Biochemical Pharmacology, Vol. 23, pp. 1871–1876. Pergamon Press, 1974. Printed in Great Britain.

REVERSIBILITY OF HEPATIC CHANGES CAUSED BY ETHOXYQUIN

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(*Received* 16 *August* 1973; *accepted* 24 *November* 1973)

Abstract—Ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) was administered to rats at a dose level of 0·5 per cent in the diet for 14 days. It caused a marked increase in liver weight and induction of microsomal drug metabolizing enzymes. In contrast glucose-6-phosphatase activity was depressed. During a 30-day recovery period after removal of ethoxyquin the following parameters were monitored at intervals of 0, 3, 7, 14 and 30 days: liver weight, microsomal protein, cytochrome P-450, cytochrome b_5, biphenyl-4-hydroxylase, ethylmorphine-*N*-demethylase, glucose-6-phosphatase and DNA. Recovery appeared to be biphasic, specific enzyme activity and DNA concentration returning to normal in 3–7 days but, as a result of the slower return to normal liver size, *total* hepatic enzyme activity did not return to control levels until the 30th day.

INTRODUCTION

ETHOXYQUIN is a synthetic antioxidant permitted for use in the U.K. in the prevention of scald on apples and pears; it is also used as a preservative in animal feedstuffs. It prevents some vitamin E deficiency symptoms such as encephalomalacia,[1,2] exudative diathesis and muscular degeneration,[3] hepatic lipid peroxidation[4] and loss of fertility[5] in chicks, muscular dystrophy in lambs[6] and steatitis in swine.[7] Ethoxyquin also protects against loss of vitamin A *in vivo* in growing chicks[8] and turkeys.[9]

Parenterally administered ethoxyquin was found to be non-carcinogenic to Swiss mice[10] and, orally administered, it inhibited the carcinogenicity of benz(a)pyrene or 7,12-dimethylbenz(a)anthracene (DMBA) in mice and rats.[11] It has been reported to protect against dietary liver necrosis[12] and the hepatotoxicity of carbon tetrachloride in rats and sheep.[13–15] Ethoxyquin is rapidly metabolised and excreted by rats,[16] it has low oral toxicity[17] and has even been reported to prolong life-span in mice.[18,19]

In view of these many beneficial effects, ethoxyquin is likely to receive wider application in the preservation of food and feedstuffs. However, Cawthorne *et al.*[13] reported that on administration to rats, ethoxyquin caused liver enlargement and induction of some hepatic drug-metabolizing enzymes, and similar observations have been made by Parke *et al.*[20] It is important, therefore, to know whether these hepatic changes are adaptive and reversible or manifestations of a toxic response.

MATERIALS AND METHODS

Ethoxyquin was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

Weanling male Wistar rats were given a diet containing 0·5% ethoxyquin for 14 days using a pair-feeding experimental protocol similar to that previously reported

with ethoxyquin and nordihydro-guaiaretic acid.[20,21] Two groups of 18-day old male Wistar albino rats were kept with their dams for a 3-day weaning period during which control diet was available *ad lib*. The control diet was Spillers small-animal diet, meal form, to which was added 2·5% v/w antioxidant-free arachis oil (Saladin Brand, Craigmillar Ltd.). At the end of this period, the weanlings were caged singly and the experimental animals fed, *ad lib*., a diet containing 0·5% ethoxyquin, the arachis oil being used as the vehicle. Control rats, paired by weight with experimental partners, were pair-fed with control diet and every day given an amount equal to that consumed during the previous 24 hr by their partners. Fresh diet was prepared at not more than weekly intervals to minimise the possible accumulation of toxic lipid peroxidation products. Body weights were checked daily.

