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Biodehalogenation. Epoxidation of Halohydrins, Epoxide Opening, and Transhalogenation by a *Flavobacterium* sp.*

C. E. Castro and E. W. Bartnicki

ABSTRACT: Cell-free extracts of a *Flavobacterium* sp. efficiently convert 2,3-dibromopropanol into glycerol *via* the sequence: dibromopropanol → epibromohydrin → dihydroxybromopropane → glycidol → glycerin. The reactivity of a partially purified halohydrin epoxidase toward a variety of substrates has been assessed. In the presence of chloride ion, epibromohydrin is rapidly converted into epichlorohydrin by the enzyme.

Carbon–halogen bonds are present in a wide array of biocides and anesthetics. Moreover, α-halo acids are widely employed as enzymatic inhibitors (Webb, 1966; Leasure, 1964). Indeed, because of the toxicity of organic halides and their inhibitory capacities it is understandable that the full scope of enzymatic dehalogenation remains to be portrayed. Nevertheless some remarkable transformations have been recorded. Thus, carbon tetrachloride is reduced to chloroform by dogs (Butler, 1961) and converted into carbon dioxide by monkeys (McCollister *et al.*, 1951) and rat liver homogenates (Rubinstein and Kanics, 1964). The insecticide DDT undergoes dehydrohalogenation (Lipke and Kearns, 1960) and reductive dehalogenation (Bunyan *et al.*, 1966; Kallmann and Andrews, 1963) in a variety of organisms. More typically, alkyl halides are metabolized in animals to the corresponding alkylmercapturic acid (Barnsley, 1966). In addition to *N*-acetyl-3-(2-propyl)cysteine, the propyl halides and 1-chloro-2-hydroxypropane are converted into *N*-acetyl-3-(2-hydroxypropyl)cysteine (Barnsley, 1966). This latter conversion of a halohydrin represents the extent of knowledge of the fate of such compounds in biological systems. Chloroethanol undergoes a prior oxidation to the aldehyde in rats before the carbon chlorine bond is attacked (Johnson, 1967). Bacterial hydroxylation of both α-halo (Leasure, 1964; Hirsch and Stellmach-Helvorg, 1961; Davies and Evans, 1962; Goldman, 1965) and β-halo acids (Castro and Bartnicki, 1965) with whole cells has been noted, as has the cell-free hydroxylation of the substi-

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TABLE I: Growth[a] of the *Flavobacterium* sp. and Br^- Release on Several Substrates.

Substrate (4×10^{-3} M)	OD[b] 500 mμ		Br^-[c]
	20 hr	40 hr	40 hr
2,3-Dibromopropanol	0.25	0.75	3.5×10^{-3}
1-Bromo-2,3-dihydroxypropane	0.34	>1.0	2.5×10^{-3}
1,3-Dibromopropanol	0.18	0.18	1.6×10^{-3}
2,3-Dibromopropionaldehyde	0.03	0.03	1.6×10^{-3}
2-Bromo-1,3-dihydroxypropane	0.28	0.47	1.25×10^{-3}
1-Bromo-2-propanol	0.11	0.15	8.0×10^{-4}
3-Bromopropanol	0.20	0.19	2.8×10^{-4}
2-Bromoallyl alcohol	0.07	0.24	8.0×10^{-5}
2,3-Dibromopropionic acid	0.06	0.10	$<5.5 \times 10^{-5}$
Glycerol	0.43	>1.0	
1,2-Dibromo-3-chloropropane	0.04	0.15	$<5.5 \times 10^{-5}$
1,2-Dibromopropane	0.04	0.10	$<5.5 \times 10^{-5}$
0.02% yeast extract	0.22	0.18	

[a] Salts medium plus substrate listed at pH 8.0. [b] Optical density at 500 mμ. [c] Blanks are not subtracted out.

tution labile α-halo acids (Goldman and Milne, 1966; Goldman *et al.*, 1968).

As a part of our study of the biological cleavage of carbon–halogen bonds, we sought to examine the enzymatic dehalogenation of vicinal dihalides. Although mixtures of soil organisms will cleanly dehalogenate ethylene dibromide and 2,3-dibromobutane to the corresponding olefins (Castro and Belser, 1968), the more water-soluble and less volatile 2,3-dibromopropanol was chosen for closer scrutiny. The present work delineates the path of conversion of 2,3-dibromopropanol into glycerol by cell free extracts of a *Flavobacterium* sp. The chemical capacities of a partially purified halohydrin epoxidase obtained from these extracts are outlined.

