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DETERMINATION OF OXIDE SYNTHETASE AND HYDRATASE ACTIVITIES BY A NEW HIGHLY SENSITIVE GAS CHROMATOGRAPHIC METHOD USING STYRENE AND STYRENE OXIDE AS SUBSTRATES *

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Summary

A new highly sensitive gas chromatographic method for the separate measurement of microsomal oxide synthetase (aniline, reduced-flavoprotein:oxygen oxidoreductase (4-hydroxylating), EC 1.14.14.1) and oxide hydratase (glycol hydro-lyase (epoxide-forming), EC 4.2.1.63) activities by using styrene or styrene oxide as substrate is presented.

The assay has been developed in order to permit the evaluation of the true kinetic parameters of the two enzymes, respectively, catalysing the activation and desactivation processes of styrene.

In the presence of a microsomal suspension and a NADPH generating system, styrene is converted, under the influence of oxide synthetase, into styrene oxide, a certain amount of which is hydrated into styrene glycol, either spontaneously or enzymatically by the microsomal oxide hydratase; the remaining fraction of the oxide is chemically hydrated in acidic conditions.

The total amount of styrene glycol formed is quantitated by electron-capture gas chromatography after its conversion into pentafluorobenzoyl derivative. For the measurement of oxide hydratase activity, styrene glycol formed after incubation of styrene oxide with the microsomal fraction is estimated as before.

The kinetic data obtained in this study demonstrate that, at low protein concentrations, the affinity of oxide synthetase for styrene is about 20 times as low as that of the oxide hydratase for styrene oxide.

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Abbreviation: PFB, pentafluorobenzoyl.

Introduction

Styrene, (vinylbenzene) is extensively used as a monomer for the manufacture of plastic materials. It contains a vinylic group susceptible to generate a reactive epoxide under the influence of the cytochrome *P*-450-dependent mixed function oxidases. The formation of this intermediate epoxide by the microsomal enzymatic system has been demonstrated [1]. Recently, it has been reported that styrene is mutagenic towards *Salmonella thyphimurium* [2] and able to bind to macromolecules [3] after its activation into styrene oxide by the microsomal enzymes. Styrene oxide is subsequently detoxified into styrene glycol by a microsomal epoxide hydratase [1]. In order to evaluate the relative importance of the respective activation and deactivation processes of styrene, as well as the influence of various pretreatments of the animals on the true kinetic parameters of the two enzymatic activities, we developed a new gas chromatographic method which enables us to separately measure extremely low levels of oxide synthetase (aniline, reduced-flavoprotein:oxygen oxidoreductase (4-hydroxylating), EC 1.14.14.1) and oxide hydratase (glycol hydro-lyase (epoxide-forming), EC 4.2.1.63) by using styrene or styrene oxide as substrates. The classical radiometric method developed by Oesch et al. [4] is very sensitive but restricted to the measurement of styrene oxide hydratase activity. Recently, Belvedere et al. [5] described a gas chromatographic method applicable to the measurements of both activities but its sensitivity was not sufficient for our purposes.

Experimental

Reagents and chemicals. All chemicals were of reagent grade and used without further purification. Styrene, styrene oxide and allylbenzene were purchased from Aldrich-Europe (Beerse, Belgium) and pentafluorobenzoyl (PFB) chloride from Mackeray-Nagel (Düren, West Germany). Glucose 6-phosphate and NADH were obtained from Sigma (St. Louis, U.S.A.), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and $NADP^+$ from Boehringer (Mannheim, West Germany). Other chemicals were obtained from Merck (Darmstadt, West Germany).

Apparatus and conditions. Gas chromatography: A Hewlett-Packard model 5750 G gas chromatograph equipped with a ^{63}Ni electron-capture detector was employed. A spiral borosilicate-glass column (2 m × 4 mm internal diameter) packed with 3% OV1 on Supelcoport (80—100 mesh) was used. The operating conditions were as follows: column temperature, 210°C; injector and detector temperatures, 250°C; carrier gas, argon/methane (95 : 5, v/v) at a flow rate of 50 ml/min.

