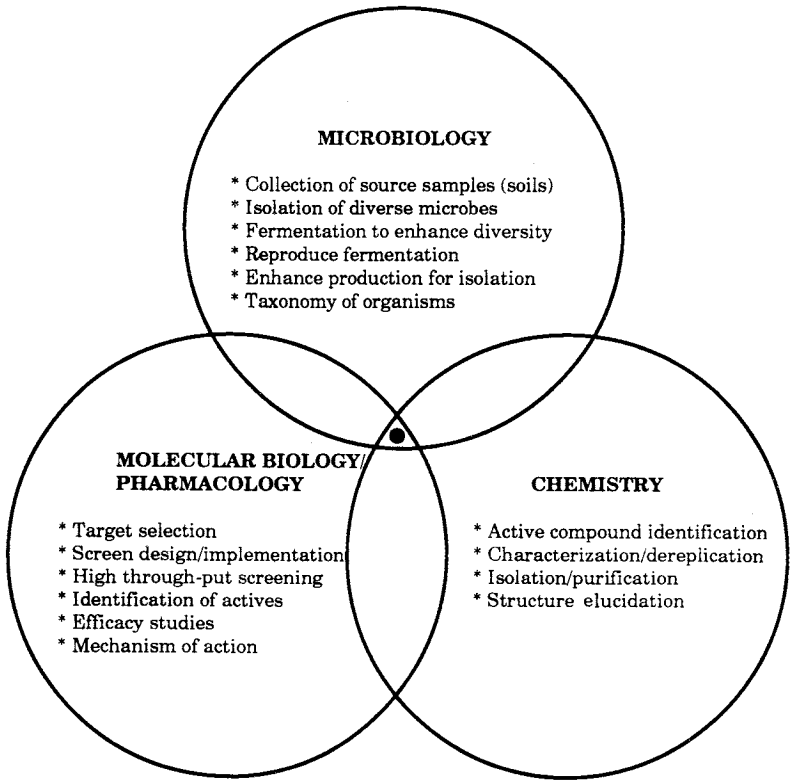
Organizational Issues

Significant operational constraints face most organizations wishing to engage in a microbial metabolite screening program. These relate primarily to a critical mass of scientific and fiscal resources and to the fostering of cooperation across diverse scientific disciplines. Fig. 1 is a representation of the major scientific disciplines which ideally should be integrated to drive a successful screening program. These include microbiology, pharmacology and natural product chemistry. However, even when an organization has these scientific and technical resources, it is not a trivial endeavor to coordinate the efforts of these generally disparate scientific disciplines.

Depending on the therapeutic objectives or institutional strengths and histories, one or two of these disciplines usually emerge as the "driving force" of a discovery program. In random screening for new antibiotics from microbial sources, it is logical that a high degree of microbial chemistry diversity, both in terms of microbial ecology and optimization of novel metabolite production (*i.e.* microbiology), and rapid lead dereplication (*i.e.* chemistry) represent the driving forces. This is because thousands of structures have already been described which possess antimicrobial activity[7]. In other instances, the driving force is the identification of novel structures where associated biological activity may be of secondary interest.

More recently, the integration into microbial screening of pharmacology and molecular biology have

Fig. 1. Integration of scientific disciplines in the screening of microbial metabolites.



● Integrated team effort leading to discovery of novel therapeutic leads.

focused efforts on novel molecular targets and the development of new screening technologies. This has altered the approach to screening microbial metabolites, thus bringing new perspectives into what historically has been the domain of the microbiologist and natural product chemist. By expanding the targets and ability to detect novel activities, this new perspective on screening dramatically expands the potential return on investment. This integration is, in fact, the major driving force throughout the industry for the continuing expansion of interest in microbial chemistry as a source of new therapeutics.

Technical Aspects of the Laboratory Operations

Optimizing Secondary Metabolite Diversity

Secondary metabolites are, by one definition, molecules that are synthesized by microorganisms but are not required for growth. It is not known why microorganisms produce these compounds. Relevance to past evolutionary survival pressures or current needs for competition in their ecological niche are usually invoked as possible explanations[8]. Antecedent to discovery, it is desirable to introduce as much metabolite diversity into a screening program as is possible. In addressing this objective, three major elements are discussed below.

