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# Effect of Aging on Mixed-Function Oxidation and Conjugation by Isolated Perfused Rat Livers

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**ABSTRACT.** Aging is known to decrease hepatic cytochrome P450 content in rats. However, limited information is available on the effects of aging on mixed-function oxidation and conjugation in intact liver. The purpose of these studies was to determine the effects of aging on oxidation and conjugation of *p*-nitrophenol (pNP) in perfused livers from male Sprague–Dawley rats. Livers from senescent (22–24 months) or young adult (3–6 months) rats were perfused in a nonrecirculating hemoglobin-free system and supplemented with pNP (60 μM). Glucuronide and sulfate conjugates of the oxidation product, 4-nitrocatechol, in effluent perfusate were cleaved enzymatically and 4-nitrocatechol was determined colorimetrically. Rates of 4-nitrocatechol production were decreased in senescent compared with young adult rats (0.67 ± 0.14 vs 0.92 ± 0.15 μmol/g/hr). However, the rates of oxidation of pNP in microsomes from senescent rats were similar to those in young adult rats. Hepatic malate content was decreased approximately 50% in livers from senescent compared with young adult rats in the presence and absence of pNP, suggesting that movement of reducing equivalents from the mitochondria to the cytosol, and thus cytosolic NADPH supply, may have been diminished by senescence. The rates of conjugation of 60 μM pNP in perfused livers from senescent rats were similar to those in young adult rats, but $K_m$ and $V_{max}$ values of microsomal 4-nitrocatechol glucuronyltransferase were about 2.5- and 1.6-fold higher, respectively, in livers from senescent compared with young adult rats. Hepatic glycogen content was about 50% lower in livers from senescent compared with young adult rats, but the contents of UDP-glucose and UDP glucuronic acid were similar between the two groups. Taken together, the data are consistent with the hypothesis that rates of mixed-function oxidation are decreased in intact livers from senescent compared with young adult rats, due possibly to age-related changes in cofactor supplies. Glucuronidation of low, but not high, concentrations of substrates may be affected by age-related changes in $K_m$ and $V_{max}$ values of microsomal glucuronyltransferase. BIOCHEM PHARMACOL 54;1:159–164, 1997. © 1997 Elsevier Science Inc.

**KEY WORDS.** liver; cytochrome P450; mixed-function oxidation; glucuronidation; senescence; *p*-nitrophenol

Various factors that may have a significant impact upon drug metabolism are affected by aging. For example, absorption may be altered by increased gastric pH or decreased splanchnic flow [1, 2]. In addition, distribution may be altered by age-related decreases in lean body mass relative to total body mass [3–5] or decreases in intracellular water content from 42% in young adults to 33% in the elderly [6]. Hepatic clearance of drugs can decrease with age [7, 8], and it has been estimated that age-related decreases in xenobiotic metabolism can account for over 80% of toxic reactions to drugs observed in elderly populations [9].

A number of studies investigating the effects of aging on xenobiotic metabolism in rats have focused upon age-related changes in enzyme content or activity of hepatic microsomes [10–12]. Both decreases [10] or no changes [11] in hepatic cytochrome P450 content have been observed. More recently, decreased microsomal activity of selective isozymes of cytochrome P450 (i.e. 2B1, 2B2, 2C11, and 2E1) as well as increased activity (2A1, testosterone 5α-reductase) in livers from senescent (22-months-old) male Fischer rats compared with young adults (2-months-old) has been reported [12]. In contrast, limited information exists on the effects of aging on rates of mixed-function oxidation in intact rat liver. The O-demethylation of *p*-nitroanisole is decreased by 41% in isolated hepatocytes from senescent compared with young adult male Fischer rats [13]. The cause of the decrease was not determined in that study, but was postulated to be due, at least in part, to alterations in NADPH supply.

Conjugation of xenobiotics and oxidative metabolites is also affected by aging. For example, sulfation of *p*-nitrophenol is decreased in hepatocytes from senescent compared with young adult male Fischer rats [13]. However, hepatic sulfotransferase and glucuronyltransferase activities are reported to be unaffected by aging [14].

