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EFFECT OF THE BRACHYMORPHIC TRAIT IN MICE ON XENOBIOTIC SULFATE ESTER FORMATION*

STEWART D. LYMAN† and ALAN POLAND‡
The McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI 53706, U.S.A.

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Abstract—Mice carrying the recessive mutation brachymorphic have been shown previously to have a reduced capacity to synthesize 3′-phosphoadenosine-5′-phosphosulfate (PAPS), the required coenzyme in sulfation reactions [K. Sugahara and N. Schwartz, *Proc. natn. Acad. Sci. U.S.A.* **76**, 6615 (1979)]. The capacity of the liver cytosol fractions from brachymorphic (bm/bm) mice or their phenotypically normal littermates (+/+ or +/bm) to catalyze the formation of sulfate esters of [^{3}H]estrone and [^{14}C]*p*-nitrophenol *in vitro* was determined. When PAPS was added to the reaction, the rates of sulfate ester formation catalyzed by the two cytosol fractions were similar. In contrast, when PAPS was generated *in situ* from ATP and SO_4^{2-}, the rates of sulfate ester formation catalyzed by the brachymorphic cytosol were only 4–22% of the rates catalyzed by the cytosol fraction from normal mice. The hepatic cytosol fraction from brachymorphic mice incorporated less $^{35}SO_4^{2-}$ into PAPS than that catalyzed by cytosol of normal mice. [^{14}C]*p*-Nitrophenol (1.5 μmoles/kg) was eliminated from brachymorphic and normal mice as urinary conjugates; in normal mice, 73% of the urinary radioactivity was *p*-nitrophenyl sulfate, while in the brachymorphic mice only 33% of the urinary excretion was the sulfate ester. Brachymorphic mice have a reduced capacity for synthesizing sulfate esters of xenobiotics *in vitro* and *in vivo*, which is attributable to their reduced synthesis of PAPS.

Sulfation is one of the two major competing pathways for the conjugation of many foreign chemicals, the other being glucuronidation [1]. The addition of the negatively charged sulfate group increases the polarity and water solubility of the chemical, usually reduces its biological activity, and hastens its excretion from the body [2]. Many endogenous compounds are also conjugated with sulfate; examples include bile salts [3], steroids [4], catecholamines [5], peptide hormones [6], glycosaminoglycans [7], and proteins [8].

The sulfate group in all sulfation reactions is donated by the coenzyme 3′-phosphoadenosine 5′-phosphosulfate (PAPS)§ [7]. The biosynthesis of PAPS occurs in two steps: ATP and inorganic sulfate first combine to form APS (5′-adenosine phosphosulfate), and APS is then phosphorylated at the 3′-position to yield PAPS [7]. Transfer of the sulfate group from PAPS to an acceptor group is catalyzed by the sulfotransferase enzymes, a group of enzymes with distinct (but sometimes overlapping) substrate specificities [3]. The sulfotransferases that catalyze the sulfation of foreign chemicals and steroids are found in highest concentration in the liver [9].

Analysis of the sulfation pathway has been facilitated by the use of a genetic mutant, the brachymorphic mouse. Mice that carry this trait have a distinctive short stature [10], caused by the undersulfation of their cartilage glycosaminoglycans [11]. Sugahara and Schwartz [12–14] have shown that cartilage (and liver) homogenates prepared from brachymorphic mice have a reduced capacity to synthesize PAPS *in vitro* compared with homogenates prepared from normal mice. In this report we demonstrate that the reduction in PAPS formation in brachymorphic mice results in a decreased capacity to form sulfate esters of xenobiotics *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

