Advantages and Perspective of Fortified Agarised Media Application

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With mechanical fortifying, the solid (agarised) media layers become more suitable and efficient in realising their application purposes. Essentially, the fortified agarised media layers become more suitable for applications mainly because they enable the use of both layer sides to start microbial cultures, or to realise the contacts with other reaction systems. The fortifying can be performed by applying the appropriately designed nets made from inert material, based on the direct use of commercially available wire nets, or on the weaving of metallic or other appropriate filaments, or on the application of other procedures of working out net tissues and perforated sheets as well as devices based on them. Advantages and perspective of the application of fortified agarised media layers are discussed on the basis of author's own partly already published and discussed experimental data, with taking into account the expected further applications. Authors recommend the further studies and applications, especially because of supposed more efficient realisation of series of research goals (*e.g.* of microorganism screening and bioactive substance recognition) and expected discovery of relationships generally applicable for microbial populations and populations of other organisms.

Key words:
Mechanically fortified solid media, application advantages, microorganism screening, bioactive substance recognition

Introduction

Petri-dishes containing agarised media layers are frequently applied in cultivating microorganisms for different research purposes. In addition to Petri-dishes of different sizes, plates of other form can also be used to prepare agarised media layers. Large quadratic dishes (300 × 300 mm) with appropriate agarised media, *e.g.,* they are commonly used to assay antibiotic concentrations in different samples. In general, determination of specific microbial activities and screening of microorganisms are often the main goals of studies which can commonly be successfully attained by applying plates with agarised media. However, a disadvantage of the use of common agarised media layers in mentioned dishes is that such layers do not enable a simple use of both layer sides for cultivation of microorganisms. Recently,[1–3] it was shown that this disadvantage can be overcome by inserting the net (screen) of an inert metal (*e.g.* stainless steel) into agarised medium layer. As demonstrated, in such a way the layer becomes fortified and therefore suitable for manipulation. The convenience of the use of fortified agarised media layers was investigated comparing antibiotic productivities of different microbial strains[2,3] as well as when testing the presence of bioactive substances in samples of different reaction products (*M. Bošnjak, I. Crnolatac, D. Hranueli,* unpublished results). To shed more light on substance diffusion relationships, kinetics of citric acid diffusion through the common and fortified agarised media layers were compared.[3] Some physical properties of agarised media layers prepared with different agar fractions (*e.g.* break loads of tested layer slices) were also investigated to find the best solutions for preparation of fortified media. Assuming the characteristics of inserted metallic screens can influence the properties of prepared fortified media layers, different inert metallic nets were applied to estimate their convenience for preparation of agarised media layers.[3] The study was not limited only to the use of commercially available screen nets. Additionally, different possibilities of preparing the corresponding screens for their inclusion in the structure of fortified media layers were analysed with proposed solutions for application purposes.[4] Although, the application of metallic nets and nets of some other materials is already common in a series of different engineering fields (even in surgery) and, although, the use of aluminium grids (thickness 0.2 cm, perforations 1.27

Table 1 – *Kinetics of citric acid diffusion through common and fortified agarised media layers Agarised citric acid solution layer discs of Φ =7 mm were put on the agarised media layers in Petri-dishes to enable citric acid diffusion*

Agarised medium layer type	Diffusion time [mm]	Diffusion zone diameter [mm]		Layer height [mm]	
		on top side	on bottom side	total	of diffusion zone
Common, with 3 % agar	120	15.0	–	7.0	6.0
Common, with 3 % agar	220	18.0	12.0	7.0	7.0
Fortified, with 3 % agar and with inserted metallic net (holes of 4mm^2)	220	18.0	12.0	7.0	7.0
Common, with 2 % agar	126	16.0	7.0	7.5	7.5
Common, with 2 % agar	182	18.0	12.0	7.5	7.5
Fortified, with 2 % agar and with inserted metallic net (holes of 4mm^2)	182	18.0	11.0	7.5	7.5

cm · 1.27 cm) fixed to an aluminium sheet (thickness 0.63 cm) for preparation of supported microbial film reactor has long been recognized,[5] the recent information on properties of fortified agarised media layers, formed by inserting inert metallic screen nets and the application of these layers in microbiological laboratories to study the behaviour and activities of different microorganisms, is a rather new one[13] and should be analysed in more detail in order to estimate the perspective of the further use of fortified media layers and to evaluate their significance in studies of the behaviour and activities of different microorganisms and/or even of different cells of other organisms.