Four animals from the experimental group with their respective controls were killed by cervical fracture at the end of the 14-day period of pretreatment with ethoxyquin (zero day) and thereafter at intervals of 3, 7, 14 and 30 days. Immediately after killing, the livers were rapidly excised, freed from adhering connective tissue, washed in ice-cold 1·15% KCl solution, blotted and weighed. Liver homogenates, 10,000 *g* supernatants and microsomal preparations were then prepared by the method of Basu *et al.*[22]

Portions of the homogenates were used to separate the DNA[23] and determine this quantitatively by the diphenylamine reaction.[24] The 10,000 *g* supernatant was used to assay biphenyl-4-hydroxylase activity by the method of Creaven *et al.*,[25] ethylmorphine-*N*-demethylase activity by the method of Holtzman *et al.*[26] and glucose-6-phosphatase activity by the method of de Duve *et al.*[27] The microsomal suspension was used to determine the concentrations of cytochrome P-450, cytochrome b_5 and microsomal protein by the methods of Sladek and Mannering,[28] Schenkman *et al.*[29] and Lowry *et al.*[30] respectively.

RESULTS AND DISCUSSION

It can be seen (Table 1) that, after 14 days pretreatment with ethoxyquin, the liver weight was increased by 90 per cent compared with pair-fed controls while the body weights were not significantly different. The *decrease* of 35 per cent in DNA *concentration* compared with the 25 per cent *increase* in *total* hepatic DNA at this time suggests that liver enlargement may have resulted both from cell hypertrophy and from hyperplasia. Light microscopy of liver sections stained with haematoxylin and eosin did not reveal any obvious increase in nuclear size or in polynucleated cells and other workers found that induction of microsomal enzymes with phenobarbital did not produce an increase in nuclear volume.[31] On this basis it was calculated from the results of the DNA analyses that the increase in liver mass was due to a 25 per cent increase in cell number and a 50 per cent increase in cell mass.

Concomitant increases both in concentration and total amount in the liver were observed with microsomal protein, cytochrome P-450 and cytochrome b_5 after pretreatment with ethoxyquin and there was a similar increase in biphenyl-4-hydroxylase activity. However, the specific activity of ethylmorphine-*N*-demethylase in the microsomes was depressed by approx. 13 per cent but, due to the increased liver mass, the total activity in the liver increased by 65 per cent relative to pair-fed controls. This observation agrees with the earlier report[20] that ethoxyquin did not consistently increase the specific activity of this enzyme.

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TABLE 1. RECOVERY FROM THE EFFECTS OF DIETARY ETHOXYQUIN OF SOME HEPATIC MICROSOMAL ENZYMES AND OTHER PARAMETERS

