DRUG METABOLIZING FUNCTION OF
ISOLATED PERFUSED LIVER

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Abstract—How closely the isolated liver of the rat would simulate the in vivo function of
the organ in terms of the metabolic pattern of the compounds such as bromosulpho-
phthalein, p-nitrophenol, hexobarbital, and indocyanine green was investigated. In
order to produce tissue with the stimulated function, the animal was pretreated with
phenobarbital and, for the reverse purpose, with ethionine. Some of the indices of
the function employed herein, such as the appearance pattern of the compound, the
rate of biochemical transformation or the biliary excretion, showed that the perfused
liver would generally well reflect the in vivo situation. The method with isolated and
perfused liver could exclude the participation of other organs and also the influence
of the factors unavoidable in an in vivo experiment. Thus, it is suggested that the
isolated perfused liver is useful for studying directly the functional level of the organ
as drug metabolizing tissue.

The biological transformation of the foreign compounds and the excretion into bile
are the important functions assigned to the liver. Employment of appropriate compounds
and testing of the metabolism provide reliable information about the functional state of
the organ and also allow for further selection and classification of the compounds as a
diagnostic tool of the liver function. Such should be achievable using intact animals but
the validity of the obtained results is reduced by uncontrollable factors such as accumula-
tion of the compound in the other tissues (1, 2) or the compound induced changes in the
flow rate of blood through the liver tissue or of the bile (1).

On the other hand, more detailed information about the biochemical aspects of the
metabolic pathway of a given compound would be provided by simplifying the experi-
mental system to slices, isolated cells, etc. (3, 4), but on the contrary, the simplicity of the
experimental system cannot always correctly reflect the state of normally functioning liver.

The isolated liver perfusion technique not only circumvents the unwanted factors
in the in vivo experiment but also provides a more physiological milieu than the simplified
system. The method originally devised by Miller et al. (5) for studying the role of liver
in blood protein synthesis, has been used for examining drug metabolism by the liver (6–8),
but the actual applicability of the method as a functioning model of the liver has rarely
been tested (6).

The major criteria employed herein included how closely the perfused liver can simu-
late the in vivo situation in terms of the disappearance patterns of the foreign compounds
from the perfusion circuit, and how the patterns would change by the drug pretreatments known to stimulate or depress liver function.

MATERIALS AND METHODS

Preparation of rats with modified liver function: Male Wistar strain rats (200-20 g, body weight) were daily treated with phenobarbital Na (80 mg/kg, i.p.) for 4 days or with ethionine (100 mg/kg, i.p.) for 2 days, and 24 hr after the last scheduled treatment, the animals were used for experiment. The animals treated with saline alone (5 ml/kg) for 2 or 4 days, served as the controls.

Liver perfusion: A Natsume TN liver perfusion apparatus used is in principle the same as the one originally composed by Miller et al. (5). A glass electrode for pH measurement and a flow cell for spectrophotometric measurement were inserted into the perfusion circuit. The volume of the perfusion medium for a liver was 120 ml, which contained 312 mg of glucose in Krebs-Henseleit buffer (9). The pH of the medium was adjusted to 7.4 by passing a gas mixture of O\textsubscript{2} and CO\textsubscript{2} (95.5, v/v) and when it fell below 7.4 during experiment, was maintained by adding NaHCO\textsubscript{3}. For operation, the animal was anesthetized with ether and, according to the procedure described by Hems et al. (10), the bile duct, the portal vein and the vena cava were cannulated respectively. Perfusion with the cold medium was began after cannulation of the portal vein and was continued until the blood color had disappeared from the perfusate. Thereafter, the liver was connected to the perfusion circuit and warm medium was utilized. The time spent from start of operation till that of perfusion with the warm medium was less than 20 min. The direction of perfusion was from the portal vein to the thoracic vena cava and the hydrostatic pressure 20 cm at the portal vein level. This condition gave a flow rate of 25-30 ml/min. The temperature of the cabinet and the circulating medium was maintained at 37 ± 0.5°C.

