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COMMENTARY

THE RADIOCHEMICAL ASSAY FOR MONOAMINE OXIDASE ACTIVITY

PROBLEMS AND PITFALLS

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There are many different ways for determining the activity of monoamine oxidase (EC 1.4.3.4; MAO§), which have been reviewed in detail [1, 2]. The present review will concentrate on the radiochemical assay procedures that have been widely used for determining the activities and kinetic behavior of MAO preparations. The popularity of the radiochemical assay stems from its apparent simplicity, high sensitivity and productivity, but its injudicious use has also been responsible for much controversial or invalid data in the literature.

The radiochemical assay procedures have been described in detail elsewhere [3, 4]. Briefly, they involve incubation of the enzyme preparation with radioactively labeled substrate for a fixed time after which the reaction is stopped by acidification or rapid cooling and the products formed are separated from the unchanged substrate by extraction into an organic solvent or by ion-exchange chromatography and determined by liquid scintillation counting. Several common substrates for MAO are available in appropriate ^{14}C- or ^{3}H-labeled forms at specific radioactivities that are suitable for use in such assays. The simplicity of the assay procedure allows large batches of samples to be assayed at the same time. The number of individual determinations that can be made in one working day is often considerably greater than the number of rates that can be measured by use of a continuous assay in which the time–course of the reaction is followed polarographically, spectrophotometrically or fluorimetrically. However, this greater productivity is more apparent than real because of the necessity to perform a number of control experiments with each set of samples to ensure that the results are valid. Unfortunately, such control experiments have not been performed in many studies, rendering the results of doubtful validity. Another apparent advantage of the radiochemical assay is its high sensitivity and applicability to crude tissue preparations that are less easy to assay spectrophotometrically or fluorimetrically because of the high background absorbance and the light-scattering due to the presence of particulate material [see Ref. 5].

With these apparent advantages it is not surprising that the radiochemical assay has enjoyed widespread popularity. However, although the procedure may yield valid results, its improper use can result in misleading data, and without appropriate controls and procedures the results obtained all too often have no validity. Some of the problems that can be encountered with the radiochemical assay are common with other discontinuous assays, whereas others are peculiar to the assay itself.

Alternative assay procedures

Enzyme activities may be assayed by following the utilization of substrate or the appearance of product continuously either directly, as in the case of polarographic determination of oxygen consumption or monitoring the absorbance or fluorescence changes in the oxidation of specific substrates, or indirectly by coupling the formation of product to a reaction that can be monitored continuously, such as in the coupled assays for aldehyde, hydrogen peroxide or ammonia formation. Some of the assays that have been commonly used for the determination of MAO activities are summarized in Table 1. These have been reviewed in detail [1, 2], and the special precautions that are necessary to ensure the validity of coupled assays have been discussed elsewhere [5, 6]. Generally, continuous assays are always preferable to discontinuous ones because they allow the time–courses of reactions to be followed directly, which, in turn, allows the initial rate of the reaction to be determined accurately.

Polarographic determination of oxygen consumption and determination of hydrogen peroxide formation have the advantage of being applicable to the determination of any MAO substrate, whereas

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§ Abbreviations: MAO, monoamine oxidase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium; and SSAO, semicarbazide-sensitive amine oxidase.

Table 1. Some assay procedures for determining MAO activity

Substrate/product	Determination	Type	Refs.
Oxygen	Oxygen electrode	Direct continuous	[7]
Ammonia/ammonium	Glass or ammonia electrode	Direct continuous or discontinuous	[8, 9]
	Glutamate dehydrogenase-coupled*	Indirect continuous or discontinuous	[11]
	Color reaction†	Indirect discontinuous	[12]
H_2O_2	Peroxidase coupled*	Indirect continuous or discontinuous	[13–16]
Aldehyde	Aldehyde or alcohol dehydrogenase-coupled*	Indirect continuous	[17]
	HPLC or GLC	Indirect discontinuous	[2, 18–23]
	Colorimetric†	Indirect discontinuous	[24]
Specific substrates:			
Benzylamine, MPTP	Spectrophotometric	Direct continuous	[11, 17, 25]
Kynuramine	Spectrophotometric/fluorimetric	Direct continuous or discontinuous	[26–28]
Labeled substrates	Radiochemical	Direct discontinuous	This paper

* Determined by both spectrophotometric and fluorimetric assays. Further details of the procedures used in these assay methods are also to be found in Refs. 1, 2, and 10.
† Determined by spectrophotometric assay.

the determination of ammonia formation is restricted to primary amines. Similarly, the determination of the aldehyde formed by coupled assay with aldehyde or alcohol dehydrogenase is restricted to amines which produce aldehyde products that are substrates for one or another of these enzymes. Some substrates, such as benzylamine and kynuramine, undergo a specific absorbance change on oxidation, and this can be used to monitor the progress of the reaction spectrophotometrically, and in the case of kynuramine the reaction may also be followed fluorimetrically.

