but considerably lower than those of the primary catabolic enzyme cytidine deaminase in the leukemic cell. High levels of 5-azacytidine would have to be achieved in order to approach the K_m of U–C kinase, and in view of its rapid metabolism by cytidine deaminase [10] and its chemical lability, a high dose infusion schedule would seem justified. However, recent data [11] indicate that high dose therapy with 5-azacytidine may be associated with neurotoxicity as well as the extreme nausea and vomiting often seen at lower doses of this agent.

The affinity of U–C kinase from human leukemic cells for 5-azacytidine appears to be substantially lower than that previously reported by Liacouras and Anderson [2] for the murine mast cell tumor enzyme and by Lee *et al.* (K_m = 200 μM) for the calf thymus enzyme. The Michaelis constants for cytidine and uridine using all three sources of enzyme, mast cell tumor, calf thymus and CEM cell line, were quite similar, in the 10^{-4} to 10^{-5} M range. Lee *et al.* [5], in their studies of the calf thymus U–C kinase, estimated the relative maximum velocities for 5-azacytidine, cytidine, and uridine were approximately 0.25:0.75:1.0, respectively, values which are similar to those of the CEM enzyme except for reversal of the relative velocities of cytidine and uridine. Further studies will be required to establish whether the enzyme from human tissues has other properties differing from those of the murine and calf thymus enzymes.

It is noteworthy that despite the poor affinity of substrate for this enzyme in the human leukemic cells, the CEM cell line remained responsive to 5-azacytidine *in vitro*.

U–C kinase levels have been measured in extracts of myeloblasts obtained from previously untreated patients with acute non-lymphocytic leukemia [12], and averaged 16.4 ± 19.4 units/mg of protein (median value of 11.1 units/mg of protein), with variation over a three log range (0–75 units/mg of protein). The levels of U–C kinase in myeloblasts were considerably lower than the levels of the degradative enzyme, cytidine deaminase, in the human myeloblastic cells (mean concentration, 377 ± 530 units/mg of protein).

The poor affinity of U–C kinase for 5-azacytidine as a substrate and the low activity of the enzyme in leukemic cells in comparison to cytidine deaminase raise the possibility that metabolism of 5-azacytidine to the nucleotide form and subsequent incorporation into RNA [13] may not be the only mechanism responsible for its cytotoxicity. Other possible routes of metabolism, such as deamination to 5-azauridine [10] or ring cleavage, might be responsible for formation of an active antimetabolite. Further studies of the metabolism of 5-azacytidine in intact cells and in the whole animal are needed to determine the relative importance of nucleotide formation as compared to alternate transformations of this agent under physiologic conditions.

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Effects of luteinizing hormone and follicle stimulating hormone on hepatic drug metabolism in gonadectomized male and female rats

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Many hormones participate in the regulation of drug and steroid metabolism by rat liver microsomes [1–3]. The actions of gonadal hormones have been particularly well investigated [4–10]. Androgens increase the oxidative metabolism of various substrates including ethylmorphine, hexobarbital and testosterone [4–7]. As a result, hepatic oxidation of many substances proceeds far more rapidly in male than female rats. In contrast, reductive steroid metabolism (Δ^4-hydrogenase activity) is inhibited by testosterone and enhanced by estradiol, producing a sex difference in Δ^4-hydrogenase activity opposite that in oxidative metabolism [8–10].

Until recently, gonadal hormones were thought to act directly and independently on the liver to alter the activities of drug- and steroid-metabolizing enzymes. However, Colby *et al.* [11] demonstrated that the actions of both testosterone and estradiol on hepatic corticosteroid metabolism in rats and hamsters were not demonstrable in hypophysectomized animals, indicating a dependence on the pituitary gland. Subsequently, other investigators

reported a similar pituitary requirement for the development and maintenance of sex-dependent patterns in steroid metabolism [12–14]. In addition, we have recently noted that androgenic effects on hepatic ethylmorphine demethylation are also manifested only in the presence of the pituitary gland [15]. These observations suggest that pituitary factors have an important role in the regulation of hepatic drug and steroid metabolism. However, relatively little attention has been given to possible direct effects of pituitary hormones on hepatic enzymes. Only the actions of growth hormone on drug metabolism have been studied in detail [16–18]. The effects of follicle stimulating hormone (FSH) and luteinizing hormone (LH) are of particular interest in relation to gonadal hormone actions on hepatic mixed function oxidases, since the secretion of both is acutely influenced by testosterone and estradiol. The studies presented in this report indicate that both FSH and LH affect drug metabolism in gonadectomized rats and that the effects are substrate dependent.

