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# DIETARY MODULATION OF PLASMA BILIRUBIN AND OF HEPATIC MICROSOMAL LIPID PEROXIDATION IN THE GUNN RAT

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An in vitro assay for the simultaneous measurement of lipid peroxidation (LPO) and bilirubin degradation (BRD) activities in rat liver microsomes has been developed; a good correlation between the 2 activities was observed ($r = 0.78$). In the Gunn rat a lipid free diet caused an increase in plasma bilirubin (62.4 ± 25.8%, $n = 6$) and a concomitant decrease in both hepatic microsomal LPO and BRD to zero. In contrast, on a 25% lipid diet there was a decrease in plasma bilirubin (46.1 ± 3.6%; $n = 8$) associated with an increase in LPO (1.26 ± 0.11 nmol/min/mg protein, and BRD (0.21 ± 0.6 nmol/min/mg protein). Therefore, in the absence of bilirubin glucuronidation, dietary modulation of plasma bilirubin and lipid peroxidation appear to be closely associated.

KEY WORDS: Lipid peroxidation, bilirubin degradation, Gunn rats, plasma bilirubin, dietary lipid.



## 1. INTRODUCTION

Gunn rats lack functional UDP-glucuronyltransferase (E.C.2.4.1.95) activity and consequently have a marked unconjugated hyperbilirubinaemia. Under constant dietary conditions, their plasma bilirubin is maintained at a stable high level, apparently by alternate, but less efficient, pathways(s) for bilirubin elimination.[1]

It has been shown that dietary lipid lowers the plasma bilirubin in Gunn rats, while a lipid-free diet elevates the plasma bilirubin.[2,3] These findings have so far not been satisfactorily explained.

Recent *in vitro* studies have shown an association between hepatic microsomal bilirubin degradation and lipid peroxidation.[4] Experiments were therefore performed in which the lipid composition of the diets of Gunn rats was altered, to determine whether changes in the microsomal bilirubin degradation and lipid peroxidation could account for the dietary modulation of plasma bilirubin.

## 2. MATERIALS AND METHODS

### *Materials*

All chemicals (Analar grade) were purchased from either British Drug Houses or Sigma.

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Solkafloc, a wood cellulose product used as a source of dietary fibre, was obtained from Special Diets Services Ltd., Witham, Essex.

*Dietary Regimen*

Jaundiced male Gunn rats, from the Royal Free Hospital School of Medicine colony, weighing 280–380 g were used in all experiments. For the initial *in vitro* studies they received a standard 41B diet ad libitum. For the *in vivo* studies they were fed for 2 weeks on diets containing either 0% or 25% w/w corn oil, with carbohydrate in the form of corn starch contributing 69% or 44% of the diets respectively. Otherwise, both diets were identical containing 20% casein and balanced amounts of vitamins, minerals and Solkafloc.[2] Blood samples (1 ml) were obtained under halothane anaesthesia by cardiac puncture at the beginning and end of the test period, and plasma bilirubin concentrations were estimated by a standard diazo method with caffeine as the accelerator;[5] the initial bilirubin concentations ranged from 129 to 310 $\mu$M; (mean 179 $\pm$ 8, $n$ = 24). After 14 days, the rats were sacrificed and liver microsomes were prepared by a rapid calcium aggregation method[6] and stored at $-70$°C.

*Bilirubin Degradation and Lipid Peroxidation*

Bilirubin (BDH) was dissolved in DMSO and the volume adjusted with 40 mM (3-[N-morpholino]propanesulphonic acid (MOPS) buffer pH 7.4 and sodium taurocholate to give a solution containing 216 $\mu$M bilirubin, 620 mM DMSO and 9.6 mM taurocholate which was suitably diluted for the incubation studies.

