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# Demethylation Studies. IV. The *in vitro* and *in vivo* Cleavage of Alkyl- and Arylalkyl-*p*-nitrophenyl Ethers

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The oxidative O-dealkylation of a series of alkyl and arylalkyl *p*-nitrophenyl ethers by rat liver microsomes has been studied. For saturated alkyl groups the rate tends to decrease with increasing bulk of the group. Certain electron donating groups such as ethylene, cyano, and phenyl when attached to the reactive methylene increase the rate of reaction. Ring substitution of any type in the benzyl group of *p*-nitrophenyl benzyl ether tends to reduce the rate. This is thought to be simply a steric effect. *In vivo* dealkylation rates correlated well with *in vitro* results.

Huggins, Jensen, and Cleveland[1] studied the *in vivo* metabolism of *p*-nitroanisole and *p*-nitrophenetole in the rat and found them to be extensively cleaved to *p*-nitrophenol. Using tissue homogenates, they found that both liver and kidney could carry out this cleavage. In a study of the *in vitro* conversion of codeine to morphine Axelrod[2] found the enzymatic activity responsible resided in liver microsomes and required NADPH and oxygen. The methyl group appeared as formaldehyde. Thus the reaction was shown to be one of the several oxidative reactions that are catalyzed by mammalian liver microsomal enzymes and which possess a requirement for NADPH and oxygen.[3] Axelrod[4] also studied *in vitro* O-dealkylation of a variety of ring substituted anisoles and phenetoles by the microsomal system.

Although the cleavage of a wide variety of arylalkyl ethers have been reported,[5] none have been investigated in which the alkyl group was other than methyl or ethyl. In the present study the aryl group was held constant (*p*-nitrophenyl) and the nature of the alkyl group varied over a wide range.

## Experimental

**Materials.**—Substituted *p*-nitrophenyl ethers needed for this study were prepared by standard organic synthesis procedures. Paper chromatography was used to show that each ether was free of *p*-nitrophenol. The preparation of $C^{14}$ labeled *p*-nitrophenyl benzyl ether will serve to illustrate the method used.

A mixture of 330 μmoles of sodium *p*-nitrophenoxide and 200 μmoles of benzyl-1-$C^{14}$ chloride in 3 ml. of absolute ethanol was refluxed 16 hr. and then evaporated to dryness. The product was taken up in ether and unreacted phenol removed by extraction with alkali. The ether solution was reduced to dryness and the product recrystallized from methylcyclohexane. The product weighed 20 mg. (43%) and melted at 103–105° (reported[3] 106°). Paper chromatography was used to demonstrate that no unreacted *p*-nitrophenol or benzyl-1-$C^{14}$ chloride remained in the product.

**Methods. Microsomes.**—The livers of normal male rats were quickly removed, following decapitation, and placed in ice cold 0.1 *M* phosphate buffer, pH 7.4. The livers were weighed, minced, and put into a cold Potter–Elvehjem homogenizer. Phosphate buffer was added in the ratio of 4:1 and the livers were homogenized with a Teflon pestle for 1–1.5 min. The pooled homogenates were centrifuged at 20,000*g* for 30 min. in a Spinco Model L centrifuge at 0°. The resulting supernatant was then recentrifuged at 80,000*g* for 30 min. and an aliquot of the clear supernatant, free of visible fat, was removed and saved. The remainder of the supernatant was decanted and discarded. The microsomes were resuspended by homogenizing the microsome pellet with suitable volumes of buffer and 80,000*g* supernatant to yield a final suspension containing in each ml. the microsomes from 200 mg. of liver and 80,000*g* supernatant from 40 mg. of liver.

*In Vitro* **Dealkylations.**—Varying amounts of substrate dissolved in 0.2 ml. of polyethylene glycol (PEG 400) were placed in 20 ml. beakers and to this was added 1 ml. of microsome suspension, 50 μmoles of nicotinamide, 50 μmoles of magnesium chloride, 10 μmoles of glucose-6-phosphate, 0.5 μmole of NADP, and sufficient buffer (pH 7.4) to bring the final volume to 3 ml. In the runs involving inhibitors the inhibitor was added with the substrate.

