# Regulation and expression of four cytochromes P-450 isoenzymes, NADPH-cytochrome P-450 reductase, the glutathione transferases B and C and microsomal epoxide hydrolase in preneoplastic and neoplastic lesions in rat liver

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## Summary

Nitrosamine-induced hepatocarcinogenesis has been used to investigate the regulation and expression of different drug-metabolizing enzymes in preneoplastic and neoplastic lesions in the female Wistar rat. The enzymes investigated were two phenobarbital-inducible cytochrome P-450 (cyt. P-450) isoenzymes ($PB_1$ and $PB_2$, mol. wt. 52000 and 53500, respectively), two 3-methylcholanthrene-inducible forms ($MC_1$ and $MC_2$, mol. wt. 54500 and 57000, respectively), NADPH-cytochrome P-450 reductase, the cytosolic glutathione transferases (GSTs) B and C and the microsomal epoxide hydrolase with broad substrate specificity ($mEH_b$).

Carcinogen-induced lesions were identified by use of the known markers of hepatocarcinogenesis adenosintriphosphatase and γ-glutamyl transpeptidase. While the GSTs and $mEH_b$ were increased in all preneoplastic and neoplastic lesions, the levels of the individual cyt. P-450 isoenzymes were characteristically different from each other. In many of the early ATPase deficient islets $PB_1$ was elevated, whereas the content of the other cyt P-450 forms and NADPH-cytochrome P-450 reductase was either unchanged or slightly lowered. At later stages of hepatocarcinogenesis $PB_1$ returned to the levels of the surrounding tissue, while the other cyt. P-450 isoenzymes were decreased, the most prominent reduction being found in $MC_1$. In neoplastic nodules all cyt. P-450s and NADPH-cyt. P-450 reductase were diminuished, some of them dramatically.

These findings indicate that in spite of a common response of groups of P-450s to inducing agents, individual P-450 isoenzymes are also regulated separately. Moreover, the constant elevation of $mEH_b$ and GSTs in all lesions investigated in this study demonstrates that these enzymes, which are largely involved in deactiviation, are regulated in a different fashion from the predominantly carcinogen-activating monooxygenases. The observed differences in enzyme pattern may provide a useful method for subdividing and categorizing preneoplastic and neoplastic lesions.

## Introduction

Chemically induced hepatocarcinogenesis is associated with the sequential appearance of phenotypically altered cell populations, which can be characterized by changes in the expression of different marker enzymes such as canalicular adenosinetriphosphatase (ATPase) (1), 7-glutamyl transpeptidase (γ-GT) (2), glucose-6-phosphatase (3), glucose-6-phosphate dehydrogenase (4) and the microsomal epoxide hydrolase with broad substrate specificity ($mEH_b$) (5, 6). In contrast to $mEH_b$, microsomal epoxide hydrolase with narrow substrate specificity ($mEH_{ch}$) (for characterization see Discussion) remains unchanged in hyperplastic nodules (7). There is increasing evidence that at least some of these enzyme altered foci are precursor lesions, which are causally related to malignant transformation (8, 9). This is substantiated by the observation that neoplastic nodules and hepatocellular carcinoma show enzyme patterns similar to those seen in preneoplastic foci (10, 11). Moreover, strong quantitative correlations between the volume of these foci and subsequent development of liver tumors have been demonstrated (12-15).

The molecular basis of the alterations in enzyme expression in preneoplastic and neoplastic cells remains unclear. Recent investigations showed that the preneoplastic lesions are monoclonal in origin (16, 17) and grow faster than the surrounding liver tissue (18,19). This increased cell proliferation may be due to genetic changes leading to inherently altered growth properties. Alternatively, it has been suggested that different susceptibility of normal and preneoplastic cells to hepatotoxins will lead to selective growth of preneoplastic and neoplastic cells (20). This concept was substantiated by the observation that the levels and activities of microsomal monooxygenases and NADPH-cytochrome P-450 reductase are reduced in hyperplastic nodules and hepatomas (21-25). On the other hand, $mEH_b$ (5, 6), cytosolic glutathione transferases (GSTs) (24,26) and UDP glucuronyl transferase (24, 27), which play largely detoxifying roles, are increased in preneoplastic and benign neoplastic lesions. In contrast to the observations made in neoplastic tissues, very little information is available on the expression of monooxygenase enzymes in early preneoplastic stages and on the behavior of individual isoenzymes (28). We therefore studied the levels of four different cyt. P-450 isoenzymes ($PB_1$, $PB_2$, $MC_1$, $MC_2$), NADPH-cytochrome P-450 reductase, the GSTs B and C and $mEH_b$ in preneoplastic and neoplastic lesions in rat liver by immunohistological techniques. A preliminary report of these results has appeared elsewhere (29).