Sodium [^{35}S]sulfate (99% radiochemically pure, sp. act. 941 mCi/mmole) and [6,7-^{3}H]estrone (98% radiochemically pure, sp. act. 44 Ci/mmole) were purchased from the New England Nuclear Corp. Boston, MA. Uniformly labeled [^{14}C]*p*-nitrophenol (98% radiochemically pure, sp. act. 24 mCi/mmole) was purchased from the California Bionuclear Corp., Sun Valley, CA. Estrone, estrone-3-sulfate, estrone-β-D-glucuronide, *p*-NPS, *p*-NPGA, APS, ATP, β-glucuronidase type IX, and sulfatase type H-1 were brought from the Sigma Chemical Co., St. Louis, MO, and *p*-NP was purchased from the Aldrich Chemical Co., Milwaukee, WI. PEI cellulose TLC sheets were obtained from Brinkmann Instru-

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‡ Burroughs Wellcome Scholar in Toxicolgy. Author to whom all correspondence should be addressed.
§ Abbreviations: PAPS, adenosine 3′-phosphate-5′-phosphosulfate; PEI, polyethyleneimine; *p*-NP, *p*-nitrophenol; *p*-NPGA, *p*-nitrophenyl glucuronide; *p*-NPS, *p*-nitrophenyl sulfate; ES, estrone-3-sulfate; APS, adenosine 5′-phosphosulfate; MOPS, 3-[*N*-morpholino]propanesulfonic acid.

ments, Inc., Westbury, NY, and silica gel TLC plates (Whatman LK5DF) were purchased from the Anspec Co., Inc., Ann Arbor, MI. QAE Sephadex A-25 was purchased from Pharmacia Fine Chemicals, Piscataway, NJ. PAPS was purchased from P-L Biochemicals, Inc., Milwaukee, WI, or was prepared by the method of Irving *et al.* [15].

Animals

C57BL/6J, C3H/HeJ, and brachymorphic mice (bm/bm) were obtained from the Jackson Laboratory, Bar Harbor, ME, and subsequently bred in our laboratory. The brachymorphic trait is recessive and determined by the *bm* locus on chromosome 19 [10]. Mice that are homozygous for the *bm* allele at this locus are characterized by their disproportionately short stature, dome-shaped skulls, and short, thick tails. The brachymorphic trait is maintained by crossing brachymorphic mice with (C57BL/6J × C3H/HeJ)F_1 mice (B6C3F_1) and then intercrossing the offspring to produce two phenotypic classes: brachymorphic mice (bm/bm) and normal-appearing mice (+/+ or +/bm, denoted here as +/? mice). These phenotypically normal mice and B6C3F_1 mice were used as controls for brachymorphic mice in our experiments.

Tissue preparation

Mice were killed by cervical dislocation. Their livers were removed, weighed, and homogenized in 3 vol. of 0.15 M KCl. The homogenate was then centrifuged at 10,000 *g* for 20 min at 4°, and the supernatant fraction was then centrifuged for 1 hr at 100,000 *g* at 4°. The supernatant layer (cytosol fraction) was used immediately or stored at −70°; no loss of enzyme activity was observed after storage.

Measurement of the rates of estrone-3-sulfate and p-nitrophenyl sulfate formation

The *in vitro* rate of sulfate ester formation of [^{3}H]estrone and [^{14}C]*p*-nitrophenol catalyzed by hepatic cytosol was determined under two conditions: (1) the addition of PAPS, which provides a direct measure of sulfotransferase activity, and (2) the addition of SO_4^{2-} and ATP, where the synthesis of sulfate conjugates reflects the synthesis of APS and PAPS, and the sulfotransferase activity. Hence, these direct and indirect measurements of sulfate ester formation have different optimal conditions, most notably in the pH optima, and linearity with time. The incubation mixture had a total volume of 100 μl, consisting of 25 μl of the appropriate buffer, 45 μl of liver cytosol, 5 μl containing the radiolabeled substrate, and 25 μl of a cofactor solution consisting of 0.4 μmole $MgCl_2$, 0.24 μmole 2-mercaptoethanol, and either 0.05 μmole PAPS or 2.4 μmoles K_2SO_4 and 0.8 μmole ATP. Following incubation at 37° in a shaker water bath for the appropriate time, the reaction was ended by the addition of an organic solvent, which extracted most of the unmetabolized substrate. The radiolabeled sulfate ester in the aqueous phase was separated from remaining substrate by TLC (Whatman LK5DF TLC plates) and quantified by liquid scintillation spectrometry. The blank (zero-time control) was determined by adding the denaturing organic solvent to the reaction mixture prior to the cytosol fraction, and incubated samples were corrected by the amount of radioactivity in the zero-time blank.

