American Journal of Biochemistry and Biotechnology 1 (2): 115-120, 2005
ISSN 1553-3668

A New Method to Estimate Intrinsic Parameters in the Ping-pong Bisustrate Kinetic: Application to the Oxipolymerization of Phenol

José L. Gómez, Antonio Bódalo, Elisa Gómez, Asunción M. Hidalgo and María Gómez
Department of Chemical Engineering, University of Murcia, Campus de Espinardo, 30071 Murcia, Spain

Abstract: A new method for determining the intrinsic parameters of reaction in processes involving a high initial rate has been developed. The usefulness of this alternative, which consists of determining several sets of apparent parameters at different times and then extrapolating these to time zero, is demonstrated proved by the linear dependence obtained between the apparent parameters and the reaction time. The method permitted the values of the intrinsic parameters (enzyme specific activity and Michaelis-Menten constants of both substrates) to be obtained for the system under study and was checked with experimental reaction rate data for the soybean peroxidase/phenol/hydrogen peroxide system.

Key words: Bisubstrate kinetic, intrinsic parameters, soybean peroxidase enzyme, phenol, hydrogen peroxide, wastewater treatment

INTRODUCTION

Usually, the intrinsic parameters of enzymatic systems with soluble enzymes are determined from experimental data of the reaction rate in the first instants of the process. Under these conditions, the rate varies lineally with time and the initial value can be determined by extrapolating the values of the reaction rate to zero time. This is possible with most of the systems, regardless of their mechanism.

However, the reaction rates of some systems show no such lineal variation in the first instants of the reaction, which it impossible to apply this procedure. Among such systems is the oxipolymerization reaction of the phenol with hydrogen peroxide in the presence of soybean peroxidase. This process is very important, since it constitutes an alternative for the elimination of phenol from wastewaters.

Phenolic compounds are present in widely varying concentrations in the wastewaters of oil refineries and numerous other industries, including the plastics, resins, textiles, iron, steel and forestry industries[1-5]. So that they can be eliminated from the medium, these compounds must previously undergo some treatment that will reduce their concentrations to the limits established by environmental legislation. Until now no such treatment has shown itself to be better than any other, although the most studied has been peroxidase-catalysed oxidation by hydrogen peroxide.

In the bibliography numerous studies exist which propose the kinetic mechanisms for peroxidase/phenolic compounds systems. Most have been developed by Nicell *et al.*[6-10] and consist of complex equations which include enzymatic inhibition and de-activation terms.

Other authors[11-14] have described the behavior of the enzymatic reaction that takes place during the elimination of phenolic compounds, such as phenol, 4-chlorophenol and 2-chlorophenol, in processes in which horseradish peroxidase and hydrogen peroxide intervene, and some authors[15-17] have proposed a bisubstrate kinetic that follows a ping-pong mechanism.

This study forms part of a project "Biotreatment of contaminating phenolic effluents" being carried out by the University of Murcia, in which two phenolic substrates (phenol and 4-chorophenol) and two enzymes (soybean and horseradish peroxidase), both in soluble and immobilized form, have been studied[18-20].

The previous studies carried out with soybean peroxidase (enzyme) and phenol (substrate) enabled us to establish the optimal conditions of pH, temperature and the influence of the reagent concentrations used in the reactor on the elimination of phenol, as a previous step to the industrial application of the process. The object of the present work was to study the kinetic of the SBP/phenol system in a discontinuous reactor, decreasing the buffer load and without adding polyethylene glycol, in an attempt to confirm whether or not the above system follows a bisubstrate ping-pong kinetic.

In such enzymatic systems, the reaction rate in the first minutes is very high and its variation is not linear with the time, which makes it impossible to obtain reliable data for the values of the kinetic constants. Here, we develop an alternative method which permits us to obtain the intrinsic parameters from three sets of parameters calculated from the mean rates at 5, 10 and 15 min.