Experimental Section

Materials

All substances employed in this work had physical constants which checked the literature or they were distilled before use. Purity was assessed by gas chromatographic analysis. All reagents were analytical grade. 2,3-Dibromopropanol-2,3-^{14}C was prepared from ethylene dibromide-1,2-^{14}C in the manner previously described (Castro and Belser, 1968). 1-Bromo-2,3-dihydroxypropane was prepared from epibromohydrin by warming in 1% HBr. The twice-fractionated material had bp 94° (0.9 mm), lit. bp 90° (0.8 mm). The substance gas chromatographed as one peak and had the correct infrared spectrum.

Methods

Isolation and Growth of the Organism. A solution of approximately 5×10^{-3} M 2,3-dibromopropanol was continually circulated through a 100-g column of soil which had been obtained from an alfalfa field. The pH was maintained at 7.0 by periodic addition of sodium hydroxide. The perfusate was sampled biweekly for bromide release employing an acidic (HNO_3) silver nitrate solution. After 1 month appreciable quantities of Br^- could be detected. The perfusate (1 ml) was inoculated into a liquid salts medium (Castro and Bartnicki, 1965) that was 4×10^{-3} M in substrate and contained 0.01% yeast extract. After 4 days, Br^- was at a high level, and the solution was streaked on agar plates of the same medium. Of several macroscopically different colonies that were inoculated into liquid medium, only the small round yellow colonies consistently produced Br^-. These colonies were composed of gram-negative rods which were classified as a *Flavobacterium* sp. Cultures were routinely grown in fernbach flasks containing 500 ml of the salts medium with 0.02% yeast extract, 1% glycerol, and substrate at 5×10^{-3} M. These were incubated in a New Brunswick gyrorotating shaker at 28°. The rate of growth of the organism and its dehalogenating capacity are indicated in Table I.

Cells grown with 2,3-dibromopropanol as the substrate were harvested when Br^- reached 3×10^{-3} M. The cells were then centrifuged (5°) for 15 min at 10,000 rpm, washed twice with 0.1 M phosphate buffer at pH 7.0, and resuspended in 100 ml of buffer solution. Portions (25 ml) of the suspension were sonicated for 15 min in a Raytheon sonic oscillator at maximum power. The combined sonicates were centrifuged at 17,000 rpm for 15 min. The enzymatic activity of the supernatant solution was the same as that of the corresponding cell suspension over a 12-hr period, whereas the boiled supernatant solution was inactive. The extract was most effective on 2,3-dibromopropanol at pH 9.0. However, in order to minimize any basic hydrolysis or nonenzymatic conversion assays were run at pH 8.0. Only negligible chemical production of Br^- occurred at this pH over a 24-hr period.

Activity Assay. Bromide ion was followed potentiometrically employing a silver–silver bromide working

electrode and a calomel reference electrode with a Beckman research potentiometer in a manner similar to that previously described (Castro and Bartnicki, 1965). In the present work the potentiometer output was fed to a Sargent recorder. The instrument was usually set such that a change in Br^- from 3×10^{-5} to 3×10^{-4} M was graphed full scale and corresponded to a 1-mV output of the potentiometer. The rate of production of Br^- was calculated from the time required to reach a Br^- concentration of 2×10^{-4} M. Typically a 10-ml solution of 10^{-3} M substrate in 0.01 M phosphate buffer at pH 8.0 was magnetically stirred at room temperature for 1 min to allow the electrodes to equilibrate. The enzyme (0.2–0.5 mg of protein) in 0.5 ml of buffer solution was added to the stirred solution. Reaction commenced immediately. The electrode was quite reliable under these conditions and there was no need to maintain high ionic strengths. A freshly plated Ag–AgBr electrode (Castro and Bartnicki, 1965) was employed for approximately 1 month. Appropriate blanks without enzyme or with boiled enzyme resulted in no enhanced Br^- production.

Protein was assayed in the crude supernatant (or extract) by the method of Lowry (Lowry *et al.*, 1951). Once the nucleic acid content of the supernatant (or extract) was diminished, spectrophotometric determinations of the protein by measuring the 260 mμ/280 mμ absorbance were reliable. Activity was calculated as micromoles of Br^- per liter per minute per milligram of protein. For our system, this corresponds to 100 μmoles of Br^-/min per mg of protein.

