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Studies on Liver Arginase

III. Its Activation and Inhibition Reactions

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The properties of arginase especially these pertaining to its activation and inhibition represent an interesting aspect in the field of enzymes. They have been the subject of numerous investigations which, unfortunately, have yielded quite conflicting results. In the following is a brief summary of some of the various results previously reported.

The activation of arginase was first attributed specifically to sulfhydryl compounds [1–3]. This view was modified later, as Edlbacher, Kraus and Walter [4], and Klein and Ziese [5] found that the effect of the sulfhydryl group was not absolute, but depended on the purity of the enzyme preparation as well as on the hydrogen ion concentration. Further work, by Waldschmidt-Leitz, Scharikova and Schäffner [6], revealed that sulfhydryl compounds, combined with heavy metals (Fe or Cu), could function as specific activators of arginase. Waldschmidt-Leitz and Kocholaty [7] visualized the activated arginase as only an oxidized form of the enzyme. This was confirmed by Purr and Weil [8] who added that many products of the intermediary metabolism when combined with Fe, such as alloxan-Fe, ascorbate-Fe, and methyl glyoxal-Fe, could also activate arginase. The conclusions reached by these authors were that the activation process required a certain definite oxidation-reduction potential. Quite contradictory results were reported by Leuthardt and Koller [9], as they stated that all activators of arginase must be reducing agents, whose action was actually a protection of the enzyme against oxidizing agents, and mainly oxygen. Using partially purified arginase, Hellerman and Perkins [10], found that neither mercaptide forming organomercurials (*e. g.* C_6H_5HgOH), nor such potent reducing agents as bisulfite, cysteine, cyanide or hydrogen sulfide had any effect on its action. On the other hand Weil [11] observed activation by cysteine-Fe under several conditions.

The first reference to the activation of arginase by metallic ions was made by Hellerman and Perkins [10]. They visualized the arginase molecule as being a metal-protein complex from which the metal ion could be easily dissociated. Klein and Ziese [12] observed that Mn^{++} ions were essential for obtaining maximum activity only in the further stages

of purification, and that they were without effect in crude liver extracts. The mechanism of activation was explained by Hellerman [13], as due to formation of enzyme-metal-substrate coordination complex. Edlbacher and Zeller [14], however, proposed that arginase consists of a metal with a protein carrier. The latter could be digested by trypsin except in the presence of an excess of Mn^{++} ions. This was later modified by Edlbacher and Pinösch [15] who assumed arginase to be a conjugated protein with a Mn-containing prosthetic group.

Rossi [16] rejected the idea of manganese being an actual constituent of arginase. The same author and Pontecorno [17] suggested the presence of a coenzyme, coarginase, that contains no manganese. This was followed later by a report made by Rossi and Ruffo [18] claiming that arginase, completely inactivated by prolonged dialysis, could not be reactivated by addition of Mn ions alone or by readmixture with Mn free diffusate, but only by both added together. Recently, Thompson [19] described a procedure for obtaining a coarginase which could activate his purified arginase a further 50 per cent.

The effect of manganese, cobalt and nickel was interpreted by Mohamed and Greenberg [20, 21], as a catalytic reaction, influenced by time, temperature, pH, and type and concentration of the activating ion. Bach [22], using a partially purified arginase, reported it to be unaffected by such metal activators. This was later disproved by Mohamed [23] who obtained 2—3 fold activation of the same preparation if incubated with Mn^{++} ions at 40° C for one hour.

The activation of arginase by the metal ions was observed by Hellerman and Stock [24] to be accompanied by a shift in the pH optimum. The same results were obtained by Mohamed and Greenberg [20].

Edlbacher and Bauer [25] observed that dialysis of both yeast and liver arginase resulted in complete inactivation, but it could be restored by adding manganese, cobalt, nickel, cadmium, and vanadium ions, the manganese ions being the most effective. Similar results were reported by Kocholaty and Kocholaty [26] who found that maximum activation by Mn^{++} ions required about fifteen minutes and that other ions, such as Fe^{++}, Ni^{++}, and Co^{++} also activated arginase but to a less extent than Mn^{++}. The activating action of cobalt was studied by Hunter and Downs [27] who found it a slow reaction at 20° and 37° but proceeding quite rapidly at 50° reaching completion after twenty minutes. This difference in ability of activation by the different metals was also observed by Anderson [28] on Jack bean arginase.

