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# The Effect of Age and Sex on Glutathione Reductase and Glutathione Peroxidase Activities and on Aerobic Glutathione Oxidation in Rat Liver Homogenates

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1. Changes in liver glutathione reductase and glutathione peroxidase activities in relation to age and sex of rats were measured. Oxidation of GSH was correlated with glutathione peroxidase activity. 2. Glutathione reductase activity in foetal rat liver was about 65% of the adult value. It increased to a value slightly higher than the adult one at about 2–3 days, decreased until about 16 days and then rose after weaning to a maximum at about 31 days, finally reaching adult values at about 45 days old. 3. Weaning rats on to an artificial rat-milk diet prevented the rise in glutathione reductase activity associated with weaning on to the usual diet high in carbohydrate. 4. In male rats glutathione peroxidase activity in the liver increased steadily up to adult values. There were no differences between male and female rats until sexual maturity, when, in females, the activity increased abruptly to an adult value that was about 80% higher than that in males. 5. The rate of GSH oxidation in rat liver homogenates increased steadily from 3 days until maturity, when the rate of oxidation was about 50% higher in female than in male liver. 6. In the liver a positive correlation between glutathione peroxidase activity and GSH oxidation was found. 7. It is suggested that the coupled oxidation–reduction through glutathione reductase and glutathione peroxidase is important for determining the redox state of glutathione and of NADP, and also for controlling the degradation of hydroperoxides. 8. Changes in glutathione reductase and glutathione peroxidase activities are discussed in relation to the redox state of glutathione and NADP and to their effects on the concentration of free CoA in rat liver and its possible action on ketogenesis and lipogenesis.

The activities of many enzymes in liver have been studied during the development of the rat (Herrmann & Tootle, 1964; Dawkins, 1966; Taylor, Bailey & Bartley, 1967; Vernon & Walker, 1968), but glutathione reductase and glutathione peroxidase have not been studied in this context. Glutathione reductase (Rall & Lehninger, 1952) is an important enzyme for the maintenance of glutathione in the reduced form, and possibly for controlling the redox state of NADP in tissues if GSSG is available. GSH oxidation in rat liver homogenates (Pinto, 1961) possibly involves a reaction catalysed by xanthine oxidase (Jocelyn, 1964) and is concerned with peroxidation in rat liver microsomes and mitochondria (Christophersen, 1966, 1968). Rat liver contains a highly active glutathione peroxidase that catalyses GSH oxidation by hydrogen peroxide (Mills, 1960) or by linoleic acid hydroperoxide (O'Brien & Little, 1967; Little & O'Brien, 1968*a*). Glutathione peroxidase could therefore be involved in the formation of GSSG in rat liver.

The present paper reports changes in glutathione reductase and glutathione peroxidase activities in rat liver from foetal to adult life and also correlates glutathione peroxidase activity with the rate of aerobic oxidation of GSH in rat liver homogenates.

A preliminary communication of some of these results has been published (Pinto & Bartley, 1968).

## MATERIALS AND METHODS

*Materials.* All the chemicals used were of A.R. grade or the purest available. GSH, GSSG and yeast glutathione reductase were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, and NADPH was obtained from Sigma (London) Chemical Co., London S.W.6. All the other reagents used were supplied by British Drug Houses Ltd., Poole, Dorset. Full-cream powdered cow's milk was supplied by Cow and Gate Ltd., Guildford, Surrey.

*Animals and diets.* Wistar rats from the Sheffield University Animal House colony were used in all the experiments. Adult rats were divided into two groups: one, referred to as 'young adults', was of male or female rats

about 4 months old, and the other group, referred to as 'old adults', was of male rats older than 18 months, weighing 470–540g. Foetuses were taken from pregnant females at 1 and 2 days before expected delivery; these are referred to as −1 and −2 days old. Pools of five or six foetuses or newborn animals and two to five rats (1–70 days old) were used in each experiment. Adult rats were used alone, or pairs were pooled. Rats were fed *ad libitum* with pellets (diet 1) or solid artificial rat milk (diet 2). Diet 1 was Oxoid diet 86 [Herbert C. Styles (Bewdley) Ltd., Bewdley, Worcs.], a high-carbohydrate diet containing carbohydrate, protein and fat in the proportions 14:5:1 (by wt.). Diet 2 had the same proportions of carbohydrate, protein and fat as those of rat milk (13:37:50, by wt.) and was thus a high-fat diet. Diet 2 was prepared by adding butter, casein and salts to powdered cow's milk in which the proportions of carbohydrate, protein and fat were 36:25:29 (by wt.). The composition of the salts mixture was given by Bartley, Dean, Taylor & Bailey (1967).

