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Spurious isotope effects in enzymatic reactions with Tritium Labelled Substrates

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SUMMARY

Tritium labelled substrates are commonly used in enzyme assays on account of their high specific activity, cheapness and availability. Because of certain special effects occurring with tritium labelled compounds, isotope effects may erroneously be inferred from rate measurements using these compounds. These special effects, and recommended methods of detecting and eliminating them, are discussed.

INTRODUCTION.

An isotope effect may be defined as any difference in chemical or physical behaviour between two compounds which differ only in their isotopic composition.

The large mass difference between hydrogen and tritium makes large isotope effects possible and many examples have been described [1].

Most reported isotope effects have been concerned with differences in reaction rate constants for two isotopically different species of the same compound. These rate differences may be classified into primary and secondary isotope effects.

Primary isotope effects are demonstrated by the difference, in reaction rates of two isotopic species of the same compound, due to the formation or breaking of isotopically different bonds in a rate determining step. As might be expected, from the large mass difference between hydrogen and tritium, quite large isotope effects have been reported for this type of reaction [1].

Secondary isotope effects arise when isotopically different bonds are not formed or broken in a rate determining step and they should be much smaller than the primary isotope effect. For example, the rate of hydrolysis of methyl

p-methyl-T benzoate [2] is about 5 % slower than that of the unlabelled compound in basic solution but proceeds at the same rate in acidic solution. This difference in isotope effect in basic and acidic solution is explained by the differing reaction mechanisms under these conditions. Similarly, it has been shown [3] that, in the acetolysis of deuterated methyl-*p*-tolyl carbinyl chloride, secondary isotope effects cause the rate to be about 5 % lower for each deuterium atom substituted in the methyl group.

It can, therefore, be predicted that isotope effects in reactions in which no bond to tritium is formed or broken in a rate determining step, will be quite small. Such isotope effects are likely to be of the same order of magnitude as the accuracy of the assay method and care is necessary when measuring small rate differences before ascribing them to isotope effects.

Quite large isotope effects have been postulated with tritium labelled substrates in circumstances where these rate differences might be due to special effects which are not isotope effects in the usual sense.

Five situations where these effects have been, or might be, wrongly interpreted as isotope effects will be discussed.

Discussion.

1. — *Apparent low rates due to labilization of tritium.*

When tritium labelled compounds are used as substrates for enzymes the tritium label is nearly always being used as an auxiliary label for carbon. The assumption is made that the tritium label is stable under the experimental conditions. If a proportion of the label is lost during the reaction, or isolation of products, any measurement of enzyme rates, involving a radioactive measurement, will be proportionally low. This could be mistakenly interpreted as an isotope effect.

Such labilization of tritium can occur in several ways : chemically or enzymatically during the reaction, chemically during the separation and isolation of products, or during storage in solution before use.

Suppliers of tritium labelled compounds remove readily labile tritium under relatively mild conditions during manufacture but, because the conditions during reaction are likely to be more drastic, a loss of tritium can occur from the substrate, product or both.

Many tritium labelled compounds, supplied in aqueous solution, slowly release small amounts of tritium into solution [4]. This loss can amount to several percent on prolonged storage and, unless it is removed before use, it could result in an apparently low rate of reaction.

A few reports of enzymatic labilization of tritium have been published. For example Evans and co-workers [5] found that tritium was lost from generally labelled L-amino acids when they were treated with D-amino acid oxidase and no degradation of the amino acid occurred during this treatment.

Loss of tritium label during the isolation of products has occurred, for example, in studies of the metabolism of histidine where the product, histamine, is separated from histidine by ion exchange [6]. Satisfactory results were obtained with carbon-14 labelled histidine but experiments with tritium labelled histidine showed apparently very low conversion to histamine. This could have been ascribed to an isotope effect. It was, however, shown to be due to the chemical labilization of tritium during the acid elution of histamine from the ion-exchange column and the subsequent processing before counting.

Erroneous conclusions resulting from tritium labilization can, and should be, eliminated by simple procedures.