Analytical procedures: Indocyanine green (ICG) and sulphobromophthalein (BSP) were used mainly for measuring the biliary function of the liver. p-Nitrophenol (PNP) was employed as substrate of glucuronide formation and hexobarbital (HEX) for measuring hydroxylation activity. Five \textmu moles of ICG were added to the perfusion medium and the disappearance rate was continuously followed by direct recording of absorption at 780 nm of the perfusion medium. In order to estimate the excretion rate of the dye into the bile, the bile was collected for each 10 min after addition of the dye and the amount of the dye estimated from absorbancy at 790 nm since the wave length for the maximum absorption shifts in bile. BSP was added in the concentration of 6 \textmu moles/120 ml. The amount of the dye in 0.5 ml of the perfusate sample taken at various time intervals or in the 10 min sample of the bile was estimated as described by Nishikaze (11). In the case of PNP, the perfusate contained 32 \textmu moles of the dye at start and the disappearance rate from the perfusate was measured directly as decrease of absorbancy of the perfusate at 400 nm (12). The dye maximally absorbs at this wave length only when the pH of the solution is in the range of 9.5-12 (13) and then, the dye concentration in the perfusate was read from the standard curve run at pH 7.4. p-Nitrophenol-glucuronide (PNPG) in the
perfusate or in the bile was isolated after the termination by the method of Yeh and Woods (14). The pH of the total amount of bile collected during the experimental period or of 50 ml of the perfusate was adjusted to 2.0 with 0.1 N HCl and washed with chloroform three times to remove PNP and other interfering substances. The sample was hydrolyzed with glucuronidase (Worthington Biochemical Corp.) and estimated as PNP according to the method of Fishman et al. (15). The disappearance rate of HEX from the perfusate which originally contained 100 μmoles, was monitored by measuring the concentrations in the samples collected at various time intervals according to the method of Cooper and Brodie (16). For determination of HEX trapped by the liver, the whole liver was homogenized with 4 volumes of 1.15% KCl and an aliquot (2 ml) of the homogenate analyzed.

RESULTS

Disappearance patterns of the compounds from the perfusate and effects of drug pretreatments

Fig. 1 compares the disappearance patterns of four types of compounds and effects of phenobarbital and ethionine pretreatments. In the phenobarbital pretreated liver, the disappearance rates of PNP and HEX were significantly accelerated. In contrast, the ethionine pretreatment invariably decreased the rates of ICG, HEX and BSP. Either phenobarbital or ethionine, when added directly to the perfusate of the liver from the naive rat, failed to exert any significant influence on the disappearance pattern of BSP (Fig. 2).

![Graphs showing disappearance patterns](image)

**Fig. 1.** Disappearance patterns of various compounds from the perfusion medium of the isolated liver and effects of phenobarbital or ethionine pretreatment.

The liver was taken from the animal pretreated with saline alone as the control (××××), phenobarbital (○○○○) or ethionine (●●●●). The vertical scale indicates the concentration of each compound in the perfusate at the time on the horizontal scale after addition of the compound at Time 0. Each point represents the mean of 5 preparations. Significant difference from the control was marked; *(P<0.05) or **(P<0.01).
FIG. 2. Effect of phenobarbital or ethionine directly added to the perfusion medium on the disappearance pattern of BSP.

The liver of the naive rat was perfused with the normal medium (×···×), or the medium containing $6.5 \times 10^{-4}$ M of phenobarbital (○○○) or $1.0 \times 10^{-3}$ M of ethionine (●●●). BSP was added at Time 0. Each point represents the mean of 5 preparations.

Biliary excretion of the compounds and effects of drug pretreatments

When the control liver was perfused with the medium containing BSP, more than 20% of added dye was recovered in the bile within 1 hr. The phenobarbital pretreatment markedly accelerated the excretion rate of BSP, while in the liver of the ethionine pretreated rat, the rate decreased to about one half of the control liver (Figs. 3 and 5). In the case of ICG, only a small fraction of the added amount was recovered in the bile, 2% of ICG after 60 min perfusion, and the excretion was not affected with phenobarbital pretreatment but lowered with ethionine (Figs. 4 and 5). PNP was poorly excreted in the bile, 3% in the form of PNPG after 40 min perfusion. The phenobarbital pretreatment ac-
FIG. 4. Biliary excretion of ICG from perfused liver and effect of phenobarbital or ethionine pretreatment.

