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EFFECT OF FASTING ON SUBSTRATE SPECIFICITY OF RAT LIVER UDP-GLUCURONOSYLTRANSFERASE

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Summary

The effect of a 3-day period of complete starvation on the hepatic UDPglucuronosyltransferase activity was studied in the rat. The substrate specificity of the enzyme was assayed with bilirubin as a carboxylic acceptor, and phenolphthalein and *p*-nitrophenol as phenolic acceptors.

Starvation increased the bilirubin UDPglucuronosyltransferase specific activity by 33%, whereas no increase in specific activities appeared when the phenolic substrates were used. However, on a total liver weight basis, all three activities were significantly lower than those of the controls. Kinetic studies of activated microsomal bilirubin UDPglucuronosyltransferase showed that apparent K_m values were similar; fasting acted only by increasing V.

The results suggest that the changes in bilirubin glucoronosyltransferase activity provoked by starvation may reflect actual enzyme induction; they favour the multiplicity of the UDPglucuronosyltransferase system.

Introduction

Starvation may have opposite effects on the hepatic activity of microsomal enzymes, causing an increase in some enzymes and a decrease in others [1]. However, the effect of complete starvation on the UDPglucuronosyltransferase activity (UDPglucuronate β-glucuronosyltransferase, (acceptor-unspecific), EC 2.4.1.17), has been quoted in only a few reports [2—4]; moreover, these are hampered by the possibility of glucuronosyltransferase being a heterogeneous group of closely related enzymes with different specificities for various substrates [5—7]. Clearly, direct evidence for the multiplicity of UDPglucuronosyltransferase could be only obtained by its total solubilization and purification; this however never completely succeeded up to the present time, in part probably because the properties of the transferase are closely influenced by the membrane environment [8], especially by the phospholipids

[9,10]. Nevertheless, an understanding of the influence of fasting on bilirubin UDPglucoronosyltransferase could have a practical outcome, for the enzyme may play a role in the fasting hyperbilirubinemia which occurs in various species [2,11], including man [2,4,12].

Methods

Male Charles River CD rats, weighing 200—300 g, were kept under similar conditions and fed ad libitum for two weaks prior to the experiments. At the end of this period, the rats were paired, and housed individually in screen-bottom cages; one group underwent a total starvation whereas both groups had free access to tap water. After 72 h, the rats were anesthetized with sodium pentobarbital (Nembutal, Abbott), 3—5 mg/100 g body weight intraperitoneally; portal vein was catheterized at once, the liver washed with 20 ml of 0.15 M ice-cold KCl, then quickly excised and weighed. All subsequent manipulations were done at 0—4 °C. The liver was homogenized in 5 vol. of 0.25 M sucrose containing 1 mM EDTA (disodium salt), (pH 7.4). When microsomes were to be obtained, the homogenate was centrifuged at 10 000 × g_{max} for 15 min; the 10 000 × g supernatant was then spun at 100 000 × $g_{av.}$ for 60 min. The resulting microsomal pellet was resuspended in 0.25 M sucrose/EDTA (pH 7.4) so as to achieve a protein concentration of about 10—15 mg/ml. Homogenates and microsomal suspensions were stored at −20 °C prior to enzymic assays: no detectable loss affected the activities to be measured for a period up to two months. Protein concentration was determined according to the method of Lowry et al. [13] using human serum albumin as reference. Prior to all enzymic assays, homogenates or microsomal suspensions were diluted with an equal volume of 2% (w/v) digitonin in 0.25 M sucrose/EDTA (pH 7.4). The Student t test was used to compare the means of the results.

Reagents

Bilirubin was obtained from British Drug Houses Ltd., Poole, England; when controlled, its molar extinction was found to be greater than 58 000 and could not be further improved by recrystallisation. Phenolphthalein, disodium salt, was purchased from RAL, Kuhlmann, Paris, France; *p*-nitrophenol from Koch-Light Laboratories Ltd., Colnbrook Bucks, England; UDPglucuronic acid, triammonium salt, and bovine crystalline albumin, Fraction V, from Sigma, St. Louis, Mo., U.S.A.; human serum albumin from CDTS, Paris, France; ethyl anthranilate from Eastman Kodak Co., Rochester, N.Y, U.S.A.; all other reagents were from Merck, Darmstadt, Germany, and of analytical grade.

UDPglucuronosyltransferase assay

Bilirubin UDPglucuronosyltransferase was measured in the homogenates according to the method of Van Roy and Heirwegh [14] and Black et al. [15], i.e. a system using diazotized ethyl anthranilate and in which the excess of unconjugated bilirubin does not react [16]. The incubation mixture contained 1.25 μmol of $MgCl_2$, 10 μmol of triethanolamine buffer (pH 7.4), 1.2 mg of human serum albumin, 43 nmol bilirubin and 0.4 μmol UDPglucuronic acid, in 80 μl which were added with 50 μl of homogenate to start the reaction.