Incubations were carried out in a shaker at 37° in an atmosphere of oxygen for 30 min. The presence of oxygen completely inhibited the activity of the microsomal reductase[7] and *p*-nitrophenol carried through the incubation could be recovered quantitatively. The incubation was stopped by the addition of 1 ml. of 10% trichloroacetic acid and the precipitated protein removed by centrifugation. After adding 1 ml. of 0.75 *N* NaOH to 3 ml. of supernatant, unreacted substrate was removed by extraction with 5 ml. of methylene chloride. Removal of unreacted substrate was necessary since its presence interfered with the *p*-nitrophenol reading. *p*-Nitrophenol was determined spectrophotometrically at 400 mμ. That the product was indeed *p*-nitro-

(1) C. Huggins, E. V. Jensen, and A. S. Cleveland, *Proc. Soc. Exp. Biol. Med.*, **68**, 477 (1948).

(2) J. Axelrod, *J. Pharmacol. Exptl. Therap.*, **115**, 259 (1955).

(3) B. Brodie, J. Axelrod, J. R. Cooper, L. Gaudette, B. N. LaDu, C. Mitoma, and S. Udenfriend, *Science*, **121**, 603 (1955).

(4) J. Axelrod, *Biochem. J.*, **63**, 634 (1956).

(5) R. T. Williams, "Detoxication Mechanisms," John Wiley and Sons, New York, N. Y., 1959, p. 324.

(6) C. Kumpf, *Ann.*, **224**, 123 (1884).

(7) J. R. Fouts and B. B. Brodie, *J. Pharmacol. Exptl. Therap.*, **119**, 197 (1957).

phenol was established by ultraviolet absorption studies and by paper chromatography (1:1 BuOH–5 *N* $NH_4OH$ system). *p*-Nitrophenol carried through the incubation and analytical procedure served as a standard. An incubation mixture containing all components except substrate served as a blank.

*In Vivo* **Dealkylations.**—The ethers were dissolved in PEG 400 (100 $\mu M$/ml.) and 1 ml. was given by intraperitoneal injection into 200-g. male rats. Water (5 ml.) was then given by stomach tube. The rats were kept in metabolism cages with free access to water and a 24-hr. urine sample was collected.

A 1-ml. aliquot of the urine collection was incubated overnight with 0.05 ml. of Glusulase solution (a mixture of $\beta$-glucuronidase and sulfatase, Endo Products) at pH 5. The sample was adjusted to pH 5.5 following incubation and was extracted 3 times with ether (3 ml.). The ether from the combined extracts was evaporated under nitrogen and the residue dissolved in 4 ml. of 0.75 *M* NaOH. These solutions were extracted once with 4 ml. of dichloromethane. Aliquots of water layer were used to determine the *p*-nitrophenol content spectrophotometrically. All samples were read against the same dilution of a urine extract from a rat receiving only PEG 400.

**Metabolism of *p*-Nitrophenyl Benzyl-1-$C^{14}$ Ether in Rats.**—Labeled *p*-nitrophenyl benzyl ether in PEG 400 was administered to 3 male rats by intraperitoneal injection at a dose rate of 100 mg./kg. Urine was then collected for 24 hr. and its radioactive content determined by standard methods. The nature of the radioactive metabolites was established by paper chromatography on Whatman No. 1 paper using 1:1 BuOH–5 *N* $NH_4OH$ as the developing solvent system.

## Results and Discussion

*In Vitro.*—In studies on N-dealkylation it has been reported that in addition to methyl and ethyl groups, butyl,[8,9] allyl,[10] and phenethyl (J. Cochin, private communication) groups can be removed enzymatically from nitrogen. In the case of O-dealkylation only demethylation and deethylation have been reported. The purpose of this study was to explore substrate specificity with respect to permitted structural variation in the alkyl group. To this end the oxidative cleavage of a variety of alkyl and arylalkyl *p*-nitrophenyl ethers was studied in the rat liver microsomal system. In these studies advantage was taken of the *p*-nitrophenoxide anion chromophore as was done earlier by Huggins, *et al.*,[1] in their *in vitro* studies of *p*-nitroanisole. Recently Netter[11] has followed the cleavage of *o*-nitroanisole by rat liver microsomes by determining the extent of *o*-nitrophenol formation.