Purification of the Protein. The crude extract (*vide supra*) was lyophilized and combined batches were stored at 0°. Over a 1-year period, activity decreased from 50 to 30 units. The nucleic acid content of the crude extract was about 20%. The crude lyophilized mixture (4.0 g) was taken up in 200 ml of water, stirred, and treated dropwise with 4 ml of a neutralized solution of protamine sulfate (0.4 g) and streptomycin sulfate (0.1 g) at 5°. The mixture was allowed to stand for 10 min in an ice bath and was then centrifuged at 1500 rpm for 10 min. This procedure decreased the nucleic acid content of the supernatant solution to approximately 12%. Ammonium sulfate was slowly added to this solution until its concentration reached 50%. The solution was maintained at 5° for 1 hr and centrifuged. The supernatant solution was brought to 80% ammonium sulfate and the above process was repeated. The precipitate from this fraction contained the bulk of the activity. Usually 100 mg of protein from this fraction in 5 ml of buffer was placed on a refrigerated Sephadex G-200 or 150 column at 5° that was 1 M long and 2.5 cm in diameter. The protein was eluted with 0.01 M phosphate buffer (pH 7.0) containing 2×10^{-4} M mercaptoethanol. The active fraction emerged in about 30 hr, free of nucleic acids. Solutions of activity >300 could not be stored more than 3 days without appreciable loss of activity. The procedure is summarized in Table II.

Product Characterization. Final detection and monitoring of products and derivatives was accomplished by direct gas chromatography of reaction solutions. An Aerograph A600C unit equipped with a flame ionization detector was employed. In general, substances were characterized by their coemergence with authentic samples and by the coemergence of a hydrolytic derivative with an authentic sample of it. Both epibromohydrin and glycerol were further characterized by the additional techniques described later. Salient gas chromatographic data are presented in Table III. The hydrolytic characterization of epichlorohydrin and glycidol in product solutions was accomplished as follows. After completion of a reaction, determined by the amount of Br^- released and the lack of substrate apparent in the gas chromatogram, a 1-ml sample of the product solution was warmed with 1 ml of 1.0 NaOH at 90° for 12 hr in sealed tubes. Upon rechromatography, this hydrolysate no longer showed glycidol or epichlorohydrin but a glycerol peak was apparent. Appropriate blanks of authentic samples of epichlorohydrin and glycidol at these concentrations behaved identically.

TABLE II: Purification of the Protein.

	Protein (mg)	Act.[a]
Crude lyophilized extract	1200	30
Protamine sulfate and streptomycin sulfate	800	44
$(NH_4)_2SO_4$ (50–80%)	175	154
Sephadex G-200	36	340[b]

[a] Activities are typical of several runs. [b] A composite activity; a broad fraction ranged from 300 to 500. Activity = 100 μmoles of Br^-/min per mg of protein.

Similarly the gas chromatographic detection of propylene oxide in product solutions was substantiated by warming 1 ml of the reaction solution with 5 μl of 2 N H_2SO_4 for 15 min at 98°. Rechromatography afforded a propylene glycol peak. This method converts authentic propylene oxide into propylene glycol at these concentrations.

Enzymatic Conversions. A. WITH CRUDE EXTRACT. A reaction mixture composed of 1 l. of 10^{-3} M 1,2-dibromopropanol-1,2-^{14}C (3.4×10^7 dpm), 0.01 M phosphate buffer at pH 7.0, and 0.6 g of lyophilized supernatant (activity ~30) was stirred at room temperature for 8 hr. At this time bromide concentration was 1.3×10^{-3} M, corresponding to 65% conversion. The water was stripped on a rotary evaporator until the phosphate salts began to crystallize. Unlabeled glycerol (5 ml) was added to the salts concentrate and the mixture was extracted with methanol. The methanol extract was stripped to dryness and taken up in 5 ml of pyridine to which 3 ml of benzoyl chloride was slowly added with cooling. After 2 hr the crystals were filtered and recrystallized from 80% ethanol to yield glyceryl tribenzoate with mp and mmp 73°. *Anal.* Calcd for $C_{24}H_{21}O_6$: C, 71.28; H, 4.98. Found: C, 71.27; H, 5.05. The ester had an activity of 600 dpm/mg. Thus, by isotopic dilution the yield of glycerol was at least 85%.

2,3-Dibromopropanol-2,3-^{14}C. In a run with an ammonium sulfate fraction of the protein (activity ~70)

TABLE III: Gas Chromatographic Analysis of Products, Derivatives, and Substrates.