Arginase is quite stable even in the purified state. It is unaffected by a variety of agents, such as heat, urea, most heavy metals with the exception of Ag and Hg [20]. However, the enzyme is very sensitive to pH values lower than 5 and the effect is irreversible.

Lenti [29] reported that the activity of guinea pig and rat liver arginase was inhibited by F^- ions. The effect was reversible, *i. e.* on dialysis and addition of a trace of $MnSO_4$ the activity was restored.

Karrer and Zehender [30] found that cysteine and lactoflavin inhibited arginase and that the addition of F^{++} or Fe^{++} ions in small concentration protected the enzyme against this inhibition. Other inhibitors, namely quinone, Fe^{++}, iodine, and H_2S were reported by Hellerman and Perkins [10]. In all cases they could restore the lost activity by Mn^{++}, Co^{++} and Ni^{++} ions.

Kitagawa [31] studied the action of various compounds on arginase. He found *d,l-α*-amino valeric acid the most inhibitory. Fatty acids, acyl derivatives and esters of *α*-amino acids and urea had no effect. He concluded that the inhibition was due to a combination of arginase with the amino acids. Hunter and Downs [27] investigated the inhibitory effect

of a large number of amino acids and their derivatives and of some proteins. The enzyme was only inhibited in varying degrees, by the α-amino acids having the *l*-configuration, but not by the *d*-isomers, or by mono amino acids having the amino group in positions other than the α-position. The inhibition was non-competitive except in the cases of ornithine and lysine which exhibited a clearly competitive type of inhibition.

MATERIALS AND METHODS

Horse liver was used as the source for arginase throughout the work. The liver was obtained fresh from the sacrificed animal, chopped and mixed with several volumes of cold acetone (at 3° C). The mixture was stirred several times during the period of one hour. The acetone was then decanted and more fresh acetone was added. This procedure was repeated several times and then the mixture was filtered on a large Büchner funnel by suction. Several washings with fresh cold acetone were applied to the liver residue on the filter. The suction was continued until the residue became dry. It was then put in a desiccator under vacuum and stored in the cold room (at 3° C).

Arginase activity was measured according to a procedure previously published by Mohamed and Greenberg [20]. The activity was always reported as mg urea produced in 30 minutes.

EXPERIMENTAL

The effect of borate ion on arginase

Arginase has been shown to be partially depressed when the activity measurement was made using borate buffer [20]. This was explained as due to the borate forming a complex with the metal activator thus rendering it unavailable for the enzyme. A further attempt to study this effect was made in the following manner:

Two 100 ml volumes of a 2.5 % sodium acetate extract of the acetone dried liver (prepared by extracting 100 g liver powder in 1 liter sodium acetate) were treated each with 120 ml cold acetone at 3° C. Each mixture was centrifuged and one of the precipitates was taken up in 20 ml borate buffer (pH 8.0) and the other precipitate was taken up in the same volume of phosphate buffer at the same pH. The resulting extracts were centrifuged and the residues were discarded. The pH of the extracts were checked and adjusted to 7.2. A portion of each extract was dialyzed against running distilled water for 48 hours. Another portion of each was dialyzed for 24 hours against KCN solution (.05 *M*) adjusted to pH 8.0.