Discontinuous assays

Any assay that involves the determination of the amount of product formed (or substrate used) after a fixed time will only yield a meaningful estimation of the activity of the enzyme if the product formation proceeds linearly for this time. If this is not the case, the values obtained will be an underestimate of the activity. There are many possible causes of the time–course of product formation departing from linearity [see Refs. 5 and 29 for detailed discussions], and depletion of substrate is only one of these. Thus, the procedure of restricting the measuring period to a range where only a small percentage (often less than 10–20%) of the substrate has been converted cannot be regarded as being adequate to ensure that initial rates are being determined.There is no substitute for determining the time–course of the reaction, by stopping it after various times and measuring the amount of product formed in order to ensure that the assay time chosen corresponds to the initial-rate period of the reaction.

It should be emphasized that time–courses will have to be redetermined each time the conditions are changed. This applies to variations of the enzyme concentration or the nature of the preparation, changes of assay medium (e.g. pH, ionic strength, buffer or temperature) as well as changes in the substrate or its concentration. At low substrate concentrations, the depletion of substrate during the reaction can result in non-linear time–courses. For example, if the initial substrate concentration is much less than the K_m value of the enzyme, the time–course of the reaction will be given by [see Ref. 30]:

$$\ln[s/(s-p)] = (V_{max} \cdot t)/K_m$$

where s and p are the initial substrate concentration and the product concentration at time t, respectively. Thus, the time–course will describe a smooth curve and it will not be possible to determine the initial rate from the quantity of product formed after any single fixed time. The substrate 2-phenylethylamine has been shown to act as a time-dependent inhibitor at higher concentrations, whereas at lower concentrations of this substrate, where these time-dependent inhibitory effects are less important, the progress curves are non-linear because of substrate depletion [31]. Failure to measure initial rates at higher concentrations of this substrate led to erroneous reports that it gave rise to high substrate inhibition, whereas no such inhibition is seen if care is taken to ensure that initial rates are measured at all substrate concentrations [31]. This type of behavior emphasizes the necessity of checking the linearity of progress curves over the full range of substrate concentrations that are used, not just at the lowest substrate concentration that is to be used.

Departure from linearity will also be observed if one of the reaction products is a potent inhibitor of the enzyme. This is seen, for example, in the oxidation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) by MAO-A, where the potent product inhibition of the enzyme by the products 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) and 1-methyl-4-phenylpyridinium (MPP^+) can result in a rapid departure from linear, initial-rate, conditions [32].

In using the radiochemical assay, it should also be remembered that the K_m values of MAO towards oxygen can be relatively high. These will depend on the amine substrate used and its concentration. However, the values can approach the concentration of oxygen in air-saturated water (230 μM at 30°),

and thus departure from linearity can occur due to depletion of this substrate, unless the reaction mixture is adequately and continuously shaken to ensure continued equilibration with atmospheric oxygen.

With any discontinuous assay it is essential to ensure that the procedure used to terminate the reaction does so instantaneously. Rapid mixing with acid to alter the pH to a value where the enzyme is inactive is usually effective, but methods involving transfer of the reaction vessel to an ice or boiling-water bath may be less satisfactory if the volume of the assay mixture is relatively large. It is essential to check that the method used does, in fact, stop the reaction instantaneously. This can be done by comparing the results given by samples in which the reaction is stopped at zero-time with those given by samples from which either the enzyme or one of the substrates has been omitted or the enzyme has been inactivated in some way, such as by heat treatment or incubation with an irreversible inhibitor, before it is added to the assay mixture. The importance of ensuring that the reaction mixture is equilibrated to the desired temperature before the reaction is started must also be stressed. Failure to do this can lead to curvature of the progress curve as the temperature changes during the course of the reaction [see Ref. 5].

Studies with inhibitors

Some substrates for monoamine oxidase also act as time-dependent irreversible inhibitors. Examples include MPTP, $MPDP^+$ [32, 33] and MD 780236 [34] as well as the case of 2-phenylethylamine discussed above. The time–course of the reaction with such "suicide substrates" will describe a smooth curve and it will not be possible to determine the initial rate of reaction accurately from a single time-point discontinuous assay. The time–course of an assay in the presence of an irreversible monoamine oxidase inhibitor, such as clorgyline, deprenyl or pargyline, will also describe a first-order curve [see Refs. 35–37]. In studies on the effects of such mechanism-based irreversible inhibitors it may be possible to minimize the degree of curvature by diluting the enzyme–inhibitor mixture before assay, to reduce the inhibitor concentration to a level where its reversible effects are relatively small, and also, where possible, by using relatively high substrate concentrations to displace any reversibly-bound, competitive, inhibitor from the enzyme. Such procedures are, of course, also necessary if one is attempting to measure the irreversible inhibition in the absence of any contribution from non-covalent (reversible) inhibitory effects [see Ref. 38]. However, it would be necessary to determine time–courses of product formation in all such cases to ensure that time-dependent effects are minimized and that the values obtained in the assay approximate to the true initial rate.