1. Diversity and Novelty of Microorganisms

To achieve metabolite structural diversity, it is generally agreed that a diverse and novel repertoire

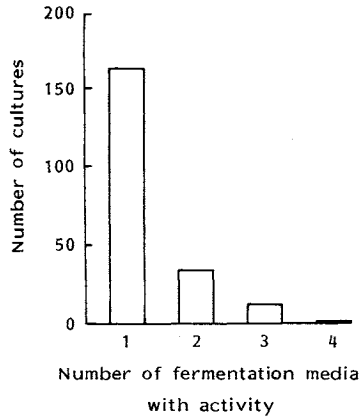
of microbes is desirable. The issue of which, if any, microorganisms should be favored in the selection process for screening has been discussed extensively (reviewed in reference)[5]. Filamentous fungi, actinomycetes, unicellular bacteria and others have all yielded successes. Fungi and actinomycetes are usually targeted because most of the microbially derived antibiotics and therapeutic drugs manufactured today are produced using members of these taxonomic groups. In order to obtain a novel and diverse collection, soils are routinely sampled from different geographical areas and ecological habitats. Treatments of the soils (*e.g.* heat, chemical) and selection pressures (*e.g.* enrichments for the growth of certain microbes by adding antibacterial and antifungal compounds to isolation media) are routinely applied in the initial isolation and incubation procedures to favor the growth of desired organisms. At this early stage, some taxonomic characterization may help ensure that the same common organisms are not being routinely selected and re-introduced into the program. This is part of the overall effort to limit the rediscovery of similar metabolites. Differences of opinion exist as to the level of effort appropriate and necessary in obtaining "exotic" or highly unusual microbes. Additionally, it is the opinion of the authors that freshly obtained, wild organisms yield greater microbial metabolite diversity than isolates held in culture collections. Organisms which are maintained in culture collections and subjected to repeated passages (*i.e.* regrowth in the same fermentation media) may lose some of their original capacity for gene expression and are not as robust in their level of production of secondary metabolites[9].

Once isolated, these microbial cultures must be preserved. Any activity observed in initial testing must be reproduced in subsequent fermentations, often weeks and months later. A number of preservation options are available with lyophilization, freezing at $\leq 70°C$ in liquid media, or frozen storage in the vapor phase of liquid nitrogen being the preferred methods for storage of actinomycetes and fungi[10,11].

2. Fermentation of Microorganisms

Metabolite diversity and production are functions of the biosynthetic capabilities of the organism and the fermentation conditions which allow for the expression of this capacity. Fermentation parameters can be manipulated to encourage the production of diverse secondary metabolites. Usually, the biosynthetic pathways for the production of these molecules are activated in the late logarithmic to stationary stage of culture development, after cell division and biomass accumulation have largely ceased. The time needed for appearance of secondary metabolites in the fermentation medium can be reduced by first growing the organism in a seed medium which has been optimized to promote cell growth. Once the organisms have been grown in the seed medium, inocula can be transferred to multiple production media. These media, differing in carbon and nitrogen sources, and other media components, promote the production of different metabolites (as illustrated in Fig. 2). At periodic intervals in a

Fig. 2. Media dependence for production of active metabolites.



These data are derived from 760 microorganisms, each fermented in four different media, and screened in several assays. The number of cultures determined to be active were then compared to determine the number of media in which that activity was produced. The bars indicate the number of broths with activity in a single media (1), two of the four media (2), three of the four media (3), and in all four media (4). 75% of the activities were produced in only one of the four media.

screening program, media are routinely rotated to optimize metabolite diversity.

Numerous other fermentation parameters, such as temperature, pH and aeration, also influence metabolite yields and diversity. These and other variables can be considered in an attempt to optimize diversity. One commonly discussed issue is the size and shape of the fermentation vessel, that is the use of test tubes *versus* shake flasks for fermentation. With appropriate care and diligence, test tube fermentations can be acceptable, but it is generally easier to optimize metabolite production and to obtain more reproducible conditions in shake flasks. Some of the assumptions in this endeavor are empirical and difficult to quantify or statistically validate. The approaches tend to be a compromise between or a combination of travelling to the "ends of the earth" to collect "exotic" organisms *versus* using a few organisms fermented in a wide variety of media under various conditions. There is, however, little debate that efforts taken to enhance the quality and diversity of the microbial metabolites produced by these "chemical factories" are important contributors to success.

3. Preparation of Fermentation Samples for Screening

The next consideration relates to the optimal method to capture the secondary metabolites in preparation for screening. The metabolites of interest may include intracellular molecules as well as those secreted into the fermentation media. The primary consideration is generally to capture and concentrate as many diverse metabolites as possible using a method which will not interfere with the assay.

