The effects of dietary lipid and phenobarbitone on the production and utilization of NADPH in the liver

A combined biochemical and quantitative cytochemical study

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The validity of the concept that cellular NADPH utilization in the cytoplasm can, by quantitative cytochemical procedures, be classified into two pathways (Pathway I, in which NADPH is oxidized via the microsomal electron-transport system, and Pathway II, in which NADPH supplies reducing equivalents for biosynthetic processes) was tested. The amount of NADPH, generated by glucose 6-phosphate dehydrogenase, entering Pathways I and II in the centrilobular and periportal regions was measured by quantitative cytochemistry, and the values obtained were compared with biochemical measurements of mixed-function oxidase and fatty acid synthetase activity after the administration of sodium phenobarbitone or by altering the quantity and nature of the dietary lipid. Phenobarbitone stimulates hepatic mixed-function oxidation measured biochemically and Pathway I, but not Pathway II. Variation in the type and quantity of dietary lipid can also regulate the activity in mixed-function oxidation and alter the amount of NADPH entering Pathways I and II. It is concluded that, in general, the concept of two main pathways of NADPH utilization in the liver is valid, but that the ratios of NADPH utilization in the two pathways gives a better indication of the use of NADPH *in vivo* than is obtained for absolute values for the two pathways. Moreover, the centrilobular and periportal hepatocytes showed different patterns in their response to changes in dietary lipid and the administration of phenobarbitone. These results indicate the different metabolic roles that these two groups of cells may play in the metabolism of foreign compounds.

The two quantitatively most important uses of cytoplasmic NADPH in hepatocytes are the mixed-function oxidation of drugs and other foreign compounds, involving cytochrome *P*-450, and the biosynthesis *de novo* of fatty acids. Cytoplasmic NADPH may also be used for the biosynthesis of cholesterol, the reduction of oxidized glutathione and the reduction of folic acid to tetrahydrofolate. These three processes, along with the biosynthesis of fatty acids, may be classified as synthetic uses of NADPH. On the basis of these concepts it is possible to classify pathways of NADPH utilization in the liver into two main categories (Scheme 1). The first, designated Pathway I (Altman & Chayen, 1970; Chayen *et al.*, 1973), involves the transfer of reducing equivalents from NADPH along an electron-transport chain to the terminal oxidase, cyto-

chrome *P*-450, whereas the second, designated Pathway II (Altman & Chayen, 1970; Chayen *et al.*, 1973), involves the direct use of NADPH in cytosolic biosynthetic processes (Scheme 1). In liver cells, the major use of Pathway-I NADPH will be for mixed-function oxidase activity, whereas the major use of Pathway-II NADPH will be for fatty acid biosynthesis.

These two pathways of NADPH utilization may be distinguished by quantitative cytochemical procedures involving the use of the intermediary electron carrier, phenazine methosulphate (Altman & Chayen, 1970; Altman, 1972; Chayen *et al.*, 1973). Subsequent experimental evidence has supported the existence of these two pathways of NADPH utilization in rat liver and the ability of phenazine methosulphate to distinguish between them (Altman, 1971, 1972; Chayen *et al.*, 1973, 1974; Weisz & Zoller, 1979). However, no direct comparison of the amount of NADPH entering

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Scheme 1. Schematic representation of the uses of NADPH generated by the pentose-shunt dehydrogenases in hepatocytes

Pathways I and II, determined by quantitative cytochemistry, and conventional biochemical measurements of the rate of NADPH utilization in either biosynthetic processes or mixed-function oxidase activity has yet been carried out.

In the present investigation the activity of the mixed-function oxidase system and the rate of fatty acid biosynthesis in rat liver have been systematically varied by the administration of the inducing drug sodium phenobarbitone, and by altering the fatty acid composition of the dietary lipid (Rosmos & Leveille, 1974; Rowe & Wills, 1976; Wade & Norred, 1976; Triscari *et al.*, 1978; Smith & Wills, 1981a). The amount of NADPH, generated by glucose 6-phosphate dehydrogenase, entering Pathways I and II in different regions of the liver has then been measured by quantitative cytochemistry and the results obtained have been compared with conventional biochemical measurements of mixed-function oxidase and fatty acid synthetase activity in subcellular fractions of rat liver.

