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Histochemical Demonstration of Enzymes Related to NADPH-Dependent Hydroxylating Systems in Rat Liver after Phenobarbital Treatment

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Summary. Male rats were given 100 mg phenobarbital for three days intraperitoneally. Biochemically an increase was found in activity of nitro-anisole demethylation and in the content of cytochrome P-450. Enzymhistochemically an increase in activity was noted for NADPH tetr. red., G6PD, ICD, and Naftol AS-D-esterase; a decrease was seen in G6Pase and glycogen, but no difference was found in NADH tetr. red. From these results it has been suggested that NADPH tetr. red. is directly involved in the hydroxylation chain, while G6PD and ICD are more indirectly involved.

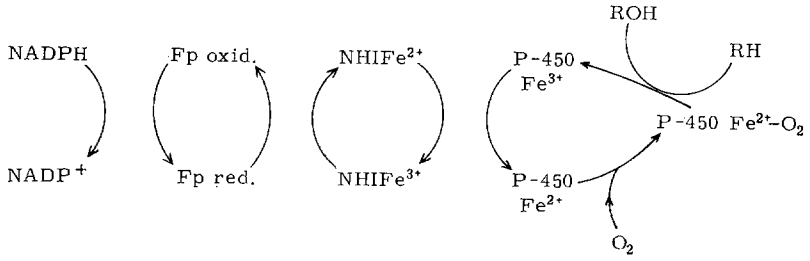
Introduction

A number of steroids and foreign compounds (drugs) can be metabolized by livermicrosomes in the presence of NADPH[1] and O_2. Reactions catalyzed by these enzymes include N-dealkylation, ether cleavage and steroid-hydroxylations (GILETTE, 1966). It seems to be likely that normal substrates for these enzymes are steroids (KUNTZMAN *et al.*, 1964). In the presence of NADPH these enzyme systems catalyze the introduction of one atom of atmospheric oxygen into the substrate, while reducing the other to water.

In steroid producing cells of adrenal, ovary and testes a similar oxygen activating system is present in microsomal as well as in mitochondrial fractions. In the microsomal fraction 17 and 21 hydroxylations are present (TAMAOKI and SHIKITA, 1966; COOPER *et al.*, 1963), while in the mitochondrial fraction 11 and 18 hydroxylations are found (KIMURA and SUZUKI, 1967; RAMAN *et al.*, 1966). In liver drug metabolizing enzymes are found in microsomes. Microsomes, as isolated by differential centrifugation, appear to consist of fragments arising from endoplasmic reticulum and other membranes during cell rupture. The drug metabolizing enzymes appear to be concentrated in the lighter particles that are derived from the smooth surfaced endoplasmic reticulum (JONES and FAWCETT, 1966). An increase in the activities of drug metabolizing enzymes has been shown to be induced by a number of drugs, polycyclic hydrocarbons, testosterone (CONNEY and BURNS, 1962), and progesterone (EMANS and JONES, 1968). After phenobarbital or progesterone treatment a hypertrophy of liver cell smooth endoplasmic reticulum is seen (JONES and FAWCETT, 1966; EMANS and JONES, 1968). In the last years it has become clear that NADPH dependent oxidative enzymes

1. *List of Abbreviations*; NADH = nicotinamide adenine dinucleotide. NADPH = nicotinamide adenine dinucleotide phosphate. NADPH tetr. red. = NADPH tetrazolium reductase. G6PD = glucose-6-phosphate dehydrogenase. ICD = iso-citric acid dehydrogenase. G6Pase = glucose-6-phosphatase. PAS = periodic acid-Schiff method.

are complex systems. The system consists of a flavoprotein, a non haem bound iron protein and a cytochrome called P-450. The chain given in Scheme 1 has been generally accepted (WILSON *et al.*, 1968; PERON and CALDWELL, 1968; ELEMA, 1969).



