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Reduced nicotinamide-adenine dinucleotide phosphate (NADPH) enhancement of *p*-nitrophenol glucuronidation*

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NADPH is known to be required by certain hepatic microsomal enzyme systems involved in oxidations, reductions and hydrolyses, but it has not been thought to be involved in conjugations.[1–3] UDP-glucuronyltransferase (UDP-GT, EC 2.4.1.17), the enzyme system that conjugates various endogenous and exogenous compounds with glucuronic acid, has been shown to have enhanced activity in the presence of Mg^{2+},[4–6] ATP, and UDP-*N*-acetyl glucosamine.[7,8]

During fractionation of rat liver, we noted a loss of *p*-nitrophenol (PNP)-conjugating activity in the 105,000 *g* microsomal pellet and found that the activity could be restored by addition of the 105,000 *g* supernatant, which by itself had negligible activity. Because the 105,000 *g* supernatant is known to contain a NADPH-generating system, we decided to measure the effect of NADPH on microsomal UDP-GT activity, although to our knowledge enhancement of UDP-GT activity by NADPH has not been reported. In this paper, we report the effect of NADPH on rat liver microsomal PNP UDP-GT activity.

Adult male Holtzman rats, ages 100–220 days and weighing 300–500 g, were used in these experiments. Animals were not fed for 16–20 hr but allowed water *ad lib.* prior to being sacrificed by cervical fracture. Livers were perfused *in situ* with cold 0·25 M sucrose solution, then excised and placed in cold 0·25 M sucrose solution. All subsequent manipulations were carried out at 0–4°. Each excised liver was pressed through a Lucite seive to remove connective tissue and the resulting tissue mash was homogenized in 0·25 M sucrose solution (6 ml/g of mash) with a Potter–Elvehjem glass-Teflon homogenizer (one stroke/g of mash). The liver homogenate was first centrifuged at 9000 *g* for 15 min in a Sorvall RC-2 centrifuge (SS-34 rotor, 25 ml/tube). The 9000 *g* supernatant was centrifuged in a Spinco Model L preparative ultracentrifuge at 105,000 *g* for 60 min (No. 50 rotor, 9·5 ml/tube). The 105,000 *g* supernatant was removed by aspiration and the microsomal pellet resuspended in either 0·25 M sucrose or 0·15 M KCl solution so that each milliliter contained the equivalent of 167 mg of liver mash.

Reaction mixtures contained liver fractions isolated from 100 mg liver mash, 0·2 μmole PNP, 5·0 μmoles uridine diphosphoglucuronic acid (UDPGA), 0·1 ml of 0·5 M Tris buffer, pH 7·4, and cofactors as indicated in Table 1. The final volume of the reaction mixtures was 1·0 ml. Mixtures were incubated for 10 min with shaking at 37° in open flasks in a Dubnoff metabolic shaker. During this period the relationship between the amount of substrate conjugated and the duration of the incubation was linear. Glucuronide formation was estimated by measuring the disappearance of PNP from the reaction mixture by the method described by Isselbacher.[9] The formation of PNP glucuronide was verified according to the criteria suggested by Dutton,[3] with the use of β-glucuronidase and its inhibitor, saccharo-1,4-dilactone. All assays were compared to controls containing all of the reaction mixture components except UDPGA. Protein determinations were made by the method of Lowry *et al.*[10]

PNP, UDPGA, NAD, NADH, NADP, NADPH, and G 6-P were obtained from Sigma Chemical Co.; G 6-PD from Boehringer Mannheim Corp.; β-glucuronidase from Warner–Chilcott Laboratories; and saccharo-1,4-dilactone from Calbiochem.

The UDP-GT activity and the protein content of each liver fraction are shown in Table 1. The sum of the UDP-GT activity recovered from the 9000 *g* supernatant and the 9000 *g* pellet was approximately equivalent to that of the whole homogenate. Further fractionation of the 9000 *g* supernatant into a 105,000 *g* pellet (microsomes) and supernatant resulted in a considerable loss of PNP UDP-GT activity. However, when the 105,000 *g* pellet was resuspended in the 105,000 *g* supernatant, the combination was as effective as the 9000 *g* supernatant in conjugating PNP. These results demonstrate that one or more unknown factors in the 105,000 *g* supernatant increases microsomal UDP-GT activity.