## Materials and methods

Goat anti-rabbit IgG antibody labelled with horseradish peroxidase was obtained from Medac (Hamburg, FRG), 3,3'-diaminobenzidine-tetrahydrochloride from Polysciences (Warrington, USA), γ-L-glutamyl-4-methoxy-ß-naphthylamide from Bachem (Bubendorf, Switzerland), and p-rosaniline from Serva (Heidelberg). Nitro blue tetrazolium hydrochloride was purchased from Merck (Darmstadt, FRG), while NADPH was obtained from Boehringer (Mannheim, FRG). All other chemicals were of the highest grade available from commercial sources.

*Purification of proteins and preparation of antibodies*

Cyt. P-450 isoenzymes ($PB_1$, $PB_2$, $MC_1$ and $MC_2$), NADPH-cyt. P-450 reductase, the glutathione transferases B and C and microsomal epoxide hydrolase ($mEH_b$) were purified

from the livers of male Sprague Dawley rats (180-200 g) using methods reported previously (30-33). Antisera were also prepared using procedures previously described (30).

*Treatment of animals with carcinogens*

Female Wistar rats were obtained from Zentralinstitut für Versuchstiere (Hannover, FRG) and kept on a standard diet (Altomin pellets, Altromin, Lage, FRG) and water *ad libitum* with a daily light and dark cycle of 12 h each. The animals were allowed to acclimatize to their environment for at least 1 week prior to the start of the experiments. Animals weighing an average of 70 g at the beginning of the experiment were treated with diethylnitrosamine (DEN) at a dose level of 50 or 100 p.p.m. in the drinking water for 10 days. Total carcinogen uptake was reckoned to be 125 mg/kg and 200 mg/kg, respectively. To study the development of enzyme-altered foci in liver, groups of animals were sacrificed at various time-points after cessation of carcinogen treatment, as indicated in the legends to the figures.

In an additional experiment rats were treated continuously with either DEN (10 mg/kg for 8 weeks) or dimethylnitrosamine (DMN, 3 mg/kg for 22 weeks) by stomach tube (3 ml/kg in olive oil) on 5 days of each week. Carcinogen treatment was stopped 14 days before the preparation of tissue samples.

*Preparation for histochemistry*

The abdomen was opened under ether anaesthesia and livers were carefully removed. The large median lobe was excised and immediately frozen. Serial sections of 10 μm were prepared at -15°C on a cryostat microtome and used for enzyme histochemical and immunohistochemical procedures. The first two sections were stained for ATPase and γ-GT activity, while the following four sections were used for immunohistochemical incubation with antisera against four different isoenzymes of cyt. P-450. The next section was stained for ATPase activity, and the following four sections were used for the immunohistochemical demonstration of NADPH-cyt. P-450 reductase, GST B, GST C, and $mEH_b$.

*Enzyme histochemistry and immunohistochemistry*

ATPase activity was demonstrated according to the method of Wachstein and Meisel (34), γ-GT activity according to Lojda *et ad.* (35) using γ-L-glutamyl-4-methoxy-β-naphthylamide and p-rosaniline as the coupling agent. The slides were counterstained with hemalum. NADPH-tetrazolium reductase activity, which is predominantly catalyzed by NADPH-cytochrome P-450 reductase, was demonstrated by the use of nitro blue tetrazolium (NBT). Freshly prepared liver sections were mounted on albumin-coated slides, air dried, washed with PBS (see below), and incubated at room temperature in PBS containing 1.2 mM NBT and 7.2 mM NADPH until an intense staining was visible (5-10 min). Following a wash in PBS the sections were dehydrated and mounted under cover slips.

