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Kinetic isotope effects in the enzymic reactions of tritium- and deuterium-containing substrates

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ABSTRACT. The kinetic differences between the enzymic reactions involving substrates containing heavy hydrogen isotopes are examined. Theoretical estimates and numerous examples of experimentally measured kinetic isotope effects in various enzymic reactions are presented. The kinetic isotope effects are widely used in studies of the mechanisms of enzymic reactions and of the structures of the enzyme–substrate complexes, for the enrichment of labelled compounds in heavy hydrogen isotopes, and also in certain fields of medicine and biochemistry.

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I. Introduction

The use of labelled compounds as radioactive tracers is based on the hypothesis of the identity of the physicochemical properties of the labelled and unlabelled molecules of the given compound.[1] This assumption is to some extent arbitrary. The introduction of an isotope label and especially of heavy hydrogen isotopes induces the appearance of isotope effects—definite changes in the physical, chemical, and biochemical, pharmacokinetic, and certain other properties of the labelled compounds.[2]

Among the numerous studies devoted to isotope effects, a large proportion referred to studies of the kinetic differences between the chemical reactions of isotopic molecules, i.e. to kinetic isotope effects (KIE).[3] KIE referring to enzymic reactions have been studied to a lesser extent, although recently there has been a continuous growth of interest in such effects, which is indicated by the constantly increasing number of published (mainly abroad) review and original (see, for example, Refs. 17–21) papers. Unfortunately, one must conclude that this field of enzyme kinetics has as yet attracted little attention by the investigators in our country.

The current interest in studies on KIE in enzymic reactions is caused, on the one hand, by the fact that the KIE may serve as a potential source of possible errors in the use of labelled compounds in radiochemical studies on enzymic reactions[22,23] and, on the other hand, KIE can serve as a powerful means of investigating a number of important problems in enzymology.[5, 11–14]

While our aim is not a detailed examination of the negative aspect of KIE, we may note, for example, that the difference between the rates of enzymic reactions of labelled and unlabelled substrates can be the cause of errors in radiometric measurements of the catalytic activities of enzymes, in the isotopic determinations of the rates of individual stages of the process, and in the establishment of the kinetic orders of the reactions of the substrates. Failure to take into account KIE in the studies on metabolism and pharmacokinetics with the aid of labelled preparations can also lead to serious errors. These and certain other complications associated with KIE become particularly significant in polyenzymic processes and also when substrates with a high degree of isotope substitution (multiply labelled compounds) are used. Comprehensive study of KIE makes it possible not only to foresee the possibility of such errors but also to avoid them by selecting conditions in the use of labelled compounds under which KIE are minimal or are fully excluded.

As regards the positive aspect of KIE, one should note in the first place the enormous importance which they have in studies on the mechanisms of enzymic reactions and the structures of enzyme–substrate complexes. One of the promising fields also involves the use of KIE for the enrichment of labelled compounds in heavy isotopes.

II. Theoretical estimates of kinetic isotope effects

The ratios of the rate constants for the corresponding pairs of isotopic molecules have come to be used for the quantitative characterisation of the kinetic differences in reactions of labelled and unlabelled compounds. Since these differences are determined primarily by the relative differences between the masses of the isotopes, the maximum KIE are to be expected for compounds labelled with hydrogen isotopes, for which the mass ratios are maximal ($^1H:^2H:^3H = 1:2:3$).

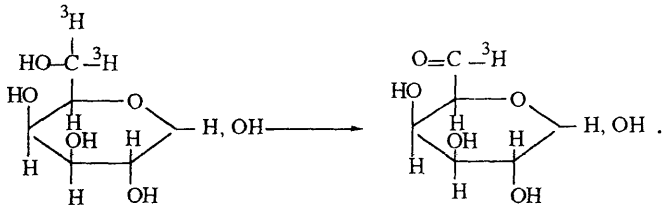
Theoretical calculations of KIE are based on the kinetic transition state theory or the theory of rate processes.[24] Without dwelling on the details of the conclusions which have been described quite fully in the familiar monograph by Melander and Saunders,[3] we shall quote the principal predictions by these authors concerning the values of the KIE for reactions involving compounds labelled with hydrogen isotopes.

The simplest calculations of KIE in which the absence of differences between the energies of the zero-point vibrations of the C–H, C–D, and C–T bonds in the transition complexes is assumed yield the quantities $k_H/k_D = 6.9$ and $k_H/k_T = 16$. In calculations where the disappearance of the deformation vibration

frequencies in the transition state is assumed, the following limiting isotope effects were obtained: from 15 to 20 for k_H/k_D and from 50 to 75 for k_H/k_T. However, the available data show that even if the deformation modes are weakened, this occurs to a very slight extent and the appreciably higher values of the KIE sometimes observed in practice compared with the above results of semiclassical calculations (6.9 for deuterium and 16 for tritium) is more likely to be attributable to quantum mechanical tunnelling. Melander and Saunders believe[3] that the isotope effects k_H/k_D below 12 and k_H/k_T below 36 may contain a definite contribution due to tunnelling, while higher values of the KIE cannot be explained at all without resorting to the tunnel effect.

The experimental protium–deuterium and protium–tritium KIE for many chemical and enzymic reactions are close to the values predicted by semiclassical calculations. There are numerous examples in the literature of a significant retardation (sometimes by several tens) of a wide variety of chemical (see, for example, Rosenberg[25]) and enzymic[26,27,34–42] reactions involving deuteriated and tritiated substrates.