*Preparation of homogenates.* Rats less than 15 days old and foetuses were killed by decapitation and bled, and the livers were removed and chilled to 0° in 0·154M-KCl. Animals 15 days old or more were killed by stunning and decapitation, and the livers were removed and chilled to 0° in 0·154M-KCl and perfused with chilled iso-osmotic KCl. Livers were dried between filter paper, weighed, minced with scissors, homogenized in 4vol. of ice-cold 0·154M-KCl in a chilled Potter–Elvehjem homogenizer and filtered through a double layer of nylon cloth. This homogenate was diluted threefold in water (homogenate 1) for the measurement of glutathione reductase activity, diluted fourfold with 0·154M-KCl (homogenate 2) for glutathione peroxidase activity measurement, or diluted twofold with 0·25M-sucrose (homogenate 3) for the measurement of GSH oxidation.

*Incubations.* For the determination of aerobic GSH oxidation, incubations were carried out in Warburg manometers at 30°: the vessels were gassed with $O_2$ and the centre wells contained 0·2ml. of 4M-NaOH and filter paper. The incubation mixture consisted of 3·0ml. of homogenate 3, 0·3ml. of 32·5mM-GSH, 0·5ml. of 0·25M-potassium phosphate buffer, pH7·4, and 0·2ml. of 0·154M-KCl (total volume 4·0ml.). The vessels were kept at 0° after the addition of the homogenate, before gassing and incubation. The GSH was tipped in from the side arm after 10min. incubation. At the end of the incubation the vessels were cooled in ice and the reaction was stopped by the addition of 1·0ml. of 10% (w/v) metaphosphoric acid. The precipitated proteins were removed by centrifugation at 25000*g* at 2° for 15min. in a refrigerated centrifuge, and the supernatant was analysed for GSH and GSSG.

*Determination of GSH.* This was done by potentiometric titrations with 0·1M-$HgCl_2$ by using a mercury–thiol electrode (Cecil, 1955) as modified by Pinto (1961).

*Determination of GSSG.* This was done by a modification of the method of Klotzsch & Bergmeyer (1963). The cuvette contained 0·05ml. of 3·7mM-NADPH, 0·1–0·25ml. of the deproteinized sample and 0·25M-phosphate buffer, pH7·1, to give a final volume of 1·25ml.; 0·01ml. of yeast glutathione reductase (diluted fourfold with water) was added and the decrease in $E_{340}$ was measured in a Zeiss PMQII spectrophotometer coupled to a Varicord 43 recorder.

*Measurement of enzyme activities.* Glutathione reductase (EC 1.6.4.2) was assayed at 30° in a Beckman DB spectrophotometer and the decrease in $E_{340}$ was followed on a Sargent SR recorder. The experimental cuvette contained 0·5ml. of 0·25M-potassium phosphate buffer, pH7·4, 0·1ml. of 25mM-EDTA, 0·1ml. of 7·5mM-NADPH, 0·25ml. of 25mM-GSSG, usually 0·05ml. of enzyme sample (homogenate 1) and water to 2·5ml. final volume. A control with water instead of GSSG was used.

Glutathione peroxidase (EC 1.11.1.9) was assayed at 30°. The assay mixture contained 3·0ml. of homogenate 2, 0·5ml. of 0·25M-phosphate buffer, pH7·4, 0·1ml. of 25mM-EDTA, 0·1ml. of 0·4M-$NaN_3$, 0·3ml. of 50mM-GSH (freshly dissolved) and 0·1ml. of 50mM-$H_2O_2$. The mixture, with no GSH or $H_2O_2$, was equilibrated for 10min. at 30°, and then the GSH solution was added and mixed. At zero time the $H_2O_2$ was added and the reaction was stopped with 1ml. of 10% (w/v) metaphosphoric acid 10sec. after the addition of the $H_2O_2$. Each reaction was carried out in duplicate. After centrifugation as described above a sample was taken for GSSG measurement. Incubations for 10sec. were considered preferable to longer incubations with less-concentrated homogenates because the non-enzymic reaction of GSH with $H_2O_2$ gives only a small blank in 10sec. Allowance was made for endogenous GSSG plus the GSSG contained in the GSH solution.

