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# IDENTIFICATION AND QUANTITATION OF 1,2-EPOXYBUTENE-3 AS THE PRIMARY METABOLITE OF 1,3-BUTADIENE

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## SUMMARY

1,3-Butadiene was incubated in the presence of rat liver microsomes supplemented with an NADPH-generating system. One of the major metabolites of butadiene was found to be 1,2-epoxybutene-3, which was analysed by electron-capture gas–liquid chromatography after its derivatization with pentafluorophenylhydrazine. The effects of variation of several incubation parameters and of different pretreatments of the animals on its formation kinetics were evaluated.

## INTRODUCTION

Butadiene is widely used as a polymer component in the manufacture of synthetic rubber and copolymeric plastics such as ABS (acrylonitrile–butadiene–styrene)[1].

A previous report from our laboratory[2] has demonstrated that butadiene and 1,2-epoxybutene-3 are direct mutagens towards *Salmonella typhimurium* strains TA 1530 and TA 1535. Moreover, 1,2,3,4-diepoxybutane has been shown to be a mutagenic compound in several test systems[3–6]. In addition, both of those likely oxidative metabolites of butadiene (1,2-epoxybutene-3 and 1,2,3,4-diepoxybutane) possess carcinogenic properties[7–10]. Because numerous workers are regularly exposed to butadiene, especially by inhalation in industrial atmospheres, it was of obvious interest to study its possible *in vitro* biotransformation into one or several of those carcinogenic epoxides under the influence of the microsomal cytochrome-P450 linked mixed function oxidases.

## EXPERIMENTAL AND RESULTS

### *Reagents and chemicals*

1,2-Epoxybutene-3 (purity 97%) and pentafluorophenylhydrazine were purchased from Aldrich Europe (Beerse, Belgium). Butadiene (purity 99.5%) was obtained from Matheson Gas Products (Oevel, Belgium). *n*-Hexane was obtained from Merck (Darmstadt, G.F.R.) and stored over molecular sieves (5 Å). All sta-

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tionary phases for gas–liquid chromatography were purchased from Applied Science Labs. (State College, Pa., U.S.A.).

*Instrumentation and operating conditions*

The formation of 1,2-epoxybutene-3 from butadiene was assessed by gas–liquid chromatography using different column phases and mass fragmentography.

Gas–liquid chromatographic determinations were performed on different instruments. A Hewlett-Packard Model 5750G was equipped with a $^{63}$Ni electron-capture detector and a borosilicate-glass column, 2 m × 4 mm I.D., and packed with 3% OV-1 on Supelcoport (80–100 mesh). Samples were injected into the gas chromatograph with an injector temperature of 250°, an oven temperature of 140°, a detector temperature of 250° and an argon–methane (95:5) carrier gas flow-rate of 60 ml min$^{-1}$. A Perkin-Elmer 3920B gas chromatograph equipped with a similar electron-capture detector was used under the same instrumental conditions except for the column phases and temperatures, which were 175° (3% XE-60) 165° (3% OV-17) and 170° (3% OV-25), respectively. A capillary gas–liquid chromatograph (Pye 104) equipped with an electron-capture detector ($^{63}$Ni), a glass solid injector and a wall-coated open-tubular (WCOT) glass capillary column (21 m × 0.5 mm I.D.) (SE-30, 0.3 $\mu$m) or (10 m × 0.5 mm I.D.) (OV-225, 0.3 $\mu$m) was used with an oven temperature of 164°, an inlet pressure (0.3 bar) of helium carrier gas and an argon–methane (95:5) detector make-up gas flow-rate of 50 ml min$^{-1}$.

Mass spectrometric analysis was carried out with an LKB 9000S instrument.

All of the derivatives were injected into the gas chromatograph with a flash heater temperature of 250°, a helium carrier gas flow-rate of 30 ml min$^{-1}$ and an oven temperature of 140°. The column was a standard LKB coiled glass gas chromatographic column (2.2 m × 0.5 cm O.D.) packed with 1% OV-1 on 60–80-mesh Chromosorb W. The retention time of the pentafluorophenylhydrazone of 1,2-epoxybutene-3 was 40 sec.

All mass spectra were recorded at electron energy 70 eV with accelerating voltage 3500 V, trap current 60 $\mu$A and ion source at 270°.

A multiple ion detection accessory (MID) was used to detect the formation of 1,2-epoxybutene-3 from butadiene in the microsomal suspension.