Fundamental characteristics of agarised media and comparison of different agarised media properties

Effect of agar concentration on properties of agarised media layers

Depending on the aim of use, agarised media can be prepared to contain different agar concentrations, even those below the limit, above which agar concentrations can lead to medium solidification. However, when preparing standard agarised media, one commonly adds 20–30 g of agar to the medium before it is sterilised. Disregarding this fact, in the course of developing the method of fortified agarised media preparation it appeared necessary to investigate the effects of agar concentrations on properties of agarised media layers. One considered the diffusion permeability and solidity of prepared agarised media layers to be the most relevant characteristics, and therefore the corresponding experiments have been performed.[3] The fortified layers were compared with the common ones, with respect to their diffusion kinetics profiles, their strength and their convenience for testing antibiotic activity. Kinetics of citric acid diffusion through the agarised layers containing traces of methyl red were studied. In the first series of experiments citric acid was incorporated in agar layer discs (Table 1), whereas in the second series of experiments the amounts of 0.1 mL of 1 mol L^{-1} citric acid solution were put into agarised medium layer holes of 7.5 mm diameter (Table 2). To investigate the effect of agar concentration on the agarised medium layer tenacity agarised media layer slices (50 · 10 · 3.5, mm^3 in size) were prepared. Slices were put onto small laboratory stand walls having a 33 mm free interval. Small pieces of lead were then put on a particular layer slice until it broke. Experimental data are disclosed in Table 1 and Table 2. The data in Table 1 clearly show that diffusion kinetics profiles of the fortified agarised media layers do not markedly differ from those of the common agarised media layers. The data also suggest the effects of investigated wire nets (range of sizes of net holes: 1 – 25 mm^2) as diffusion obstacles can be ne-

Table 2 – *Effect of agar concentration on properties of agarised media layers. Diameters of citric acid diffusion zones (after 2 hour diffusion) and threshold layer loads as functions of agar concentration*

Agar concentration [g/L]	20	30	40	50	60	70
Diffusion zone diameter [mm]	25.0	24.0	22.0	21.0	20.5	20.0
Break load of tested layer slice [gm/s^2]	0.0	39.53	108.60	215.82	326.28	–

glected. As demonstrated by the data in Tab. 2, properties of the agarised media layers can be markedly improved by the increased agar concentration, although a concentration increase causes a slight reduction in diffusion rates. Agarised medium layer improvement was especially expressed with respect to its tenacity.

Formation of inhibition zones, recognition of microorganisms with antibiotic activities and evaluation of strain selection criteria

As has been known for a long time, antibiotics inhibit the growth of sensitive microorganisms used as test organisms in assaying antibiotic quantities in different samples of investigated substances of various purity degrees as well as of mixtures of different substances of more or less complex material sources. Liquid samples containing substances with antibiotic activity, after being put in agarised medium layer holes, as well as agar layer discs or paper discs when they contain antibiotic substance, after being put on agarised medium layer surface, inhibit the growth of test microorganism, upon this being inoculated and dispersed in agarised medium layer. Due to growth inhibition caused by antibiotic diffusion the inhibition zones, *i.e.* surface area without observable test microorganism microcolonies, are formed. Microbial colonies of antibiotic producing microorganism can also cause a formation of inhibition zones, after the agarised medium layer where colonies grow has been overlaid with a suspension of test microorganism cells. Since antibiotic diffusion occurs in all directions, antibiotic molecules will appear after some time also on the other side of the agarised medium layer. Therefore, the other side of the agarised medium layer can be used to test antibiotic activity. Indeed, this was confirmed experimentally.[2,3] The convenience of fortified agarised media layers was investigated comparing antibiotic production efficiency of different microbial strains, particularly of different derivatives of the *Streptomyces rimosus* R6-500 strain. Small circular sterile stainless steel wire nets with wire supports on their edges were used to prepare the fortified sterile agarised media layers. After the sterile fortified agarised media layers have been prepared, cultures of strains chosen to be studied were used to start the growth of their colonies on the surface of the upper sides of the fortified layers. The transfer of cultures to inoculate the upper layer surface parts was performed by means of a sharp needle, glass rod or a pipette, depending on the design of the experiment. After the layers in Petri-dishes were inoculated, their incubation was performed at 28 °C and/or 37 °C for a given time. The fortified layers were then reversed using the corresponding tweezers, in order to make the surface of the opposite layer side available for inoculation with the corresponding test microorganism. After seeding the mentioned layer surface with the test microorganism, the dishes with seeded layers were incubated at the same temperature for 20 h (23 h, in one series of experiments). All incubated agarised media layers were then examined to evaluate the formed inhibition zones. The series of experiments was performed and experimental results analysed.[2,3] Some typical results are demonstrated in Fig. 1 and Tab. 3.