It has therefore been possible to investigate the validity of the concept of two pathways of NADPH utilization in rat liver, the ability of quantitative cytochemical procedures to distinguish between them and the effects of drug induction and changes in nutritional status on the amount of NADPH available for mixed-function oxidation and biosynthetic processes in different regions of the rat liver lobule.

Materials and methods

Materials

NADP⁺ (disodium salt) and glucose 6-phosphate (disodium salt) were obtained from Boehringer-Mannheim G.m.b.H., Mannheim, West Germany. Pure neotetrazolium chloride was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Phenazine methosulphate, malonyl-CoA (trilithium salt) and acetyl-CoA (lithium salt) were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

Polyvinyl alcohol (G04/140 grade) was obtained from Wacker-Chemie G.m.b.H., München, West Germany. All other compounds and reagents were obtained from BDH, Poole, Dorset, U.K. The n-hexane was free from aromatic hydrocarbons, boiling range 67–70°C.

Preparation of diets of known lipid composition

Purified diets containing lipids of known composition were freshly prepared in a powdered form as previously described (Hammer & Wills, 1978; Smith & Wills, 1981a). The dietary lipids used were herring oil, mackerel oil (Marfleet Refining Co., Hull, U.K.) and corn oil (Mazola, Brown and Polson, C.P.C., Esher, Surrey, U.K.). It was necessary to replace herring oil with mackerel oil in part of this study because herring oil became unavailable during the investigation. The fatty acid composition of mackerel oil is, however, generally similar to that of herring oil, but contains less C_{20:5} (1.2% as compared with 7.6% for herring) and less C_{22:1} (6.5% as compared with 15.8%). Its addition to the diet is therefore likely to have effects which are similar to those which follow addition of herring oil to the diet (Hammer & Wills, 1979).

Feeding of experimental animals

Groups of three to six male albino Wistar rats, each weighing 130–150 g, were transferred from a stock diet (no. 1; Spratts, Central House, Barking, Essex, U.K.) to synthetic diets containing 10% corn oil, 10% herring oil, 10% mackerel oil or no lipid (fat-free diet) for 10–24 days before killing by cervical dislocation. For comparison, other groups of rats were maintained on the stock diet. After 10 days on synthetic diets the fatty acid composition of the endoplasmic reticulum stabilizes and remains unchanged for the period of the experiment (Hammer & Wills, 1979). The fatty acid compositions of the purified and stock diets have been described previously (Hammer & Wills, 1978).

Administration of phenobarbitone

One group of four rats was also maintained on the stock diet but was given a solution of sodium phenobarbitone, containing 1 g/litre of water, as their sole source of drinking water for 5–7 days before they were killed.

Preparation of the liver microsomal fraction

This was prepared by the calcium precipitation method described by Kamath & Rubin (1972). The fraction was suspended in 125 mM-KCl (2 ml/g of liver used in preparation) and is described as a '50% microsomal suspension'.

Determination of the activity of the microsomal mixed-function oxidase system

The cytochrome *P*-450 content of the microsomal fraction was determined by the method of Omura & Sato (1964), and the rate of aminopyrine *N*-demethylation in this fraction was also determined by the method described by Wills (1969), with minor modifications. The reaction mixture contained 2 mg of microsomal protein and 0.4 mM-NADPH in a total volume of 3.0 ml, but concentrations of other reagents were as described previously.

Determination of fatty acid synthetase activity in the cytosolic fraction of rat liver

The activity of fatty acid synthetase was measured in the dialysed cytosolic fraction of liver prepared by centrifuging at 100 000 *g* for 1 h, by following the rate of NADPH oxidation at 340 nm with a SP8-100 u.v. spectrophotometer, essentially by the method of Saggerson & Greenbaum (1970).