Often, biochemical assays employing peptide or protein ligands in conjunction with cellular or sub-cellular target proteins are used in screening programs. Thus, some degree of initial broth preparation is desirable to minimize protease activity and interference by non-specific cellular components, while simultaneously achieving the maximally tolerated "dose" of metabolites. Depending on the number of samples to be processed and the type of assay involved, a choice can generally be made from the following options:

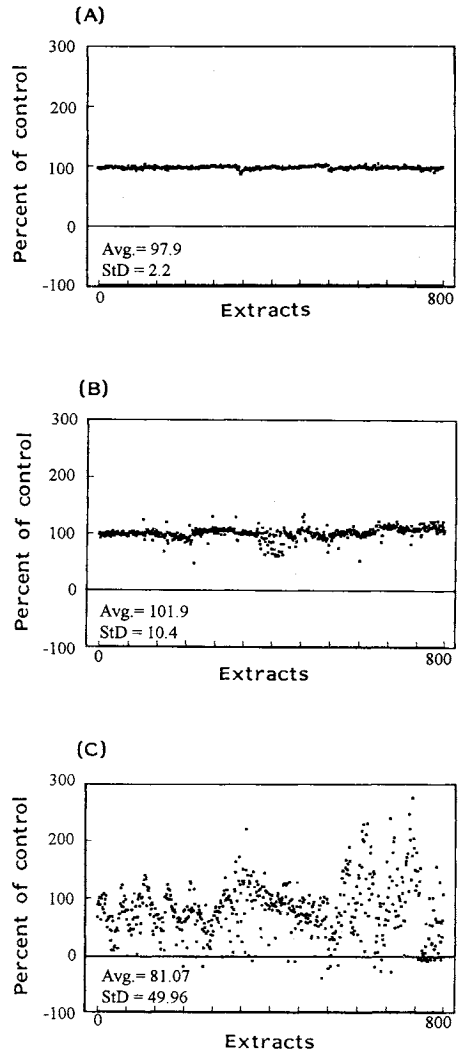
1) Heating of whole broth (may inactivate labile metabolites);
2) Filtration of broth supernatant through a high molecular weight exclusion filter with subsequent freeze-drying of the filtrate;
3) Extraction of either whole broth or broth supernatant with organic solvents of varying polarities (*e.g.* methanol or ethyl acetate) followed by evaporation of the extracts to dryness.
4) Mixing whole broth with polystyrene resin. The resin is then washed with water and bound materials eluted with either methanol or acetone. The solvent is then removed by evaporation.

Each strategy has limitations. Efficiency and cost, assay compatibility, and provision of an initial basis for more rapid isolation and purification of molecules of interest all become important factors in deciding which extraction/purification approach to employ. With any given assay, predictions are difficult at best; experience is usually the best guide. For example, with a particular radioligand binding assay, we found that fermented media extracted with organic solvents of substantially different polarities (*i.e.* methanol and ethyl acetate) both exhibited high levels of background activity, thus presenting a low signal to noise "window" for detecting a lead. When fermented broths were filtered with a molecular weight sieve device, the background noise in the assay was dramatically reduced. Thus, among the sample preparation procedures tested in this particular assay, the molecular filtration method of sample preparation was the best approach. If the same broths are to be tested in several assays, one must ask if it is economically justifiable to subject fermentation broths to different extraction procedures for different assays. Such questions must be answered on a pragmatic and operational basis. Additionally, it may be reasonable to systematically rotate

or alternate between extraction procedures, in a manner similar to rotating media for promoting metabolite diversity. This is particularly true with low yielding screens in which few leads result.

Finally, not only is it important to consider how the sample is prepared but also the concentration or amount of extract to be tested. The magnitude of effect that extract concentration has on assay standard deviation varies with different assays. However, as illustrated in Fig. 3, a general correlation between increasing extract concentration and increasing assay standard deviation holds. Thus, the choice of the extract concentration used in each assay is a compromise between having enough extract present to detect potentially active compounds and the effect of higher extract concentrations on the standard deviation of the assay.

Fig. 3. Effect of extract concentration on assay standard deviation.



A panel of 800 different extracts was tested in a typical biochemical assay at three concentrations: 0.05× (A), 0.1× (B), and 0.2× (C). The response of individual extracts are plotted as a percent of control. Inhibition of the assay is indicated by values less than 100% and enhancement of activity by values greater than 100%. These results show a typical relationship between extract concentration and assay standard deviation.