*Units of enzyme activity.* A unit of glutathione reductase was defined as 1$\mu$mole of GSSG reduced/min. at 30° at pH7·4, and a unit of glutathione peroxidase as 1$\mu$mole of GSSG formed/min. at 30° at pH7·4.

## RESULTS

*Changes in liver glutathione reductase activity in relation to age.* Immediately before birth the glutathione reductase activity (Fig. 1) of the foetal liver was about 65% of the adult value. After birth the activity increased to a maximum slightly higher than the adult value at about 2–3 days, then it declined to a minimum (approximately to the foetal value) at about 16 days, and after weaning the activity increased to another maximum at about 31 days. The high activity after birth was somewhat variable and changed rapidly with time; it is therefore impossible to give a precise value of the maximum activity at about this time. The activity at the second maximum was about 25% higher than the adult value (10·4 ± 0·8$\mu$moles of GSSG reduced/min./g. of fresh liver; mean ± S.D.), which was reached at about 45 days. The value for young adult female rats (4 months old) was 10% lower than that for males of the same age ($P \approx 0·05$). Changes in diet affected the glutathione reductase activity, as illustrated by the fact that during the suckling period, when the diet is rat milk, the activity decreased, whereas it increased after the change to a solid diet high in carbohydrate (diet 1).

Fig. 2 shows the effect on liver glutathione reductase of weaning rats on to diet 1 (high carbohydrate content) and on to diet 2 (artificial rat milk), and of separating rats from the mother at 19–20 days. In the liver of rats fed with diet 1,

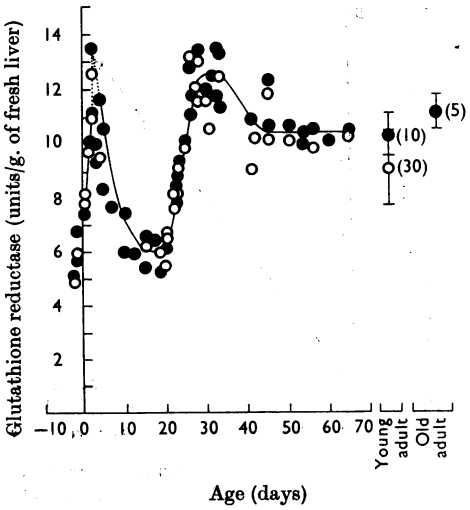



Fig. 1. Activities of hepatic glutathione reductase in relation to age in male (●) and female (○) rats. Enzyme activity was measured spectrophotometrically as described in the Materials and Methods section. The ages of the foetuses are referred to as −1 and −2 days old; young adults and old adults were rats 4 months old and more than 18 months old respectively. Most analyses are from pooled samples of tissue. Where more than one analysis was made the number of analyses is indicated in parenthesis, and the vertical line indicates ± S.D.

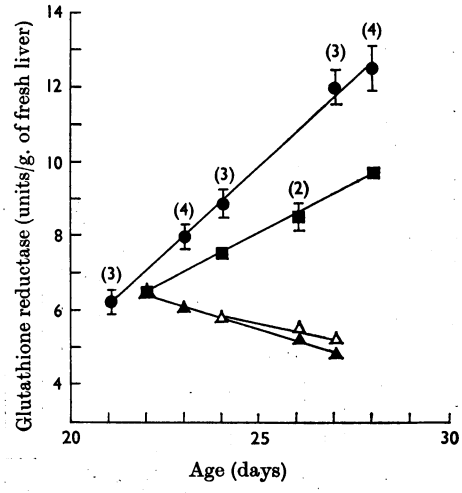



Fig. 2. Effects on hepatic glutathione reductase of weaning rats on to high-carbohydrate (diet 1) and artificial rat-milk (diet 2) diets. Results are shown for rats fed on diet 1 separated from the mother (●) and not separated from the mother (■), and for rats fed on diet 2 separated (▲) and not separated from the mother (△). Other details are as given in Fig. 1.