Microsomes (1.5–2 mg protein ml$^{-1}$) were incubated aerobically at 37°C in dim light in 8 ml of 40 mM MOPS buffer pH 7.4 with 1.6 mM ADP, 18 $\mu$M ferrous sulphate, 35 $\mu$M unconjugated bilirubin (100 mM DMSO, 1.55 mM taurocholate) and an NADPH generating system (0.18 mM $NADP^+$, 4.03 mM glucose-6-phosphate and 0.19 IU ml$^{-1}$ glucose-6-phosphate dehydrogenase), in order to initiate lipid peroxidation[7] and bilirubin degradation enzymatically. To initiate lipid peroxidation and bilirubin degradation non-enzymatically, the NADPH generating system was replaced with 0.2 mM ascorbic acid. Reactions were started by the addition of microsomes and samples were taken at zero time and various time intervals to follow the rate of disappearance of bilirubin and the progress of lipid peroxidation. In some experiments bilirubin was added as sodium bilirubinate[8] or as bilirubin in DMSO at pH 8.2 in the presence of EDTA.[9] In other incubations, microsomal enzymes were denatured by heating the microsomal suspension at 83°C for 5 min prior to addition to the incubation mixture.

Control experiments were performed in which enzymatically initiated lipid peroxidation activity was determined with and without DMSO/taurocholate. In the absence of bilirubin, in microsomes from four animals the mean activities were 1.29 $\pm$ 0.10 and 1.64 $\pm$ 0.11 nmol min$^{-1}$ mg protein$^{-1}$ respectively. This indicates a 21% inhibition of lipid peroxidation activity by DMSO/taurocholate. Bilirubin did not influence DMSO/taurocholate inhibition of lipid peroxidation (activity = 1.23 $\pm$ 0.11 nmol min$^{-1}$ mg protein$^{-1}$), nor did DMSO/taurocholate appear to influence rates of bilirubin degradation (see below).

Bilirubin degradation activity was assayed by measuring the rate of decrease in bilirubin concentration using a diazo method.[10] Results were expressed as nmol of bilirubin degraded min$^{-1}$ mg microsomal protein$^{-1}$.

Lipid peroxidation activity was determined spectrophotometrically by measuring the rate of formation of thiobarbituric acid reactive products (TBA-RP).[11] Results were expressed as nmol of TBA-RP formed $min^{-1}$ mg $protein^{-1}$. 1 mM butylated hydroxytoluene was added to the reaction mixture which was heated at 80°C. Zero time values were routinely measured and subtracted from all TBA-RP assays: in 5 consecutive experiments, these averaged only 16% ± 4% of the total TBA-RP formed in 10 min. Evidence that biliverdin, derived from bilirubin, did not interfere significantly in the TBA-RP assay was obtained by shaking the butanol extracted TBA-RP with 10 M NaOH, when no blue pigment was formed.[12]

Microsomal protein was assayed by a modification of the Lowry method.[13]

### *Statistics*

All results have been expressed as means ± SEM and statistical analysis was performed using the unpaired t-test.

## 3. RESULTS AND DISCUSSION

### *In vitro Studies*

Table I shows that NADPH, ADP chelated iron and oxygen were essential for the enzymatically initiated microsomal degradation of bilirubin (BRD) and lipid peroxidation (LPO). A significant correlation between BRD and LPO activities was observed ($r = 0.78$, $n = 23$), suggesting that bilirubin is oxidised as lipid peroxidation proceeds.

When exogenous iron was omitted from the incubation mixture, degradation of bilirubin was not detected, thereby indicating that its oxidation by the cytochrome $P_{450}$ system was negligible. This was also the case when bilirubin was added as sodium bilirubinate, pH 7.4[8] or dissolved in tris/KCl buffer containing DMSO and EDTA pH 8.2.[9]

TABLE I
NADPH induced lipid peroxidation and bilirubin degradation activities in Gunn rat hepatic microsomes: co-factor requirements.

| | $n$ | Mean activity ± SEM ($nmol.min^{-1}$ mg $prot^{-1}$) Lipid peroxidation | Bilirubin degradation |
|---|---|---|---|
| Complete system*: | | | |
| Native microsomes | 23 | 1.26 ± 0.09 | 0.40 ± 0.04 |
| – Minus NADPH | 3 | 0.02 ± 0.02 | 0.00 |
| – Minus $FeSO_4$, ADP | 3 | 0.00 | 0.00 |
| – Minus $O_2$ (100% $N_2$) | 3 | 0.13 ± 0.04 | 0.01 |
| Heat denatured microsomes | 6 | 0.06 ± 0.08 | 0.00 |

*Microsomes (1.5–2 mg protein $ml^{-1}$), 35 μM bilirubin, NADPH generating system, 18 μM $FeSO_4$, 1.6 mM ADP, 40 mM MOPS pH 7.4. Incubation for 10 min at 37°C.

