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EFFECT OF LIPID-DEPLETION ON THE DIFFERENT FORMS OF MONOAMINE OXIDASE IN RAT LIVER MITOCHONDRIA

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Abstract—In order to study the possible role of phospholipids in the genesis of different forms ('A' and 'B') of monoamine oxidase (MAO), rat liver mitochondrial monoamine oxidase was compared in mitochondria before and after lipid-depletion by extraction with aqueous methyl ethyl ketone with respect to substrate specificity and inhibitor sensitivity. With serotonin (substrate for the 'A form' of the enzyme) 7 per cent of the activity in the mitochondrial preparation was recovered after extraction, while 80 per cent was recovered with β-phenylethylamine (substrate for the 'B form' of the enzyme) and 3[illegible] per cent with tyramine (which is supposed to be a substrate for both forms). A comparison of the sensitivity to the inhibitors clorgyline ('A form' inhibiting) and deprenil ('B form' inhibiting) before and after extraction also showed that the 'B form' of the enzyme was almost exclusively recovered in the lipid-depleted residue. From extraction experiments performed on mitochondria with either the 'A' or the 'B form' of the enzyme selectively inhibited with clorgyline or deprenil, respectively, it could be concluded that no transformation of the 'A form' into 'B form' occurred as a result of the extraction. After extraction of mitochondria in which both forms of monoamine oxidase had been labelled with the irreversible inhibitor [^{14}C]pargyline most of the radioactivity was found in the lipid-depleted residue. This indicates that the 'A form' was not liberated from the membranes by the extraction, but was still present in the membrane residues in an inactivated state. The results do not support the hypothesis that the multiple functional forms of monoamine oxidase are explained by the binding of different amounts of membrane material to one single enzyme species.

Monoamine oxidase (monoamine:O_2 oxidoreductase, EC 1.4.3.4) has been studied in different tissues from several species and results have been obtained which have been interpreted as indices for the existence of multiple forms of the enzyme in many tissues (for reviews see Refs. 1 and 2). The question of multiple forms of the enzyme is of great importance for the development of new antidepressive drugs of the monoamine oxidase inhibitor type with fewer unwanted side-effects due to a greater selectivity in their monoamine oxidase inhibiting capacity. The evidence for the existence of multiple forms is mainly based on differences in substrate specificity, sensitivity to inhibitors and electrophoretic mobility (see Ref. 1). Due to the results obtained with the inhibitor clorgyline, Johnston [3] suggested that monoamine oxidase might exist in an 'A form' which is highly sensitive and a 'B form' which is less sensitive to clorgyline. Later Knoll and Magyar [4] introduced deprenil, an inhibitor which preferentially inhibits the 'B form' of the enzyme. The distribution and substrate specificities of the two forms have recently been reviewed by Neff and Yang [5]. Thus the 'A form' metabolizes e.g. nor-epinephrine and serotonin and the 'B form' β-phenylethylamine and benzylamine, while dopamine, tyramine and tryptamine are metabolized by both forms.

Recently, Tipton and coworkers [6, 7] have suggested that the differences between the two forms may be explained by different amounts of membrane material, i.e. phospholipids, bound to the enzyme. They showed that after treatment of monoamine oxidase from rat liver or human brain with the chaotropic agent sodium perchlorate the electrophoretic mobility and the sensitivity of the enzyme to clorgyline changed as to reveal only one single form of the enzyme. These results supported the contention that the multiple forms of monoamine oxidase were due to the differential binding of membrane material to a single enzyme species, conferring allotropic properties upon it.

Our group has developed a method for liberation of monoamine oxidase from pig liver mitochondria by extraction of phospholipids [8]. In this method the phospholipids are effectively removed by extraction with aqueous methyl ethyl ketone with most of the monoamine oxidase activity retained. In a first step, about 90 per cent of the phospholipids are extracted, however, without liberation of the enzyme [9]. In the present investigation we have used this first step of the procedure to study the role of the phospholipids for the existence of multiple forms of rat liver monoamine oxidase. We thus have compared the properties of monoamine oxidase in the mitochondrial preparation with those of the enzyme in the lipid-depleted residue with regard to substrate specificity and sensitivity to the inhibitors clorgyline and deprenil.

