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THE EFFECT OF PHENOBARBITONE TREATMENT OF RATS AND OF PROTEIN DEPRIVATION ON THE CAPACITY OF LIVER SLICES TO CONJUGATE BILIRUBIN

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Abstract—The capacity of liver slices to conjugate and excrete bilirubin was increased by 45 per cent on treatment of rats with oral phenobarbitone for 3 weeks but was not significantly altered on feeding a protein-free diet for 4 days. These responses are different from those reported by other workers for liver microsomal drug-hydroxylating enzymes. Studies with inhibitors and stimulators of the liver slice system conjugating bilirubin indicated that the characteristic effects of these compounds were not altered by pre-treatment of rats with phenobarbitone or with a protein-free diet.

MANY workers[1–6] have reported that microsomal, NADPH-dependent, drug-metabolizing and steroid-hydroxylating enzymes are induced by treatment of animals with phenobarbitone. Although the induction is associated with an increase of smooth endoplasmic reticulum, there are differences in the response of different enzymes. The UDP glucuronyl-transferase (UDPglucuronate glucuronyl-transferase, acceptor unspecific E.C. 2.4.1.17) activity of liver homogenates or microsomes using bilirubin[7] or *p*-nitrophenol[8] as substrates, is increased by treatment of rats with phenobarbitone. Barbitone treatment of mice leads to increased UDPglucuronyl-transferase activity of microsomes using bilirubin as substrate, but not with either phenolphthalein or *o*-aminophenol as substrate.[9] The development of UDPglucuronyl-transferase activity in newborn mice is accelerated by treatment with barbitone.

Attempts have been made to apply these observations, therapeutically, in chronic unconjugated hyperbilirubinaemia due to defective conjugation.[10, 11] Administration of phenobarbitone to two infants, resulted in a decrease in the serum unconjugated bilirubin concentration. There may be other explanations of this result.

In contrast to these findings, rabbits treated with phenobarbitone, did not have increases in the UDPglucuronyl-transferase activity in liver microsomes, using *p*-nitrophenol, *o*-aminophenol and phenolphthalein as substrates.[12]

In view of the possible role of endoplasmic reticulum in conjugation of bilirubin and secretion into bile, we have examined the effect of atrophy (protein deprivation) and hypertrophy (phenobarbitone treatment) of the endoplasmic reticulum on conjugation and secretion of bilirubin by rat liver slices. Previous studies on the effect of phenobarbitone on UDPglucuronyl-transferase used liver homogenates or microsomal preparations to which the coenzyme UDPglucuronic acid (UDPGA) must be added for substantial enzyme activity. The slice system has some other feature which make it more like the intact liver.

Inhibitors and stimulators of the conjugation of bilirubin in the slice system are

being studied currently, as a means of defining the biochemical mechanism of conjugation and secretion. We have examined the effect of four compounds, which seem to act in different ways, under conditions of enhanced and reduced endoplasmic reticulum.

MATERIALS AND METHODS

Bilirubin was purchased from British Drug Houses Ltd., pregnanolone (5β-pregnane-3α-ol,20-one), from Koch-Light Laboratories Ltd., was recrystallized once and was chromatographically homogenous. Synthetic sodium taurocholate was a gift from Dr. T. G. Richards. All other chemicals were from British Drug Houses Ltd., and were of analytical grade.

The experiments were based on 18 male Wistar rats, divided into three groups of 6 animals each, so that the mean weight (about 220 g.) and the distribution of weights were approximately the same in each group. All animals had been on MRC 41B diet and water *ad libitum* before the experiment. The control group was continued on this regime. A second group were given a solution of phenobarbitone (1 mg/ml) to drink [13]during the 3 weeks before being examined. The daily intake was estimated at less than 50 mg, from the reduction of drinking water in bottles. A third group was fed a protein-free diet, similar to that described by McLean and McLean,[14] and were given water *ad libitum* for 4 days prior to sacrifice. Following the above treatment one animal from each group was examined each day for 6 days.

Bilirubin conjugation by liver slices was determined from the amount of direct-reacting bilirubin in the incubation medium by the method of Lathe and Walker[15] with two modifications: a 5-min "direct" reaction time was used, instead of 30 min, and 0·2% sodium azide solution was used to neutralize the excess diazo reagent, instead of ascorbic acid solution.[16] In order to minimize variation in thickness of slice and speed of slicing one person prepared tissues from all animals. Conjugated bilirubin in the liver slices was determined by the method of Hargreaves.[17] The total quantity of bilirubin conjugated during a two-hour incubation is the sum of the quantities measured in the medium and slices.

