Results and discussion

Induction of transferase bands. Transferase purified from phenobarbital-treated B6 mice was homogeneous by SDS-polyacrylamide gel electrophoresis and had a subunit Mr of 51000. Antibodies against the purified transferase immunocomplexed a 51 kDa and a 54 kDa protein (Fig. 1). Both complexes were recovered by binding to protein A-Sepharose. After the various treatments described in Fig. 1, it can be seen that the 51 kDa and 54 kDa proteins were induced to the greatest extent in B6 mice by phenobarbital and 3-methylcholanthrene and were affected by the effects of these compounds in D2 mice. TCDD induced both bands in both strains equally. In each case with these three inducers, the best response was in the 54 kDa band. Benz[a]pyrene preferentially induced the 54 kDa band, whereas benzanthracene preferentially induced the 51 kDa protein in B6 mice. trans-Stilbene oxide did not affect either protein.

Protease and endoglycosidase digestions. In order to determine whether the two bands contained similar peptides, each band excised from disc gels was digested separately with Staphylococcus aureus V8 protease during electrophoresis on a 12% polyacrylamide/SDS slab gel. The peptide maps were similar, suggesting that both the 51 kDa and 54 kDa proteins were indeed transferase. Both proteins were resistant to trypsin digestion. Since the two proteins had similar peptide fragments and both proteins were recognized by antibodies raised against the 51 kDa protein, it was considered possible that at least a core of primary structure is similar for the two proteins, and that one or both may be further modified by glycosylation and/or proteolytic processing to produce mature proteins with different Mr values. Endoglycosidase H treatment of labelled microsomal membranes released the 51 kDa but not 54 kDa protein. Endoglycosidase D did not affect either protein.

Translation of transferase mRNA. Immunoprecipitation of protein translated in vitro with anti-transferase immunoglobulin G by using total mRNA from phenobarbital-treated B6 mice again generated two protein bands, of about 50 kDa and 54 kDa. After inclusion of dog pancreatic microsomes during translation, the 50 kDa band increased to 51 kDa, and the 54 kDa band was unchanged. The two products translated in vitro had a pattern similar to that in microsomes. These results indeed indicate that the 51 kDa protein in microsomes is post-translationally modified by glycosylation to a mature protein.

The observation that two proteins were generated during the translation in vitro demonstrates that two different mRNA species must code for them. Thus the two proteins exist in microsomes such that one is not a digested product of the other. Furthermore, it is likely that the glycosylated 51 kDa protein is inserted in the luminal side of the endoplasmic reticulum, since that is the only site in the cell known to glycosylate proteins (Boulan et al., 1978). The 54 kDa transferase, however, appears to be unglycosylated and most probably would be located at a different site in the membrane.

The differences in location of these two transferase enzymes in the endoplasmic-reticulum membrane most probably dictates the accessibility of aglycones to the enzymes' active sites and, ultimately, their participation in glucuronidating activity.

DUNDEE MEETING

Differeital induction of UDP-glucuronosyltransferases and their 'permanent induction' in pre-neoplastic rat liver

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Conjugation with glucuronic acid is a major pathway in the metabolism and elimination of drugs and endogenous compounds such as steroids and bilirubin. This reaction is probably catalysed by a family of closely related enzyme forms of UDP-GT with different but overlapping substrate specificities. The enzyme has attracted many scientists because of a variety of interesting features, such as the phospholipid-dependence of its catalytic activity, latency of UDP-GT activity in the microsomal membrane and differential inducibility of the multiple enzyme forms by hormones and xenobiotic inducers (Dutton, 1980). Because of its complex regulation, UDP-GT activity has been difficult to interpret. However, recent advances in the isolation and purification of several enzyme forms of UDP-GT (for references, see Bock et al., 1983) and the availability of antibodies against the enzyme greatly helped elucidation of some of the ambiguities about enzyme induction.

Differential induction of UDP-glucuronosyltransferase activities

Hormones and xenobiotic inducers have been known as regulatory factors of UDP-GT activity since the report of Inscoe & Axelrod (1960). This regulatory control seemed to be remarkably similar to that found previously for cytochrome P-450-dependent mono-oxygenases (Conney et al., 1956; Quinn et al., 1958). Later it was found that the two prototypes of xenobiotic inducers, 3-methylcholanthrene and phenobarbital, differentially increased UDP-GT activities (Bock et al., 1973; Wishart, 1978a). The model of differential induction of rat liver microsomal UDP-GT activities has been extended to a variety of other inducers (Lilienblum et al., 1982; Watkins et al., 1982). It has been
used to classify UDP-GT reactions. At least three groups of UDP-GT activities can be clearly distinguished (Fig. 1). Conjugation of group-1 substrates (mostly planar phenols) is markedly (>2-fold) inducible by 3-methylcholanthrene. Independent regulatory control of group-1 activities is also observed in studies on the perinatal development of rat liver UDP-GT activities (Wishart, 1978b; Dutton & Leakey, 1981). In the latter model group-1 activities appear before birth (late-foetal cluster), whereas all other UDP-GT activities develop after birth (neonatal cluster). Independent regulation as well as co-purification of group-1 activities during enzyme purification (Bock et al., 1979) suggested that group-1 activities may be catalysed by a distinct enzyme form. This enzyme form appears to show some regioselectivity for planar phenolic substrates. Whereas 3-hydroxybenzo[a]pyrene has been classified as a typical group-1 substrate, UDP-GT activity towards 9-hydroxybenzo[a]pyrene was only moderately induced by either 3-methylcholanthrene (1.6-fold) or phenobarbital (1.8-fold). This suggests that 9-hydroxybenzo[a]pyrene is mainly conjugated by enzyme forms different from the 3-methylcholanthrene-inducible UDP-GT.