Scheme 1. Scheme of components of the electron transport system in hydroxylations. *Fp oxid.* oxidized flavoprotein, *Fp red* reduced flavoprotein, *NHI* non-haem bound iron protein, *P-450* cytochrome P-450, *RH* substrate, *ROH* hydroxylated substrate

Until now only one histochemical method has been described for NADPH-dependent hydroxylation reactions (WATTENBERG and LEONG, 1962). In this report evidence is given that one of the enzymes in the NADPH dependent hydroxylating system, possibly the flavoprotein, is responsible for the reduction of nitro-BT. Further we noticed a close relationship between Naftol AS-D esterase and hydroxylating capacity.

Materials and Methods

Animals used in this study were male Wistar rats weighing 250—275 g from the Centraal Proefdieren Bedrijf T.N.O. Zeist, the Netherlands. Ten animals were given injections of phenobarbital-Na intraperitoneally for three days, 100 mg/kg in physiologic salt solution, at half past ten a.m. Ten controls received physiologic salt solution only. Twenty-four hours after the last injection animals were decapitated and exsanguinated. A portion of the liver was frozen with CO_2; 10 μ sections were cut in a cryostat for enzyme histochemistry. The rest of the liver was used for biochemical investigations.

Biochemical Methods. The liver was homogenized in 0.75 M sucrose; microsomes were prepared by centrifugating the 9,000 g supernatant at 105,000 g for 60 minutes. Hydroxylations were carried out as described by JANSON (1968). A typical reaction mixture contained: 0.5 ml enzyme preparate ($\pm$ 50 mg microsomes), 0.1 ml NADP (6.1 mg/ml), 0.1 ml G-6-Phosphate (30.4 mg/ml), 0.1 ml substrate ($\pm$ 25 μmol/ml), 0.1 ml magnesium sulphate (24.6 mg/ml) and 10 μl G6PD (Boehringer 10 mg/ml). Aniline hydroxylation was measured by coupling the formed p-amino-phenol with phenol and reading the extinction at 630 nm after 30 minutes (IMAI and SATO, 1966). Nitro-anisole demethylation was measured by reading the colour at 415 nm of the formed p-nitro-phenolate at P_H 10. Cytochrome P-450 was determined as described by OMURA and SATO (1964).

Staining Reactions. The cryostat sections were stained with the periodic acid Schiff reaction (PAS). The following enzyme reactions were performed: NADPH tetr. red. (NACHLAS *et al.*, 1958b), NADH tetr. red. (NACHLAS *et al.*, 1958a), G6PD, ICD (NACHLAS *et al.*, 1958b), G6Pase (WACHSTEIN and MEISEL, 1957), Naftol AS-D esterase (GÖSSNER, 1958) and alkaline phosphatase (BURSTONE, 1961; GOMORI, 1959). In the alkaline phosphatase and esterase methods the sections were fixed in a mixture of 10 ml formaldehyde (40%), 90 ml dextran (6%), and 1 g calcium chloride for 10 minutes. After washing the staining reactions were carried out according to the methods described above. The G6Pase was performed on unfixed sections. Prior to incubation the sections for the dehydrogenase

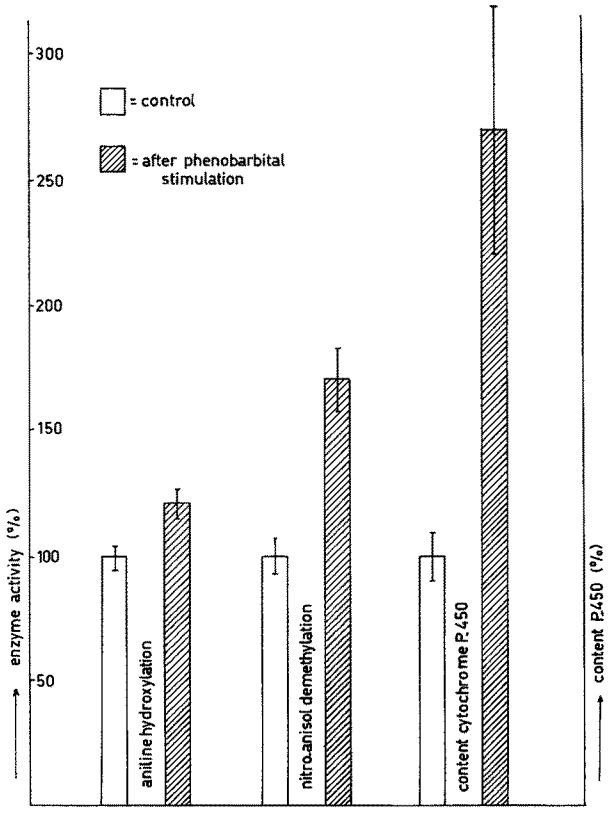


Fig. 1. Activities of aniline hydroxylation, nitro-anisole demethylation and content of cytochrome P-450. Activities are given as percentages of controls. Figures are given $\pm$ 1 standard deviation

