The Distribution of Glutathione in the Rat Liver Lobule

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A quantitative cytochemical method was developed for measuring the GSH (reduced glutathione) content of hepatocytes in different regions of the rat liver lobule. Use of this method enabled us to show that GSH is not evenly distributed within the rat liver lobule. The hepatocytes located within 100 μ m of the central vein contain much less GSH than do those in other regions of the rat liver lobule. We suggest that this partially explains the peculiar susceptibility of these cells to electrophilic attack by toxic metabolites formed via the microsomal cytochrome *P*-450 system.

Many otherwise inert xenobiotics are converted into hepatotoxic metabolites by the microsomal cytochrome *P*-450 system. A peculiar feature of this conversion is that the resultant liver necrosis is usually localized in the region of the lobule surrounding the central vein (McLean *et al.*, 1965; Brodie *et al.*, 1971). Mitchell *et al.* (1973*b*) have shown that the liver appears to be protected against this conversion by GSH.

GSH is particularly abundant in the liver and forms covalent conjugates with many xenobiotics and their metabolites, which are then excreted as mercapturic acids (Bray *et al.*, 1969; Boyland & Chasseaud, 1969). When the availability of glutathione is decreased to a very low value through conjugation, hepatotoxic metabolites, formed by the cytochrome *P*-450 system, cause cellular injury by binding covalently to vitally important hepatic macromolecules (Jollow *et al.*, 1973; Mitchell *et al.*, 1973*a*). The susceptibility of the centrilobular hepatocytes to damage by this mechanism could be partially explained by a gradient of concentration of GSH across the liver lobule.

It is therefore desirable to quantify the GSH content of hepatocytes in the different regions of the liver lobule. One approach to this problem would be the development of a rigorous quantitative cytochemical method for the determination of GSH in rat liver.

There are, however, two major problems in developing such a method. Firstly, when unfixed tissue sections are immersed in reaction media, GSH rapidly diffuses from its original site. Secondly, most chromogenic reactions are not specific for GSH.

Ashgar *et al.* (1975) have used the stain Mercury Orange in an attempt to develop a qualitative histochemical method for GSH. The response of this stain to GSH is slow, allowing time for the reaction product

to diffuse, and the reaction is not very specific. It therefore produces a very weak reaction for GSH uniform throughout the liver lobule. Loveridge *et al.* (1975) have shown that, in the presence of a certain high concentration of Fe^{3+} ions, ferricyanide is reduced more specifically by GSH than by other thiol compounds or other reducing agents and thus brings about the precipitation of the very insoluble dye ferric ferrocyanide (Prussian Blue), which can be measured by microspectrophotometry. Moreover, this reaction, at a high concentration of the reactants, occurs in a matter of seconds. We have developed a quantitative cytochemical method based on this reaction for measuring the GSH content of hepatocytes in different regions of the rat liver lobule.

Materials and Methods

Animals

Adult male albino Wistar rats (185–270 g) were killed by cervical dislocation 1 h after intraperitoneal administration of either diethyl maleate (0.7 ml/kg body wt.) or saline (0.9% NaCl).

Preparation of liver homogenates

Liver (2 g) was mildly homogenized in approx. 10 ml of ice-cold 5% (w/v) trichloroacetic acid. The homogenate was then made up to 20 ml with fresh ice-cold 5% trichloroacetic acid to give a 10% (w/v) suspension. After 5 min had been allowed for complete deproteinization, the suspension was centrifuged at 1000*g* for 15 min and the supernatant used for analytical procedures.

Determination of GSH and ascorbic acid in liver supernatant

Acid-soluble thiols, 95% GSH in rat liver (Jocelyn, 1972), were determined in the protein-free supernatant

Abbreviation used: GSH, reduced glutathione.

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by the method of Beutler *et al.* (1963) by using Ellman's (1959) reagent. Ascorbic acid was determined in the supernatant by the method of Zannoni *et al.* (1974). This method is a micro-modification of a previously described macro-method which relies on the reduction of ferric iron by ascorbic acid followed by the formation of a complex of the ferrous iron product and $\alpha\alpha$ -bipyridine (Sullivan & Clarke, 1955; Maickel, 1960).