Male and female Sprague–Dawley rats (obtained from Zivic–Miller Laboratories, Pittsburgh, Pa.) were maintained under standardized conditions of light (0600–1800) and temperature (22°) on a diet of Purina Laboratory Chow and water *ad lib.* Orchiectomies and ovariectomies were performed on rats approximately 60 days of age and hormonal treatment was initiated approximately 48 hr later. Follicle stimulating hormone (FSH) (NIH-FSH-B1, 0.49 units/mg) and luteinizing hormone (LH) (NIH-LH-B9, 0.70 units/mg) were administered by subcutaneous injection in 0.9% saline at a dose of 125 μg/rat twice daily. All rats were sacrificed after 7 days of hormonal treatment.

Animals were decapitated between 9:00 and 10:00 a.m. and livers quickly removed and homogenized in cold 1.15% potassium chloride. Homogenates were centrifuged at 9000 *g* for 20 min in a Sorvall refrigerated centrifuge. Aliquots of the supernatant were removed for enzyme assays and the remainder was centrifuged at 105,000 *g* for 60 min in a Beckman preparative ultracentrifuge. All steps in the preparation of microsomes were performed with the tissue kept at 0–4°. Microsomal pellets were resuspended immediately prior to use in 1.15% potassium chloride containing 0.05 M Tris–HCl (pH 7.4) at a concentration of 3–4 mg protein/ml. Microsomal cytochrome P-450 was measured as described by Omura and Sato [19] using a millimolar extinction coefficient of 91. NADPH-cytochrome *c* reductase activity was assayed by the method of Phillips and Langdon [20] using a millimolar extinction coefficient of 18.7. Microsomal protein was determined by the method of Lowry *et al.* [21]. All assays (except protein determinations) were performed on the same day the animals were sacrificed.

The demethylation of ethylmorphine or aminopyrine and the hydroxylation of aniline were assayed as the rates of formation of formaldehyde [22] or paraminophenol [23], respectively, by 0.5 ml of the liver 9000 *g* supernatant (200 mg/ml) incubated with glucose-6-phosphate (9.0 μmoles), $MgSO_4$ (24.2 μmoles), Tris–HCl (0.02 M, pH 7.4) and ethylmorphine–HCl (12 μmoles), aminopyrine (12 μmoles) or aniline-HCl (6 μmoles) in a final volume of 3.0 ml. Semicarbazide–HCl (25 μmoles) served as a trapping agent for formaldehyde produced from ethylmorphine and aminopyrine. Incubations were carried out in a Dubnoff metabolic incubator at 37° for 15 min in air. All samples were read against appropriate tissue blanks and standards. Enzyme activities are expressed as nmoles product formed/min/g of liver. However, since none of the treatments affected hepatic microsomal protein concentration, the effects reported are equally valid when expressed as nmoles product/mg of microsomal protein. The data presented in Tables 1 and 2 each represent the pooled observations from two different experiments.

Administration of either LH or FSH to orchiectomized male rats had no effects on body or liver weights (Table 1). Similarly, neither microsomal protein nor cytochrome P-450 content was significantly affected by either hormone. Nonetheless, both LH and FSH, when given to castrated male rats, increased the rate of ethylmorphine demethylation (Table 1). The rates of aniline hydroxylation and aminopyrine demethylation were not affected by administration of either hormone.

Treatment of castrated female rats with FSH or LH did not affect body weight, liver weight, microsomal protein concentration, cytochrome P-450 content or NADPH-cytochrome *c* reductase activity (Table 2). Aniline hydroxylation was also unaffected by either hormone. In contrast, FSH treatment increased the rates of metabolism of both ethylmorphine and aminopyrine. LH increased ethylmorphine demethylase activity in females but did not affect aminopyrine metabolism. Direct addition of FSH or LH to hepatic tissue *in vitro*, in concentrations as high as 1.5 μg/ml, had no effect on the metabolism of aniline, ethylmorphine or aminopyrine.

These observations indicate that LH and FSH have effects on hepatic microsomal mixed function oxidases independent of the gonadal hormones. The actions of FSH and LH are probably not mediated by adrenal androgens, since recent studies indicate that neither hormone affects adrenal testosterone production in rats [24]. The effects of both hormones clearly depend upon the substrate employed and the sex of the experimental animal. Aniline hydroxylation was not affected by either hormone in males

Table 1. Effects of FSH and LH administration on hepatic oxidative metabolism in gonadectomized male rats*

	Saline	LH	FSH
Body weight (g)	258.3 ± 6.0	258.8 ± 4.5	257.7 ± 5.9
Liver weight (g)	10.9 ± 0.4	10.6 ± 0.3	10.1 ± 0.4
Microsomal protein (mg/g liver)	34.0 ± 0.9	32.3 ± 0.7	32.6 ± 0.8
Cytochrome P-450 (nmoles/mg protein)	0.64 ± 0.02	0.69 ± 0.02	0.69 ± 0.02
Aniline metabolism (nmoles/min/g liver)	41.2 ± 1.9	42.4 ± 1.3	42.5 ± 1.5
Ethylmorphine metabolism (nmoles/min/g liver)	410.4 ± 40.0	545.1 ± 48.7†	590.4 ± 24.5†
Aminopyrine metabolism (nmoles/min/g liver)	374.9 ± 16.5	375.7 ± 16.7	382.7 ± 17.8

* Values are expressed as mean ± S.E.; there were 16 animals/group.
† $P < 0.05$ (vs saline-treated group).

