Localization of Mycobacterial Enzymes.*† (23245)

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Schneider and Hogeboom(1) pointed out the need for quantitative studies in order to ascertain the true localization of enzymes in particulate and soluble fractions of mammalian cells. It is their opinion that enzymatic activity of isolated fractions should be compared quantitatively with total activity of the cells. Only from such a comparison can conclusions be drawn regarding intracellular distribution of enzymes. Two criteria often used for localization of enzymes are percent recovered activity and specific activity (activity per unit of nitrogen). If a high percentage of enzyme activity is recovered in a fraction and if concentration of enzyme (specific activity) is sufficiently high when compared with total cell activity then that fraction is considered the site of enzyme activity. The results of a quantitative enzymatic localization study of *Mycobacterium tuberculosis* var. *hominis* strain H37Ra have been presented(2). Terminal respiratory enzymes were localized either in a supernatant or one or more particulate fractions. In this investigation the criterion used for localization was dependent on the total quantity of enzyme recovered in a fraction. The fraction containing the highest activity of enzyme was considered the locus of this enzyme within the cell.

It is the purpose of this work to present results of quantitative studies of enzymes of the tricarboxylic acid cycle and of the terminal electron transport system in 5 strains of mycobacteria using the two criteria mentioned above. We hope to show that these criteria often lead to contradictory results which makes necessary some modification.

Materials and methods. This study was carried out with the following organisms: *Mycobacterium tuberculosis* var. *hominis* strains H37Rv, H37Ra and H37RaN;‡ *Mycobacterium tuberculosis* var. *bovis* strain BCG-4;§ and *Mycobacterium smegmatis*. Throughout this paper the strains of *Mycobacterium tuberculosis* will be referred to by the abbreviations H37Rv, H37Ra, H37RaN and BCG. All organisms except *M. smegmatis* were grown as described previously(2). *M. smegmatis* was grown as a pellicle on Difco nutrient broth and harvested after 4 to 7 days incubation at 37°C by centrifugation and/or filtration through medium sintered glass filters. After harvesting, the cells were prepared and fractionated as reported previously (2). All precipitates were homogenized with a hand driven glass homogenizer to insure a homogeneous suspension, and the nitrogen content of each fraction was determined by the micro-Kjeldahl method of Ma and Zuazaga(3). The fractions were labeled L, A, B, C and S as previously reported(2). The L fraction contained particles approximately 100-200 mμ in diameter, the A contained particles approximately 50 mμ in diameter, the B contained particles approximately 25 mμ in diameter, the C contained particles approximately 19 mμ in diameter and the S represented supernatant after the removal of all particulate fractions. With fractions from H37Rv, BCG and *M. smegmatis* the presence of aconitase, fumarase, malic and succinic dehydrogenases was tested by the methods described by Colowick and Kaplan(4); catalase by the method of Lawrence and Halvorson(5); DPNH oxidase by the method of Green, Mackler *et al.*(6); isocitric dehydrogenase by the method of Hogeboom and Schneider(7); and alpha-ketoglutaric dehy-

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‡ A mutant of *M. tuberculosis* var. *hominis* strain H37Rv transferred on a nitrogen deficient medium for over 6 years.

§ Kindly supplied by Dr. Sol Roy Rosenthal, Tice Clinic, University of Illinois, Chicago.

TABLE I. Localization of Cytochrome Oxidase in Mycobacteria.

	CE	Fraction L	A	B	C	S	Total
Units of activity*							
H37Ra	4,742	1,697	693	173	31	1,175	
BCG	1,536	215	156	147	34	482	1,034
M. smegmatis	2,072	163	680	155	51	1,174	2,223
H37RaN	2,235	602	143				745
H37Rv	2,937		211	246	147	2,204	2,808
% CE							
H37Ra	(100)	35.8	14.6	3.7	.7	24.8	79.6
BCG	"	14.0	10.2	9.6	2.2	31.4	67.4
M. smegmatis	"	7.9	32.8	7.5	2.5	56.7	107.4
H37RaN	"	27.0	6.4				33.4
H37Rv	"		7.2	8.4	5.0	75.0	95.6
Specific activity							
H37Ra	(1)	2.41	4.76	1.75	1.03	.50	
BCG	"	2.23	2.08	6.33	2.38	.39	
M. smegmatis	"	1.94	9.24	3.90	2.50	.87	
H37RaN	"	2.34	1.08				
H37Rv	"		1.57	1.92	1.74	1.03	

