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EXCRETION OF BENZOATE IN BOVINE URINE AFTER THE ADMINISTRATION OF THIOPENTAL*

R. P. SHARMA, L. E. OLSON and C. M. STOWE

Department of Veterinary Science and Department of Food Science and Industries, Utah State University, Logan, Utah 84321 and Department of Physiology and Pharmacology, College of Veterinary Medicine, University of Minnesota, St. Paul, Minn. 55101, U.S.A.

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Abstract—Bovine urine, collected over a 4-day period after the administration of thiopental, contained an appreciable amount of a compound later identified as benzoic acid. The compound was characterized by ultraviolet, infrared and mass spectroscopy. The excretion of this compound was greatest on the second and third day after the administration of thiopental; it declined on the fourth day. Although rumen microorganisms may synthesize benzoic acid from a variety of sources, benzoic acid is not normally synthesized by animals, and ingested benzoic acid is excreted in herbivorous animals after conjugation, as hippuric acid. *In vitro*, conjugation of glycine and hippuric acid by bovine liver homogenate was inhibited by the presence of thiopental. It is concluded that the presence of thiopental interfered with the conjugating enzymes in the liver, thus causing the urinary excretion of unconjugated benzoic acid.

WHILE isolating the various metabolites of thiopental from bovine urine, an unknown compound was isolated primarily on the second and third day after a single administration of the drug. A strong absorption peak in the ultraviolet region and the pattern of daily excretion of this compound, which quantitatively appeared to be a major fraction of thiopental injected, suggested that it was possibly a metabolite of the drug. The chemical characteristics of this compound were not similar to those of any known metabolite of thiopental reported earlier in the literature.[1–6] The quantitative pattern of its excretion over a 4-day period after the injection of thiopental and its chemical characteristics are described in this report.

METHODS

Five female, sexually matured Holstein calves, weighing approximately 250 kg were used. Three animals were given a 10% solution of thiopental sodium,† 20 mg/kg body weight, as a slow intravenous injection in a cannulated jugular vein over a period of 30 min. The purity of thiopental was verified by recrystallization and ultraviolet spectra. The other animals were used as controls and were not given any injection, while they were given the same feed as the treated animals and were kept under identical conditions throughout the 4-day period. Control blood and urine samples were obtained periodically from the opposite cannulated jugular vein throughout the period of the experiment. The urine was collected by cementing a rubber conduit

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† Pentothal Sodium, from Abbott Laboratories, Chicago, Ill. The drug included sodium carbonate, 60 mg/g of thiopental sodium, as a stabilizer.

around the vulva, with the other end attached to a collecting bottle. The animals usually urinated upon the stimulation of their external genital organs. The urine obtained was quickly stored in a refrigerator at 4°. The urine from each animal was pooled each day for 4 days. The plasma levels of thiopental were determined by a method described by Brodie *et al.*,[1] and modified by Frey *et al.*[7] The details of the method and its validity have been described earlier.[8]

Each urine sample was placed in a large separatory funnel and acidified to pH 2 by the addition of dilute hydrochloric acid. Chloroform, 100 ml for each liter of urine, was added and the sample was shaken vigorously for 10 min. The chloroform layer was separated and the extraction was repeated with the same amount of fresh chloroform. The two chloroform fractions were combined and centrifuged. After the second extraction, a third extraction with chloroform or isoamyl alcohol did not show an appreciable absorption in the ultraviolet region, suggesting that the double extraction with chloroform removed most of the barbiturate compounds or other extractable organic materials present.

The chloroform extract was re-extracted with 1 N sodium hydroxide; 10 ml was used for each 100 ml of chloroform fraction. The alkaline extract was separated, reacidified with an excess of 1 N hydrochloric acid, and re-extracted with 100 ml of fresh chloroform. This chloroform extract was stored in a refrigerator at 0° until used.