glutathione reductase activity increased by about 100% from 21 days (1–2 days after they were separated from the mother) until 28 days. In rats kept with the mother liver glutathione reductase also increased, but to a final value about 25% less than that in rats separated from the mother. Weaning on to diet 2 (artificial rat milk) slightly decreased the enzyme activity both in rats separated and in those not separated from the mother. Thus the artificial rat milk, with its high fat content, completely suppressed the usual rise in liver glutathione reductase activity that was found in animals weaned on to a high-carbohydrate diet (see also Bartley *et al.* 1967).

*Age- and sex-linked changes in liver glutathione peroxidase activity.* Liver glutathione peroxidase activity (Fig. 3) in the foetus was about 35% of the young adult male values. The activity of this enzyme increased steadily after birth until about 55 days; at this time the activity was approximately that of the young adult male rat ($49 \pm 5$ μmoles of GSSG formed/min./g. of fresh liver; mean ± S.D.). After this age in the male the activity still increased but much more slowly (mean daily increase about 0·03 μmole of GSSG formed/min./g. of fresh liver), and in the old rats (older than 18 months and

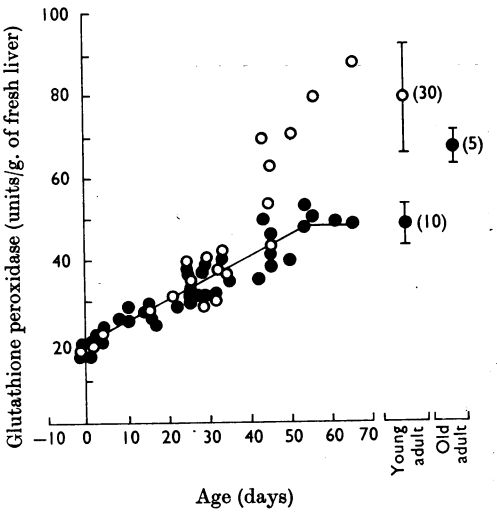



Fig. 3. Activities of hepatic glutathione peroxidase in relation to age in male (●) and female (○) rats. Enzyme activity was measured as described in the Materials and Methods section. The ages of the foetuses are referred to as −1 and −2 days old; young adults and old adults were rats 4 months old and more than 18 months old respectively. Other details are as given in Fig. 1.

weighing approx. 490g.) it was 40% higher than the activity in young adults. Already at 45 days female rats showed a higher glutathione peroxidase

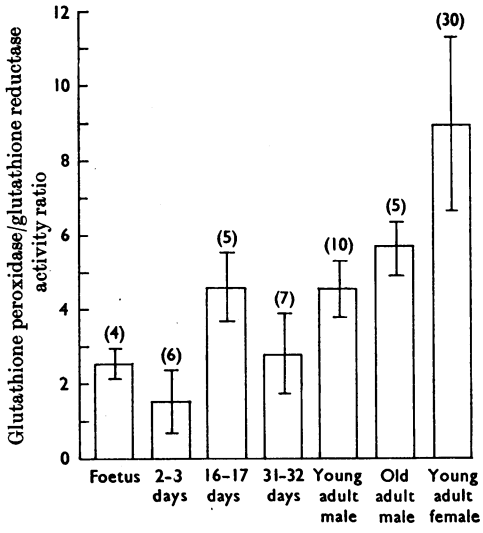



Fig. 4. Glutathione peroxidase/glutathione reductase activity ratios in foetuses, in adult male rats and in rats at ages when glutathione reductase activities in relation to the age showed maximum or minimum values. Young adults and old adults were rats 4 months old and more than 18 months old respectively. The number of animals used is indicated in parenthesis and the vertical line indicates ±S.D.

