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Hepatic drug metabolism *in vitro* in the horse

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IN RECENT years much attention has been directed toward species variation in drug metabolism. However, comparatively little is known about drug metabolism in large domestic animals. This paper is a report on hepatic drug metabolism *in vitro* in the horse.

Preparation of tissues. Fresh liver was obtained immediately after evisceration of horses in an abattoir. The tissues were identified by sex and an estimate of age was made by examination of the teeth. No attempt was made to determine breed. The males were geldings and most of the horses were at least 10 years of age. Part of the excised tissue was submerged into iced 0·25 M sucrose and the remainder was wrapped in aluminum foil and iced for transportation to the laboratory. The foil-wrapped liver was frozen for glycogen determination.

The liver was weighed and 1·0 or 3·3 g liver was passed through a cold tissue press. Sufficient cold 0·25 M sucrose was added to make a 10 or 33% (w/v) homogenate. The liver was homogenized with four or five passes in a motor-driven Potter–Elvehjem tissue homogenizer with a Teflon pestle and packed in ice prior to use. A 10% whole liver homogenate was used for measuring UDPglucuronyltransferase activity. The remainder of the enzyme assays were conducted with a 9000 *g* supernatant prepared from a 33% whole homogenate by centrifuging for 20 min at 4°. Because of the labile nature of UDPglucuronyltransferase, this determination was one of three (*p*-nitrophenol glucuronidation, *p*-nitrobenzoic acid reduction, procaine hydrolysis) initiated as soon as the tissues were received in the laboratory. Preliminary studies indicated that there was no loss of activity from the 9000 *g* supernatant for oxidative enzymes when the supernatant was refrigerated at 4° for at least 30 hr. Consequently, the oxidative enzyme reactions were conducted on the following day with the refrigerated 9000 *g* supernatant.

Measurement of enzyme activity. Six substrates were used to measure major metabolic pathways. These were: (1) glucuronide conjugation of *p*-nitrophenol; (2) oxidation of zoxazolamine; (3) oxidation of hexobarbital; (4) *N*-dealkylation of aminopyrine; (5) hydrolysis of procaine; and (6) reduction of *p*-nitrobenzoic acid.

p-Nitrophenol glucuronidation was determined by the method of Isselbacher *et al.*[1] using 0·4 μmole of recrystallized *p*-nitrophenol as a substrate. The 0·3 μmole of uridine diphosphoglucuronic acid (UDPGA) was not rate limiting in a final volume of 3·0 ml. The reaction flasks were flushed with nitrogen, stoppered, and incubated for 15 min at 30°. We obtained better activity with 10% whole homogenate than with 9000 *g* supernatant, possibly because some of the enzyme was lost in the sediment.

The incubation mixture for the oxidative reactions contained an NADPH-generating system consisting of: 0·6 μmole NADP; 25 μmoles glucose 6-phosphate; 25 μmoles $MgCl_2$; 0·1 M potassium phosphate buffer, pH 7·35; 100 μmoles nicotinamide; 1·0 ml of tissue preparation; and substrate in a total incubation mixture of 5·0 ml. Incubations were made at 37° in a metabolic shaking incubator operated at 100 oscillations per min. For oxidative reactions, the oxygen flow rate was 1 l./min.[2]

Oxidation of zoxazolamine was determined using 3 μmoles substrate and measuring substrate disappearance according to the method of Conney *et al.*[3] Oxidation of hexobarbital was determined by measuring substrate disappearance by the method of Cooper and Brodie,[4] using 3 μmoles hexobarbital. *N*-dealkylation of aminopyrine was measured by the formation of 4-aminoantipyrine from 10 μmoles of recrystallized aminopyrine by a modification of the methods of La Du *et al.*[5] and of Brodie and Axelrod.[6] The concentration of 4-aminoantipyrine was determined by measuring absorbance at two wavelengths, 540 and 400 mμ, and solving the simultaneous equation for analysis of a two-component system.[7] The incubation time of each of the preceding substrates was 1 hr.

Procaine hydrolysis was measured by the formation of *p*-aminobenzoic acid. The optimum pH was 9·3. Two μmoles of procaine hydrochloride was incubated with 1·0 ml of a 9000 *g* fraction of a 33% homogenate and 1·5 ml of 0·1 M tris–HCl buffer, pH 9·3, for 10 min at 37°. Meaurement of the *p*-aminobenzoic acid formed was by the method of Brodie *et al.*[8] Nonenzymatic hydrolysis did not occur under the conditions employed in this procedure.

Reduction of *p*-nitrobenzoic acid was determined by the formation of *p*-aminobenzoic acid by the method of Fouts and Brodie.[9] Five μmoles *p*-nitrobenzoic acid in a total of 5 ml was incubated under nitrogen for 1·5 hr. The rate of reduction was linear throughout this period.

Because of the uncertainty of the nutritional status of the horses and the reported implications of the relationship between starvation and drug-metabolizing enzyme activity,[10] liver glycogen was determined with anthrone reagent as described by Hassil and Abraham.[11]

Liver specimens were obtained from 34 horses. All six enzymatic pathways were determined on 21 of the specimens and one or two pathways were not examined for 13 of the horses. The protein content of tissue preparations was analyzed by the method of Lowry *et al.*[12]

The results are summarized in Table 1. It is difficult to compare absolute values for drug-metabolizing enzyme activity from one laboratory to another because of the inevitable variations in animals and procedures, and the lack of uniformity in expressing enzyme activity. However, it would appear from the data reported by Fouts and Brodie[9] that the horse has much greater *p*-nitroreductase activity than the dog, rat, rabbit, guinea pig or mouse. With the exception of *p*-nitrobenzoic acid reductase activity, the values obtained were generally lower than those reported for commonly used laboratory animals.[5,9,13]

