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USE OF KINETIC ISOTOPE EFFECTS IN ENZYMATIC OXIDATION OF D-[6-^{3}H]GALACTOSE FOR INCREASING ITS MOLAR ACTIVITY

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SUMMARY

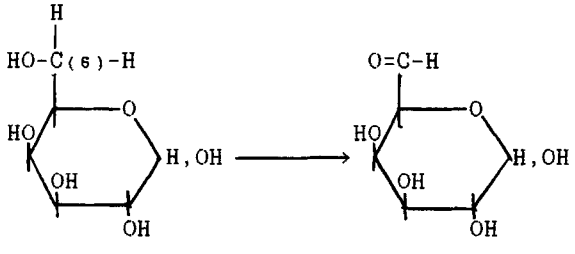
Large kinetic isotope effects have been observed in D-[6-^{3}H]galactose enzymatic oxidation reactions catalyzed by the galactoseoxidase. It is possible to use these effects for considerable increase of the molar activity of D-galactose labelled with tritium in the position 6.

Key Words: D-[6-^{3}H$_1$]galactose, kinetic isotope effect, enzymatic oxidation

INTRODUCTION

The use of kinetic isotope effect (KIE) is one of the worth-while ways for increasing the molar activity of labelled compounds. In particular it is possible to use the differences in enzymatic reaction rates for substrates with isotopically different composition. However, the literature mainly deals with only theoretical aspects of such KIE application (1-3) and there are only few examples of practical effect employment for fractionation and concentration of isotopes and labelled compounds (4).

This paper describes the results of our investigation of KIE in D-galactose oxidation with galactoseoxidase (EC 1.1.3.9)



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and application of this effect for increasing the molar activity of D-galactose labelled with tritium in the 6th position.

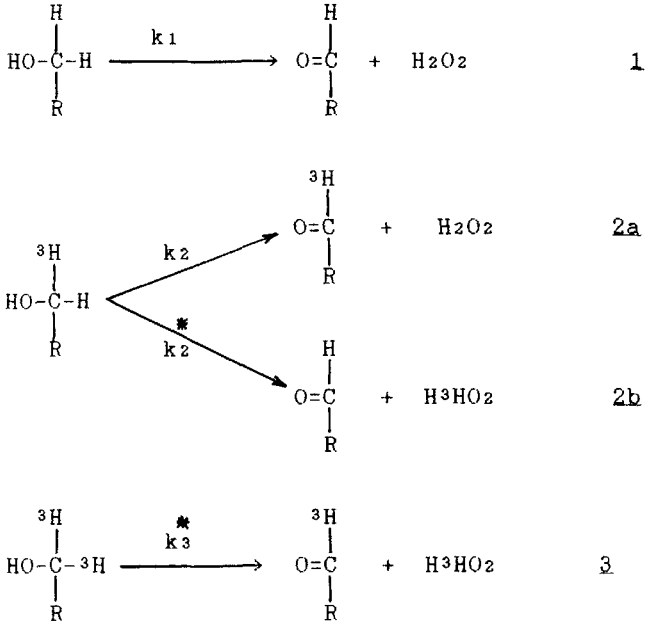
RESULTS AND DISCUSSION

The isotope effects were determined by the oxidation rates of the three isotopically different forms of D-galactose:

a) D-galactose with ordinary isotope composition (D-Gal);

b) D-galactose with single tritium label in position 6 D-[6-$^3H_1$]Gal and c) D-galactose with two tritium labels in position 6 (D-[6-$^3H_2$]Gal).

For the convenience of further interpretation the enzymatic oxidation reactions investigated are presented on the following schemes:



where R - pyranose part of carbohydrate; k_1, k_2, k_2^* and k_3^* - rate constants of corresponding reactions and the asterisk marks the transformation of the C-^3H bond.

Measurement of separate reaction rate constants makes it possible to calculate the following isotope effects:

k_1/k_2^* and k_2/k_3^* - primary isotope effects caused by different isotope composition of oxidizing bond while the neighbouring C-H bond has the same isotope composition; k_1/k_2 and k_2^*/k_3^* - secondary isotope effects caused by different isotope composition of the neighbouring C-H bond while the oxidizing bonds have the same isotope composition; k_1/k_3^* - primary isotope effect complicated by secondary isotope effect; this effect caused by different isotope composition of both the oxidizing and the neighbouring C-H bonds; k_2/k_2^* - intramolecular isotope effect (or intramolecular competition factor) caused by different isotope composition of both C-H bonds in the same molecule. The latter value may be regarded as the primary isotope effect k_1/k_2^* reduced by the secondary isotope effect.