Isolation of the compound present in major amounts in the extracts of acidified urine

The primary objective of the investigation was to isolate and identify any major-metabolite obtained from thiopental. The urine extracts were submitted to countercurrent distribution and ion-exchange chromatography. While scanning the alkaline solutions of various fractions obtained from these processes, a peculiar compound was observed that had an absorbance maximum at approximately 220 mμ. The presence of this compound was appreciable in the urine extracts obtained on the second and third day (see Table 1). The quantities declined on the fourth day of urine collection. Since the plasma levels of thiopental fell to undetectable amounts by the methods used here, no urine was collected after 4 days.

The countercurrent distribution was performed with aliquots of each chloroform extract (1–5 ml) in a 15 plate system, using 25 ml each of chloroform (as a mobile phase) and 0·05 N borate buffer at pH 9·75 (both phases mutually saturated with each other).[3] The distribution was performed in 125-ml separatory funnels, as described by Craig.[9] The quantities of thiopental and its desulfurated product, pentobarbital, were calculated by the expansion of the binomial term[10] from the quantities of these compounds in the tubes showing the maximum absorption at 303 and 240 mμ respectively.[3] The positive identification of thiopental and pentobarbital using this countercurrent distribution system has been reported earlier.[4,11] The quantity of the unknown compound was obtained by the absorbance (220 mμ) present in the first two tubes and comparing it with suitable standard curves prepared from the isolated and purified crystals.

For ion-exchange chromatography, the residue from the evaporated chloroform extract was dissolved in a small amount of methanol and deposited on a 22 × 1·2 cm Dowex-1 (X4, 50–100 mesh, Cl^- form) column. The column was then eluted with a gradient of 0·05 to 1 N ammonium acetate in 90% methanol. The flow rate was maintained at 60–80 drops/min and fractions of 160 drops each were collected with a

Beckman automatic fraction collector. About 75 fractions were collected in this way, followed by the elution with 1 N acetic acid in 90% methanol, until a total of 110–120 fractions were collected.[6] From each fourth fraction, 0·025 ml of eluted solution was added to 5 ml of 0·5 N sodium hydroxide and its absorbance was recorded from 200 to 330 mμ in a Beckman DB spectrophotometer with an automatic recorder. In some cases, further dilution with 0·5 N sodium hydroxide was necessary to attain the proper absorbance.

Thirty ml of the chloroform extract was used for primary isolation of the unidentified compound excreted in the urine. The acidic derivatives present were transferred to 25 ml of 0·05 N borate buffer at pH 9·75. This alkaline solution was washed nearly 15 times with 25 ml chloroform to remove all thiopental and pentobarbital. The remaining solution was acidified with 1 N hydrochloric acid to pH 2 and re-extracted with 25 ml chloroform. The chloroform was evaporated and the solid residue was sublimed to obtain needle-shaped white crystals, which were vacuum-dried and stored in a glass-stoppered bottle in a desiccator at 5°.

After the primary characterization, the same compound was also isolated from another fraction of chloroform extract of acidified urine by dissolving the residue after evaporation of the chloroform in hot water and recrystallizing from water. These crystals were purified by sublimation.

Characterization of the unknown compound excreted in the urine

The purified crystals were analyzed for nitrogen and sulfur. The pK'_a of the compound was determined by the partition method of Butler.[12] The compound in 90% ethanol was titrated potentiometrically with standard sodium hydroxide.[13] The anesthetic activity of this compound was estimated by injecting several female mice (approximately 30 g body weight) intraperitoneally with a dose of 200 mg/kg and observing the animals closely for 2 hr. The determinations of melting point and reactive group analysis were not conclusive of the definite chemical identity of this compound. The mass spectrum and elemental analysis of this yet unknown compound suggested its structural nature, which was further identified by comparing its infrared and ultraviolet spectra with those of an authentic sample of benzoic acid.