As an example of such high KIE, one may quote the results of kinetic studies of the oxidation reaction of tritium-labelled galactose catalysed by galactose oxidase:[27]



It has been established that galactose doubly labelled with tritium in the 6-position is oxidised at a rate which is lower almost by a factor of 150 than the rate of oxidation of normal galactose. Such a high KIE is due both to the different isotopic composition of the bond subjected to chemical change and to the different isotopic composition of the neighbouring bond.

There exists a fairly well justified theoretical and experimentally confirmed relation between the protium–deuterium and protium–tritium KIE. It was first established by Swain, et al.[28] in the form of the equation

$$\frac{\ln (k_H/k_T)}{\ln (k_H/k_D)} = 1.44$$

or

$$\frac{k_H}{k_T} = \left(\frac{k_H}{k_D}\right)^{1.44}.$$

These relations are used widely to predict the KIE in the reactions of substrates labelled with one of the heavy hydrogen isotopes, provided that the KIE for the other isotope has been established experimentally.

The same relations can be used to confirm the reliability of the experimental results. For example, markedly different results of spectrophotometric measurements of the KIE in D-[1-^{2}H]glucose enzymic oxidation reaction have been published: the protium–deuterium KIE has been estimated as 10–15 by Bright and Gibson[29] and as 2.2–2.5 by Hauge et al.[30] Radiometric measurements of the protium–tritium KIE in a similar D-[1-^{3}H]-glucose oxidation reaction yielded k_H/k_T = 3.0–3.1.[31] When the latter was used to calculate the expected protium–deuterium effect from the equations presented above, it was found that the KIE is 2.2, which confirms the reliability of the value found by Hauge, et al.[30]

III. Classification of kinetic isotope effects in enzymic reactions

The classification adopted for chemical processes[3] can be used for the KIE in enzymic reactions. As in chemical reactions, the KIE can be divided into primary and secondary and intramolecular and intermolecular. The primary isotope effects are determined by the different isotopic compositions of the bond being dissociated with the same isotopic composition of the neighbouring bond. The secondary isotope effect is determined by the different isotopic compositions of the neighbouring bond for the same isotopic composition of the bond being dissociated. The combined isotope effect is determined simultaneously by the different isotopic compositions of the bonds being dissociated and the neighbouring bond. The intramolecular isotope effect (or the intramolecular competition factor) is determined by the different isotopic compositions of two chemically equivalent bonds in the same molecule. The intermolecular isotope effect is determined by the different isotopic compositions of molecules in the same incubation system.

The characteristic features of the kinetics of enzymic reactions, associated primarily with the multistage nature of the process and the phenomenon involving the saturation of the enzyme by the substrate, require the expansion of the classification of the KIE using special kinetic parameters which are not used in the kinetics of chemical reactions. Such a classification has been mainly developed by Northrop.[5,7]

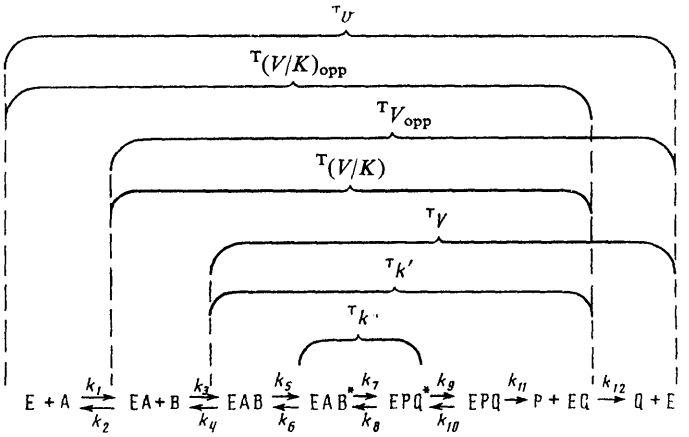


Figure 1. Classification of kinetic isotope effects in a two-substrate enzymic process.[5,7]

In the multistage enzymic process illustrated schematically in Fig. 1, the presence of a tritium (or deuterium) label in one of the substrates (for example, A) can affect the rates of individual stages

and the kinetic parameters entering into the main kinetic equation for enzymic processes (the Michaelis–Menten equation):[32]

$$v = \frac{V\,[S]}{K + [S]},$$

where v is the rate of reaction, V the maximum rate for the complete saturation of the enzyme by the substrate, [S] the substrate concentration, and K the Michaelis–Menten constant. The braces in the scheme enclose the stages in which the KIE can be determined experimentally. The symbols above the braces denote the KIE in terms of definite kinetic parameters and the superscript T means that in the given instance the isotope effect refers to tritium, i.e. one is dealing with the ratio of the corresponding parameter for the protium form to the parameter for the tritium form of the substrate.

The KIE indicated have been given the following names:[7] the isotope effect ^{T}v is the "observed isotope effect", the isotope effects $^{T}(V/K_{app})$ and $^{T}V_{app}$ are the "apparent isotope effects" (they are obtained by extrapolating the observed isotope effects to infinitely small and infinitely large concentrations of the substrate A for a fixed concentration of the cosubstrate B), the isotope effects $^{T}(V/K)$ and ^{T}V are the "limiting isotope effects" (they refer respectively to low and high concentrations of the cosubstrate B), the isotope effect $^{T}k'$ is the "internal isotope effect", and finally the isotope effect ^{T}k is the "true isotope effect" (it is analogous to the KIE in chemical reactions). The true isotope effects in the forward and reverse reactions may not be the same, which leads to the appearance of an isotope effect in the equilibrium constants.

IV. Methods for the determination of kinetic isotope effects in enzymic reactions

The observed KIE is determined from v as the ratio of the observed rates of conversion of the unlabelled and labelled substrates under identical conditions.