As shown in Table 1, microsomal (105,000 *g* pellet) UDP-GT activity was increased by the addition of NADPH or a NADPH-generating system (NADP + G 6-P + G 6-PD) to the reaction mixture. Similar results were obtained with double or half the indicated amount of NADPH, and with NADP, isocitrate and isocitrate dehydrogenase employed as a NADPH-generating system. These observations suggest that NADPH may be involved in the microsomal conjugation of PNP. However,

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TABLE 1. UDP-GLUCURONYLTRANSFERASE ACTIVITY IN RAT LIVER FRACTIONS

Additions to reaction mixture*	Total activity†	Per cent activity‡	Protein§	Specific activity‖
Whole homogenate	11·2 ± 1·2(5)	100	20·4 ± 0·6	0·55
9000 *g* Pellet	4·9 ± 0·8(5)	44	14·5 ± 1·0	0·34
9000 *g* Supernatant	4·8 ± 0·2(10)	43	9·8 ± 0·5	0·49
105,000 *g* Supernatant	0·9 ± 0·3(6)	8	7·0 ± 0·5	0·13
105,000 *g* Pellet	2·2 ± 0·3(8)	20	3·7 ± 0·2	0·61
105,000 *g* Pellet + 105,000 *g* supernatant	5·1 ± 0·7(8)	45	10·5 ± 0·7	0·48
105,000 *g* Pellet + NADPH	3·9 ± 0·4(7)	35	3·7 ± 0·2	1·08
105,000 *g* Pellet + NADP, G 6-P, G 6-PD	3·8 ± 1·2(5)	33	3·7 ± 0·2	1·05
105,000 *g* Pellet + NADP	1·4 ± 0·4(2)	12	3·7 ± 0·2	0·39
105,000 *g* Pellet + NADH	2·0 ± 0·3(4)	18	3·7 ± 0·2	0·56
105,000 *g* Pellet + NAD	1·9 ± 0·2(3)	17	3·7 ± 0·2	0·53

* All reaction mixtures contained 0·2 μmole PNP, 5·0 μmoles UDPGA, 0·1 ml 0·5 M Tris buffer, pH 7·4, tissue fraction equivalent to 100 mg liver mash, and, as indicated, 2·0 μmoles NADPH, NADP, NADH or NAD, 20·0 μmoles G 6-P, 3 enzyme units G 6-PD, or 105,000 *g* supernatant equivalent to 100 mg liver mash.

† Activity as mμmoles PNP conjugated per min per 100 mg liver mash; values are means ± S.E. with the number of determinations in parentheses.

‡ Percentages relative to whole homogenate activity.

§ Expressed as mg of protein per 100 mg liver mash; values are means ±S.E.

‖ Expressed as mμmoles of PNP conjugated per min per mg protein.

since NADPH did not restore activity to the level obtained with the 9000 *g* supernatant or the recombined 105,000 *g* pellet and supernatant, the activity lost during fractionation cannot be completely ascribed to NADPH.

The enhancement of microsomal UDP-GT activity toward PNP by NADPH was not affected by $MgCl_2$; no enhancement occurred if NAD, NADP, or NADH was used in place of NADPH. The addition of NADPH to reaction mixtures containing the 9000 *g* supernatant had no effect on UDP-GT activity. No conjugation took place in reaction mixtures containing microsomes and a NADPH-generating system if either the UDPGA was omitted, or the microsomes were boiled prior to incubation.