The addition of bilirubin to the standard incubation mixture as sodium bilirubinate gave a comparable value for BRD activity ($0.37 \pm 0.06$; $n = 7$ mnol.min$^{-1}$ mg protein$^{-1}$). This shows that DMSO/taurocholate does not affect bilirubin degradation activity (Table I).

A good association was also found between bilirubin degradation and lipid peroxidation when the latter was initiated non-enzymatically with 0.2 mM ascorbic acid ($r = 0.71$; $n = 11$). In contrast to the enzymatically initiated system, both activities (BRD and LPO) were comparable in heat-denatured and native microsomes (Table II). These *in vitro* studies indicate that the degradation of bilirubin is an oxidative process dependent on lipid peroxidation.

TABLE II
Non-enzymatic lipid peroxidation and bilirubin degradation activities.

| | $n$ | Mean activity ± SEM (nmol.min$^{-1}$ mg prot$^{-1}$) Lipid peroxidation | Bilirubin degradation |
|---|---|---|---|
| Complete system*: | | | |
| Native microsomes | 11 | 0.98 ± 0.12 | 0.25 ± 0.06 |
| – Minus ascorbate | 4 | 0.11 ± 0.05 | 0.00 |
| Heat denatured microsomes | 11 | 1.24 ± 0.13 | 0.26 ± 0.13 |
| – Minus ascorbate | 4 | 0.03 ± 0.01 | 0.00 |

*Microsomes (1.5–2 mg protein ml$^{-1}$), 35 μM bilirubin, 0.2 mM ascorbic acid, 18 μM $FeSO_4$, 1.6 mM ADP, 40 mM MOPS pH 7.4. Incubation for 10 min at 37°C.

As the result of studies in which the oxidation of linoleic acid or soybean phosphatidylcholine was initiated by either 2,2′-azobis (2-4-dimethylvaleronitrile) (AMVN)[14] or 2,2′-azobis (2-amidinopropane) hydrochloride (AAPH),[15,16] Stocker and his associates have proposed an anti-oxidant role for unconjugated bilirubin,[14]

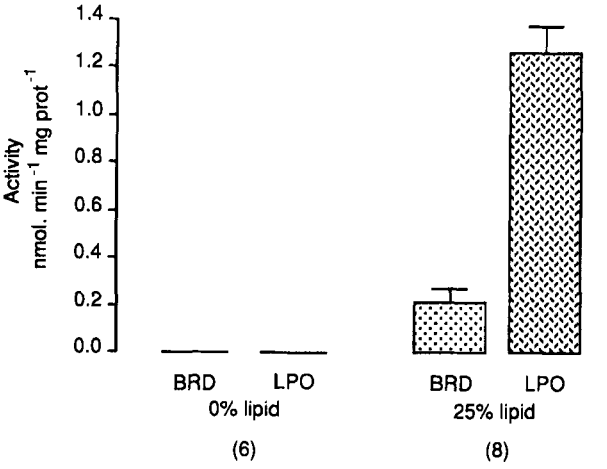


FIGURE 1 Effect of dietary lipid on enzymatically initiated lipid peroxidation (LPO) and bilirubin degradation (BRD) activities in Gunn rat liver microsomes. Activities are expressed as mean ± SEM; numbers in each group are in parenthesis.

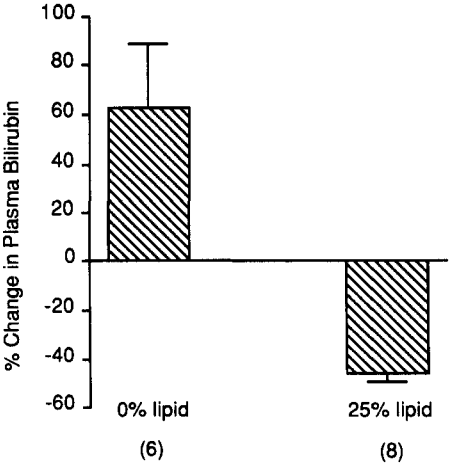


FIGURE 2 Effect of dietary lipid on plasma bilirubin in the Gunn rat (expressed as mean percentage change in concentration after 14 days).

albumin-bound bilirubin,[15] and conjugated bilirubin.[16] To investigate this hypothesis further, microsomal lipid peroxidation was initiated enzymatically in 2% or atmospheric oxygen, at different concentrations of bilirubin (1–65 $\mu$M) using the incubation conditions described above. The lipid peroxidation activity was not inhibited (data not shown). Moreover, biliverdin formation was not detected which is contrary to the findings obtained with AAPH induced oxidation of bilirubin-albumin.[15] Further studies are, therefore, required to ascertain whether bilirubin acts as a physiological antioxidant.