The rates of cleavage of the various ethers are summarized in Table I. Dealkylation rates were determined at three different substrate concentrations to make sure that the effects of possible substrate inhibition did not prejudice the results.

The dealkylation of ethers containing saturated alkyl groups (Table I, 1–7) was first investigated. If one excludes the methyl group from discussion, it is seen that there is a clear correlation between molecular weight and rate of dealkylation: the larger the group, the slower the rate. The ethyl derivative is the most active substrate, followed by propyl and isopropyl. The chloroethyl group which is structurally similar to propyl does not differ in rate from the two isomeric propyl ethers. The *n*-butyl compound dealkylates only slowly and the *n*-hexyl derivative appears to be without activity.

(8) B. N. LaDu, L. Gaudette, N. Trousof, and B. B. Brodie, *J. Biol. Chem.*, **214**, 748 (1955).
(9) R. McMahon, *J. Pharmacol. Expl. Therap.*, **130**, 383 (1960).
(10) J. Cochin and J. Axelrod, *ibid.*, **121**, 107 (1957).
(11) K. J. Netter, *Arch. Expl. Pathol. Pharmakol.*, **238**, 202 (1960).

Table I

*In Vitro* Cleavage of *p*-Nitrophenyl Ethers by Rat Liver Microsomes[a]

| | Substrate: $O_2N$–C$_6$H$_4$–OR, R | Rate of dealkylation (nmoles/min./g. liver) at substrate concentration 1.67 × $10^{-3}$ *M* | 5 × $10^{-4}$ *M* | 1.67 × $10^{-4}$ *M* |
|---|---|---|---|---|
| 1 | $CH_3$ | 3.4 | 8.5 | 19.6 |
| 2 | $C_2H_5$ | 13.9 | 30.4 | 48.5 |
| 3 | *n*-$C_3H_7$ | 5.8 | 12.2 | 17.9 |
| 4 | *i*-$C_3H_7$ | 1.8 | 11.5 | 21.2 |
| 5 | *n*-$C_4H_9$ | 2.4 | 4.6 | 6.8 |
| 6 | *n*-$C_6H_{13}$ | 0 | 0 | Trace |
| 7 | $ClCH_2CH_2$ | 6.2 | 11.5 | 18.2 |
| 8 | $NCCH_2$ | 9.4 | 15.8 | 25.5 |
| 9 | $CH_2{=}CHCH_2$ | 12.4 | 22.4 | 35.2 |
| 10 | $HOCOCH_2$ | 0 | 0 | 0 |
| 11 | $(CH_2)_5NCH_2CH_2$ | 0 | 0 | Trace |
| 12 | $C_6H_5CH_2$ | 22.5 | 22.4 | 46.4 |
| 13 | *o*-$FC_6H_4CH_2$ | 9.0 | 10.7 | 12.2 |
| 14 | *o*-$ClC_6H_4CH_2$ | 6.2 | 7.4 | 5.5 |
| 15 | *o*-$BrC_6H_4CH_2$ | 5.0 | 6.0 | 5.8 |
| 16 | *o*-$CH_3C_6H_4CH_2$ | 3.3 | 7.2 | 8.4 |
| 17 | *m*-$CF_3C_6H_4CH_2$ | 3.4 | 4.6 | 4.8 |
| 18 | *p*-$ClC_6H_4CH_2$ | 3.9 | 4.0 | 4.4 |
| 19 | *p*-$CH_3C_6H_4CH_2$ | Trace | Trace | Trace |
| 20 | *p*-$NO_2C_6H_4CH_2$ | 0 | 0 | Trace |
| 21 | $C_6H_5CH_2CH_2$ | 2.4 | 2.9 | 3.9 |
| 22 | $C_6H_5$ | 7.2 | 5.2 | 3.2 |

[a] Incubation conditions are described under methods (see text).

Table II

$V_{max}$ and $K_m$ Values for the Dealkylation of *p*-Nitrophenylalkyl Ethers[a]

| $NO_2$–C$_6$H$_4$–OR, R | $V_{max}$, mμmoles/min./g. liver | $K_m$, *M* |
|---|---|---|
| $CH_3$ | 143 | 167 × $10^{-4}$ |
| $CH_3CH_2$ | 72 | 6.8 × $10^{-3}$ |
| *i*-$C_3H_7$ | 38 | 12.0 × $10^{-3}$ |
| *n*-$C_3H_7$ | 34 | 6.4 × $10^{-3}$ |
| *n*-$C_4H_9$ | 8 | 4.2 × $10^{-3}$ |

[a] Incubation conditions are described under methods. The $V_{max}$ and $K_m$ values were approximated by the method of least squares from data collected over the concentration range of 167 × $10^{-5}$ to 1.67 × $10^{-3}$ *M*. Rates at 5 concentrations were used in the calculation.