As clearly shown in Fig. 1, the convenience of the application of fortified agarised media layers in screening antibiotic producing strains is beyond doubt. Evidently the differences between particular strains can be well defined, if applying the fortified agarised media layers. The advantages of the forti-

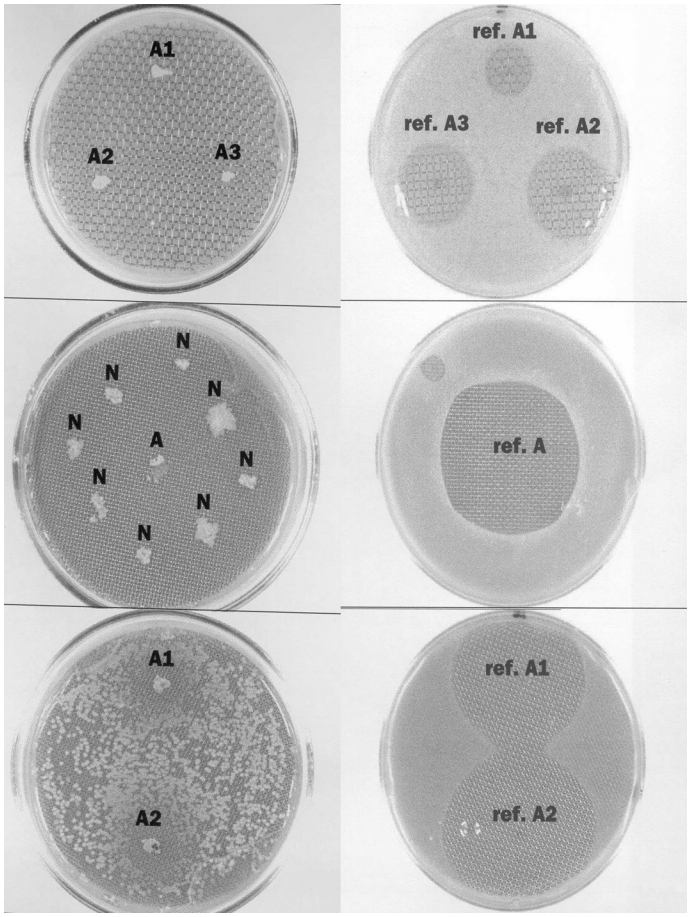

Fig. 1 – *Fortified agarised medium layers in Petri dishes applied for testing of antibiotic productivities of microbial strains. Left : microbial colonies of S. rimosus R6-MV9R1 (antibiotic producing strain,* ***A****) and S. rimosus R6-MV25W (antibiotic nonproducing strain,* ***N****) seeded on front layer side; Right : reverse layer side seeded with Bacillus cereus as test microorganism. Remark: small inhibition zone in the second row resulted due to a contamination with active strain*

Table 3 – *Inhibition zones of Streptomyces rimosus R6-500 colonies. Malt -agar (2%) medium applied*

Type of agarised medium layer	Incubation time [hours]		Test microorganism	Diameter [mm] of:	
	Colony growth	Colony and test microorg. growth		Colony	Inhibition zone
Common	48; 37 °C	23; 37 °C	*Bacillus subtilis* ATCC 6633	11	*53 (56)
Fortified, with inserted metallic net, 1 mm^2 holes	48; 28 °C	23; 37 °C	*Micrococcus luteus* ATCC 9341	10	*36 (44)
Fortified, with inserted metallic net, 4 mm^2 holes	48; 28 °C	23; 37 °C	*B. subtilis* ATCC 6633	9	*33 (42)
Fortified, with inserted metallic net, 25 mm^2 holes	48; 37 °C	23; 37 °C	*Micrococcus luteus* ATCC 9341	11	*46 (46)