The experimental conditions and expression of the obtained results are the same as those in Fig. 3, except that ICG was used instead of BSP.

FIG. 5. Comparison of effect of drug pretreatment on the biliary excretion of three dyes from perfused liver.

Effects of drug pretreatments (phenobarbital; \( \text{□□□} \) and ethionine; \( \text{○○○} \)) on the biliary excretion of three dyes were compared in terms of % increase or decrease of the amount of dyes excreted into the bile for 1 hr infusion period. The control value was taken as 0% and the values for BSP and ICG calculated from the results in Fig. 3 and 4, respectively. Each value represents the mean of 5 preparations and the vertical bar S.E. Significant difference from the control was marked; *\((P<0.05)\) or **\((P<0.01)\).

celerated the biliary excretion of PNPG significantly (Fig. 5). The \textit{in vitro} addition of either drug had no effect on the excretion pattern of BSP (Fig. 6).

\textbf{Metabolism of HEX and PNP in the perfused liver and effects of drug pretreatments}

Four days treatment with phenobarbital significantly increased both liver weight and the disappearance rate of HEX from the perfusate (Table 1), but in terms of \( \mu \)moles/tis-
FIG. 6. Effect of phenobarbital or ethionine directly added to the perfusion medium on the biliary excretion of BSP.

The liver of the naive rat was perfused with the normal medium ( ), or the medium containing $6.5 \times 10^{-4}$ M of phenobarbital ( ) or $1.0 \times 10^{-2}$ M of ethionine ( ). The other experimental conditions were the same as those in Fig. 3.

**Table 1.** Effect of phenobarbital or ethionine pretreatment on the hexobarbital metabolism by perfused liver

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver weight (g)</th>
<th>Hexobarbital metabolism</th>
<th>Mole/g liver/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>saline</td>
<td>$6.7 \pm 0.2^*$</td>
<td>$58.5 \pm 3.3$</td>
<td>$8.7 \pm 0.8$</td>
</tr>
<tr>
<td>phenobarbital</td>
<td>$8.7 \pm 0.4^{**}$</td>
<td>$75.4 \pm 3.0^{**}$</td>
<td>$8.6 \pm 0.4$</td>
</tr>
<tr>
<td>ethionine</td>
<td>$7.9 \pm 0.7$</td>
<td>$48.5 \pm 4.5$</td>
<td>$6.3 \pm 0.9$</td>
</tr>
</tbody>
</table>

$^*$: Each value represents the mean ± S.E. from 5 rats.

**Table 2.** Effect of phenobarbital pretreatment on the glucuronidation of PNP and its excretion into bile in the perfused liver

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Phenobarbital pretreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNPG in bile</td>
<td>$1.47 \pm 0.13^*$</td>
<td>$2.42 \pm 0.21^{**}$</td>
</tr>
<tr>
<td>PNPG in perfusate</td>
<td>$24.9 \pm 2.1$</td>
<td>$22.2 \pm 0.9$</td>
</tr>
<tr>
<td>PNPG in bile and perfusate</td>
<td>$26.4 \pm 2.1$</td>
<td>$24.6 \pm 1.0$</td>
</tr>
</tbody>
</table>

$^*$: Each value represents the mean ± S.E. from 5 rats.

**Biliary flow rate and effects of drug pretreatments**

Pretreatment of rat with phenobarbital is known to increase the biliary flow rate in

sue weight there was no increase in phenobarbital treated group. On the other hand, ethionine pretreatment did not induce changes in either liver weight or the disappearance rate of HEX. When the effect of phenobarbital pretreatment on the PNPG formation by liver and excretion into bile of the metabolite was examined, it was found that the treatment had no influence on the formation step but almost doubled the excretion rate of PNPG into the bile (Table 2).
FIG. 7. Effect of phenobarbital or ethionine pretreatment on the bile flow rate of perfused liver.

Effects of drug pretreatments on the bile flow were compared in terms of the volume of bile excreted for 1 hr perfusion. The dye which the perfusion medium contained, is indicated below each group of blocks. The data for BSP, PNP or ICG were obtained with the corresponding group of livers in Fig. 5.