Determination of protein

The protein concentrations of the liver microsomal and cytosolic fractions were determined by the method of Lowry *et al.* (1951), with bovine plasma albumin as a standard.

Preparation of tissue sections

Sections (10 μ m) were prepared from chilled blocks of rat liver (4 mm³) by using a Bright's automated cryostat as previously described (Smith *et al.*, 1979a).

Determination of glucose 6-phosphate dehydrogenase activity

The activity of glucose 6-phosphate dehydrogenase in rat liver sections was determined by the method of Altman (1972). NADPH generated by the enzyme activity reduces neotetrazolium chloride via the intermediary electron carrier phenazine methosulphate and causes the precipitation in the tissue section of the coloured neotetrazolium formazan, which can be measured by microdensitometry

(Butcher, 1972; Altman, 1976). Liver sections (10 μ m) were incubated for 5–10 min at 37°C in 50 mM-glycylglycine/NaOH buffer, pH 8.0, containing 5 mM-neotetrazolium chloride, 5 mM-glucose 6-phosphate, 3 mM-NADP⁺ and 30% polyvinyl alcohol (G04/140 grade). This grade and concentration of polyvinyl alcohol has been shown to retain all the glucose 6-phosphate dehydrogenase activity within liver sections (Henderson *et al.*, 1978). Just before use, the incubation medium was saturated with O₂-free N₂ and 0.25 g of phenazine methosulphate/litre was added. All procedures involving phenazine methosulphate were performed in subdued light. The incubations were performed in Perspex rings maintained in an atmosphere of N₂ as previously described (Altman, 1972).

For each rat, four to six sections, prepared from two blocks of liver, were incubated in the presence of substrate (glucose 6-phosphate), and two control sections were incubated in its absence. Controls in the absence of NADP⁺ were also performed. The control activity was subtracted from the activity produced in the presence of exogenous substrate.

Determination of the amount of NADPH entering Pathways I and II

Pathway I is equivalent to the enzyme system often referred to as 'the NADPH diaphorase' or 'the NADPH neotetrazolium reductase' (Novikoff, 1963; Koudstaal & Hardonk, 1969). In the absence of an intermediary electron carrier, such as phenazine methosulphate, reducing equivalents from NADPH pass along the microsomal electron-transport chain and reduce neotetrazolium to its formazan (Altman, 1972). The activity of glucose 6-phosphate dehydrogenase in the absence of phenazine methosulphate is directly proportional to the amount of NADPH, generated by glucose 6-phosphate dehydrogenase activity, entering Pathway I.

The amount of NADPH entering Pathway II was determined by subtracting the amount of NADPH entering Pathway I from the total amount of NADPH produced by glucose 6-phosphate dehydrogenase activity.

Microdensitometry

Neotetrazolium chloride is a ditetrazolium salt and produces two formazans, which have different absorption characteristics (Butcher & Altman, 1973). Measurements were therefore made with a Vickers M85 scanning and integrating microdensitometer at the isosbestic wavelength of 585 nm, where both formazans have the same absorption coefficient (Altman & Butcher, 1973; Altman, 1976). The absorption of the neotetrazolium formazan was determined in individual hepatocytes by using $\times 40$ objective, scanning spot size 1 (being 0.5 μ m in diameter in the optical plane of the

specimen) and a field size of 17 μm diameter. Measurements were made in hepatocytes located within 100 μm of centrilobular veins of less than 40 μm diameter.

Data analysis and statistics

The data produced from the microdensitometric measurements were processed by a PET 2001 microcomputer interfaced to the microdensitometer as previously described (Smith *et al.*, 1979b, 1980). A Petsoft statistics program (Petsoft, P.O. Box 9, Newbury, Berks., U.K.) was used to calculate the means, variance, S.D., S.E.M. and S.D. as a percentage of the mean. Some 10–15 absorbance measurements were made in each section for the region of the liver under investigation. The mean blank reading was subtracted from each absorbance reading and the mean absorbance reading calculated.