In studies with reversible inhibitors, it should be remembered that any systematic errors arising from inappropriate assay procedures will be greatly compounded when the determination of ratios of kinetic constants is involved, such as when K_i values are calculated.

Specific problems with the radiochemical assay

Absolute activities. In many cases such as the simple assessment of inhibitor potencies, the determination of the absolute activity of MAO is not necessary since velocities may be expressed in arbitrary units or sometimes as per cent inhibition. However, if the activity of the enzyme is to be expressed in absolute units, such as micromoles of product formed per milligram, or moles of enzyme per minute, it is necessary to calculate the recovery of product in the procedure. This represents the proportion that is recovered from the product extraction or chromatographic step. There have been several published calculations of extraction efficiencies into different organic solvents and mixtures [4, 39–42]. These differ considerably for different substrates and, indeed, for the same substrate in different reports. As an example, reported extraction efficiencies into toluene:ethyl acetate (1:1, v/v), a commonly used solvent mixture, are shown in Table 2. In one study [40], two different methods were used to determine upper and lower limits for the extraction efficiencies, and the differences between these values were considerable for some substrates (see Table 2). Furthermore, although the authors of these papers have stressed that these values should not be regarded as constants under all conditions, this warning has been ignored all too frequently.

The binding of the aldehyde products to tissue components has been reported [4, 39, 43, 44], and this may decrease the recovery of product. Clearly, this problem might be expected to depend on the nature of the enzyme preparation used and would be more serious with crude tissue preparations. The possibility that the relative proportion of such tissue binding might vary in a complex manner with enzyme preparation or product concentration requires careful control experiments. Indeed it has been shown that the presence of tissue preparations decreases the recovery of the reaction products from ion exchange columns [4, 39, 45] in a manner that depends on the preparation used [39].

The aldehyde product of the reaction may be further metabolized to the corresponding carboxylic acid in the presence of aldehyde dehydrogenase and NAD^+ or to the primary alcohol in the presence of aldehyde reductase and NADPH [see Ref. 46]. Such further reactions could directly affect product recovery and also decrease tissue binding of the aldehyde. Indeed, the presence of the MAO preparation has been shown to decrease the chromatographic recovery of the acid product to a lesser extent than the aldehyde and to have no significant effect on the extraction of the alcohol [see Refs. 10 and 39].

Other components of the reaction mixture may also affect extraction. For example the semicarbazide-sensitive amine oxidase (EC 1.4.3.6; SSAO) from some tissues is active towards some MAO substrates, and it is common practice to assess its contribution by performing assays in the presence and absence of high concentrations of semicarbazide [see Ref. 42]. However, high concentrations of this reagent prevent the extraction of the aldehyde products

Table 2. Reported extraction efficiencies of products of the MAO reaction into toluene:ethyl acetate

Substrate	Percentage products extracted		
	Ref. 40		
	Upper limit	Lower limit	Ref. 42
Benzylamine	99	74	101
Dopamine	80	54	46
5-Hydroxytryptamine (5-HT)	92	57	
2-Phenylethylamine	99	86	
Tryptamine	98	39	
Tyramine	96	89	93

derived from some MAO substrates resulting in an underestimation of MAO activity and a corresponding overestimation of the activity due to SSAO [47]. In such cases, the problem would be revealed by control experiments in which semicarbazide is added after termination of the reaction or by studying the effects of semicarbazide on the assay of pure MAO preparations that did not contain SSAO. Such behavior might also be expected to occur with other compounds that react with aldehydes.

A general point to be remembered in comparing the behavior of the pure enzyme with those of crude preparations is that activity and kinetic data may not be strictly comparable. In the mitochondrial outer-membrane, the enzyme exists in a hydrophobic environment where the apparent behavior may also be affected by the surface charge on the membrane [48]. This might be expected to affect local pH, ionic strength and substrate concentration. Indeed the inhibitor sensitivity, substrate specificity [see, for example, Refs. 48–54] and kinetic behavior [55, 56] of MAO have all been reported to be influenced by its membrane-bound environment.

The importance of quench correction for any liquid scintillation assay should not need stressing. However, it should be remembered that the degree of quenching may differ with the nature of the enzyme sample or if additional compounds that may extract or elute with the assay product are present.