Production of tissue sections

Blocks of liver, approx. 5 mm³ in size, were chilled in n-hexane maintained at -70°C by an outer bath of crushed solid CO_2 and ethanol. After 1 min the tissue blocks were removed, shaken free of excess hexane, and stored temporarily in a pre-cooled glass tube at -70°C . Removal from the coolant and all subsequent manoeuvres involving the cold tissues were performed with forceps pre-cooled to -70°C .

Sections (10 μm) were cut on a Bright's cryostat, at a cabinet temperature of -25°C to -30°C , with the knife cooled to -70°C by surrounding the shaft with crushed solid CO_2 (Chayen *et al.*, 1973). The sections were flash-dried on to glass slides and stored for up to 1 h in the cryostat cabinet until required. Sections prepared in this manner have been shown, by electron microscopy, to be free of ice damage (Altman & Barnnett, 1975).

Production of gelatine sections containing specific concentrations of reducing substances

GSH was dissolved in solutions of gelatine (20%, w/v) to give final concentrations of 2, 5 and 10 mM. Ascorbic acid and cysteine were separately dissolved in solutions of gelatine (20%) to give a final concentration of 10 mM. The gels were allowed to set for 30 min in a refrigerator. Blocks of these gels (5 mm³) were then chilled and sectioned at 10 μm in the manner described for the blocks of tissue.

Preparation and use of the reaction medium

The reaction medium contained ferric and ferricyanide ions in a molar ratio of 4:3 and was prepared by mixing equal volumes of 33% (w/v) FeCl_3 and 30% (w/v) $\text{K}_3\text{Fe}(\text{CN})_6$ just prior to use. Sections were immersed in this medium for the specific reaction time, washed briefly in water, air-dried and mounted in Farrants' medium.

Microdensitometry

Measurements were made by means of a Vickers M85 scanning and integrating microdensitometer. The use of this machine to overcome the problems of

measuring heterogeneously distributed reaction products in tissue sections has been discussed fully by Bitensky *et al.* (1973) and by Chayen (1978).

The coloured cytochemical reaction product was measured in 20 different fields with a $\times 20$ objective, scanning spot size 2 (being 2 μm in diameter in the optical plane of the specimen) and a field size of 20 μm diameter. By histological inspection the relative absorptions of ten groups of cells surrounding the central vein and ten groups close to the portal tract were measured in each tissue section. For each rat the mean relative absorption was determined in this way in both regions in four duplicate sections cut from two tissue blocks. By suitable calibration (Bitensky *et al.*, 1973), these values were converted into absolute units of mean integrated absorption.

Chemicals

Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid)] and crystalline GSH were obtained from the Sigma Chemical Co., Poole, Dorset, U.K. Farrants' medium was obtained from Hopkin and Williams, Romford, Essex, U.K. All other compounds and reagents were obtained from BDH, Poole, Dorset, U.K. The n-hexane was free from aromatic hydrocarbons, boiling range $67-70^{\circ}\text{C}$.

Statistics

Student's *t* test was used to test the significance of the results. The level of significance was taken as 5%. Grouped results from individual rats or sections were used.

Results

Absorption curve of Prussian Blue in liver sections

The mean integrated absorption of the insoluble reaction product, Prussian Blue, at wavelengths between 450 and 700 nm was determined by microdensitometry in liver sections from the control group of rats. The absorption curve has a maximum at 675 nm and a minimum at 475 nm. Compensation for light scatter was achieved by separate measurement of the A_{675} and A_{475} ; the specific absorption of the Prussian Blue is then given by $A_{675} - A_{475}$.

Tests on the specificity of the reaction

Loveridge *et al.* (1975) have found that, in the presence of Fe^{3+} ions at a concentration of 0.6 M, ferricyanide (0.45 M) is more specifically reduced by GSH than by other thiols or by ascorbate.

The specificity of this reaction has been further tested by incubating sections (10 μm) of gelatine, containing GSH or ascorbic acid or cysteine, each

at 10mM, for up to 20s. After 5s incubation both GSH and cysteine formed approximately twice as much reaction product as did ascorbic acid (see Table 1).

