ENZYME LOCALIZATION IN THE INNER AND OUTER MITOCHONDRIAL MEMBRANES

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There is general agreement that the mitochondrion has an outer and inner membrane system but there is disagreement as to which enzymes are localized in which of the two mitochondrial membranes. Data are now available to resolve some of these controversies, and to trace the errors of assumption and experimentation that have led to the present state of confusion.

Liver or heart mitochondrial suspensions as prepared by standard methods catalyze a rotenone-insensitive DPNH cytochrome c reductase activity (RIDCR activity). However, mitochondria are less active per mg of protein than are the microsomes with respect to RIDCR activity.[1,2] This activity is assumed by some investigators to be intrinsic to the mitochondrion[2-6] and it has, in fact, been selected by these investigators as a marker activity of the outer mitochondrial membrane. However, when suspensions of beef or rat liver mitochondria are washed several times under conditions which we shall describe in detail later on, RIDCR activity is reduced to less than 5% of the corresponding microsomal activity (see Table I). The loss of RIDCR activity is paralleled by the loss of glucose-6-phosphatase (GP) activity. The mitochondria so treated are indistinguishable from the original suspension in respect to respiratory control, electron transfer capability, and other criteria of mitochondrial function, such as citric cycle activities.[1] Electron microscopic examination clearly shows

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TABLE I

The Removal of Glucose-6-phosphatase and DPNH-cytochrome c Reductase Activity from Beef and Rat Liver Mitochondria by Successive Washings

Source of liver particles	Particles	No. of washes[a]	Rotenone-insensitive DPNH-cytochrome c reductase		Glucose-6-phosphatase	
			(mμmoles/min/mg protein)[b]	Activity ratio ($\frac{\text{mitochondrial}}{\text{microsomal}}$ x 100)	(mμmoles/min/mg protein)[c]	Activity ratio ($\frac{\text{mitochondrial}}{\text{microsomal}}$ x 100)
Beef	Mitochondria	0	710	30	180	74[d]
Beef	Mitochondria	2	170	7.2	30	12
Beef	Mitochondria	3	110	4.2	15	6.3
Beef	Mitochondria	4	60	2.5	4.7	1.9
Beef	Microsomes	0	2350	----	244	----
Rat	Mitochondria	0	500	29	39	24
Rat	Mitochondria	1	465	26	28	19
Rat	Mitochondria	2	355	20	17	10
Rat	Mitochondria	3	218	12	15	9
Rat	Mitochondria	4	123	7	6	3
Rat	Microsomes	0	1750	--	160	--

[a]Beef and rat liver mitochondria and microsomes were prepared, washed, and assayed as described previously.[1] In the first two washes of rat liver mitochondria, sedimentation was carried out for 10 min at 10,000 x g in a No. 40 rotor of the Model L Spinco ultracentrifuge; in the last two washes, sedimentation was carried out for 10 min at 15,000 x g in a No. 40 rotor.

[b]DPNH-cytochrome c reductase activity is expressed as mμmoles of cytochrome c reduced per min per mg of protein in the presence of 5 μg of rotenone.

[c]Glucose-6-phosphatase activity is expressed as mμmoles of P_i released per min per mg of protein.

[d]See Allmann et al.[1] for a detailed explanation of the high ratio.

that mitochondria which have been washed free of RIDCR activity have normal morphology; the outer membranes are intact, and the cristae show no evidence of modification in size or shape. We have concluded that the RIDCR activity of the original unwashed mitochondrial suspension is referable to a contaminating membrane fragment, and have identified this contaminant as a microsomal membrane on the basis of four lines of evidence. (a) The RIDCR activity of the microsomal fraction of beef heart, beef liver, or rat liver, is three to ten times higher than that of the unwashed mitochondrial fraction derived from the same tissue.[1] (b) RIDCR activity has been correlated with cytochrome b_5--a cytochrome known to be present in microsomes.[7-9] (c) The loss of RIDCR activity in mitochondrial suspensions runs parallel with the loss of GP activity--an activity universally accepted as nonmitochondrial and known to be present in microsomes.[10] (d) The leaching out of RIDCR activity from mitochondrial suspensions can be correlated with the concentration of microsomal membrane fragments in the fluffy fractions discarded during the extraction procedure (see Figure 1a).