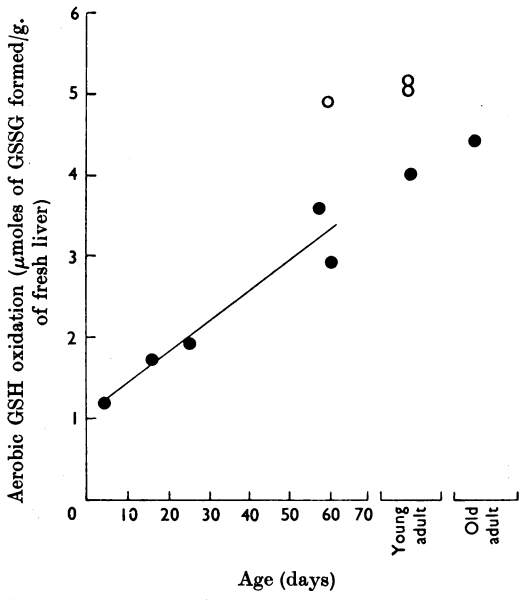



Fig. 5. Rate of aerobic GSH oxidation in relation to age in male (●) and female (○) rats. For details of the assay see the Materials and Methods section; other details are as given in Fig. 1.

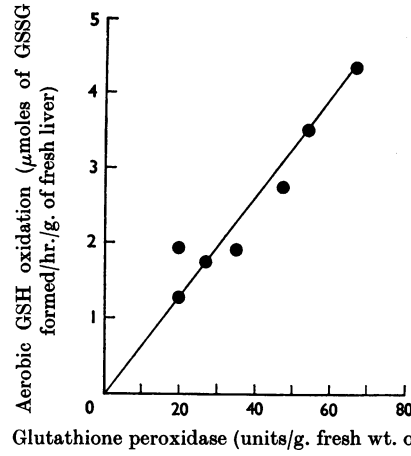



Fig. 6. Positive correlation between glutathione peroxidase activity and rate of aerobic oxidation of GSH in male rats. Details are as given in Figs. 1 and 5.

activity than males, and the young adult females (4 months old) had an activity about 80% higher than that in males of the same age. Although there was a change in diet from the mother's milk during the suckling period to the normal solid diet this did not affect the glutathione peroxidase activity. Changes in the activity of this enzyme appeared to be mainly due to age and sex.

*Changes in the glutathione peroxidase/glutathione reductase activity ratio in the liver in relation to age and sex* (*Fig.* 4). This ratio in the foetus was about 50% of that at about 16 days, which is the same as in young adult rats (4·6 ± 0·7; mean ± S.D.). After birth the lowest ratio (1·7) was at about 2–3 days and the highest (5·7 ± 0·8) was in old male rats. In the young adult female the ratio was about 8·9 ± 2·2, which was almost double that in the male of the same age.

*Changes in the rate of aerobic oxidation of GSH in the rat liver in relation to age and sex.* The rate of aerobic oxidation of GSH in male rat liver homogenates (Fig. 5) increased steadily from 3 days after birth until about 55 days (3·2 μmoles of GSSG formed/hr./g. of fresh liver). At this age females showed activity about 50% higher than males. The rate in the old adult male (4·4 μmoles of GSSG formed/hr./g. of fresh liver) was higher than that in the young adult male and lower than that in the young adult female (5·1 μmoles of GSSG formed/hr./g. of fresh liver). These changes paralleled those found in glutathione peroxidase activities. Fig. 6 correlates glutathione peroxidase activity and the rate of GSH oxidation in the liver of male rats from very young to old adults.

The correlation of the rate of GSH oxidation

with glutathione peroxidase activity strongly suggests that GSH oxidation in rat liver homogenates (Pinto, 1961) probably involves hydroperoxides, since they are the only compounds known to oxidize GSH through glutathione peroxidase. This conclusion agrees with the findings of Christophersen (1966) and of Jocelyn (1964) on the nature of GSH oxidation in rat liver.

Some haemoproteins, such as cytochrome *c*, could also be partially responsible for the oxidation by organic peroxides, since cytochrome *c* stimulates the oxidation of GSH by linoleic acid hydroperoxide (Little & O'Brien, 1968*a*). This reaction produces both GSSG (60%) and the sulphonic acid derivative of GSH (40%), whereas that catalysed by glutathione peroxidase gives only GSSG. The aerobic rate of disappearance of GSH in rat liver homogenates and GSSG formation are almost quantitatively related (Table 1) and therefore under these conditions the reaction catalysed by haemoproteins can only play a minor part in the oxidation of GSH.