The reaction with cysteine was then virtually complete, but both GSH and ascorbic acid continued to react (see Table 1). Seeing that a greater amount of reaction product was formed in the sections containing GSH after 20s, we decided to use this incubation time in the present studies. Therefore, although some specificity is lost, less error is incurred in measuring any changes in GSH concentration because the specific absorption corresponding to GSH is relatively high. Moreover, in rat liver, GSH is present at almost 4 times the concentration of ascorbic acid (see Table 2) and at approx. 70 times the concentration of cysteine (Gaitonde, 1967; Jocelyn, 1972).

Table 1. Mean specific absorption of gelatine sections (10 μ m) containing GSH, ascorbic acid or cysteine (each at 10mM) after incubation in the specified reaction medium for 5 and 20 s

Duplicate sections were used. Ten measurements of the specific absorption ($A_{675}-A_{475}$) were made in each section. The values shown are the means $\pm$ S.E.M. of 20 measurements.

Substrate added	Mean specific absorption of reaction product formed	
	After 5s incubation	After 20s incubation
GSH	0.221 $\pm$ 0.002	0.329 $\pm$ 0.008
Cysteine	0.182 $\pm$ 0.005	0.206 $\pm$ 0.002
Ascorbic acid	0.119 $\pm$ 0.007	0.233 $\pm$ 0.005

Effect of diethyl maleate administration on the amount of reaction product formed in sections of rat liver

Two groups of male rats, one consisting of five control rats, designated C1–C5, and the other of four rats treated with diethyl maleate, designated D1–D4, were used. Diethyl maleate (0.7ml/kg body wt.) or saline was administered 1 h before killing. This dose of diethyl maleate selectively depletes GSH to insignificant concentrations (Boyland & Chasseaud, 1970).

Liver sections (10 μ m) were incubated for 20s in freshly prepared reaction medium, washed briefly, air-dried and mounted in Farrants' medium. The specific absorption ($A_{675}-A_{475}$) of the Prussian Blue deposited in these sections was measured by microdensitometry in groups of periportal and centrilobular hepatocytes. The mean specific-absorption values in both the periportal and centrilobular regions of each individual rat liver are shown in Table 2, together with the acid-soluble thiol content and ascorbic acid content of the individual rat livers; the mean group values of these parameters are also shown in Table 2.

These results show that the administration of diethyl maleate (0.7ml/kg body wt.) had no significant effect on the ascorbic acid content of rat liver ($P>0.5$). However, the liver acid-soluble thiol content which is mainly GSH (Jocelyn, 1972) and was determined by means of Ellman's reagent, was depleted to less than 10% of the control value by the administration of diethyl maleate.

In contrast with this almost complete loss of GSH from the livers of the rats treated with diethyl maleate, there was only a 50% decrease in the amount of

Table 2. Effect of diethyl maleate administration on the mean specific absorption ($A_{675}-A_{475}$) of the periportal and centrilobular regions, liver acid-soluble thiol content and ascorbic acid content of the individual rats

The mean group values of these parameters are also shown. All values are given as the mean $\pm$ S.E.M. Mean specific absorption is expressed in units of integrated absorption $\times$ 1000 and calculated from 40 individual measurements. Acid-soluble thiol and ascorbic acid contents are expressed in mg/100g of liver

Treatment	Rat	Mean specific absorption in periportal region	Mean specific absorption in centrilobular region	Liver acid-soluble thiol content	Liver ascorbic acid content
Saline (control)	C 1	409 $\pm$ 7	244 $\pm$ 13	140.1	27.0
	C 2	391 $\pm$ 5	155 $\pm$ 10	142.4	22.2
	C 3	380 $\pm$ 8	214 $\pm$ 14	162.7	24.3
	C 4	410 $\pm$ 15	260 $\pm$ 14	167.2	29.3
	C 5	406 $\pm$ 15	219 $\pm$ 14	149.2	29.2
Mean group values		399 $\pm$ 6	218 $\pm$ 8	152.3 $\pm$ 6.1	26.4 $\pm$ 1.6
Diethyl maleate (0.7ml/kg)	D 1	158 $\pm$ 14	91 $\pm$ 6	13.1	27.5
	D 2	223 $\pm$ 7	156 $\pm$ 6	18.1	34.0
	D 3	150 $\pm$ 8	103 $\pm$ 4	11.3	26.3
	D 4	165 $\pm$ 7	107 $\pm$ 5	12.6	25.2
Mean group values		174 $\pm$ 17	114 $\pm$ 14	13.8 $\pm$ 1.5	28.3 $\pm$ 2.0