Studies in vitro *of hippuric acid formation*

Enzymatic formation of hippuric acid by conjugation of glycine and benzoic acid, using enzymes obtained from bovine liver, was followed in the presence of various concentrations of thiopental. The incubating system was modified from that of Chantrenne.[14] The mixture consisted of: 8 μmoles magnesium sulfate; 100 μmoles each of benzoic acid, glycine and adenosine triphosphate; and 50 μmoles cysteine in 2·5 ml of 20% calf liver homogenate in 0·02 M phosphate (pH 7·5) buffer. Thiopental was added to provide the final concentrations ranging from 10^{-6} to 10^{-3} M. The mixture was incubated at 39° for 3 hr in a shaking water bath. The reaction was stopped by adding 1 ml of 10 N sulfuric acid. The mixture was extracted twice with equal amounts of isoamyl alcohol. The isoamyl alcohol was evaporated to dryness and the residue was hydrolyzed with 6 N HCl at 110° in a sealed tube for 16 hr. The solution was evaporated to dryness and the resulting glycine was analyzed spectrophotometrically by ninhydrin reaction and compared with a glycine standard. Corrections were made for a tissue blank in which the reaction was stopped immediately after the

addition of enzyme to substrate. Such blank values were comparatively very low. The recovery of hippurate through the extraction process has been reported to be almost quantitative.[14]

RESULTS

The white, fluffy, needle-shaped crystals of the compound obtained from urine and purified by sublimation had a peculiar odor. The crystals were only slightly soluble in cold water at pH 7, but were soluble in organic solvents (chloroform, diethyl ether, acetone). Tests for sulfur and nitrogen were negative. The pK'_a of the compound, as obtained by the partition method of Butler,[12] was 4·4, which suggested that it was a carboxylic acid. Potentiometric titration of the compound in 90% ethanol yielded an inflection point at a pH of 6·9 similar to that of a monocarboxylic acid in alcohol solution.[13] This compound had no anesthetic properties.

The compound has a strong absorption peak at 220 mμ in alkaline solutions. This property was the means of quantitation of this compound in daily urine samples after the administration of thiopental. The first two plates of countercurrent distribution, from which all of thiopental and pentobarbital had been removed, showed an appreciable amount of absorption at 220 mμ, and the difference of absorbance at 220 and 330 mμ was compared with the standard curves prepared from purified crystals. The daily and total urinary excretion of thiopental, its desulfurated product pentobarbital, and the unknown compound in the three treated heifers are shown in Table 1. The unknown compound appeared in large amounts on the second and third day, and declined on the fourth day. None of the above three compounds was found in the urinary extracts of the control animals or even in the urine of experimental animals, obtained before the administration of thiopental. The absence of this compound from the first-day urine of the calves after the administration of thiopental and from all control urines was also ascertained by ion-exchange chromatography. A noticeable feature in the comparisons of Fig. 1a and 1b is the absence of a major peak in the former, in fractions 15–55, having an absorption maximum at 220 mμ. The drug and its metabolites are represented by the other peaks in Fig. 1.

The decline in the amounts of this unknown compound on the fourth day after treatment was associated with the fact that the blood levels of thiopental fell almost to undetected levels after this day. The plasma disappearance of thiopental in treated animals versus the cumulative excretion of this compound isolated from urine extracts is shown in Fig. 2.

The carbon and hydrogen analysis* results indicated a highly unsaturated compound. Although benzoic acid was not a probable compound to expect, the suggestion was provided by the mass spectra of this unidentified compound. The mass spectra suggested a molecular ion at m/e 122, a base ion at m/e 105 with major peaks at m/e 77, 51, 44, 28 and 18 (Fig. 3). Metastable peaks at m/e 56·5 and 33·8 confirmed the presence of an aromatic carbonyl fragment in the molecule.[15] On the basis of these spectra, the unknown compound extracted from the urine was tentatively identified as benzoic acid. This was also supported by element analysis. *Anal.* calc. for $C_7H_6O_2$: C, 68·84; H, 4·95. Found (for isolated compound): C, 68·94; H, 5·14.

* Analyses by Huffman Microanalytical Laboratories, Wheatridge, Colo.