Rat liver mitochondria washed twice by sedimentation at 100,000 *g* x min still retain an amount of RIDCR and GP activity corresponding to contamination of the mitochondria by microsomes to the extent of 20% and 10% respectively (see Table I). If the washing procedure is continued at 150,000 *g* x min, the washed mitochondrial pellet is found to be essentially free of both contaminant activities. Atop the mitochondrial pellet at the end of the washes is a fluffy layer that is discarded. This fraction shows relatively high GP and RIDCR activities. Electron microscopic examination of negatively stained specimens of the fluffy layer discloses membrane fragments that are ultrastructurally indistinguishable from authentic microsomal membranes. Figure 1b is a micrograph of rat liver microsomes and Figure 1a a micrograph of the fluffy fraction obtained above the mitochondrial pellet after the second 150,000 *g* x min centrifugation of rat liver mitochondria.

It is instructive to compare a micrograph of twice washed rat liver mitochondria that are still heavily contaminated with RIDCR activity (Figure 2a)

with a micrograph of well-washed liver mitochondria in which RIDCR and GP activities are greatly reduced (Figure 2b). Structures resembling microsomal membrane fragments are numerous in Figure 2a and sparse in Figure 2b. Also, the mitochondrion shown in Figure 2b clearly shows the intactness of the outer membrane as well as the normalcy of the cristael membranes. If the enzyme catalyzing RIDCR activity were an integral component of the mitochondrial outer membrane, then removal of this activity by continuous washing of the mitochondrial suspension should lead to removal of the outer membrane in 95% or more of the mitochondrial population. This, however, is clearly not the case.

If the assumption be made that RIDCR activity is a marker for the outer membrane, as others have assumed,[2-6] the removal of RIDCR activity by washing the mitochondrial suspension could be ascribed to the instability and fragmentation of the outer membrane. Accordingly, the activities of this fragmented fraction would correspond to those of the mitochondrial outer membrane, and indeed the conclusion must be drawn that the citric cycle enzymes would be present only in the cristael membranes or in the matrix since none are found in the fragmented "outer" membrane fraction. If a wrong assumption in assigning a marker enzyme activity to a particular membrane is made, and this marker activity is used as the criterion of membrane identification, then inevitably one will end up isolating a membrane other than the one desired. In this case the RIDCR activity is in fact associated with the microsomal membrane. It has been assumed without adequate evidence or proof, that RIDCR activity is intrinsic to the mitochondrial outer membrane.

We have described methods for the isolation of the outer mitochondrial membranes and have shown that these membranes contain the complete set of enzymes that implement the citric cycle and fatty acid oxidation.[1,11-14] The electron micrographs of specimens of the outer membrane stained with phosphotungstate show smooth membranes (inset Figure 1c). By contrast the membranes removed from the mitochondrion by repeated washing are enriched in RIDCR and GP activities and show segmented multipartite repeating units (inset Figure 1a), which are