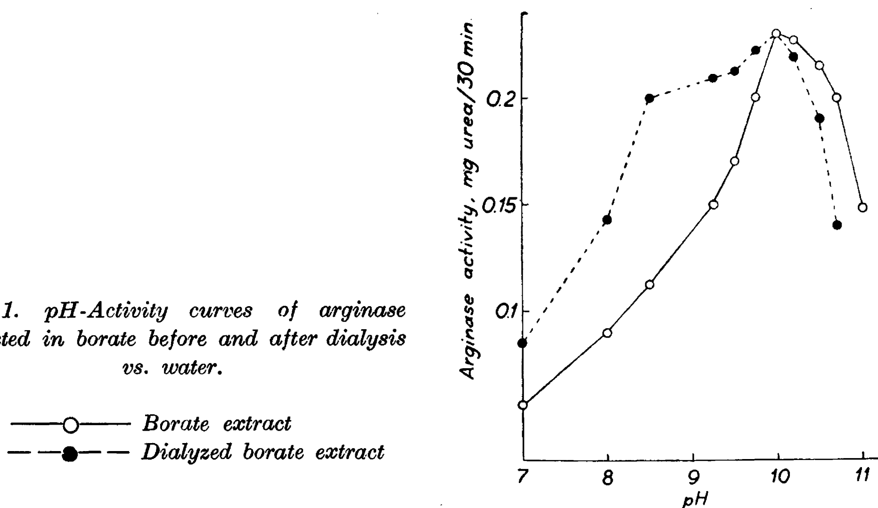


Fig. 1. pH-Activity curves of arginase extracted in borate before and after dialysis vs. water.

—○— *Borate extract*
– –●– – *Dialyzed borate extract*

The pH-activity curve of the borate and water-dialyzed borate extract are shown in Fig. 1. Only phosphate and glycine buffers were used in determining the pH-activity relationship. The pH-activity curve of borate extract essentially resembles the curve for Mn treated arginase [20] except for the difference in the extent of activity. On dialysing the borate out an extension of the activity to the less alkaline side is observed thus becoming similar to the Co-activated enzyme. In the following communication this is given as an evidence for the presence of two enzymes with arginase activity.

Comparison of the arginase activity of the borate, phosphate, water dialyzed and CN^--dialyzed extracts of both was made at both pH 7.2 (phosphate) and 9.2 (glycine) in presence and absence of Mn^{++} and Co^{++} ions. The results of these tests are recorded in Table 1.

The results show almost a complete inhibition of activity in the less alkaline pH 7.2 in the borate extract while the phosphate extract shows a good deal of activity. This inhibition is not released by Co^{++} ions which, in the phosphate extract, show high activating power. Manganous ions activate in the more alkaline side even in presence of borate.

On dialysis the borate ions are removed and we find that practically all the activity in the less alkaline region is restored to the normal and Co^{++} exerts again its activating effect.

Table 1. Comparison between borate and phosphate extracted arginase and the effect of dialysis and activating metals thereon.

	Arginase activity * (mg urea / 30 min)											
	Borate extract						Phosphate extract					
	no metal		Mn^{++}		Co^{++}		no metal		Mn^{++}		Co^{++}	
	pH 7.2	pH 9.2	pH 7.2	pH 9.2	pH 7.2	pH 9.2	pH 7.2	pH 9.2	pH 7.2	pH 9.2	pH 7.2	pH 9.2
Undial. extract	.08	.14	.08	.26	.08	.15	.17	.28	.21	.33	.23	.28
Ext.dial vs. H_2O	.14	.20	.16	.29	.23	.30	.17	.27	.19	.32	.22	.30
Ext. dial. vs. CN^-	.15	.22	—	.28	.21	.28	.15	.26	—	.29	—	—

* Arginase activity was measured in the usual manner at 40° C for 30 minutes, .05 ml of the extract was used.

When Mn^{++} or Co^{++} ions were used, incubation of the extract with the metal (conc. 2.5×10^{-3} *M*) at 40° C for half an hour preceded the activity determination.

Dialysis was carried out in cellophane bags at room temperature for 24 hours.

The effect of high temperature on arginase under different conditions

Arginase is quite stable towards high temperatures, a property which has been utilized as a means of purification by several workers [33, 22, 19, 34].

A study was conducted to see the effect of heating different arginase extracts at different temperatures. Crude extracts were prepared by suspending 25 g portions of acetone dried liver in 250 ml aliquots of the extracting solution. The mixtures were adjusted to pH 7.0 and placed in a shaker for two hours, after which they were centrifuged discarding the residues. The extractants used were as follows: 2.5 % sodium acetate, .01 *N* KCN, .005 *N* KCN, and 0.1 *M* cysteine hydrochloride.

Aliquots of each extract (10 ml) were heated at 30°, 40°, 50°, 55°, 60°, 65° and 75° C each for exactly 5 minutes, with continuous mixing. Each extract was then cooled at once under running water, centrifuged and arginase activity of the supernatent was determined. The same extracts were stored at 3° C and the enzyme activity was again measured after seven and fifteen days of

Fig. 2. The effect of high temperatures on arginase activity in different extracts.

