adrenal activation as long as 6–8 h post-injection. The absence of significant elevations in corticosterone with 40 μg/kg soman may be attributable to the early occurrence of death and time required for stimulation of the hypothalamo-pituitary-adrenal axis. It is significant in this regard that the augmentation of plasma corticosterone evidenced with 30 μg/kg is of approximately equal magnitude (about 5-fold) to that occurring with exposure to OP-insecticides or various other forms of stress[2]. Successful adrenal activation in the presence of diminished proteo-synthetic capacity may be a manifestation of the magnitude and/or nature of specific metabolic impairments. Adrenocortical cell RNA and protein depletion was not more severe with 40 μg/kg than with 30 μg/kg (table 1), although the latter produced near-maximal adrenal activation. Both RNA and protein synthesis are required for continued expression of the ACTH-induced secretory response, but severe inhibition of macromolecular synthesis is required to diminish actual corticoid secretion; moreover, the acute steroidogenic response does not appear to require newly synthesized RNA or protein[16,17]. Additional studies are required to determine whether soman-induced impairments in adrenal metabolism translate into diminished corticoid secretion in vivo during later stages of intoxication.

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Inhibition of sarcolemmal Na, K, Mg ATPase from the guinea pig heart is not compatible with a homogeneous population of non-interacting ouabain receptors[1]

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Summary. The inhibition of sarcolemmal Na, K, Mg ATPase from the guinea pig heart by ouabain was evaluated with a coupled enzyme assay. Models of negative cooperativity and of two independent receptors fitted the inhibition data equally well. The analysis was not compatible with a homogeneous population of non-interacting ouabain receptors.
Key words. Na, K, Mg ATPase; ouabain; negative cooperativity; multi-site ouabain binding.

In various preparations exhibiting Na, K, Mg ATPase activity two ouabain binding sites were shown repeatedly[2–6]. However, it has been questioned whether both sites contribute to the inhibition of the Na pump, since in the rat heart only the site with the low affinity correlated with inhibition of ^{86}Rb uptake[6,7]. Recently we described two ouabain binding sites in the intact guinea pig papillary muscle[8]. Occupation of either site with ouabain elicited a positive inotropic effect. The common cause of the inotropic effects was proposed to be the inhibition of the Na pump. It was, therefore, of interest to evaluate whether inhibition of Na, K, Mg ATPase from the guinea pig heart by ouabain would be compatible with different ouabain binding sites of the enzyme.

Materials and methods. Sarcolemmal vesicles were prepared from the hearts of five guinea pigs (circa 150 g) by a procedure similar to that of Jones et al.[9]. After excision the hearts were cooled immediately in a buffer containing 0.75 moles/l KCl and 5 mmoles/l histidine, pH 7.4, 4 °C. After the connective tissue had been removed, the ventricles (circa 3 g) were minced in 10 volumes of the buffer with a Waring Blendor 8011 C at full speed. The disrupted tissue was further homogenized with a Potter Elvehjem S (three times for 10 s). This homogenate was then passed through four layers of gauze. The filtrate was at first centrifuged at 500 × g for 5 min to remove the large fragments, then at 14,000 × g for 20 min. The resulting pellet was resuspended in 80 ml of the above medium, and resedimented at the same speed. The pellet was washed similarly at first in 80 ml of a buffer containing 10 mmoles/l $NaHCO_3$ and 5 mmoles/l histidine, pH 7.4, then in 80 ml of 3 mmoles/l Tris buffer, pH 7.0. Thereafter the contractile proteins were extracted with 0.4 moles/l LiBr in 10 mmoles/l Tris, pH 8.2 at 0 °C for 60 min, maintaining a ratio of 2 mg protein/mmole LiBr. After a threefold wash at 500 g for 20 min the last pellet with the Na, K, Mg ATPase activity (0.21–0.47 μmoles/min/mg protein) was stored in Tris buffer at a concentration of 2.5 mg protein/ml. The yield of sarcolemmal vesicles was 2 mg per g wet wt. of cardiac tissue. Cytochrome-c-oxidase and glucose-6-phosphatase activity of the preparation were 3- and 10-fold lower than in a mitochondrial or microsomal preparation. These enzyme activities indicate low contamination of the sarcolemmal membranes with subcellular organelles. Protein was determined by the method of Lowry et al.[10].