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TABLE 1. DAILY AND TOTAL URINARY EXCRETION OF THIOPENTAL, ITS DESULFURATED METABOLITE, PENTOBARBITAL, AND THE UNKNOWN COMPOUND IN HOLSTEIN HEIFERS DURING 4 days AFTER ADMINISTRATION OF THIOPENTAL

Animal	Body wt. (kg)	Total dose* (g)	Compound recovered	Compound recovered (mg)				
				Day 1	Day 2	Day 3	Day 4	Total
Calf 1	281	5·62	Thiopental	67·4	90·9	10·1	2·3	170·7
			Pentobarbital	168·0	106·8	84·0	12·3	371·1
			Unknown†	0·0	90·0	825·0	366·0	1191·0
Calf 2	218	4·36	Thiopental	23·6	10·1	4·0	1·4	39·1
			Pentobarbital	53·4	43·8	20·3	9·8	127·3
			Unknown†	176·5	1149·0	277·5	487·0	2190·0
Calf 3	241	4·82	Thiopental	10·1	20·2	4·0	2·0	36·3
			Pentobarbital	325·5	88·4	20·3	13·3	447·5
			Unknown†	0·0	257·5	1490·0	250·0	1997·5

* Thiopental (20 mg/kg) was used as a slow single intravenous injection.
† The compound isolated from urine extracts in large amounts.

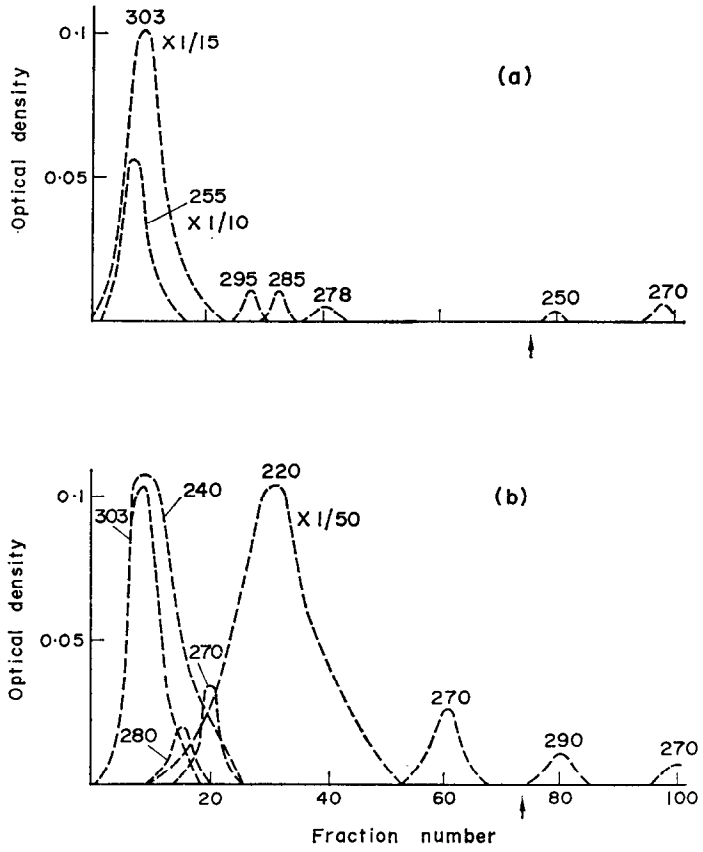

FIG. 1. Dowex-1 chromatograph of calf urine extract. (a) Urine samples from the first day, and (b) urine samples from the second day of thiopental administration. The absorbance of the peaks (wavelengths indicated) are plotted. The fractional numbers indicate the factor used to accommodate the plots on a uniform scale. Arrow on abscissa indicates completion of the gradient. A major peak having an absorption maximum at 220 mμ is absent in Fig. 1(a), but is prominent in Fig. 1(b).