 UDP-GT activities towards group-2 substrates, including bulky non-planar phenols such as morphine and 4-hydroxybiphenyl, are induced by phenobarbital. Although morphine UDP-GT has been isolated and purified (Bock et al., 1979), the isolated enzyme did not conjugate 4-hydroxybiphenyl. Hence it is questionable whether all group-2 activities are catalysed by a single enzyme form. UDP-GT activities towards bilirubin and testosterone are only moderately inducible by phenobarbital treatment. Selective inducibility of bilirubin UDPGT activity by clofibrate (Lilienblum et al., 1982) and pregnenolone 16a-carbonitrile (Watkins et al., 1982), as well as enzyme purification (Burchell, 1982), suggest that bilirubin UDPGT may be a separate enzyme form. Furthermore, testosterone UDPGT has also been identified as a separate entity (Matern et al., 1982). UDP-GT activities towards group-3 substrates, including oestrone and a number of angiotensins, are not markedly inducible (<2-fold) by either 3-methylcholanthrene or phenobarbital.

Functional heterogeneity of UDP-GT activities, indicated by their differential inducibility, and the results of enzyme purification suggest at least five different enzyme forms of UDP-GT, with 4-nitrophenol, morphine, bilirubin, testosterone and oestrone as typical substrates (see Bock et al., 1983). Genetic evidence suggested that 3-methylcholanthrene-inducible enzyme forms of UDP-GT is co-ordinately induced with cytochrome P450 (Owens, 1977). This co-ordinate induction of some metabolic pathways may facilitate detoxification of arylhydrocarbons (Bock et al., 1981, 1984).

Purification of the major 3-methylcholanthrene-inducible enzyme form of UDP-glucuronosyltransferase: properties of rabbit antibodies against this enzyme form

To be able to distinguish changes in the enzyme amount (induction) and modulation of enzyme activity by alterations of its membrane environment, we purified the 3-methylcholanthrene-inducible enzyme form and raised antibodies against it in rabbits (Pfeil & Bock, 1983). The purified enzyme had a subunit Mr of 54000 (determined by sodium dodecyl sulfate/polyacrylamide-gel electrophoresis) and a Mr of about 220000 (determined by gel chromatography), suggesting the formation of tetramers. This oligomeric structure may greatly influence the incorporation and properties of the enzyme in the microsomal membrane. Rabbit antibodies against UDP-GT showed strong continuous precipitation lines between the purified enzyme and solubilized microsomal proteins. The antibody precipitated and inhibited UDP-GT activities (Fig. 2), but did not precipitate NADPH-cytochrome c reductase. Qualitatively similar results were obtained with antisera or when the amount of IgG was varied. Induction was greater with the solubilized than with the membrane-bound enzyme. Nevertheless the membrane-bound enzyme activity was clearly inhibited by antibody, indicating that antigenic sites of the enzyme are exposed at the cytoplasmic site of the microsomal vesicle. The antibody showed some specificity for the 3-methylcholanthrene-inducible enzyme form, since group-1 activities were precipitated to greater extents than those of other groups (Pfeil & Bock, 1983). With these antibodies, an electroimmunochemical assay was developed to quantify the enzyme. These studies substantiated that the administration of 3-methylcholanthrene, but not of phenobarbital, led to a selective increase in the amount of the enzyme. In studies with isolated hepatocytes from phenobarbital- and 3-methylcholanthrene-treated rats it could be demonstrated that, apart from cofactor supply, the amount of enzyme is a major factor determining glucuronide formation in the intact cell (Ullrich & Bock, 1983).

'Permanent induction' of UDP-glucuronosyltransferase in pre-neoplastic liver lesions and its modulation by the tumour promoter phenobarbital