- ● *Fresh 0.01 N KCN extract.*
- ○ *0.01 N KCN extract after 15 days at 3° C.*
- ■ *Fresh 0.005 N KCN extract.*
- □ *0.005 N KCN extract after 15 days at 3° C.*
- ◑ *Fresh 0.1 M cysteine-HCl extract.*

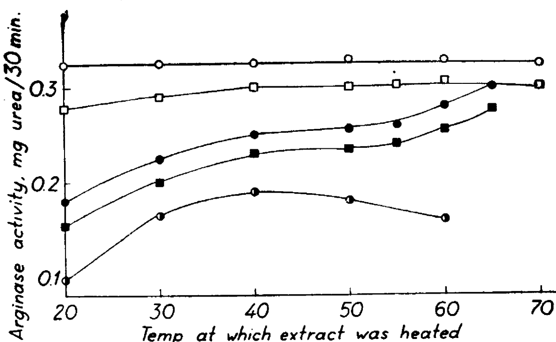
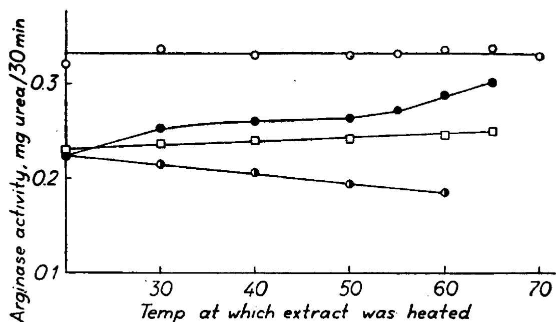


Fig. 3. The effect of high temperatures on arginase activity in different extracts.

- ● *Fresh KCN extract.*
- ◑ *Dialysed KCN extract.*
- ○ *KCN extract stored at 3°C for 15 days.*
- □ *NaAc extract stored at 3°C for 15 days.*



storage at 3° C. Plots of the activities against the temperatures at which the extracts were heated are shown in Figs. 2 and 3.

Portions of the .01 CN^- extract were heated at 65° C for various periods; namely 2, 4, 6, 8, 10, and 12 minutes. This was followed by measurement of arginase activity in the resulting supernatents. Plots of activity vs. time of heating are shown in Fig. 4.

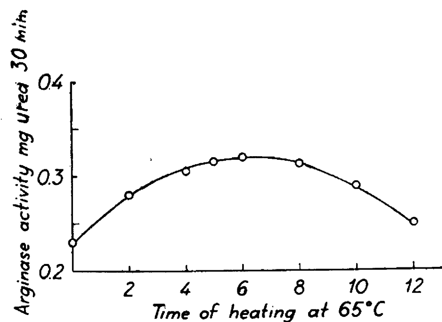


Fig. 4. The effect of duration of heating on arginase activity in 0.01 N CN^-.

Table 2. Arginase activity in cyanide and acetate buffers, as affected by heating, storage at 3° C, dialysis and treatment with Mn^{++} ions.

	Total N	Arginase activity *	
	mg/ml	day of extraction	after 2 weeks at 3° C
Sodium acetate extract	1.9	0.195	0.23
» » » + Mn^{++}	1.9	0.26	0.30
» » » + H_2S	1.92	0.25	0.29
» » » heated at 40° C for one hour	1.8	0.26	0.27
» » » heated at 65° C for 5 minutes	1.4	0.24	0.28
cyanide extract (.01 *N*)	2.1	0.22	0.34
» » + Mn^{++}	2.0	0.32	0.38
» » heated at 40° C 1 h	2.0	0.30	0.35
» » » » 65° C 5 min.	1.3	0.30	0.38
» » » » 65° C 5 min. + Mn added	1.35	0.30	0.38
» » dialysed vs. water	2.0	0.23	0.20
» » » » » and heated at 65° C for 5 minutes	1.4	0.19	0.14
Cyanide extr. heated at 40° C for 1 h and dialyzed vs. H_2O	1.0	0.16	0.195
Same as above + Mn^{++}	1.0	0.34	—

* Reported as mg urea produced in 30 minutes. Detns. were conducted at 40° C, pH 9.9. Incubation with Mn^{++} (2.5×10^{-3} *M*) was made at pH 7.0, and 40° C for one hour prior to the addition of substrate.