For further confirmation, the ultraviolet spectra of the compound isolated from urine and that of benzoic acid in 95% ethanol were compared. In each compound, the major absorption peaks occurred at 203 and 226 mμ, and minor absorption peaks appeared at 272 and 277 mμ, as shown in Fig. 4.

The infrared spectra of the compound from urine and of authentic benzoic acid in potassium bromide pellets are shown in Fig. 5. From these ultraviolet and infrared spectra, the two compounds appear to be identical.

Studies *in vitro* indicated that thiopental was an inhibitor of glycine conjugation of benzoic acid by bovine liver homogenates (Fig. 6). In our studies, the crude bovine liver homogenate converted approximately 6·6 μmoles of benzoic acid to hippuric acid/g of original tissue/hr. The formation of hippurate was directly proportional to the amount of tissue added, and the reaction was linear throughout the 3-hr incubation period.

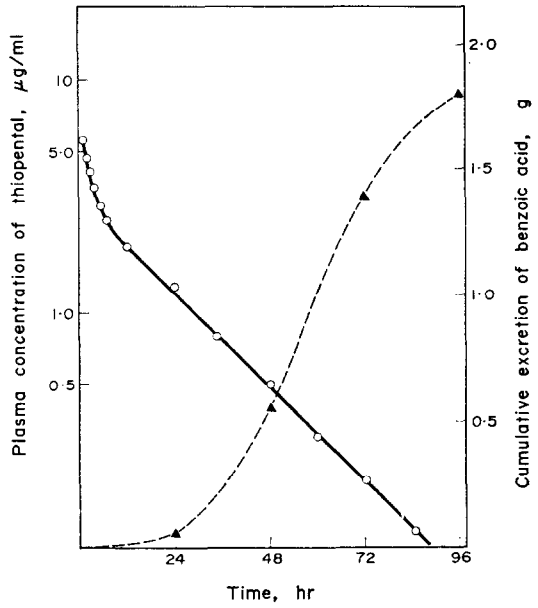

FIG. 2. Plasma disappearance of thiopental (○—○) and the cumulative excretion of the isolated compound (▲—▲). Each value is an average of three animals.

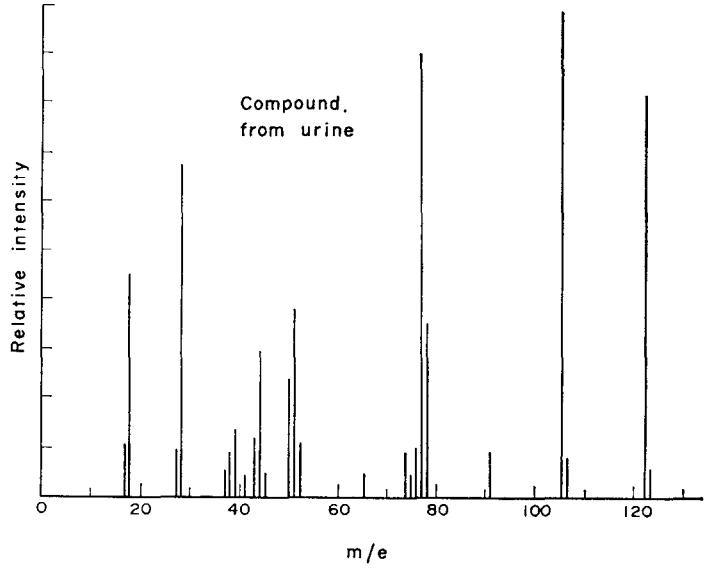

FIG. 3. Mass spectra of the compound obtained from bovine urine after administration of thiopental.