In order to determine the apparent or limiting isotope effects in terms of V and V/K, the concentration variations of the observed rates of conversion of the labelled and unlabelled substrates are investigated. Figure 2*a* presents typical relations of this type. For a high substrate concentration, where the enzyme is saturated by the substrate and the order of the enzyme reaction approaches zero, the labelled and unlabelled molecules are converted at the maximum rates V_H and V_T. The KIE ^{T}V corresponds to this boundary condition (Fig. 2*b*).

In the other boundary situation—at low substrate concentrations—where the process is of first order with respect to substrates, the slope of the initial sections of the curves is equal to the ratio V/K according to the Michaelis–Menten equation. The KIE $^{T}(V/K)$ corresponds to this condition.

The isotope effects in the Michaelis constants can be calculated from the KIE in terms of V and V/K obtained in this way.

Such concentration variations of the KIE can be investigated for the majority of labelled substrates and enzymes. However, limitations are possible for tritium-containing substrates. This applies to reactions for which the Michaelis constants are large and when the attainment of saturation by the enzyme requires the introduction of an appreciable amount (frequently unavailable) of the labelled substrate into the incubation system. We encountered such a case (see, for example, Akulov and Smetkova[31]) in a study of the KIE in the oxidation of glucose labelled with tritium in the 1-position in the presence of glucose oxidase. For this reaction, K has been estimated as 3.3×10^{-2} mol litre^{-1}.[33] In this case, the saturation of the enzyme by the substrate requires the use of a labelled substrate having an activity amounting to tens of Curies. Furthermore, difficulties associated with radiation processes arise under these conditions.

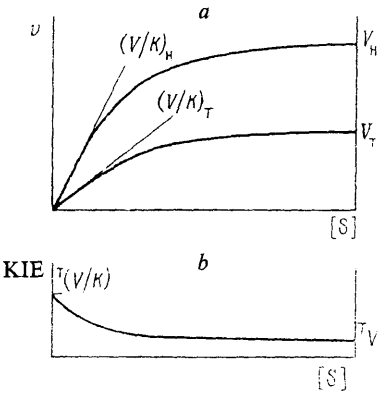


Figure 2. Determination of KIE from the concentration variations of the observed rates of reaction (*a*) and the variation of the KIE with substrate concentration (*b*).

The true ^{T}k or ^{D}k, which reflect the influence of the isotopic composition of the substrate on the true chemical reaction within the activated enzyme–substrate complex, cannot be measured experimentally but they can be calculated from the protium–deuterium and protium–tritium isotope effects in terms of V/K.[7] Thus the true protium–deuterium KIE ^{D}k can be determined with the aid of the equation

$$\frac{^{D}(V/K) - 1}{^{T}(V/K) - 1} = \frac{^{D}k - 1}{^{D}k^{1,44} - 1}.$$

The equation for the calculation of the true tritium KIE has a similar form.

Thus, in order to determine the individual KIE, it is necessary to carry out enzymic reactions of labelled substrates with complete isotope substitution at the reaction centre. Whereas for deuterium such compounds are entirely available, for tritium the production of preparations with complete isotope substitution at a given position within the molecule has begun comparatively recently. Studies in which the KIE in enzymic reactions were investigated using substrates containing exclusively tritium atoms in this position within the molecules reacting chemically in the given process began to be published only in the very latest years. However, there are still frequent claims in the literature that, owing to the absence of tritiated preparations without carriers, the protium–tritium KIE cannot be determined from the individual rates of reaction of the labelled and unlabelled substrates and that the only method which can be used in such investigations is that of competitive reactions in which the incubation system contains simultaneously labelled and unlabelled substrate molecules [Melander and Saunders (Ref. 3, p. 312) and Cleland[14]].

Owing to the difference between the rates of reaction of the labelled and unlabelled molecules, the specific radioactivity of the substrate changes in such competitive experiments. In the determination of the KIE by the competitive method, the reaction is stopped at specified time intervals and the degree of enzymic reaction and the specific radioactivity of the unreacted substrate or product are determined. The kinetic isotope effect in terms of the V/K ratio is calculated from the formula (Ref. 3, p. 105).

$$^{T}(V/K) = \frac{\lg(1-F)}{\lg[1-(FA_p/A_S^0)]},$$

where F is the extent of reaction and A_S^0 and A_p are the initial molar radioactivity of the substrate and the specific radioactivity of the product. In order to achieve the maximum accuracy, it is recommended to determine the limiting value of the function for F tending to zero:

$$^{T}(V/K) = \left|(A_S^0/A_p)\right|_{F\to 0}.$$

Thus the competitive method makes it possible to measure only the KIE in terms of V/K. This constitutes its disadvantage.

The methods for the determination of KIE examined require specially synthesised labelled substrates enriched in deuterium or tritium. However, in certain cases it is possible to use preparations with the natural isotopic composition for such purposes.

All hydrogen-containing compounds are known to contain approximately 0.015% of natural deuterium and 4×10^{-15}% of natural tritium. Unfortunately, modern experimental techniques do not allow the employment of natural tritium for the measurement of KIE; however, in the case of deuterium highly sensitive NMR spectrometers permit the measurement of the content of such deuterium in various positions within molecules and to follow its variation in chemical and biochemical processes.

It has been shown with the aid of NMR analysis that the distribution of deuterium can differ fairly appreciably as a function of the source of the preparation. For example, it has been established that specimens of synthetic and natural anethole[43] and camphor,[44] differing in their chemical history, have different distributions of natural deuterium. The cause of this difference is the KIE, which lead to the enrichment of specific positions within the molecules in deuterium. Using the NMR method based on natural deuterium, Pascal, et al.[45] established that C(10) position is appreciably enriched in deuterium in the biosynthesis of β-pinene from mevalonic acid. The KIE calculated from this enrichment proved to 2.1.