This procedure was repeated for four to six sections and mean absorbance ($\pm$ S.D.) calculated.

The whole procedure was repeated on four to six rats, and the means $\pm$ S.D. and S.E.M. were calculated. The S.E.M. values quoted in all Figures and Tables therefore represent the variation between animals in each experimental group. Student's *t* test was used to calculate the significance of the results, and the level of significance was taken as 5%.

Results

Studies of the effects of dietary lipid and phenobarbitone on the concentration of cytochrome P-450 and the mixed-function oxidase activity in rat liver microsomal fraction by biochemical methods

Biochemical measurements were made so that the results could be directly compared with those obtained by quantitative cytochemistry under the same experimental conditions. Phenobarbitone administration caused a 3-fold increase in both the concentration of cytochrome P-450 and the rate of aminopyrine *N*-demethylation in the rat liver microsomal fraction (Table 1).

When rats were transferred from a stock diet to a diet containing 10% herring oil or corn oil for 10–24 days, the rate of aminopyrine *N*-demethylation was significantly increased ($P < 0.03$) (Table 1). The cytochrome P-450 content of the microsomal fraction was also increased, but only significantly in rats eating the 10%-herring oil diet ($P < 0.02$). The cytochrome P-450 content of liver microsomal fraction from corn-oil-fed rats was, however, significantly higher ($P < 0.05$) than in those of rats fed on a fat-free diet. A fat-free diet decreased both the liver microsomal cytochrome P-450 content and the rate of aminopyrine *N*-demethylation in comparison with rats fed on a stock diet (Table 1). The decrease in the rate of aminopyrine *N*-demethylation was statistically significant ($P < 0.04$), but the decrease in cytochrome P-450 content was not ($P > 0.30$).

Effect of dietary lipid and phenobarbitone on fatty acid synthetase activity in rat liver

The activity of fatty acid synthetase in the liver cytosolic fraction of phenobarbitone-pre-treated rats was not significantly different from that found in stock-diet-fed rats, given water alone ($P > 0.09$) (Table 2). In contrast, variations in the fatty acid composition of the dietary lipid do markedly affect the activity of this enzyme in rat liver (Muto & Gibson, 1970; Rosmos & Leveille, 1974).

When rats were transferred from a stock diet to a fat-free diet for 10–24 days the fatty acid synthetase activity in the liver cytosol was significantly increased ($P < 0.006$), approx. 4-fold (Table 2). However, when rats were transferred from a stock diet to diets containing 10% corn oil or 10% mackerel oil the synthetase activity in the liver cytosol was significantly decreased ($P < 0.03$ and $P < 0.009$ respectively) (Table 2).

Effect of dietary lipid and phenobarbitone on the activity of glucose 6-phosphate dehydrogenase in different regions of liver

Two groups of four rats were transferred from a stock diet to powdered diets containing either 10%

Table 1. *Effect of dietary lipid and phenobarbitone on the concentration of cytochrome P-450 and the rate of aminopyrine N-demethylation in rat liver microsomal fraction*

Diets were given for 10–24 days. Phenobarbitone was administered in the drinking water at a concentration of 1 g/litre for 5–7 days. Results are expressed as means $\pm$ S.E.M. for four rats in each experimental group.

Diet or treatment	Microsomal cytochrome	Rate of aminopyrine
	P-450 content (nmol/mg of microsomal protein)	<i>N</i> -demethylation (nmol demethylated/min per mg of protein)
Fat-free diet	0.47 $\pm$ 0.06	2.58 $\pm$ 0.14
Stock diet	0.56 $\pm$ 0.09	3.20 $\pm$ 0.14
10% Corn-oil diet	0.74 $\pm$ 0.12	4.68 $\pm$ 0.42
10% Herring-oil diet	1.07 $\pm$ 0.04	5.92 $\pm$ 0.48
Phenobarbitone + stock diet	1.77 $\pm$ 0.04	11.40 $\pm$ 0.73

Table 2. *Effect of dietary lipid and phenobarbitone on the activity of fatty acid synthetase in rat liver*

Diets were given for 10–24 days. Phenobarbitone was administered in the drinking water at a concentration of 1g/litre for 5–7 days. Results are expressed as means $\pm$ S.E.M. for three rats in each experimental group.