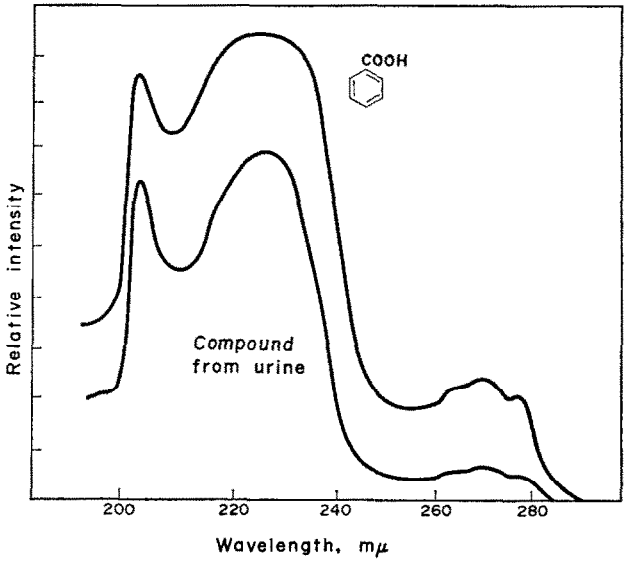

FIG. 4. Ultraviolet spectra in 95% ethanol of benzoic acid (upper) and compound obtained from bovine urine (lower) after administration of thiopental.

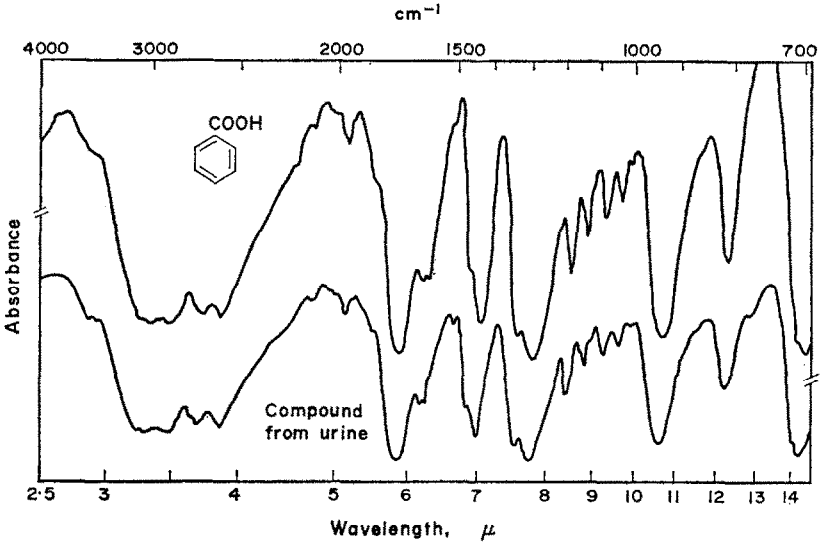

FIG. 5. Infrared spectra in KBr pellets of benzoic acid (upper) and compound obtained from bovine urine (lower) after administration of thiopental.

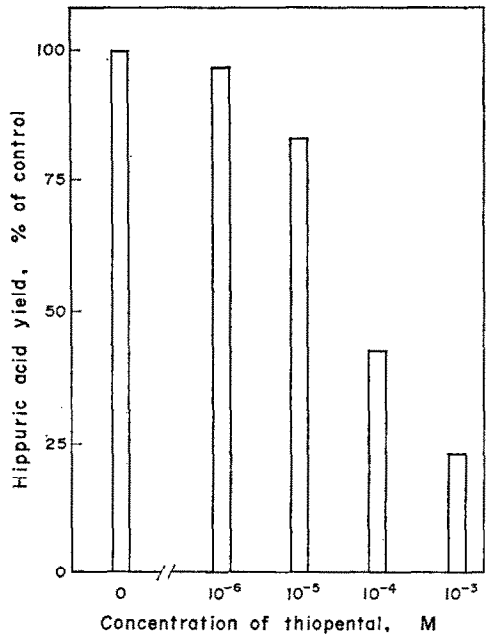

FIG. 6. Inhibition of benzoic acid and glycine conjugation by bovine liver homogenates by the presence of various concentrations of thiopental. Details mentioned in Methods.