* 1 unit of enzyme is that amt of fraction causing uptake of 1 μl O_2 in 1 hr.

drogenase by the method of Lindstrom(8). Cytochrome oxidase was determined by the method described previously(2). With fractions from H37RaN and H37Rv a modified Thunberg technic was used for determining malic, isocitric and alpha-ketoglutaric dehydrogenases. This was necessary because of the virulence of H37Rv and the potential virulence of H37RaN. Eight ml screw cap tubes were employed. To each tube was added 1.6 ml of 0.5M phosphate buffer pH 7.2, 0.1 mg methylene blue, 1 ml of 0.2M substrate adjusted to pH 7.2 with KOH, and fractions. When testing for isocitric dehydrogenase 5 μmoles of $MgSO_4$; 5 μmoles of $MnCl_2$ and 1 mg TPN‖ were added. When testing for malic dehydrogenase 5 μmoles of $MgSO_4$ and 1 mg DPN¶ were added. When testing for alpha-ketoglutaric dehydrogenase 5 μmoles of $MgSO_4$, 5 μmoles of $MnCl_2$, 1 mg DPN, and 0.2 mg Co A concentrate (Armour, 13.0 Lipmann units/mg) were added. The final volume of each tube was made up to 8 ml with distilled water and the tubes were then incubated in a 37°C water bath. Rate of reduction of methylene blue was observed and recorded. One unit of the enzyme was that amount of fraction causing the trans-

TABLE II. Localization of DPNH Oxidase in Mycobacteria.

	CE	Fraction L	A	B	C	S	Total
Units of activity*							
H37Ra	24,100	7,360	3,180	348	84	1,100	12,072
BCG	1,670	920	897				1,817
% CE							
H37Ra	(100)	30.5	13.2	1.4	.4	4.6	50.0
BCG	"	55.0	53.7				108.8
Specific activity							
H37Ra	(1)	11.47	6.38	2.21	.89	.13	
BCG	"	4.00	6.55				

* 1 unit of enzyme is that amt of fraction causing decrease of 0.001 optical density unit in 1 min.

‖ TPN: triphosphopyridine nucleotide.

¶ DPN: diphosphopyridine nucleotide

TABLE III. Localization of Succinic Dehydrogenase in Mycobacteria.

	CE	Fraction L	Fraction A	Fraction B	Total
Units of activity*					
H37Ra	76	53	14		67
BCG	61	21		1.5	22.5
M. smegmatis	2,627	1,302	1,449		2,751
H37RaN	306	66	46		132
H37Rv	68	14	25	16	55
% CE					
H37Ra	(100)	69.7	18.4		88.1
BCG	"	34.4		2.5	36.9
M. smegmatis	"	49.6	55.2		104.8
H37RaN	"	21.6	15.0		36.6
H37Rv	"	21.0	36.7	23.5	81.2
Specific activity					
H37Ra	(1)	4.33	5.32		
BCG	"	4.01		1.35	
M. smegmatis	"	80.71	13.67		
H37RaN	"	1.89	2.28		
H37Rv	"	5.10	2.78	2.51	

* 1 unit of enzyme is that amt of fraction causing a transfer of 1 μl H_2 to methylene blue in 1 hr.

fer of 1 μl of hydrogen to methylene blue/hour. The localization of fumarase, aconitase, and DPNH was not determined. When an enzyme could not be localized by the methods described, the crude extract was tested for its presence by the standard Warburg manometric procedure before concluding that no activity was present. To the Warburg reaction vessels were added 5 μmoles each of $MgSO_4$ and $MnCl_2$, 0.1 mg DPN (or TPN in the case of isocitric dehydrogenase), 0.1 mg ATP,** 0.5 ml of 0.1M substrate adjusted to pH 7.2 with KOH and 0.1 ml of the crude extract. To the center well was added 0.2 ml of 20.0% KOH. In each case the endogenous respiration was measured and subtracted from that of the test vessel.