Labile tritium formed on storage can easily be removed before use by lyophilisation.

The chemical lability of the tritium label should be tested, with both substrate and product, under the experimental conditions to be used.

Enzymatic labilization of the tritium label might best be checked by distilling a sample of water from the reaction mixture and measuring its tritium content.

Where appropriate, further checks may be carried out by comparing the initial specific activity of the substrate with that of the product; or by carrying out duplicate experiments with carbon-14 labelled substrate.

In many experiments a correction can be applied if the extent of labilization is not too great. Because variations in the labelling pattern of generally labelled tritiated compounds can occur from batch to batch, it is better to use specifically labelled rather than generally labelled substrates to make such corrections easier and more accurate.

Alternatively, since the extent of labilization will vary from position to position, it should be possible to select a position of labelling where labilization losses are minimal. For example, in the example above [5], tritium was labilized from the α-position of the amino acids. The use of L-amino acids labelled in positions other than the α-position should result in a lower rate of labilization.

2. — *Apparent false rates due to uncertainty about the specific activity of the substrate.*

This can apply to all assays with labelled substrates but is more common with tritium labelled compounds.

In particular, confusion is likely to result when a labelled substrate, which has undergone marked decomposition on storage, is diluted with inactive substrate before use. In such a situation the radiochemical purity of the labelled substrate must be known and taken into account when calculating the specific activity of the diluted material which is used as substrate in the enzyme assay. Failure to make this correction will result in an apparently low rate of reaction.

A recent report [7] of isotopic discrimination between tritium and car-

bon-14 labelled thymidines might be accounted for in this way. The authors found that the rate of phosphorylation, using thymidine labelled with tritium, both in the 6 position and in the methyl group, was from 60 %-80 % of that with carbon-14 labelled thymidine. They tentatively ascribed this difference to an isotope effect. However, on allowing the reaction to go to completion, they found that essentially 100 % conversion to thymidylate occurred with carbon-14 labelled thymidine but only *ca* 75 % conversion with tritiated thymidine. This implies that only *ca* 75 % of the activity in the tritiated substrate was, in fact, thymidine and consequently the tritiated thymidine rates should be multiplied by a factor 100/75 to correct for this. This correction reduces the rate difference between tritium and carbon-14 labelled substrates from the stated 60 %-80 % to 80 %-107 %.

In these experiments thymidine labelled with tritium in two different positions, and obtained from different sources, was used. The purity, and hence the correction factor, must have varied from experiment to experiment and so the corrected range of 80 %-107 % should be taken only as an indication that any isotope effect is quite small and probably lies within the normal range of experimental variation.

It may not be essential to use absolutely pure substrates in enzyme assays although the sensitivity will, in general, be greater the purer the substrate is. If the substrate is not pure, its purity must be determined so that the true specific activity may be calculated after dilution with inactive substrate. Alternatively, provided the analytical method used separates substrate, products, and impurities, the true rate of reaction may be calculated from the ratio of product to substrate.

For absolute measurements of enzyme rates it is necessary to know accurately the specific activity of the substrate. Because of the difficulties associated with the accurate measurement of high specific activities, the specific activities of many labelled compounds are known with only *ca* 5 % accuracy. If greater accuracy is required it might be best to prepare lower specific activity substrates by taking a known amount of the labelled substrate, of known radiochemical purity, and diluting it, at least tenfold, with pure inactive material. The effect of any small error in the specific activity of the labelled compound will then be reduced considerably. The accuracy with which the specific activity of the substrate is known will then depend on the chemical purity of the inactive substrate used for dilution. Often, however, this purity is known less accurately than is the specific activity of the original labelled compound.