The perfused rat liver is an experimental model in which

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‡ *Abbreviation:* UDPGA, uridine diphosphoglucuronic acid.

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hepatic biochemistry and architecture are intact and, thus, is well-suited to the study of phase I and phase II reactions involved in xenobiotic metabolism [15–17]. The purpose of the present work was to determine the effects of aging on metabolism and conjugation of the model substrate, *p*-nitrophenol, in perfused livers from young adult and senescent rats.

## MATERIALS AND METHODS

### *Animals*

Male Sprague–Dawley rats (CD-VAF) were obtained from Charles River, Raleigh, NC. All rats were maintained on a 12-hr light/dark cycle and had access to food and water *ad lib*. Young adult rats were 3–6 months of age and senescent rats were 22–25 months of age at the time they were killed. Experiments were approved by the SmithKline Beecham R&D Animal Use and Care Committee.

### *Measurement of Mixed-Function Oxidation and Conjugation of p-Nitrophenol by Perfused Rat Livers*

Rats were anesthetized with 50 mg Nembutal/kg and livers were removed and perfused with Krebs–Henseleit buffer, pH 7.4, 37° in a nonrecirculating system as described elsewhere [16]. Flow rates were 4–5 mL/min/g wet weight liver. In some experiments, *p*-nitrophenol (final concentration, 60 μM) was infused by a precision pump for 20–30 min. Under these conditions, the rates of oxygen uptake increased by 4–6 μmol/g/hr in livers from both young adult and senescent rats (data not shown). Samples of effluent perfusate were collected every 3 min, and concentrations of unconjugated 4-nitrocatechol formed from the hydroxylation of *p*-nitrophenol by perfused livers were determined spectrophotometrically at 546 nm following the addition of 10 N NaOH [17]. To determine the total (free + conjugates) rates of 4-nitrocatechol formation, glucuronide and sulfate conjugates of 4-nitrocatechol in effluent perfusate were cleaved by incubation of 1 mL of perfusate with 250 U glucuronidase/25 U sulfatase for 90 min before detection of 4-nitrocatechol spectrophotometrically. Sulfate conjugates accounted for less than 5% of the total amount of conjugated products (data not shown). Rates of 4-nitrocatechol production were calculated based on effluent 4-nitrocatechol concentrations, flow rates, and liver wet weights. Mean rates were calculated based on rates at steady-state conditions, typically 12–30 min after the initiation of infusion of *p*-nitrophenol.

Rates of conjugation of *p*-nitrophenol by perfused rat livers were determined by incubating 1.0 mL of effluent perfusate with 250 U of β-glucuronidase/25 U sulfatase for 90 min to determine glucuronide and sulfate conjugates of *p*-nitrophenol spectrophotometrically at 405 nm in effluent perfusate [17–19]. Free and total (free + conjugated) *p*-nitrophenolate were measured spectrophotometrically in samples collected every 3 min for 15–18 min. Rates of conjugation were calculated from concentrations of *p*-nitrophenolate and *p*-nitrophenol glucuronide and sulfate conjugates, flow rates, and liver wet weights and were corrected for hydroxylation of *p*-nitrophenol to 4-nitrocatechol by subtracting total rates of formation of 4-nitrocatechol from total rates of *p*-nitrophenol conjugate formation. Mean rates were calculated from rates at steady-state conditions.

### *Measurement of Microsomal Oxidation and Supernatant Glucuronyltransferase Activities*

Microsomes were prepared by differential centrifugation, washed in 0.1 M sodium pyrophosphate buffer, and resuspended in 0.1 M Tris acetate buffer, pH 7.4, containing EDTA and glycerol as described elsewhere [20]. *p*-Nitrophenol hydroxylase activity was determined by incubating approximately 10 mg of microsomal protein (measured by using the method of Bradford [21]) for 30 min with 200 μM *p*-nitrophenol in the presence of an NADPH-generating system consisting of 100 mM glucose-6-phosphate, 100 mM $NADP^+$ and 50 IU/mL glucose-6-phosphate dehydrogenase in a final volume of 0.5 mL. Reactions were terminated by the addition of 1 mL of 10 N NaOH, and, following additional centrifugation, 4-nitrocatechol was measured spectrophotometrically in supernatants at 546 nm [19]. Under these conditions, reactions were linear for up to 30 min.