Corresponding Author: Antonio Bódalo, Department of Chemical Engineering, University of Murcia, Campus de Espinardo, 30071 Murcia, Spain, Tel: +34968-367354, Fax: +34968-364148

THEORY: Because the proposed new method will be checked with the soybean peroxidase / phenol / hydrogen peroxide system, the theoretical background will be developed in terms of this system. In agreement with the bibliography[15-17], a bi-substrate ping-pong mechanism is assumed for the reaction of phenol with hydrogen peroxide in the presence of soybean peroxidase. The initial rate of such a system would be given by:

$$r_0 = \frac{V_{max} \cdot [Ph]_0 \cdot [H_2O_2]_0}{K_M^{H_2O_2} \cdot [Ph]_0 + K_M^{Ph} \cdot [H_2O_2]_0 + [Ph]_0 \cdot [H_2O_2]_0} \quad (1)$$

Where, r_0 is the initial rate of the reaction, V_{max} is the maximum rate, $[Ph]_0$ and $[H_2O_2]_0$ are the initial concentrations of phenol and hydrogen peroxide, respectively, and K_M^{Ph} and $K_M^{H_2O_2}$ are the Michaelis constants for phenol and hydrogen peroxide, respectively.

Kinetic parameters determinations: usual method: For a kinetic of this type, the bibliography recommends a study based on an analysis of particular situations, as described follows.

Determination of V_{max}: When the initial concentrations of both substrates are equal, Eq. (1) reduces to Eq. (2):

$$r_0 = \frac{[Ph]_0}{\left(\frac{K_M^{H_2O_2}}{V_{max}} + \frac{K_M^{Ph}}{V_{max}}\right) + \frac{1}{V_{max}} \cdot [Ph]_0} \quad (2)$$

This equation can be linearised, as in Eq. (3):

$$\frac{1}{r_0} = \frac{1}{V_{max}} + \left(\frac{K_M^{H_2O_2}}{V_{max}} + \frac{K_M^{Ph}}{V_{max}}\right) \cdot \frac{1}{[Ph]_0} \quad (3)$$

and the value of V_{max} is obtained from the inverse of the intercept. Also, we can obtain, from de slope, the value of the sum $\left(\frac{K_M^{H_2O_2}}{V_{max}} + \frac{K_M^{Ph}}{V_{max}}\right)$, but not the individual values of both Michaelis constants.

Determination of $K_M^{H_2O_2}$: When the experiments are carried out with the same initial concentration of phenol but varying the concentration of hydrogen peroxide, the usual way of linearising Eq. (1) is that indicated in Eq. (4), which provides families of parallel straight lines with ordinates on the origin as a linear function of the inverse of the phenol concentration.

$$\frac{1}{r_0} = \left(\frac{1}{V_{max}} + \frac{K_M^{Ph}}{V_{max}} \cdot \frac{1}{[Ph]_0}\right) + \frac{K_M^{H_2O_2}}{V_{max}} \frac{1}{[H_2O_2]_0} \quad (4)$$

From the slope of the Eq. (4) and taken into account the obtained value for V_{max}, $K_M^{H_2O_2}$ can be obtained.

Determination of K_M^{Ph}: Similarly to the above, for a series of experiments involving constant initial concentrations of hydrogen peroxide and different concentrations of phenol, Eq. (1) leads to Eq. (5) and parallel straight lines whose ordinate on the origin varies linearly with the inverse of the hydrogen peroxide concentration:

$$\frac{1}{r_0} = \left(\frac{1}{V_{max}} + \frac{K_M^{H_2O_2}}{V_{max}} \frac{1}{[H_2O_2]_0}\right) + \frac{K_M^{Ph}}{V_{max}} \cdot \frac{1}{[Ph]_0} \quad (5)$$

from this we can obtain K_M^{Ph} in the same way that $K_M^{H_2O_2}$ was obtained above.

Kinetic parameter determinations: proposed method: Differentiating both parts of the Eq. (4) with respect to the initial variable concentration of hydrogen peroxide gives

$$\frac{d}{d[H_2O_2]_0}\left(\frac{1}{r_0}\right) = -\frac{K_M^{H_2O_2}}{V_{max}} \frac{1}{[H_2O_2]_0^2}$$

This equation reveals the limitations of this type of representation for the system under study. Thus, for low concentrations of hydrogen peroxide, large variations in the inverse of the initial rate are obtained. However, in these conditions, the hydrogen peroxide becomes the limiting reagent and, given the high initial rate of this system, it is consumed within the first few moments, hindering the correct measurement of the initial rate. For the same reason, the results are no better when the hydrogen peroxide concentration is much higher than that of phenol; furthermore, in this situation, the inverse values of the initial rate give rise to a cloud of very close points in the region of the small values given by the inverse of the hydrogen peroxide concentration.