At the present time we are unable to explain the results obtained with NADPH within the framework of known mechanisms of glucuronidation. Pogell and Leloir[7] observed enhancement of UDP-GT activity when dialyzed 18,000 *g* supernatant was added to guinea pig liver microsomes; they attributed this to "protein activation". They also demonstrated activation by ATP and UDP-*N*-acetyl glucosamine, and they attributed this to protection of UDPGA from destruction by UDPGA pyrophosphatase. Since UDPGA was in great excess in our studies and was not rate limiting, protection of UDPGA is not the mechanism of NADPH enhancement.

Other workers have also measured UDP-GT activity in liver fractions.[9,11] In contrast to the present findings, no striking loss of activity with fractionation was observed. This apparent discrepancy could simply be a reflection of the methodological differences in the studies. In this regard it should be noted that various substrates have been employed to study glucuronidation. Our results may be uniquely applicable to PNP glucuronidation because we have been unable to demonstrate a similar NADPH activation of *o*-aminophenol. Other substrates are currently under investigation.

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Departments of Pediatrics and Pharmacology,
University of Utah College of Medicine,
Salt Lake City, Utah 84112, *U.S.A.*

ANTHONY R. TEMPLE
DAVID J. GEORGE*
ALAN K. DONE

* D. J. George is a National Aeronautics and Space Administration Trainee (1967–1970).

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Metabolism of propranolol by rat liver microsomes and its inhibition by phenothiazine and tricyclic antidepressant drugs*

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PROPRANOLOL appears to be extensively metabolized in animals and man, only small amounts of the drug being excreted unchanged.[1,2] In the present study the metabolism *in vitro* of propranolol by rat liver microsomes has been investigated and the inhibitory effects of certain phenothiazine and tricyclic antidepressant drugs tested.

Male Sprague–Dawley rats (150–200 g) were decapitated and the livers removed and homogenized in 2 vol. of ice-cold isotonic KCl in a motor-driven, Teflon-glass homogenizer. The 9000 *g* supernatant, the 100,000 *g* supernatant (soluble fraction) and the microsomal pellet were obtained by differential centrifugation. Various fractions of the liver preparation (in 1·5 ml) were incubated in a medium containing $MgCl_2$ (150 μmoles), glucose-6-phosphate (50 μmoles), nicotinamide (100 μmoles), and 1·8 ml of 0·2 M potassium phosphate buffer (pH 7·4) and brought to a final volume of 4 ml with water containing any drugs used. NADPH (12 μmoles) was added to the microsomal pellet or, in the case of the 9000 *g* and 100,000 *g* supernatant, generated from NADP (0·5 μmoles) and the glucose 6-phosphate in the presence of the glucose 6-phosphate dehydrogenase present in the soluble fraction. Incubations were carried out in a Dubnoff shaker and the reaction terminated by the addition of 1 ml of 2·5 N NaOH.

Propranolol metabolism was determined by measuring the disappearance of the drug using a method previously described.[2] Briefly, the alkalinized reaction mixture was extracted with 30 ml of heptane containing 1·5% isoamyl alcohol, a 10-ml aliquot of which was extracted into 3 ml of 0·1 N HCl. Their resulting fluorescence was read in an Aminco–Bowman spectrophotoflurometer (excitation at 295 mμ; emission at 360 mμ-uncorrected). The assay was linear over the range of substrate concentration used and 80–85 per cent recovery of the drug was obtained after correcting for the proportion of the organic phase transferred. Also, none of the drugs used interfered with the assay.

Propranolol was metabolized by the 9000 *g* supernatant fraction in the presence of NADPH-generating system, but not by the soluble fraction or microsomes alone (Table 1). However, the microsomes were active when NADH or especially NADPH was added. There was no metabolism in an atmosphere of nitrogen. These findings indicate that propranolol is metabolized by a microsomal enzyme system requiring NADPH and oxygen.

Pretreatment of rats with chlorpromazine or desmethylimipramine (DMI) markedly inhibited the metabolism of propranolol by the 9000 *g* supernatant fraction (Table 2). These drugs have also been shown to inhibit the metabolism of amphetamine[3,4] and guanethidine.[5] DMI can inhibit the metabolism of pentobarbital, tremorine and oxotremorine.[6,7]

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