Hepatic and Extrahepatic Metabolism of "C-Styrene Oxide

by Adrian J. Ryan,* Margaret O. James,* Zvi Ben-Zvi,* Francis C. P. Law,* and John R. Bend*

With 8-"C-styrene oxide as substrate, specific glutathione S-transferase and epoxide hydrase activities were determined in subcellular fractions of liver, lungs, kidney, and intestinal mucosa from rabbit, rat, and guinea pig. Liver had the highest enzyme activities in each species. Rat and guinea pig had higher glutathione S-transferase activity in both liver and kidney than rabbit. Rat testis also had appreciable glutathione S-transferase activity.

The perinatal development of epoxide hydrase and glutathione S-transferase was followed in liver and several extrahepatic tissues of fetal and neonatal guinea pigs and rabbits. The rates at which enzyme activities reached adult levels in the extrahepatic tissues differed from the liver in both species. Epoxide hydrase and glutathione S-transferases developed at different rates in each organ, demonstrating that the relative importance of these two detoxifying pathways for styrene oxide may shift before and after birth.

The effects of pretreating male and female rats with phenobarbital (PB), 1,2,3,4-dibenzanthracene (DBA), pregnenolone-16α-carbonitrile (PCN), or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on hepatic and extrahepatic epoxide hydrase and glutathione S-transferase activities toward styrene oxide were determined. PB increased both enzyme activities in liver of both sexes. PCN induced only glutathione S-transferase activity in female liver. Extrahepatic epoxide hydrase and glutathione S-transferase activities were unaffected except that TCDD doubled female renal epoxide hydrase activity and PB increased intestinal epoxide hydrase activity in both sexes.

Styrene oxide biotransformation was studied in isolated, perfused rat liver and rabbit lung preparations. Conjugation with glutathione was a major metabolic pathway although significant amounts of diol were also formed in each instance. In rat liver, 27-40% of the administered styrene oxide was excreted via the bile mainly as S-(1-phenyl-2-hydroxyethyl)glutathione.

Introduction

The cytochrome P-450-dependent mixed-function oxidase system found in the microsomal fraction of hepatic and, to a lesser extent, other tissues, is able to metabolize a wide variety of endogenous and exogenous substrates. Of particular importance is its ability to oxidize olefins and aromatic hydrocarbons to epoxides and arene oxides, respectively (1, 2). These metabolites are chemically reactive and, in fact, are covalently bound to cellular macromolecules (3, 4). The binding of arene oxides derived from polycyclic hydrocarbons to DNA appears to be a critical biochemical event in carcinogenesis and mutagenesis (5-7).

Styrene oxide, which is thought to be the initial oxidative metabolite of styrene, is more toxic than styrene in the rat (8); it has been shown to be carcinogenic in mouse skin (9) and is also mutagenic (10). It is, therefore, a useful model compound for study of the detoxifying systems which remove the highly electrophilic epoxides from body tissues.

Epoxides are metabolized principally by two enzymatic systems. Microsomal epoxide hyd-
gates which are the precursors of urinary mercapturic acids (13). Styrene oxide is a substrate for both enzymes and is converted by epoxide hydrase to styrene glycol and by glutathione S-transferase to S-(1-phenyl-2-hydroxyethyl) glutathione (Fig. 1).

This paper summarizes some of our studies on the \textit{in vitro} metabolism of styrene oxide by hepatic and extrahepatic subcellular fractions from various rodent species and in isolated perfused rabbit lung and rat liver preparations.

**Tissue Distribution of Epoxide-Metabolizing Enzymes**

\textsuperscript{14}C-Styrene oxide was used to determine the tissue distribution of both glutathione S-transferase and epoxide hydrolase in the guinea pig and rabbit (14). Table 1 shows the data for glutathione S-transferase activity. In all tissues the guinea pig showed much higher specific activities than the rabbit. Activity in guinea pig skin was not determined. In both species, liver and kidney extracts showed the highest specific activities.