Mass spectrometry: Mass spectrometric analyses were carried out on a LKB 9000 S instrument.

Microsomes preparation. Liver microsomes were prepared from adult male Wistar rats weighing between 200 and 250 g, according to the method of de Duve as described by Amar-Costesec et al. [6]. Animals were fasted 24 h before killing. Microsomal concentrations were expressed in mg protein/ml, as deter-

mined by the method of Lowry et al. [7].

Synthesis of allylbenzene glycol. This glycol was prepared in order to be used as an internal standard in the gas chromatographic method.

30 ml 90% formic acid and 7 ml 30% H_2O_2 were placed in a 3-necked flask, equipped with a mechanical stirrer, thermometer and dropping funnel. Allylbenzene (6 g) was then added slowly and the temperature was maintained at 40—45°C by cooling in an ice bath. Reaction was continued at 40°C for 1 h and then overnight at room temperature. The excess of reagent was removed by evaporation and 7.5 ml 13.4 M NaOH added in order to hydrolyse the formyl esters of allylbenzene glycol.

The glycol was then extracted by 7 × 20 ml ethyl acetate. The combined extracts were evaporated to 15 ml and, after cooling at 0°C, the glycol was recovered by filtration and distilled under vacuum.

Statistical analysis. Two types of computer-programmable analysis were performed, according to the procedure of Cumps [8]: (a) A descriptive statistical analysis which included: (i) the determination of major errors; (ii) the determination of position parameters (mean value) and dispersion parameters (S.D.) for the reaction velocity at each substrate concentrations; (iii) decision tests concerning the effect of the controlled factors (substrate and protein concentrations) on reaction velocity and the separation of the controlled factors in significantly different groups by a two-way variance analysis.

(b) A deductive statistical analysis according to the Michaelis-Menten model including: (i) determination of position parameters (V, K_m) and their standard deviations by an iterative process of non-linear regression [9] further corrected by using a Taylor series linearization [10]; (ii) decision tests (t tests) to check whether position parameters differ significantly from zero; (iii) comparison tests to detect significant differences between position parameters (K_m) at different protein concentrations.

Methods

Assay of oxide synthetase. A NADPH-generating system (4.25 ml) containing 0.18 mM $NADP^+$, 0.20 mM NADH, 5 mM $MgCl_2$, 8 mM glucose 6-phosphate, 0.025 mM $MnCl_2$ and 0.35 unit/ml of glucose-6-phosphate dehydrogenase in 100 mM Tris · HCl buffer (pH 7.2) was preincubated at 37°C; 0.75 ml of the microsomal suspension (0.8 mg protein in the standard procedure) was then added and the reaction was initiated by the addition of 10 μl of an ethanolic solution of styrene (final concentration 50—2000 μM). Incubations were carried out in oxygenated glass-stoppered flasks placed in a shaking bath at 37°C.

The enzymatic reaction was stopped after 4 min, unless otherwise indicated, by addition of 1 ml 0.3 M H_2SO_4. Subsequent standing at room temperature for 30 min ensured a complete transformation of styrene oxide into the corresponding glycol. Samples were then neutralized with 1 ml 0.6 M NaOH and washed once with 2 ml hexane in order to eliminate impurities which could interfere in the gas chromatographic analysis.

Styrene glycol was then determined as described below. Each determination was performed in triplicate.

Assay of oxide hydratase. 0.1 M phosphate buffer, pH 7.5 (0.2 ml) was maintained for 2 min at 37°C. After addition of 0.05 ml of the microsomal sus-

pension (0.07 mg protein in standard procedure), the reaction was initiated by the addition of 10 µl of a solution of styrene oxide in acetonitrile, in order to obtain final concentrations of 10—1000 µM in substrate.

The reaction was stopped after 10 min (unless otherwise indicated) by adding 2 ml ice-cold 0.1 M phosphate buffer (pH 7.5) followed by immediate extraction of the residual substrate with 2 ml hexane. For each determination, control experiments were conducted with boiled microsomes in order to evaluate the chemical spontaneous hydratation which was subtracted in calculations. Styrene glycol was then evaluated as described below. Each determination was performed in triplicate.