- : saline alone as the control
- - - : phenobarbital
** - - : ethionine

vivo (17). From the results in Fig. 7, it can be seen that the pretreatment increased the flow rate of perfused liver when BSP or PNP was added to the perfusate. However, such effect of the pretreatment was masked in the presence of HEX or ICG in the medium. On the other hand, ethionine pretreatment uniformly reduced the flow rate and the effect was not influenced by the compound added to the perfusate.

DISCUSSION

In the liver perfusion experiment, the following factors would regulate the disappearance rate of a foreign compound added to the perfusion medium; uptake, storage and metabolism by the liver and excretion into bile. In order to estimate the applicability of the method as the functional test of the organ, therefore, the compounds used should be those in which the extent of contribution of the individual factors and modification by the drug pretreatment are relatively well known. In the present experiment, four compounds were selected for the following reasons: 1) BSP is known to be conjugated with glutathione in the liver and both the compound and the conjugate to be excreted into bile (18), 2) ICG is not metabolizable, for excretion of which the biliary route is major (19), and directly measurable by its color, 3) PNP is transformed to PNPG, the excretable form through the biliary route (20) and both are measurable by color, and 4) HEX disappears from the perfusate mainly by oxidation in the liver (21) and, in either form, is not excreted into bile.

As shown in Fig. 1, phenobarbital pretreatment significantly accelerated the disappearance rate of PNP or HEX from the perfusate. In the case of PNP, this effect can be explained by assuring that a larger portion of the dye itself or in the form of PNPG is
retained by the liver enlarged by the drug pretreatment because the treatment failed to change the PNPG content in the perfusate plus bile (Table 2). In contrast, the amount of unchanged HEX found in the control liver after 1 hr perfusion, was higher than that in the liver treated with drug. The increased rate of disappearance seems to reflect the increased ability to metabolize the compound (Table 1). Though the in vivo disappearance rates of BSP and ICG have been reported to increase in the phenobarbital treated animals (22), our experiment failed to reproduce the in vivo results. Oinhaus et al. (23) have reported that the drug treatment increased the blood flow rate of the liver by 33 to 175%. Throughout our experiment, however, the flow rate was maintained at a constant level and the difference of the flow rate through the liver may explain the discrepancy between our results and previous reports. Thus, by the same reason, the results obtained for PNP and HEX might be underestimated in the present experimental system.

During 1 hr perfusion, about 20% of BSP came out into the bile and with phenobarbital pretreatment the rate almost doubled. A similar result was obtained in PNPG excretion. In contrast, the excretion of ICG was not affected by the pretreatment and, in either case of the latter two compounds, the amount found in the bile corresponded to only a small fraction of the dye originally added to the perfusate. According to Klassen (22), ICG is mainly excreted by the biliary route in vivo and within 1 hr after administration of 25 mg/kg, more than 20% is excreted into the bile. He also reported that ICG inhibits the biliary flow rate in the same concentration as was used in this experiment. The discrepancy between the in vivo and the perfusion experiment observed in biliary excretion of ICG might be due to the inhibitory effect of the dye on the biliary flow.

Levine et al. (24) proposed that phenobarbital pretreatment would accelerate the excretion of only the compounds which are metabolized in the liver and excretable by the biliary route. In fact, BSP and PNP, both of which meet the requirements, were found to be excreted more rapidly in the drug pretreated liver than in the control. In addition, the biliary flow rate itself was enhanced by the drug treatment, when BSP or PNP was used. It is considered therefore that the increased excretion rates of these two compounds from the liver of phenobarbital treated rat may to a certain extent be explained by the increase of the biliary flow rate itself.

On the other hand, ethionine pretreatment uniformly depressed various measures of the liver function employed in this experiment, though the animals were treated with the drug at a relatively low dose level since treatment with higher doses was found to make the liver too fragile to conduct the perfusion experiment (25, 26). The disappearance rate of these dyes, the biliary excretion of BSP and ICG, or the biliary flow rate, all these measures were found to be in subnormal levels in the ethionine pretreated liver.

Though differences between the in vivo and the perfusion experiment were evident, the results of the present study generally showed a good parallel with the in vivo function of the liver.
REFERENCES

11) NISHIKAZI, O.: Igaku no Ayumi 9, 551 (1965) (in Japanese)