DISCUSSION

The pattern of urinary excretion of the isolated compound from acidified extracts indicated that it could have been a metabolite of thiopental. It seemed improbable that this drug could be metabolized to benzoic acid, since animal tissues are not capable of synthesizing a benzene ring. Such a transformation could possibly be carried out by the microorganisms in the rumen, since some benzoic acid is usually produced in the rumen by bacterial action. It is probable that the benzoic acid was not derived from thiopental, but came from other sources, and perhaps the excretion of benzoic acid was caused by the presence of thiopental.

Normally all benzoic acid ingested or produced in herbivorous animals is excreted in the form of hippuric acid.[16] This transformation, conjugation with glycine, is carried out in the liver or kidneys. Even young calves have the capability of conjugating benzoic acid.[17] Hippuric acid is a reasonably stable compound and will not be hydrolyzed by the extraction processes used in our methods. No heat or strong acids were used for extraction processes described earlier, since such conditions might have destroyed even a malonylurea ring present in thiopental or hydrolyzed other conjugated metabolites. Similarily, the absence of the benzoic acid in any of the control urine samples and its pattern of excretion, as related to the levels of thiopental, in treated animals support the view that it is not a normal compound excreted in bovine urine. Regardless of whatever may be the source of benzoic acid, the results mentioned here indicated that the compound was excreted in urine in an unconjugated form. The benzoic acid excreted in the urine would be in the form of a benzoate because of the alkaline nature of the urine samples (pH 7·2–7·6). The urinary excretion of benzoic

acid on the second and third day after the administration of thiopental and the declining during the fourth day indicate that this event was related to the presence of thiopental in the animal body.

The excretion of benzoate in urine was in addition to the other metabolites of thiopental. The other metabolites of thiopental were similar to those described earlier for other mammalian species; these have been reported elsewhere.[11] Considerable amounts of thiopental and its desulfurated product, pentobarbital, were obtained from the urine.

It is well known that the bovine urine is a rich source of hippuric acid.[16] This is derived by the conjugation of benzoic acid and glycine in the animal, primarily in the hepatic and renal tissues.[16] The same organs are also the site of thiopental metabolism.[18] It is not probable that the benzoate was derived from the drug. The ruminants have a considerable amount of benzoate, obtained by the microbial digestion of dietary lignin. The amount of benzoate produced will be variable from day to day and will depend on factors like the dietary composition and the nature of bacterial action in the rumen. In ruminants, all benzoate thus produced is excreted as hippurate. In the present report, it has been shown that the presence of thiopental in the animal body prevented the conjugation of benzoate, which was excreted unconjugated in the urine.

The above observation was also supported by the findings *in vitro*. The rate of hippurate formation by the bovine liver homogenate was comparable to (or in excess of) those reported earlier for other mammalian species.[19,20] The systems involved in the conjugation of benzoic acid and glycine have been described earlier.[21,22] Energy-rich phosphates are required for the formation of benzoyl CoA, which is necessary for subsequent peptide bond formation. A soluble enzyme, glycine *N*-acylase, has been characterized.[22] The present work indicates that the conjugation of benzoate was inhibited by the presence of thiopental. At this point, it is difficult to say whether the interference with glycine conjugation was caused by the inhibition of the enzyme required for the formation of benzoyl CoA or by inhibiting the supply of some other cofactor, e.g. CoA, ATP or cysteine, etc.

No report of this nature is available in the literature regarding other species. Although the laboratory animals do not normally excrete hippuric acid, the effect of thiopental on the conjugation of exogenous benzoate is worth determining. Similarly, the excretion of benzoate in ruminants after thiopental injection, after their ruminal microbial activity has been interfered with (e.g. by the use of antibiotics), would be of interest. In the present report, no effort was made to determine the source of benzoate. The emphasis has been directed toward identifying the benzoic acid, which is not a normal excretory product of the bovine, and verifying by experiments *in vitro* that the conjugation of benzoic to hippuric acid by bovine liver enzymes is interfered with by the presence of thiopental.

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