*Microsome preparation*

Male Wistar rats (200–250 g) were given free access to commercial food pellets and fasted for 24 h before decapitation. The microsomes were prepared according to the procedure described by De Duve[11]. Protein concentrations were determined by the method of Lowry *et al.*[12].

The NADPH-generating system contained the following constituents in a final volume of 4 ml: 0.05 *M* Tris–hydrochloric acid (pH 7.8), 0.5 m*M* $NADP^+$, 8 m*M* glucose-6-phosphate, 0.2 m*M* manganese chloride and glucose-6-phosphate dehydrogenase (3U).

*Incubation system*

The incubation system is shown in Fig. 1. After passage through a molecular sieve 5Å filter, butadiene was incubated in glass-stoppered tubes. The gas pressure was monitored with a mercury manometer. The microsomal suspension was injected

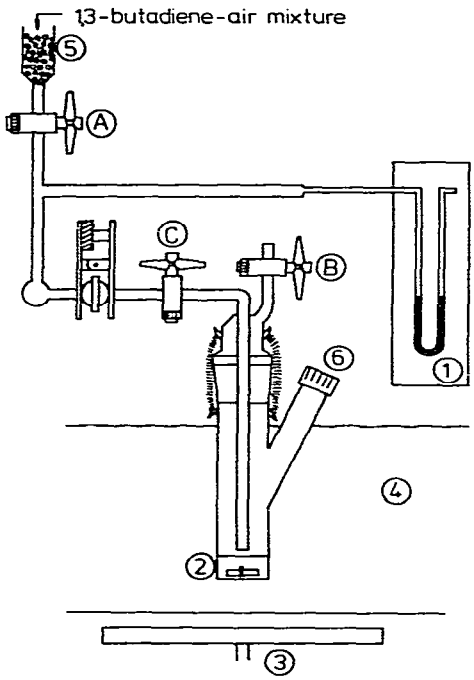


Fig. 1. Apparatus for incubation of 1,3-butadiene. 1 = Mercury manometer; 2 = magnet for stirring incubation mixture; 3 = magnetic stirrer; 4 = water-bath at 37°; 5 = molecular sieve; 6 = septum.

through a septum and stirred magnetically in the incubation vial. The incubation system was designed to permit six incubation mixtures to be exposed to a definite pressure of air–1,3-butadiene mixture in a reproducible manner, each incubation vial being removable. The temperature of the water bath (37°) was regulated with a contact thermometer.

*Method*

The NADPH-generating system was pre-incubated at 37° for 15 min. An air–1,3-butadiene mixture (35:65) was injected into the tube, via stopcocks A and C. Atmospheric air was driven out of the tube via stopcock B, which was then closed; when the gas pressure in the system rose to 10 cmHg, stopcocks A and C were closed. The reaction was initiated by the addition of 0.3 ml of the microsomal suspension, corresponding to 0.3 mg of protein per millilitre of incubation mixture. Incubation was carried out at 37°.

Twelve minutes later, the reaction was stopped by the addition of 0.3 ml of 60% perchloric acid to the incubation mixture. A fixed amount (100 $\mu$l of a $9 \cdot 10^{-5}$ *M* aqueous solution of chloral hydrate (used as the internal standard) was added. The mixture was vortexed and the tubes were centrifuged at 2000 *g* for 10 min; 1.2 ml of a 0.09 *M* solution of pentafluorophenylhydrazine in 2 *N* hydrochloric acid was added to 2 ml of the supernatant and the mixture was left for 30 min at 32–33°. The derivatives of both butadiene monoxide and the internal standard were extracted by shaking for 1.5 min with 1.5 ml of *n*-hexane and aliquots (1–2 $\mu$l) were injected on to the gas chromatograph.

Typical gas chromatograms are shown in Fig. 2.

The concentrations of 1,2-epoxybutene-3 formed after 1,3-butadiene incubation with male Wistar rat liver microsomal preparations were calculated from a calibration graph obtained by introduction of known amounts of 1,2-epoxybutene-3 and a fixed amount of the internal standard (370 ng $ml^{-1}$) in the same biological medium.