*Dietary Modulation*

Significant enzymatically initiated bilirubin degradation and lipid peroxidation activities were evident during 10 min incubation of microsomes from rats fed the high lipid diet (Figure 1). Contemporaneously, there was a significant decrease in plasma bilirubin of 46.1 $\pm$ 3.6% ($n$ = 8) over the two week dietary period (Figure 2). In contrast, microsomes from the lipid free diet, did not produce appreciable bilirubin degradation or lipid peroxidation up to 60 min incubation (Figure 1). Others have reported a similar lack of lipid peroxidation in rats fed a lipid-free diet.[17] The lipid free diet also resulted in a significant increase in the plasma bilirubin of 62.5 $\pm$ 25.8% ($n$ = 6) over the dietary period (Figure 2); this could be due to a decrease in the oxidative destruction of bilirubin as a result of diminished lipid peroxidation.

Similar activities for LPO and BRD were obtained in microsomes from the two dietary groups, when ascorbic acid was used to initiate lipid peroxidation by the non-enzymatic mechanism (data not shown).

In order to explore the mechanisms underlying these diet modulated effects, lipid peroxidation was initiated non-enzymatically with 0.2 mM ascorbate in heat treated microsomes from both dietary groups. If the high lipid diet promotes greater lipid peroxidation activity by induction of microsomal enzymes then heat treatment of

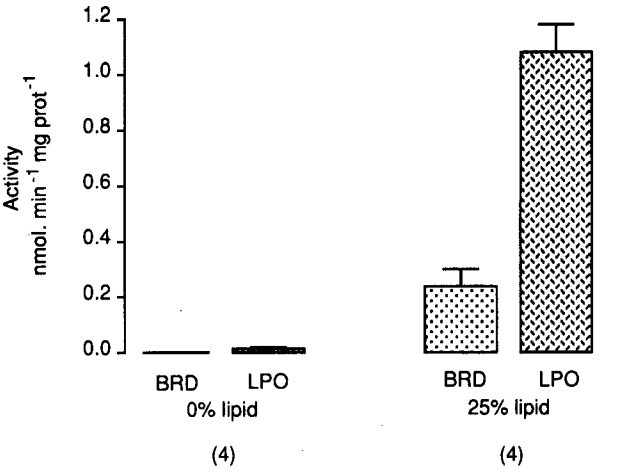


FIGURE 3 Effect of dietary lipid on non-enzymatically initiated (0.2 mM ascorbic acid) LPO and BRD activities in heat denatured Gunn rat hepatic microsomes.

microsomes would be expected to destroy these enzymes. Similarly, if the decreased BRD and LPO activities in the lipid free group are due to other enzymatic pathways, these would also be destroyed. The observed differences in lipid peroxidation and bilirubin degradation activities between the two groups of microsomes, as described for both the NADPH and ascorbic acid initiated systems, would therefore be eliminated. However, if the basis of these differences is not enzymatic, then they would persist in heat denatured microsomes as indeed is shown in Figure 3. This suggests that LPO and BRD must be influenced by other factors, such as the lipid composition or the lipid antioxidant levels in microsomes from the two dietary groups.

*In conclusion* these dietary studies have shown that alterations in the dietary lipid content have a marked effect on the hepatic microsomal lipid peroxidation activity which is inversely associated with changes in the plasma bilirubin in the Gunn rat. Similar findings would be expected in patients with the Crigler-Najjar and Gilberts syndrome, who also have a deficiency in bilirubin glucuronidation and respond to dietary changes in a similar manner.[18,19] It remains, however, to be established whether there is a causative relation between lipid peroxidation and the plasma bilirubin level in the Gunn rat and to what extent it is an important factor in the alternate pathway(s) of bilirubin metabolism employed by these animals for the elimination of bilirubin.

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