It is necessary to note that for tritium labelled compounds there are difficulties in direct KIE measurement using rate constants because of inadequate accessibility of "pure" isotope form substrates. This is the reason that almost all the authors of reviews and studies devoted to KIE (for example (1,2)) say that for tritium labelled compounds (but not for deuterium labelled ones) the isotope effects can be determined by the competitive reaction method only. But the last years achievements in the field of tritium labelled compounds synthesis allow to solve the problem of isotope effect determination in noncompetitive conditions.

For the case under consideration this problem has been solved by us with specially synthesized samples of D-[6-^{3}H$_1$]galactose with the molar activity 28-29 Ci/mmol and D-[6-^{3}H$_2$]galactose with the molar activity 55-58 Ci/mmol. The oxidation rate of D-galactose with ordinary isotope composition (reaction 1) was measured using the D-[1-^{3}H]galactose as the radioactive tracer. The presence of label in the 1st position of this galactose isotope form shouldn't cause a change in reaction rate because the tritium atom in this case is far enough from the oxidizing

C6-H bond. As it is known from literature for such enzymatic reaction the bonds to the 1st position do not arise during formation of a substrate-enzyme complex (only bonds to the 6th and the 4th positions arise) (5).

The rate constants for reactions 1, 2a and 3 were determined by the accumulation rate of the labelled oxidation product (D-[^{3}H]galactohexodialdoze). This rate was controlled by the growing of the corresponding peak on the thin layer radiochromatogram. The rate constants of the 2b reaction were determined by the accumulation rate of the second reaction's product - labelled hydrogen peroxide. In the presence of catalase (EC 1.11.1.6) wich had been added to the incubation mixture for shifting the reaction to the right, labelled hydrogen peroxide turned into water and its radioactivity was measured after lyophilization. The initial parts of the kinetic curves were used for calculation of the rate constants.

The substrate concentration in the enzymatic reactions is usually much higher than the enzyme concentration (the enzyme is saturated by the substrate) and therefore zero order is observed in relation to the substrate. But for labelled substrates the concentrations are very low and the reaction rate depends on the substrate concentration. That is why the oxidation processes for different isotope forms of D-galactose were carried out with the same substrate concentration ($2.5*10^{-3}$ mol/l). The enzyme concentration also was constant (60 - 70 a.u./ml). Incubation was carried out in presence of catalase at 30 °C.

In kinetic studies we have found the following rate constants values for enzymatic oxidation of different isotope forms of D-galactose: $k_1=3.7*10^{-4}$ s^{-1}, $k_2=4.9*10^{-5}$ s^{-1}, $k_2^*=1.3*10^{-5}$ s^{-1} and $k_3^*=2.6*10^{-6}$ s^{-1}. The KIE values calculated on the base of these data are: primary isotope effects $k_1/k_2^*=28.5$ and $k_2/k_3^*=20$; secondary isotope effects $k_1/k_2=7.5$ and $k_2^*/k_3^*=5.2$; intramolecular

isotope effect k_2/k_2^*=3.8; primary isotope effect complicated by secondary isotope effect k_1/k_3^*=148.

The obtained data show that the C-H bond oxidation rate with the same isotope composition of the neighbouring bond is essentialy higher than that of the C-^{3}H bond. The primary isotope effect value, corresponding to the rate differencies, is 28 and 20 in dependence on the isotope composition of the neighbouring bond.

The secondary isotope effects k_1/k_2 and k_2^*/k_3^* as it had been expected, turned out to be close to each other.

Large value of the k_1/k_3^* isotope effect is caused by simultaneous action of the primary (k_1/k_2^*) and secondary (k_2^*/k_3^*) isotope effects and is equal to the product of these last values.

The intramolecular isotope effect k_2/k_2^* is equal to the ratio of the primary isotope effect k_1/k_2^* to the secondary isotope effect k_1/k_2.

The kinetic data obtained were taken as the principle of the enzymatic method for increasing the molar activity of D-galactose with single or double tritium label in the position 6. When the samples of isotopically different forms of labelled galactose are treated by galactoseoxidase the unlabelled molecules are oxidized more rapidly as compared with the D-[6-^{3}H$_1$]Gal molecules.
The result is an isotope enrichment. Let us assume that the initial sample consists of once and double labelled molecules and has molar activity A_0. It is easy to show that after some incubation period in presence of galactoseoxidase when the extent of transformation of the unlabelled molecules is F, the molar activity of D-[6-^{3}H$_1$]Gal will be described by the equation

$$A=A_0(1-F)^{(k_2/k_1)-1} \qquad 4$$

where k_1 and k_2 are the rate constants of the reactions 1 and 2. The figure (solid curve) presents a dependence of the enrichment extent A/A_0 on the F value, calculated on the basis of the obtained primary KIE value k_1/k_2=28.