**Table 1. Hepatic and extrahepatic glutathione S-transferase activities in the guinea pig and the rabbit.**

\begin{tabular}{|l|c|c|}
\hline
Tissue & Glutathione conjugate formed, nmole/min-mg protein \hline
Liver & 30.5 ± 1.6 (15) & 236.5 ± 49.3 (9) \\
Lung & 6.5 ± 0.6 (15) & 18.6 ± 3.8 (9) \\
Kidney & 7.9 ± 0.9 (15) & 73.5 ± 10.6 (9) \\
Intestinal mucosa & 4.4 ± 0.4 (15) & 24.6 ± 2.7 (9) \\
Skin\textsuperscript{c} & 0.7, 1.2 & - \hline
\end{tabular}

\textsuperscript{a} Data from James, Fouts, and Bend (14).
\textsuperscript{b} Mean ± S.D. (N).
\textsuperscript{c} Pohl and Bend, unpublished data.

Table 2 shows the results for the analysis of epoxide hydrolase activity in tissues of both species. Except for kidney, guinea pigs show higher activity in each tissue studied. Again, both species showed highest activity in the liver with the intestinal mucosa next.

**Table 2. Hepatic and extrahepatic epoxide hydrolase activities in the guinea pig and the rabbit.**

\begin{tabular}{|l|c|c|}
\hline
Tissue & Styrene glycol formed, nmole/min-mg microsomal protein \hline
Liver & 5.6 ± 0.3 (15) & 13.7 ± 3.1 (9) \\
Lung & 0.17 ± 0.02 (15) & 0.47 ± 0.12 (9) \\
Kidney & 1.4 ± 0.1 (15) & 1.1 ± 0.3 (9) \\
Intestinal mucosa & 2.8 ± 0.3 (15) & 5.5 ± 1.1 (9) \\
Skin\textsuperscript{c} & 0.05, 0.02 & - \hline
\end{tabular}

\textsuperscript{a} Data from James, Fouts, and Bend (14).
\textsuperscript{b} Mean ± S.D. (N).
\textsuperscript{c} Pohl and Bend, unpublished data.

The low activity for both hydrolase and glutathione S-transferase in rabbit skin is interesting. Styrene oxide has been shown to be carcinogenic to mouse skin (9). If, as seems likely, only small amounts of the detoxifying enzymes are present in this tissue, these would be easily
saturated leaving the tissue with no defense against the toxic action of the epoxide.

Table 3. Hepatic and extrahepatic glutathione S-transferase activity in male and female rats with styrene oxide as substrate.

| Tissue              | GSH conjugate formed, nmole/min-mg protein* |
|---------------------|-------------------------------------------|
|                     | Male                        | Female                      |
| Liver               | 197.9 ± 15.9                | 123.7 ± 5.6b               |
| Lung                | 19.5 ± 3.3                  | 20.0 ± 1.7                 |
| Kidney              | 114.7 ± 19.2                | 105.9 ± 13.8               |
| Intestinal mucosa   | 14.5 ± 1.5                  | 14.9 ± 2.5                 |
| Testis              | 91.2 ± 3.5                  | —                          |

* Mean ± S.D. (N = 3).

b $p < 0.01$, male vs. female (two-sided Students' t test).

Similar studies have been carried out comparing the distribution of these two enzyme systems in the tissues of male and female adult rats. Table 3 summarizes the glutathione S-transferase activities. The results clearly show a sex difference in hepatic activities, with livers from female animals showing about 60% of the activity found in male tissues. Other tissues common to both sexes displayed comparable activities with a relative order similar to rabbit and guinea pig (Table 1).

Table 4. Hepatic and extrahepatic epoxide hydrolase activity in male and female rats with styrene oxide as substrate.

| Tissue              | Styrene glycol formed, nmole/min-mg microsomal protein* |
|---------------------|--------------------------------------------------------|
|                     | Male                        | Female                      |
| Liver               | 5.25 ± 0.72                 | 4.94 ± 0.60                 |
| Lung                | 0.77 ± 0.13                 | 0.37 ± 0.09                 |
| Kidney              | 1.30 ± 0.21                 | 1.16 ± 0.34                 |
| Intestinal mucosa   | 0.35 ± 0.09                 | 0.38 ± 0.02                 |
| Testis              | 2.10 ± 0.31                 | —                           |

* Mean ± S.D. (N = 3).

Epoxide hydrolase does not show any statistically significant sex difference in activity in any tissue except lung. In tissues common to both sexes, highest activity was found in the liver and lowest activity in the intestinal mucosa (Table 4).