methods were placed in acetone at $-20°$ C for 30 minutes. The sections were then dried in air and incubated in the incubating media. After staining the sections were fixed in the formaline dextran mixture and embedded in glycerol gelatine.

Results

Biochemical Findings

The biochemical findings are given Fig. 1. Activities of enzymes and content of cytochrome P-450 are given as percentages of controls. The activities are calculated as product formed per g wet liver per 15 min. No difference in the ratio of activities was found if we calculated the conversion per mg microsomal protein. As can be seen from the figure no significant increase was found for aniline hydroxylation, as was found by ARCASOY *et al.* (1968).

Histochemical Findings

NADPH tetr. red. In controls the activity is predominantly localized pericentrally. After phenobarbital treatment there is an increase in activity in the pericentral area (Fig. 2 and 2a).

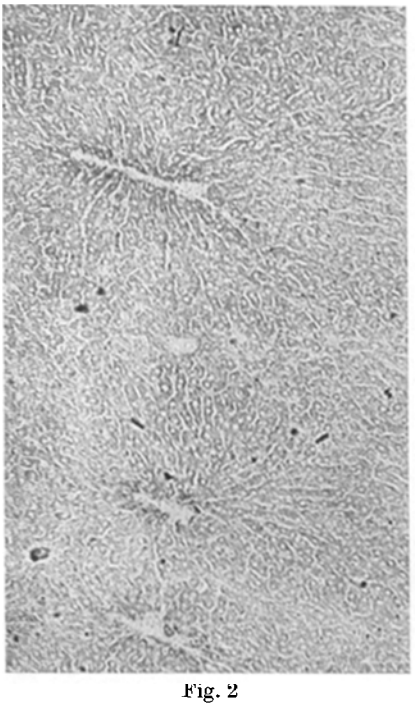


Fig. 2

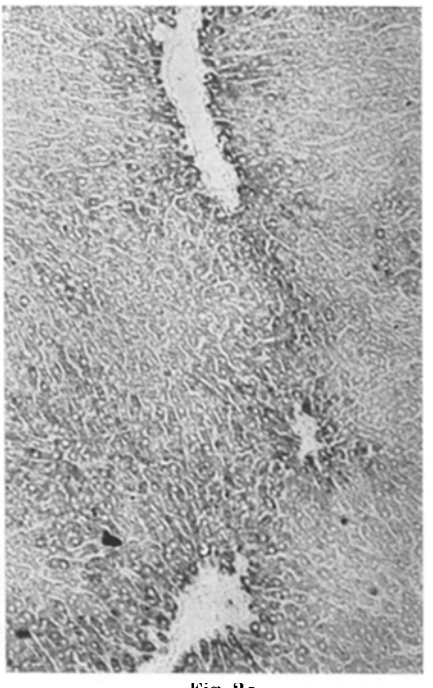


Fig. 2a

Fig. 2 and 2a. NADPH tetr. red. for and after phenobarbital stimulation. × 80

NADH tetr. red. In controls the activity is more diffusively distributed over the whole liver lobule than NADPH tetr. red. After phenobarbital treatment there is no or little difference to control. Only a few cells in the pericentral area gave a more intense blue staining after phenobarbital (Fig. 3 and 3a).

Naftol AS-D Esterase. In controls the activity is predominantly localized pericentrally. After phenobarbital treatment there is an increase in activity pericentrally (Fig. 4 and 4a).

ICD. In controls the activity is predominantly localized pericentrally. After phenobarbital treatment there is an increase in activity in the pericentral area (Fig. 5 and 5a).