Diet or treatment	Fatty acid synthetase activity (μ mol of NADPH oxidized/min per g of cytosolic protein)
Fat-free diet	19.60 $\pm$ 1.99
Stock diet	4.94 $\pm$ 0.23
Phenobarbitone + stock diet	4.71 $\pm$ 0.24
10% Corn-oil diet	3.73 $\pm$ 0.13
10% Mackerel-oil diet	2.66 $\pm$ 0.29

corn oil or 10% herring oil for 10–24 days before they were killed. Another group, consisting of six rats, was transferred from a stock diet to a fat-free diet for 10–24 days. In addition, one group of four stock-diet-fed rats was given a 0.1% (w/v) solution of sodium phenobarbitone as their sole source of drinking water for 5–7 days before they were killed. For comparison, one other group of four rats was maintained on stock diet and given water alone.

Glucose 6-phosphate dehydrogenase activity was not evenly distributed in the livers of the control stock-diet-fed rats and was approximately twice as high in the centrilobular hepatocytes as in the periportal hepatocytes (Fig. 1). Administration of sodium phenobarbitone significantly increased glucose 6-phosphate dehydrogenase activity only in the centrilobular hepatocytes ($P < 0.0005$) and had no significant effect in the periportal hepatocytes ($P > 0.05$) (Fig. 1). Transferring rats from a stock diet to a fat-free diet, however, caused a significant increase in the activity of the dehydrogenase in both regions of the liver ($P < 0.0005$), but this increase was much more marked in the periportal hepatocytes (Fig. 1).

The activity of glucose 6-phosphate dehydrogenase in rats transferred from a stock diet to a diet containing 10% herring oil was significantly decreased in both regions of the liver ($P < 0.04$) (Fig. 1). Addition of 10% corn oil to the diet caused only a small decrease in the activity of the dehydrogenase in both regions of the liver (Fig. 1), which was not significant ($P > 0.15$).

Effect of dietary lipid and phenobarbitone on the amount of NADPH entering Pathways I and II in different regions of rat liver

To investigate how the NADPH was divided between Pathways I and II, the activity of glucose 6-phosphate dehydrogenase was measured in the presence and absence of phenazine methosulphate in

Fig. 1. *Effects of dietary lipid and phenobarbitone on the activity of glucose 6-phosphate dehydrogenase in different regions of rat liver*

Diets were given for 10–24 days. Phenobarbitone was administered in the drinking water at a concentration of 1g/litre for 5–7 days. The activity of glucose 6-phosphate dehydrogenase was determined by the method of Altman (1972). Numbers of rats used are shown in parentheses and results are expressed as the mean $\pm$ S.E.M. (bars) for each experimental group. Region: $\square$, centrilobular; $\square$, periportal.

the centrilobular and peripheral regions of liver sections (10 μ m) prepared from each group of rats fed on the different diets or pretreated with phenobarbitone.

In control stock-diet-fed rats the amount of NADPH entering Pathway I was approximately 2.5 times greater in the centrilobular hepatocytes than in the periportal hepatocytes (Fig. 2a), but the amount of NADPH entering Pathway II was approximately the same in both regions (Fig. 2b).

Phenobarbitone administration caused a highly selective and significant increase in the amount of NADPH entering Pathway I in the centrilobular hepatocytes ($P < 0.0001$), but had no significant effect on the amount of NADPH entering Pathway I in the periportal hepatocytes ($P > 0.30$) (Fig. 2a). Phenobarbitone pretreatment also had no significant effect on the amount of NADPH entering Pathway II in either region of the liver ($P > 0.43$) (Fig. 2b).