Results. Tables I-III and Figs. 1-6 show the results of enzyme localization studies with 5 mycobacteria. These results are reported both as percent of recovered activity (the percent of activity in a fraction compared with that found in the unfractionated crude extract) and as specific activity (activity per mg nitrogen). Specific activities of one or more were considered significant since this represented either the same concentration or a greater concentration of enzyme found in the crude extract (arbitrarily assigned a value of 1.0).

With fractions of H37Ra, BCG, *M. smegmatis*, and H37RaN both criteria indicate that cytochrome oxidase activity is localized in the particulate fractions (Table I). A high percentage of recovered activity was found in the particulate fractions and these fractions possessed significant (above 1.0) specific activities. With strain H37Rv this enzyme appeared to be localized in the supernatant fraction which possessed 75% of recovered activity and in addition a significant specific activity. Although the supernatant fraction of all strains tested possessed a high percentage of recovered activity this fraction of H37Rv only possessed a significant specific activity.

Table II shows the results of localization studies of DPNH oxidase with strains H37Ra and BCG. There was good agreement between the 2 criteria for localization. This enzyme, by both criteria, was localized in the 2 larger particulate fractions L and A.

The results with succinic dehydrogenase (Table III) again demonstrated good agreement between the 2 localization criteria. This enzyme was localized in the particulate fractions L and A, or L, A and B of all strains tested. With strains BCG and H37RaN rather poor total recoveries were obtained (36.1 and 36.6% respectively). However, the high concentration of enzyme activity in the particulate fractions makes the presence of undetectable enzyme in the supernatant fractions doubtful.

Figs. 1-6 show that aconitase, catalase, fumarase, isocitric, alpha-ketoglutaric and malic dehydrogenases could not be localized because of the lack of correlation between the 2 criteria used. In many cases where the supernatant fractions possessed the highest % of recovered activity they did not possess significant specific activities. On the other hand, many fractions possessed significant specific activities and very low or insignificant percentages of recovered activity. Further complicating the localization attempt was the fact that some fractions which pos-