G6PD and G6Pase. In controls large individual variations were found for both enzymes. The activity of G6PD was predominantly localized pericentrally, while G6Pase activity was mainly present in the periportal area. The activities of the enzymes were inversely related to each other. G6Pase seemed to be related to glycogen storage. If there was a lot of glycogen in the section the activity of G6Pase was high and the activity of G6PD was low. As in the method we used for the determination of G6PD the slices are incubated for 30 minutes in cold acetone, which destroys all the activity of G6Pase (MANNS, 1968), it is unlikely that the inverse relationship we noted is due to the fact that both enzymes concur for the same substrate. After phenobarbital treatment there is an increase of G6PD and a decrease of G6Pase in the pericentral part of the lobule (Fig. 6 and 6a; Fig. 7 and 7a).

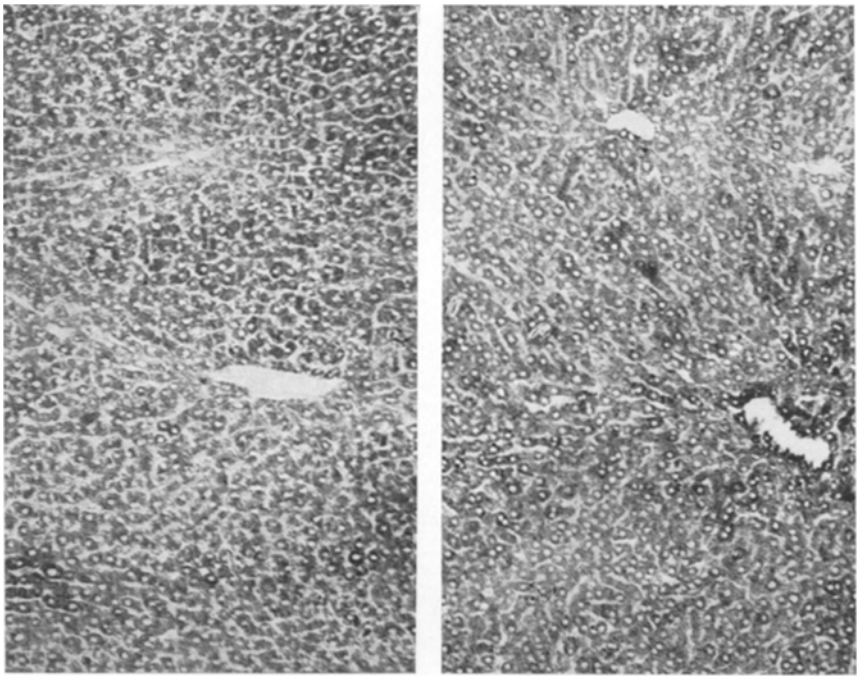


Fig. 3 Fig. 3a

Fig. 3 and 3a. NADH tetr. red. for and after phenobarbital stimulation. ×80

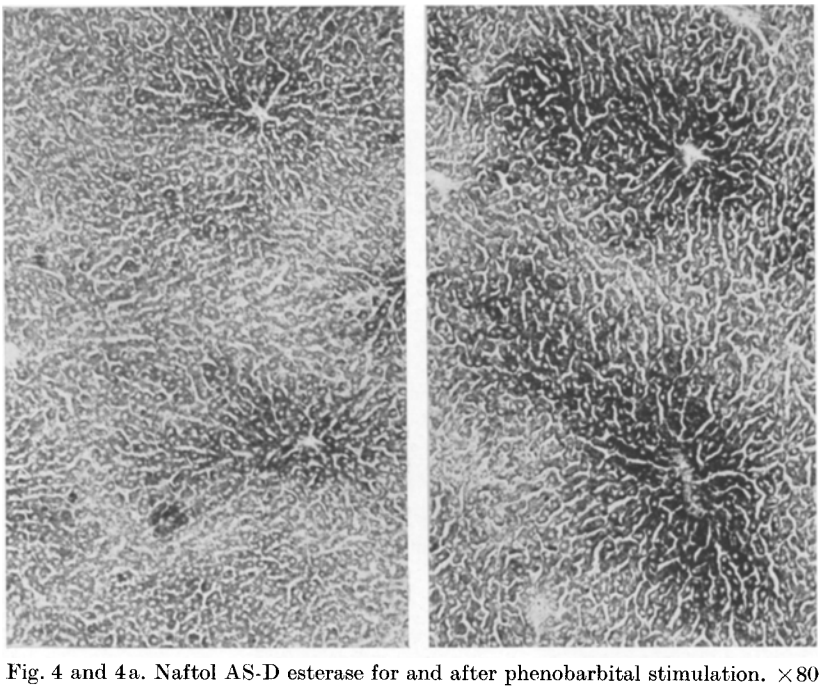


Fig. 4 and 4a. Naftol AS-D esterase for and after phenobarbital stimulation. ×80

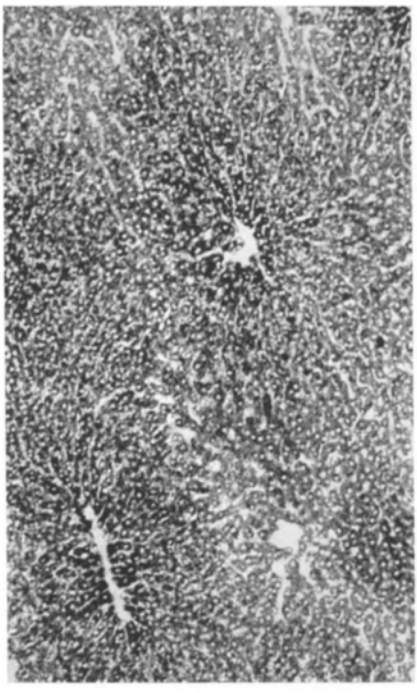


Fig. 5