Substance	Column: Length (ft)[a]	Column: Packing[b]	Column: Temp (°C)	Column: Time (min)	Hydrolysis Product
Epichlorohydrin	5	Porapak P (3% DEGS)	140	6.5	Glycerol
Glycidol	5	Porapak P (3% DEGS)	140	7.5	Glycerol
Epibromohydrin	1	Porapak P	125	2.5	Glycerol
Propylene oxide	3	Porapak P	110	0.4	Propylene glycol
Propylene glycol	3	Porapak P	110	3.1	
Glycerol	1	Porapak P	150	9.0	
1-Bromo-2,3-dihydroxypropane	1	Porapak P	150	12.0	
2,3-Dibromopropanol	1	Porapak P	150	18.0	
	5	(5% DEGS) Chromosorb W HMDS treated	145	7.0	
1,3-Dibromopropanol	1	Porapak P	150	16.0	
1-Bromo-2-propanol	3	Porapak P	110	5.0	

[a] All columns were made of glass and were 3 mm in diameter. [b] DEGS, diethylene glycol succinate; Porapak and Chromasorb packings were obtained from the Varian-Aerograph Co. Inc.

the epibromohydrin peak reached a maximum in 15 min. Reaction was stopped by the addition of 4 ml of nonlabeled epibromohydrin. The mixture was shaken and the resulting emulsion was allowed to separate in the refrigerator. From the organic phase radioactive epibromohydrin was isolated by trapping the peak from repeated gas chromatographic injections. An Aerograph A-90 P chromatograph with a 6-ft Porapak P column at 180° was employed. The trapped substance (0.5 ml) was hydrolyzed in sealed tubes with 2 ml of 0.1 M NaOH at 90° for 20 hr. The hydrolysate was injected on an 8-ft Porapak P column and showed peaks coemergent with 1-bromo-2,3-dihydroxypropane and glycerol. The radioactive dihydroxybromopropane peak was trapped and it had an infrared spectrum identical with authentic material.

The rate of consumption and the path of conversion of 2,3-dibromopropanol into glycerin were discerned from small-scale runs like the one above. Typically, 50 mg of crude extract in 10 ml of 0.01 M phosphate buffer at pH 7.0 that was 10^{-3} M in substrate was allowed to react at room temperature.

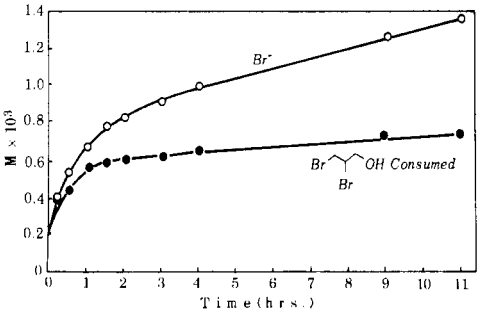


FIGURE 1: Rates of Br^- production and 2,3-dibromopropanol consumption by a crude extract activity 10. Initial concentration of substrate was 1×10^{-3} M.

For the kinetics (Figure 1) dibromopropanol was followed gas chromatographically on the 5% DEGS column by employing succinonitrile as a marker. Bromide was determined as noted above.

Qualitative gas chromatographic monitoring of the reactions with the crude extract afforded the following observations.

2,3-Dibromopropanol. Epibromohydrin is the first produced substance. It vanishes. A trace of glycidol is seen followed by an increasing amount of glycerol.

Epibromohydrin. 1-Bromo-2,3-dihydroxypropane first appears and diminishes followed by glycidol and glycerol.

1-Bromo-2,3-dihydroxypropane. Glycidol appears and diminishes as glycerol increases.

Glycidol. Glycerol is the only observable product.

B. WITH PARTIALLY PURIFIED ENZYME ($(NH_4)_2SO_4$ AND SEPHADEX G-150). General reaction conditions paralleled those for activity determinations. Thus, 100 ml of 10^{-3} M substrate solution (10^{-4} mole) containing 0.01 M phosphate buffer at pH 7.0 and 5 mg of protein was allowed to react at room temperature. The enzyme in the product runs had an activity of 200–300 on 1,2-dibromopropanol at pH 8.0.

2,3-Dibromopropanol and 1,3-Dibromopropanol. These substances are quantitatively converted into epibromohydrin.