Immunohistochemical demonstration of the different drug-metabolizing enzymes was performed by the 'Sandwich' technique using the following procedure. Air dried cryostat sections mounted on albumin-coated slides were washed with 0.05 M phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS) for 5 min, and then fixed with a p-benzoquinone solution [0.5% in 0.02 M $CaCl_2$, 0.2 M sodium cacodylate, pH 7.4 (36)] for 5 min. Sample preparation was followed in the sequence: PBS wash (2 x 5 min), graded methanols, 95% methanol containing 0.01% $H_2O_2$ (30 min, to suppress endogenous peroxidase activities), graded methanols and PBS wash (2 x 5 min). The sections were then washed with PBS/S (PBS supplemented with 1% bovine serum albumin and 0.35 M NaCl, 5 min) and incubated with non-immune goat serum (diluted 1:30 with PBS/S) for 5 min. Subsequently they were

incubated with antisera to the different enzymes (diluted with PBS/S to suitable extents in the range of 1:700 to 1:1600) in a humidified chamber at 4°C for 24 h. The following washing with PBS/S (3x5 min), treatment with non-immune goat serum (5 min), and incubation with goat anti-rabbit IgG antibodies labelled with horseradish peroxidase (1:20 in PBS/S) for 20 min was performed at room temperature. Sections were washed with PBS (3 x 5 min) to remove unbound immunoglobulins, and peroxidase activity was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris-HCl buffer (37). After washing with PBS (3 x 5 min), sections were treated with 0.1% $OsO_4$ in $H_2O$ (1 min), dehydrated with graded ethanols, and mounted under cover slips. Control incubations were performed either by sbstitution of the first antiserum with non-immune rabbit serum or by omission of anti-rabbit IgG antibodies. The incubation with non-immune rabbit IgG gave a slight staining which was uniform over the entire liver lobule from untreated rats. In carcinogen-treated rats, however, the non-specific staining was slightly lowered in preneoplastic and neoplastic lesions.

All sections were examined by transmitted light microscopy using a comparative microscope, where overlays of two sections can be examined simultaneously. Semi-quantitative analysis of antibody binding to liver tissues was performed by microscope photometry of the peroxidase-staining product using a Leitz microscope photometer 'MPV compact' equipped with an interference filter (S 433-20). The staining intensity of islet tissue was measured and related to that of the surrounding normal tissue. No interference of cellular particles with the DAB staining was observed.

## Results

The expression of two phenobarbital-inducible cytochrome P-450 isoenzymes ($PB_1$ and $PB_2$), two 3-methylcholanthrene-inducible forms ($MC_1$ and $MC_2$), NADPH-cytochrome P-450 reductase, the glutathione transferases B and C and microsomal epoxide hydrolase ($mEH_b$) were studied by immunohistological techniques at various stages of nitrosamine-induced hepatocarcinogenesis. Changes in the activities of the known markers ATPase and γ-GT were used to identify carcinogen-induced lesions. The immunohistochemical technique employed has the advantage that it enables one to demonstrate expression and localisation of several enzymes in very small lesions which appear early on, but the limitation is that it recognizes amounts of immunoreactive protein and not necessarily enzymic activities. Moreover, it has to be borne in mind that cross-reaction with closely related proteins cannot be excluded.

Distinct changes in the expression of all enzymes relative to the surrounding tissue were observed at different stages of hepatocarcinogenesis (Figures 1-3, *cf. to original paper*). The ATPase deficient islets shown in Figure 1 are characterized by an increased cyt. P450 $PB_1$ level, whereas the other cyt. P-450 isoenzymes are either unchanged or slightly decreased. In the focus shown in Figure 2, the level of $PB_1$ is within the range observed in the surrounding normal tissue, while $MC_1$, $MC_2$ and $PB_2$ are clearly lower. In the neoplastic nodule shown in Figure 3 all cyt. P-450 isoenzymes are decreased, some down to very low levels. In all of these lesions $mEH_b$, GST B and GST C were increased. Interestingly, the reduction in immuno-staining of $PB_2$ closely matched that of NADPH-cyt. P-450 reductase. As Figure 2 shows, the activity of cyt. P-450 reductase demonstrated by means of the NADPH-dependent reduction of NBT corresponded to the content of immunoreactive protein.