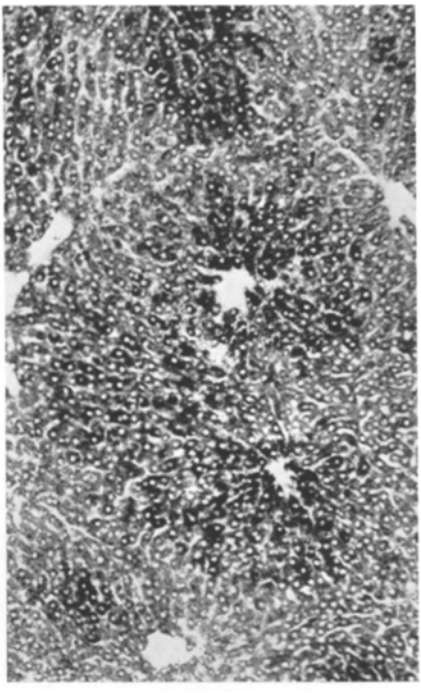


Fig. 5a

Fig. 5 and 5a. ICD for and after phenobarbital stimulation. ×80

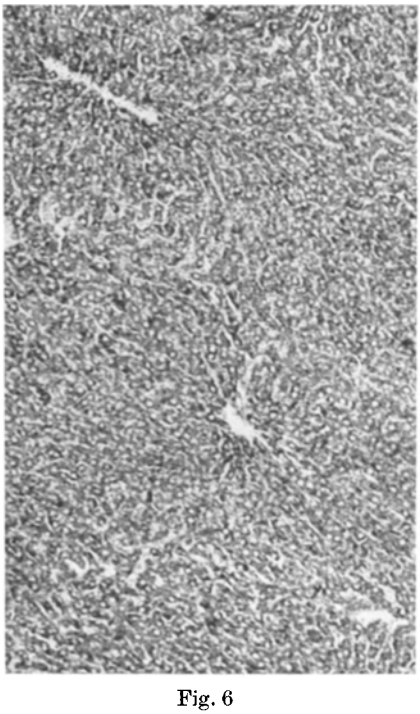


Fig. 6

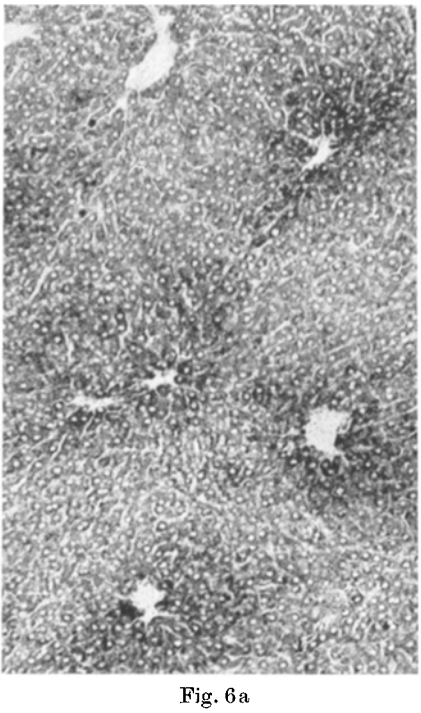


Fig. 6a

Fig. 6 and 6a. G6PD for and after phenobarbital stimulation. ×80

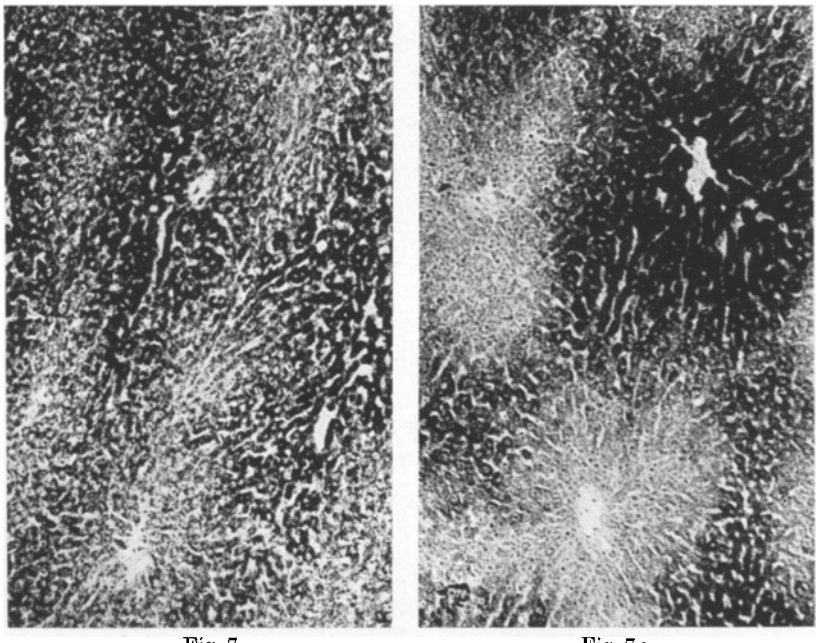


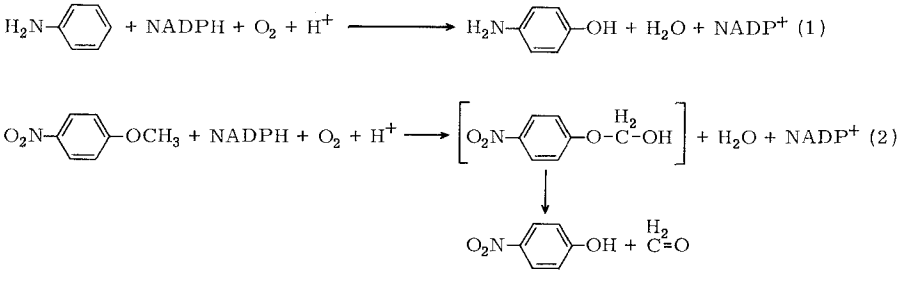
Fig. 7 Fig. 7a

Fig. 7 and 7a. G6Pase for and after phenobarbital stimulation. ×80

Alkaline phosphatase. After phenobarbital stimulation an increase in alkaline phosphatase was noted at the bile canaliculus side of the liver parenchym cell.