TABLE 1. HEPATIC DRUG-METABOLIZING ENZYME ACTIVITY *in vitro* IN THE HORSE*

Substrate	Incubation time (min)	Enzyme activity (Mean ± S.E.) Males (μmoles/g liver)	Females (μmoles/g liver)
p-Nitrophenol	15	0·980 ± 0·126 (13)†	1·043 ± 0·121 (21)
Zoxazolamine	60	0·525 ± 0·124 (10)	0·499 ± 0·075 (21)
Hexobarbital	60	0·933 ± 0·150 (11)	1·414 ± 0·164 (21)
Aminopyrine	60	0·073 ± 0·019 (7)	0·065 ± 0·012 (16)
Procaine	10	2·823 ± 0·401 (8)	2·146 ± 0·296 (14)
p-Nitrobenzoic acid	90	6·012 ± 0·643 (13)	4·631 ± 0·721 (21)

* Protein content per milliliter of 10% homogenates was 54·4 ± 1·06 mg; protein content per milliliter of 9000 *g* supernatant from 33% homogenates was 36·5 ± 0·83 mg.

† No. of animals.

The procaine esterase activity was similar to that reported for 10-week-old pigs by Short and Davis.[13] The values for hexobarbital and zoxazolamine were approximately one-fifth and one-tenth, respectively, of their value for pigs.

The rate of oxidation of hexobarbital was significantly ($P < 0.05$) greater in female than in male horses. Sex-related differences for other substrates were not statistically significant. There was wide variation in liver glycogen, ranging from 2·1 to 62·8 mg glycogen per g of liver with a mean of 18·7 and a standard deviation 13·4. Liver glycogen values did not correlate with drug-metabolizing enzyme activity.

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Effect of antimalarial drugs on the efflux of K^+ from *Streptococcus pyogenes*

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ANTIMALARIAL drugs such as primaquine, quinacrine, quinine and chloroquine possess antibacterial properties *in vitro*.[1–5] Hahn *et al.*[6,7] have demonstrated that the antibacterial action of chloroquine against *Bacillus megaterium* is due to the formation of drug–DNA complexes. In other studies, primaquine, quinacrine and quinine were found to alter the permeability of the red cell membrane for K^+.[8] The present study was designed to investigate the effect of the above antimalarial compounds on the efflux of ^{42}K from bacteria.

In preliminary experiments, the antibacterial action of primaquine diphosphate, quinacrine hydrochloride, quinine hydrochloride and chloroquine diphosphate against some common bacteria isolated from hospitalized patients was assessed. One ml of a 1 to 1000 dilution of a 24-hr beef heart broth culture was added to decreasing concentrations of drug and then incubated at 37° for 48 hr. The test tubes were then examined visually for signs of bacterial growth. Primaquine and quinacrine inhibited the growth of two strains of *Streptococcus pyogenes* (group A) at concentrations of 0·1 mM; quinine did the same at concentrations of 0·1 and 0·25 mM, respectively, whereas chloroquine was inactive at 1 mM, the highest concentration tested. The four drugs demonstrated a similar antibacterial effect on two strains of *Diplococcus pneumoniae* and all were inactive against a number of gram-negative enteric organisms at concentrations of 1 mM.

S. pyogenes (group A) was employed in the studies of ^{42}K efflux, since they best withstood the manipulations associated with ^{42}K loading. One ml of a 24-hr culture of *S. pyogenes* was added to 150 ml of beef broth containing 1 mc ^{42}K and the contents were then incubated at 37° for 6–7 hr. The ^{42}K-laden bacteria were washed three times and then resuspended in broth. Aliquots (25 ml) of the well mixed bacterial suspension containing approximately $1·3–1·8 \times 10^7$ organisms were added to glass-stoppered flasks. Four-ml aliquots from each flask were quickly filtered through a 5-ml syringe attached to a Swinnex filter unit containing a pre-filter and 25 mm millipore filter with pores measuring 0·22μ. The bacteria trapped in the pre-filter and filter were washed with 4 ml broth. A 4-ml aliquot of a pH 7·3 phosphate buffer solution of drug was added to each of the flasks, with buffer to the controls. The flasks were agitated at 23° with 4-ml aliquots removed at 5, 10, 15 and 20 min and treated as described above. The bacteria were incubated at 23° rather than 37°, since the lower temperature slowed the efflux of ^{42}K and thus permitted more accurate measurement of this parameter. Bacterial growth during incubation was assessed by the measurement of the optical density of zero-time and 20-min samples using a Klett colorimeter with a red filter. The millipore filter and pre-filter containing the ^{42}K-laden bacteria were placed into plastic test tubes and counted in a plastic well counter.[9] The counting error was less than 2 per cent.

The effect of antimalarial drugs on the efflux of ^{42}K from a strain of *S. pyogenes* (group A) is shown in Fig. 1. With the data plotted on semilogarithmic paper, the efflux of ^{42}K from control cells approximated a first-order process with a half-time of 16 min. In four other similar experiments, the half-time ranged from 16 to 19 min. Primaquine, quinacrine and quinine produced an approximately equivalent increase in ^{42}K efflux over that observed in control cells. In contrast to the control, the efflux of ^{42}K from these cells was not a linear process, but consisted of a rapid initial component with a half-time of about 6 min and a slower one commencing at 5 min with a half-time of about 11 min. The reason for the decrease in drug effect on ^{42}K efflux continued incubation with is unclear. Chloroquine, unlike the above three drugs, did not affect the efflux of ^{42}K. In four other experiments with