Transferring rats from a stock diet to a fat-free diet for 10–24 days before they were killed significantly increased the amount of NADPH enter-

Fig. 2. Effect of dietary lipid and phenobarbitone on the amount of NADPH entering Pathways I (a) and II (b) in different regions of rat liver

The amount of NADPH entering Pathways I (a) and II (b) was determined by the method of Altman (1972). Numbers of rats used are shown in parentheses and results are expressed as the mean $\pm$ S.E.M. for each experimental group; Region: $\square$, centrilobular; $\square$, periportal.

Table 3. Effect of dietary lipid and phenobarbitone on the ratios of Pathway-I to Pathway-II NADPH in different regions of rat liver

(a) Ratios of the group means calculated from data shown in Figs. 2(a) and 2(b). (b) Ratios calculated from 16–24 individual sections from each group. Means $\pm$ S.E.M. are shown.

Diet or treatment	Pathway I/Pathway II			
	Centrilobular region		Periportal region	
	(a)	(b)	(a)	(b)
Fat-free diet	3.38	4.34 $\pm$ 0.44	0.85	0.97 $\pm$ 0.09
Stock diet	2.30	2.50 $\pm$ 0.18	1.26	1.35 $\pm$ 0.11
10% Corn-oil diet	1.96	2.11 $\pm$ 0.17	1.87	1.95 $\pm$ 0.14
10% Herring-oil diet	3.09	3.17 $\pm$ 0.15	1.62	1.69 $\pm$ 0.12
Phenobarbitone + stock diet	3.92	4.52 $\pm$ 0.48	1.15	1.20 $\pm$ 0.11

ing Pathway I in both the centrilobular ($P < 0.002$) and periportal ($P < 0.021$) hepatocytes (Fig. 2a). However, the amount of NADPH entering Pathway II was substantially increased in the periportal hepatocytes ($P < 0.01$), but was not significantly affected in the centrilobular hepatocytes ($P > 0.65$) (Fig. 2b). Consuming a fat-free diet, which stimulated the rate of fatty acid biosynthesis (Table 2), causes a highly selective increase in the amount of NADPH entering Pathway II in the periportal hepatocytes. This selective increase is similar to the effect of phenobarbitone on the amount of NADPH entering Pathway I in the centrilobular hepatocytes (Fig. 2a) and suggests that the centrilobular and periportal hepatocytes perform different functions.

Eating a 10%-corn-oil diet for 10–24 days had no significant effect on the amount of NADPH entering Pathway I or Pathway II in either region of the rat liver lobule as compared with the group fed on stock diet ($P > 0.10$) (Figs. 2a and 2b), but a diet containing 10% herring oil caused the amount of NADPH entering Pathway I to decrease in both regions of the liver (Fig. 2a). This decrease was significant in the centrilobular hepatocytes ($P < 0.009$), but not in the periportal hepatocytes ($P > 0.15$). Moreover, eating a 10%-herring-oil diet caused only small decreases in the amount of NADPH entering Pathway II in both the centrilobular and periportal hepatocytes, as compared with eating a stock diet, which were not statistically

significant ($P > 0.12$). It was evident from these results that changes in the total activity of glucose 6-phosphate dehydrogenase were probably masking the effects of the different diets on the amount of NADPH entering Pathways I and II.

Ratios of Pathway I to Pathway II may therefore be a more useful indication of the utilization of NADPH than absolute values because they will be independent of changes in the total activity of the enzyme. The direct group ratios of Pathway I to Pathway II in the centrilobular and periportal hepatocytes are shown in Table 3 and are discussed fully in the Discussion section.