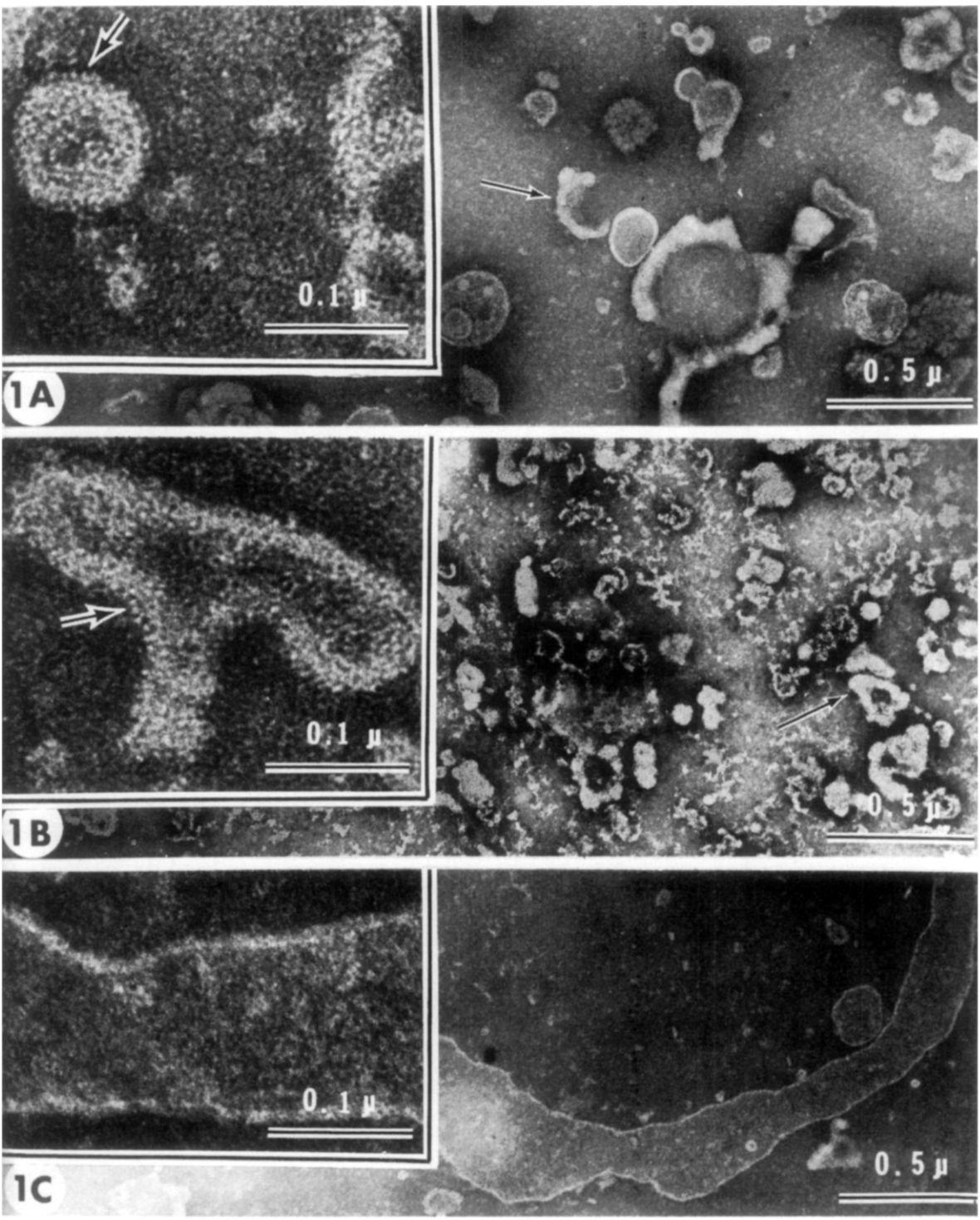
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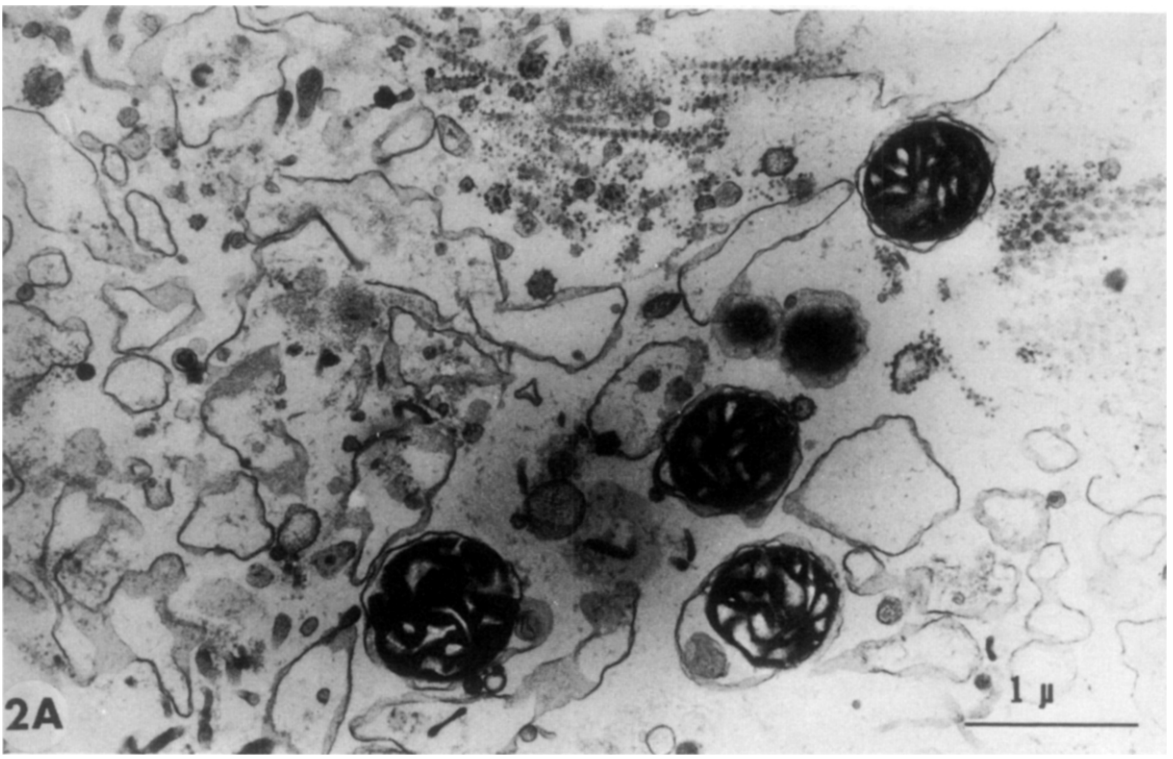
Fig. 1. a) An electron micrograph of a negatively stained specimen of the fluffy fraction which is separated from the mitochondrial pellet during washing of rat liver mitochondria. Arrows indicate multipartite repeating units; inset shows one fragment at high magnification. b) Electron micrograph of rat liver microsomes, negatively stained with phosphotungstate. Arrows indicate membrane fragments with multipartite repeating units; inset shows one such fragment at high magnification. c) An electron micrograph of a negatively stained mitochondrial outer membrane fraction; inset shows a portion of this membrane at high magnification.