Estrone-3-sulfate sulfotransferase activity. Measurement of this activity was based on the method of Singer *et al.* [16] using the following conditions: (1) 25 μl buffer containing 10 μmoles KPO_4, pH 7.5 (37°), (2) the cytosol was diluted 1:1 with 0.15 M KCl, (3) 0.05 μmole PAPS, (4) 5 μl ethanol containing 3.2 nmoles [^{3}H]estrone (3×10^6 dpm/assay), and (5) the incubation time was 5 min.

Estrone-3-sulfate formation. ES formation from ATP and SO_4^{2-} was determined in a similar manner, with the following modifications: (1) 25 μl buffer containing 10 μmoles glycine, pH 9.0 (37°), (2) the hepatic cytosol was not diluted, (3) the cofactor mixture contained K_2SO_4 and ATP instead of PAPS, and (4) the incubation time was 30 min.

The reactions were stopped by the addition of 3 ml of toluene, the mixture was shaken, and the phases were separated by centrifugation. The organic layer was discarded and the extraction with toluene repeated. A single toluene extraction removed 98% of [^{3}H]estrone and only 1% of [^{3}H]estrone-3-sulfate. Fifty microliters of the aqueous phase was added to an equal volume of ice-cold acetone and, following mixing, the precipitate was removed by centrifugation. The supernatant fraction (25 μl) and unlabeled ES (5 μg) were applied to a TLC plate, and the chromatogram was developed in chloroform–ethyl acetate–ethanol (5:5:1). The ES band was identified by a vanillin reagent [17] and removed, and the radioactivity was quantified. ES had an R_f of 0.05 and estrone 0.59.

p-*Nitrophenol sulfotransferase activity.* Measurement of this activity was determined in the standard 100 μl assay using the following conditions: (1) 25 μl buffer containing 10 μmoles MOPS, pH 6.5 (37°), (2) the hepatic cytosol was diluted 1:1 with 0.15 M KCl, (3) the cofactor mixture contained 0.05 μmole PAPS, (4) 5 μl H_2O containing 54.5 nmoles [^{14}C]*p*-nitrophenol (2.5×10^5 dpm/assay), and (5) the reaction was incubated for 5 min.

p-*Nitrophenyl sulfate formation. p*-NPS formation from ATP and SO_4^{2-} was assayed in a similar manner except (1) the buffer solution contained 10 μmoles MOPS, pH 7.5 (37°), (2) the cytosol was undiluted, (3) the cofactor solution contained K_2SO_4 and ATP in place of PAPS, and (4) the incubation time was 10 min.

The incubation was stopped by the addition of 3 ml of water-saturated diethyl ether, containing 5 μl of glacial acetic acid and, after mixing, the phases were separated by centrifugation. The ether layer extracted 99% of the *p*-NP and only 3% of the sulfate conjugate. Fifty microliters of the aqueous phase was added to 100 μl of methanol, and the precipitate was removed by centrifugation. Twenty-five microliters of the supernatant fraction, along with 10 μg of unlabeled *p*-NP and *p*-NPS, were applied to a TLC plate and developed in chloroform–methanol (4:1). It was necessary to include these unlabeled carrier compounds to avoid artifacts in the migration of [^{14}C]*p*-NPS. The *p*-NPS band was identified by u.v. light, removed, and its radioactivity quantified. The R_f of *p*-NPS was 0.19 and the R_f of *p*-NP was 0.65.