It is noteworthy that the method for the determination of KIE in terms of natural deuterium examined above is based on the competitive principle and therefore it permits the determination of KIE solely in terms of the V/K ratio.

V. Applications of kinetic isotope effects

1. Kinetic and chemical mechanisms of enzymic reactions

One of the important problems in enzyme kinetics is the determination of the kinetic mechanism of reactions occurring with participation of two or more substrates (or products), i.e. the specification of the order of the addition of substrates and of the liberation of the products. In order to estimate the kinetic order by classical methods, use is normally made of the technique involving inhibition or equilibrium exchange.[46] However, for enzyme systems, in which the conversion of the substrate into the product via a ternary complex is irreversible, such methods proved to be extremely inconvenient. Additional experimental difficulties arise when one of the substrates is oxygen and one of the products is water.

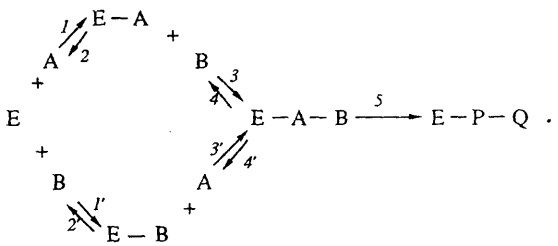
Unique possibilities in the study of the kinetic order of enzymic reactions are provided by the apparent and limiting isotope effects in terms of the V/K ratio, which are determined, as noted above, from the concentration variations of the rate of the process at a fixed concentration of one of the substrates.

Suppose that a substrate A, containing a tritium (or deuterium) label, and substrate B participate in the reaction. The order of the addition of the substrates to the enzyme involves one of the two ordered mechanisms

$$E + A \underset{2}{\overset{1}{\rightleftharpoons}} E-A + B \underset{4}{\overset{3}{\rightleftharpoons}} E-A-B \xrightarrow{5} E-P-Q, \quad (1)$$

$$E + B \underset{2}{\overset{1}{\rightleftharpoons}} E-B + A \underset{4}{\overset{3}{\rightleftharpoons}} E-B-A \xrightarrow{5} E-P-Q \quad (2)$$

or can occur at random:



Kinetic analysis of mechanism (1) yields the following expression for the KIE:[12]

$$^{T}(V/K) = \frac{^{H}k_5/^{T}k_5 + [^{H}k_5(1+k_3[B]/k_2)/k_4]}{1 + [^{H}k_5(1+k_3[B]/k_2)/k_4]}.$$

At an infinitely high concentration of the substrate B, the terms of the equation in square brackets become dominant and the KIE tends to unity.

On the other hand, in those cases where the addition of the substrate A precedes the addition of the substrate B [mechanism (2)], the KIE is defined by the equation

$$^{T}(V/K) = \frac{^{H}k_5/^{T}k_5 + ^{H}k_5/k_4}{1 + ^{H}k_5/k_4},$$

i.e. is independent of the concentration of the substrate B. In this case, the KIE can assume values from unity to the theoretical maximum value and depends on the ratio $^{H}k_5/k_4$. Here it is important that the ratio is the same for all concentrations [B]. Thus, if it has been established that the concentration of B does not affect the KIE, it follows that B is bound to the enzyme after A.

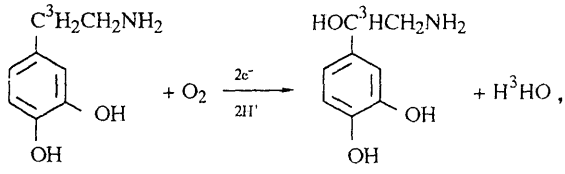
For a random sequence of the addition of the substrates A and B, the expression for the KIE becomes more cumbersome[12] but significantly simplified when the concentration [B] tends to zero:

$$^{T}(V/K) = \frac{^{H}k_5/^{T}k_5 + ^{H}k_5/(k_4+k_{4'})}{1 + ^{H}k_5/(k_4+k_{4'})}$$

or to infinity:

$$^{T}(V/K) = \frac{^{H}k_5/^{T}k_5 + ^{H}k_5/k_4'}{1 + ^{H}k_5/k_4'} .$$

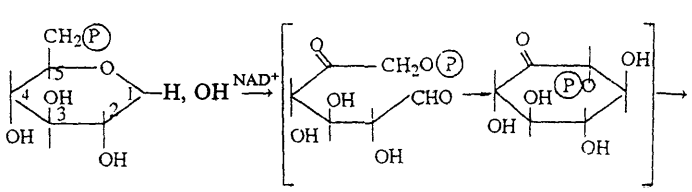
Using the KIE, Miller and Klinman established[10,47] that the oxidation of tritium-labelled dopamine catalysed by the enzyme dopamine β-monooxygenase, i.e.



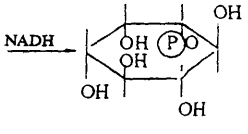
is accompanied by an appreciable KIE (V/K) which decreases from 8.8 at an oxygen concentration of 0.02 mM to 4.1 at $[O_2]$ = 1 mM. Analysis of the experimental dependences of the KIE on the oxygen concentration yielded the following limiting values of the KIE: $^{T}(V/K)_{O_2\to 0} \simeq 15$ and $^{T}(V/K)_{O_2\to\infty} \simeq 3.6$. The data obtained indicate a non-ordered kinetic mechanism of this reaction.