The photomicrographs provide typical examples of lesions with alterations in the expression of the various drug metabolizing enzymes. However, not all islets showed significant changes with regard to their monooxygenase contents. To analyze the frequency of cyt. P-450 alterations during nitrosamine-induced hepatocarcinogenesis, all ATPase-deficient lesions which appeared following limited or chronic carcinogen exposure were graded as possessing increased, unchanged or decreased levels of each of the different isoenzymes. The relative proportions of islets with changes in the expression of the individual cyt. P-450 isoenzymes are depicted in Figure 4. Following short-term DEN exposure, a considerable number of ATPase-deficient islets with increased $PB_1$ levels was observed to appear early on. At this time the levels of the other cyt. P-450 isoenzymes were mainly unchanged or slightly decreased. Further in the course of hepatocarcinogenesis the relative incidence of $PB_1$-elevated islets continuously diminished, whereas the frequency of ATPase-deficient lesions with lowered levels of the other cyt. P-450 forms increased. This was most pronounced with respect to $MC_1$ indicating that this isoenzyme is most rapidly lost in preneoplastic lesions, followed by $MC_2$, $PB_2$ and NADPH-cyt. P-450 reductase. A reduction in the content of all cyt. P-450 isoenzymes occurred in neoplastic nodules. By contrast with limited carcinogen exposure, a focal elevation of $PB_1$ was not found during continuous treatment of animals with DMN or DEN, whereas foci which displayed a decrease in $MC_1$ $MC_2$ and $PB_2$ isoenzymes were observed much earlier on.

The extent of changes in cytochrome P-450 concentrations within the enzyme-altered lesions was analyzed semiquantitatively by microscope-spectrophotometry of the peroxidase product. Staining intensity measured in islet tissue was expressed relative to that of the surrounding tissue (Figure 5). These data confirm the observation that following the initial increase in $PB_1$ the levels of all cyt. P-450 isoenzymes steadily decreased as the lesions progressed. In close agreement with the data shown in Figure 4, $MC_1$ was the most decreased of all, $MC_2$ and $PB_2$ were less decreased, while $PB_1$ was only slightly lowered.

In contrast to the differential expression of cyt. P-450 isoenzymes during hepatocarcinogenesis, both GST groups and $mEH_b$ were increased in all the preneoplastic and neoplastic lesions investigated. As demonstrated schematically in Figure 6 (*cf. to original paper*), this elevation was most pronounced with respect to $mEH_b$, but also clearly evident for the two GST groups, the GST C levels being usually increased to a greater extent than those of GST B.

In general, focal alterations in the phenotypical expression of the P-450s, GSTs and $mEH_b$ were associated with ATPase deficiency. In a few cases, however, an elevation of either $PB_1$ $PB_2$ or $mEH_b$ without clear changes in the activities of the marker enzymes ATPase or γ-GT was observed. The nature and significance of these alterations remains to be clarified.