## DISCUSSION

*Possible implications of the dependence of GSH oxidation on glutathione peroxidase.* The oxidation of GSH by glutathione peroxidase coupled with the reduction of GSSG through glutathione reductase is probably of importance in determining the redox state of glutathione. The GSH/GSSG concentration ratio must be a function of the activities of both these enzymes and also of the concentration of thiols and disulphides other than glutathione. The concentrations of non-protein thiols and disulphides in the liver are low and possibly do not affect the GSH/GSSG concentration ratio, but interaction with proteins might be of importance in altering it. However, as the glutathione reductase and glutathione peroxidase activities are high, it is probable that these enzymes, and their activity ratio, are most important for deciding the GSH/GSSG concentration ratio, which in turn influences the thiol/disulphide concentration ratio of other compounds dependent on the redox steady state of the glutathione (see Scheme 1).

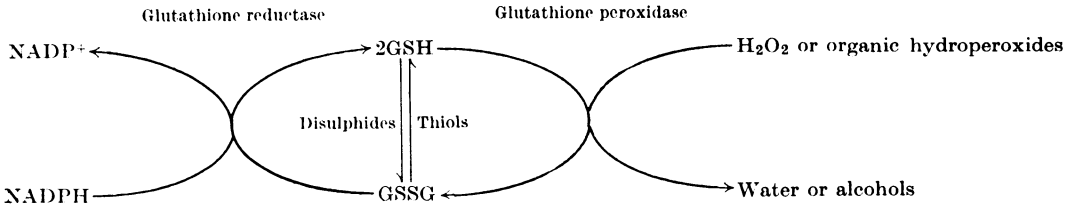
Another feature of the coupled oxidation–reduction of glutathione through glutathione reductase and glutathione peroxidase is the oxidation of NADPH. The $K_m$ of rat liver glutathione reductase for NADPH is 3$\mu$M and that for GSSG is 55$\mu$M (Mise & Langdon, 1962). The concentration of NADPH in rat liver is about 0·3mM (Glock & McLean, 1955; Jacobson & Kaplan, 1957; Bassham, Birt, Hems & Loening, 1959); that of GSSG is not well known but may be assumed to be about the same as the $K_m$ for GSSG (Bhattacharya, Robson & Stewart, 1956). Thus the rate of formation of $NADP^+$ by glutathione reductase is expected to be almost entirely dependent on the GSSG concentration. As glutathione is synthesized metabolically in the reduced form (Bloch, 1949; Snoke & Bloch, 1952), the rate of NADPH oxidation by the pathway shown in Scheme 1 depends on the rate of GSSG formation, which is probably a function of hydroperoxide concentration.

The participation of glutathione peroxidase in the mechanism for removal of peroxides in cells has been suggested (Neubert, Wojtczak & Lehninger, 1962; Little & O'Brien, 1968*a*). The work of Christophersen (1968) suggests that protection by GSH against peroxidation is an enzymic process, and since there is a positive correlation between the rate of oxidation of GSH and glutathione peroxidase activity this enzyme is probably responsible, at

Table 1. *Aerobic oxidation of GSH and formation of GSSG in rat liver homogenate*

For details of the assays see the Materials and Methods section.

| Expt. no. | Incubation time (min.) | GSH used ($\mu$moles/g. of fresh liver) | GSSG formed ($\mu$moles/g. of fresh liver) |
|---|---|---|---|
| 1 | 20 | 3·7 | 2·3 |
| | 60 | 7·0 | 4·0 |
| | 120 | 13·3 | 6·0 |
| 2 | 20 | 6·7 | 2·7 |
| | 60 | 10·0 | 4·0 |
| | 120 | 13·0 | 6·7 |




Scheme 1.