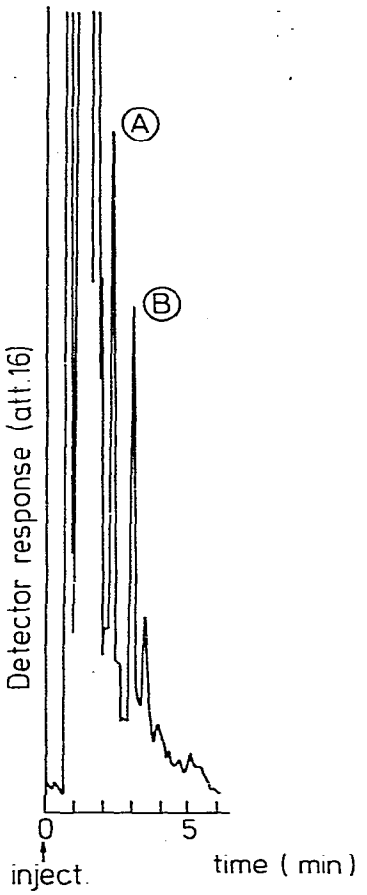


Fig. 2. Typical gas chromatogram of the derivatives of components in the 1,3-butadiene epoxidase assay, as extracted from the incubation mixture. Separation of the pentafluorophenylhydrazine derivatives of (A) chloral and (B) 1,2-epoxybutene-3. Conditions: 3% OV-1 on Supelcoport (80–100 mesh); column temperature, 140°.

*Identification of 1,2-epoxybutene-3 as a butadiene metabolite*

When 1,2-epoxybutene-3 reacts with pentafluorophenylhydrazine under the conditions defined above, a pentafluorophenylhydrazone derivative is formed; its mass spectrum (Fig. 3) shows a molecular ion of *m/e* 250 and a series of relevant fragmentation ions of *m/e* 235 ($M-CH_3^{\bullet}$), 223 ($M-CH_2-CH^{\bullet}$), 195 ($C_6F_5-N^+\equiv N$), 183 ($C_6F_5-NH^{+\bullet}$), 182 ($C_6F_5-NH^+$) and 155 ($C_6F_5^+$), as expected for this kind of compound.

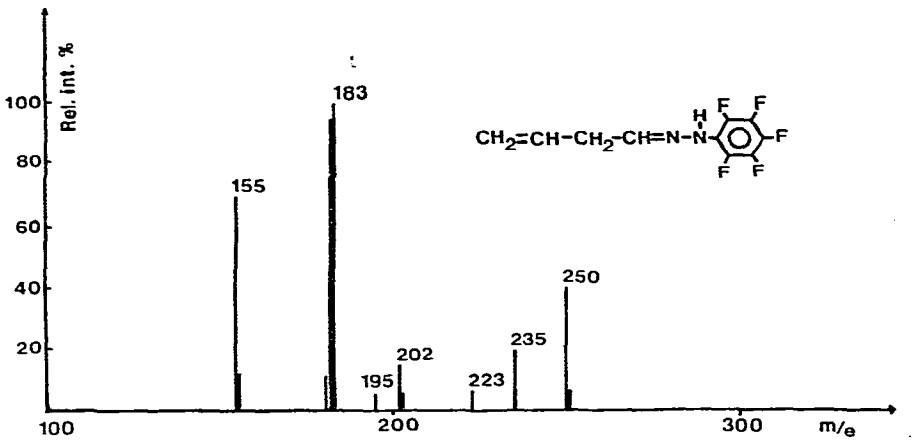


Fig. 3. Mass spectrum of the pentafluorophenylhydrazone derivative of 1,2-epoxybutene-3.

The fragmentogram where two masses (*m/e* 250 and 235) are focused in order to detect extremely small amounts of the pentafluorophenylhydrazine derivative of 1,2-epoxybutene-3 formed from butadiene incubated with the liver microsomal suspension is shown in Fig. 4.

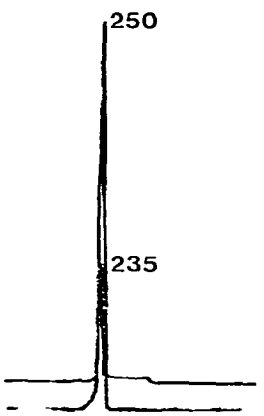


Fig. 4. Fragmentogram of the pentafluorophenylhydrazone derivative of 1,2-epoxybutene-3.

When injected on to gas chromatographs equipped with columns containing several different phases, as indicated under *Instrumentation and operating conditions*, the retention time of the pentafluorophenylhydrazine derivative of the butadiene metabolite is, in every instance, identical with that of the pentafluorophenylhydrazone derivative of authentic 1,2-epoxybutene-3. When butadiene is incubated with boiled microsomes, no 1,2-epoxybutene-3 is formed, as evidenced by the absence of the corresponding peak in the respective gas chromatograms and of the ions of *m/e* 250 and 235 in the mass fragmentogram.