MATERIALS

[^{14}C]serotonin, [^{14}C]tyramine and [^{14}C]β-phenylethylamine were purchased from New England Nuclear, Boston, Mass., and the corresponding unlabelled substrates from Sigma Chemical Co., St. Louis, Mo.

Deprenil (phenylisopropylmethylpropionylamine hydrochloride, E-250) was a kind gift from Dr.

Magyar, Budapest, Hungary through Dr. Kinemuchi, Tokyo and clorgyline (N-methyl-N-propargyl-3 (2,4-dichlorophenoxy)-propylamine hydrochloride), M & B 9302 from May & Baker Ltd., Dagenham, England (Dr. R. A. Robinson). [^{14}C]pargyline was a kind gift from Abbot Laboratories, North Chicago, Ill. (Dr. R. G. Wiegand and Dr. R. C. Sonders).

METHODS

Preparation of rat liver mitochondria and of lipid-depleted mitochondrial membrane residue. Male Sprague–Dawley rats were killed by a blow on the head and the livers were quickly removed and chilled. Mitochondria were prepared in 0·25 M sucrose by the method of Thompson *et al.* [10]. To prepare lipid-depleted mitochondrial membrane residue the mitochondrial preparation (about 50 mg protein/ml) was extracted with aqueous methyl ethyl ketone as described earlier for pig liver mitochondria [8]. Briefly, 8 volumes of methyl ethyl ketone were added slowly under rapid stirring to one volume of the mitochondrial preparation. The ketone extract was poured off and the residue suspended in four times the original volume of 0·1 M potassium phosphate (pH 7·2) containing 0·001 M EDTA and then spun down. The residue from this centrifugation was suspended in the original volume of 0·01 M potassium phosphate (pH 7·2). In the following this suspension is referred to as the lipid-depleted residue.

Assay of monoamine oxidase. Monoamine oxidase was estimated according to the principles of Wurtman and Axelrod [11] with [^{14}C]tyramine, [^{14}C]β-phenylethylamine and [^{14}C]serotonin as substrates at a final concentration of 20 μM. Since many pitfalls are involved in the estimation of monoamine oxidase using labelled substrates [12, 13] we routinely controlled the results obtained by using an oxygen polarographic method [14]. In all cases tested there was an excellent agreement between the two methods.

Separation of phospholipids on thin layer chromatography (t.l.c.) and determination of lipid phosphorus. Aliquots of the mitochondrial preparation, the methyl ethyl ketone extract, the buffer extract and the lipid-depleted residue were extracted with 20 vol chloroform–methanol (2:1, v/v). The extracts were shaken with 0·4 vol 0·9% NaCl. After separation the chloroform layer was dried with anhydrous Na_2SO_4 and evaporated to a small volume. Samples were then taken for determination of phosphorus and for two-dimensional t.l.c. separation of phospholipids on silica acid H (E. Merck AG, Darmstadt). The mixture for the development in the first direction consisted of chloroform–methanol water ammonia (25%), 130:110:8:0·5 (v/v). The plates were dried and then developed in the second direction with a mixture of chloroform–acetone methanol–acetic acid water (75:35:15:12:7, v/v). After drying, the spots were visualized by spraying with iodine vapor and then scraped off. The individual phospholipids were identified by comparing the R_f-values with those of standard samples chromatographed on different plates at identical conditions. The phospholipids were extracted with 3 ml of chloroform methanol–acetic acid water (50:39:1:10, v/v) and 2 ml of the extract evaporated to dryness. Phosphorus was then estimated according to Chen *et al.* [15]. The recovery of lipid phosphorus after the separation on t.l.c. was always more than 85 per cent.

Protein was estimated according to Lowry *et al.* [16] with human serum albumin as a standard.

Radioactivity was estimated in a Packard Tri-Carb liquid scintillation spectrometer with Aquasol (New England Nuclear, Boston, Mass.) as scintillation media. Quenching was, when necessary, corrected for by using an internal standard.

RESULTS

The composition of phospholipids in the mitochondrial preparation, the methyl ethyl ketone extract and in the lipid-depleted residue is shown in Table 1. It can be seen that the extraction procedure removed about 80 per cent of the lipid phosphorus, thereby lowering the ratio phospholipid:protein from 0·093 (3·73 μg lipid P/mg protein) in the mitochondrial preparation to 0·029 (1·15 μg lipid P/mg protein) in the residue. As regards to individual phospholipids, lecithin and phosphatidyl ethanolamine were preferentially extracted with the ketone, while mainly cardiolipin accumulated in the residue to constitute about one-third of the total phospholipid content as compared to about 10 per cent found in the mitochondrial preparation.