Microsomal glucuronyltranferase activity was determined by measuring the disappearance of 4-nitrocatechol as described for *p*-nitrophenol by Reinke *et al.* [17]. Briefly, 30 μL of a microsomal suspension (0.4 mg protein) was incubated with 200 μM 4-nitrocatechol in 100 μL of 0.05 M sodium phosphate buffer, pH 7.0, containing 0.2% bovine serum albumin, 1.0 mM $MgCl_2$, and 0.5 mM dithiothreitol at room temperature. The reaction was linear over 30 min of incubation. Reactions were stopped after 20 min by adding 1 mL of 0.02 M sodium carbonate, pH 10.2, and the remaining 4-nitrocatechol was measured spectrophotometrically at 546 nm [19]. $K_m$ and $V_{max}$ values were calculated for individual liver preparations by using Lineweaver–Burk plots of substrate–velocity curves, or mean velocities were calculated and plotted.

### *Metabolite Measurements*

In some experiments, livers were freeze-clamped with tongs chilled in liquid nitrogen during perfusion with or without *p*-nitrophenol (60 μM). Samples of frozen liver were ground with a mortar and pestle under liquid $N_2$ and then were treated with 0.4 M $HClO_4$. Extracts were centrifuged to remove proteins, and then supernatants were neutralized with 2 M $KHCO_3$ and stored at −80°. Malate and UDP-glucose were measured as described elsewhere [22, 23]. UDPGA‡ was measured via HPLC analysis by using a Whatman SAX-12.5, 5 μm particle size column and a linear gradient of 0.2 to 0.5 M $KH_2PO_4$, pH 5.5, and KCl

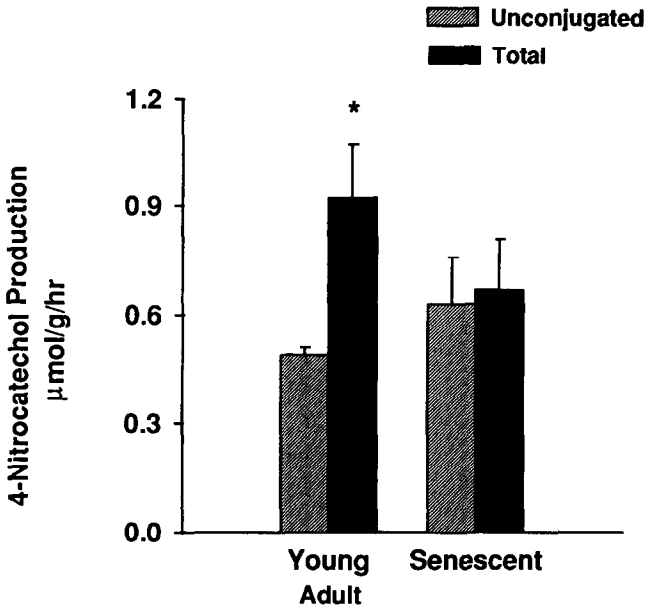


FIG. 1. *p*-Nitrophenol hydroxylation by perfused livers from young adult and senescent male Sprague–Dawley rats. Livers from young adult and senescent male Sprague–Dawley rats were perfused in the presence of 60 μM *p*-nitrophenol, and 4-nitrocatechol in effluent perfusate was measured spectrophotometrically as described in Materials and Methods. Glucuronide and sulfate conjugates of 4-nitrocatechol were cleaved with β-glucuronidase/sulfatase for 90 min prior to analysis of 4-nitrocatechol. Data are means ± SEM for 4 livers per group. Key: (*) $P < 0.05$ as compared with senescent rats. Hatched bars, unconjugated 4-nitrocatechol; solid bars, sum of production of unconjugated and conjugated 4-nitrocatechol.