Studies on the simple alkyl derivatives was extended and an estimate of the maximum rate at infinite substrate concentration ($V_{max}$) and of the Michaelis Menton constant ($K_m$) was made (Table II).[12]

Again excluding the methyl group from consideration, it is seen that the $K_m$ value does not vary appreciably among the compounds. This result seems reasonable if the assumption is made that in these systems $K_m$ measures affinity, and if one assumes that the oxygen atom is of primary importance for the binding of substrate to enzyme. The availability (for binding) of the electron pair on oxygen would be influenced almost entirely by ring substitution in the aromatic portion of the molecule. In the present case substitution has been limited to *p*-nitro so that very little difference in binding among the various compounds would be expected. The fact that the isopropyl binds somewhat more poorly than does *n*-propyl however does suggest that steric factors on the alkyl group can also influence binding.

(12) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

The apparent discrepancy shown by the methyl compound most probably relates to the appreciable water solubility of this ether. The microsomal fraction is the major lipid bearing subfraction of the liver cell, and in the incubation mixture one has in effect a two-phase system, *i.e.*, an aqueous phase and a lipid phase (microsomes). N-Dealkylation studies[13-15] have led to the belief that only those substrates which can penetrate into the lipid phase will interact with enzyme. If this speculation were correct it would mean that a true measure of $K_m$ would be that concentration in the microsome fraction which results in one-half maximum rate. In a well defined group of water insoluble lipid soluble compounds such as those under discussion, the apparent $K_m$ values measured, though not the true values, would relate to each other in a proportional manner. This would not however be true in the case of a water soluble compound such as the *p*-nitrophenyl methyl ether, and the $K_m$ value found would be deceptively high.

When the maximum rates ($V_m$, Table II) are compared, it is seen that the methyl ether now falls in line and is the most active, with the others showing decreased reactivity as the size of the alkyl groups increases. The inverse relationship between rate and the size of the alkyl group may result simply from a decreasing chemical reactivity with increasing bulk. It might also be merely a matter of increasing steric hindrance to reaction.

The effect of incorporating various functional groups into the alkyl group was investigated next. The incorporation of a double bond into the three-carbon side chains (9) resulted in a considerable increase in activity in dealkylation compared to the corresponding saturated ether (3). If, as is likely,[16] the dealkylation reaction involves electrophilic attack upon the methylene carbon, then any electron donor group in an adjacent position would be expected to increase the rate of reaction. The same considerations also explain the increased rate shown by the cyanomethyl ether (8).

As would be expected *p*-nitrophenoxyacetic acid (11) is inactive. It should be completely ionized at pH 7.4 and not available for distribution into the microsomes. The reason for the lack of activity of the β-piperidinoethyl ether (12) is not clear.

Another electron donor group which, when attached to the active methylene carbon, would be expected to lead to increased reactivity is the phenyl group. This is the case. Indeed, *p*-nitrophenyl benzyl ether (12) is the most active substrate at low concentrations. Unfortunately this interesting ether exhibited substrate inhibition of dealkylation rate even at relatively low concentration. As can be seen from the data presented in Fig. 1 the rate reached a maximum at a concentration of less than $3 \times 10^{-4}$ *M*. Actually substrate inhibition was observed with nearly all of the substituted benzyl ethers studied and it was not possible to study enzyme binding and $V_{max}$ in this important series.

