Substrate capacity of chemically esterified oxidized ribonuclease

Compound	Treatment	Methyl-^{14}C incorporated (cpm)
I	None	4,130
II	Partially hydrolyzed[a]	16,450

2 mg of each substrate protein were incubated for 15 min under the standard incubation condition with purified calf thymus protein methylase II. The reaction was terminated with 15% Cl_3CCOOH and radiomethyl incorporated into protein was recovered as previously described[3].

[a] Partial hydrolysis of chemically esterified oxidized ribonuclease was carried out in 0.1 *M* sodium phosphate buffer pH 7.2 at 37 °C for 60 min.

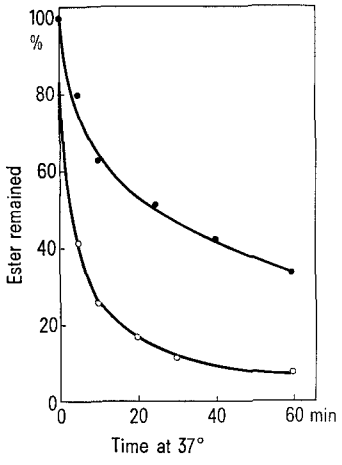

Fig. 3. The rate of hydrolysis of enzymatically esterified oxidized ribonuclease at pH 7.1 and 8.6: The general experimental conditions are the same as in Figure 2. ●—●—●, in 0.1 *M* Na phosphate pH 7.1; ○—○—○, in 0.1 *M tris* HCl pH 8.6.

Substrate capacity of chemically esterified oxidized ribonuclease. The substrate capacity of chemically esterified oxidized ribonuclease is presented in the Table. Compound I, in which 90% of free carboxyl groups was chemically esterified, was incubated at pH 7.2 and 37 °C for 60 min for partial ester hydrolysis. This mild hydrolysis is shown to remove 65% of enzymatically esterified methyl groups (Figure 3). As shown in the Table, when 4000 cpm of methyl groups were enzymatically incorporated into the compound I, 16,450 cpm were incorporated after the hydrolysis into compound II. This increase methylation is due to the selective de-esterification of the substrate protein which was previously esterified non-selectively. These results are consistent with the previous observation in which glycine-methyl ester was used to block free carboxyl groups of the same substrate[5], and thus further confirm that the free carboxyl is the site for protein methylase II, and the methyl-esters formed enzymatically at specific sites are more labile than those formed non-enzymatically as evident from Figure 1.

Discussion. Protein methylase II and 'methanol-forming enzyme' have recently been identified to be the same enzyme[6,12,13]. The enzyme transfers the methyl group of S-adenosyl-L-methionine to substrate protein. This methyl group is unstable in aqueous alkaline solution. Thus the enzymatic product can be recovered either as the protein-methyl ester or as methanol, depending on the assay method[14]. In the present report the comparison of two protein-methyl esters, prepared chemically and enzymatically, clearly shows that the enzymatic product is extremely unstable. This difference may be explained by the possibility that the enzyme modifies only specific residues in the protein and that these residues may be under the influence of the proximal amino acid side chains in a way that would cause instability of the ester bond.

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Kinetic Studies on Soluble and Membrane-Bound Dopamine β-Hydroxylase Isolated from Storage Vesicles of Heart and Adrenal Medulla of Different Species

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Summary. Identical K_m-values for soluble and membrane-bound dopamine β-hydroxylase isolated from adrenal medullary vesicles of different species were obtained; the same holds true for both forms of the enzyme of heart vesicles.

It is well known that dopamine β-hydroxylase (DBH), the enzyme which catalyzes the final step of the noradrenaline biosynthesis, is located within the catecholamine-storing vesicles. It is present in these vesicles in two forms; one of them is readily solubilized by osmotic lysis, while the other appears to be firmly membrane-bound. The distribution of both forms of the vesicular DBH seems to be species-dependent; in the chromaffin vesicles of the bovine adrenal medulla, 40–50% of DBH remains with the membranes after several washes, while in the rat, 80–85% of the DBH remain with the medullary vesicles[2]. The question arises whether these two forms of the DBH are isoenzymes or not. Gel electrophoretic studies of the membrane-bound and soluble DBH of different species showed no differences in mobility between the two forms[3]. According to HÖRTNAGL et al.[4] the amino acid composition of both forms of the bovine adrenal medullary DBH are similar; the same results were obtained by AUNIS et al.[5], while the studies of the amino acid composition of the DBH by CRAINE et al.[6] showed several significant differences. Recently, we have shown[7] that during cold exposure the activity of the soluble DBH of the vesicles of the sympathetic nerve terminals of the rat heart is increased, while simultaneous-

ly the activity of the membrane-bound DBH is decreased. Since it is generally accepted that during nerve stimulation soluble DBH is released from the vesicles by an exocytotic mechanism, we concluded from our results that membrane-bound DBH might have been transformed into soluble DBH to substitute for the losses of soluble DBH. Since no reports exist in which the K_m-values of both forms of the enzyme are compared, we determined the Michaelis-Menten constants of soluble and membrane-bound DBH of the vesicles of the adrenal medulla and heart of different species in order to get more detailed information about the two forms of the enzyme.