Na, K, Mg ATPase activity was determined by a method similar to the coupled enzyme assay of Schwartz et al.[11]. The reaction medium contained, in a final volume of 2.6 ml, 5.9 mmoles/l KCl, 140 mmoles/l NaCl, 5 mmoles/l $MgCl_2$, 5 mmoles/l NaN_3,

50 mmoles/l Tris, 3 mmoles/l Tris ATP (Merck, Darmstadt), 0.5 mmoles/l phosphoenolpyruvate (tricyclohexylamine salt; Boehringer, Mannheim), 0.26 mmoles/l NADH (disodium salt; Boehringer, Mannheim), 5.6 U pyruvate kinase and 8 U lactate dehydrogenase (PK/LDH mixture; Sigma); pH 7.5, 35 °C. After preincubation for 3 min the reaction was started by the addition of 10 µg of the enzyme protein. The Na, K, Mg ATPase activity was monitored at 334 nm. Ouabain was added after 10 min.

Results and discussion. The enzyme activity is proportional to the concentration of the enzyme-substrate complex when the equilibrium of enzyme catalysis is established. The linear reduction of a substrate with time indicates this condition. In the coupled enzyme assay of Na, K, Mg ATPase the control activity of the enzyme remained roughly constant as extinction declined linearly with time (fig. 1). The stability of the preparation excluded any artifacts which might be due to a time-dependent deterioration of the enzyme[12]. When ouabain was added after 10 min preincubation, the onset of its inhibitory effect was earlier and its extent greater with the higher concentrations. However, from t = 20 min on, i.e., 10 min after the adding of ouabain, all time courses were approximately linear. So we selected the slope between 20 and 25 min as the measure of enzyme activity. When the individual data on enzyme activity, as a percentage of the control value of the respective sarcolemmal preparation, were plotted in dependence on ouabain concentration (fig. 2), the inhibitory effect was found to extend over several decades of molar ouabain concentration. This flat curve suggests that ouabain binding to Na, K, Mg ATPase might differ from a simple bimolecular reaction. Two models could possibly account for this observation 1) negative cooperativity, i.e., interdependent binding sites, or 2) at least two independent sites. To examine the first assumption we fitted to the data a modified Hill equation[13]

$$\text{Effect} = 100 - K_1 A^{K_2}/(A_2^{K_2} + K_3^{K_2}); \quad (1)$$

100 = control activity in the absence of ouabain; K_1 = maximal inhibition of ouabain as a percentage of the control; K_2 = parameter related to the steepness of the curve (Hill coefficient); K_3 = concentration for half-maximal inhibition; A = ouabain concentration. With the Gauss-Newton method we computed the following estimates ± SE; $K_1 = 89.2 \pm 7.8\%$, $K_2 = 0.58 \pm 0.11$, $K_3 = 1.95 \pm 0.8$ µmoles/l ouabain. The broken line in figure 2 reflects this fit. The estimate of K_1 showed that about 90% of Na, K, Mg ATPase activity could be inhibited by ouabain. The value of K_2 indicated a Hill coefficient which was significantly lower than unity (95% confidence intervals 0.36–0.80). A Hill coefficient of 1 would have been expected for a homogeneous population of non-interacting ouabain receptors, whereas the actual value suggested negative cooperativity of the binding sites.