Discussion

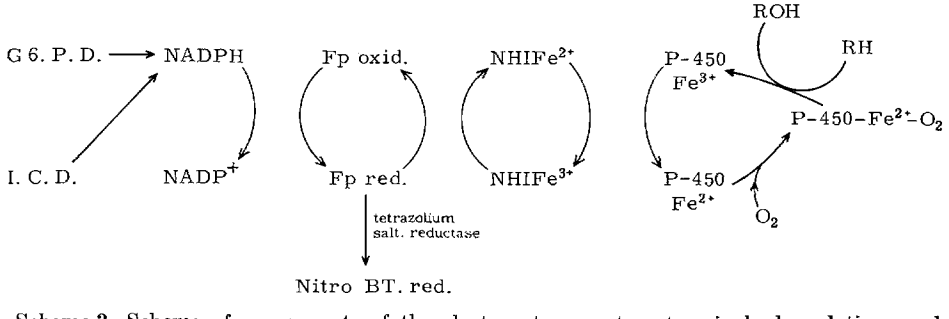
After phenobarbital treatment we found an increase in O-demethylating activity in liver microsomes as was found by NETTER and SEIDEL (1964). No increase was found for aniline hydroxylating capacity in agreement with ARCASOY *et al.* (1968). It is likely that there are several hydroxylating systems in liver microsomes each more or less specific for a certain group of related compounds. Aniline hydroxylation stands for aromatic hydroxylations (reaction 1, Scheme 2), while aliphatic hydroxylations are represented by the nitro-anisole demethylation (reaction 2, Scheme 2). The increase we noted in



Scheme 2. Scheme of aniline-hydroxylation (reaction 1) and nitro-anisole demethylation (reaction 2)

NADPH tetr. red. is in agreement with the biochemical findings of ARCASOY *et al.* (1968), who found an increase in neo-tetrazolium salt reductase. There is also an increase in the content of cytochrome P-450. So looking at Scheme 1 there is an increase in total hydroxylating capacity, an increase in a part of the chain (cyt. P-450), while the increase in NADPH-tetr. red. could be related to an increase in the content of the flavoprotein (Scheme 3). If there is an increase in hydroxylating capacity, there will be need for more NADPH, so the increase in G6PD and ICD can be explained. Naftol AS-D esterase can be used as a microsome marker (JANSON, 1968), so the increase can be correlated to the hypertrophy of the smooth endoplasmic reticulum It is of interest to note that in steroid producing cells, where hydroxylations are present, also non-specific esterases can be detected (KOUDSTAAL *et al.*, 1966, 1967, 1968). In how far nonspecific esterases are involved in hydroxylations is not clear. Our histochemical findings are in good agreement with the electronmicroscopic findings of other investigators. Electronmicroscopically a depletion of glycogen has been noted after phenobarbital treatment (JONES and FAWCETT, 1966). In the PAS reaction we also did notice a depletion of glycogen after phenobarbital treatment. If the liver lobule is divided into thirds as suggested by NOVIKOFF (1959), the altered cells after phenobarbital treatment are predominantly found in the pericentral part of the lobule. The individual hepatocytes of the central third of the liver lobule demonstrated maximal proliferation of smooth endoplasmic reticulum, while those found in other areas demonstrate lesser proliferation only in scattered cells (BECKER and LANE, 1968). The hypertrophy of smooth endoplasmic reticulum can be correlated to the increase in hydroxylating capacity of liver microsomes (JONES and FAWCETT, 1966).

From the facts that: a) 3,4-benzpyrene hydroxylase (WATTENBERG and LEONG, 1962) and NADPH tetr. red. is located pericentrally in rat liver, b) an increase in hydroxylating capacity correlates with an increase in NADPH tetr. red. biochemically (ARCASOY *et al.*, 1968) and histochemically, c) the localization of NADPH tetr. red. in the rat liver lobule correlates with the electron microscopic distribution of smooth endoplasmic reticulum, d) no increase in NADH tetr. red. is seen after phenobarbital treatment (NADH tetr. red. is probably involved in the respiration chain (LESTER and SMITH, 1961)), we suggest that NADPH tetr. red. is directly involved in the hydroxylating chain. In analogy to NADH tetr. red. where the reduction is probably due to the flavoprotein (LESTER and SMITH, 1961), we suggest the following scheme (Scheme 3).



Scheme 3. Scheme of components of the electron transport system in hydroxylations and the possible role of G6PD, ICD, and NADPH tetr. red.

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