3. — *Intramolecular migration of tritium, the "NIH shift".*

A very rapid and simple assay method for aromatic amino acid hydroxylases has been developed in which amino acids, labelled with tritium in the position of hydroxylation, are used as substrates. The rate of tritium release into solution is then used as a measure of the reaction. Measurement of tyro-

sine hydroxylation by this method showed that tritium release occurred at *ca* 85 % of the rate measured using a carbon-14 labelled substrate [8]. This difference could be ascribed either to an isotope effect or to non-specific labelling. It was found that the hydroxylated product contained tritium, and it was concluded that the substrate had only *ca* 85 % of its tritium in the specified position. Later experiments with phenylalanine hydroxylase, using phenylalanine-4-T, showed that the rate of tritium release was only 8 % of the true rate of hydroxylation [9]. Since it had been shown that the phenylalanine-4-T was labelled exclusively in the 4 position, the retention of tritium in the product is the result of an intramolecular migration of tritium. This has been called the "NIH Shift" and has been well described elsewhere [10, 11]. It has now been suggested [12] that this shift is a common feature of all enzymic hydroxylations of aromatic amino acids.

There is a possibility of similar shifts occurring in other reactions which are followed by tritium release. Both thymidylate synthetase and hydroxymethyl deoxycytidylate synthetase have been assayed using dUMP-5-T and dCMP-5-T as substrates [13, 14, 15]. Measurement of tritium release gave rates varying from 26 % to 86 % of the true rate. As no analyses were performed to measure the extent of any tritium retention in the products, one can only suggest the possibility of an intramolecular shift occurring in these reactions. The occurrence of such a shift does not, however, detract from the value of this method as a very rapid and simple enzyme assay. In most reactions a simple correction can be applied, if necessary, to convert the rate of tritium release to the true rate. In other reactions, for example the hydroxylation of phenylalanine [9], where the effect of the shift is quite large, chemical methods have been used to displace the tritium which is retained in the product after migration. In this way the measurement of the rate of tritium release becomes a true measure of the enzyme rate.

This type of intramolecular shift can easily be detected and corrected for by measuring the amount of tritium retained in the product.

4. — *Errors resulting from the use of specifically labelled substrates in which the position of labelling is not exactly as specified.*

When measuring the rate of tritium release from a specifically labelled substrate, an apparently low rate of reaction will result if the position of labelling is not exactly as specified [14].

In some reactions it may also be necessary to know the stereospecificity of the tritium label [16]. For example, in a study of the enzymatic hydroxylation of proline it was found [17] that the use of *cis*-L-proline-4-T resulted in the complete retention of tritium label during hydroxylation whereas complete loss of tritium occurred when *trans*-L-proline-4-T was used.

This effect may easily be detected, and corrected for, by isolating the product and determining its radioactivity.

Differentiation between this effect and the "NIH Shift" can be made by

determining the position of labelling in the original substrate by degradation [9], Nuclear Magnetic Resonance Spectroscopy [10], or other methods [11].

5. — *Isotope effects on the Michaelis constant.*

In discussing isotope effects during enzyme reactions most authors refer to the effect on the maximum velocity, V_{max}, and most enzyme assays are carried out at substrate concentrations high enough to cause saturation of the enzyme with substrate so that V_{max} is measured. Isotope effects can also affect the binding of the substrate to enzyme, and hence the Michaelis constant, K_M. With many enzymes, hydrogen bonds are involved in this binding and one might predict fairly high isotope effects for these.

It has been shown [18] that, during the reaction of yeast alcohol dehydrogenase, V_{max} for isotopically normal ethanol is, as might be expected, 1.8 times as great as V_{max} for deuterated ethanol. At the same time, K_M for ethanol is 2.3 times as great as K_M for deuterated ethanol. As a result, at high substrate concentrations where the enzyme is saturated with substrate, there is a large isotope effect and the deuterated substrate reacts more slowly than the isotopically normal substrate. At low substrate concentrations, however, the enzyme is more saturated with the deuterated substrate than with the isotopically normal substrate. As a result the deuterated compound can react faster than the isotopically normal substrate under these conditions.

This isotope effect on the Michaelis constant can occur with many reactions and should be tested for either by measuring K_M and comparing it with K_M for the unlabelled substrate; or by varying the substrate concentration both with labelled and unlabelled substrate to confirm that the enzyme is fully saturated with substrate under the assay conditions.

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