** ATP: adenosine triphosphate.

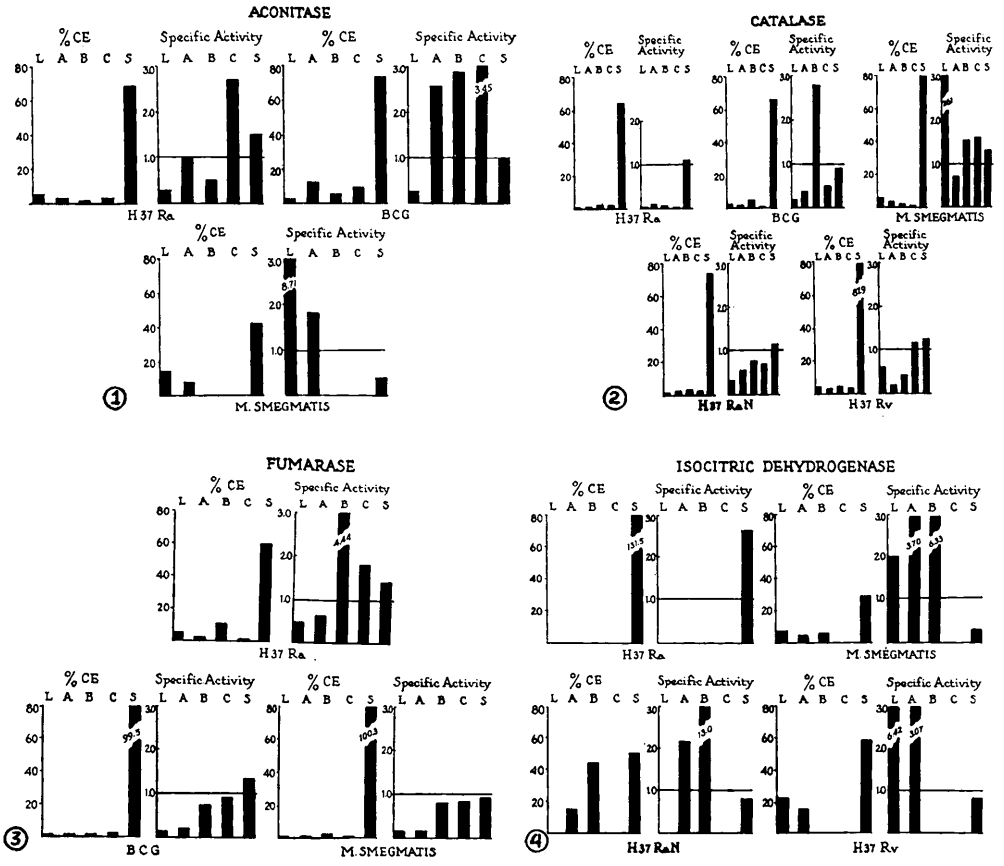

FIG. 1 (top left). Comparison of percent recovered activity (% CE) and specific activity of aconitase. Specific activities less than 1 (horizontal line) are considered insignificant.

FIG. 2 (top right). Comparison of percent recovered activity (% CE) and specific activity of catalase. Specific activities less than 1 (horizontal line) are considered insignificant.

FIG. 3 (bottom left). Comparison of percent recovered activity (% CE) and specific activity of fumarase. Specific activities less than 1 (horizontal line) are considered insignificant.

FIG. 4 (bottom right). Comparison of percent recovered activity (% CE) and specific activity of isocitric dehydrogenase. Specific activities less than 1 (horizontal line) are considered insignificant.

sessed the highest percentage of recovered activity and a significant (above 1.0) specific activity often possessed lower specific activities than other fractions of the same organism with insignificant percentages of recovered activity.

Isocitric, alpha-ketoglutaric and malic dehydrogenases could not always be found in fractions of all organisms tested although often positive Warburg manometric tests of the crude extract proved their presence. Isocitric dehydrogenase (Fig. 4) could not be found in extracts of BCG whereas the crude extract showed an average of 88 μl of oxygen uptake over that of the endogenous. Alpha-ketoglutaric dehydrogenase (Fig. 5) could not be localized in fractions of either BCG or *M. smegmatis* whereas the crude extract of only *M. smegmatis* showed an uptake of 16 μl/ml over that of the endogenous. Malic dehydrogenase (Fig. 6) could not be found in fractions of *M. smegmatis* and manometric tests of the crude extract produced negative results.

One possible explanation for the lack of correlation between the 2 criteria and the fact that catalase, fumarase, isocitric dehydrogenase, alpha-ketoglutaric dehydrogenase and

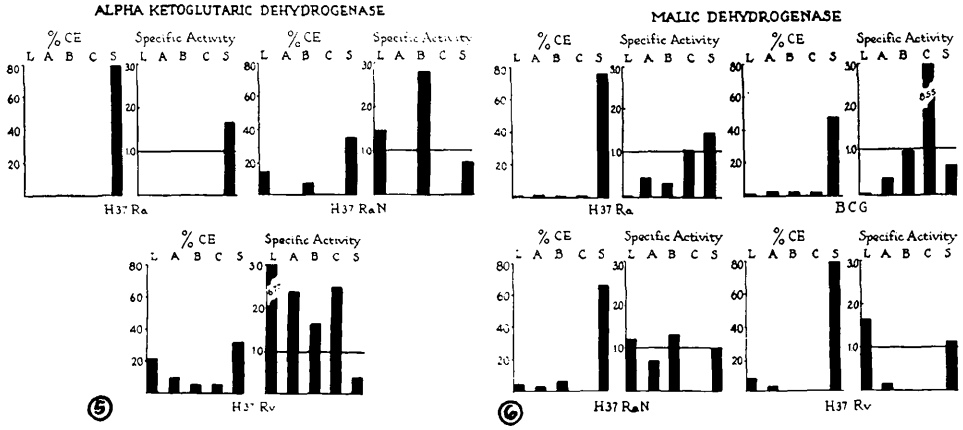

FIG. 5 (left). Comparison of percent recovered activity (% CE) and specific activity of alpha-ketoglutaric dehydrogenase. Specific activities less than 1 (horizontal line) are considered insignificant.