For these reasons, we propose another form of linear representation, (Eq. 6), which has proved to be less sensitive to errors in measuring the initial rate:

$$\frac{[Ph]_0 \cdot [H_2O_2]_0}{r_0} = \frac{K_M^{H_2O_2}}{V_{max}} \cdot [Ph]_0 + \left(\frac{K_M^{Ph}}{V_{max}} + \frac{1}{V_{max}} \cdot [Ph]_0\right) \cdot [H_2O_2]_0 \quad (6)$$

As can be seen, when the equation is differentiated,

$$\frac{d}{d\left[H_2O_2\right]_0}\left(\frac{\left[Ph\right]_0\cdot\left[H_2O_2\right]_0}{r_0}\right)=\frac{K_M^{Ph}}{V_{max}}+\frac{1}{V_{max}}\cdot\left[Ph\right]_0$$

which indicates that the successive variations in hydrogen peroxide produce an effect of constant magnitude on the product of the inverse of the initial rate and initial concentrations of both reagents. From the intercept of Eq. (6), the $K_M^{H_2O_2}$ value can be obtained.

For the same reasons as mentioned above, Eq. (7) is proposed to obtain the K_M^{Ph} value from series with variable phenol concentration:

$$\frac{\left[Ph\right]_0\cdot\left[H_2O_2\right]_0}{r_0}=\frac{K_M^{Ph}}{V_{max}}\cdot\left[H_2O_2\right]_0+\left(\frac{K_M^{H_2O_2}}{V_{max}}+\frac{1}{V_{max}}\cdot\left[H_2O_2\right]_0\right)\cdot\left[Ph\right]_0 \quad (7)$$

MATERIALS AND METHODS

Materials (chemical and equipment): Soybean peroxidase enzyme (SBP) (EC 1.11.1.7, 25,000 Units), catalase enzyme (EC 1.11.1.6, 2,860 units mg^{-1} of protein), hydrogen peroxide (35% w/v), phenol (molecular mass 94.1, minimum purity 99%), 4-aminoantipyrene (AAP), potassium ferricyanide reagent and aluminium potassium sulphate (dodecahydrate) were purchased from Sigma-Aldrich Fine Chemicals.

An Eppendorf (MiniSpin) centrifuge was used to separate the precipitates in the samples and a Shimadzu UV-160 spectrophotometer was used for all absorbance measurements.

Experimental system: The experimental system used in the different assays consisted of a sleeved reactor tank of 50 cm^3 maximum capacity. The reaction mixture was continuously stirred using magnetic stirrers and Teflon-coated stir bars. Aliquots were taken at different time intervals and were mixed with catalase (1 cm^3 of sample + 1cm^3 of catalase). Each sample was treated with 0.2 cm^3 of 40 g dm^{-3} of $(AlK(SO_4)_2\ 12H_2O)$ and then centrifuged for 30 min at 10,000 rpm. The supernatant was analyzed to determine residual phenol concentration by the colorimetric method described above.

Experimental planning: The following experimental conditions, established in previous assays[20], were maintained in the reactor: pH 7.0, temperature 30 ºC and a reaction time of 90 min. The enzyme concentrations was also established in previous works as $4\cdot10^{-2}$ $mg\cdot cm^{-3}$ (SBP).

In all the experiments, both the enzyme and the phenol were dissolved in 100 mM phosphate buffer and the hydrogen peroxide was dissolved in distilled water. Finally the concentration in the reactor was 70 mM in phosphate buffer.

Three series of experiments were carried out:

Varying the concentrations of phenol and hydrogen peroxide: These experiments were carried out at 30 ºC, pH 7.0 and using an enzyme concentration of $4\cdot10^{-2}$ $mg\cdot cm^{-3}$ (SBP), for six concentrations of phenol and a hydrogen peroxide (0.5, 0.7, 1.0, 1.5, 2.0 and 3.0 mM). The initial concentration ratio was constant 1:1 for both substrates.

Varying the concentration of hydrogen peroxide: These experiments were carried out in the same conditions of pH, temperature and enzyme concentration of (SBP) as those used in the above assays and a phenol concentration of 2.0 mM. Eight hydrogen peroxide concentrations, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mM, were assessed.