The recovery of the monoamine oxidase activity in the buffer extract and in the lipid-depleted residue is shown in Table 2. In the ketone extract it was not possible to measure activity. In the residue the recovery was dependent upon the substrate used for the estimation. Thus, with serotonin as substrate about 7 per cent, with tyramine 39 per cent and with β-phenylethylamine 80 per cent of the activity in the mitochondrial preparation was recovered. In the buffer extract less than 1 per cent of the activity in

Table 1. Phospholipids* in the mitochondrial preparation and in the different fractions obtained after extraction

	Total	Start	Lysolecithin	Sphingomyelin	Lecithin	Phosphatidyl serin	Phosphatidyl inositol	Phosphatidylethanolamin	Cardiolipin	Front
Mitochondrial preparation	100·0†	0·1	2·2	2·9	55·4	2·0	3·4	22·9	10·2	0·8
Methyl ethyl ketone extract	77·4	0·0	0·9	1·4	41·5	1·2	2·9	25·3	3·8	0·5
Buffer extract	5·5									
Lipid-depleted residue	16·8‡	0·2	0·7	0·6	4·7	0·6	1·0	3·6	4·8	0·9

* All values are expressed as per cent of the total amount of lipid phosphorus in the mitochondrial preparation.
† 3·73 μg lipid phosphorus/mg protein.
‡ 1·15 μg lipid phosphorus/mg protein.

Table 2. Monoamine oxidase activity* remaining after extraction† of rat liver mitochondria with methyl ethyl ketone

	Substrate		
	Serotonin	Tyramine	β-phenylethyl-amine
Mitochondrial preparation	100	100	100
Buffer extract	0·3 ± 0·1	0·05 ± 0·02	0·15 ± 0·03
Lipid-depleted residue‡	6·5 ± 0·7	39·2 ± 1·9	80·1 ± 2·3

* Expressed as per cent of the activity in the mitochondrial preparation before extraction. The values are the mean ± S.E.M. of ten experiments.

† The extraction procedure was carried out as described in the text.

‡ Protein content 59·9 ± 1·4 of that in the mitochondrial preparation.

the mitochondria was found, irrespective of the substrate used.

In order to investigate whether the removal of phospholipids transformed the 'A form' of monoamine oxidase (serotonin-oxidizing activity) into the 'B form' (β-phenylethylamine-oxidizing activity), mitochondria were inhibited by either clorgyline or deprenil and then extracted. When monoamine oxidase in the mitochondrial preparation was inhibited by clorgyline only 2·5 per cent of the activity with serotonin as substrate but 84·4 per cent of the β-phenylethylamine oxidizing activity remained (Table 3). After the extraction with ketone the recovery of the activity against β-phenylethylamine was about 70 per cent of that in the clorgyline-treated mitochondrial preparation.

When deprenil was used to inhibit the 'B form' activity in the mitochondria, essentially no activity with β-phenylethylamine as substrate but 45 per cent of the activity with serotonin remained (Table 3). When this selectively inhibited mitochondrial preparation was extracted and the activity against β-phenylethylamine was estimated still no activity was found in the lipid-depleted residue.

The effect of various concentrations of clorgyline on the monoamine oxidase activity in the mitochondrial preparation and in the lipid-depleted residue is shown in Fig. 1. With serotonin as substrate both the activity in the mitochondrial preparation and the remaining activity (7%) in the lipid-depleted residue were highly sensitive to the inhibitor. The activity