It was thought that a study of the effect of ring substitution upon rate would be instructive in attempts to understand the mechanism of the reaction. However,

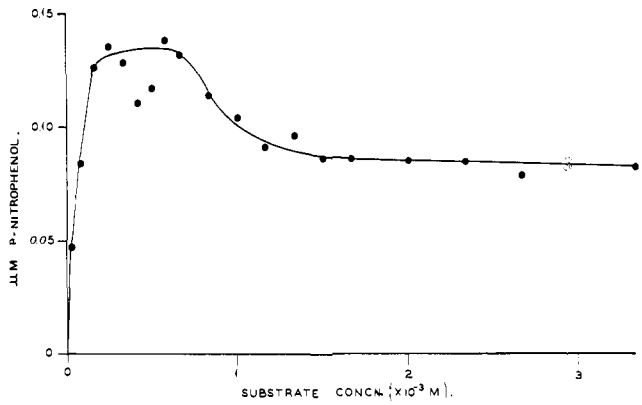



Fig. 1.—The effect of substrate concentration upon the rate of cleavage of *p*-nitrophenyl benzyl ether. Incubation conditions are described under methods.

it was found that without exception all of the ring substituted benzyl ethers were cleaved at a slower rate than the parent compound. For example, both the *p*-nitro group, which should decrease the electronegativity of the active methylene group, and the *p*-methyl group, which should increase it, lead to substrates which are essentially inactive. An examination of the *o*-halo derivatives yields a clue to the nature of the effect of substituents. It was found that in going from benzyl to *o*-fluorobenzyl (13) to *o*-chlorobenzyl (14) to *o*-bromobenzyl (15) the rate decreased in that order, *i.e.*, the rate decreases with increasing bulk of the substituent. The methyl group which is similar in size to bromine yields an *o*-methyl derivative (16) which differs little in rate from the *o*-bromo compound. Substitution in the *para* position seems to be even more detrimental. The bulky *p*-nitro group (20) and *p*-methyl group (19) yield derivatives which are without activity as substrates. The smaller chloro substituent has a greater depressant effect in the *para* position than in the *ortho* position.

Thus the unsubstituted benzene ring seems to be the optimal configuration for this series of ethers. Any substitution on the ring reduces substrate activity possibly through steric interference with the approach of the ring to the enzyme surface.

The phenethyl ether (21) in which the benzene ring is too far removed from the active methylene to affect its electronegativity is predictably much less active than the benzyl ether. *p*-Nitrodiphenyl ether is also a substrate for this enzyme thus confirming the earlier *in vivo* observations of Huggins, *et al.*[1]

The effect of various inhibitors upon the cleavage of *p*-nitrophenetole was also studied (Table III). DPEA (2,4-dichloro-6-phenylphenoxyethylamine) which is a competitive inhibitor of N-demethylation[17] is an effective inhibitor although relatively high concentrations are required. Cupric ion also inhibits effectively. It is possible that copper ion inhibits by blocking NADPH oxidase thus depressing the rate of formation of 'active hydroxyl.'[18] Cyanide, dipyridyl and iodoacetate did not inhibit at the concentrations used.

*In Vivo*.—The cleavage of this interesting series of ethers was also studied in the whole animal. Since

(13) L. Gaudette and B. B. Brodie, *Biochem. Pharmacol.*, **2**, 89 (1959).
(14) R. E. McMahon, *J. Med. Pharm. Chem.*, **4**, 67 (1961).
(15) R. E. McMahon and N. R. Easton, *ibid.*, **4**, 437 (1961).
(16) J. R. Gillette and J. J. Kamm, *J. Pharmacol. Exptl. Therap.*, **130**, 262 (1960).
(17) R. E. McMahon and J. Mills, *J. Med. Pharm. Chem.*, **4**, 211 (1961).
(18) J. R. Gillette, B. B. Brodie, and B. N. LaDu, *J. Pharmacol. Exptl. Therap.*, **119**, 532 (1957).

Table III

The Effect of Inhibitors on the Cleavage of *p*-Ethoxynitrobenzene[a]

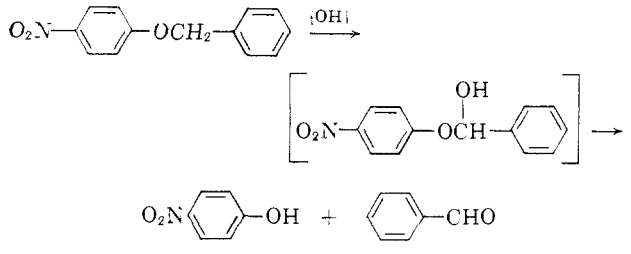
| Inhibitor ($3.3 \times 10^{-4}$ *M*) | % Inhibition |
|---|---|
| DPEA | 87 |
| Copper sulfate | 80 |
| Potassium cyanide | 7 |
| $\alpha,\alpha'$-Dipyridyl | 0 |
| Sodium iodoacetate | 0 |

[a] Substrate concentration: $1.7 \times 10^{-3}$ *M*. Incubation conditions are described under methods.