Incorporation of $^{35}SO_4^{2-}$ into APS, PAPS, and estrone-3-sulfate in vitro

The assay was based on the method of Sugahara and Schwartz [12]. The 100-μl reaction mixture comprised 3.2 nmoles estrone (added in 5 μl ethanol); 1.8 nmoles carrier-free $Na_2{}^{35}SO_4$ (3.77×10^6 dpm/assay) in 5 μl H_2O; 25 μl of 0.4 M glycine buffer (pH 9.0) (37°) containing 800 nmoles ATP, 400 nmoles $MgCl_2$, and 240 nmoles 2-mercaptoethanol; and 65 μl of liver cytosol. The reaction was started by addition of the cytosol and incubated at 37°. At appropriate time points, 25-μl aliquots of the reaction mixture were removed and transferred to test tubes containing 25 μl of ice-cold acetone. The contents of the tubes were mixed, and the precipitate was removed by centrifugation. Five microliters of the supernatant fraction was spotted on a PEI cellulose TLC sheet and chromatographed together with authentic standards in 2 M LiCl (containing 10 mM Na_2EDTA). The R_f values were: estrone-3-sulphate, 0.08; PAPS, 0.44; and APS, 0.62. Sections of the TLC sheet containing APS, PAPS, and estrone-3-sulfate were cut out, and their radioactivity was quantitated by liquid scintillation spectrometry. Recovery of radioactivity from the PEI cellulose was about 98%. Control assays (blanks) were carried out as described above except that the cytosol was incubated separately from the other components and then added in the presence of the acetone.

Collection and analysis of urinary metabolites of [^{14}C]p-nitrophenol

Mice were injected intraperitoneally with [^{14}C]*p*-nitrophenol (dissolved in saline) and then placed in rodent metabolism cages (three mice/cage), and their urines were collected for 48 hr over dry ice. The cage surfaces were then rinsed with distilled water, and this was added to the urine. Aliquots (1 ml) of the diluted urine were lyophilized, and the lyophilizate was resuspended in 1 ml of methanol, sonicated for 5 min, and centrifuged. The clarified supernatant fraction was transferred to a second test tube containing 200 μg each of unlabeled *p*-NP, *p*-NPGA, and *p*-NPS. The methanol was removed under a nitrogen stream, and 3 ml of 10 mM pyridine–HCl buffer, pH 5.4, was added. An aliquot of this solution was placed on a QAE Sephadex A-25 column (2.5×1.4 cm) that had been equilibrated with the pyridine buffer. The column was then washed with this buffer until [^{14}C]*p*-NP eluted. The [^{14}C]*p*-NPGA and [^{14}C]*p*-NPS were then sequentially eluted with pyridine buffer containing either 30 mM NaCl (*p*-NPGA) or 100 mM NaCl (*p*-NPS). Radioactivity was quantitated by liquid scintillation spectrometry. Recovery of radioactivity from the column was 93–100%, with glucuronide and sulfate conjugates accounting for 93–98% of the total radioactivity. The eluted compounds were identified by u.v. spectra analysis, thin-layer chromatography, and β-glucuronidase and sulfatase treatment.

Protein measurements

Proteins were measured by the method of Lowry *et al.* [18], with bovine serum albumin as a standard.

Statistical analysis

Student's *t*-test was used, and the level of significance was set at $P < 0.05$.

