Biochemical Pharmacology, Vol. 22, pp. 743–747. Pergamon Press, 1973. Printed in Great Britain.

URINARY D-GLUCARIC ACID EXCRETION AND TOTAL LIVER CONTENT OF CYTOCHROME P-450 IN GUINEA-PIGS: RELATIONSHIP DURING ENZYME INDUCTION AND FOLLOWING INHIBITION OF PROTEIN SYNTHESIS

JOHN HUNTER,* J. DOUGLAS MAXWELL, DAVID A. STEWART and ROGER WILLIAMS

Medical Research Council Group on the Metabolism and Haemodynamics of Liver Disease, King's College Hospital, London, S.E.5, England

(*Received* 12 *June* 1972; *accepted* 25 *September* 1972)

Abstract—In guinea-pigs treated with phenobarbitone 50 mg/kg for up to 5 days a highly significant correlation ($r = 0{\cdot}61$, $P < 0{\cdot}002$) was found between the total liver content of the microsomal enzyme cytochrome P-450 and the daily urinary excretion of D-glucaric acid. A significant correlation still obtained in animals pretreated with an inhibitor of protein synthesis (Actinomycin D) in addition to phenobarbitone. These results provide further evidence that measurement of D-glucaric acid excretion provides a quantitative although indirect estimate of hepatic enzyme activity.

DRUGS such as phenobarbitone which cause induction of hepatic microsomal enzymes also stimulate the activity of the glucuronic acid pathway of the liver (Fig. 1), increasing the urinary excretion of its end products, L-ascorbic acid,[1] D-glucaric acid,[2] and in

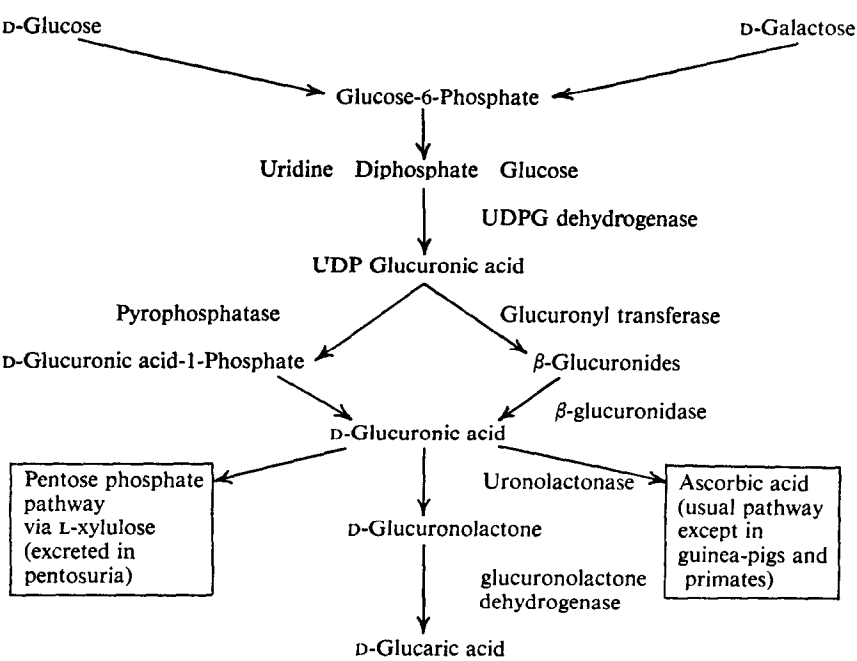

FIG. 1. The glucuronic acid pathway.

* Present address: Addenbrooke's Hospital, Hills Road, Cambridge.

patients with essential pentosuria, L-xylulose.[3] Conney and Burns[4] suggested that measurement of urinary ascorbic acid might be of value in the rat as an indirect index of the activity of hepatic microsomal enzymes, and Aarts[5] proposed that D-glucaric acid excretion might similarly be used in man and other animals such as the guinea-pig which are unable to synthesize ascorbic acid. In recent studies we have found D-glucaric acid excretion to be significantly related to the dose of inducing agent, for instance in epileptic subjects on anticonvulsant therapy, and to correlate well with the blood level of substances known to be metabolized by microsomal enzymes.[6,7] However, Aarts[8,9] claimed that there were important differences between the effects of drugs on the glucuronic acid pathway and on hepatic microsomal enzymes, and in particular that inhibitors of protein synthesis, such as actinomycin-D and puromycin, did not prevent stimulation of the pathway. In this paper we report a direct comparison between D-glucaric acid excretion in guinea-pigs and the total liver content of the microsomal enzyme cytochrome P-450, following administration of phenobarbitone and actinomycin-D.