Two points of interest arise from the data in Tables 3 and 4. First, both male and female rats have relatively low activities of both epoxide-metabolizing systems in the lung. This limits the ability of the lung to detoxify epoxides, and, consequently, it may be an important factor in the formation of pulmonary tumors after the administration of certain polycyclic hydrocarbons.

The second point concerns the relatively high glutathione S-transferase and epoxide hydrolase activities in testes. The presence of large amounts of epoxide-detoxifying enzymes in reproductive tissue may well be an important defense mechanism against chemical mutagens.

Perinatal Development of Epoxide-Metabolizing Enzymes

The ability of neonatal tissues to detoxify epoxides and other reactive toxicants is a topic of considerable importance. Animals before and shortly after birth are often deficient in drug-metabolizing enzymes (15). The development of the enzyme systems which are responsible for xenobiotic transformation is a major concern in our laboratories since neonates may be especially sensitive to chemical toxicity.

We have examined the development of microsomal epoxide hydrolase and cytosolic glutathione S-transferase (with styrene oxide as substrate) in hepatic and extrahepatic tissues of both the rabbit and guinea pig (15, 16). Age-related alterations in rabbit pulmonary glutathione S-transferase activity toward styrene oxide (labeled epoxide transferase) and 1,2-dichloro-4-nitrobenzene (labeled aryl transferase) are shown in Figure 2. At the earliest time point at which sufficient tissue was available for assay, specific activities were already greater than 50% adult levels. The apparent decrease in specific activity near birth appeared to be due to the synthesis of cytosolic protein which did not have glutathione S-transferase activity since there was no concomitant decrease in whole tissue activity. In contrast, the development of transferase activity occurred substantially more slowly in liver (Fig. 3). In hepatic tissue, activity was very low until after birth, and at 30 days of age liver specific activity was still only about 50% adult levels (with styrene oxide as substrate). These data are representative of those observed in the various organs with both glutathione S-transferase and epoxide hydrolase. Thus, the rates at which adult activities are reached in

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extrahepatic tissues differ from liver for both rabbits and guinea pigs. Moreover, epoxide hydrase and glutathione S-transferase activities develop with age at different rates in any single organ. Thus, the balance in epoxide metabolism by hydration or by conjugation with glutathione shifts in vitro before and after birth and these alterations could be important in epoxide-mediated toxicity (teratogenesis, carcinogenesis) to fetal or neonatal tissues.

With discovery of the relatively high glutathione S-transferase and epoxide hydrase activities in the testes of rats (Tables 3 and 4), we decided to compare the development of these two styrene oxide detoxifying systems in this germinal tissue to that of liver (G. L. Foureman, I. P. Lee, and J. R. Bend, unpublished data). At the earliest time point at which sufficient testis could be obtained for assay (7 days, Table 5), appreciable glutathione S-transferase activity (> 50 nmole/min-mg protein) was observed. The time course of the specific transferase activity showed an apparent decline from 20 to 45 days after birth and then a steady increase up to about 200 days. However, a steady increase in total enzyme activity was observed with age up to about 50 days of age, at which time near-adult levels were reached (Table 5).

Similar studies with microsomal epoxide hydrase (Table 6) demonstrated that testis had low activity until about 30 days postpartum. Thereafter, activity increased rapidly until adult levels were reached by 45–50 days of age. The slower development of epoxide hydrase activity in testis could potentially be important, for example, if an immature animal were exposed to a toxic epoxide that was a good substrate for epoxide hydrase but a poor substrate for the glutathione S-transferases. Such compounds are known. Thus, the carcinogen cholesterol-5a,6a-epoxide is hydrated by microsomal epoxide hydrase (17, 18) but is not a substrate for the glutathione transferases (A. J. Ryan, Z. Ben-Zvi, and J. R. Bend, unpublished observations).

**Figure 2.** Perinatal development of glutathione S-transferase activity toward styrene oxide (labeled epoxide transferase) and 1,2-dichloro-4-nitrobenzene (labeled aryl transferase) in 176,000 g supernatant fraction of rabbit lung homogenates. Data are expressed as mean ± S.D., N = 3 litters (up to 33 days of age; tissues from one litter pooled prior to homogenization) or 3 individuals (80 days of age and older, all males).