RESULTS

Sulfate ester formation in vitro

The primary biochemical defect in brachymorphic mice is a greatly reduced capacity to synthesize PAPS [12], which causes the undersulfation of cartilage glycosaminoglycans [11] and abnormal bone formation [19]. The diminished availability of PAPS in these mice might also affect their capcity to form sulfate conjugates of steroids and xenobiotic compounds. We measured the capacity of the liver cytosol fractions prepared from brachymorphic mice

Table 1. Sulfate ester formation catalyzed by liver cytosol fraction*

			Estrone-3-sulfate formation (pmoles/mg protein/min)		*p*-Nitrophenyl sulfate formation (nmoles/mg protein/min)	
Mouse	Sex	N	ATP and SO_4^{2-} addition (pH 9.0)	PAPS addition (pH 7.5)	ATP and SO_4^{2-} addition (pH 7.5)	PAPS addition (pH 6.5)
bm/bm	Female	5	0.54 ± 0.14†,‡	9.11 ± 1.40†	0.031 ± 0.004†,‡	1.98 ± 0.26
+/?	Female	5	3.37 ± 1.43	14.70 ± 1.09‡	0.518 ± 0.060	1.82 ± 0.15
B6C3F$_1$	Female	5	2.46 ± 0.35	9.85 ± 1.63	0.523 ± 0.097	2.09 ± 0.21
bm/bm	Male	4	0.30 ± 0.13†,‡	4.74 ± 0.61†,‡	0.017 ± 0.005†,‡	1.12 ± 0.13†,‡
+/?	Male	5	1.54 ± 0.36	7.20 ± 0.95	0.310 ± 0.070‡	1.49 ± 0.17
B6C3F$_1$	Male	5	1.60 ± 0.18	7.85 ± 1.79	0.411 ± 0.036	1.44 ± 0.11

* Assays were performed as described in Materials and Methods on 10-month-old mice. The assays to determine estrone-3-sulfate formation were done on freshly prepared liver cytosol, and the assays of *p*-nitrophenyl sulfate formation were performed on the same liver cytosol samples after storage at −70° for 3 weeks. N is the number of animals in each group. Statistical comparisons are made within each column between animals of the same sex. It is not valid to compare the values obtained with the ATP and SP_4^{2-} addition assay with those from the PAPS addition assay because these assays were done at different pHs. Each value is the mean ± S.D.

† Significantly different from +/? at $P < 0.05$.

‡ Significantly different from B6C3F$_1$ at $P < 0.05$.

(bm/bm), their phenotypically normal littermates (+/?), and B6C3F$_1$ mice to catalyze the formation of sulfate esters of two model substrates, estrone and *p*-nitrophenol. PAPS, the sulfate-donating coenzyme in the reaction, either was added directly into the reaction or was generated *in situ* from ATP and SO_4^{2-}. When PAPS was added directly to the reaction, the rates of estrone-3-sulfate and *p*-nitrophenyl sulfate formation were similar, whether catalyzed by the cytosol fraction from brachymorphic mice, their phenotypically normal littermates, or B6C3F$_1$ mice (Table 1). In contrast, when ATP and SO_4^{2-} were added in place of PAPS, the rates of formation of estrone-3-sulfate and *p*-nitrophenyl sulfate catalyzed by the brachymorphic cytosol fraction were only 4–22% of the rates catalyzed by the cytosol fractions of the two control groups (Table 1). Therefore, the reduced rates of sulfate ester formation catalyzed by the bm/bm cytosol fraction appear to be due to a reduced capacity to generate PAPS and not to a decrease in sulfotransferase activity.

Incorporation of radioactive sulfate in vitro

We incubated the liver cytosol fraction obtained from brachymorphic mice or their normal littermates with $^{35}SO_4^{2-}$, ATP, and estrone, and compared the capacity of these fractions to catalyze the incorporation of radiolabel into APS, PAPS, and estrone-3-sulfate. Since the successive transfer of the radiolabeled sulfate group from APS to PAPS to estrone-3-sulfate cannot be analyzed kinetically without separating the enzyme activities that catalyze these reactions, we compared the quantity of each compound present at the indicated time points. Throughout the incubation period, the concentration of radiolabeled APS was greater in bm/bm cytosol compared with +/? cytosol (Fig. 1). In contrast, the amounts of radiolabeled PAPS and estrone-3-sulfate measured were lower in the bm/bm cytosol than in the +/? cytosol. Sugahara and Schwartz [12] obtained similar results with cartilage homogenates. Our data therefore support their suggestion that the primary enzymatic defect in bm/bm mice is in the conversion of APS to PAPS. Incubation of equal amounts of bm/bm and +/? liver cytosol fractions with $^{35}SO_4^{2-}$, ATP, and estrone produced a pattern of radiolabel incorporation similar to that seen with the +/? cytosol fraction alone, indicating that there was no diffusible inhibitor in the bm/bm cytosol fraction (data not shown).