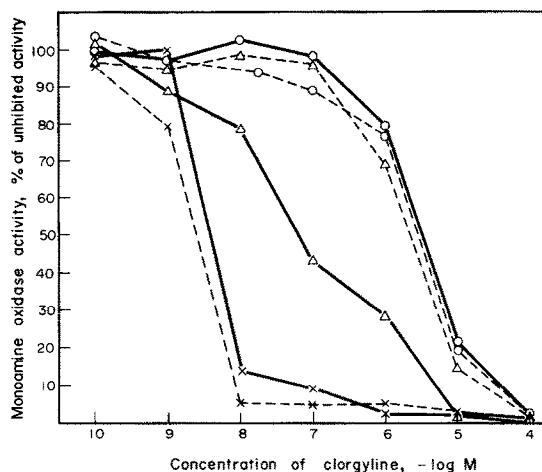

Fig. 1. Inhibition by clorgyline of monoamine oxidase in the mitochondrial preparation and in the lipid-depleted residue. Samples were preincubated at 25° in the presence of clorgyline at the concentrations indicated in a total volume of 275 μl of 0·5 M potassium phosphate (pH 7·4). After 20 min, the assay was started by adding 25 μl substrate and carried out as described in the text. The activity is expressed as per cent of the activity in the absence of clorgyline. Mitochondria with serotonin as substrate: ×——×——×; residue with serotonin: ×- -×- - -×; mitochondria with tyramine: △——△——△; residue with tyramine: △- - -△- - -△; mitochondria with β-phenylethylamine: ○——○——○ and residue with β-phenylethylamine: ○- - -○- - -○.

towards β-phenylethylamine was about 1000 times less sensitive both in the mitochondrial preparation and in the lipid-depleted residue. When tyramine was used as substrate, a plateau-shaped inhibition curve was obtained with increasing concentrations of clorgyline. This is in agreement with previous reports that tyramine is oxidized both by the clorgyline-sensitive 'A form' and the less sensitive 'B form' of the enzyme [3]. After the lipid-depletion, however, only the less sensitive part of the curve remained.

The effect of various concentrations of the preferentially 'B form' inhibiting drug, deprenil, is shown in Fig. 2. The activity against β-phenylethylamine was highly sensitive to deprenil both in the mitochondrial preparation and in the lipid-depleted residue. The activity against serotonin, on the other hand, was considerably less sensitive in both preparations, and when tyramine was used as substrate for the mito-

Table 3. Monoamine oxidase activity* remaining after extraction of rat liver mitochondria inhibited by clorgyline and deprenil, respectively†

	Clorgyline			Deprenil		
	Serotonin	Tyramine	β-phenylethylamine	Serotonin	Tyramine	β-phenylethylamine
Mitochondrial preparation	2·5	41·0	84·4	45·3	39·4	1·3
Lipid-depleted residue	1·4	23·0	58·4 (69·2‡)	6·7	4·8	0·7

* Expressed as per cent of activity in the mitochondria before inhibition.

† The mitochondrial preparation (158 mg) was incubated 1 hr at 37° in the presence of 1·75 nmoles clorgyline and 3·50 nmoles deprenil, respectively, in a total volume of 3·85 ml of 0·25 M sucrose. The inhibited preparations were then extracted as described in the text for uninhibited mitochondria.

‡ Expressed as per cent of the activity of the clorgyline-inhibited mitochondrial preparation.

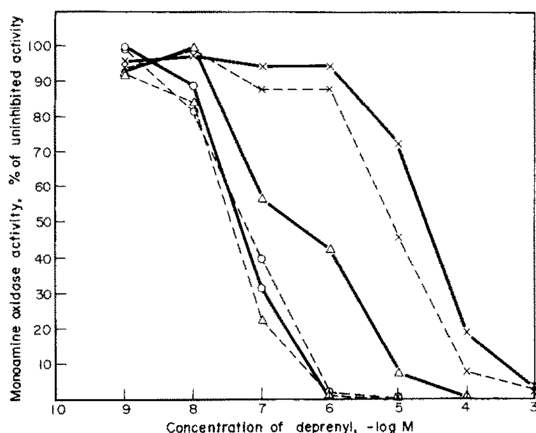

Fig. 2. Inhibition by deprenil of monoamine oxidase in the mitochondrial preparation and in the lipid-depleted residue. The experiments were performed as described in the legend to Fig. 1, but with deprenil instead of clorgyline as the inhibitor. The signs represents the same sources of enzyme and substrates as indicated in Fig. 1.

chondrial preparation again one sensitive and one less sensitive form of the enzyme could be distinguished. After extraction, however, only the highly deprenil-sensitive part of the enzyme activity remained.