(0.1 to 0.5 M) at a flow rate of 0.5 mL/min [24]. Detection of UDPGA was at 254 nm.

### *Statistics*

Data are expressed as means ± SEM for 4 livers or preparations per group. Data were analyzed for statistical differences with Student's *t*-test [25].

## RESULTS

### *Effect of Senescence on Mixed-Function Oxidation of p-Nitrophenol by Perfused Rat Livers and Hepatic Microsomes and on Hepatic Malate Content*

*p*-Nitrophenol is hydroxylated in perfused rat livers primarily via cytochrome P450 2E1 to 4-nitrocatechol, with subsequent conjugation to glucuronide and sulfate adducts. In livers from young adult and senescent rats, the rates of production of unconjugated 4-nitrocatechol were 0.49 ± 0.02 vs 0.63 ± 0.13 μmol/g/hr, respectively (Fig. 1). In contrast, rates of unconjugated and conjugated (total) 4-nitrocatechol production in livers from senescent rats were significantly less than in livers from young adult rats (0.67 ± 0.14 vs 0.92 ± 0.15 μmol/g/hr, respectively; $P < 0.05$). However, rates of *p*-nitrophenol hydroxylation by microsomes from senescent rats (1.96 ± 0.09 nmol/min/mg protein) were significantly greater than rates by microsomes from young adult rats (1.46 ± 0.10 nmol/min/mg protein; Fig. 2). The overall yield of microsomal protein per gram wet weight of tissue was decreased about 10% in livers from senescent compared with livers from young adult rats (data not shown).

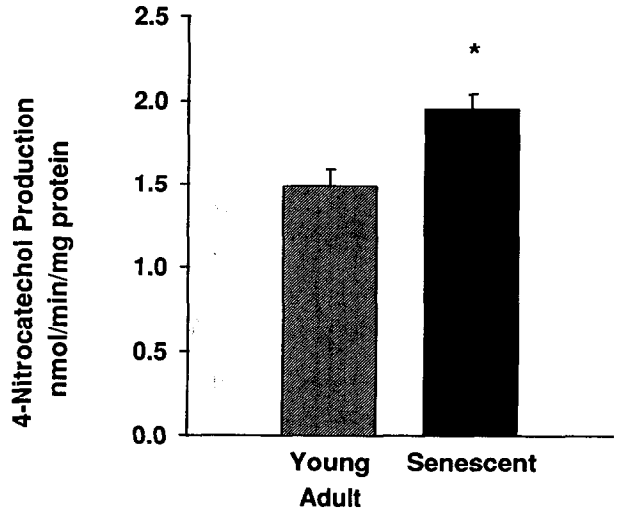


FIG. 2. Hydroxylation of *p*-nitrophenol by hepatic microsomes from senescent and young adult rats. Microsomes were prepared by differential centrifugation from livers of senescent and young adult rats as described in Materials and Methods. 4-Nitrocatechol production was measured spectrophotometrically over time. Data are means ± SEM for 4 livers per group. Key: (*) $P < 0.05$ as compared with young adult males.

To investigate whether or not senescence could affect intermediary metabolism, hepatic malate contents were measured. Basal malate contents in the presence or absence of *p*-nitrophenol (60 μM) were decreased approximately 50% in livers from senescent rats (Table 1).

### *Effects of Senescence on Conjugation of p-Nitrophenol by Perfused Livers and Hepatic Microsomes and Intermediates Involved in Glucuronidation*

Perfused livers from senescent rats did not form conjugates of 4-nitrocatechol, which was produced from the hydroxylation of *p*-nitrophenol (Fig. 1). In contrast, rates of

TABLE 1. Effects of age on hepatic malate content

| | Malate (μmol/kg wet weight) | |
|---|---|---|
| Age | Basal | +*p*-Nitrophenol |
| Young adult | 49.1 ± 8.8 | 34.6 ± 5.2 |
| Senescent | 28.9 ± 4.1* | 15.1 ± 3.4* |

Livers from young adult or senescent male Sprague–Dawley rats were perfused as described in Materials and Methods. *p*-Nitrophenol (60 μM) was infused after 20 min of perfusion. Then livers were frozen rapidly by freeze-clamping with tongs chilled in liquid nitrogen after an additional 15 min of perfusion. Cellular malate content was determined as described in Materials and Methods. Data are means ± SEM for 4 livers per group.