Epibromohydrin. In the absence of Cl^- this substance is very slow to react with purified protein. Only a trace of glycidol could be discerned after 20 hr. However, in

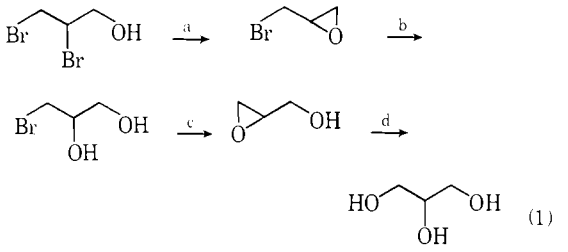
the presence of 10^{-2} M KCl, epibromohydrin was consumed in 0.5 hr and epichlorohydrin was produced. The yield at all conversions (vapor phase chromatography, cyclohexane marker) was quantitative. The potentiometric assay of enzymatic activity for epibromohydrin, because of Cl^- leakage from the calomel electrode, also results in the production of epichlorohydrin. Thus, an initial concentration of 1.17×10^{-3} M epibromohydrin resulted in 0.8×10^{-4} M Br^- in 2 hr (71% conversion). The yield of epichlorohydrin was 96%. It should be emphasized here and throughout this work that blanks containing all ingredients at the same concentrations except without enzyme or with boiled enzyme showed no conversion of substrate, production of Br^-, or any transformation.

1-Bromo-2,3-dihydroxypropane. This halide was partially converted into glycidol (~10-30%) upon gas chromatography of an aqueous solution by a variety of column packings. Thus, it was not possible to quantitate this reaction. The calculated yield at 50% conversion (1 hr) of the substrate was ~130%. Qualitatively the glycidol did increase with Br^- production. It was the sole discernible product.

1-Bromo-2-hydroxypropane. Bromide production was complete in 1 hr. Propylene oxide was the sole product.

Results and Discussion

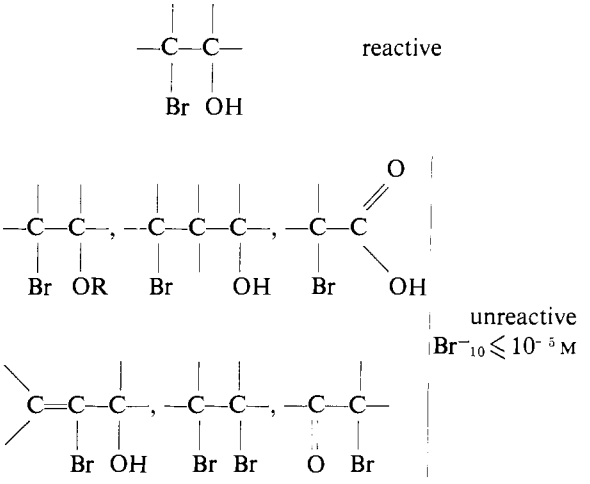
Typical over-all kinetics for the consumption of 2,3-dibromopropanol and the production of bromide by the crude extract are traced in Figure 1. It will be noted that the stoichiometric ratio of rates is not approached until after 4 hr. These results bespeak a multistep path of conversion into glycerol in which dibromopropanol is consumed more rapidly than Br^- is released. The sequence determined for this process (*vide supra*) (eq 1)



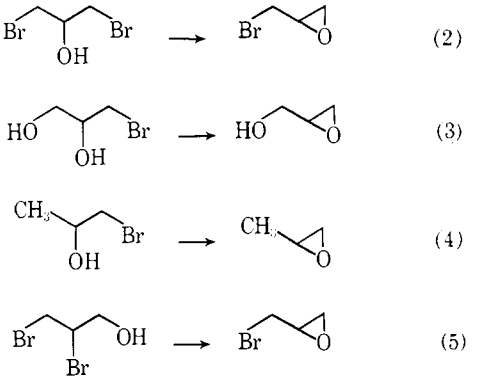
(1)

fits well with the kinetics. Although no quantitative comparison of rates was made with the crude extract, the epoxidation steps (1a and 1c) were qualitatively more rapid than the epoxide opening steps (1b and 1d). Thus dihydroxybromopropane concentrations never became large.