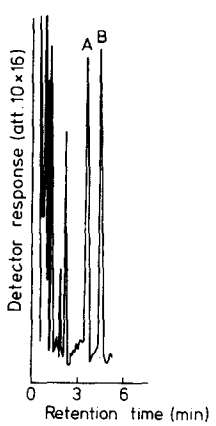
Quantitative determination of styrene glycol. After the addition of an adequate amount of allylbenzene glycol, used as internal standard, the samples were extracted with 2 ml ethyl acetate by shaking for 30 s on a Vortex mixer. The organic phase was then evaporated to dryness under a stream of nitrogen and then under vacuum for 20 min.

The residue was dissolved in 500 µl toluene containing 0.4% pyridine; 1 µl PFB chloride was added to the solution which was then heated at 50°C for 20 min. The excess of reagents was removed by evaporation under nitrogen and afterwards under vacuum for 20 min. The residue was dissolved in 500 µl 90% methanol and extracted with 1 ml hexane. A 1-µl aliquot of the hexane phase was injected into the gas chromatograph.

Results and Discussion

Gas chromatography

Typical gas chromatograms are shown in (Fig. 1). PFB derivatives of styrene glycol and allylbenzene glycol are well separated and no interference from



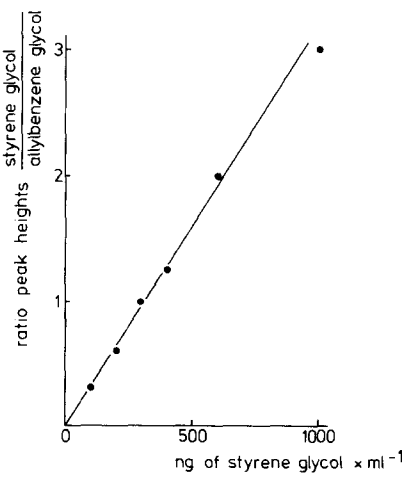


Fig. 1. Typical gas chromatogram of the PFB derivatives of styrene glycol (A) and allylbenzene glycol (B), as extracted from the incubation mixture.

Fig. 2. Calibration curve for the determination of styrene glycol in microsomal suspension.

impurities contained in incubation mixtures was observed. The sensitivity of the method allows us to detect quantities of styrene glycol as low as 10 pg.

The identities of PFB derivatives of both glycols were established by mass spectrometry. The molecular ion has an *m/e* ratio corresponding to the monoester of styrene glycol which has loosed one molecule of water by 1,2-elimination. Similar results were obtained for the allylbenzene derivative.

The concentration of styrene glycol formed in the incubation mixtures was calculated from a standard graph (Fig. 2) constructed from chromatograms of biological samples containing varying amounts of styrene glycol and a fixed amount of internal standard. Ratios of the peak heights of derivatized glycols were plotted vs. concentration.

Effects of pH, incubation time and protein concentration on oxide synthetase and oxide hydratase activities

The pH-dependence curves (not shown) of oxide synthetase and oxide hydratase activities using styrene or styrene oxide as substrate display broad maxima centered on pH 7.2 for the former and 7.7 for the last one. However, a better sensitivity was obtained by measuring oxide hydratase at pH 7.5, due to the slower chemical hydratation rate observed at that pH.

The oxide synthetase activity is linearly dependent on the incubation time for up to 5 min, at a styrene concentration of 50 μM (lowest substrate concentration used in further studies) and for a microsomal protein concentration of 0.21 mg/ml. The oxide hydratase activity is time-linear up to 20 min at a styrene oxide concentration of 20 μM and a protein concentration of 0.15 mg/ml. The longest incubation times used in subsequent experiments were 4 and 15 min, respectively.

In order to verify if enzyme activities are linearly dependent on the concentration of microsomes, oxide synthetase and oxide hydratase activities have been measured at a substrate (styrene and styrene oxide, respectively) concentration of 1 mM. Fig. 3 shows that both enzyme activities are linear with respect to protein concentration up to 0.25 mg/ml.