Prussian Blue deposited in liver sections prepared from these rats when compared with the amount deposited in liver sections of control rats. The mean values of specific absorption in the periportal and centrilobular regions of sections prepared from the livers of the diethyl maleate-treated group of rats (shown in Table 2) are therefore equal to the mean amount of Prussian Blue formed in these regions by substances other than GSH, such as ascorbic acid and protein thiol groups.

Subtraction of these values from the mean values of specific absorption of both regions of the lobule in sections prepared from the livers of the saline-control group of rats therefore gives an estimation of the mean amount of GSH present in the different regions of the rat liver lobule.

The mean GSH content ($\pm$ S.E.M.), expressed as specific absorption in units of integrated absorption $\times 1000$ in a number of regions of $20\mu\text{m}$ diameter in the periportal and centrilobular regions, were 225 ± 5 ($n = 200$) and 104 ± 13 ($n = 200$) respectively. Thus, by Student's t test, the specific absorption corresponding to GSH was significantly higher in the periportal region ($P < 0.0001$).

Determination of the absolute GSH content of cells in the different regions of the lobule

Duplicate sections ($10\mu\text{m}$) of gelatine containing GSH at concentrations of 2, 5 and 10mm were incubated for 20s in the reaction medium. The mean amount of specific absorption ($A_{675} - A_{475}$) was measured in each section by microdensitometry (Fig. 1).

By reading directly from this curve, the values of specific absorption owing to GSH in the different lobular regions were converted into absolute values

Fig. 1. Standard curve of mean specific absorption against GSH concentration

Duplicate sections ($10\mu\text{m}$) of gelatine containing GSH at concentrations of 2, 5 and 10mm were incubated for 20s in the reaction medium. The mean specific absorptions $\pm$ S.E.M. were calculated from 15 microdensitometric measurements in each section.

in units of μmol of GSH/ cm^3 of liver. The GSH content of the cells close to the portal tract was $7.25\mu\text{mol}/\text{cm}^3$ of liver, and those surrounding the central vein were found to contain $3.35\mu\text{mol}/\text{cm}^3$ of liver.

Determination of the total liver GSH content by microdensitometry

The mean specific absorptions of liver sections produced from the control rats, C2 and C4, and from the rats treated with diethyl maleate, D1 and D3, were determined by making random measurements of integrated absorption by using a large field size ($160\mu\text{m}$ diameter), so that measurements were made in virtually all areas of each section. The values of mean specific absorption ($\pm$ S.E.M.) in units of integrated absorption $\times 1000$ were 334 ± 18 in the control rats and 126 ± 6 in the diethyl maleate-treated rats.

The specific absorption corresponding to GSH, obtained by random measurement, is therefore equal to $334 - 126 = 208$ units of integrated absorption $\times 1000$. Thus, from Fig. 1, the mean total liver GSH content of the control rats was $6.70\mu\text{mol}/\text{cm}^3$. This value is in close agreement with the GSH content of male rat liver obtained by highly specific biochemical assays, which give values between 6 and $9\mu\text{mol}/\text{g}$ of liver (Jocelyn, 1972; Sies *et al.*, 1974).

Further studies of GSH distribution in the liver lobule

The intralobular distribution of GSH was further investigated by making a series of individual measurements of the specific absorption of areas $20\mu\text{m}$ in diameter, at $40\mu\text{m}$ intervals, between selected portal tracts and central veins in liver sections of the control rat, C4, and the diethyl maleate-treated rat, D3 (Fig. 2). These results show that the distribution of GSH within the rat liver lobule is not a simple linear concentration gradient from portal tract to central vein. The hepatocytes located within $100\mu\text{m}$ of the central vein appear to contain much less GSH than those in other regions of the liver lobule.

