METABOLISM OF GAMMA-LACTONES BY RAT LIVER PREPARATIONS *IN VITRO**

D. H. DE KOCK AND H. G. RAUBENHEIMER, *Department of Chemistry, University of Stellenbosch*

SUMMARY

As part of an attempt to elucidate the mechanism of carcinogenesis by αβ-unsaturated γ-lactones, a systematic study of the metabolism of a group of saturated and αβ-unsaturated lactones was performed. It was shown that the saturated γ-lactones are much more rapidly enzymatically hydrolysed than their αβ-unsaturated analogs, while the latter compounds are also conjugated with glutathione which could eventually lead to the excretion of mercapturic acids. On the whole, however, the saturated lactones are more rapidly metabolized.

Among the series of γ-lactones, marked biological activities are often associated with the presence of unsaturation in the lactone ring.[1] Apart from β-propiolactone, a compound with a highly strained ring structure, and aflatoxin, a complicated δ-lactone, some γ-lactones possess carcinogenic activity.[2-5] It appeared that αβ-unsaturation of the γ-lactones is also desirable for this activity. In the present work a systematic study of the metabolism of a group of saturated and αβ-unsaturated γ-lactones (Fig. 1) by

I. R = R′ = H
II. R = H; R′ = CH_3
III. R = R′ = CH_3
IV. R = R′ = H
V. R = H; R′ = CH_3
VI. R = R′ = CH_3

Fig. 1. γ-lactones employed in this study: I. γ-butyrolactone; II. γ-valerolactone; III. γ-isocaprolactone; IV. γ-crotonolactone; V. 4-hydroxypent-2-enoic acid γ-lactone (β-angelica lactone); VI. 4-hydroxy-2-enoic acid γ-lactone.

rat liver preparations was undertaken. Metabolic reactions which were studied were hydrolysis of the lactones, formation of conjugates with glutathione which is the initial reaction leading to the formation of mercapturic acids,[6] and hydroxylation of the unsaturated lactones.

*Date received: 15 October 1970.

MATERIAL AND METHODS

Lactones I, II and V were obtained commercially while lactones III, IV and VI were synthesized as described by Haynes and Jones.[7]

Lactone Hydrolysis Assay

A rat liver homogenate (10% w/v) in 0·15M tris-histidine buffer (pH 7·4) was centrifuged at 10 000 G to remove mitochondria and nuclei which contained no lactone-hydrolysing activity. Of the supernatant 1·0 ml was added to 1·0 ml 10 mM lactone solution in de-ionized water and the mixture incubated at 37°C for the appropriate time. Thereupon the concentration of the unhydrolysed lactone was determined by the reaction with alkaline hydroxylamine followed by colorimetry in acid ferric chloride.[8] For this purpose 1·0 ml 2M hydroxylamine was added to each incubation mixture followed by 0·1 ml 3N sodium hydroxide and 0·6 ml of a reagent consisting of a 1:1 mixture of 96% ethanol and 10% ferric chloride in 4N hydrochloric acid. The proteins were precipitated by addition of 1·0 ml 0·1M trichloroacetic acid to the mixtures and subsequent centrifugation. Finally the optical density of the purple supernatant was measured at 510 nm. Control determinations in which the liver preparation was boiled before incubation were also carried out.

Reaction with Glutathione

A rat liver homogenate (10% w/v) in 0·05M phosphate buffer (pH 7·4) was centrifuged at 700 G for 10 minutes to remove nuclei and unbroken cells followed by centrifugation of the resulting supernatant at 120 000 G for 40 minutes. Of this supernatant samples of 2·5 ml were added to a mixture of 2·5 ml 10 mM lactone solution in water and 5·0 ml 5 mM glutathione in 0·05M phosphate buffer (pH 7·4). These mixtures were incubated at 37°C for the appropriate time and the residual glutathione concentration determined as described by Woodward and Fry.[9]

RESULTS AND DISCUSSION

It was confirmed by inhibition studies with EDTA and p-chloromercuribenzoate as well as subcellular localization experiments that the decrease in lactone concentration by incubation with the 10 000 G supernatant of a rat liver homogenate is due to hydrolysis by the γ-lactonase described by Fishbein and Bessman.[10] The decrease in glutathione concentration when incubated with the γ-lactones in the presence of the 120 000 G rat liver supernatant fraction is due to conjugation of glutathione with the latter compounds, catalysed by the enzyme described by Boyland and Chasseaud.[6] The latter reaction is probably addition of the sulphydryl group of glutathione to the $\alpha\beta$ double bond of the lactones.