FIG. 6 (right). Comparison of percent recovered activity (% CE) and specific activity of malic dehydrogenase. Specific activities less than 1 (horizontal line) are considered insignificant.

malic dehydrogenase could not be definitely localized in either particulate or supernatant fractions could be that soluble enzymes from the supernatant fractions became adsorbed to the particles of the particulate fractions during preparation. If the major portion of the particulate nitrogen could be attributed to adsorbed enzymes then this could account for high specific activities along with low % recoveries in many of the particulate fractions. To allow for this possibility modifications in criteria were made. An arbitrary figure of 10% was adopted as the maximum amount of enzyme that might be adsorbed from the su-

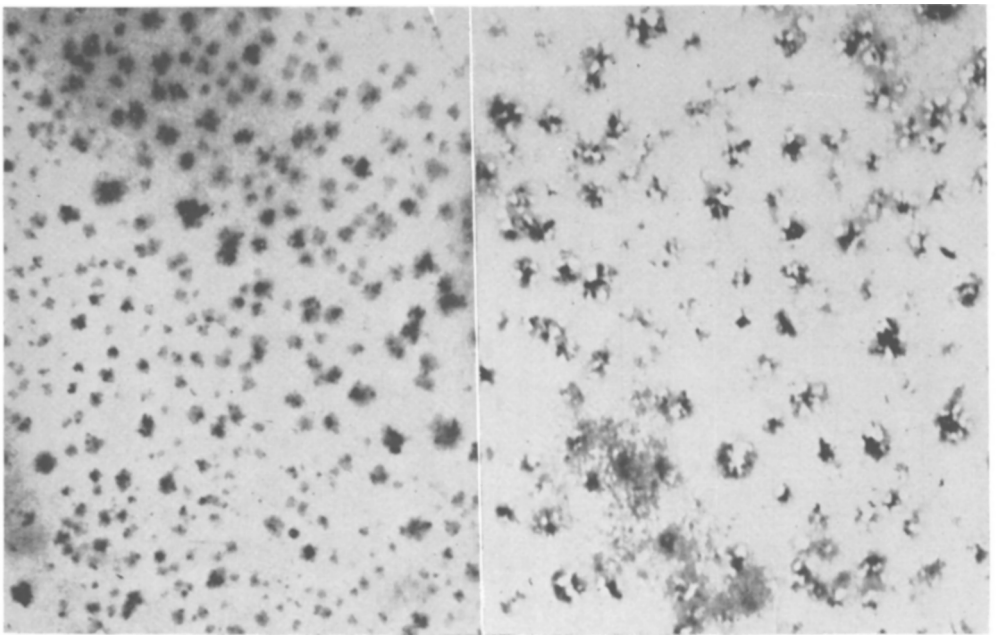

FIG. 7a (left). Crude extract of *Mycobacterium tuberculosis* var. *hominis* strain H37Ra showing aggregates of varying sized particles. × 5080.

FIG. 7b (right). Same field as in Fig. 7a after 45 sec. exposure to electron beam. × 8620.

pernatant under the conditions of our fractionation scheme. Criteria were modified therefore, so that only fractions containing 10% of recovered activity or higher and at the same time specific activities of 1.0 or greater were accepted as possible enzyme loci.

Using this new criterion it was found that most of the tricarboxylic acid cycle and terminal electron transport enzymes could be localized in one or more fractions of the strains of mycobacteria tested. The exceptions were catalase, and malic dehydrogenase in BCG and fumarase and isocitric dehydrogenase in *M. smegmatis*. In all probability fumarase is localized in the supernatant fraction of *M. smegmatis* as was true with the other organisms tested since this fraction of *M. smegmatis* possessed not only most of the total recovered activity but a specific activity of 0.93. The total recovery of isocitric dehydrogenase from fractions of *M. smegmatis* was poor (47.2%) which might explain the failure to localize this enzyme. We can find no explanation for the failure to localize catalase in fractions of BCG.

Discussion. It becomes apparent from the results that the fraction showing the greatest percentage of recovery does not always coincide with that showing the greatest concentration of specific activity. It seemed logical to expect a fraction to contain at least 10% of enzyme activity in order to be accepted as a possible locale for this enzyme. A specific enzyme activity of 1.0 or greater should also be expected because the consideration of only those fractions which contain greatest percentages of recovered activity as enzyme loci automatically eliminates the possibility of multiple loci. A single locus appears unlikely since several fractions possessed moderate percentages of recovered activity for a single enzyme. Furthermore, eliminating fractions with less than 10% of recovered activity from consideration as enzyme loci would more than compensate for adsorption as a source of error. Therefore, the finding of at least 10% of recovered activity with a specific activity of 1.0 or more in a given fraction was adopted as a new criterion for enzyme localization.