Michaelis-Menten's kinetic analysis: effect of microsome concentration

A detailed Michaelis-Menten analysis of both oxide synthetase and oxide

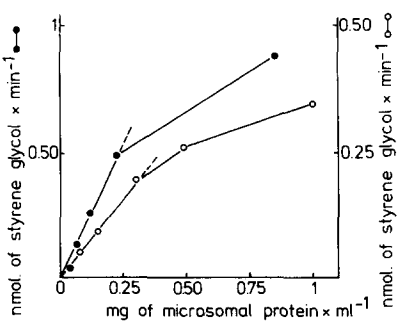
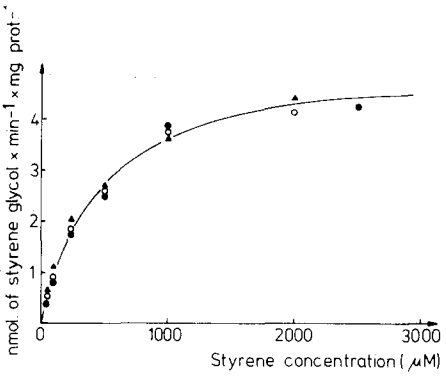


Fig. 3. Effect of microsomal protein concentration on the activities of oxide synthetase (●——●) and oxide hydratase (○——○). The concentrations of styrene and styrene oxide were 1 mM and the incubation times were 4 and 10 min, respectively.



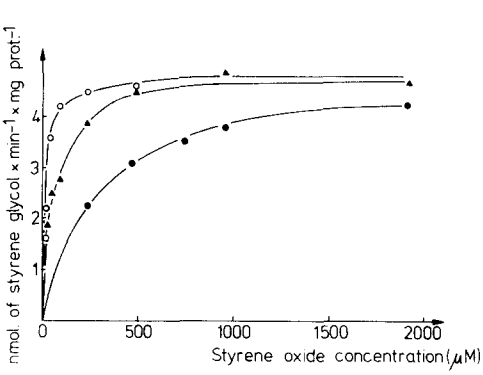


Fig. 4. Effect of microsomes concentration on the styrene concentration dependence of oxide synthetase activity. ▲———▲, 0.06 mg protein/ml; ○———○, 0.15 mg protein/ml; ●———●, 0.65 mg protein/ml.

Fig. 5. Effect of microsomes concentration on the styrene oxide concentration dependence of oxide hydratase activity. ○———○, 0.16 mg protein/ml; ▲———▲, 0.69 mg protein/ml; ●———●, 0.85 mg protein/ml.

hydratase activities of rat liver microsomes by using styrene or styrene oxide as substrate was performed. Since it has been reported previously, that the kinetic parameters of both benzpyrene hydroxylase [11] and benzpyrene oxide hydratase [12] are influenced by the concentration of the microsomes in the incubation system, the Michaelis-Menten kinetic analysis of both oxide synthetase and oxide hydratase was carried out at different concentrations of microsomal proteins ranging from 0.06 up to 1.50 mg/ml for oxide synthetase and from 0.07 up to 1.27 mg/ml for oxide hydratase.

Fig. 4 shows the effect of varying the concentration of microsomes (as expressed as mg of protein/ml) on the substrate concentration dependence velocity of oxide synthetase when styrene is the substrate. The concentration of the microsomes does not significantly affect the kinetic parameters of that enzyme and, on the basis of a statistical analysis of the results obtained at six different protein concentrations (0.06, 0.09, 0.15, 0.65 and 1.50 mg protein/ml), the following kinetic parameters and their standard deviation were estimated: $K_m = 563 \pm 36$ μM and $V = 5.29 \pm 0.15$ nmoles · min^{-1} · mg $protein^{-1}$.

The effect of the concentration of the microsomes on oxide hydratase was inferred from a similar type of analysis. Fig. 5 reports the effect of the concentration of styrene oxide on the initial velocity of the hydratase at three different concentrations of the microsomal protein. The curves have been plotted using the computed K_m and V values for each individual experiment. Increasing the concentration of the microsomes in the incubation mixture influences the kinetic properties of oxide hydratase by significantly increasing its K_m; there is no significant effect on V.