MATERIALS AND METHODS

Twenty male guinea-pigs weighing 400–1000 g were fed on Oxoid diet SG 1 with greenstuffs and water *ad lib.* They received phenobarbitone 50 mg/kg by intraperitoneal injection daily for 0–5 days and on the last day were placed in individual metabolic cages so that a complete 24 hr collection of urine could be obtained. The animals were then killed by a blow on the head and the liver rapidly removed. All subsequent manipulations were carried out at 4°. The gall bladder, bile ducts and any fibrous tissue were dissected away and the liver washed in ice-cold 0·25 M sucrose, blotted and weighed. A 25 per cent homogenate from 4·5 g portions taken from the centre of the hepatic lobes was prepared in 0·25 M sucrose, using a glass vessel with a PTFE pestle. After centrifugation at 10,000 *g* for 20 min the pellet was homogenized again in a volume of sucrose four times the weight of the original portion of the liver and recentrifuged. Finally, the pooled supernatants (now 12·5%, w/v) were then centrifuged at 105,000 *g* for 60 min and the microsomal pellet suspended in buffered sucrose solution (0·25 M sucrose:0·1 M Tris–HCl buffer at pH 7·4, 1:2, v/v).

Cytochrome P-450 was estimated immediately after preparation of the microsomal suspension by the method of Omura and Sato,[10] protein concentration being determined by Hess and Lewin's modification[11] of the method of Lowry *et al.*[12] As the sizes of the animals varied considerably the total liver content of cytochrome P-450 was calculated from the following formula:

$$\frac{\text{Specific content P-450} \times \text{protein concn} \times \text{volume microsomal suspension} \times \text{liver weight}}{\text{Weight of liver portion from which microsomes prepared}}$$

Urine was centrifuged to remove particulate matter, before determination of its D-glucaric acid content by the method of Marsh[13] in which the glucaro(1, 4) lactone produced by boiling D-glucaric acid at pH 2 is estimated by its inhibitory effect on β-glucuronidase. Results are expressed as μmoles glucaro(1, 4) lactone per 24 hr.

The effect of actinomycin-D was studied in a further group of 12 animals, according to the regime described by Kuntzman[14] which has been shown to prevent induction of cytochrome P-450 by phenobarbitone. Animals were placed in metabolic cages for urine collection immediately after an intraperitoneal injection of actinomycin-D,

1 mg/kg. One hr later phenobarbitone 50 mg/kg was given and 5 hr after this the injection of actinomycin was repeated. Six control animals received saline instead of actinomycin. A complete 24 hr urine collection was obtained and at the end of this time the animals were killed, microsomes prepared and cytochrome P-450 assayed as previously described.

RESULTS

D-Glucaric acid excretion increased with length of the period of phenobarbitone administration, the range being from 0·22 μmole/day in animals killed on the first day to 1·54 μmole in those killed after 5 days. Total liver content of cytochrome P-450 also showed an increase with the duration of phenobarbitone adminstration with values corresponding to the times just given of 101 mμmole to 358 mμmole. When values for D-glucaric acid excretion were compared with those for cytochrome P-450 in the complete group of 20 animals, the relationship was statistically significant ($r = 0{\cdot}671$, $P < 0{\cdot}002$, Fig. 2).

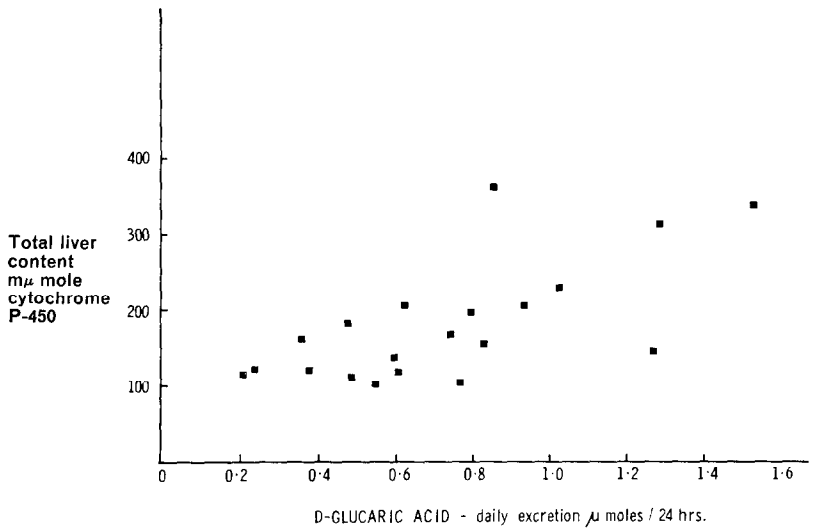

FIG. 2. Relationship between daily excretion of D-glucaric acid and total liver activity of cytochrome P-450 in 20 guinea-pigs receiving phenobarbitone (50 mg/kg) for 0–5 days.