*$P < 0.05$ as compared with values for young adults.

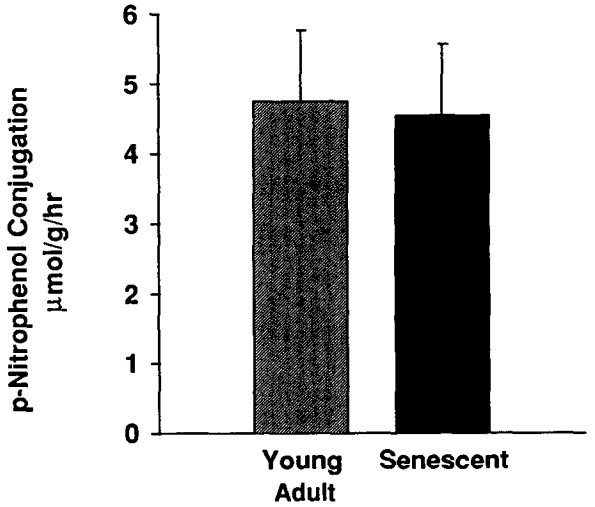


**FIG. 3. Conjugation of *p*-nitrophenol by perfused livers from senescent and young adult rats. *p*-Nitrophenol (60 μM) was infused into perfused livers from senescent and young adult rats, and conjugation was measured by differences in influent and effluent *p*-nitrophenol concentrations as described in Materials and Methods. Data are means ± SEM for 4 livers per group.**

conjugation of 60 μM *p*-nitrophenol were similar in livers from young adult and senescent rats (Fig. 3). Under these conditions about 95% of the conjugates formed were glucuronides (data not shown). Because of the differences between conjugation of optimal concentrations of *p*-nitrophenol and lesser concentrations of 4-nitrocatechol formed by mixed-function oxidation, the kinetics of 4-nitrocatechol glucuronyltransferase were determined (Fig. 4). $K_m$ Values for microsomal 4-nitrocatechol glucuronyltransferase were increased about 3-fold in senescent compared with young adult rats from 0.017 ± 0.008 to 0.045 ± 0.013 mM (Table 2). These values are about 10-fold lower than $K_m$ values for *p*-nitrophenol observed by others [26]; the reasons for the differences are unknown. Values for $V_{max}$ were also increased about 2-fold in senescent compared with young adults from 1.60 ± 0.077 to 2.52 ± 0.067 nmol/min/mg protein (Table 2).

The supply of UDPGA is a major rate determinant in glucuronidation in perfused rat livers [17]. Accordingly, it was of interest to investigate the effects of senescence on intermediates involved in UDPGA formation, namely glycogen, UDP-glucose, and UDPGA contents. Hepatic glycogen contents were decreased about 50% in livers from senescent compared with young adult rats (Table 3). In contrast, UDP-glucose and UDPGA contents were in livers from senescent rats were similar to those in young adult rats (Table 3).

## DISCUSSION

Decreases in the clearance of xenobiotics in senescent animals and humans have been documented extensively (for review, see Ref. 27). One problem in the interpretation of metabolism or clearance data generated *in vivo* is in