Analysis of the KIE in the enzyme oxidation reactions of 2-[2-^{2}H]propanol and [1-^{2}H]cyclohexanol made it possible to establish the kinetic mechanisms of processes catalysed by alcohol dehydrogenases isolated from both yeast and liver.[48–50] When liver alcohol dehydrogenase is used, the liberation of NAD from the ternary complex E–NAD–cyclohexanol takes place at a negligible rate, while cyclohexanol is liberated much faster than it reacts with formation of products, i.e. the process is ordered. In the 2-propanol oxidation reaction catalysed by yeast alcohol dehydrogenase, the liberation of 2-propanol and NAD from the complex E–NAD–propanol takes place at approximately the same rates, i.e. the reaction is random. In the study of the mechanisms of the same reactions, use was also made of secondary KIE.[51] Together with the determination of the kinetic mechanisms of enzymic reactions, the KIE can yield valuable information about their chemical mechanisms.[12,52–55]

For example, the study of the KIE in the reactions involving the conversion of *D*-[5-^{3}H]-, *D*-[3-^{3}H]-, and *D*-[4-^{3}H]-glucose-6-phosphate[34,56,57] and also *D*-[5-^{2}H]- and *D*-[G-^{2}H]- glucose-6-phosphate[58,59] into the corresponding tritiated and deuteriated isotopic forms of myoinosotol-1-phosphate, catalysed by myoinositol-1-phosphate syntheses of plant and animal origin, established the following process mechanism:



D-glucose-6-phosphate

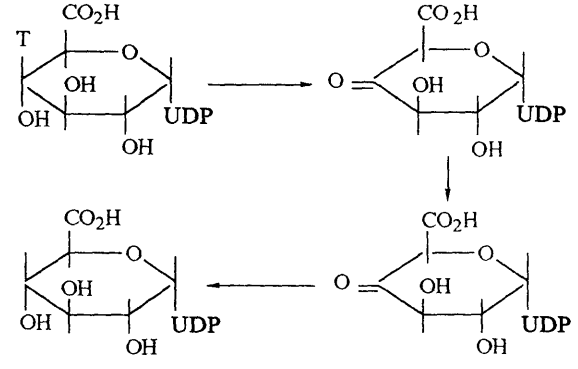


myoinosotol-1-phosphate

Isotope effects were observed only in those cases where tritium or deuterium was present in the initial substrate in the 5-position, while in the remaining cases the labelled substrates reacted at the same rate as the unlabelled *D*-glucose-6-phosphate. This shows that only hydrogen atoms in the 5-position participate in the oxidation stage and in the subsequent cyclisation of *D*-glucose-6-phosphate to myoinositol-1-phosphate.

A difference between the mechanisms of two parallel transmethylation reactions catalysed by catechol *O*-methyltransferase, involving the transfer of [^{3}H]methyl groups from the labelled *S*-adenosylmethionine to the *m*- and *p*-hydroxy-groups of dopamine, has also been established with the aid of KIE.[42] It was found that for *meta*-methylation, the secondary KIE is 1.16, whereas for *para*-methylation it is 1.35. The latter value is close to the value of 1.29 determined previously for the S_N2 transfer of the methyl group in the methylation of 3,4-dihydroxyacetophenone in the presence of the same enzyme.[60] On this basis, it was concluded[42] that the methylation of the *para*-position of dopamine proceeds via an S_N2 mechanism. The lower value of the KIE for *meta*-methylation indicates the influence of other stages on the rate of the process as a whole.

An appreciable KIE (3.1) has been observed in the decarboxylation of uridine-diphospho-*D*-[4-^{3}H]glucuronic acid in the presence of UDP-*D*-glucuronate decarboxylase, whereas the presence of the tritium label in the 3- and 5-positions in the initial compound did not affect the rate of the process.[61,62] These results show that the decarboxylation reaction is accompanied by oxidation at C(4):



Similar conclusions concerning process mechanisms have been drawn from the KIE in the reaction involving the conversion of UDP-*D*-[4-^{3}H]glucose into UDP-*D*-[4-^{3}H]galactose catalysed by UDP-*D*-glucose 4-epimerase.[63]

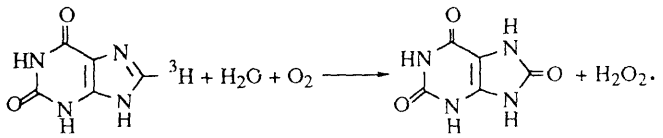
KIE have also been used in the study of the mechanisms of reactions catalysed by fumarase,[64] protease,[17] alcohol dehydrogenase,[62,65–67] aminoxidase,[68,69] chorismate synthase,[19] yeast hexokinase,[70] lacate dehydrogenase,[71] phosphoribosyltransferases,[72] succinoxidase,[73] adenosine deaminase,[74] liver monoxygenase,[75] and lipoxygenase.[54]

Useful information about the mechanisms of enzymic reactions is also provided by KIE when deuteriated solvents are used (the dependence of the KIE on the solvent).[76–78]

2. Determination of the rate-limiting stage in the enzymic process

Analysis of the principal types of KIE makes it possible to discover the rate-determining stage in enzymic reactions. An example of such use of KIE is provided by a study[21] in which kinetic

differences in the oxidation of the protium and tritium forms of xanthine catalysed by xanthine oxidase were investigated.



A marked dependence of the KIE on the xanthine concentration in the incubation mixture was observed. If the kinetic data are presented graphically using the method of the double reciprocal quantities 1/v and 1/[S], then linear relations are observed for all the cases investigated, as can be seen from Fig. 3, which is consistent with the Lineweaver–Burk equation:

$$\frac{1}{v} = \frac{K}{V[S]} + \frac{1}{V} \quad .$$

Here it is significant that all the experimental straight lines have different slopes but intersect the ordinate axis at virtually the same point at a given pH. It follows from the equation that the slopes of the straight lines are equal to the ratios K/V; the intercept on the abscissa axis is $-1/K$, while that on the ordinate axis yields $1/K$.