The activity was measured after a 30-min incubation period. Results were expressed as nmol of bilirubin conjugated per mg of protein per h at 37 °C. For kinetic studies, only microsomal suspensions were used, in order to avoid the possible inhibiting or stimulating factors that the homogenates might contain; the final substrate concentration varied from 0.03 to 0.11 mM with a fixed concentration of UDPglucuronic acid: 3 mM. Incubation periods were 15 min, since activity was still linear with time at this point. In order to avoid deviation from the Michaelis-Menten kinetics due to variation in the free-to-bound bilirubin ratio [9,17,18], albumin was omitted in these kinetic studies. The pH of these assays was 8.0, in order to ensure solubilization of bilirubin as well as an optimal reaction when albumin is absent [19,20]. Under these conditions, and with respect to the substrate concentration, hyperbolic curves were always obtained. The final extraction volume of butyl acetate/methylpropylketone (3 : 17, v/v) was reduced to 500 μl owing to the low yield of azopigments used for the calculation of the initial velocities; all other steps were those described by Van Roy and Heirwegh [14].

Phenolphthalein UDPglucuronosyltransferase was measured in the homogenates by a method derived from that of Halac and Reff [21]. The volume of the incubation mixture was 25 μl, containing 6 μmol of Tris/HCl buffer (pH 8.0), 0.6 mg of bovine serum albumin, 80 nmol of mercaptoethanol, 20 nmol of phenolphthalein, and 160 nmol of UDPglucuronic acid. The reaction was started by adding 20 μl of the enzyme preparation. It was incubated for 15 min, and stopped with 20 μl of 10% (w/v) tricholoroacetic acid.

For *p*-nitrophenol, the total volume of the incubation mixture was 15 μl, containing 3 μmol of Tris/HCl buffer (pH 8.0), 0.125 mg of bovine albumin, 40 nmol of mercaptoethanol, 0.4 μmol of $MgCl_2$, 30 nmol of *p*-nitrophenol, and 160 nmol of UDPglucuronic acid. The reaction was started with the addition of 10 μl of homogenate. It was stopped after 10 min with 10 μl of 10% trichloroacetic acid.

For both the phenolphthalein and *p*-nitrophenol assays, after trichloroacetic acid had been added and in order to eliminate any remaining binding of the substrates to the proteins, the mixtures were incubated for 2 min in boiling water. Then 1 ml of 1.6 M glycine (pH 10.7) was added to each assay; the tubes were spun for 2.5 min at 8000 × *g* (Eppendorf centrifuge), and the supernatants read at 555 nm for phenolphthalein and 400 nm for *p*-nitrophenol. The enzymic activities were expressed as nmol of substrate conjugated per mg of protein per h at 37 °C.

Results

After complete starvation for 72 h, the weight of the liver decreased by about 43%, i.e. more than the average decrease of body weight which was about 19% (Table I). The protein concentration in the homogenates however was not significantly altered by starvation: 47.16 ± 5.96 (S.D.) mg/ml of homogenate in the fasting animals, as against 44.16 ± 5.68 in the control group. This corresponds to about 270 mg of protein per g of wet liver in both groups of rats.

Bilirubin UDPglucuronosyltransferase specific activity (Table I) raised in

TABLE I

EFFECT OF A 72-h STARVATION ON THE LIVER WEIGHT AND BILIRUBIN UDP-GLUCURONOSYLTRANSFERASE ACTIVITY IN THE LIVER HOMOGENATES

Results are means ± S.D.; n is the number of rats in each group.

	Liver weight	$\frac{\text{Liver weight}}{\text{Body weight}} \times 100$	Bilirubin UDPglucuronosyltransferase	
			nmol/mg protein per h	μmol per whole liver per h
Controls (n = 23)	9.38 ± 0.97	3.70 ± 0.31	6.66 ± 1.95	17.16 ± 4.20
Fasted (n = 21)	5.39 ± 0.83	3.01 ± 0.34	8.85 ± 1.93	13.58 ± 4.29
P	< 0.001	< 0.001	< 0.001	< 0.01

fasting rats at 72 h by about 33%. However on a whole liver basis there was a marked decrease of the bilirubin enzyme, which was significant at the 1% level.

In contrast to the bilirubin transferase, the specific activities of the phenolphthalein enzyme did not differ significantly in the two groups of rats (Table II); for p-nitrophenol, the fasting animals had even lower figures than the controls. On a total liver weight basis, and for both phenolphthalein and p-nitrophenol, there was a marked decrease of the transferase which roughly paralleled the liver weight changes. As a significant increase in the specific enzymic activities was observed only with bilirubin as the acceptor substrate, kinetic studies were restricted to this carboxylic substrate.