All 12 guinea-pigs given actinomycin D became generally ill with diarrhoea and occasional wheezing. Three died before completion of the experiment. The mean value for total cytochrome P-450 content in the remaining 9 was 121·1 mμmole (S.E.M. 9·2) which was less than in the 6 controls of 151·8 mμmole (S.E.M. 9·6). Similarly D-glucaric acid excretion was lower with a mean rate of 0·64 μmole/day (S.E.M. 0·165) as compared to the corresponding value of 0·78 (S.E.M. 0·125) μmole/day in control animals. Because of the wide range of values for both measurements neither of these decreases from control animals were statistically significant. However, over the range of values obtained there was a significant correlation between P-450 and glucaric acid levels ($n = 15$, $r = 0{\cdot}612$, $P < 0{\cdot}025$).

DISCUSSION

It seems likely that drugs such as phenobarbitone which are known to cause induction of microsomal enzymes increase D-glucaric acid excretion as a result of a similar effect on the glucuronic acid pathway. However, this pathway is complex and several of the enzymes involved are not found in the microsomes but in the cytosol. Furthermore, the rate-limiting step of the pathway is unknown. According to Aarts[9] urinary D-glucaric acid excretion rises before induction of UDP glucose dehydrogenase can be detected and although Hänninen[15] claimed that activity of the final enzyme in the chain, glucuronolactone dehydrogenase, rose rapidly after phenobarbitone, other workers, using different techniques, were unable to confirm this.[2,9] The microsomal uronolactonase which converts D-glucuronic acid to glucuronolactone can be induced[16] but this would not explain the increase in glucuronic acid synthesis after barbiturates which has long been recognized.[1]

Nevertheless, increased D-glucaric acid excretion has now been shown following administration of a wide range of drugs known to induce hepatic enzymes, including barbiturates, diphenylhydantoin and primidone,[6] antipyrine,[5] phenylbutazone,[9] progesterone,[17] oral contraceptives[18] and organochlorine pesticides.[7] Excretion runs in parallel with the natural variations in microsomal enzyme activity, being low in the neonate and rising with maturity.[19] We have found (unpublished data) that peak excretion in the urine is in the afternoon and early evening which is known to be the time when drug metabolizing enzymes are most active.[20] Furthermore, an increase in D-glucaric acid excretion can be demonstrated between 4 and 6 hr after a single dose of an enzyme inducing drug, which is similar to the time response of cytochrome P-450.[21] In the present study we were able to show a statistically significant correlation between the total liver content of cytochrome P-450 and D-glucaric acid excretion. Cytochrome P-450 was chosen for the study as it plays a central role in hydroxylation of compounds such as drugs and steroids[22] on which many of the clinical and pharmacological effects of enzyme induction depend.[23] Moreover, as cytochrome P-450 is determined by measurement of its ability to bind carbon monoxide[10] the concentration of the enzyme is obtained rather than its activity.

The relationship between D-glucaric acid excretion and cytochrome P-450 was still present after administration of doses of actinomycin-D sufficient to impair induction of the cytochrome by phenobarbitone. This differs from the findings of Aarts[8] who reported increases in D-glucaric acid excretion in rats after inducing drugs despite pretreatment with puromycin and actinomycin-D, although the effect on marker enzymes was not studied. However, the use of inhibitors of protein synthesis is difficult and requires careful attention to dosage. Batches of puromycin may vary in potency.[24] Kuntzman[14] showed that in guinea-pigs actinomycin-D 1 mg/kg could prevent induction of cytochrome P-450 by phenobarbitone but not by 3-methylcholanthrene. Differences in drug response between animal species and strains are well known. Thus it cannot be assumed with certainty that a particular dose of these drugs will inhibit protein synthesis, and claims that a dose is sufficient to prevent enzyme induction must be substantiated by measurement of a marker enzyme. The dose of actinomycin-D we found necessary to affect induction (1 mg/kg) was 25 per cent greater than that used by Aarts[8] and is the most likely explanation for the difference in our findings.

Acknowledgements—We are grateful to Dr. M. Carrella, and the staff of the animal house for their assistance.

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