Table 2. Effects of FSH and LH administration on hepatic oxidative metabolism in gonadectomized female rats*

	Saline	LH	FSH
Body weight (g)	233.2 ± 4.4	233.6 ± 5.2	225.5 ± 4.4
Liver weight (g)	9.0 ± 0.3	9.4 ± 0.3	8.8 ± 0.2
Microsomal protein (mg/g liver)	39.3 ± 1.8	38.4 ± 2.1	41.6 ± 2.1
Cytochrome P-450 (nmoles/mg protein)	0.36 ± 0.02	0.34 ± 0.02	0.34 ± 0.02
Cytochrome *c* reductase (nmoles/min/g liver × 10^{-2})	32.0 ± 1.8	34.8 ± 2.7	31.9 ± 2.3
Aniline metabolism (nmoles/min/g liver)	22.5 ± 1.7	24.3 ± 1.6	23.2 ± 2.1
Ethylmorphine metabolism (nmoles/min/g liver)	126.3 ± 5.9	173.5 ± 10.8†	195.8 ± 9.0†
Aminopyrine metabolism (nmoles/min/g liver)	204.2 ± 19.0	243.4 ± 14.4	289.4 ± 16.4†

* Values are expressed as mean ± S.E.: there were 11 animals/group.
† $P < 0.05$ (vs saline-treated group).

or females. Ethylmorphine metabolism, in contrast, was enhanced by both hormones in animals of both sexes. FSH and LH had differing effects only with aminopyrine as substrate. Demethylation of aminopyrine was increased by FSH but not LH and only in female rats. The divergence of hormonal effects on ethylmorphine and aminopyrine metabolism is consistent with the existence of multiple microsomal demethylases.

Gustafsson and Stenberg [25] have recently noted that FSH and LH administration to castrated rats also affects androgen metabolism by hepatic microsomes. They concluded that FSH increased the activity of androgen-dependent hydroxylases but not androgen-independent enzymes. Our findings are consistent with that conclusion, since the metabolism of ethylmorphine and aminopyrine is profoundly affected by testosterone in rats whereas regulation of aniline hydroxylation is relatively independent of gonadal hormones. Furthermore, some of the effects reported by Gustafsson and Stenberg were also dependent upon the sex of the animal being treated. In contrast to our observations, Gustafsson and Stenberg [25] found that LH decreased the activity of several steroid-metabolizing enzymes, further illustrating the substrate dependence of hormonal actions.

The actions of FSH and LH on hepatic oxidative metabolism may be even greater than reported here or by Gustafsson and Stenberg, since in both studies hormones were administered under conditions of already elevated gonadotropin secretion, i.e. in castrated animals. Administration of FSH and LH to hypophysectomized-gonadectomized rats may result in changes of greater magnitude. In any case, these observations indicate that care must be taken to consider the effects of changes in gonadotropin secretion when studying the effects of testosterone or estradiol on hepatic drug metabolism in animals with intact pituitary glands. Depending upon the relative effects of FSH, LH, testosterone and estradiol on metabolism, gonadotropins may enhance or obscure the direct actions of androgens and estrogens on liver. Further studies are now needed to determine the mechanism(s) of action of FSH and LH on hepatic drug- and steroid-metabolizing enzymes and to study possible interactions between the gonadal hormones and gonadotropins in the regulation of these enzymes.

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Influence of sex and Freund's adjuvant on liver *N*-acetyltransferase activity and elimination of sulphadimidine in urine of rats

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Two distinct acetylation phenotypes, characterized by high or low percentage of acetylated product of isoniazid and sulphadimidine eliminated in urine, can be distinguished in man [1–6] and rabbits [7–10]. The difference between them is believed to be due to differences in the activity of *N*-acetyltransferase [10–16]. In these two species no sex difference in the two phenotypes exists, but in rats we have found [17] that the high elimination of acetylsulphadimidine (Ac-S) occurs in females (about 75 per cent of sulphadimidine in urine is present as Ac-S), and low elimination in males (about 46 per cent). The aim of this work was to investigate whether this sex difference is also associated with corresponding differences in liver *N*-acetyltransferase activity. It has been found in rats [18] that Freund's adjuvant which is a potent stimulant of the reticuloendothelial system, presumably the main site of drug acetylation [19], increases the percentage of Ac-S in urine of males, but not of females. Similar elevation of Ac-S only in males was observed also after castration and treatment by estrogen [20]. Therefore, it was of interest to find out whether these changes in males were also accompanied by corresponding changes in enzyme activity.