Varying the concentration of phenol: These experiments were carried out in the same conditions of pH, temperature and enzyme concentration of (SBP) as those used in the above assays and a hydrogen peroxide concentration of 2.0 mM. Seven phenol concentrations 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mM were assessed.

Analytical procedure: The phenol concentration was determined using a colorimetric assay as recommended by Standard Methods[21], in which the phenolic compounds within a sample react with 2.08 mM AAP in the presence of 8.34 mM potassium ferricyanide reagent. The reaction product absorbs light at a wavelength of 505 nm with an extinction coefficient of 10.235 $mM^{-1}cm^{-1}$. The assay mixture consisted of 0.3 cm^3 of ferricyanide solution, 0.3 cm^3 of 4-aminoantipyrine solution and 2.4 cm^3 of phenol sample.

RESULTS AND DISCUSSION

To check the validity of the proposed mechanism, the initial rate of the enzymatic reaction was represented as in Eqs. (3), (6) and (7). Because the system studied does not permit reliable extrapolation to time zero, the mean rate in the first five min of the reaction was taken an approximation of the initial rate

$$r_0=-\left(\frac{\Delta\left[Ph\right]}{\Delta t}\right)_5 \text{ mM min}^{-1}$$

With these approximations, the initial rate values for the three series of experiments were obtained. For each individual assay duplicate runs were made and triplicate when necessary. The experimental reaction rate values used for checking the method are the mean values; the estimated maximum error was 3.25 % and the average error was 1.12 %.

By fitting these values to Eq. (3), (6) and (7), Figures 1, 2 and 3, and the following lineal relationship was obtained, with the indicated values of R^2:

Series 1:

$$\frac{1}{r_0} = 2.676 + 9.375 \cdot \frac{1}{[Ph]_0}\ ;\ R^2 = 0.9864.$$

Series 2:

$$\frac{[Ph]_0 \cdot [H_2O_2]_0}{r_0} = 1.696 + 12.910 \cdot [H_2O_2]_0\ ;\ R^2 = 0.9812.$$

Series 3:

$$\frac{[Ph]_0 \cdot [H_2O_2]_0}{r_0} = 19.337 + 2.390 \cdot [Ph]_0\ ;\ R^2 = 0.9420.$$

So, in accordance with that established in Eq. (3) for series 1, we obtain:

$$\left(\frac{1}{V_{max}}\right)_5 = 2.676\ mM^{-1}\ \text{min}$$

and the estimated $(V_{max})_5$ value is 0.374 mM min^{-1}.

From the fitting of series 2 and 3 we obtain:

$$\left(\frac{K_M^{H_2O_2}}{V_{max}}\right)_5 = 0.848\ \text{min}\ ;\ \left(\frac{K_M^{Ph}}{V_{max}}\right)_5 = 9.669\ \text{min}$$

This result and the calculated value for $(V_{max})_5$, give the following values for the Michaelis constants:

$$(K_M^{H_2O_2})_5 = 0.317\ \text{mM}\quad (K_M^{Ph})_5 = 3.616\ \text{mM}$$

These values will be used in the intrinsic parameter determination.

Intrinsic kinetic parameters: The above results confirmed that the process under study follows a bisubstrate ping-pong mechanism. We obtained approximate values for the kinetic constants, calculated from the mean rate of the reaction during the first five min. The definitive values of these constants, known as the intrinsic kinetic parameters, will depend on the availability of rate data at time zero, which is not possible in the present system because the reaction rate is not linear at the outset and cannot, therefore, be extrapolated to time zero.

As an alternative, we chose to determine three sets of parameters calculated from the mean rates at 5, 10 and 15 min, which were then extrapolated to time zero. We used the experimental data from series 1, in which the phenol and hydrogen peroxide concentrations were the same at the outset. The respective representations of the inverse of the rate versus the inverse of the initial concentration, for the mean rates at 5, 10 and 15 min, are depicted in Fig. 4.

As can be seen from Fig. 4, a good linear fit is obtained. This result provides the three pairs of kinetic parameters depicted in Table 1, where the coefficients of the linear correlation of the respective fits are indicated.