*Ref. to clear zone

fied media layers are so evident that one can recommend their application in the screening procedures of streptomycetes. Data in Tab. 3 support such a conclusion. In addition, data disclosed in Tab. 3 demonstrate that culture conditions influenced the colony growth and antibiotic production kinetics and therefore reflected on colony and inhibition zone sizes. Although, data in Tab. 1 and Tab. 3 suggest the inhibition zones on the reverse side appear with some delay and are slightly smaller in size, the advantages of fortified agarised media layers cannot be neglected. Tab. 4 undoubtedly confirms such a statement. When three investigated strains were cultivated in both common and fortified agarised media layers in order to compare their effects on the basis of the same classification criterion, similar relations between activities of compared strains resulted. More over, obtained results suggest that the application of fortified agarised media layers can lead to more reliable data and therefore to more reliable conclusions. In the case of the application of common agarised media layers it is not possible to mark inhibition zones smaller than surface area occupied by microbial colony of investigated microorganism.[6–8] This problem disappears if fortified agarised media layers are applied.

Microbial morphology significance

In colonies of mycelial microorganisms one can distinguish aerial and substrate mycelia. Particular strains can differ with respect to fractions of their aerial and substrate mycelia, as observed in the case of two different *Streptomyces rimosus* derivative strains, nominated as isolates "1001" and "1003" (*M. Bošnjak* and *J. Pigac*, unpublished data). Colonies of the isolate 1001 showed to be more efficient with respect to antibiotic biosynthesis than colonies of the isolate 1003.[7,8] Since it was observed that the fraction of substrate mycelium was more pronounced at the isolate 1003 than at the isolate 1001, the lower efficiency of colonies of the isolate 1003 with reference to antibiotic biosynthesis could be due to more expressed oxygen insufficiency and probably higher inorganic phosphate availability around substrate mycelia. Substrate mycelium consumes dissolved oxygen from the medium much faster than the oxygen from the air can be dissolved into medium and transferred from upper layer surface to the medium part close to the substrate mycelium. Since the antibiotic biosynthesis rate is closely connected with the inorganic phosphate exhaustion rate,[8] higher inorganic phosphate availability, due to the higher fraction of the substrate mycelium, could be one of the causes for reduced antibiotic biosynthesis rate by microbial colonies characterised by higher fractions of substrate mycelia. If one would apply fortified agarised media layers instead of common agarised media layers, then it would be possible to enable additional oxygen absorption and transfer through the opposite layer side surface and, therefore, to improve conditions for colony biomass growth and colony efficient biosynthetic activity.

Table 4 – *Activities of investigated S. rimosus strains expressed as average potency indexes. (Potency index = inhibition zone diameter/colony diameter)*

Agarised media layers	*S. rimosus* R6-500	*S. rimosus* R6-ZGL1	*S. rimosus* R6-MV25W	Remarks
Common media	2.230	1.757	1.0	Average values for 16 different media 6
Fortified standard media	4.250*	2.125*	1.0; 0.0*	*Reverse layer side testing