Discussion

Many otherwise inert xenobiotics, such as bromobenzene, as well as pharmacologically active agents such as paracetamol, are metabolized to highly reactive electrophiles by the microsomal cytochrome P-450 system (Brodie *et al.*, 1971; Mitchell *et al.*, 1973a). One mechanism by which these potentially toxic metabolites are rendered harmless is by their reaction with the nucleophilic thiol group of GSH to form a water-soluble conjugate, the reaction being catalysed by a glutathione S-transferase enzyme (Boyland & Chasseaud, 1969; Chasseaud, 1973).

Fig. 2. Distribution of reaction product across the liver lobule of a diethyl maleate-treated and a control rat. Individual measurements of the specific absorption of areas $20\mu\text{m}$ in diameter were made at $40\mu\text{m}$ intervals between selected portal tracts and central veins in liver sections of the control rat, C4 (●), and the diethyl maleate treated-rat, D3 (■). The mean values of specific absorption are shown.

However, if tissue GSH is depleted to a very low concentration through conjugation, these highly reactive metabolites are able to bind covalently to vitally important macromolecules, such as nucleic acids and proteins, so initiating tissue damage (Jollow *et al.*, 1973). Such considerations led Mitchell *et al.* (1973b) to propose that GSH has a fundamental role in protecting tissues against electrophilic attack by xenobiotic metabolites and other alkylating agents.

Hepatocytes located in the centrilobular region of the liver lobule are peculiarly susceptible to injury caused by toxic metabolites formed via the microsomal cytochrome *P*-450 system (McLean *et al.*, 1965; Koudstaal & Hardonk, 1969; Altman, 1971; Butcher, 1971; M. T. Smith & E. D. Wills, unpublished work). By microspectrophotometry, Gooding *et al.* (1978) have also shown that the centrilobular hepatocytes contain more cytochrome *P*-450 than do those in the periportal region. This evidence suggests that during

the oxidative metabolism of certain xenobiotics, more toxic metabolite is formed in these cells than in the periportal hepatocytes. This, however, only partially explains the susceptibility of the centrilobular hepatocytes to injury, because a certain amount of toxic metabolite must also be formed by this system in the periportal cells, but these metabolites do not appear to injure these periportal cells.

In the present paper a quantitative cytochemical method has been used to determine the GSH concentration of hepatocytes in different regions of the rat liver lobule. So far it has not been possible to do this by conventional biochemical techniques. Tests of the specificity of the staining procedure showed that the reaction was extremely rapid, taking only 20s for all the GSH present in the section to react, but that it was not specific for GSH alone.

This lack of specificity has been overcome by measuring the amount of reaction product formed in liver sections prepared from male rats pretreated with diethyl maleate to deplete selectively GSH from these livers. Subtraction of the mean values of specific absorption in the periportal and centrilobular regions of sections prepared from these rats from

mean values of specific absorption determined in both regions of liver sections prepared from a saline-control group of rats therefore gave an estimation of the amount of GSH present in the different regions of the liver lobule.

The determination of liver GSH content by this method gave results which were in close agreement with previously reported values obtained by highly specific conventional biochemical assays (Jocelyn, 1972; Sies *et al.*, 1974).

Use of this method has enabled us to show that GSH is not evenly distributed within the rat liver lobule. The hepatocytes of the periportal region contain approximately twice as much GSH as those in the centrilobular region. We therefore suggest that the centrilobular hepatocytes are peculiarly susceptible to electrophilic attack by toxic metabolites formed via the microsomal cytochrome P-450 system, for two major reasons. Firstly, a higher rate of oxidative xenobiotic metabolism occurs in the centrilobular cells. Hence, more of the potentially toxic metabolite will be formed in these cells. Secondly, the centrilobular cells contain less GSH than those in other regions of the liver lobule. They therefore have less protection against electrophilic attack by toxic metabolites. Therefore the cells most potentially at risk are the least protected. We propose that this explains the peculiarly centrilobular hepatic necrosis resulting from the conversion of inert substances into highly electrophilic and therefore potentially toxic metabolites by the microsomal cytochrome P-450 system.

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