TABLE I. PERCENTAGE γ-LACTONE METABOLIZED BY LIVER PREPARATION *in vitro* (INCUBATION TIME: 20 MINUTES)

γ-lactone	% *Hydrolysis*	% *Conjugation with glutathione*
γ-butyrolactone	73 ± 6·5 (3)	0
γ-valerolactone	83 ± 6·2 (3)	0
γ-isocaprolactone	64 ± 2·4 (3)	0
γ-crotonolactone	24 ± 9·3 (4)	7·2 ± 1·3 (4)
4-hydroxypent-2-enoic acid γ-lactone	14 ± 1·6 (5)	15·5 ± 3·0 (4)
4-hydoxy-isohex-2-enoic acid γ-lactone	22 ± 12 (5)	1·6 ± 0·3 (5)

Figures in parentheses indicate the number of rats utilized

In Table I the amounts of lactone metabolized by hydrolysis and conjugation with glutathione are given. From these results it is clear that the $\alpha\beta$-unsaturated γ-lactones are much more slowly enzymatically hydrolysed than the saturated lactones. Since enzymatic hydrolysis is inhibited by the sulphydryl reagent, p-chloromercuribenzoate,[10] and as the sulphydryl group reacts with unsaturated lactones, the possibility that the slower hydrolysis of the latter may be attributed to substrate inhibition was studied. By employing lactone V as substrate and setting up a Lineweaver-Burke plot, a straight line was obtained, indicating that no substrate inhibition occurs. In this experiment the rates of enzymatic hydrolysis at different substrate concentrations were determined after 5, 10 and 15 minutes of incubation. The rates were linear for at least 10 minutes.

The relative small differences between the amounts of lactone hydrolysed in the saturated and unsaturated groups of lactones respectively are evidence that steric factors due to the γ-substituents do not have a significant influence on enzymatic hydrolysis. Since γ-valerolactone is a racemic mixture of optically active isomers and is hydrolysed to near-completion under the above experimental conditions, it further appears that this reaction is not stereo-specific for one optical isomer in so far as substituents at the γ-position of the lactone ring are concerned. It is therefore more likely that enzymatic hydrolysis of the lactones is controlled by nucleophilic attack by the enzyme on the carbonyl carbon atom. Due to the conjugation effect of the $\alpha\beta$ double bond the carcinogenic lactones would have a relative higher electron density at the carbonyl carbon atom than the saturated lactones and would therefore be relatively resistant towards nucleophilic attack by this enzyme.

As far as the further metabolism of the resulting 4-hydroxy fatty acids are concerned, it was previously shown that the saturated 4-hydroxy fatty acids are converted to their corresponding saturated fatty acids by rat liver slices but that this does not occur in the case of their $\alpha\beta$-unsaturated derivatives.[11]

On the other hand conjugation with glutathione occurs only with the unsaturated lactones. From the results given in Table I it is clear that substituents at the γ-position have a considerable effect on the extent of the reaction. Since the ratio of the lactone concentration to the amount of liver from which the liver fractions were prepared was the same in the glutathione conjugation experiments as in the experiments on hydrolysis, it furthermore appears that the saturated lactones are more easily and rapidly metabolized by hydrolysis than their $\alpha\beta$-unsaturated analogs are by hydrolysis and conjugation with glutathione.

No metabolites were detected by thin-layer chromatography employing cyclohexane-ethyl acetate (2:8 v/v) as solvent system of ethyl acetate extracts obtained after incubation of the unsaturated lactones with the microsomal hydroxylating system described by Boyland *et al.*[12]

We wish to thank the South African Council for Scientific and Industrial Research for financial support for this study.

REFERENCES

1. Haynes, L. J. (1948): Quart. Med. Rev., **46**, 46.
2. Dickens, F. and Jones, H. E. H. (1961): Brit. J. Cancer, **15**, 85.
3. *Idem* (1963): *Ibid.*, **17**, 100.
4. *Idem* (1963): *Ibid.*, **17**, 691.
5. *Idem* (1965): *Ibid.*, **19**, 392.
6. Boyland, E. and Chasseaud, L. (1967): Biochem. J., **104**, 95.
7. Haynes, L. J. and Jones, E. R. H. (1946): J. Chem. Soc., Part 2, 954.
8. Goddu, R. F., Le Blanc, N. F. and Wright, C. M. (1955): Anal. Chem., **27**, 1251.
9. Woodward, G. E. and Fry, E. G. (1932): J. Biol. Chem., **97**, 465.
10. Fishbein, W. N. and Bessman, S. P. (1966): *Ibid.*, **241**, 4835.
11. De Kock, D. H. and Raubenheimer, H. G. (1969): S. Afr. Med. J., **43**, 959.
12. Boyland, E., Kimura, M. and Sims, P. (1964): Biochem. J., **92**, 631.