Table 1: Values of the kinetic parameters obtained with r_0 (5 min), r_0 (10 min) and r_0 (15 min)

t (min)	$(K_M^{H_2O_2} + K_M^{Ph}) / V_{max}$ (min)	$1/V_{max}$ (mM^{-1}.min)	R^2
5	9.374	2.677	0.9863
10	15.129	3.100	0.9805
15	21.373	3.261	0.9813

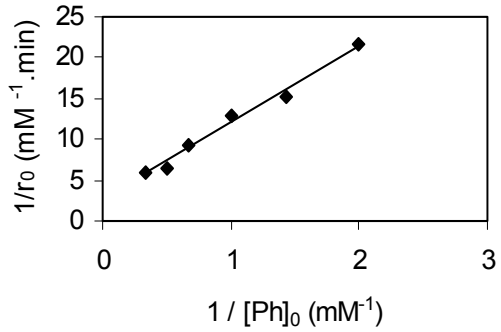

Fig. 1: Fit of r_0 (5 min) to Equation (3) for equal concentrations of hydrogen peroxide and phenol

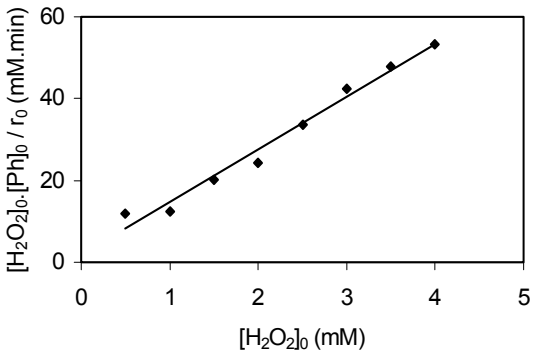

Fig. 2: Fit of r_0 (5 min) to Equation (6) for varying hydrogen peroxide concentrations and a constant phenol concentration of 2 mM

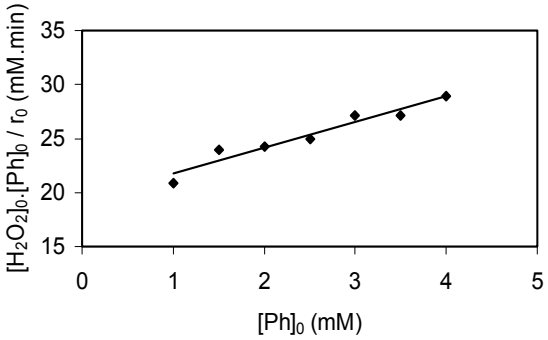

Fig. 3: Fit of r_0 (5 min) to Equation (7) for varying phenol concentrations and a constant hydrogen peroxide concentration of 2 mM

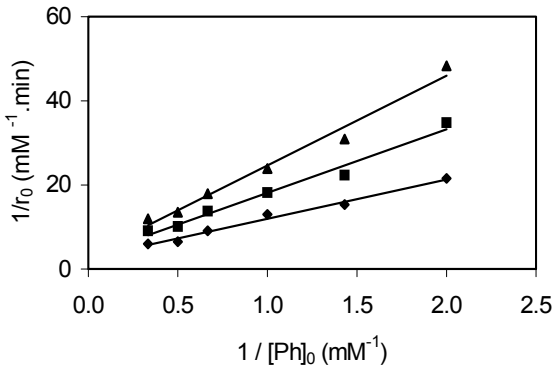

Fig. 4: Fit of r_0 ((♦) 5, (■) 10 and (▲) 15 min) to Equation (3) for equal concentrations of hydrogen peroxide and phenol

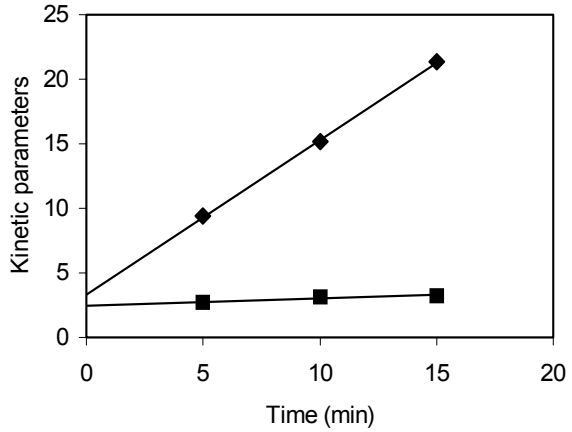

Fig. 5: Determination of the intrinsic kinetic parameters,

$$(\blacklozenge) = \left(\frac{K_M^{H_2O_2}}{V_{max}} + \frac{K_M^{Ph}}{V_{max}} \right) \; ; \; (\blacksquare) = \frac{1}{V_{max}}$$