The considerations discussed above and the assumptions used in the calculation of extraction efficiencies and recoveries from ion exchange resins [see Refs. 4, 10 and 39] mean that activity values determined in this way can at best be regarded as being only approximations. It has been pointed out before [3, 4, 10] that an unambiguous procedure for relating the values obtained in a radiochemical assay to the absolute activity of the enzyme is to compare the results obtained with those given by a direct assay such as the polarographic determination of oxygen consumption or the coupled assay for aldehyde consumption and this has been done in a few cases [see Ref. 57]. However, it should be remembered that any "conversion factor" determined in such a way will not necessarily remain valid if the conditions are changed. Perhaps it would be reasonable to suggest that if it is necessary to do this, it might be easier to use a direct assay for all studies where absolute activities were required.

Radiochemical purity. Although the unlabeled substrates for MAO are generally available in sufficient amounts to allow their purification where necessary, the expense and small quantities of radioactively labeled substrates normally purchased mean that their purity is often assumed to be adequate. This can be a hazardous assumption. Not only may the radioactively labeled substrates deteriorate upon storage but the supplier's initially stated purity may not always be entirely reliable. As an extreme example, we spent a confused time trying to make sense of the results obtained with a batch of radioactively labeled dopamine which the suppliers eventually admitted was not dopamine at all.

Contamination of the substrate would result in an underestimation of the real activity of the enzyme if the contaminating material was inert. However, the possibility that it might be an inhibitor of the enzyme cannot be excluded. In such cases, the effects would depend on the type of inhibition. Contamination with a competitive inhibitor would decrease *both* the apparent K_m and maximum velocity, whereas contamination with an uncompetitive inhibitor would give apparent high-substrate inhibition and, since noncompetitive (mixed) inhibition can be regarded as a combination of competitive and uncompetitive effects, the contaminant would result in a decrease in both K_m and maximum velocity values as well as the appearance of inhibition at high substrate concentrations [58]. These effects have been applied in the form of a kinetic procedure for determining the presence of inhibitory contaminants in radiochemical substrates [59]. In this method, differences in the apparent initial rate kinetic parameters when the behavior is determined in assays where the radioactive substrate is varied as a fixed proportion of the unlabeled substrate (constant specific radioactivity) with that observed when the amount of radioactivity is kept constant whilst the total substrate concentration is varied (constant radioactivity) will indicate the presence of an inhibitory contaminant.

Generally, the radioactive substrate used should be present at rather low concentrations relative to

the unlabeled substrate to minimize any inhibitory problems. However, this will not deal with the problem of an inactive contaminant which will simply mean that the amount of labeled substrate present will be less than that believed to be present from the amount of radioactivity added. Clearly, if the extent of contamination depends on the batch of radioactive material and the length of time for which it has been stored, this could introduce considerable variations in the results from different studies performed under otherwise identical conditions.

In kinetic studies with samples that have low enzyme activities, it can be tempting to reduce the relative proportion of the unlabeled substrate. This will, of course, increase the radioactivity of the sample and thus the sensitivity of the assay, but it will accentuate any problems arising from contamination of the labeled substrate. Furthermore, reduction of the quantity of unlabeled substrate to increase the sensitivity of the assay may result in high radioactivity but very low substrate concentrations with consequent problems of non-linear time–courses preventing accurate determination of initial rates. If the radiochemical assay has to be used for kinetic studies, the procedure of constant specific radioactivity probably has fewer disadvantages than the use of the constant radioactivity approach. However, for accurate work, assessment of the purity of radioactively labeled substrates, and their purification if necessary, will be required.

Conclusions

For accurate kinetic work it is generally accepted that the use of continuous assays that allow the initial rates of the reaction to be unambiguously determined are preferable to discontinuous assays. For some enzymes and tissue preparations there may be no satisfactory alternative to a discontinuous assay. When such assays are to be used, it is essential to perform adequate control experiments to ensure that initial rates are being determined under all conditions to be used. The radiochemical assay for monoamine oxidase is beguilingly simple and has the superficial advantage of allowing large batches of samples to be assayed together. However, it has a number of inherent problems, some of which are common to all discontinuous assays and others are specific to assays of this type. Although it is possible to obtain valid results, within the limitations discussed above, using this assay procedure, control experiments are necessary to ensure this, and these should be repeated for any change in assay conditions. This will, of course, considerably reduce the number of new samples that can be assayed in any batch and thus the productivity of correctly used radiochemical assays may not be much greater than those of continuous methods. Unfortunately, in many published studies that have used the radiochemical assay it is not clear that adequate controls have been done, and thus the significance of the results reported cannot be assessed.

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