Pargyline is an inhibitor of both the 'A' and the 'B form' activity of monoamine oxidase [5, 17]. It has been shown to bind irreversibly to the active site of the enzyme in a ratio of 1:1 [14, 18, 19]. To exclude the possibility that the elimination of the 'A form' activity by the extraction was due to extraction of this form of the enzyme into the ketone phase, the enzyme in the mitochondrial preparation was labelled with [^{14}C]pargyline and then extracted as described for unlabelled mitochondria. The main part of the radioactivity was recovered in the residue and less than 10 per cent was found in the ketone extract (Table 4), indicating that the 'A form' of the enzyme was most likely present in the residue after the extraction, however, in an inactivated state.

Table 4. Distribution of radioactivity after extraction of [^{14}C]pargyline-labelled mitochondria

	Per cent of radioactivity
Washed mitochondria before extraction	100
Methyl ethyl ketone extract	4·4
Buffer extract	1·9
Residue after extraction	71·9
Yield	78·2

The mitochondrial preparation (526 mg protein) was incubated in the presence of 24 nmoles [^{14}C]pargyline (51,000 cpm) in a total volume of 12·0 ml at 37° for 5 hr. After the incubation, the mitochondria were spun down at 85,000 *g* for 15 min. The sediment was suspended in 20 ml distilled water and was again spun down. After this wash the sediment was suspended to 9·5 ml with distilled water. The radioactivity remaining after the washings amounted to 16,000 cpm, and the monoamine oxidase activity to 7·0 and 3·2 per cent of that in the uninhibited mitochondrial preparation with serotonin and β-phenylethylamine, respectively, as substrates. Extraction of 6 ml of the labelled mitochondria was performed as described in the text for unlabelled mitochondria. Each sample was incubated with 1 ml of NCS tissue solubilizer (Amersham/Searle) for 1 hr at 37° before measuring radioactivity.

DISCUSSION

It is a problem of lively current interest whether there are multiple forms of monoamine oxidase or not. The indices for multiple forms are mainly based on electrophoretical separation of membrane fragments containing monoamine oxidase with different properties and on kinetic data without prior separation (See Refs. 1 and 20). As regards to the electrophoretic separation Youdim [21] has separated rat liver monoamine oxidase on polyacrylamide electrophoresis into five bands with different substrate and inhibitor specificities. It may be noted, however, that the differences between these bands do not appear to be as distinct as might be expected if they represented the different functional forms reviewed by Neff and Yang [5] and also demonstrated in this paper (Table 2). In an attempt to elucidate the nature of the multiple forms of the enzyme Houslay and Tipton [6] recently showed that different amounts of phospholipids were bound to these electrophoretically separated bands. They also showed that treatment of the preparation with the chaotropic agent sodium perchlorate prior to electrophoresis decreased the amount of phospholipid bound and that after this treatment only one band of monoamine oxidase activity was found. Accordingly, they suggested that different amounts of phospholipids bound were responsible for the separation on electrophoresis.

The kinetic evidences for multiple forms without prior separation of the enzyme are not conclusive, however, since the possibility cannot be ruled out that the results obtained may be explained by the existence of different regions with different affinities of the active site of one single enzyme as proposed by Severina [22].

In this paper we have investigated the role of phospholipids for substrate specificity and inhibitor sensitivity by using extraction with aqueous methyl ethyl ketone for lipid-depletion of the mitochondrial preparation. This extraction removed about 80 per cent of the total amount of phospholipids, preferentially the zwitterionic phospholipids lecithin and phosphatidyl ethanolamine, whereas mainly the acidic phospholipid cardiolipin accumulated in the residue (Table 1). These results are in agreement with those of Oreland and Olivecrona for pig liver mitochondria [9].

As can be seen in Table 2 most of the activity towards β-phenylethylamine ('B form' activity) but only a minor part of the activity towards serotonin ('A form' activity) remained after extraction. With tyramine ('A' and 'B form' substrate) about half of the activity remained. These results are most easily explained by the assumption that most of the 'A form' activity was eliminated upon extraction whereas that of the 'B form' remained. Another possibility, however, would be that the 'A form' was transformed to the 'B form' by lipid-depletion but this seems less probable since the recovered activity with β-phenylethylamine did not exceed that of the mitochondria before

extraction. If the 'B form' activity decreased by extraction, however, it would still be possible that the 'A form' was transformed to the 'B form'. To investigate this, the 'A form' activity in the mitochondrial preparation was inhibited by cloryline and then extracted. As can be seen in Table 3 this treatment did not significantly change the recovery after extraction; 69 per cent remained after extraction as compared to 80 per cent when uninhibited mitochondria were used (Table 2). When the 'B form' of the enzyme in the mitochondrial preparation was inhibited by deprenil, no return of the activity towards β-phenylethylamine was found after extraction (Table 3). These results make it unlikely that the 'A form' of the enzyme was transformed into the 'B form' by the extraction procedure.

