was operated manually following a standard procedure as closely as possible (20 passes of the piston including a twisting motion). The homogenate along with 2 washings of the homogenizer were transferred to tubes and centrifuged for 15 min at 1000 *g*. The supernatant along with 2 further washings of the 1000 *g* pellet were transferred to glass ampoules and lyophilized. During the total procedure from dissection to lyophilization, the tissue was kept on ice or held at 4 °C. For enzyme assay, the lyophilizate was reconstituted in distilled water, between 0.5 and 3.0 ml according to anticipated activity. Assay tubes containing 250 µl of the incubation medium (see below for composition) were brought to 37 °C, and 10 or 25 µl of tissue extract was added and incubated for 60 min. Under these conditions substrate utilization did not exceed 20% and the rate of inorganic phosphate liberation was constant for at least 90 min. Reaction was stopped by the addition of 2 ml 10% trichloroacetic acid and the tubes were transferred to ice. Non-incubated controls were run in parallel for each experiment. Each sample and non-incubated control consisted of 3 replicates. Inorganic phosphate was determined according to BONTING[7] using a Zeiss PM 4 Spectrophotometer. ATPase activity was determined at pH 7.4 in a medium containing 90 m*M* *Tris*, 0.1 m*M* EDTA, 2 m*M* Mg^{2+}, 60 m*M* Na^+, 5 m*M* K^+ and 2 m*M* Na_2ATP. Chloride was the predominant anion. A series of preliminary experiments had shown that either the elimination of sodium, potassium or both from the above medium, or the addition of 1 m*M* ouabain all resulted in equivalent degrees of inhibition. Thus for the following experiments the ouabain-sensitive component alone was used as the measure of Na, K-ATPase. Known volumes of enzyme solution were placed in pre-weighed glass tubes, thoroughly dried at 100 °C and weighed on a Mettler M5 microbalance to within 2 µg. After correction was made for the small amount of *Tris* known to be in solution, the dry weight of tissue per enzyme sample could be calculated. Enzyme activity is expressed as m*M* ATP hydrolyzed/h/g dry weight of tissue at 37 °C.

Results. In the present study, ouabain-sensitive ATPase of the salivary glands of feeding females was 34.5 ± 2.5% (SE of mean, $n = 15$) of the total ATPase (range = 15 to 50%). The average Na, K-ATPase activity for females weighing more than 170 mg was 8.3 m*M* ATP · g^{-1} dry weight · h^{-1}. The water content of this tissue is high – 82 ± 1.2% (SE of mean, $n = 14$), therefore the activity per g wet weight is about 1.5. Experimental results are presented in the Figure. We summarize as follows: Salivary glands from male ticks, whether unfed or fed for up to 12 days, possess a very low ATPase activity. Ouabain appeared to stimulate the activity slightly, and thus Na, K-ATPase could not be identified in this study. Salivary glands from unfed females behaved in much the same manner as those from males. In feeding females the ATPase activity increased steadily, and a ouabain-sensitive component appeared. By the time the females had attained 200 mg a plateau was reached for both ATPase components.

Discussion. In the present work we demonstrate a development of salivary gland Na, K-ATPase, the specific activity of which increases with feeding duration. Maximum activity of the enzyme is achieved when the female tick's fed: unfed weight ratio is approximately 8. As would be expected for an actively secreting tissue, the level of this plateau is comparatively high. For example, an extensive study of the quantitative distribution of Na, K-ATPase in tissues of the cat[8] showed highest activity in brain gray matter and in kidney medulla (respectively 1.52 and 0.44 m*M* ATP split/g wet weight/h at 37 °C.). Our high figure of 1.5 cannot be compared directly with the above since our extracts were partially purified to the extent of a single centrifugation, whereas their figures were for crude homogenates. We do, however, have a comparative figure for one crude homogenate of salivary glands – 0.62 m*M* ATP/g wet weight/h.

Concommittent with the increase of Na, K-ATPase, there is an enhanced ability to secrete fluid in vitro[6], also reaching a maximum at a fed: unfed ratio of 8. Moreover, current work reveals that the cell-type of the salivary gland in *A. hebraeum*, which is homologous to the 'water cell' of *Dermacentor andersoni*[9], undergoes radical ultrastructural changes during feeding, notably an enormous development in plasma membrane invagination and mitochondrial multiplication[10]. We feel that these correlations are related functionally. In harmony with this hypothesis, we note that salivary glands from well-fed males 1. possess an Na, K-ATPase activity which rests at a level below the sensitivity of the present procedure, 2. secrete fluid in vitro only feebly[6] and 3. undergo ultrastructural modifications in the secretory cell-type which are insignificant when compared to those occurring in salivary glands from females[10].

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Novel Metabolite of Nitrazepam in the Rabbit Urine

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Summary. Two novel metabolites appearing mainly as conjugated form in the urine of rabbits fed nitrazepam have been isolated as deconjugated form. From the data of elemental and spectral analysis, the structure was confirmed as 2-amino-3-hydroxy-5-nitrobenzophenone (M-I) and 2'-benzoyl-4'-nitro-2-hydroxyacetanilide (M-II).

Nitrazepam is widely used in clinical practice as a sleep-inducing agent. In the previous studies of nitrazepam metabolism, it has been reported that 7 metabolites were identified in man[1], 7-amino nitrazepam [I], 7-acetamido nitrazepam [II], 3-hydroxy-7-amino nitrazepam [III], 3-hydroxy-7-acetamido nitrazepam [IV], 2-amino-5-nitrobenzophenone [V] and 2-amino-3-hydroxy-5-nitrobenzophenone [VI]. And in rats[2], 4'-hydroxy nitrazepam [VII] and 2-amino-4'-hydroxy-5-nitrobenzophenone [VIII], in rabbits[3], 3-hydroxy nitrazepam [IX] were identified in addition to [I], [II], [III] and [V].