Screening Strategies and Tactics

The application of modern molecular biology to the study of basic physiological and immunological processes have revealed a large number of new and potentially important targets for drug discovery. These include receptor sub-types, enzyme isoforms, cellular carbohydrate and lipid molecules, and protein-protein interactions in complex signal transduction pathways. Also, the power of molecular biology and recombinant DNA (rDNA) technology increasingly is "enabling" by providing access to cloned and expressed proteins for use in developing novel screening approaches to search for small molecule therapeutics[12,13]. HOBDEN and HARRIS[14] have recently provided an analysis of the increasing and varied impact of molecular biology in natural product screening wherein they document and address four major functions fulfilled by rDNA technology to the screening enterprise: "1) provision of recombinant proteins for assays, 2) analysis of receptor structure and function and production of cell lines expressing individual receptors, 3) construction of screening organisms containing genes which alter phenotype, and 4) construction of cell lines for measuring alteration of gene transcription." So now the question increasingly is shifting from "can it be done?" to "should it be done?" The plethora of available targets and the uncertainties regarding the relative contribution that any specific target gene product makes

to various pathologies will continue to fuel debates as to whether screening efforts should focus on highly specific "mechanism defined" targets using purified sub-cellular ligands and reagents, or be based on functional activity at the cellular, tissue or whole animal levels. For example, KUMAGAI *et al.*[15] have argued for the rationale of using whole cell assays as opposed to cell-free systems when screening for enzyme inhibitors. Additionally, philosophical differences exist with regard to the desirability of focusing screening efforts in a fairly limited number of therapeutic areas *versus* screening against a broad range of therapeutic targets. Researchers involved in the algae metabolite screening program at Merck & Co. have stated their conviction that the latter approach is likely to prove more productive[16].

In screening microbial metabolites, which are impure mixtures and where active compounds may be present in low amounts, there is a general consensus that screening strategies most likely to be successful use assays with certain characteristics and common tactics for identifying and reproducing leads. The following discussion will focus on some these issues.

1. Characteristics of Suitable Assays

Sensitivity: If the assumptions are made that 1) broth concentrations of active metabolites are in the range of $1 \sim 10\,\mu g/ml$, 2) the average molecular weight of a metabolite is 500 D, and 3) there is a $20 \sim 100$ fold dilution in the concentration of metabolite in the final assay, then the required detection limits of the assay are in the $20 \sim 200$ nM range. These are well within the limits of radioligand binding, cell-based function, signal transduction and most enzyme inhibition assays, but normally this would not be applicable to conventional tissue or *in vivo* pharmacological assays. Obviously, the greater the sensitivity of the assay, the better are the chances of detecting activity from a crude fermentation extract where active metabolites might be present in very low concentrations or the affinity and/or intrinsic activity of a lead molecule might be low (but subject to synthetic enhancement later).

Selectivity: When employing more than one assay in the primary screening of the same fermentation samples, empirical judgements can be made with regard to lead selection. Thus, even though the assays may be highly sensitive with a high signal to noise ratio, the crude or partially purified samples being tested should show relatively selective activity among unrelated assays or the activity otherwise can be dismissed as non-specific. Assays that are sensitive to ubiquitous primary or secondary metabolites will be problematic. With any screening endeavor, a balance is sought to avoid false positives and not to exclude false negatives. Since, false negatives rarely get a second chance sensitivity and selectivity need to be considered in relative terms. These concerns are especially important in efforts to discover leads where no prototypic reference agents or pre-existing leads are known.

High throughput: With sufficient resources, including time, most assays can be made to process large numbers of samples. However, the efficiency of an assay (*i.e.* throughput *versus* cost) is the concern in the real world. For example, some cell-based assays may be labor intensive and relatively slow if the cells have a long doubling time in culture. With miniaturization, automation, the use of robotics and electronic data collection and analysis, most assays can be made extremely efficient. It is very often the inefficiencies of generating the metabolites for screening that impede throughput rather than the assay itself.

2. Identification of Lead Cultures

When can a fermentation sample be considered a "lead"? Having once satisfied potency and selectivity criteria (however defined), a predominant concern, unique to metabolite screening, is fermentation reproducibility. The activity must be confirmed in subsequent fermentations. Although not generally appreciated by pharmacologists and chemists, experienced microbiologists sometimes must accept

Table 1. Criteria for lead selection: potency, selectivity, reproducibility.

Fermentation/ media	Radioligand binding assay (% inhibition)		
	Assay 1	Assay 2	Assay 3
1st/A	15	12	9
1st/B	0	27	13
1st/C	52	23	1
1st/D	23	9	10
2nd/C	48	15	13

Fermentation broth extracts, produced by a single culture fermented using four media, A, B, C and D, were screened in three radioligand binding assays. Although activity (% inhibition) can be detected in many of the assays, the potencies were insufficient to be considered significant (*i.e.* $\geq 50\%$ for assay 1, $\geq 75\%$ for assay 2, and $\geq 90\%$ for assay 3) except for Assay 1 in medium C. Thus, potency and selectivity criteria were satisfied. Refermentation in medium C indicated that the activity was reproducible and although it was slightly below the cut off for significance. It was considered adequate to merit further evaluation.