**Figure 3.** Perinatal development of glutathione S-transferase activity towards styrene oxide (labeled epoxide transferase) and 1,2-dichloro-4-nitrobenzene (labeled aryl transferase) in 176,000g supernatant fraction of rabbit liver homogenates. Data are mean ± S.D. as described in Fig. 2.
The stimulation of xenobiotic metabolism by pretreatment of animals with a large number of chemical compounds is a well documented phenomenon (19). Both hepatic epoxide hydrase (20) and glutathione S-transferase activity (21) are induced in the rat in response to phenobarbital administration. We have studied the effects of administering phenobarbital (PB), 1,2,3,4-dibenzanthracene (DBA), pregnenolone-16α-carbonitrile (PCN), and 2,3,7,8-tetrachlorodibenzop-dioxin (TCDD), compounds which are known to induce the microsomal mixed-function oxidase system in rats, on hepatic and extrahepatic (lung, kidney, small intestinal mucosa, and testis) epoxide hydrase and glutathione S-transferase activities in male and female rats. Styrene oxide was used as the epoxide substrate. The xenobiotic...

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pretreatments had almost no effect on epoxide-metabolizing enzyme activities in the extrahepatic tissues, except that epoxide hydride activity was increased in small intestine of both male and female rats following phenobarbital treatment and renal epoxide hydride doubled in female rats, but not in male rats, following TCDD administration.

Table 8. Effect of pretreatment of male rats on hepatic 176,000g supernatant glutathione S-transferase activity. 

| Inducer                    | Control     | Treated     |
|----------------------------|-------------|-------------|
| Phenobarbital              | 186.5 ± 24.3| 367.3 ± 4.8 |
| Dibenzanthracene           | 188.0 ± 36.7| 180.1 ± 16.4|
| Pregnenolone-16α-carbonitrile | 182.4 ± 8.7 | 194.0 ± 16.0 |
| TCDD                       | 146.4 ± 16.1| 210.1 ± 20.7 |

* Phenobarbital and dibenzanthracene were given at 80 mg/kg for 3 days; pregnenolone-16α-carbonitrile was given at 20 mg/kg for 2 days; 2,3,6,7-tetrachlorodibenzo-p-dioxin was given once at 10 µg/kg. All compounds were given by IP injection. Enzyme fractions were prepared 24 hr after the last dose, except with TCDD, where males were sacrificed 6 days after TCDD administration and females 10 days after treatment.

* Mean ± S.D. (N = 3).

* p < 0.01, control vs. treated.

The hepatic enzyme activities from female and male rats treated with the various xenobiotics are outlined in Tables 7–11. DBA failed to increase glutathione S-transferase activity, but significant increases in specific activity were observed with PB, PCN, and TCDD in females (Table 7). In male rats (Table 8), PB and TCDD also increased transferase activity toward styrene oxide, but PCN and DBA did not increase enzyme activity. In the case of PCN, this parallels its activity as an inducer of microsomal mixed-function oxidase activity in female, but does not in male, rats (19).

There is some variability in the induction of glutathione transferase in male rat liver by PB. Table 9 shows the extent of induction in three groups of rats treated under identical conditions. In each experiment statistically significant increases in the specific activity of the enzyme were obtained. The relative increases, however, varied markedly between experiments. Currently, we have no explanation for this behavior.

Of the various compounds tested, only PB caused substantial induction of hepatic microsomal epoxide hydride in female rats (Table 10). PCN and TCDD tended to increase specific activity, but the effect was not statistically significant for TCDD. Male rats (Table 11) gave results essentially identical to those obtained with females.

The data obtained allow a few conclusions to be drawn. Thus, PB pretreatment increased both epoxide metabolizing enzymes in both sexes, whereas the polycyclic aromatic hydrocarbon tested (DBA) did not affect either enzyme. It was especially interesting that TCDD

Table 9. Effect of phenobarbital pretreatment on glutathione S-transferase activity of male rat liver 176,000g supernatant fraction.

| Trial | Control     | Treated     | Induction, |
|-------|-------------|-------------|------------|
|       | Specific activity, n mole GSH conjugate formed/min-mg protein |
|       |             |             |            |
| A     | 186.5 ± 24.3(3) | 367.3 ± 4.8(3) | 100        |
| B     | 214.0 ± 35.3(3) | 347.6 ± 10.5(3) | 60         |
| C     | 186.9 ± 16.5(4) | 234.4 ± 20.0(4) | 30         |

* Rats were treated (IP) with 80 mg/kg Na phenobarbital in saline once daily for 3 consecutive days. Animals were sacrificed 24 hr after the last injection.