Pregnanolone, phenolphthalein and sodium taurocholate were added to the incubation flasks as methanolic solutions, which were left to evaporate overnight. Disodium ethylenediamine tetraacetate (EDTA) was added as a solution in Krebs–Ringer bicarbonate buffer.

RESULTS

Animals treated with oral phenobarbitone for 3 weeks had body weights, liver weights and liver weight/body weight ratios which were significantly greater than those of control animals (Table 1). Animals fed a protein-free diet for 4 days had liver weights and liver weight/body weight ratios which were significantly smaller than those of control animals (Table 1).

Phenobarbitone treatment resulted in a highly significant but not very large increase in the amount of conjugated bilirubin formed by liver slices, whereas feeding a protein-free diet had no significant effect (Table 2). Neither treatment altered the amount of conjugated bilirubin remaining in the liver slices at the end of the two hour incubation. The effects of pregnanolone, phenolphthalein, taurocholate and EDTA in the groups on control diet and phenobarbitone are given in Table 3.

TABLE 1. EFFECT OF PHENOBARBITONE TREATMENT AND A PROTEIN-FREE DIET ON BODY WEIGHT, LIVER WEIGHT AND THEIR RATIO

Each value is the mean of six animals ± S.D. The P value (Student's *t* test) is for the significance of the difference from the control group.

Treatment	Mean body wt. (g)	P	Mean liver wt. (g.)	P	Mean liver wt. (% of body wt.)	P
Control	304 ± 17	—	13·3 ± 1·8	—	4·4 ± 0·4	—
Oral phenobarbitone 3 weeks	347 ± 26	<0·02	21·0 ± 2·1	<0·001	6·0 ± 0·2	<0·001
Protein-free diet 4 days	284 ± 20	>0·05	11·0 ± 1·3	<0·05	3·8 ± 0·2	<0·02

TABLE 2. EFFECT OF PHENOBARBITONE TREATMENT AND A PROTEIN-FREE DIET ON THE CONJUGATION OF BILIRUBIN BY LIVER SLICES

Each value is the mean of six animals ± standard deviation. The P value (Student's *t* test) is for the significance of the difference from the control group.

Treatment	Rate of bilirubin conjugation (μg/g/hr)	P	Conjugated bilirubin in slices (μg/g)	Total bilirubin conjugation (μg/g/2 hr)	P
Control	62 ± 8	—	22 ± 3	146 ± 16	—
Oral phenobarbitone 3 weeks	90 ± 11	<0·001	22 ± 2	202 ± 22	<0·001
Protein-free diet 4 days	59 ± 6	>0·05	24 ± 4	142 ± 13	>0·05

TABLE 3. EFFECT OF INHIBITORS AND STIMULATORS ON THE LIVER SLICE SYSTEM CONJUGATING BILIRUBIN

Each value is the mean of 6 animals ± S.D. An asterisk indicates that the value is significantly different (P = 0·05 according to Student's *t* test) from the value obtained for the incubation with no additions to the medium. Incubation was for 2 hr.

Treatment	Addition to incubation medium	Rate of bilirubin conjugation (μg/g/hr)	Conjugated bilirubin in slice (μg/g)	Total bilirubin conjugation (μg/g/2hr)
Control diet	None	62 ± 8	22 ± 3	146 ± 16
	Pregnanolone ($2·5 \times 10^{-5}$M)	31 ± 8*	37 ± 5*	99 ± 17*
	Phenolphthalein (5×10^{-4} M)	16 ± 2*	17 ± 2*	49 ± 4*
	Taurocholate (1×10^{-3} M)	82 ± 12*	15 ± 3*	179 ± 24*
	EDTA (5×10^{-4} M)	50 ± 7*	38 ± 4*	138 ± 14
Oral phenobarbitone, 3 weeks	None	90 ± 11	22 ± 2	202 ± 22
	Pregnanolone ($2·5 \times 10^{-5}$ M)	60 ± 17*	34 ± 6*	154 ± 34*
	Phenolphthalein (5×10^{-4} M)	16 ± 7*	20 ± 1	52 ± 14*
	Taurocholate (1×10^{-3} M)	109 ± 19	16 ± 1*	234 ± 38
	EDTA (5×10^{-4} M)	96 ± 11	35 ± 6*	227 ± 23

DISCUSSION

The NADPH-dependent, drug-metabolizing and steroid-hydroxylating enzymes show great changes in "substrate-induced" enhancement, and in dietary depression. Our studies of the changes in bilirubin conjugation and secretion show that these are altered, little or not at all, under conditions which produce several fold changes of the hydroxylating enzymes.