Methods. Chromaffin vesicles of the adrenal medulla were obtained by differential centrifugation according to SCHÜMANN[8], and the vesicles of the sympathetic nerve terminals of the heart were prepared as previously described[9]. For the preparation of soluble and membrane-bound DBH, the vesicles were lysed for 30 min with ice-cold water; after centrifugation, the supernatants containing the soluble DBH were carefully decanted. The sediments were homogenized by sonication for 30 sec and diluted; this fraction represents the membrane-bound DBH. For isolation of the total DBH, the vesicles were homogenized by sonication for 30 sec and diluted[7,10]. DBH was determined according to the method of MOLINOFF et al.[11] with at least 5 different concentrations of phenylethylamine (adrenal medulla) or tyramine (heart). The K_m-values were graphically determined according to LINEWEAVER and BURK[12]. Throughout the paper, the values given are means ± SEM of n observations.

Table I. Soluble DBH in percent of the corresponding total DBH of adrenal medullary vesicles

Species	DBH (%)	n	Results of literature (%)
Ox	59.02 ± 3.71	4	50–60[13–15]
Rabbit	33.55 ± 0.24	4	22–52[16,17]
Guinea-pig	32.94 ± 0.37	4	–

Table II. Michaelis-Menten constants for DBH of adrenal medullary vesicles

	K_m ($M \times 10^4$)		
Species	Soluble DBH	Membrane-bound DBH	Total DBH
Guinea-pig	6.52±0.35	5.85±0.19	6.34±0.74
Rabbit	6.19±0.53	6.16±0.24	5.82±0.33
Ox	6.36±0.18	6.63±0.53	6.68±0.61

K_m-values were determined for phenylethylamine at pH 6 according to the method of LINEWEAVER and BURK[12] at an ascorbic acid concentration of 3.9 mM and a fumarate concentration of 40.4 mM and 5 concentrations of substrate ranging from 1.33×10^{-4} M to 2.67×10^{-3} M. Each K_m-value represents the mean ± SEM of the intercepts generated from 4 separate lines.

Table III. Michaelis-Menten constants for DBH of heart vesicles

	K_m ($M \times 10^4$)		
Species	Soluble DBH	Membrane-bound DBH	Total DBH
Rat	2.02±0.15	2.16±0.11	2.62±0.42
Rabbit	2.75±0.73	2.24±0.20	2.40±0.21

K_m-values were determined for tyramine at pH 6 as described in the legend to Table II. The concentrations of tyramine ranged from 1.18×10^{-4} M to 2.35×10^{-3} M.
Given are the means ± SEM of 4 different experiments.

Results and discussion. The percentage degree of the adrenal medullary soluble DBH ranged from 30–60% for the 3 species studied (Table I). There was no significant difference in the apparent K_m-values for the soluble and membrane-bound as well as for the total DBH of the three species (Table II). The apparent K_m-value of the adrenal medullary DBH (substrate: phenylethylamine at pH 6) amounted to about 6×10^{-4} M. Similar values were found by MOLINOFF et al.[11] for the total DBH of the rat stellate ganglion, and by FILLENZ and WEST[18] for the total DBH of the rat heart vesicles.

We have recently shown[7] that, in the vesicles of the sympathetic nerve terminals of the rat heart, about 17% of the DBH are soluble. The same holds true for the vesicles of the rabbit heart, which contain 14.02 ± 0.79% (n = 5) of the DBH in soluble form. The apparent K_m-values for the soluble, membrane-bound and total DBH are not significantly different for the two species (Table III). They amounted to about 2×10^{-4} M (substrate: tyramine at pH 6) for the DBH of the heart vesicles. These results show that tyramine in vitro is a better substrate for the DBH than phenylethylamine is, which is in accordance with observations of CREVELING et al.[19]. A K_m-value of about 2×10^{-4} M for tyramine was also described by WINKLER et al.[15] for the membrane-bound DBH of the bovine adrenal medulla.

The properties of the soluble and membrane-bound DBH of the bovine adrenal medulla have been studied

[1] The excellent technical assistance of Miss SABINE KLEMT is gratefully acknowledged.