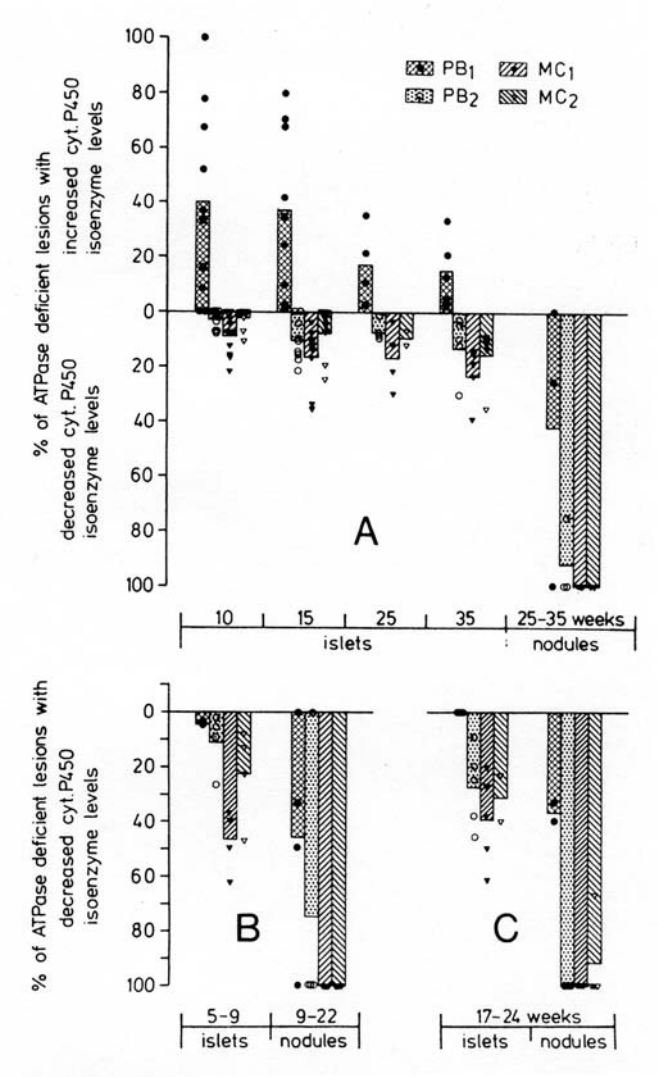


**Fig. 4.** Relative incidence of ATPase-deficient foci with altered levels of the different cyt. P-450 isoenzymes. The number of lesions with altered levels of each individual isoenzyme per liver section was determined using a comparative microscope and related to the total number of ATPase deficient lesions.

**A**: Animals were treated with DEN (50 or 100 p.p.m. in the drinking water for 10 days). Since there were no obvious differences between the two treatment groups, the values obtained were combined.

**B**: Animals were treated with DEN (10 mg/kg) on 5 consecutive days per week for up to 8 weeks. After treatment had finished, animals were examined for nodules for a further 14 weeks.

**C**: Animals were treated with DMN (3 mg/kg) on 5 consecutive days per week for up to 22 weeks. Carcinogen treatment was stopped 14 days before preparation of tissue samples. Time (weeks) gives the observation period after the start of carcinogen treatment. Each point represents a value from one animal, while columns indicate mean values of 4-10 animals.

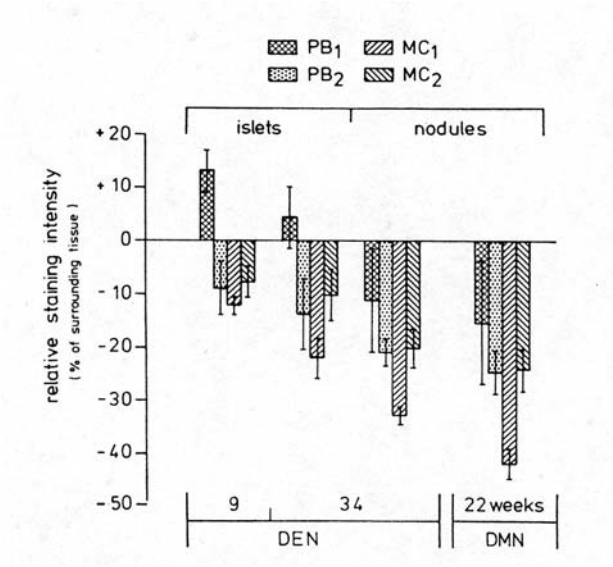


**Fig. 5.** Extent of changes in the levels of the different cyt. P-450 isoenzymes in preneoplastic and neoplastic lesions. Semi-quantitative microscope photometric measurements of the immunoperoxidase-DAB staining were performed as described in Materials and methods.

**DEN**: animals were treated with DEN (100 p.p.m. in the drinking water) for 10 days.

**DMN**: animals were treated with DMN (3 mg/kg by stomach tube) on 5 consecutive days per week.

Tissue samples were taken 2 weeks after treatment ended. Each value represents the mean ± SD of 3-5 measurements within 5-10 lesions.