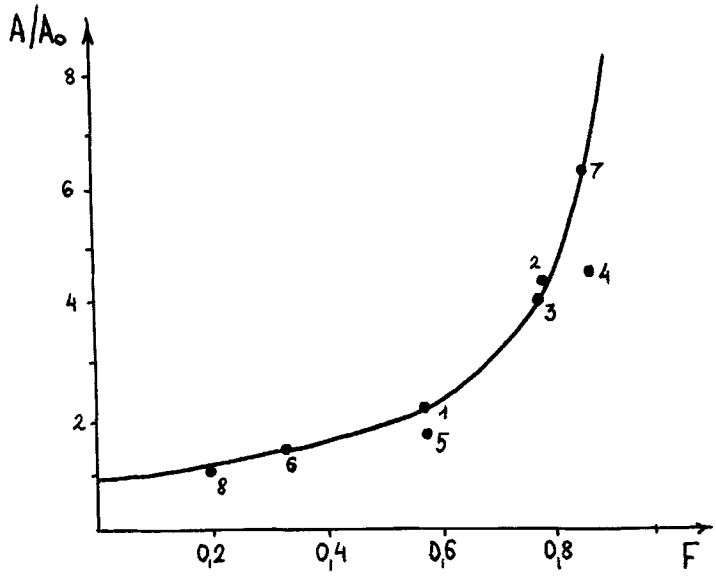


Fig. Calculated (solid curve) and experimental (points) dependences of the enrichment extent A/A0 on the transformation extent. Figures correspond to the run numbers in the table.

The presented dependence shows, for example, that the molar activity of D-[6-$^3H_1$]Gal samples should increase almost tenfold when the enzymatic oxidation extent is equal to 0.9. Using the obtained values of k_1 it is possible to calculate the time period after which such transformation extent will be reached. This period will be about 40 minutes for the case of D-[6-$^3H_1$]Gal and D-Gal separation and about 30 hours for the case of D-[6-$^3H_2$]Gal and D-[6-$^3H_1$]Gal separation. But it is necessary to take into account that the k values were obtained using the initial parts of the kinetic curves where the processes of enzyme deactivation are negligible. Under real conditions essentially longer time period is necessary for reaching such an extent of the labelled compounds enrichment. Experimental determination of transformation extents is a complicated problem therefore in the presented experiments on the D-galactose molar activity increasing the F value wasn't measured. It was evaluated on the basis of the experimental kinetic curves obtained for reactions 1 and 2 in the course of rate constants measurement for unlabelled and once

tritium labelled D-galactose.

TABLE
Results of the enzymatic increasing of the molar activity of tritium labelled D-galactose samples

Run No.	Initial mixture of isotope forms	Initial molar activity Ao, Ci/mmol	Incubation time period, min	Oxidation extent, F	Final molar activity A, Ci/mmol	Enrichment extent A/Ao
1	[6-$^3H_1$]Gal, + Gal	0.15	60	0.57	0.32	2.2
2	[6-$^3H_1$]Gal, + Gal	4.6	90	0.75	20.3	4.4
3	[6-$^3H_1$]Gal, + Gal	4.6	90	0.75	18.2	4.0
4	[6-$^3H_1$]Gal, + Gal	4.6	240	0.85	21.3	4.6
5	[6-$^3H_1$]Gal, + Gal	14.4	60	0.57	24.5	1.7
6	[6-$^3H_2$]Gal, + Gal	4.2	30	0.32	6.5	1.5
7	[6-$^3H_2$]Gal, + Gal	4.2	240	0.85	26.2	6.2
8	[6-$^3H_2$]Gal, +[6-$^3H_1$]Gal	48.0	240	0.20	53.0	1.1

The table presents the conditions and results of enzymatic enrichment of the few D-galactose samples with tritium label in the position 6 and with different molar activity. It is clear that the use of KIE in oxidation reactions with galactoseoxidase makes it possible to increase the molar activity of D-[6-^{3}H$_1$]Gal samples considerably. The extent of enrichment A/Ao achieved in most cases submits to equation 4 and this is also clear from Fig., where the points reflect the experimental data. There is a good agreement of the calculated curve and experimental points.

The deviation from the theoretical dependence A/Ao=f(F) was observed for runs 4 and 5. It can be explained by the assumption during derivation of equation 4 that the labelled form's

content in the initial sample is sufficiently lower than that of the unlabelled form. In such a case and at the F aproaching to unit, the enrichment factor A/Ao tends to infinity. But in practice such conditions don't always exist and it is necessary to consider that the maximum A/Ao value is limited by the top value of the molar activity - 29 Ci/mmol for D-[6-$^{3}H_1$]Gal and 58 Ci/mmol for D-[6-$^{3}H_2$]Gal.

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