Discussion

By using tetrazolium salts, which are converted into formazan salts by reduction, and the intermediary electron carrier phenazine methosulphate, Altman & Chayen (1970) proposed that systems utilizing NADPH could be placed into two main categories, termed Pathways I and II (Chayen *et al.*, 1973). They suggested that in Pathway I the NADPH was utilized by an electron-transport chain to reduce cytochrome *P*-450 and thus to take part in the oxidative metabolism of drugs, steroids and carcinogens, whereas the NADPH entering Pathway II was used for synthetic reactions, especially fatty acid biosynthesis.

Major supporting evidence was of two kinds. Firstly, the amount of NADPH entering the two pathways differed with the tissue. In adipose tissue it was low for Pathway I and high for Pathway II, indicating fat synthesis; in the adrenal, site of extensive steroid hydroxylation, the converse occurred. Secondly, and more relevant to the present study, phenobarbital pretreatment (Altman, 1972) increased NADPH utilization 3-fold in Pathway I, and negligibly in Pathway II.

In the present investigation we have attempted to validate quantitative cytochemical procedures for the study of NADPH utilization and the concept of Pathways I and II by comparing the results obtained with those from conventional biochemical methods.

Direct comparison is, however, complicated by the fact that the cytochemical methods permit assessment of metabolic activity in different regions of the liver, the centrilobular and periportal regions, whereas biochemical methods only give overall values.

Use of cytochemical methods has also provided further evidence for the centrilobular hepatocytes being the major site of drug metabolism in the liver.

In untreated stock-diet-fed rats the ratio of Pathway I to Pathway II was approximately twice as high in the centrilobular hepatocytes as in the periportal hepatocytes (Table 3). This result, together with the finding that the activity of glucose

6-phosphate dehydrogenase was approximately twice as high in the centrilobular hepatocytes as in the periportal hepatocytes of stock-diet-fed rats (Fig. 1) indicates that approximately four times as much NADPH is available to the mixed-function oxidase system as a result of glucose 6-phosphate dehydrogenase activity in the centrilobular hepatocytes as compared with the periportal hepatocytes. The fact that the centrilobular hepatocytes have a better supply of NADPH for mixed-function oxidation and contain more cytochrome *P*-450 (Gooding *et al.*, 1978; Smith & Wills, 1981a) than the periportal hepatocytes implicates these cells as being more important in the oxidative metabolism of foreign compounds than the periportal cells.

Biochemical determinations showed that in microsomal preparations from livers of rats fed on stock diet, phenobarbitone causes the concentration of cytochrome *P*-450 to increase by just over 3-fold and the rate of oxidative demethylation to increase by approx. 3.5-fold (Table 1).

Phenobarbitone caused a large selective increase in Pathway I in the centrilobular hepatocytes but had no significant effect on this Pathway in the periportal hepatocytes (Fig. 2a). Very small increases were observed in Pathway II in both regions of the liver (Fig. 2b). These results are thus in general agreement with those of Altman (1971) and are in accord with the highly selective increases in cytochrome *P*-450 concentration that have been found in the centrilobular hepatocytes after pretreatment of rats with phenobarbitone (Gooding *et al.*, 1978; Smith & Wills, 1981a). They indicate that more reducing equivalents from NADPH are channelled towards cytochrome *P*-450 in the centrilobular hepatocytes after phenobarbitone induction, and could explain phenobarbitone's ability to potentiate markedly the centrilobular hepatic necrosis caused by many foreign compounds (Smith & Wills, 1981b).

Conventional biochemical methods have been used to show that the concentration of cytochrome *P*-450 and rates of oxidative drug metabolism are much greater when polyunsaturated fats such as corn oil or herring oil are incorporated into the diet than when fat-free diets or saturated fats are given (Rowe & Wills, 1976; Wade & Norred, 1976; Hammer & Wills, 1979).