Typical reciprocal curves of initial rates of hepatic bilirubin UDPglucuronosyltransferase obtained in both control and starved rats are shown in Fig. 1. In four control animals, the apparent K_m values ranged from 0.178 to 0.250 (mean: $0.226 \cdot 10^{-3}$ M), vs 0.196, 0.217 and 0.286 (mean: $0.233 \cdot 10^{-3}$ M) in three fasted rats; the respective figures for V ranged from 172 to 244 (mean: 215) nmol/h per mg protein in the controls, and were 250, 286 and 303 (mean: 280) in the fasted animals. Thus, there were no obvious differences in the apparent K_m values; the increase in the bilirubin transferase which follows starvation appeared to be primarily related to the rise of V.

TABLE II

EFFECT OF A 72-h STARVATION ON PHENOLPHTHALEIN AND p-NITROPHENOL UDP-GLUCURONOSYLTRANSFERASE ACTIVITIES IN THE LIVER HOMOGENATES

Results are means ± S.D.; n is the number of rats in each group.

	UDP-glucuronosyltransferase (nmol conjugated per h)			
	Phenolphthalein		p-Nitrophenol	
	per mg protein	per whole liver	per mg protein	per whole liver
Controls (n = 22)	68.4 ± 19.3	641 ± 181	502.2 ± 90.0	4710 ± 844
Fasted (n = 18)	70.8 ± 16.6	381 ± 89	423.8 ± 72.9	2284 ± 393
P	N.S.	< 0.001	< 0.05	< 0.001

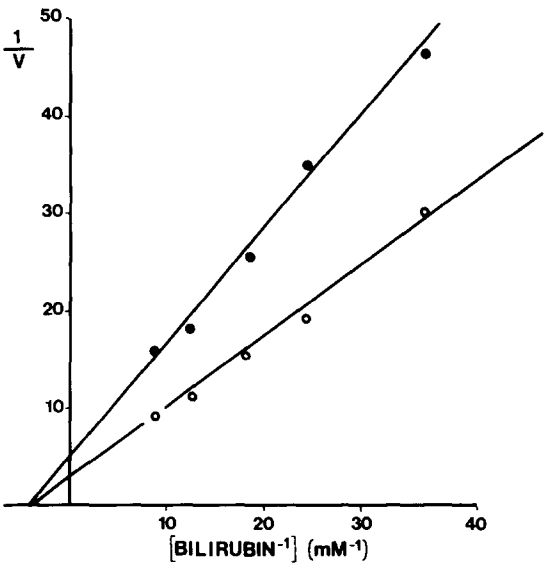

Fig. 1. Double-reciprocal plots of initial rates of bilirubin conjugation by hepatic microsomes as a function of bilirubin concentration in one control rat (•) and one having been fasted for 72 h (○). Reaction velocities are expressed as μmol of bilirubin conjugated per h per mg of microsomal protein. Assays as described in the text.

Discussion

The bilirubin UDPglucuronosyltransferase enhancement by fasting is probably not related to simple enzyme activation, for this should not reflect on the present experiments which were systematically conducted on digitonin-activated preparations. Actual enzyme induction seems a more likely explanation: (1) our kinetic data are compatible with an induction process; (2) induction is already known to occur after starvation for several enzymes, some of which being even related with the normal bilirubin pathway, such as the microsomal enzyme heme oxygenase [22]. It is also to be noticed that the increase in the specific activity of the bilirubin transferase that we observed in the present series of fasting rats is of an order of magnitude similar to that reported for the microsomal cytochrome P-450 [3].

It remains of course hazardous to extrapolate from our results to the in vivo situation. Firstly, the induction of UDPglucuronosyltransferase and the regulation of its substrate specificity seems to be species-dependent [23]. Secondly, the increased activity as measured in vitro, does not necessarily imply that it exists in vivo, as the enzyme might not be the only limiting step in the hepatic transport of bilirubin. For example, the enzyme UDPglucose dehydrogenase, which falls following starvation [24], could be limiting as well; the decrease of UDPglucuronic acid which would ensue might therefore be responsible for an in vivo decrease of glucuronidation activity which would not be detected in vitro, as exogenous UDPglucuronic acid is added. However, from our experiments, an obvious result is the dramatic decrease of the bilirubin transferase when calculated on the total liver weight basis in the fasting rats.

This has also been recently noticed by Fevery et al. [4] in Wistar rats. It leads to emphasize that such a way of calculating the results of the bilirubin transferase is probably the most rational with regard to a possible link between bilirubin conjugation and fasting hyperbilirubinemia.

The differences observed in the present work with regards to different substrates, occurred all in digitonin-treated preparations, i.e. the activated form which probably best reflects the actual amount of enzyme. Although our results do not definitely rule out the role of membrane environment, they can most easily be explained by assuming different UDPglucuronosyltransferases. Bock et al. [5] recently showed that *p*-nitrophenol conjugation was preferentially stimulated in the rat by 3-methylcholantrene, whereas chloramphenicol and bilirubin glucuronidation was increased mainly after treatment with phenobarbital. Fasting therefore appears as a condition favouring similarly the multiplicity of the enzymes responsible for glucuronidation.

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