Parameters	Period after withdrawal of ethoxyquin (days) 0 Control	0 Test	3 Control	3 Test	7 Control	7 Test	14 Control	14 Test	30 Control	30 Test
1. Body wt (g)	98 ± 3	105 ± 3	122 ± 10	128 ± 7	149 ± 0	154 ± 7	182 ± 3	183 ± 3	293 ± 7	300 ± 14
2. Liver wt (g)	3·6 ± 0·1	7·0 ± 0·1‡	6·0 ± 0·1	8·0 ± 0‡	7·1 ± 0·2	8·8 ± 0·4†	7·9 ± 0·6	9·2 ± 0·5	12·3 ± 0·7	12·7 ± 0·9
3. DNA content										
(a) mg/g liver	3·2 ± 0·1	2·1 ± 0·1‡	2·8 ± 0·1	2·7 ± 0·1	3·5 ± 0·3	3·1 ± 0·1	3·1 ± 0·1	2·9 ± 0·1	2·3 ± 0·1	2·3 ± 0·1
(b) mg/liver	11·5 ± 0·4	14·7 ± 1·3	16·8 ± 1·0	21·6 ± 0·8†	24·9 ± 1·0	27·3 ± 2·0*	24·5 ± 2·8	26·7 ± 2·6	28·3 ± 1·3	29·2 ± 2·4
4. Microsomal protein content										
(a) mg/g liver	28·4 ± 0·6	40·5 ± 1·9	29·4 ± 1·0	32·9 ± 0·8*	25·1 ± 0·8	26·1 ± 0·9	26·8 ± 2·4	26·4 ± 2·0	29·4 ± 0·9	27·7 ± 1·6
(b) mg/liver	102 ± 4	284 ± 4‡	176 ± 15	263 ± 9‡	178 ± 2	230 ± 16†	212 ± 35	243 ± 17	362 ± 16	352 ± 43
5. Cytochrome P-450 content ($91 mM^{-1} cm^{-1}$)										
(a) nmoles/g liver	21·9 ± 0·8	44·0 ± 3‡	20·6 ± 1·4	27·5 ± 1·9*	24·5 ± 0·3	26·4 ± 1·8	24·3 ± 0·9	23·5 ± 2·2	29·4 ± 1·5	28·0 ± 2·9
(b) nmoles/liver	79 ± 2	308 ± 8‡	124 ± 16	220 ± 10‡	174 ± 7	232 ± 1†	192 ± 12	216 ± 33	362 ± 26	356 ± 52
6. Cytochrome b_5 content ($185 mM^{-1} cm^{-1}$)										
(a) nmoles/g liver	14·4 ± 0·8	25·4 ± 0·8‡	11·4 ± 1·2	17·5 ± 0·7†	10·1 ± 0·5	14·6 ± 0·4‡	12·3 ± 0·8	14·2 ± 0·8*	12·6 ± 0·6	13·6 ± 0·4
(b) nmoles/liver	52 ± 2	178 ± 2‡	68 ± 12	140 ± 6‡	72 ± 5	128 ± 8†	97 ± 14	131 ± 14	155 ± 6	173 ± 19
7. Biphenyl-4-hydroxylase activity										
(a) μmoles/hr/g liver	5·1 ± 0·5	8·0 ± 0·9‡	3·3 ± 0·1	4·6 ± 0·1‡	3·2 ± 0·3	3·6 ± 0·1	3·6 ± 0·2	3·4 ± 0·1	3·0 ± 0·1	3·1 ± 0·2
(b) μmoles/hr/liver	18 ± 1	56 ± 5‡	20 ± 1	37 ± 1‡	23 ± 1	32 ± 2†	28 ± 2	31 ± 3	37 ± 1	39 ± 1
8. Ethylmorphine-*N*-demethylase activity										
(a) μmoles/hr/g/liver	38 ± 1	33 ± 1*	33 ± 3	39 ± 6	44 ± 7	42 ± 11	50 ± 2	48 ± 7	34 ± 3	33 ± 1
(b) μmoles/hr/liver	137 ± 3	231 ± 13†	198 ± 32	312 ± 60	312 ± 43	370 ± 38	395 ± 47	442 ± 86	418 ± 52	419 ± 33
9. Glucose-6-phosphatase activity										
(a) μmoles/min/g liver	12·2 ± 0·1	5·5 ± 0·1‡	11·4 ± 0·1	9·7 ± 0·2*	10·6 ± 0·7	9·0 ± 0·5	11·3 ± 0·4	12·0 ± 0·5	11·2 ± 0·3	11·0 ± 0·3
(b) μmoles/min/liver	44 ± 1	39 ± 5	68 ± 4	78 ± 5†	75 ± 3	79 ± 6	89 ± 7	110 ± 9	138 ± 10	140 ± 8

Ethoxyquin was administered at a dietary concentration of 0·5 per cent during the 14-day pretreatment period. Values given are the means ± S.E.M. Each group (control or test) consisted of four animals. Significant effects are shown: * $P < 0·5$; † $P < 0·01$; ‡ $P < 0·001$.