least partially, for maintaining cell integrity by protecting it against the deleterious effects of peroxides (Ottolenghi, Bernheim & Wilbur, 1955; Tappel & Zalkin, 1959; Hunter *et al.* 1964; Waravdekar, Saslaw & Jones, 1964; May, Poyer & McCay, 1965; Roubal & Tappel, 1966; Little & O'Brien, 1968*b*) and may also represent 'auto-protection' since peroxides appear to produce hydrogen sulphide at the expense of thiol groups (Roubal & Tappel, 1966) or partially oxidize GSH to the sulphonic acid, or both. Oxidation of GSH and reduction of hydroperoxides catalysed by glutathione peroxidase produces hydroxylated lipids or hydroxylated free fatty acids and GSSG that can be reduced to GSH through glutathione reductase.

Besides polyunsaturated lipid or free fatty acid hydroperoxides, tetrahydropteridines and reduced flavins seem also to give hydroperoxides by autoxidation. The flavin hydroperoxide may be transformed to an unstable alcohol that splits off water and regenerates the reduced flavin (Magger & Berends, 1966). If flavin hydroperoxides are substrates for glutathione peroxidase this enzyme could be related to some flavin or flavoprotein function. Autoxidation of flavins accelerates the oxidation of reduced compounds such as NADPH and thiols (Magger & Berends, 1964, 1966). Therefore the lipid alterations and peroxidation in microsomes in the presence of NADPH and oxygen (May *et al.* 1965) and also the glutathione-induced peroxidation in mitochondria (Hunter *et al.* 1964) may involve flavin compounds.

In mitochondria, swelling due to glutathione was associated with depletion of 'contraction factor I', identified as glutathione peroxidase (Neubert *et al.* 1962), and correlated with peroxidation (Hunter *et al.* 1964). When depletion of glutathione peroxidase occurs, mitochondrial capacity for protection against damage to proteins or other cell components diminishes and swelling of mitochondria occurs. Contraction of the mitochondria can be brought about by the action of glutathione peroxidase and ATP (Neubert *et al.* 1962). The formation of stable hydroperoxides in proteins or enzymes responsible for swelling and requiring ATP for contraction may represent the reversible damage, and the enzymic reduction of the hydroperoxide to alcohol, followed or not by dehydration, may be the way of restoring the contraction capacity.

*Possible influence of the changes of glutathione reductase and glutathione peroxidase activities on ketogenesis, lipogenesis and the NADPH/NADP+ concentration ratio.* Changes in the glutathione peroxidase/glutathione reductase activity ratio in rat liver may affect the GSH/GSSG concentration ratio, which could be important for controlling the concentration of free CoA in this tissue through the disulphide–thiol interchange:

$$GSSG + CoASH \rightleftharpoons GSH + GS\cdot S\cdot CoA$$

since the equilibrium constant of this reaction is near unity (Chang & Wilken, 1966). Changes in the GSH/GSSG concentration ratio are expected to produce approximately equal changes in the CoASH/GS·S·CoA concentration ratio. Such changes in the tissue content of free CoA could have important effects on fatty acid metabolism; for example, increased concentration of free CoA might accelerate lipogenesis and slow ketogenesis.

Besides the possible effect of the glutathione peroxidase/glutathione reductase activity ratio in determining the redox state of glutathione and of CoA, the effect of glutathione reductase on the NADPH/$NADP^+$ concentration ratio, and consequently on lipogenesis, may also be of importance. The changes in glutathione reductase activities in relation to age up to sexual maturity in male rats follow qualitatively a similar pattern to those of lipogenesis and of several enzymes involved in lipid biosynthesis (Taylor *et al.* 1967), including the adaptive enzymes reducing $NADP^+$. The effect of the changes in glutathione reductase activity is thus opposed to changes in the NADPH/$NADP^+$ concentration ratio brought about by glucose 6-phosphate dehydrogenase and 'malic' enzyme and so are opposed to lipogenesis. If changes in glucose 6-phosphate dehydrogenase and in 'malic' enzyme activities are higher than that required for the changes in fatty acid biosynthesis, the changes in glutathione reductase activity could tend to decrease unnecessary variations in the NADPH/$NADP^+$ concentration ratio. This may happen, as NADPH concentration is not always the limiting factor of lipogenesis (Sauer, 1960; Masoro, 1962).

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