The K_m values obtained can be separated into two groups: in the first one, where the microsomal protein concentration is higher than 0.85 mg/ml, the K_m values are in the 240 μM range, which is in good agreement with the previously reported values in similar experimental conditions [4,5]; in the second group, where the microsomal protein concentration is below 0.7 mg/ml, the K_m values

are much lower than 240 μM and they slightly decrease when the protein concentration is lowered. At the two lowest tested protein concentrations (0.07 and 0.16 mg/ml) the calculated K_m values are not significantly different from each other. The statistical analysis of the pooled values at those two protein concentrations, gives a K_m of 21.6 ± 4.1 μM. This value is of the same order of magnitude as that reported by Lu et al. [12] for the microsomal epoxide hydratase using different substrates such as dibenzo[*a*,*h*]anthracene-5,6 oxide, benzpyrene-7,8 oxide and benzpyrene-9,10 oxide. It must be pointed out however that, using styrene-7,8 oxide as a substrate, those authors did not obtain a linear Lineweaver-Burk plot.

This report is thus the first one to demonstrate that styrene oxide behaves as an usual substrate for liver microsomal epoxide hydratase. The affinity of the enzyme for this particular substrate is of the same order of magnitude as the previously reported values for the other substrates. The apparently higher K_m value previously reported was estimated from the results of experiments realized in non-Michaelis-Menten conditions.

In conclusion, the new highly sensitive gas chromatographic method for the evaluation of oxide synthetase and oxide hydratase, presented here, allows the determination of the apparent K_m for these two enzymes when styrene or styrene oxide are used as the substrate. The K_m of the epoxide hydratase for styrene oxide is dependent upon the concentration of the microsomes in the incubation medium while the K_m for oxide synthetase is not. As suggested by Parry et al. [13] an increase of the apparent K_m of a microsomal enzyme with increased microsomal concentration could be due to a partition of the substrate between the aqueous phase and the membrane lipids. The differences reported here between the behavior of styrene and styrene oxide could be explained by the difference in their binding affinity constants for the catalytic site of the microsomal enzyme which catalyzes their transformation. Indeed, if one assumes that the microenvironment of a membrane-bound enzyme influences the K_m of this enzyme for the substrate only when this K_m is of the same order of magnitude than the affinity constant of the binding of the lipids to the enzyme, the absence of effects on the K_m of the oxide synthetase could be explained by the fact that its K_m for styrene is in the 200 μM range while the

TABLE I

EFFECT OF PROTEIN CONCENTRATION ON THE K_m OF LIVER MICROSOMAL OXIDE HYDRATASE FOR STYRENE OXIDE

The mean value of V was 4.85 ± 0.15 nmol · min^{-1} · mg protein^{-1}.

Protein concentration (mg/ml)	K_m (μM)
1.27	212 ± 25
0.95	241 ± 61
0.85	269 ± 66
0.69	44.1 ± 9.6
0.27	28.9 ± 10
0.16	19.1 ± 2.8
0.07	16.9 ± 3.2

affinity of the cytochrome *P*-450 for the phospholipids is in the 10 μM range [14]. Since there is no reported value for the affinity constant of an eventual binding of lipids to epoxide hydratase, it is difficult to definitively use this argument to explain the effect of the microsomal concentration on the K_m of this enzyme. Since, however, epoxide hydratase is a membrane-bound enzyme which catalyzes the hydration of lipid-soluble substrate for which its affinity is in the 10 μM range, this possibility cannot be excluded.

The sharp inflection of the K_m values shown in Table I is on the other hand even more difficult to explain; however, it is certainly not related to the critical micellar concentration since no exogenous phospholipids were added in vitro to the incubation mixture.

Finally, our results demonstrate that the affinity of oxide hydratase for styrene oxide is about 20 times higher than the affinity of oxide synthetase for styrene. This fact reinforces the statement expressed by Stoltz and Withey [15] of a rapid detoxification of styrene oxide and could provide a possible explanation for the relatively weak mutagenic potency of styrene.

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