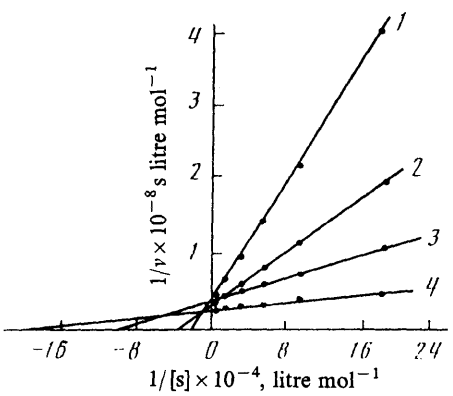


Figure 3. Graphical representation of the rates of enzymic oxidation of labelled (lines *1* and *2*) and unlabelled (lines *3* and *4*) xanthine at different pH; *1*, 10.2; *2*, 8.5; *3*, 10; *4*, 8.5.

The following conclusions can be drawn from the results: the maximum rates of oxidation of the labelled and unlabelled xanthine at the same pH are virtually identical, i.e. at high substrate concentration there is no KIE or it is insignificant; the kinetic isotope effect is manifested mainly in the value of the Michaelis constant.

The following binary mechanism has been established for the enzymic oxidation of xanthine:[79]

$$E + K \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EX \xrightarrow{k_2} E_{red} + U,$$

$$E_{red} + O_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E_{red}\cdot O_2 \xrightarrow{k_4} E + H_2O_2,$$

where E is the enzyme, X is xanthine, and U is uric acid. It is also known that the rate of oxidation of the reduced enzyme E_{red} is significantly greater than the rate of its formation and the rate of the process as a whole is therefore determined by the first two stages. At fairly high xanthine concentrations, [X] ≫ [E], the entire enzyme is bound in the enzyme–substrate complex EX and the rate of reaction has a maximum:

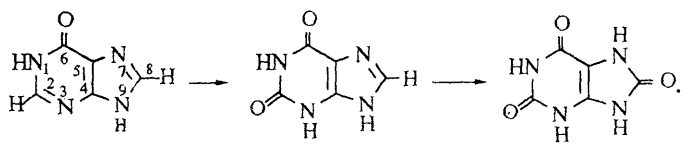
$$V = k_2[EX] = k_2[E] \quad .$$

Since the enzyme concentration was the same in the measurement of the KIE in all the experiments, the similarity of the values of V published for the protium and tritium substrates indicates a small difference between the rate constants for the decomposition of the complexes EX containing [^{1}H]- or [^{3}H]-xanthine.

On the other hand, the Michaelis constant is defined as $K = (k_1 + k_2)/k_1$. Bearing in mind that the constants k_2 have been shown to be equal, the observed difference between the Michaelis constants for the labelled and unlabelled xanthine may be explained by the difference between the rate constants for the formation and decomposition of the enzyme–substrate complexes. Thus the KIE in the reaction investigated is determined by the different rates of formation of the transition complexes of the labelled and unlabelled xanthine.

A similar discrimination between the labelled molecules was observed in the formation of enzyme–substrate complexes in the oxidation of xanthine containing deuterium in the 8-position.[79]

The rate-determining stage in the formation of the complex EX was also established in a study of the KIE in the two-stage oxidation of [2-^{3}H]-, [8-^{3}H]- and [2,8-^{3}H$_2$]-hypoxanthine catalysed by the same enzyme, i.e.



hypoxanthine xanthine uric acid

But in this case the stage involving the decomposition of the complex is partly rate-determining.[80] Isotope effects in the stage where the reaction is inhibited by the substrate were also observed under these conditions.

An effective use of KIE in order to establish the rate-limiting stages in the decarboxylation of *L*-malate catalysed by *L*-malate $NADH^+$-oxidoreductase has been reported by Schimerlik, et al.[81]

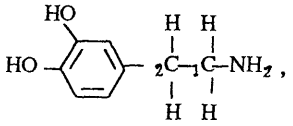
3. The structure of the transition state

The isotope effects in chemical reactions are used widely in the study of the structure of the transition state.[3] On the other hand, such use of the KIE for enzymic reaction is difficult because the chemical bond dissociation stage is usually not fully rate-determining, while other reactions such as, for example, the formation of the products or conformational changes, which precede or follow the chemical bond dissociation stage, are usually partly rate-determining. Therefore, in order to obtain information about the structure of the transition state in enzymic reactions, it is necessary to measure the true KIE, i.e. the isotope effect in the chemical bond dissociation stage. As mentioned above, this KIE can be calculated by Northrop's method. Conclusions concerning the structural characteristics of the transition state derived from the KIE

are based on theoretical considerations put forward by Melander,[3] which have been described by Cleland in relation to enzymic reactions.[11]

Unfortunately, whereas the theoretical aspect of the problem has been developed in fair detail, there have been few practical applications of KIE in the analysis of the structure of the transition state. For example, the KIE found in kinetic studies on the biosynthesis of S-adenosylmethionine from [5′-^{14}C]- and [5′-^{3}H]-ATP (catalysed by the enzyme S-adenosylmethionine synthetase) permitted the conclusion that the transition state has an S_N2-like structure in which the binding to C(5′) is symmetrical in relation to the leaving phosphate group and the arriving methionine sulphur atom.[18]