In previous studies it was recognized that some hepatomas showed high UDP-GT activity (Luenders et al., 1970; Bock & White, 1974). We previously demonstrated that UDP-GT activities towards group-1 substrates were selectively increased in hyperplastic liver nodules obtained by continuous feeding of 2-acetylaminofluorene in the diet (Eriksson et al., 1981; Bock et al., 1982). Epoxide hydrolase and glutathione S-transferase were also increased in these hyperplastic liver nodules. In contrast, cytochrome P450-dependent mono-oxygenases were found to be decreased. This altered pattern of drug-metabolizing enzymes is
This permanent alteration of UDP-GT expression (operosyltransferase activity towards I-naphthol by rabbit IgG to increase gene expression further. Administration of both DUNDEE MEETING
drawn activity could still be further induced by the tumour
guished from regular induction, which is reversible within
ationally termed 'permanent induction') has to be distin-
only N-nitrosomorpholine, UDP-GT activity was still
tumour promoter. In the group of animals which received
incremented 5-fold in focal tissue at 330 days after initiation.
ating various enzyme forms and to elucidate their
control of various endogenous substrates and detoxication
of dietary constituents such as aryl hydrocarbons. Perma-
ly increased UDP-GT activity may be a useful marker
for the altered phenotype observed in pre-neoplastic liver
lesions. It may provide clues to the altered gene expression
occurring at different stages of chemical carcinogenesis and
its modulation by tumour promoters.

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Induction of xenobiotoic-metabolizing enzymes by trans-stilbene oxide and 2-acetylaminoanilofluore: observations on enzyme induction by drugs

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During the past few years our laboratory has been interested in the induction of xenobiotoic-metabolizing enzymes by, among other substances, trans-stilbene oxide and 2-acetylaminoanilofluore (Seidégård et al., 1979; Guthenberg et al., 1980; Lind et al., 1980; Suzuki et al., 1980; Åström & DePierre, 1981; Carlberg et al., 1981; DePierre et al., 1981; Seidégård et al., 1981; Åström & DePierre, 1982; Meijer et al., 1982; Seidégård & DePierre, 1982a,b; Åström et al., 1983; Meijer & DePierre, 1983). These studies have led to certain insights about enzyme induction by drugs which we would like to share with you. In particular, it is time to dispel certain myths about this process which have been around for a long time.

Phenobarbital and 3-methylcholanthrene are representative inducers of drug-metabolizing enzymes . . . or are they?

Table 1 presents the patterns of induction by the so-called classical inducers phenobarbital and 3-methylcholanthrene and by the newly characterized inducers trans-stilbene oxide and 2-acetylaminoanilofluore. A number of characteristics of these patterns should be noted. In the first place trans-stilbene oxide has been found to induce the same isoenzyme of cytochrome P-450 as is induced by phenobarbital, whereas 2-acetylaminoanilofluore induces a form of this cytochrome which is clearly distinct from those induced both by phenobarbital and 3-methylcholanthrene. This seems to be a rather general phenomenon: many xenobiotoics induce the same isozymes of cytochrome P-450 as do phenobarbital or 3-methylcholanthrene, whereas many other xenobiotoics induce distinct isozymes. In addition, many xenobiotoics induce more than one isoenzyme of cytochrome P-450. An important task for the immediate future will be to compare the distinct isozymes induced by different xenobiotoics to determine which of them are the same and thereby classify them into different groups.

Table 1 also shows that 3-methylcholanthrene induces the phase-I P-450 system to a much greater extent than the phase-II enzymes microsomal epoxide hydroxylase and cytosolic glutathione transferases; phenobarbital induces both phase-I and -II reactions to approximately the same extent; whereas both trans-stilbene oxide and 2-acetylaminoanilofluore induce microsomal epoxide hydroxylase and cytosolic glutathione transferases to a much greater extent than they induce the total amount of cytochrome P-450. Of course, many more detailed patterns of induction will be discernible when the induction of different isoenzymes and of other enzymes such as UDP-glucuronosyltransferase, sulphotransferase and DT-diaphorase (NADPCH oxidoreductase) are also taken into account. trans-Stilbene oxide, for instance, has been found to induce all the major isoenzymes of cytosolic glutathione transferase in rat liver (Guthenberg et al., 1980) as well as to be a powerful inducer of cytosolic DT-diaphorase (Lind et al., 1980) (see also below).

It is, of course, of great importance to characterize the pattern of induction of drug-metabolizing enzymes by different xenobiotoics in such detail. Since reactive interme-
derates produced by the cytochrome P-450 system are often the direct cause of the toxic and genotoxic effects of different xenobiotoics, the differential induction of phase-I and -II activities may have a profound influence on the toxicity and genotoxicity of the inducer itself and of other xenobiotoics as well (synergistic effects). In addition, the metabolism of endogenous compounds may be strongly affected by such differential induction (Meijer & DePierre, 1983).

There are also distinct differences in the induction of drug-metabolizing enzymes in extrathepatic tissues by phenobarbital, 3-methylcholanthrene and trans-stilbene oxide (DePierre et al., 1984). For instance, 3-methylcholanthrene induces cytosolic glutathione transferase activity significantly only in the liver, and phenobarbital induces this activity in the intestine as well, whereas trans-stilbene oxide induces cytosolic glutathione transferase activity in the liver, kidney and adrenal gland. Such differences may again have a great influence on the relative susceptibility of different organs to toxic and genotoxic effects.

In view of these and other considerations, it does not seem fruitful to continue to consider phenobarbital and 3-methylcholanthrene as representative inducers with which other inducers should be compared.

It is also apparent from Table 1 that neither phenobarbital, 3-methylcholanthrene, trans-stilbene oxide nor 2-