The reactivity of the partially purified enzyme to a series of halides is portrayed in Table IV. The results were obtained from the same Sephadex G-200 fraction. Substrates are listed in a decreasing order of reactivity. In addition to those substances in the table, the following were found to be inert under these conditions: 2-bromoallyl alcohol 2,3-dibromopropionic acid, β-bromopropionic acid, *trans*-2,3-dibromocinnamic acid, 1,2-dibromo-3-chloropropane, 1,2-dibromopropane, 1,2,3-tribromopropane, *p*-bromophenacyl bromide, 2-amino-5-nitrobromobenzene, 1,1-dibromoethane, propargyl bromide, 2-bromopropane, 2-bromo-1,1-di-*n*-propoxypropane, and *N*-bromoacetamide. That is, for these materials Br^- concentration at 10 min was equal to or less than 10^{-5} M. Thus, the pattern of growth and reactivity of whole cells and the bond-type specificity of the purified protein accord with the activity of the enzyme for α-halo alcohols. The sensitivity of the purified enzyme to structural alteration of the substrate is manifest in a comparison of the potentially reactive moities examined.

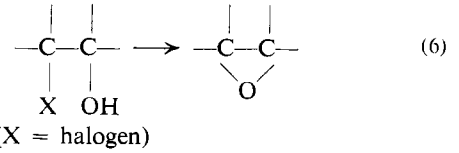


Dehalogenations effected by the halohydrin epoxidase are typified by the clean conversions observed with it (eq 2–5). The scope of activity of the enzyme to other



(2) (3) (4) (5)

molecules of this class and the stereochemical consequence of the process (eq 6) will be the subject of future work.



(6)

(X = halogen)

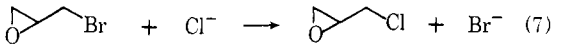
The crude extract efficiently converts epibromohydrin into glycidol (eq 1b and 1c). The enzymes responsible for this transformation have essentially been removed from the partially purified halohydrin epoxidase. Thus, best pure fractions of the enzyme with epibromohydrin afford only a trace of glycidol in 20 hr.

TABLE IV: Substrate Reactivity at pH 7.0.

Substrate (1.0–1.1 × 10^{-3} M)	Br^{-} [10] [a] (moles/l.)	Sp Act. (100 μmoles of Br^{-}/min per mg of protein)
1,3-Dibromopropanol	1.1×10^{-3}	2222
1-Bromo-2,3-dihydroxy-propane	3.1×10^{-4}	202
Epibromohydrin	4.2×10^{-4}	222
1-Bromo-2-propanol	5.9×10^{-4}	182
2,3-Dibromopropanol[b]	2.7×10^{-4}	123
Bromoethanol	6.2×10^{-5}	
2-Bromo-1,1-dimethoxy-butane	1.1×10^{-5}	
3-Bromopropanol	1.0×10^{-6}	

[a] (Br^{-} at 10 min) − (Br^{-} at time 0). [b] Activity for 2,3-dibromopropanol at pH 8.0 is 290.

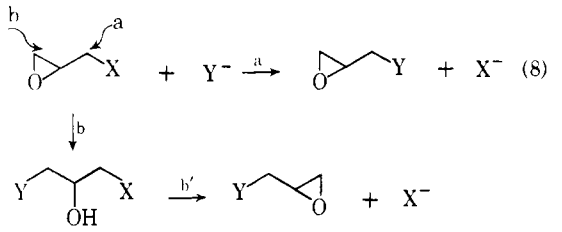
However, if chloride ion is present in the reaction solution a rapid conversion into epichlorohydrin ensues (eq 7). With 10^{-2} M KCl the transhalogenation



(7)

was complete in 0.5 hr. It should be emphasized that the specific activity noted in Table IV for epibromohydrin is an estimate for this (eq 7) process. That is, the Cl^{-} leak from the calomel reference electrode was sufficient to allow reaction to occur.

This unusual transhalogenation suggests many attractive mechanistic insights to the dehalogenation process. It is particularly reminiscent of the nonenzymatic Cu^{I}-catalyzed halogen interchange observed in aromatic



(8)

systems (Bacon and Hill, 1964). Moreover, a chemical model for hydrolytic dehalogenation (Castro and Bartnicki, 1965) may be found in the Cu^{I}-catalyzed hydrolysis of allylic halides (Hatch and Estes, 1945; Hatch *et al.*, 1952). However, a process like eq 7 may proceed by two reaction paths: attack on the C–X bond (eq 8a) or attack on the terminal epoxide carbon (eq 8b) followed by an epoxidation (eq 8b′). The general chemistry of the transhalogenation and the nature of other nucleophiles (X and Y in eq 8) which may participate in the reaction are under examination.

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