*Kinetic characteristics of the enzymatic reaction*

In order to characterize the enzymatic reaction and to establish optimal conditions for the assay of rat liver microsomal "butadiene epoxidase" activity, the effects of pH, incubation time and microsomal protein concentration on the activity were examined.

Under the conditions employed, the optimal pH was 7.85 (Fig. 5) and the time course was linear up to 12-min incubation. Fig. 6 shows that the reaction rate was linear up to 0.35 mg of microsomal protein per millilitre of incubation mixture.

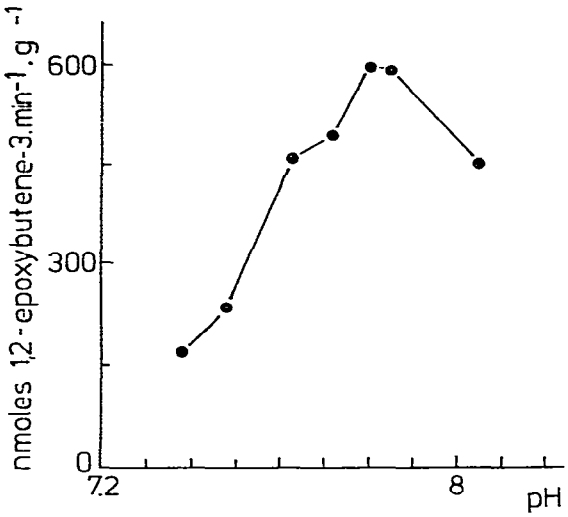


Fig. 5. pH–activity curve of butadiene epoxidase in rat liver microsomes.

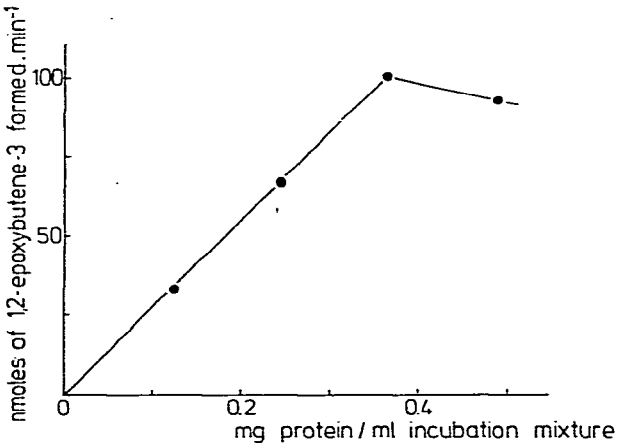


Fig. 6. Effect of microsomal protein concentration on the activity of butadiene epoxidase in liver microsomes from rat pre-treated with phenobarbital.

The pre-treatment of rats with phenobarbital (80 mg $kg^{-1}$ saline solution i.p. 48 and 24 h before killing increased the butadiene epoxidase activity approximately 2.5-fold. However, when the animals were pre-treated with 3-methylcholanthrene (40 mg $kg^{-1}$ corn oil solution i.p. for 2 days), the enzymatic activity remained unchanged (Table I).

TABLE I

INFLUENCE OF DIFFERENT PRE-TREATMENTS ON THE BUTADIENE EPOXIDASE ACTIVITY

| *Pre-treatment* | *Activity* [*nmole min*$^{-1}$ (*mg protein*)$^{-1}$] |
|---|---|
| Control | 21.65 ± 2.42 ($n = 5$) |
| Phenobarbital | 43.00 ± 4.03 ($n = 4$) |
| 3-Methylcholanthrene | 19.23 ± 2.78 ($n = 3$) |

Furthermore, SKF 525A, which is an inhibitor of the mixed function oxidase enzymatic system, added *in vitro* to the incubation mixture at a concentration of 90 $\mu M$ inhibited the butadiene epoxidase activity by 50%.

## DISCUSSION

The results demonstrate that 1,3-butadiene, which has been demonstrated to be mutagenic towards several strains and therefore a potential carcinogen, is converted into 1,2-epoxybutene-3 under the influence of the cytochrome P-450 linked mixed function oxidases of the rat liver.

Preliminary results which are not presented in this paper indicate that 1,2-epoxybutene-3 is converted into the even more carcinogenic diepoxide 1,2,3,4-diepoxybutane when incubated under similar conditions.

These two oxidative metabolites possess carcinogenic properties so that a careful examination of the possible carcinogenic effects of 1,3-butadiene, which is widely used in industry, is urgently needed.

## ACKNOWLEDGEMENT

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