The finding of an increase in the liver weight, both absolutely and as a percentage of body weight, agrees with that previously reported[7] for intra-peritoneal administration of phenobarbitone. McLean and McLean[14] reported that rats lost weight on a no-protein diet, and the weight differences between animals on control and no-protein diet in our experiments are consistent with their values. They found that at 8 days the liver weight, expressed as a percentage of body weight, was maintained; there was a small drop from 4·4 to 3·8 per cent in our 4-day experiment (Table 1).

Although oral phenobarbitone treatment of rats led to a 45% increase in the capacity of liver slices to conjugate and secrete bilirubin, this must be compared with much larger increments in microsomal enzymes under comparable conditions. Marshall and McLean[13] found that oral phenobarbitone (1 g/l. in the drinking water) or i.p. injection (100 mg/kg day) produced a 5-fold increase in microsomal cytochrome P-450 after 4 days. Schmid, Marver and Hammaker[18] found 8- and 3-fold increases of microsomal P-450 and cytochrome b_5, respectively, at 6–7 days. Ernster and Orrenius[4] noted 5-fold increases of the oxidative demethylation of aminopyrine, of P-450, and NADPH-cytochrome c reductase activity at 5 days.

The rise in liver microsomal protein, phospholipid and RNA was 50–100 per cent in the latter study and this is similar to the increased activity we found for bilirubin conjugation and secretion.

The liver-slice system behaves like intact liver, conjugating and secreting bile pigment. Hitherto studies have been made of microsomal enzymes or of intact liver in which it is difficult to take account of enlargement of the liver or increase in the amount of endoplasmic reticulum. In addition most studies have been based on the use of artificial substrates for glucuronyl transferase(s). Zeidenberg, Orrenius and Ernster[8] found, after 5 days of phenobarbitone treatment, about 60 per cent increase of glucuronyl transferase (expressed as activity per mg of microsomal protein) using *p*-nitrophenol as substrate. Catz and Yaffe[9] found increased glucuronyl transferase activity of mouse liver microsomes using bilirubin as substrate, but not with either phenolphthalein or *o*-aminophenol. De Leon, Gartner and Arias[7] studied the conjugation of bilirubin by homogenates. It increased about 70 per cent after 14 days phenobarbitone treatment. Four days treatment did not increase the activity with bilirubin or *o*-aminophenol although *p*-nitrophenol glucuronide formation was enhanced. Dutton and Lawes[19] have reviewed the evidence that there are several glucuronyl transferases. An *in vivo* study by Roberts and Plaa[20] showed an increase in secretion of administered bilirubin, of the same degree as the increase in liver weight. Our results with liver slices show that there is a consistent, but small, increase when activity is expressed per gram of liver.

The most striking dissociation between activity of the hydroxylating and glucuronylating enzymes of endoplasmic reticulum is found in the animals fed the protein-free diet for 4 days. McLean and McLean[14] found that the demethylation of pyramidon was reduced to 9 per cent of the control while hydroxylation of benzopyrine was 19

per cent. There was no reduction of the conjugation and secretion of bilirubin in our experiments. Although the rats were larger than those of McLean and McLean[14] they showed a response to the diet in other ways (loss of body and liver weights).

Inhibitors were included in the studies as it was possible that the liver, altered by phenobarbitone or by protein deprivation, might respond in a different way. We chose for study inhibitors which had different effects on the accumulation of conjugated pigment in the slices, and therefore probably affected conjugation and secretion in different ways. For instance pregnanolone increased the conjugated pigment in the slices at the end of incubation, as reported previously for pregnanediol glucuronide by Bevan, Holton and Lathe.[21] EDTA had a similar effect on conjugated pigment in the slices without much reduction of the amount secreted into the medium. The choleretic, taurocholate, on the other hand increased conjugation but reduced the amount of pigment in the slices.

Results with inhibitors are usually recorded as a percentage of uninhibited controls. We have avoided this as it is not clear that the percentage inhibition is more significant than the absolute values. Thus the percentage inhibition by pregnanolone was 50 per cent on the control diet and 33 per cent in the phenobarbitone stimulated group, but the absolute decrement was the same. Taurocholate did not increase the rate of conjugation in the phenobarbitone treated group and EDTA was not inhibitory.

Although there are a number of differences between the control and phenobarbitone stimulated group, these were not large and, taken together, the results reinforce the impression that the handling of bilirubin by liver slices from phenobarbitone treated animals was essentially normal. The detailed results of control slices and inhibited and stimulated slices, from rats on the protein-free diet, are not given in the table, as they did not differ in any significant respect from the animals on the control diet.

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