Analytical recognition of bioactive substances in complex substance mixtures

One of the advantages of microbiological assay methods is that they enable a recognition of bioactive substances independently on information referring to the substance chemical structure. After the screening of microorganisms has been performed it can happen that a more detailed investigation of selected microbial strains would be recommendable in order to determine bioactive substances produced by them in their cultures. Different analytical methods can be applied to separate substances from their mixtures, but common and frequent procedures in detecting particular substances is by applying the thin-layer chromatography and/or gel electrophoresis. If the production of antibiotic substances appears to be the subject of interest, then the common procedure for investigation of the results of thin-layer chromatography is by overlaying the applied thin-layer chromatography plates with a sterile agarised nutrient medium, containing an appropriate concentration of the test-microorganism, and then to incubate the overlaid plates at appropriate temperature for 16 – 24 h, in order to see whether the inhibition zones are formed. Since both the aseptic overlaying of chromatography plates with test-microorganism culture in agarised nutrient media and the culture aseptic incubation are accompanied with some difficulties, the technique of the use of fortified agarised media layers was developed (*M. Bošnjak, I. Crnolatac and D. Hranueli,* unpublished results). To demonstrate its advantages a few antibiotic substances (doxycycline, oxytetracycline, erythromycin, streptomycin and nystatin) were subjected to the thin-layer chromatography (silica-gel 60 F254 plates, solvent system consisting of buthanol, acetic acid and water in ratio 4:4:1 respectively). To detect the position of antibiotic substances on chromatographic plates after the chromatographic assay being performed, the layers of fortified agarised media were applied. These were prepared in an aseptic way applying stainless steel wire nets. The sterile fortified agarised media layers were put onto chromatographic plates to be in contact for a different time (10 s, 1 min, 10 min, 30 min and 1 h). To seed the test-microorganism (*Bacillus cereus, Micrococcus luteus, Escherichia coli, Sacccharomyces cerevisiae*) on agarised medium layer over the line of substance migration during the performed chromatography assay, the technique of textile tapes or cords saturated with liquid culture of the given test-microorganism was developed and applied. To increase assay efficiency the appropriate metallic frame with partition walls separating boxes with respect to particular cultures of test-microorganisms can be used. After being in contact with chromatographic plates the fortified agarised media layers were separated and then subjected to the incubation at 37 °C for 20 h under aseptic conditions. Then the layers were inspected for the presence of inhibition zones. The observed zones were more pronounced, if the contact time between agarised media layers and chromatographic plates was longer, but even short contact of only 10 min was sufficient for an inhibition zone appearance. The convenience of the use of fortified agarised media layers was especially pronounced when microorganisms were tested with respect to their ability to produce new antibiotic substances.

Insertion screen design as a function of study purpose

The design of fortified agarised media layers and, therefore, also the design of the insertion screens to form them largely depend on their application purposes. The aims of studies can be different. As already mentioned, one goal could be the screening of microorganisms with reference to their ability to produce specific bioactive substances, e.g. antibiotics. Another aim can be the analytical recognition of bioactive substances in complex substance mixtures. However, a series of research aims can be efficiently realised by the use of fortified agarised media layers, e.g. a more facilitated determination of relations between different members of mixed microbial populations, detections of probiotic and citostatic activities, etc. Different materials can be used to form insertion screens. One can consider suitable materials to be those mechanically resistant, chemically inert, and biologically non-toxic with respect to nutrient medium and culture cells. Commercially available inert metallic nets appeared to be the most appropriate material to design insertion screens for agarised media layers. Simple small circular stainless steel wire nets can be prepared in such a way. They can be of the size and form to correspond to Petri-dishes planned for the use in microorganism screening experiments. Small nets with rims and with wire supports on the edges designed to determine the agarised medium layer thickness and the layer's space from the bottom can easily be prepared (Fig. 2). Such nets are quite convenient for a simple layer formation in one step. However, a series of possible insertion screen designs and their preparation methods is adequately described.[4] Innovation was presented at "ARCA 2004", the First International Exposition of Innovations, New Ideas, Products and Technologies, Zagreb, September 14 – 19, 2004.[9] The Exposition was organised by the Croatian Community of Technical Culture, as one of the events held during the

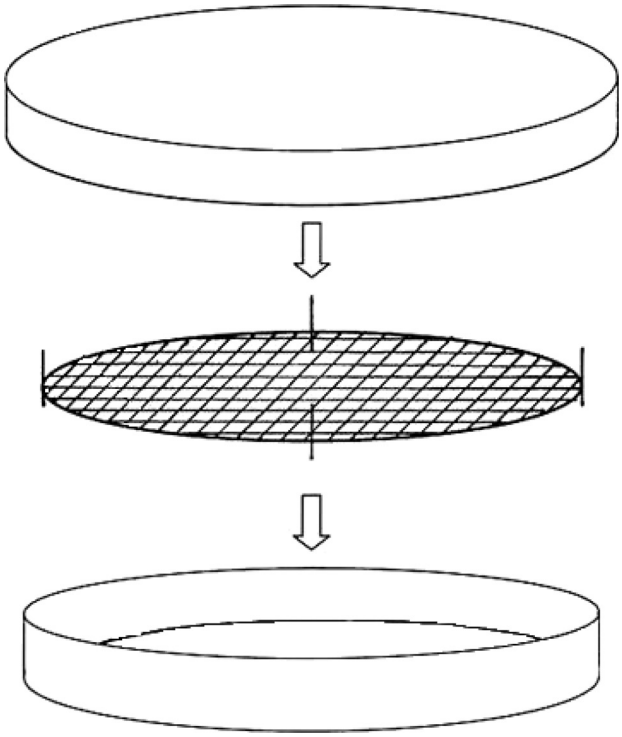

Fig. 2 – *Typical simple circular metallic insertion net applicable to form agarised media layers in Petri-dishes*