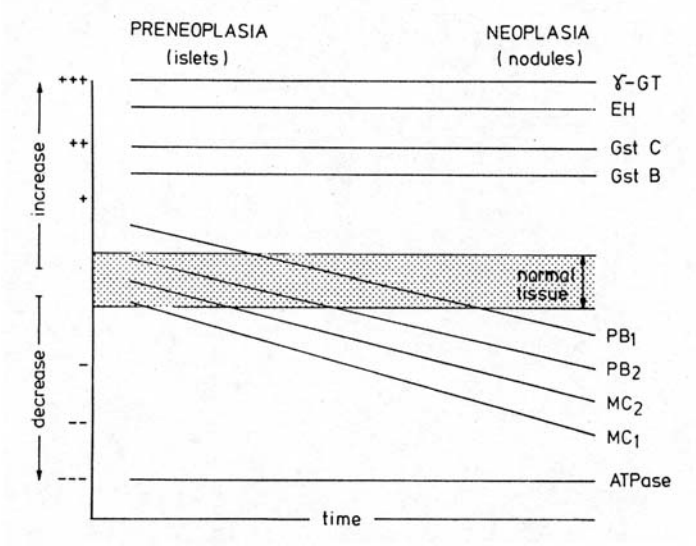


**Fig. 6.** Schematic illustration of changes in the expression of four cyt. P-450 isoenzymes ($PB_1$, $PB_2$, $MC_1$ and $MC_2$), GST B and C, $mEH_b$, and the marker enzymes ATPase and γ-GT observed in this study.

## Discussion

In the present study we have analyzed the expression of two phenobarbital-inducible and two 3-methylcholanthrene-inducible cyt. P-450 isoenzymes ($PB_1$, $PB_2$ and $MC_1$, $MC_2$, respectively), NADPH-cytochrome P-450 reductase, the glutathione transferases B and G, and microsomal epoxide hydrolase ($mEH_b$) by immunohistological techniques in order to establish the sequence and significance of alterations of these enzymes during nitrosamine-induced hepatocarcinogenesis. mEHb is a microsomal epoxide hydrolase with a broad substrate specificity which shows high activity towards benzo[a]pyrene 4,5-oxide. This form is clearly distinguishable from $mEH_{ch}$, which is characterized by a narrow specificity for cholesterol 5α,6α-oxide (38). The cyt. P-450 isoenzymes $PB_1$, $MC_1$ and $MC_2$ appear to be equivalent to those referred to by Ryan *et al.* (39) as forms b, d and c, respectively. Cyt. P-450 $PB_2$ is a novel PB-inducible enzyme with an apparent mol. wt. of 53 500, which displays a ferrous heme-carbon monoxide maximum at 447 nm (31).

A certain degree of cross-reactivity of the antibodies to the cytochrome P-450 isoenzymes, as determined by the sensitive enzyme-linked immunosorbent assay, has been reported (30, 31). However, in recent studies using the Western blot procedure, the only cross-reactivity observed was anti $MC_2$ with $MC_1$ (Adams, Seilman, Amelizad, Oesch and Wolf, in preparation). This finding, together with the previously reported differential distribution of the cyt. P-450 isoenzymes (31), indicates that cross-reactivity of the different antibodies used was not a significant contributing factor to the data described in this study. The possible reactivity with as yet unidentified cyt. P-450 forms, however, cannot be ruled out. Indeed, Western blots of liver microsomes from controls and phenobarbital-treated rats revealed cross-reactivity of anti $MC_1$ with a protein (mol. wt. 51 000) not equivalent to $MC_1$ (Adams *et al.,* in preparation).

The antibodies to GSTs used in this study had been raised against GST B (subunit structure Ya Yc) and GST C (subunit structure Yb Yb'). These antibodies are not specific for these forms as anti GST B will also react with ligandin (subunits Ya Ya) and GST AA (subunits Yc Yc), while anti GST C will also react with GST A (Yb Yb) and GST X (Yb' Yb') (for nomenclature see 32, 40, 41). Investigations using antibodies to the specific subunits will be needed to identify the individual expression of these six transferase forms.