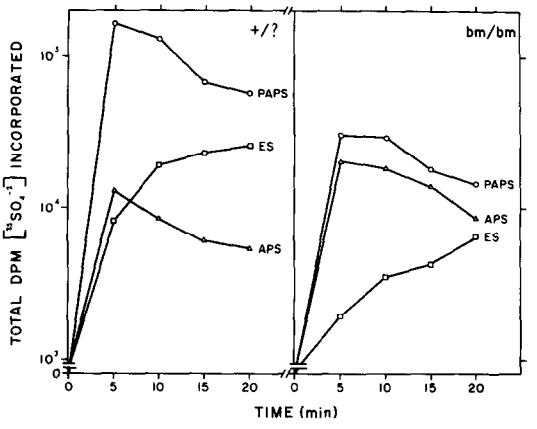

Fig. 1. Incorporation of $^{35}SO_4^{2-}$ into APS, PAPS, and estrone-3-sulfate. The capacity of the liver cytosolic fractions from brachymorphic mice (bm/bm) or their phenotypically normal littermates (+/?) to catalyze the incorporation of $^{35}SO_4^{2-}$ was measured as described in Materials and Methods. The cytosolic fractions were prepared from the pooled livers (5) from 14-week-old female mice; the protein content of these fractions was identical (19.2 mg/ml).

Conjugation of [^{14}C]p-*nitrophenol* in vivo

The decreased capacity of brachymorphic mice to synthesis PAPS, which impairs sulfate ester formation *in vitro* (Table 1), should also reduce their capacity to form sulfate conjugates *in vivo*. Brachymorphic mice and their phenotypically normal littermates were given intraperitoneal injections of [^{14}C]*p*-nitrophenol, and their urines were then collected for 48 hr. The urine contained 69–89% of the administered radioactivity, almost all of which was in the form of glucuronide and sulfate conjugates of *p*-nitrophenol (Table 2). Given a low dose of *p*-nitrophenol (1.47 μmoles/kg), *p*-nitrophenyl sulfate accounted for 73% of the urinary radioactivity excreted by the +/? mice, compared with 33% *p*-nitrophenyl sulfate excreted by bm/bm mice. The sulfate conjugation pathway is thought to be a high affinity, low capacity system, whereas glucuronide

Table 2. Urinary excretion of [^{14}C]*p*-nitrophenol conjugates*

Mouse	Dose of [^{14}C] *p*-nitrophenol (μmoles/kg)	Percent of injected radioactivity recovered in urine	Percent of urinary radioactivity†		
			p-Nitrophenol	*p*-Nitrophenyl glucuronide	*p*-Nitrophenyl sulfate
bm/bm	1.47	69	0.2	59.7	32.9
+/?		86	0.2	25.5	73.1
bm/bm	73.3	89	0.9	74.9	19.9
+/?		88	1.0	60.1	36.3

* Fourteen-week-old female mice (three mice/cage) were given an intraperitoneal injection of the indicated dose of [^{14}C]*p*-nitrophenol (approximately 7.74 × 10^7 dpm/kg), and their pooled urines were collected and analyzed as described in Materials and Methods.