Animals and experimental conditions. Random-bred albino rats (10 to 16 weeks old) obtained from the Institute of Organic Chemistry and Biochemistry, Prague, were used. They were divided into five groups (see Table 1) as follows: (1) untreated females; (2) untreated males; (3) castrated males: castration was performed when they were 6 weeks old and sulphadimidine was administered 10 weeks later; (4) estrogen-treated males: oestradiol dipropionate in oil solution (Agofollin, Spofa) was given subcutaneously in five daily doses, each containing 10,000 i.u. of the hormone, and sulphadimidine was then administered 2 days after the last dose of estrogen; (5) Freund's adjuvant- (FA-) treated males: 0.1 ml of complete FA (5 mg of *Mycobacterium tuberculosis*, strain H37Rv/1 ml of mineral oil) was injected into the skin of the foot pad and then sulphadimidine was given 21 days later when effect of the adjuvant was fully developed [21, 22].

Sulphadimidine was always administered intravenously in a dose of 40 mg/kg and in a volume of 0.2 ml/100 g body wt. Following the procedure described elsewhere [17], urine samples collected during a 24-hr interval after drug administration were analysed and sulphadimidine determined according to the method of Varley [23]. The ratio (percentage) of Ac-S to the total sulphadimidine eliminated in the urine was then calculated and statistically evaluated.

Preparation of liver cytosol. Since cells of the liver reticuloendothelial system have been shown to represent the most important site of drug acetylation [19], only this tissue was used for preparation of the cytosol. Fresh liver tissue was obtained from rats immediately after sacrifice. All samples were chilled and weighed and then homogenized at 4° in 5–10 vol of Sörensen phosphate buffer (pH 7.4) in a Teflon homogenizer. Homogenates were centrifuged at 105,000 *g* for 50 min in a Spinco ultracentrifuge. The resulting supernatant was used in the enzyme assay which allowed the estimation of total *N*-acetyltransferase activity in the absence of competing microsomal enzymes.

Incubation procedure. The method of Jenne [16] was modified to suit our purpose. The incubation mixture contained the following in a final vol of 2.0 ml: 2 m-mole of sulphadimidine as substrate, 0.5 m-mole of aqueous acetyl-CoA and liver cytosol suitably diluted in 0.2M Sörensen phosphate buffer (pH 7.4) to contain 10 mg of supernatant protein per ml. The reaction was initiated by the addition of liver cytosol and allowed to proceed at 37° for 15 min. The reaction was terminated by adding 4.9 ml of 8% (w/v) trichloracetic acid. Enzyme activity was estimated from the amount of Ac-S produced in the incubation mixture [23]. Protein concentration was determined by the technique of Lowry [24] using bovine serum albumin as a standard. Enzyme activity was expressed as μmoles of substrate acetylated per mg of supernatant protein per 15 min incubation.

The results are summarized in Table 1. The percentage of Ac-S eliminated by untreated animals corresponds well with our previous data [17, 18] showing that females eliminate a high and males a low percentage of Ac-S. The difference between untreated females and males is statistically highly significant ($t_{(16)} = 15.96$, $p \ll 0.01$). The increase in Ac-S elimination in all the groups of treated males is also statistically highly significant: $t_{(13)} = 5.12$, $p < 0.01$ for castration; $t_{(14)} = 13.49$, $p \ll 0.01$, for estrogen treatment; and $t_{(13)} = 14.12$, $p \ll 0.01$ for FA treatment.

The activity of *N*-acetyltransferase is about three times higher in untreated females than in males, the difference being statistically highly significant ($t_{(16)} = 18.16$, $p < 0.01$). However, no increase of *N*-acetyltransferase activity was brought about by castration ($t_{(13)} = 1.33$, $p > 0.10$), estrogen treatment ($t_{(14)} = 1.38$, $p > 0.10$) or administration of FA ($t_{(13)} = 0.47$, $p > 0.50$).

It should be pointed out that while all experimental treatments employed increase the percentage of Ac-S in the urine of males, they do not affect the activity of liver *N*-acetyltransferase. Obviously, the proportion of metabolized and unchanged sulphadimidine in urine is influenced by various mechanisms. The role of *N*-acetyltransferase activity seems to be decisive mainly under normal conditions, i.e. in untreated animals. The nature and interaction of other mechanisms is rather poorly understood at present but our preliminary data (unpublished results) suggest the importance of differences in the distribution of sulphadimi-