Huggins, *et al.*,[1] had reported that *p*-nitrophenol is excreted in the main in conjugated form, urine samples were treated with β-glucuronidase and sulfatase before analysis. Thus the values of *p*-nitrophenol recovery shown in Table IV represent both bound and unbound material. The correlation between the *in vivo* and *in vitro* is remarkably good indicating that studies in the isolated microsomal systems do forecast the results to be expected in the whole animal. This is also thought to be the case in *N*-dealkylations.[14]

These results are of interest to medicinal chemists since it is important to know which groups, when used to cover a phenolic hydroxyl, can be expected to be removed in the body and which can be expected to be metabolically stable. It is of particular interest that the dialkylaminoethyl ether linkage (11, 11a) which occurs so frequently in medicinals is very stable metabolically.

The course of the O-dealkylation reaction has been pictured by Brodie, Gillette, and LaDu[19] as a hydroxylation reaction




(19) B. B. Brodie, J. R. Gillette, and B. N. LaDu, *Ann. Rev. Biochem.*, **27**, 427 (1958).

Table IV

*In Vivo* Cleavage of *p*-Nitrophenyl Ethers in Rats[a]

| | Compound: $O_2N$—⟨⟩—OR, R | % recovered in urine in 24 hr. as *p*-nitrophenol |
|---|---|---|
| 1 | $CH_3$ | 76 |
| 2 | $C_2H_5$ | 78 |
| 3 | *n*-$C_3H_7$ | 86 |
| 4 | i-$C_3H_7$ | 87 |
| 5 | *n*-$C_4H_9$ | 47 |
| 6 | *n*-$C_6H_{13}$ | 16 |
| 7 | $ClCH_2CH_2$ | 74 |
| 8 | $NCCH_2$ | 72 |
| 9 | $CH_2{=}CHCH_2$ | 70 |
| 10 | $HOCOCH_2$ | 4 |
| 10a | $H_2NCOCH_2$ | 6 |
| 11 | $(CH_2)_5NCH_2CH_2$ | 5 |
| 11a | $(CH_3)_2NCH_2CH_2$ | 7 |
| 12 | $C_6H_5CH_2$ | 39 |
| 13 | *o*-$FC_6H_4CH_2$ | 36 |
| 14 | *o*-$ClC_6H_4CH_2$ | 16 |
| 15 | *o*-$BrC_6H_4CH_2$ | 6 |
| 16 | *o*-$CH_3C_6H_4CH_2$ | 4 |
| 16a | *m*-$ClC_6H_4CH_2$ | 34 |
| 17 | *m*-$CF_3C_6H_4CH_2$ | 38 |
| 18 | *p*-$ClC_6H_4CH_2$ | 14 |
| 19 | *p*-$CH_3C_6H_4CH_2$ | 2 |
| 20 | *p*-$NO_2C_6H_4CH_2$ | 2 |
| 21 | $C_6H_5CH_2CH_2$ | 18 |
| 22 | $C_6H_5$ | 10 |

[a] The dose was 500 μmoles/kg. (i.p.) except for compounds 11 and 11a where the dose was 100 μmoles/kg. (i.p.).

In the whole animal the benzaldehyde would be expected to be readily oxidized to benzoic acid and excreted as hippuric acid. This proved to be the case. *p*-Nitrophenyl benzyl ether labeled with radiocarbon in the methylene carbon of the benzyl group was administered to rats. In 24 hours, 43% of the radioactivity was recovered in urine as hippuric acid. This agrees well with the recovery of 39% of the dose as *p*-nitrophenol (Table IV).

The small amount of cleavage observed with *p*-nitrodiphenyl ether is of some interest. Since there are no α-hydrogens the reaction cannot proceed by the mechanism outlined above. In order to understand this reaction it will be necessary to identify all of the products of the reaction. In addition a study of variously substituted diphenyl ethers would be of interest.