* Mean ± SD (N).
of speculation, especially since the rate at which these oxirane metabolites are formed from the hydrocarbons is also influenced by these same inducing agents. However, it is a problem that should be clarified, at least with a few substrates.

### Metabolism of Styrene Oxide in Isolated Perfused Organs

The question of the relative importance of epoxide hydrolase and glutathione S-transferases in the metabolism of epoxides is difficult to answer from in vitro studies. The enzymes have different subcellular locations. Epoxide hydrolase is bound to the endoplasmic reticulum and the glutathione S-transferases are soluble components in the cytoplasm. It requires the use of preparations with intact cellular structure to evaluate the two pathways for epoxide or arene oxide metabolism. For these reasons the metabolism of 8-14C-styrene oxide was studied in isolated organs. Two organs have been used, the isolated perfused rat liver and the isolated perfused rabbit lung. The rat liver preparation is particularly advantageous since glutathione conjugates are preferentially excreted in the bile and are automatically separated from other metabolites in the circulating perfusate.

### Table 10. Effect of pretreatment of female rats on hepatic microsomal epoxide hydrolase activity.

| Inducer       | Control    | Treated    |
|---------------|------------|------------|
| Phenobarbital | 4.50 ± 1.1 | 14.0 ± 1.6 |
| Dibenanthracene | 5.08 ± 1.14 | 5.60 ± 2.0 |
| Pregnenolone-16a-carbonitrile | 5.22 ± 1.1 | 7.80 ± 2.2 |
| TCDD         | 4.94 ± 0.6  | 7.60 ± 1.8 |

*a Phenobarbital and dibenanthracene were given at 80 mg/kg for 3 days; pregnenolone-16a-carbonitrile was given at 20 mg/kg for 2 days; 2,3,6,7-tetrachlorodibenzo-p-dioxin was given once at 100µg/kg. All compounds were given by IP injection. Enzyme fractions were prepared 24 hr after the last dose, except with TCDD, where males were sacrificed 6 days after TCDD administration and females 10 days after treatment.

*b Mean ± S.D. (N = 3).

cp < 0.01, control vs. treated.

dcp < 0.05, control vs. treated.

### Table 11. Effect of pretreatment of male rats on hepatic microsomal epoxide hydrolase activity.

| Inducer       | Control    | Treated    |
|---------------|------------|------------|
| Phenobarbital | 5.46 ± 1.04 | 13.94 ± 0.50 |
| Dibenanthracene | 5.68 ± 1.13 | 6.80 ± 0.54 |
| Pregnenolone-16a-carbonitrile | 4.78 ± 0.43 | 5.29 ± 0.25 |
| TCDD         | 6.14 ± 2.00  | 6.85 ± 0.99 |

*a Phenobarbital and dibenanthracene were given at 80 mg/kg for 3 days; pregnenolone-16a-carbonitrile was given at 20 mg/kg for 2 days; 2,3,6,7-tetrachlorodibenzo-p-dioxin was given once at 100µg/kg. All compounds were given by IP injection. Enzyme fractions were prepared 24 hr after the last dose, except with TCDD, where males were sacrificed 6 days after TCDD administration and females 10 days after treatment.

*b Mean ± S.D. (N = 3).

cp < 0.01, control vs. treated.

dp < 0.05, control vs. treated.

induced the transferase in both males and females without appreciably affecting the epoxide hydrolase in males, although there was an apparent increase in females. The role that induction of epoxide biotransformation pathways plays in toxification-detoxification mechanisms for epoxide precursors is obviously a matter
three experiments, between 30 and 40% of the radioactivity was excreted by this pathway in 90 min. The remainder of the radioactivity was found mainly in the perfusate. Examination of the bile by TLC (Fig. 4) showed only one radio-

![Ninhydrin positive spots from bile](image1)

![Synthetic glutathione conjugate](image2)

**FIGURE 4.** Radioactivity scan of a thin-layer chromatogram of bile from rat liver perfused with $^{14}C$-styrene oxide (100 μmole). Developed in $n$-butanol-glacial acetic acid–water (4:1:1).

active component present which was ninhydrin-positive and cochromatographed with synthetic $S$-(1-phenyl-2-hydroxyethyl)glutathione (Fig. 1). Reserve isotope dilution analysis of the bile with the authentic conjugate (Table 13) confirmed that the glutathione derivative was the only metabolite excreted in the bile.