In the oxidation reactions of dopamines, chirally tritium-labelled in the 1-position {[(1*R*)-^{3}H]- and [(1*S*)-^{3}H]-dopamines}, i.e.



catalysed by ox plasma aminoxidase, strong stereochemical isotope effects were observed: 5–6 for [(1*R*)-^{3}H]- and 26–34 for [(1*S*)-^{3}H]-dopamines, which permitted a conclusion concerning the stereochemistry of the binding of dopamine to the enzyme.[68]

4. Enrichment of labelled substrates in heavy hydrogen isotopes

In those cases where the initial substrate consists of two different varieties of molecules — deuterium- or tritium-labelled molecules and the usual unlabelled molecules — the reactions of the two forms are qualitatively the same, but, by virtue of the manifestation of the KIE, are quantitatively different. The ratio of the two varieties of molecules therefore gradually changes during the reaction in favour of those labelled with heavy hydrogen isotopes.

If the initial ratio of the concentrations of the labelled and unlabelled substrate molecules $[S]_0^*/[S]_0$ is designated by R_0, while the corresponding concentration ratio after the attainment of a degree of enzymic reaction F is designated by $R = [S]^*/[S]$, the enrichment in the heavy isotope of the substrate remaining unreacted in the incubation system can be determined from the equation (Ref. 3, p. 105)

$$\frac{R}{R_0} = (1-F)^{(k^*/k)-1},$$

where k^*/k is the KIE.

When tritium-labelled substrates are used, the ratio R/R_0 in the above equation can be replaced by the ratio of the molar radioactivity of the enriched product A to the initial molar radioactivity of the substrate A_0.

This enzymic method of enriching labelled preparations in the heavy isotope is of great practical importance because the use of compounds with the maximum content of the label is of greatest interest. Furthermore, the KIE can be used to regenerate the labelled compounds after the experiments in which they were diluted by the unlabelled ("cold") compounds.

KIE have been used[20,27] in the enzymic oxidation of labelled *D*-galactose in order to increase significantly the tritium content in preparations comprising different isotopic forms of galactose. The results of these experiments are presented in Table 1.

When KIE are used to enrich substrates in heavy hydrogen isotopes, the choice of incubation conditions and primarily of the substrate concentration is important. For example, when the enzymic oxidation of xanthine is carried out at a high substrate concentration, the molar radioactivity of [8-^{3}H]xanthine does not increase appreciably since under these conditions there are virtually no KIE in terms of V.[21] On the other hand, at low xanthine concentrations, where there is an appreciable KIE in terms of V/K, it was possible to increase the molar radioactivity of the [8-^{3}H]xanthine preparations.

Table 1. Results of the enzyme-catalysed increase in the molar radioactivity of tritium-labelled *D*-galactose preparations.

Initial mixture of isotopic forms	A_0, Ci mmol^{-1}	t, min	A, Ci mmol^{-1}	A/A_0
[6-^{3}H$_1$]Gal, Gal	0.15	60	0.32	2.2
[6-^{3}H$_1$]Gal, Gal	4.6	90	20.3	4.4
[6-^{3}H$_1$]Gal, Gal	4.6	90	18.2	4.0
[6-^{3}H$_1$]Gal, Gal	4.6	240	21.3	4.6
[6-^{3}H$_1$]Gal, Gal	14.6	60	24.5	1.7
[6-^{3}H$_2$]Gal, Gal	4.2	30	6.5	1.5
[6-^{3}H$_2$]Gal, Gal	4.2	240	26.2	6.2
[6-^{3}H$_2$]Gal, [6-^{3}H$_1$]Gal	48.0	240	53.0	1.1

Note. Gal = *D*-galactose; A_0 = initial Moner activity; A = final Moner activity; t = incubation time; A/A_0 = degree of enrichment.

Examples of the analogous use of KIE in certain other enzymic reactions can be found in other studies.[31,80,82]

The observed biological fractionation of isotopes is also based on KIE.[83,84]

5. Kinetic isotope effects in pharmacology and medicine

Bearing in mind the familiar claim that enzymes are the basis of life and that the basis of biological processes is enzymic reactions, the KIE in such reactions can be regarded as the basis of the biological isotope effects, i.e. of the definite difference in the behaviour of labelled and unlabelled compounds in living organisms.

Such differences can be manifested, for example, by a change in the biotransformation of labelled products. Thus deuteriation of ethyl radicals in the anaesthetics methoxyflurane, haloctan, and enflurane leads to an appreciable slowing down of their metabolism.[85] Since the products of the biotransformation of these preparations determine to a large extent their toxicity, the weakening of the metabolism of the deuteriated compounds results in a decrease of toxicity.

The difference in the metabolic behaviour of deuteriated compounds, defined by the KIE in enzymic processes, has also been noted for certain other medicinal preparations.[86–89]

It has not been ruled out that, when deuteriated medicinal preparations are used, the KIE can in certain cases produce a greater therapeutic effect or prolong the activity of the preparations as a result of their greater concentration in certain organs.

Radioisotope diagnosis and positron emission tomography are promising fields where KIE can be applied. In such cases, it is essential to use preparations with a double isotope label: one (deuterium) label is used to achieve the KIE and the other radionuclide) label is used to investigate the distribution and location of the radiopharmaceutical preparation in the organism. In a number of instances, on the basis of the deuterium KIE it has been possible to alter the increase in the concentration in and the rate of elimination of a preparation from the test organ, achieving thereby a definite diagnostic effect. For example, such an effect has been observed by Wenzel[90] in a study of the kinetics of the process in which *N*-alkylamphetamine labelled with iodine-131 in the *para*-position was concentrated in rat and mouse brains. This radiopharmaceutical preparation is capable of being concentrated in the brain and is therefore used for various studies on this organ. It has been established that, when deuterium is introduced into the side chain of N-alkyl-*p*-[^{131}I]iodoamphetamine, the degree of concentration of this preparation in the brain increases appreciably.