Table 2. Generating a dereplication data base.

Procedure	Information available
Microbe collection	Geographical, ecological habitat
Microbe isolation	Growth characteristics, isolation conditions, morphology
Microbe fermentation	Growth characteristic in different fermentation media
Sample preparation	Crude estimates of compound polarity, acid-base status
Primary screening	Sensitivity, specificity, potency in target assays
Repeated fermentations	Reproducibility, stability characteristics
Expanded biological testing	Further information on potency, specificity, biological and microbial activity
Isolation/purification	Physical chemical characteristics, *e.g.* solvent partitioning, TLC, HPLC with diode assay spectral analysis, *etc.*
Structure determination	Known or new structure
Microbe taxonomy	Species

reproducibility rates of 50% and lower.

Problems are seen when assessing results obtained upon refermentation, especially when both significant activity and selectivity are applied simultaneously as necessary criteria for accepting a screening activity result as a reproducible lead. As an example, Table 1 presents actual results illustrating this not uncommon dilemma. In this instance, the initial screening of the sample satisfied minimal criteria regarding potency and selectivity. Upon refermentation, while the overall trend and profile of activity was consonant with the initial results, the predetermined potency criteria was not satisfied. Pragmatically, it is reasonable to accept less stringent potency and selectivity criteria to identify refermented activities as leads, especially in screens giving a low number of leads.

Dereplication

When a crude extract is found to be active in a screening assay a considerable amount of further effort is required before the chemical structure responsible for the activity is elucidated. It is important that screening "hits" due to previously discovered structures are identified quickly so that resources are not wasted. This process is referred to as dereplication. These are two types of previously discovered compounds that dereplication procedures need to identify: the first are known compounds that are expected to be found as hits in a screen and the second are compounds whose structure has been previously described but not known to be active in the screen. In both cases rapid identification of previously discovered compounds is accomplished by the combination of chemistry, bioactivity profiling, and taxonomy (see Table 2).

Efficient dereplication protocols designed to rapidly identify compounds which are expected to turn up as hits in a particular assay are based on a data base including characteristic spectral data, bioactivity profile, and producing organism taxonomy for each metabolite that is likely to be rediscovered. As an example, when protein kinase assays are in a screening program that utilizes microbial secondary metabolites

as the natural product source, it is likely that a common metabolite such as staurosporin will be rediscovered. Staurosporin can be quickly identified in crude extracts by its characteristic fluorescence excitation and emission spectra, bioactivity in a variety of tyrosine and serine/threonine kinase assays, and that it is produced by *Streptomycetes* sp. Similar protocols can be developed for other expected and common structures exposed to primary screening assays.

The identification of previously discovered compounds that are not known to be active in a particular assay can often be accomplished quickly by taking advantage of the available data bases of natural product structures. For example, after obtaining the absorbance maxima and molecular weight (from mass spectral data) for a bioactive metabolite, this information can be combined with the taxonomy of the producing organisms. Together this information can be used to generate a list of potential structures from a data base. Based on this, a series of further experiments can be designed which either confirm that the bioactivity results from a previously described structure or that it is likely a new chemical entity.

Conclusions

Microbial metabolites are a rich source of potential new therapeutic drugs. Programs designed to identify these new drugs are unique in that many scientific disciplines must be integrated to effect a successful effort. Some of the major concerns intrinsic to the process of screening soil microbial secondary metabolites, in contrast to screening pure chemicals, include:

1) fostering cooperativity among generally disparate scientific disciplines,
2) ensuring and optimizing metabolite diversity through the collection and fermentation of microorganisms,
3) balancing the inherent limitations of currently available fermentation sample extraction procedures against the issues of efficiency, assay compatibility and maximal sample capture,
4) establishing criteria for the identification of screening leads based on potency, selectivity and reproducibility, and
5) addressing the importance of efficient dereplication of leads.

Whilst to the expert engaged in this process, many of these concerns will appear obvious or even trivial, the uninitiated may underestimate the complex nature of this endeavor. The enterprise of screening microbial metabolites for new leads, first exploited by antibiotic researchers and today expanded to virtually all fields of therapeutic interest, has proven successful and will continue as an important avenue to new drug discovery.

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