Differential but characteristic changes in the expression of the different cyt. P-450 isoenzymes during nitrosamine-induced hepatocarcinogenesis were found. These changes were dependent on the duration of carcinogen administration. Since the carcinogen treatment schedule used strongly influenced the rate at which the ensuing events occurred, this schedule also proved to be an important factor. Following limited carcinogen exposure, a considerable number of those islets which made early appearances showed increased levels of $PB_1$, whereas the other cyt. P-450 forms were unchanged or slightly lowered. Later in the time course of hepatocarcinogenesis a growing number of islets and all nodules exhibited a progressive reduction in the levels of the four individual cyt. P-450 isoenzymes and NADPH-cyt. P-450 reductase. In these lesions the background staining observed using non-immune rabbit serum instead of specific antibodies was also slightly lowered. Although this may influence the absolute values of the staining intensities of the individual isoenzymes, the relative relationships are not affected. By contrast with the effects of limited carcinogen administration, in the course of continuous carcinogen exposure none of the islets showed an elevation of $PB_1$, while the decreases in all cyt. P-450 isoenzymes were more pronounced and occurred much earlier. The causes and basic mechanisms of the differences in enzymic expression between limited and continuous carcinogen exposure have proved to be complex, and will be discussed in detail elsewhere.

Increased levels and activities of various cytosolic GST isoenzyme have been reported in hyperplastic nodules (24, 26). In this study we have demonstrated that already at a much earlier stage GST B and C are increased in small preneoplastic lesions. Indeed it would appear that GSTs could be classified as preneoplastic antigens in a similar fashion to that described for $mEH_b$ (42), which is increased in preneoplastic and benign neoplastic lesions but becomes lost from these cells as they become malignant (5).

The overall alterations observed in the expression of cyt. P-450 isoenzymes appear to be compatible with current concepts of hepatocarcinogenesis. Farber showed that preneoplastic and neoplastic cells are less sensitive to the toxic action of 2-acetylamino-fluorene (2-AAF) and other hepatocarcinogens or hepatotoxins which need metabolic activation (43-45). He postulated that as a result these cells should have a proliferative advantage, provided that the proliferation of normal cells becomes suppressed by the administration of such compounds (20). The selective resistance of premalignant and malignant cells to cytotoxicity has been attributed to alterations in drug metabolizing enzymes. This assumption was substantiated by the observation that predominantly activating enzymes such as cyt. P-450 are decreased in hyperplastic nodules and hepatomas (21-25), whereas the preferentially detoxifying enzymes mEHb, GSTs and UDP glucuronyl transferase were found to be increased in premalignant lesions (5, 6, 24, 26, 27). Corresponding to these observations, our results are in accordance with the above mentioned hypothesis, although the finding that cyt. P-450 isoenzyme $PB_1$ is elevated in early preneoplastic foci may appear to be contradictory. In view of the substrate specificities of the different cyt. P-450 isoenzymes towards 2-AAF, however, it is of special interest that cyt. P-450 $MC_1$ is most rapidly lost in preneoplastic lesions. This isoenzyme (46, 47), and/or structurally related proteins (48; Robertson *et al.*, in preparation), are intimately involved in the metabolic activation of 2-AAF, whereas the PB-inducible forms do not appear to play a role in the activation of this compound, but rather in its detoxification (49, 50). Thus the pattern of the cyt. P-450 isoenzymes in the early lesions is consistent with Farber's proposal of an increased resistance of these cells to 2-acetylaminofluorene, which is used as the selective agent in his system. In this context it should be borne in mind that the immunohistochemical procedures used in this study only identify immunoreactive protein and not enzyme activities. Whether the enzyme proteins (especially $PB_1$) are functionally active or not is currently under investigation. Regarding the NADPH-cyt. P-450 reductase, where contents of immunoreactive protein and enzymic activities could be compared, concurrent changes were always found. The fact that in preneoplastic cells the contents and activities of the reductase were never increased but progressively lowered may bear directly on the question of the fünctional state of the monooxygenase system.