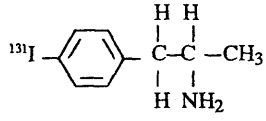


Figure 4 illustrates the kinetics of the alteration of the radioactivity in the brain of the protium form and of one of the deuteriated forms of *p*-[^{131}I]iodoamphetamine investigated by Wenzel. The figure shows that the deuteriated preparation generates a much higher concentration in the brain. This result is of practical importance, because the introduction of deuterium makes it possible to diminish the overall activity of the iodine-131 introduced into the organisms or to achieve a better diagnostic picture for the same dose. It is significant that the concentration of iodine-131 in the blood can be appreciably reduced under these conditions. The author put forward the hypothesis that the observed effect is attained as a result of the decrease in the rate of the enzymic oxidative deamination of the preparation.

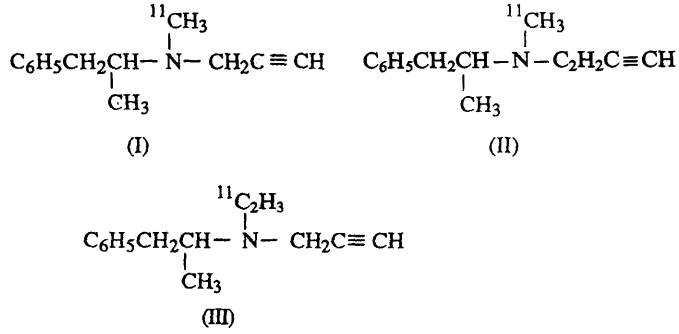


Figure 4. Kinetics of the variation of radioactivity in the brain on introduction of the protium (line *1*) and deuterium (line *2*) forms of *p*-[^{131}I]iodoamphetamine.

Analogous studies of the ruthenocene derivatives of amphetamine labelled with ^{103}Ru established that the deuteriated derivatives are also concentrated in the brain to a greater extent than the corresponding protium forms (almost to the extent of 180%).[91,92]

A decrease in the rate of metabolism in the brain has also been observed for the deuteriated preparation of *N,N*-dimethylphenylethylamine labelled with carbon-11, which is a positron emitter.[93]

In a report delivered at the VIIth International Symposium on Radiopharmaceutical Chemistry in the Netherlands in 1988, it was demonstrated that KIE can be used successfully in the solution of an important psychopharmacological problem involving the search for different kinds of monoaminoxidase inhibitors.[94] Such inhibitors include *L*-deprenyl; *L*-deprenyl containing carbon-11 is used as a radioactive tracer for the charting of aminoxidase in human and animal brains. It was postulated that *L*-[^{11}C]deprenyl is transported into the brain, where it is oxidised by monoaminoxidase. In the study of the mechanism of this inhibition process, kinetic investigations using the following three isotopic forms of *L*-deprenyl were carried out.



(I) (II) (III)

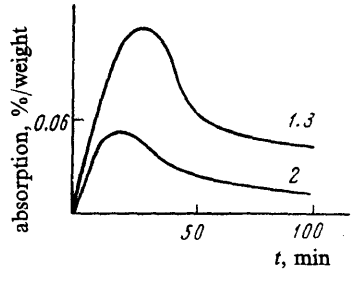


Figure 5. Kinetics of the absorption in baboon brains of the protium isotopic form of *L*-[^{11}C]deprenyl (curve *1*), the form deuteriated in the propargyl group (curve *2*), and the form deuteriated in the methyl group (curve *3*).[94]

The data for the absorption and retention of these compounds in the brains of baboons, presented in Fig. 5, were obtained by positron emission tomography.[94] An appreciably smaller absorption of ^{11}C in the brain was observed for compound (II) containing deuterium in the α-position in the propargyl group. In this case, the KIE was estimated as 3.8. The results show that monoaminoxidase

catalyses the dissociation of the C–H bond in the α-position in the propargyl group and that this is the rate-determining stage in the retention of ^{11}C in the brain.

Incubation of rat liver mitochondrial membranes in the presence of unlabelled and deuteriated serotonin showed that the activity of monoaminoxidase increases in the presence of β,β′-[$^2H_2$]serotonin, but decreases after the introduction of α,α′,β,β′-[$^2H_4$]serotonin.[85] A similar phenomenon has also been observed for dopamine. The suppression of the activity of monoaminoxidase is achieved to a greater extent in the presence of [^{2}H]phenylethylhydrazine compared with the protium form of this compound. As a consequence of this, the administration of deuteriated phenylethylhdyrazine to rats significantly increases the content of dopamine, serotonin, and *m*- and *p*-tyrosine in the brain.[95].

With the aid of fully deuteriated glucose containing simultaneously carbon-14, a significant difference was established in the distribution and metabolism of this isotopic form compared with the preparation having the usual isotopic composition.[96] This difference consists primarily in the retarded transport of deuteriated glucose into the brain, believed to be caused[96] by the isotope effects in the reaction involving glucose oxidase. The introduction of tritium instead of deuterium into glucose also alters the metabolism of this compound.[97,98] A carbon-14 KIE has been reported in the oxidation reaction of labelled *D*-glucose catalysed by glucose oxidase.[99]

There is no doubt that the examples of the use of KIE examined here do not exhaust the entire potential possibilities and that in the future kinetic differences in enzymic reactions involving labelled compounds will find new applications.

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