The almost complete elimination of the 'A form' activity by the extraction might then be due to the presence of inactive 'A form' of the enzyme in the lipid-depleted residue, or that the 'A form' was selectively extracted into the ketone. Since it was not possible to estimate monoamine oxidase activity in the ketone extract no direct evidence for the latter alternative could be obtained. The experiments performed with monoamine oxidase labelled with the irreversible inhibitor [^{14}C]pargyline, which has affinity for both the 'A' and the 'B form' of the enzyme [5, 17] indicated, however, that the former alternative was more likely. Thus only a small portion of the [^{14}C]pargyline–enzyme adduct was found in the extract (Table 4). These experiments indicate that the 'A form' of the enzyme is more vulnerable to organic solvents than the 'B form'; it might be dependent on phospholipids or some other membrane component for its activity.

Beside the differences in substrate specificities the two forms of the enzyme have also been reported to differ in their sensitivities to inhibitors as exemplified by clorgyline which preferentially inhibits the 'A form' [3] and deprenil which preferentially inhibits the 'B form' [4]. The results obtained with inhibition of the enzyme in the mitochondrial preparation and in the lipid-depleted residue by clorgyline (Fig. 1) and deprenil (Fig. 2) show that the inhibitor sensitivity of the two forms of the enzyme did not change after the extraction of phospholipids. The changed inhibition curves obtained with both inhibitors when tyramine was used as substrate are in agreement with the finding that the 'A form' activity was almost completely eliminated upon extraction.

Houslay and Tipton [6] and Tipton *et al.* [7] have reported that preparations of rat liver and human brain monoamine oxidase solubilized by detergent and sonication contained both the 'A' and 'B forms' of the enzyme as revealed by inhibition by clorgyline. When they treated the enzyme preparation with the chaotropic agent sodium perchlorate in order to remove phospholipids the sensitivity to clorgyline changed to reveal only one form of the enzyme showing no difference in sensitivity to the inhibitor with benzylamine, tyramine or dopamine as substrates. In contrast to our results, the substrate specificity did not change by this treatment. These results indicate that the effect of the treatment with perchlorate differs from that of extraction with methyl ethyl ketone. Whether these differences depend upon a change in the amount of a critical phospholipid remaining cannot be established. However, we are using different enzyme preparations and no quantitative analysis of the phospholipid composition of the preparation after the treatment with perchlorate has been performed. Furthermore, it cannot be excluded that the detergent used in the experiments with chaotropic agent might have influenced the results. The enzyme used in these experiments [6, 7] was solubilized whereas in the present experiments the enzyme after the methyl ethyl ketone extraction was still membrane-bound. This, however, does not seem to be of any importance, since in separate experiments monoamine oxidase rendered soluble by the complete ketone extraction procedure [8] behaved similarly to that in the lipid-depleted residue both with respect to substrate specificity and to sensitivity to clorgyline and deprenil (unpublished results).

One cannot discount the possibility that the present results may be explained by the existence of one single enzyme species which was changed by the extraction as to attract only the 'B form' substrates and inhibitors. If this is true the action of perchlorate might have made this change to a lesser degree with the effect noticeable only with the inhibitors.

If the different functional forms of monoamine oxidase were due to differences in the amount or composition of membrane material bound to one single molecular specie, it seems likely, however, that the different forms would be transformed to one common form by removal of phospholipids. In the present investigation we have not been able to demonstrate any transformation of the different forms after changing the amount and composition of phospholipids in the preparation. This was true with respect both to substrate specificity and to the sensitivity to clorgyline and deprenil.

Thus, the present experiments do not support the hypothesis that the different functional forms of monoamine oxidase are due to the binding of different amounts of membrane material to one single enzyme species.

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