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extensively. As discussed above, the amino acid compositions as well as the electrophoretic behaviour of both forms are not different, indicating that the two forms of the enzyme are identical. However, only few data are known about the properties of the medullary DBH of other species. Ross et al.[3] showed that DBH of crude homogenates of tissues from ox, man and rat exhibited two electrophoretically distinguishable peaks of activity. The slow-moving peak, which has been eliminated prior to electrophoresis by high-speed centrifugation, could be converted into the faster migrating peak, suggesting that this peak is corresponding to the membrane-bound DBH. Although the mobility of the main peaks from human, rat and bovine adrenal medulla differed somewhat, the K_m-values for all species were similar. This is in agreement with our results. We found no statistically significant differences between the K_m-values of the soluble and membrane-bound as well as the total DBH of the species studied. The K_m-values for tyramine are lower than those for phenylethylamine, since tyramine has a higher affinity to the DBH than phenylethylamine has. The present results strongly support the view that soluble and membrane-bound DBH are identical enzymes. However, the physiological significance of the separation of DBH into soluble and membrane-bound form is not known so far.

Na, K-ATPase in the Salivary Gland of the Ixodid Tick *Amblyomma hebraeum* (Koch) and its Relation to the Process of Fluid Secretion[1]

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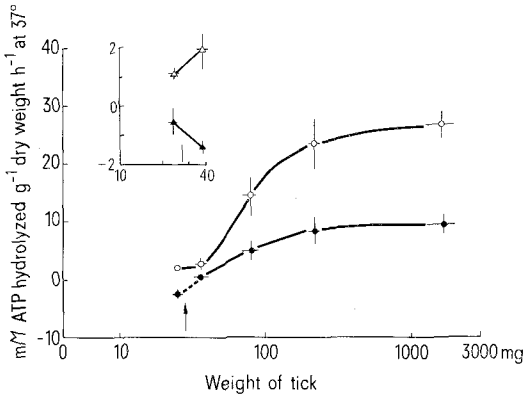
Summary. Total and ouabain-sensitive ATPase activities were determined in the salivary glands of ticks throughout the feeding cycle. Activities were very low in unfed specimens. In the glands of feeding females, the activities rose until a maximum was reached for both ATPase components at approximately 200 mg. The activities remain low in males throughout the feeding period. These findings are discussed in relation to the fluid secretory process of the salivary glands.

A common characteristic among haematophagous arthropods is the elimination of excess fluid taken in with the blood meal. Whereas in insects such as the bug, *Rhodnius*, the Malpighian tubules play the fundamental role in fluid excretion[2], in female ixodid ticks the salivary glands function in this capacity[3,4]. KAUFMAN and PHILLIPS[5] demonstrated that salivary secretion in females depended on active solute transport and that it could be inhibited by very low concentrations of ouabain. It was also shown that sodium and potassium were necessary in specific ratios for fluid secretion to proceed at a maximal rate, suggesting that an Na, K-ATPase was an important component of the secretory mechanism. KAUFMAN[6] demonstrated that salivary glands from unfed female ticks could secrete fluid only at a slow rate. Salivation augmented with increased tick weight, but the enhancement was not attributable merely to hypertrophy of the glands. The present paper confirms the existence of a Na, K-ATPase in the salivary gland of the female *Amblyomma hebraeum* (Koch); activity of this ATPase increased with time spent by the tick on its host. A similar Na, K-ATPase could not be detected in salivary glands of males.



ATPase activity in salivary glands of *A. hebraeum* plotted against logarithm of tick weight. Data for females are depicted in the main graph, those for males in the inset. Females: ○, total ATPase; ●, Na, K-ATPase. Males: △, total ATPase; ▲, Na, K-ATPase. Points appearing to the left of the arrow in each graph are for unfed specimens, those appearing to the right are for fed specimens. Horizontal and vertical bars denote SE of the mean weight and mean activity respectively, when the SE exceeds the dimension of the point. The curves have been fitted by eye. Ouabain slightly stimulated ATP hydrolysis by homogenates of unfed female glands and by unfed and fed male glands, hence the negative values for Na, K-ATPase activity in these cases. Whereas the female tick may attain a replete: unfed weight ratio approaching 100 after a normal 7 to 10 day feeding period, the male barely doubles its weight over the same time.

Material and methods. Unfed adult ticks were placed on the backs of rabbits[4] and specimens were removed at various stages of the feeding period. Salivary glands were dissected out under Hank's balanced saline (composition in g/l: 8.0 NaCl; 0.4 KCl; 0.14 $CaCl_2$; 0.06 KH_2PO_4; 0.98 $MgSO_4$; 0.048 Na_2HPO_4; 1.6 D-glucose; 0.01 phenol red) and non-salivary tissue including most of the tracheae were carefully dissected away. Preparation of the crude enzyme was essentially according to BONTING[7]. Each pair of glands was transferred to a small glass homogenizer containing 0.1 ml (equivalent to 4 or 5 volumes of tissue) of the homogenizing medium (1 m*M Tris* in distilled water adjusted to pH 7.5). The piston

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