If the concept of Pathways I and II proposed by Altman & Chayen (1970) is valid, it may therefore have been expected that giving diets high in polyunsaturated fats would increase the utilization of NADPH in Pathway I but decrease the amount entering Pathway II, because of the inhibiting effect of polyunsaturated fatty acids on fatty acid biosynthesis (Rosmos & Leveille, 1974; Triscari *et al.*, 1978). Measurements of NADPH entering Pathway I, however, showed that changing the diet from

fat-free or stock diet to one containing 10% corn oil or 10% herring oil caused decreases in the total amount of NADPH entering Pathway I in the centrilobular hepatocytes and had little effect on that entering Pathway I in the periportal hepatocytes (Fig. 2a).

The 11% difference in the calorific values of the fat-free and lipid-containing diets [1590 and 1800 J (380 and 430 cal)/100 g respectively] probably has little effect on the large changes seen in the rate of fat synthesis or in the glucose 6-phosphate dehydrogenase activity.

The interpretation of these values for the amount of NADPH entering Pathway I is, however, complicated by two considerations. Firstly the fatty acid synthetase activity in liver is very high in animals fed on a fat-free diet, but is markedly decreased by consuming corn oil or mackerel oil (Table 2). Secondly, the total activity of glucose 6-phosphate dehydrogenase is affected by the type of diet, being very high in the livers of animals given a fat-free diet and much lower in those on the corn-oil or herring-oil diets (Fig. 1).

It was evident that the changes in the total activity of glucose 6-phosphate dehydrogenase were probably masking the effects of the different diets on the distribution of NADPH between Pathways I and II, and interpretation of the measurements would be better based on the ratios of Pathway I to Pathway II, because ratios will be independent of the total activity of the enzyme (Smith & Wills, 1980). An increase in the ratio of Pathway I to Pathway II could indicate that more reducing equivalents were being channelled from NADPH towards cytochrome *P*-450, whereas a decrease in this ratio could indicate that more reducing equivalents from NADPH were being channelled towards biosynthesis. The ratios are shown in Table 3. From biochemical measurements it would be expected that eating a fat-free diet would cause a large increase in the utilization of NADPH for fatty acid synthesis (Table 3), and this is clearly shown for the peripheral hepatocytes where the ratio Pathway I/Pathway II decreased from 1.26 to 0.85 ($P < 0.13$).

However, in the centrilobular hepatocytes the ratio of Pathway I to Pathway II was significantly increased ($P < 0.002$) after consuming the fat-free diet (Table 3), and this indicates that an increased amount of NADPH is channelled towards mixed-function oxidase activity in the centrilobular hepatocytes, a result which is not in accord with biochemical measurements (Table 1).

Corn-oil diets stimulated the rate of oxidative demethylation in liver microsomal preparations (Table 1) but did not affect the ratio Pathway I/Pathway II in the centrilobular regions, which is believed to be the main site of drug metabolism. It did, however, significantly increase the ratio in the

periportal hepatocytes. This indicates that corn oil diverts NADPH from fatty acid synthesis in the periportal region, and the biochemical measurements may show increased mixed-function oxidase activity on account of the restriction of use of NADPH for synthesis.

Close agreement between quantitative cytochemical measurements and biochemical measurements were, however, obtained after eating herring-oil diets. A significant increase in the ratio Pathway I/Pathway II was observed in both regions of the liver, indicating a diversion of NADPH to mixed-function oxidase activity. Herring-oil diets are very effective in inducing the increases in the concentration of cytochrome *P*-450 (Table 1) or as measured cytochemically (Smith & Wills, 1981a) and in the rate of oxidative demethylation (Table 1).

These results further indicate that the rate of supply of reducing equivalents from NADPH may be the rate-limiting factor in controlling mixed-function oxidation and lipogenesis in whole liver cells, as has been suggested on the basis of experiments on perfused rat liver (Thurman & Scholz, 1973; Thurman *et al.*, 1977).

Moreover, quantitative cytochemical measurements of the amount of NADPH entering two separate pathways appear to be valid provided that they are interpreted in the form of ratios and not as absolute values. Measurements of the Pathway I/Pathway II ratios within different cell types in heterogeneous tissues may therefore give valuable information about the amount of NADPH available to different metabolic pathways in these cells.

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