The representation of these parameters versus time gives Fig. 5. It can be seen that the parameters in question vary linearly with time, so that they can be extrapolated to time zero, providing the intrinsic values. The respective equations are:

$$\frac{1}{V_{max}} = 0.059 \cdot t + 2.248 \text{ and}$$

$$\left(\frac{K_M^{H_2O_2}}{V_{max}} + \frac{K_M^{Ph}}{V_{max}} \right) = 1.200 \cdot t + 3.295$$

with R^2 values of 0.9368 and 0.9994, respectively.

It should be emphasized that the linear dependence is good in both cases, even if the very small variation of $1/V_{max}$ means that the respective regression coefficient is smaller. The values at t= 0 are:

$$\frac{1}{V_{max}} = 2.428 \text{ mM}^{-1} \text{ min} \text{ and}$$

$$\left(\frac{K_M^{H_2O_2}}{V_{max}} + \frac{K_M^{Ph}}{V_{max}} \right) = 3.295 \text{ min}$$

which provides the value V_{max} = 0.412 mM min^{-1} for the intrinsic maximum rate of the enzymatic reaction and $K_M^{H_2O_2} + K_M^{Ph} = 1.357$ mM for the sum of the Michaelis constants. Furthermore, in agreement with the relation $V_{max} = k_E [E]_0$ a value of k_E = 20.59 (mmols of substrate/g of enzyme·min) is obtained for the specific activity of the enzyme.

As regards the intrinsic values of the Michaelis constants, the linearity of the sum of both with respect to time permits that the following equations

$$K_M^{H_2O_2} = \left(K_M^{H_2O_2}\right)_5 \frac{\left(K_M^{H_2O_2} + K_M^{Ph}\right)}{\left(K_M^{H_2O_2} + K_M^{Ph}\right)_5} ;$$

$$K_M^{Ph} = \left(K_M^{Ph}\right)_5 \frac{\left(K_M^{H_2O_2} + K_M^{Ph}\right)}{\left(K_M^{H_2O_2} + K_M^{Ph}\right)_5}$$

can be used to obtain the respective values of the Michaelis-Menten intrinsic constant:

$K_M^{H_2O_2} = 0.109$ mM and $K_M^{Ph} = 1.248$ mM

CONCLUSION

The alternative method proposed in this work can be used to determine the intrinsic reaction parameter values an enzymatic system with a bisubstrate ping-pong kinetic.

Furthermore, the bisubstrate ping-pong mechanism assumed in this work is useful for describing the removal of phenol by soybean peroxidase in the presence of hydrogen peroxide.

ACKNOWLEDGEMENT

This work was supported by a grant, 00505/PI/04, from Fundación Séneca (Comunidad Autónoma de la Región de Murcia), Spain. During the time that this study was being carried out, María Gómez had a FPU scholarship from the Spanish Ministry of Education and Culture.

Nomenclature

$[Ph]_0$ =	initial concentration of phenol; mM
$\Delta[Ph]$ =	increase of phenol concentration; mM
$[E]_0$ =	initial enzyme concentration; g of enzyme dm^{-3}
$[H_2O_2]_0$ =	initial hydrogen peroxide concentration; mM
k_E =	specific activity of the enzyme; mmol of substrate/g of enzyme min
(K_M^{Ph}) =	Michaelis-Menten constant for phenol; mM

$(K_M^{Ph})_5$ = apparent Michaelis-Menten constant for phenol; mM

$\left(K_M^{H_2O_2}\right)$ = Michaelis-Menten constant for hydrogen peroxide; mM

$\left(K_M^{H_2O_2}\right)_5$ = apparent Michaelis-Menten constant for hydrogen peroxide; mM

mM = mmol dm^{-3}

r_0 = initial rate; mM min^{-1}

SBP = Soybean peroxidase

t = time; min

Δt = increase of time; min

V_{max} = maximum rate; mM min^{-1}

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