The reduction of the specific activity of glucose-6-phosphatase to 45 per cent of control values appears to result mainly from changes in the composition of the induced endoplasmic reticulum rather than inhibition by ethoxyquin as the *total* amount of glucose-6-phosphatase in the liver was not significantly reduced and *in vitro* studies (A. Rahim and R. Walker, unpublished) have shown that ethoxyquin is not an inhibitor of this enzyme. A fall in glucose-6-phosphatase activity frequently is observed in hepatitis and during the development of some tumours, but long-term studies in rats by ourselves[32] and others[19] using high dietary levels of ethoxyquin did not reveal any obvious signs of chronic hepatitis or tumorogenesis. From this it appears that induction of the drug metabolizing enzymes of the endoplasmic reticulum may lead to a "dilution" of the microsomal glucose-6-phosphatase. Feuer *et al.*[33] have proposed that glucose-6-phosphatase be used as a biochemical index of hepatotoxicity and demonstrated that several known hepatotoxic compounds caused a lowering in the specific activity of this enzyme. However, 2-phenyl-indole and the antioxidant butylated hydroxytoluene, which are not known to be hepatotoxic, also caused a lowering of glucose-6-phosphatase activity and ethoxyquin appears to fall into this category. The specific activity of microsomal glucose-6-phosphatase may be lowered by interference with synthesis or function of this enzyme, or by "dilution" with other enzyme proteins. The former may be considered a toxic response whereas the latter may be adaptive, and a distinction needs to be made in the interpretation of glucose-6-phosphatase activity as an indicator of toxicity.

The course of recovery after withdrawal of ethoxyquin is also shown in Table 1. By the end of the 30-day recovery period liver size and DNA content had returned to normal (not significantly different from controls) indicating that both the hypertrophy and hyperplasia were reversible as would be expected of an adaptive response. Recovery appeared to be biphasic in that recovery from cell hypertrophy was rapid and DNA concentration was normal by the third day while microsomal protein concentration and enzyme concentrations other than cytochrome b_5 were not significantly different from control levels by the 7th day. The recovery from the hyperplasia was somewhat slower, however, and the *total amount* of microsomal protein and the enzymes studied did not fall to control levels until the 30th day although in most cases the differences were not statistically significant after 14 days.

Cytochrome b_5 was unusually slow to recover, the specific activity remaining elevated even after 14 days recovery when microsomal protein concentration was normal. That cytochrome b_5 is slower to return to normal than other microsomal enzymes after induction may be due to the slower turnover rate of this cytochrome compared with cytochrome P-450 and other microsomal enzymes.[34]

The course of recovery from liver enlargement and induction of the microsomal enzymes by ethoxyquin appears to parallel that reported after induction with phenobarbital by Kurijama *et al.*[35] and Bolender and Weibel.[31] The former workers reported that enzyme activities and membranes of the smooth endoplasmic reticulum disappeared together over about eight days while the latter workers, in a detailed morphometric study, also found fairly rapid removal of the smooth endoplasmic reticulum over the course of 5 days. This compares with the time of 7 days for microsomal protein concentration to return to normal in this study and contrasts with the results of Orrenius and Ericsson[36] who found that SER membranes persisted for up to 15 days. However, as in our study microsomal protein was taken as an indicator

of the amount of SER whereas Orrenius and Ericsson used phospholipid concentration, the results are not strictly comparable in the absence of electron microscopic data.

The slow recovery of cytochrome b_5 after microsomal enzyme proteins had returned to normal indicates that some quantitative differences in enzyme activity occur between induced and recovering endoplasmic reticulum and these may be a necessary preliminary to the formation of and scavenging by aurophagic vacuoles, as observed by Bolender and Weibel.[31]

The complete reversibility of the hepatic changes produced in rats by dietary ethoxyquin suggests that the observed hepatomegaly and enzyme induction should be considered a truly adaptive response. However, some microsomal enzyme inducers, particularly phenobarbital, have been shown to potentiate the carcinogenicity of 2-acetamidofluorene when administered subsequently.[37] Conversely, the antioxidant butylated hydroxytoluene (BHT) which also induces hepatic microsomal enzymes, has been reported to inhibit the carcinogenicity of acetamidofluorene and its *N*-hydroxy derivative, but not of diethylnitrosamine or propane sultone.[38,39] No information is available concerning the potentiating or inhibitory effect of ethoxyquin on the carcinogenicity of these compounds although it has been reported that ethoxyquin protects against the carcinogenicity of benz(a)pyrene and 7,12-dimethylbenz(a)anthracene.[11] As these latter carcinogens produce tumours at sites other than the liver it would seem pertinent to investigate the effects of ethoxyquin on the activity of known hepatic carcinogens such as acetamidofluorene.

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