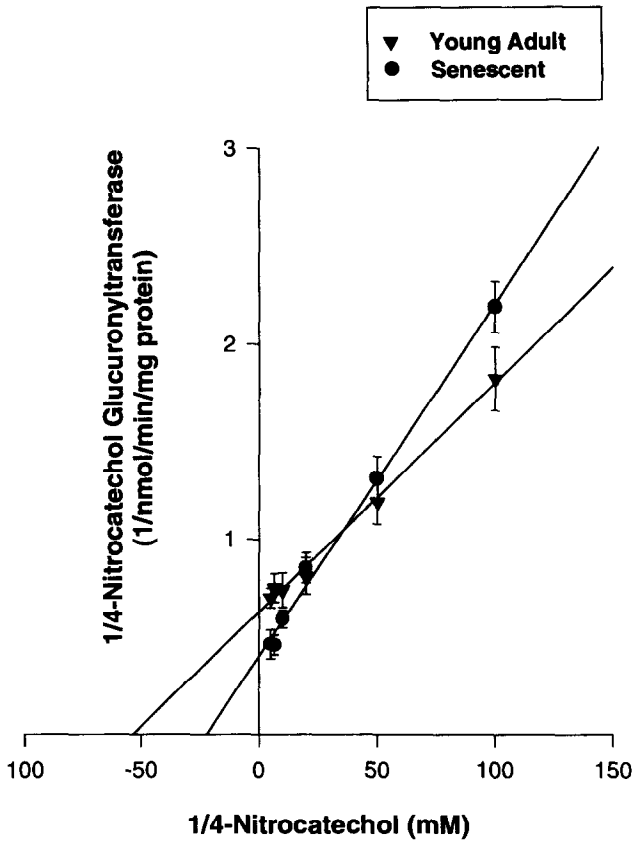


**FIG. 4. Lineweaver–Burk plots of substrate-velocity curves for 4-nitrocatechol glucuronyltransferase activity in hepatic microsomes from senescent and young adult rats. Microsomes were prepared from livers of senescent and young adult rats, and 4-nitrocatechol glucuronyltransferase activity was measured spectrophotometrically as described in Materials and Methods. Reciprocal values for substrates and velocities were then plotted, and values for $K_m$ and $V_{max}$ were calculated based on linear regressions of the data. Values are means ± SEM for 4 livers per group.**

separating pharmacokinetic differences, such as decreased hepatic blood flow [9], from changes in amounts or activities of cytochrome(s) P450 and the ability of the hepatocyte to generate reducing equivalents. The use of *in vitro* systems such as isolated hepatocytes or isolated perfused livers obviates many of these problems.

The findings that rates of *p*-nitrophenol hydroxylation by perfused livers were decreased about 33% in senescent compared with young adult rats (Fig. 1) is consistent with

**TABLE 2. Effect of age on $K_m$ and $V_{max}$ of 4-nitrocatechol glucuronyltransferase**

| Age | $K_m$ (mM) | $V_{max}$ (nmol/min/mg protein) |
|---|---|---|
| Young adult | 0.017 ± 0.008 | 1.60 ± 0.077 |
| Senescent | 0.045 ± 0.013* | 2.52 ± 0.067* |

Values for $K_m$ and $V_{max}$ were calculated based upon Lineweaver–Burk plots of substrate-velocity curves for individual liver preparations as described in Materials and Methods. Data are means ± SEM for 4 livers per group.

*$P < 0.05$ as compared with young adults.

**TABLE 3. Effects of age on glycogen, UDP-glucose and UDP-glucuronic acid contents in perfused rat livers**

| Age | Glycogen (mmol glucosyl units/kg) | UDP-glucose (μmol/kg) | UDP-glucuronic acid (μmol/kg) |
|---|---|---|---|
| Young adult | 19.6 ± 6.3 | 231.1 ± 19 | 203.7 ± 9.1 |
| Senescent | 9.2 ± 4.2* | 217.2 ± 14 | 189.4 ± 11.2 |

Livers from young adult or senescent rats were perfused with 60 μM *p*-nitrophenol after 20 min of perfusion with Krebs–Henseleit buffer. Livers were freeze-clamped after 15 min of perfusion, and glycogen, UDP-glucose and UDP-glucuronic acid were measured in extracts as described in Materials and Methods. Data are means ± SEM for 4 livers per group.