Fractionation of the perfusate radioactivity by means of XAD-2 resin and TLC indicated that this probably consisted of styrene glycol, mandelic acid, and the glutathione conjugate (Fig. 5) (22). Reverse isotope dilution studies (Table 13) confirmed the presence of approximately equal amounts of styrene oxide, mandelic acid, and $S$-(1-phenyl-2-hydroxyethyl) glutathione. Since mandelic acid is an oxidation product of styrene glycol, the data indicate that both hydration and conjugation with glutathione are major metabolic pathways for styrene oxide in the perfused liver at this epoxide concentration, although thioether metabolites may predominate slightly.

Recent reports have demonstrated that styrene oxide is mutagenic (10). Because of this, the covalent binding of styrene oxide to liver protein was assayed. However, no significant binding was detected in the livers used in these experiments.

Similar experiments were carried out in an isolated perfused rabbit lung preparation. Lungs were removed from heparinized New Zealand rabbits weighing 2–3 kg. At a styrene oxide concentration of 80 μmole styrene oxide/lung various times after addition of epoxide to the recirculating perfusate, aliquots were removed and analyzed for metabolite formation. Metabolite formation was linear for at least 45 min for both ethyl acetate-extractable (styrene glycol) and water-soluble metabolites. [Since ethyl acetate extraction was done at pH 7.4, these metabolites included $S$-(1-phenyl-2-hydroxyethyl)glutathione, $S$-(1-phenyl-2-hydroxyethyl)cysteinylylglycine, and mandelic acid. It is possible that N-acetyl-$S$-(1-phenyl-2-hydroxyethyl)cysteine is also present, but this has not been verified.] The water-soluble metabolites predominated in both the perfusate and in the lung (at the end of the experiment). However, both conjugation with glutathione and hydration were major metabolic pathways in lung. Shortly after perfusion was initiated (a few minutes), the glutathione conjugate was the only thioether metabolite present in lung. However, substantial amounts of the cysteinylglycine derivative were present in lung after 60 min. The glutathione conjugate serves as

![Styrene glycol Mandelic Glutathione acid conjugate](image3)

**FIGURE 5.** Radioactivity scan of a TLC plate of an XAD-2 resin extract of the perfusate from a rat liver treated with $^{14}C$-styrene oxide (100 μmole). Plate developed in $n$-butanol-ethanol–concentrated ammonia–water (4:1:1:1). Positions of styrene glycol, mandelic acid, and $S$-(1-phenyl-2-hydroxyethyl) glutathione on the same TLC plate are also shown.
the precursor for the cysteinylglycine derivative (Fig. 1), and the enzyme γ-glutamyltranspeptidase catalyzes the conversion. As in the liver, no evidence for covalent binding of the radioactivity associated with styrene oxide to lung protein was found under these conditions.

It is clear that in the two organs most likely to be concerned with styrene oxide metabolism, the liver and lung, both glutathione S-transferases and epoxide hydrolase play a major role. Studies in these organs with other epoxides and arene oxides can be expected to provide useful insights into the qualitative and quantitative aspects of their metabolism by these two systems and how this alters epoxide toxicity.

Concluding Remarks

Epoxide-mediated target organ toxicity is likely to be dependent on many factors. These include the site and rate of absorption into the body; the relative rates of hepatic and extrahepatic microsomal mixed-function oxidase catalyzed epoxide formation from precursors; the stability of the epoxide in the blood and the rate of blood supply to the target organ; the rate of uptake of the epoxide from the circulation by tissues; and the relative abilities of tissues to detoxify electrophilic epoxides after entry into the cell. The present paper has attempted to summarize the work of this laboratory in this last area, the assessment of the ability of hepatic and extrahepatic tissues to detoxify epoxides.

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