In Tables 2, 3 and 4 are recorded the activities of the forementioned extracts under various conditions and treatments with H_2S, Mn^{++}, Co^{++}, and Ni^{++} ions.

The common feature about Figs. 2 and 3 is that heating of freshly undialyzed arginase extracts results in enhancing the enzyme activity. In cyanide extracts a peculiar sort of an S-shaped curve results that is difficult to explain without assuming the complicated nature of arginase and probably because of an interaction with CN^- ions, and also with other components of the extract.

If, however, the extracts are stored at 3° C for two weeks their activity seems to rise to the maximum and heating has no further effect except for the denaturing of other foreign nonarginase proteins.

Table 3. The effect of heating and dialysis on cyanide extracts of arginase in presence and absence of Mn^{++}, Co^{++}, and Ni^{++} ions.

Extract	Arginase activity mg urea/30 min.						
	No activator	30 min incubation with activ.			24 h incubation with activ.		
		Mn	Co	Ni	Mn	Co	Ni
I .01 *N* CN^- (after 7 days at 3° C)	.26	.35	.28	.26	.38	.30	.29
II .005 *N* CN^- extr. (after 7 days at 3° C)	.23	.36	.30	.26	.38	.24	—
III Treatment I+heat at 65 C 5 min	.28	.35	.27	.25	.37	.29	—
IV » I+dialysis vs. H_2O	.21	.34	.25	.24	.38	.30	.28
V » III+ » » H_2O	.13	.28	.20	.21	.34	.23	.21
VI » II+heat at 65° C 5 min. followed by dialysis vs. CN^-	.19	.34	—	—	.37	.27	—

Arginase activity measurement was run at pH 9.9 and 40° C. Conc. of Mn^{++} ion was 2.5 × 10^{-3}, conc. of Co^{++} and Ni^{++} ions was 17 × 10^{-3}. Incubation was made at 40° C, pH 7.0.

Table 4. The effect of Co^{++} and Fe^{++} on cysteine extracts of arginase.

Treatment	Arginase activity mg urea / 30 min.
0.1 *M* cysteine extract	0.08
0.1 » » » + Co^{++}	0.18
0.1 » » » + Fe^{++}	0.08
Dialyzed 0.1 » » »	0.10
» 0.1 » » » + Co^{++}	0.17
» 0.1 » » » + Fe^{++}	0.06

Arginase activity was determined at pH 9.9 and 40° C. Concn. of Co^{++} and Fe^{++} was 1.7 × 10^{-3}. Incubation with the metal was made at pH 7.0 and 40° C.

If the crude extract is first dialyzed against water and then heated, a loss in its activity is gradual depending upon the temperature at which the heating was performed. Thus it seems that there is an element, most probably one of the metal activators that, although present in minute amounts, not only protects the enzyme against inactivation by heat but also causes further activation (2-fold approximately). Removal by dialysis, heating or even

storage at room temperature leads to loss of the arginase activity of the extract.

A crude extract can be heated to 70° or 80° C without any damage if the duration of heating does not exceed 5—7 minutes. If heated for longer periods a gradual loss in activity ensues as is shown in Fig. 4.

Cysteine extracts show much lower activity, and the latter is enhanced by heating to 40° C but is lowered again if the temperature exceeds 40°—50° C.

If an extract heated to 65° C for 5 minutes, thus reaching its maximum activity, then is dialyzed, it is observed to lose most of the activity. However, it can be regained by incubating the extract with Mn^{++} ions.

The activities recorded in Table 3, which have been run at pH 9.9, show definitely a response to Mn^{++} activation but not to either Co^{++} or Ni^{++} ions.

Contrary to reports by other workers neither cysteine alone nor cysteine — Fe^{++} can activate arginase.

The effect of prolonged dialysis

It has been reported that arginase activity is decreased after dialysis of the preparation. An experiment was performed to study the effect of prolonged dialysis and to correlate the loss in activity with the duration of dialysis. The effect of Mn^{++} ions, H_2S—Mn, the dialysate, and H_2S treated dialysate in restoring the lost activity and in further activation were also investigated.

An extract was prepared by mixing 40 g of acetone dried liver with 400 ml of water and the pH was adjusted to 7.0. After mixing for two hours on a shaker a clear extract was obtained by centrifugation.