*$P < 0.05$ as compared with values for young adults.

decreases of 41% in rates of *p*-nitroanisole O-demethylation observed in hepatocytes from senescent compared with young adult male Fischer 344 rats [13]. The latter observation was attributed to decreases in microsomal cytochrome P450 levels, although rates of microsomal *p*-nitroanisole O-demethylation were not measured directly. In the present study, microsomal protein yield was decreased about 10% in livers from senescent compared with young adult rats (Results), consistent with other reports of age-related decreases in hepatic cytochrome P450 content in male rats [28]. Surprisingly, rates of *p*-nitrophenol hydroxylation were increased in microsomes from senescent compared with young adult rat livers (Fig. 2). These data suggest that the decreases in rates of *p*-nitrophenol hydroxylation by perfused rat livers were not due exclusively to decreased cytochrome P450 2E1 content or activity. In intact hepatocytes, mixed-function oxidation is limited not only by the activity of cytochromes P450 but also by the supply of reducing equivalents [29]. Thus, the data are consistent with the hypothesis that the supply of reducing equivalents is diminished in livers from senescent compared with young adult rats.

The reasons for decreases in reducing equivalent supply are not clear, but may be related to the supply of NADPH from mitochondria, which is a major source of NADPH for mixed-function oxidation in rat liver [30]. It is widely accepted that reducing equivalents are transferred from the mitochondria to the cytosol via substrate shuttles [18, 31]. At least two shuttle mechanisms are postulated to be involved in the generation of cytosolic NADPH. The first entails the use of isocitrate, α-ketoglutarate, and $NADP^+$-dependent isocitrate dehydrogenases located in both the cytosolic and mitochondrial compartments. The second involves movement of reducing equivalents from the mitochondria to the cytosol by the use of malate-pyruvate exchange. In brief, oxalacetate is formed from pyruvate by pyruvate carboxylase and is then reduced to malate. The malate then moves into the cytosol, and NADPH is subsequently formed from malate via malic enzyme. The decrease in malate content in livers from senescent compared with young adult rats (Table 1) suggests that shuttle intermediates may be reduced, thereby limiting the ability of the livers from senescent rats to generate NADPH through this mechanism. Thus, it is possible that a diminished supply of reducing equivalents is responsible for decreased rates of mixed-function oxidation in livers from senescent rats.

Glucuronidation of xenobiotics has been reported to be unaffected by aging both in microsomes (for example, with acetaminophen) or in intact hepatocytes (for *p*-nitrophenol) [13, 14]. Rates of conjugation of 60 μM *p*-nitrophenol in livers from senescent rats were similar to those in young adult rats (Fig. 3). However, it was of interest that 4-nitrocatechol produced by the hydroxylation of *p*-nitrophenol was not conjugated in livers from senescent rats (Fig. 1). The metabolism of *p*-nitrophenol would produce much lower concentrations of 4-nitrocatechol (about 2.5 μM) at the rates of hydroxylation observed (Fig. 1) than concentrations of *p*-nitrophenol used to determine conjugation (60 μM). These data suggested that the kinetics of the phenol UDP-glucuronyltransferase may be altered by senescence. In fact, the $K_m$ of microsomal UDP-glucuronyltransferase was increased about 3-fold and the $V_{max}$ was increased about 2-fold in livers from senescent compared with young adult rats (Table 2), which is consistent with the hypothesis that changes in the kinetics of the glucuronyltransferase accounted for the lack of glucurondation of 4-nitrocatechol produced during mixed-function oxidation of *p*-nitrophenol.

The supply of UDPGA is a major rate-determinant for glucuronidation in perfused liver [17]. In general, changes in rates of glucuronidation correlate with changes in hepatic contents of glycogen, UDP-glucose, and UDPGA [32]. In the present study, however, although glycogen content was decreased by approximately 53% in livers from senescent compared with young adult rats (Table 3), contents of UDP-glucose and UDPGA were not decreased significantly (Table 3). These data suggest that the rates of glycogenolysis in livers from senescent rats were sufficient to provide glucose for formation of intermediates necessary for glucuronidation. Thus, alterations in UDPGA supply are most likely not involved in decreased rates of glucuronidation of 4-nitrocatechol formed during the mixed-function oxidation of *p*-nitrophenol.

Taken together, the data indicate that phase I and phase II reactions are altered in livers from senescent compared with young adult male Sprague–Dawley rats, due possibly to diminished supplies of cofactors for mixed-function oxidation and changes in glucuronyltransferase kinetics.

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