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Table I. Rf values and colour reactions of some metabolites of nitrazepam in thin-layer chromatography

Compound	Occurrence of metabolites in urine		Solvent system		Colour reaction	
	Free	Conjugated	I	II	Bratton-Marshall[a]	Phenol[b]
Metabolite I (M-I)	+	+++	0.35	0.30	brown	greyish blue
Metabolite II (M-II)	absent	+	0.50	0.66	pink-red	—

Adsorbent: Silica gel G (Merck). Solvent system: I = benzene-chloroform-methanol-28% ammonia (50:50:15:1); II = chloroform-methanol-28% ammonia (90:10:1). Detection reagent: [a]sprayed with the Bratton-Marshall reagent after hydrolysis by 2 *N* sulfuric acid. [b]Folin-Ciocalteu reagent. The number of (+) signs in this table is based on the relative colour intensity of the metabolites on the thin-layer chromatograms.

Table II. Physical properties of metabolites of nitrazepam

Compound	Formula and mp (°C)	Analysis found (%)			UV-spectrum ($\lambda_{max}^{50\%\ EtOH}$)	IR-spectrum (ν_{max} cm^{-1})	Mass spectrum (*m/e*)
		C	H	N			
M-I	$C_{13}H_{10}N_2O_4$ 208–210	59.57	3.99	10.35	255 381	(in Nujol) 3500 (OH) 3420, 3295 (NH) 1570, 1343 (NO_2)	258 (M^+) 241 (−OH)
M-II	$C_{15}H_{12}N_2O_5$ 171–172	59.87	4.16	9.14	220 267 315	(in KBr) 3530 (OH) 3275 (NH) 1708 (CO) 1637 (aromatic CO) 1518, 1346 (NO_2)	300 (M^+) 269 ($-CH_2OH$)

Elemental analysis: Calcd. for $C_{13}H_{10}N_2O_4$: C, 60.48; H, 3.87; N, 10.85 and for $C_{15}H_{12}N_2O_5$: C, 60.00; H, 4.03; N, 9.33.

The purpose of these studies was to determine the structure of new urinary metabolites isolated from rabbit urine. The rabbit urine collected during 48 h periods after a single dosage (100 mg/kg, p.o.) was extracted at pH 9.0 with ethyl acetate (1.5 volume of the urine) before and after the treatment of β-glucuronidase. The extract was dried over anhydrated sodium sulfate and evaporated to dryness. The residue was submitted to thin-layer chromatography and each metabolite of free and conjugated form was detected. As shown in Table I, M-I was excreted both as free and conjugated form, but M-II existed only as conjugated form. A sample of the urine (3.5 l) was collected for 48 h from 5 male animals after each had recieved a single oral dose of 100 mg/kg of nitrazepam. Urine was extracted twice with a 1.5 volume of ethyl acetate to remove free metabolites. The aqueous solution was passed through Amberlite XAD-2 resin column, and then the adsorbed metabolites were eluted with methanol after washing with water, and gel-filtrated through the column of Sephadex G-10. As the conjugates of M-II were eluted with water faster than that of M-I, they were fractionated separately. Each eluted fraction was concentrated by the use of XAD-2 chromatography, as described above, and the conjugates were dissolved in 0.2 *M* acetate buffer (pH 5.0). Then the incubation with β-glucuronidase at 37 °C for 18 h was followed by the extraction with an equal volume of ethyl ether. The ethereal extract of M-I was washed with 0.1 *N* hydrochloric acid and M-I was back-extracted with 0.1 *N* sodium hydroxide, and then extracted with ethyl ether after neutralization with 2 *N* hydrochloric acid. The extract of M-I was dried over anhydrated sodium sulfate and evaporated to dryness, and then recrystallized from ethanol/chloroform, 38 mg of M-I was obtained as orange needles. On the other hand, the ethereal extract of M-II was washed twice with 0.2 *N* hydrochloric acid and washed with 0.1 *N* sodium hydroxide, and then concentrated to a small volume and submitted to the preparative thin-layer chromatography with solvent system [I] in Table I. The zone of M-II was scrapped off the plate and eluted from silica gel with acetone, 12 mg of M-II was obtained as colourless needles after recrystallization from chloroform/hexane.

The Rf values and colour reactions on thin-layer chromatography and the analytical data with elemental analysis and UV-, IR- and mass spectra and melting point of isolated metabolites are presented in Tables I and II. From these data, it was confirmed that M-I was the metabolite [VI] and M-II was 2′-benzoyl-4′-nitro-2-hydroxyacetanilide.

M-I was the major metabolite excreted mainly as the conjugated form in rabbit urine, similarly as in man; it is formed probably by the further hydroxylation of metabolite [V] as similar biotransformation had been reported with metabolism of medazepam[4] and bromazepam[5,6]. A substituted benzophenone, analogous to M-II, has been detected among the transformation products of oxazepam. SINSENWINE et al.[7] have reported that 2′-benzoyl-4′-chloro-2, 2′-dihydroxyacetanilide have been isolated as the urinary metabolite of oxazepam in man and miniature swine. It is considered to be a primary metabolite probably leading to 2-amino-5-chloro-benzophenone. The metabolite is a novel type of benzodiazepine biotransformation product. M-II was assumed to be formed after ring cleavage of the diazepine of nitrazepam, and to be a precusor of metabolite [V].

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