indistinguishable from those of an authentic microsomal membrane fraction (inset Figure 1b). The membrane fragments isolated from the mitochondrial fraction by washing are indistinguishable from the "outer membrane" fraction of Parsons[15] at the ultrastructural level. Thus, the RIDCR activity in mitochondrial suspensions has to be assigned to contamination by microsomes.

The assumption of RIDCR activity as a marker activity for the mitochondrial outer membrane leads to the other serious error of localization--that which concerns the locale of the citric cycle enzymes. If it can be argued that the removal of RIDCR activity from mitochondrial particles is tantamount to stripping away the outer membrane, the residual particle fraction may be considered to have originated from the inner membrane fraction. The absence of RIDCR activity cannot be taken as a criterion of an inner membrane fraction stripped of outer membrane.[2-6] With the information now available to us we see at once that the "inner membrane" preparations described by others are in fact equivalent to a suspension of intact mitochondria. One such "inner membrane" fraction, prepared by exposure of mitochondria to digitonin, has been shown to have the capability for citric cycle oxidations,[2,5,16,17] fatty acid oxidation,[5] and electron transfer.[2,5,16-18] Moreover, the uptake of adenine nucleotides by this so-called "inner membrane" fraction is atractylate-sensitive.[18] This spectrum of properties fully confirms our prediction that a digitonin-treated mitochondrial suspension[5,16] is, in essence, equivalent to a suspension of fully competent mitochondria, containing both outer and inner membranes, and indistinguishable in capabilities from those of the mitochondrial suspension prior to exposure to digi-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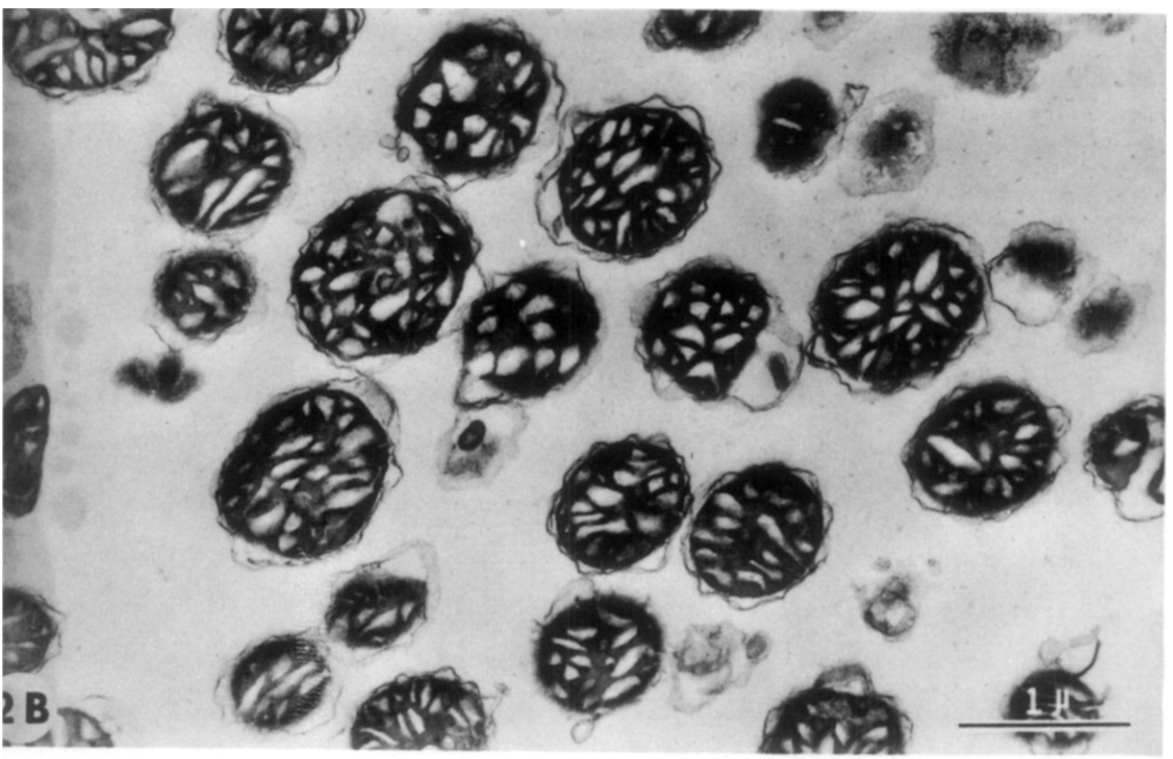
2B

Fig. 2. Electron micrographs of sections of an osmium-fixed, Epon-impregnated suspension of rat liver mitochondria: (a) unwashed rat liver mitochondria; (b) rat liver mitochondria washed four times.

tonin. Allmann et al.[19] have recently established the fact that atractylate sensitivity is the hallmark of an intact mitochondrion and this sensitivity is the clearest evidence that the outer membrane is indeed intact. Atractylate sensitivity is referable to the inhibition of a transphosphorylating enzyme localized in the outer membrane which is required for transfer of a phosphoryl group from inside the mitochondrion to the outer membrane, and again from the outer membrane to the external medium. By this rather stringent criterion of sensitivity to atractylate the identification of digitonin-treated particles of the kind used in the above-cited localization studies, as functionally intact mitochondria, can be considered to be definitive.