A 100-ml portion of this extract was dialyzed against the same volume of distilled water. The dialysis water was not changed in order to test the dialysate for its ability to restore the activity and its power of activation. This dialysis was continued for 5 days.

The rest of the extract was placed in another bag which was suspended in 4 liters of distilled water which was changed twice daily. Samples of the extract were withdrawn at intervals and tested for their arginase activity as such and after incubation with Mn^{++}, H_2S saturated Mn, phosphate buffer, dialysate, and H_2S treated dialysate.

Dialysis was performed at room temperature and with continuous stirring by hanging the bag on the stirrer. A layer of toluene was added to the water of dialysis for preservation. The results are recorded in Table 5.

The results recorded in Table 5 show that prolonged dialysis has very little effect on arginase activity measured at pH 9.9.

Table 5. The effect of prolonged dialysis and some activators on arginase activity.

	Arginase activity *, mg urea / 30 min.										
	Un-treated extr.	Incubated with Mn		Incubated with H_2S-Mn		Incubated with phos-phate bf.		Incubated with dialysate		Incubated with H_2S-dialysate	
		$\frac{1}{2}$ h	24 h	$\frac{1}{2}$ h	24 h	$\frac{1}{2}$ h	24 h	$\frac{1}{2}$ h	24 h	$\frac{1}{2}$ h	24 h
Water extract	0.18	0.28	0.45	—	—	0.22	0.31	0.2	0.2	0.18	0.18
Water extract, stored at 3° C for 192 h	0.22	0.29	0.43	0.28	0.44	0.22	0.30	—	—	0.21	—
Extr. dial. 20 h	0.17	0.27	0.38	0.23	—	—	0.22	0.18	0.11	0.19	0.03
» » 72 »	0.20	0.29	0.42	0.26	0.44	0.20	0.10	0.18	0.10	0.17	0
» » 144 »	0.18	0.26	0.41	0.25	0.43	0.18	—	0.18	0.07	—	0.04
» » 264 »	0.17	0.28	0.42	0.27	0.43	0.17	0.02	0.18	0	0.17	0.02
» » 360 »	0.17	0.26	0.41	0.25	0.44	0.15	0.01	0.14	0	0.15	0

* pH 9.9 and 40° C.

Incubation of dialyzed arginase with Mn^{++} ions alone or H_2S treated Mn^{++} ions caused a rise in the activity. This shows the importance and specificity of Mn^{++} ions in activation of arginase when the measurement is made as pH 9.9. Incubation for $\frac{1}{2}$ h with other solutions such as phosphate buffer (pH 7.0), dialysate, and H_2S-treated dialysate showed no activation. If the time of incubation was extended to 24 h the activity was measurably diminished or completely lost particularly in extracts previously dialyzed for a long time (144—360 h). This may be attributed to exposure to the high temperature (40° C) for a long time in absence of the protecting metal activator.

SUMMARY

1. Arginase activity is reversibly lost in the pH region between 7—8.5 in presence of borate ion. This effect is partially removed by dialysis. The activity in the forementioned region still remains low owing to the effect of dialysis itself in diminishing action in this pH range.

2. Mn^{++} ions have no effect whatsoever on the activity of borate extracted arginase in the pH region of 7—8.5, yet it considerably enhances the activity at pH 9.9 even in presence of borate.

3. Co^{++} ions have no effect on arginase in both pH ranges; namely, 7.0—8.5 and 9.9 if borate is present. Only when borate ions are completely removed does cobalt exert a distinct activation in the first pH range (7.0—8.5) with no effect in the second.

4. Heating fresh extracts of arginase to different temperatures results in enhancing its activity. This effect can also be reached if the extracts are merely stored at 3° C for 15 days. Any subsequent heating only exerts a denaturing action on other nonarginase proteins without any untoward effect on arginase itself.

5. The effect of duration of heating at 65° C was studied. The activation is gradual up to 7 minutes where the activity reaches maximum. If the duration is prolonged a gradual loss results.

Heating of dialyzed extracts is detrimental to the activity. The loss of activity is dependent on the temperature and duration of heating.

6. Prolonged dialysis has no deleterious action on arginase activity with a maximum at pH 9.9. Only Mn^{++} and H_2S-treated Mn^{++} ions strongly activate the extract.

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