† The numbers shown are corrected for the recovery (93–100%) of applied radioactivity from the QAE Sephadex columns. A small amount (1.2–7.2%) of the applied radioactivity eluted in the void volume of the column; no attempt was made to characterize this radioactivity.

formation is considered a lower affinity, higher capacity pathway [20]. Recent work [21] indicates that the shift from sulfation to glucuronidation with increasing xenobiotic load is due to saturation of the sulfotransferase enzymes. Following the administration of a 50-fold higher dose of *p*-nitrophenol, glucuronidation was the major conjugation route in both types of mice, but the +/? mice excreted nearly twice as much of the sulfate conjugate (36%) as did the bm/bm mice (20%).

DISCUSSION

In this report we have shown that brachymorphic mice have a reduced capacity to form sulfate esters of xenobiotics both *in vitro* and *in vivo* compared to phenotypically normal mice. The hepatic cytosolic fractions from bm/bm, +/?, and B6C3F$_1$ mice catalyzed similar rates of sulfate ester formation when PAPS was supplied as a coenzyme in the reaction. However, when PAPS was replaced by ATP and SO_4^{2-}, the soluble liver fraction from bm/bm mice formed less of the xenobiotic sulfate conjugates than the cytosol fractions from the phenotypically normal mice. In addition, bm/bm cytosol formed a higher concentration of APS and a lower concentration of PAPS compared with cytosol from +/? mice. The rates of PAPS degradation in liver homogenates from both brachymorphic and normal mice have been reported to be almost identical [13]. In summary, these results indicate that brachymorphic mice (a) possess a defect in the conversion of APS to PAPS, resulting in a deficiency of PAPS, and (b) when provided with PAPS, have sulfotransferase activity similar to those of normal mice.

Sugahara and Schwartz [12–14] first demonstrated that the primary biochemical defect in brachymorphic mice is a reduction in the enzyme activity (ATP:adenylylsulfate 3′-phosphotransferase, EC 2.7.1.25, also known as APS-kinase) that converts APS to PAPS. Our observations confirm their findings and demonstrate the effect of this trait on the metabolism of xenobiotics.

The brachymorphic mouse may be used to study the effects of sulfate conjugation of the biological activity of both foreign chemicals and endogenous compounds. For example, the biologically active form of the hypotensive drug minoxidil (2,4-diamino-6-piperidinopyrimidine 3-oxide) is thought to be its sulfate ester metabolite [22]. A reduced hypotensive response to minoxidil in brachymorphic mice compared with normal mice would support this hypothesis.

While several peptides and proteins, e.g. gastrin [6,23], leu-enkephalin [24] and fibrinogen [25], have been isolated with sulfate esters of their tyrosine residues, the biological significance of this modification is unknown. Huttner [8] suggested that tyrosine residues that are normally sulfated *in vivo* may be modified by phosphorylation, and that these improper modifications may alter cellular regulation. Brachymorphic mice might be used to explore the biological significance of peptide sulfation.

Labile sulfate esters are though to be the ultimate carcinogenic forms of several chemical carcinogens (e.g. *N*-hydroxy-2-acetylaminofluorene [26] and 1′-hydroxyalkenylbenzenes [27–29]) in rodent liver. However, *N*-hydroxy-2-acetylaminofluorene also produces tumours in other tissues of the rat, and in other species, which lack appreciable sulfotransferase activity; the principal route of metabolic activation in these tissues is thought to be via N,O-transacetylation [30]. Comparison of covalent binding and tumorigenicity in brachymorphic and normal mice provides a useful test for carcinogenic chemicals that are suspected of producing reactive electrophilic species that are labile sulfate esters. In support of this idea, we have found that the covalent binding of [^{3}H]*N*-hydroxy-2-acetylaminofluorene, [^{3}H]1′-hydroxysafrole, and [^{3}H]1′-hydroxyestragole *in vivo* to liver DNA is lower in brachymorphic mice than in their phenotypically normal littermates (E. C. Miller, J. A. Miller and A. Poland, unpublished data).

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