Beattie[5] has incorrectly concluded from studies of a digitonin particle with low RIDCR activity, that the fatty acid oxidizing enzymes are present exclusively in the inner mitochondrial membrane. The presence of fatty acid oxidizing activity in a mitochondrial particle which had not been resolved into its component membranes cannot be used as evidence to decide which one of the two mitochondrial membranes contains the fatty acid oxidizing system.

Schnaitman et al.[16] have recently announced a new marker enzymic activity for the outer mitochondrial membrane, namely monoamine oxidase (MO) activity. We have examined our resolved inner and outer membrane fractions for this activity. It is clear from the data of Table II that close to 95% of the MO activity in the original, unresolved mitochondrion is localized in the inner membrane, R_2 fraction--a conclusion also reached by Ragland.[20] The discrepancy between our results and those of Schnaitman et al.[16] may be attributed to the assay of Schnaitman et al.[16] adapted from Tabor et al.[21] which depends upon the conversion of benzylamine to benzaldehyde, measured spectrophotometrically by the increase in absorption at 250 mμ. If benzaldehyde is further oxidized to benzoate or reduced

TABLE II

The Distribution of Monoamine Oxidase Activity among Cristael and Outer Mitochondrial Membrane Fractions

Treatment of the particle	Fraction	Monoamine Oxidase		
		Specific activity (mμmoles/min/mg)	Per cent of original activity recovered in the fraction	Per cent of original protein recovered in the fraction
Exposure to phospholipase[a]	Beef liver mitochondria	3.2	100	100
	R_2 (cristael fraction)	7.2	75	38
	K (outer membrane fraction)	2.4	8	11
	S_3 (detachable sector of outer membrane fraction)	1.2	16	45
Oleate-induced swelling followed by ATP-induced contraction[a]	Rat liver mitochondria	1.13	100	100
	Residue (cristael)	3.37	98	33
	Supernatant (outer membrane)	0.073	2	66

[a]The digestive procedures and assays were described earlier.[1]

to benzyl alcohol (both of these reactions are in fact catalyzed by liver mitochondria) then the assay procedure would be inappropriate and misleading. We have determined that aldehyde removal in liver mitochondria is so much faster than aldehyde formation from benzylamine, that the values obtained for MO activity by this assay procedure are meaningless. In our own studies of the distribution of MO we have used the conversion of ^{14}C-tyramine to either the acid or the aldehyde as the basis of the assay. The MO activity present in the so-called

"outer membrane" fraction is actually referable to the microsomes present in preparations of this fraction.[10] The aldehyde dehydrogenase which complicates the assay of MO in mitochondria by the Tabor procedure is missing in the microsomes; thus, MO was found by Schnaitman et al.[16] to be localized exclusively in the so-called "outer membrane" fraction. The complication due to the oxidation of benzyaldehyde to benzoate in the assay of MO has been recognized by others. In fact, Weisbach et al.[23] explicitly recommend that the Tabor assay procedure should not be used for the assay of MO activity in crude systems.

It is clear that there can be no meaningful disagreement at present with respect to localization of enzymes in mitochondrial membranes since none of the groups referred to above has succeeded in separating the two membranes in question. They have either been studying the localization of enzymes in a membrane other than the mitochondrion or dealing with mitochondrial particles which were unresolved into the respective membranes.

The important conclusion to be drawn from the above account is that the strategy of using marker enzymes to decide localization of enzymes in a membrane is putting the cart before the horse. The first prerequisite is to establish by unambiguous evidence (ultrastructural and biochemical) that the desired membrane has in fact been separated from other membranes. Only then can the enzymic pattern be explored and only then can the question of marker enzymes be raised.

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