The significance of the differential expression of cyt. P-450 isoenzymes within preneoplastic lesions for hepatocarcinogenesis is unclear and whether the same findings would be obtained using other experimental models or not remains to be demonstrated. However, the presented data may provide a useful way of differentiating between and categorizing islet subpopulations by means of their cyt. P-450 pattern. Based on the concept of selective resistance it should be possible to characterize discrete phenotypes in terms of their proliferative potential: Islets showing increased levels of $PB_1$ combined with unchanged contents of other isoenzymes are likely to be susceptible to hepatotoxins, and consequenty should represent a slowly proliferating subpopulation. On the other hand, islets which display decreased cyt. P-450 levels may indicate resistant populations with a selective growth advantage. The question remains as to whether these considerations also apply to those experimental conditions, where short-term or even single carcinogen exposure gives rise to the neoplastic process without any further treatment. Quantitative analysis of the evolution of ATPase-deficient lesions during

the course of limited DEN exposure demonstrated that, following discontinuation of carcinogen treatment the number of ATPase-deficient islets rises for a certain period of time, and then, having reached a maximum, gradually decreases until a steady-state level is established (12-14). It is therefore conceivable that enzyme-altered cells are subjected to physiological turnover, unless they have acquired an increased proliferative potential, i.e., due to a more neoplastic character. Comparison between the time course of the proportion of $PB_1$ elevated islet populations and that of total ATPase-deficient lesions reveals similar characteristics, showing an initial maximum followed by a continuous decrease. In contrast, the proportion of lesions with reduced monooxygenase levels steadily increases in the course of hepatocarcinogenesis, the content of cyt. P-450 being generally lower in neoplastic nodules and tumors which appear later on. This process could well be explained by a selective outgrowth of those subpopulations which show the phenotype of the later stages very early on, either due to the mechanism of selective resistance as discussed above, or by the intrinsic possession of a more neoplastic character.

An alternative explanation for this process would be to assume that the levels of the individual cyt. P-450 isoenzymes within one focus become continuously decreased during its progression to malignancy. In support of this possibility is our finding that the extent of reduction of the different cyt. P-450s is generally more pronounced in nodular lesions than in early foci (see Figure 5). The observation of a gradual decrease is consistent with numerous findings regarding the behaviour of other enzyme markers during hepatocarcinogenesis (4, 5), and supports the concept that a regular sequence of alterations in enzymic expression occurs during the development of malignancy (9, 51, 52).

The molecular basis of the divergent alterations in enzyme expression during hepatocarcinogenesis is unclear. In principle these alterations could simply result from primary genotoxic effects leading to mutational events in genetic structures, which are directly related to the monooxygenase system. Preliminary studies on the inducibility of drug metabolizing enzymes in preneoplastic and neoplastic lesions have demonstrated that a considerable number of foci and nodules are capable of expressing increased levels of $PB_1$ and $PB_2$ following phenobarbital treatment (manuscript in preparation). The extent of induction was comparable with that seen in normal tissue, demonstrating that preneoplastic and neoplastic cells still contain the genetic systems required for cyt. P-450 expression. Thus the decrease in cyt. P-450s during hepatocarcinogenesis may be due to genotoxic effects of the carcinogen on regulatory systems of a higher order. The multiplicity of enzymic alterations within one and the same focus strongly supports this assumption. The various alterations may then be explained by adaptive changes in enzyme synthesis and/or turnover as a result of cellular imbalance in the physiological pattern of effectors, e.g., substrates and metabolites. Our previous investigations on the lobular localization and inducibility of drug metabolizing enzymes in normal cells produced evidence that, in spite of a common response of groups of P-450s to inducing agents, individual cyt. P-450 isoenzymes are also regulated separately (30, 31). With regard to the present study, it would appear that the non-uniform changes in the expression of the individual P-450s in early preneoplastic lesions, i.e., increase of $PB_1$ versus slight decrease of the other cyt. P-450 isoenzymes, are controlled by those mechanisms which act separately for each form. The principles which are common to all isoenzymes might be responsible for the gradual decrease in all cyt. P-450s during the further course of hepatocarcinogenesis. Moreover, there is additional evidence to suggest that the predominantly carcinogen-activating cyt. P-450s are regulated in a different fashion from the preferentially deactivating enzymes $mEH_b$ and GSTs, which are elevated in all lesions investigated. It would be interesting to obtain further information on enzyme regulation in preneoplastic cells so as to understand better enzymic involvement and significance in the process of malignant transformation.

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