International Fair of Zagreb. Since the Exposition Organiser addressed the written "Special Acknowledgement" to Innovation authors (*M. Bošnjak* and *D. Kirša*), one can conclude that the interest for more extended application of fortified agarised media layers really can be expected.

Development of research devices

The story on advantages of fortified agarised media layer application cannot be finished with the above text. As known, the initially developed biological methods of assaying antibiotic activity were based on visual inhibition zone diameter measurements. Later, the optical methods with corresponding devices were developed and introduced in the practice. Recent advances are based on computer applications. The application of fortified agarised media layers can lead to further advances, especially with reference to the development of new devices and methods for microorganism screening. One of the possibilities is to develop more sophisticated methods for screening the antibiotic producing microorganisms. Since one can optically monitor both the surface area of inhibition zones and those occupied by microbial colonies, specific biological activities of microbial colonies or of their clusters can be evaluated on the basis of relations between inhibition zone surface area and corresponding surface area occupied by microbial colonies. The appropriate methods for mathematical treatment of microbial colonies grown on agarised media layers such as classification (*e.g.* as demonstrated by unpublished data of *Ž. Bošnjak* Cihlar), clustering and other data mining methods and algorithms for estimation of specific activities of microbial colonies can be defined to develop corresponding computer software for the recognition and selection of microorganisms with demonstrated specific activity.

Relevance for the study of populations of other organisms

As known[10] and mentioned previously,[11] series of the fundamental relationships are common to all populations of live organisms. Therefore, based on studies of the behaviour of microbial populations at different culture conditions, one can discover the phenomena and relationships of general importance, i.e. the microbial populations can be used as model systems for scientific studies of population relationships in general. Advantage of microbial populations to be applied as model systems resulted due to their much faster reproduction rates in comparison to reproduction rates of populations of other organisms. Due to much faster reproduction rates, much more organism generations can be produced for a much shorter time, and therefore, it becomes possible to recognize some relationships after much shorter experimentation time. It was demonstrated how the results of simple experiments, referring to microbial cultures, can be used to explain some relationships applicable for culture systems of other organisms or their cells.[10] Since the application of fortified agarised media layers undoubtedly can accelerate and facilitate some studies referring to microbial populations, it follows that the application of fortified agarised media layers can induce or even facilitate the more efficient studies of relationships generally applicable for populations of macroorganisms, especially, when one consider the applicability of corresponding mathematical methods in defining population relationships. Computer simulation, e.g., can be applied in describing growth and product formation kinetics in microbial colonies,[7,8] growth and microorganism differentiation kinetics in submerse microbial culture,[11] process kinetics in submerse cultures of different microbial strains,[8] process kinetics of mixed microbial cultures 12, whereas the application of cluster analysis was demonstrated in explaining relations between normal and morphologically changed microbial population in their common culture.[13] Both the computer simulation and data mining techniques (clustering, classification, *etc*.) can be successfully

applied to explain relations in populations of other organisms, including those typical for human population. Of course, there is a series of other mathematical methods applicable in describing or explaining the behaviour and activities of both the microbial populations and populations of other organisms.

Whether to inform students on properties of fortified agarised media layers

Students of those faculties, where microbiology is incorporated in education programmes, certainly should be well informed on methods of performing microbiological experiments. They should be taught how to personally design and to perform experiments individually. Education programmes should be adequate to attain the defined education degree for given profession candidates, and harmonised with reference to actual society needs and perspectives of society development. Therefore, one can recommend including the information on usage possibilities of fortified agarised media layers in mentioned education programmes.

Concluding remarks

We are of the opinion that the application advantages of fortified agarised media layers are not debatable. One can even recommend their further study. However, the problem of finding the optimal solution for commercial production of insertion nets (screens) is still open. Also, the other possibilities of improving mechanical properties of agarised media layers should be considered.

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