The data presented suggest the possibility of the presence of true mitochondria in members of the family Mycobacteriaceae. The isolated particles contain enzymes of terminal electron transport as well as enzymes concerned with oxidative metabolism. Of prime interest is the fact that these particles were opaque and labile to electron bombardment. The crude extract contained a mixture of varying sized particles (Fig. 7A). The lability of the particles was evident by volatilization after 45 seconds of exposure (Fig. 7B). Mudd, Takeya *et al.*(9) recently showed the existence of rosettes of particles within BCG cells which could also be volatilized by the electron beam. These intracellular particles were of approximately the same size range as those reported here. It is entirely possible that the entire rosette reported by these workers represents the particles in our L fraction and that the particles in the A, B and C fractions represent fragments of larger particles. This fragmentation could also explain the presence of high concentrations of enzymes such as cytochrome oxidase and DPNH oxidase in the supernatant fractions. These enzymes may have been solubilized by the drastic grinding procedure necessary to break up the relatively sturdy mycobacterial cells.

The localization results are in rather close agreement with those of several other workers. Yamamura, Kusunose *et al.*(10) and Kusunose, Nagai *et al.*(11) found that succinic dehydrogenase and DPNH oxidase along with the cytochromes resided in the particulate fraction of *Mycobacterium avium*. Their fraction would probably correspond to the L and A fractions combined. They reported, however, that malic dehydrogenase was localized in the particulate fraction while with the organisms studied here it was localized in the supernatant fraction. Alexander and Wilson's(12a, b) data are also quite similar to those reported here. They reported that cytochrome oxidase, DPNH oxidase and succinic dehydrogenase were localized in the particulate fractions of *Azobacter vinelandii*. Catalase, aconitase, isocitric dehydrogenase and alpha-ketoglutaric dehydrogenase were localized in the supernatant fraction. Their data concerning localization of a malic acid oxidizing enzyme agreed with Yamamura and

his co-workers since malic oxidase was localized in the particulate fractions.

The only differences between strains which might be directly or indirectly concerned with virulence were found in the localization of cytochrome oxidase, isocitric dehydrogenase and alpha-ketoglutaric dehydrogenase. Cytochrome oxidase appeared to be soluble with the virulent H37Rv strain and particulate bound with the other strains tested. Isocitric and alpha-ketoglutaric dehydrogenases appeared to be particulate bound with the virulent H37Rv and its mutant H37RaN strain but soluble with the other strains tested. The significance of these differences must await further studies.

Summary. *Mycobacterium tuberculosis* var. *hominis* strains H37Rv, H37Ra and H37RaN; *Mycobacterium tuberculosis* var. *bovis* strain BCG-4; and *Mycobacterium smegmatis* were ground in a ball mill and fractionated according to the methods outlined earlier(2). Enzymes aconitase, fumarase, DPNH oxidase, catalase, cytochrome oxidase, isocitric, alpha-ketoglutaric, malic and succinic dehydrogenase were localized in one or more fractions using the two criteria established by Alexander and Wilson(12a, b). Cytochrome oxidase, DPNH oxidase and succinic dehydrogenase were found to be particulate bound by both criteria. However, the locus of other enzymes determined by one criterion did not always coincide with that determined by the second criterion. A modified criterion was then established and this was discussed. With the new criterion most of the enzymes of the tricarboxylic acid cycle and terminal electron transport system could be localized. The particles found within the various fractions were electron opaque and labile to electron bombardment. The presence of enzymatic activity, particularly those enzymatic activities concerned with energy yielding mechanisms lends support to the hypothesis that these particles are mitochondria.

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Host Resistance in Hemorrhagic Shock: VIII. Effect of Properdin on *in vitro* Function of Phagocytes.* (23246)

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Hemorrhagic shock in the dog lowers the Properdin titer in blood to or near zero within a few hours(1). Another effect of hemorrhagic shock is a fall in phagocytic and bacteriostatic indexes of phagocytes, which has been attributed to the presence of a leukotoxin in the plasma(2). Recently a lethal

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