Subsequently we tested the model of two independent binding sites with

$$\text{Effect} = 100 - K_1 A/(A+K_2) - K_3 A/(A+K_4); \quad (2)$$

100 and A have the same meaning as in equation 1; K_1 = maximal inhibition due to site I; K_2 = concentration for half-maximal inhibition at site I; K_3 and K_4 refer to the respective parameters of site II. Again the fitting procedure converged, yet at the expense of estimation precision as indicated by the huge error of K_4. The numerical values of the estimates were: $K_1 = 43.8 \pm 17.0\%$, $K_2 = 0.30 \pm 0.25$ µmoles/l ouabain, $K_3 = 40.8 \pm 14.9\%$, $K_4 = 9.35 \pm 9.45$ µmoles/l ouabain. The solid line in figure 2 represents the fit according to equation 2. Obviously, the two curves in figure 2 (broken vs solid line) are very similar. Indeed, values for the goodness of fit according to equations 1 or 2 did not differ significantly from each other (F-test). From a statistical point of view it appeared, therefore, that the more flexible model (four parameters of eq. 2 instead of 3 parameters of eq. 1) was not required for an adequate description of the experimental results. In addition the huge error of K_4 indicates that the pertinent information is but sparsely present in the data. The simpler model, i.e., negative cooperativity, would thus be preferable. Although it is clear that such a decision between the two models can finally be made only on the basis of experimental arguments, the data, nevertheless, support the idea that the reaction of ouabain with a homogeneous population of non-interacting receptors on Na, K, Mg ATPase is incompatible with the inhibition of the enzyme. Our binding data in the intact guinea pig papillary muscle are thus corroborated by the enzymatic analysis of the sarcolemmal particles.

In contrast to the present findings a concentration-effect curve of ouabain inhibition with a slope near unity was detected when the enzyme activity was evaluated from the amount of inorganic phosphate released from ATP after 15 min of incubation (Fiske-Subbarow[14] method; own unpublished results). Conceivably the contribution of pre-steady-state effects obscured ouabain inhibition under these conditions. Also, in preparations from rat heart, the results were quite similar[15]. Only with ^{32}P-ATP or with the coupled enzyme assay, the inhibition of Na, K, Mg ATPase

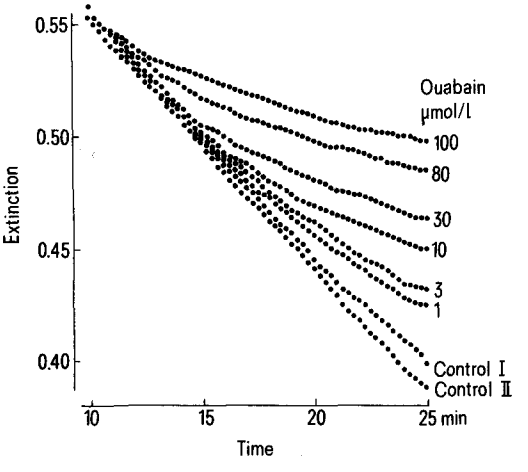

Figure 1. The time course of ouabain inhibition of sarcolemmal Na, K, Mg ATPase. As an example, the time course of ouabain inhibition of the enzyme activity is shown with the data obtained from one single sarcolemmal preparation. Na, K, Mg ATPase activity was assayed with the coupled enzyme test. The reaction was started at t = 0; ouabain was added at t = 10 min. Control I refers to ouabain concentrations ≥ 10 µmoles/l, control II to the lower concentrations. Ordinate: extinction at 334 nm wavelength. Abscissa: time in min.

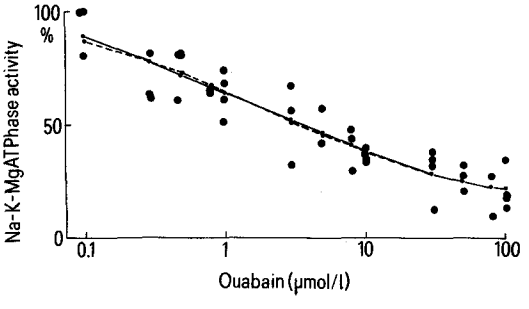

Figure 2. The inhibition of sarcolemmal Na, K, Mg ATPase in dependence on ouabain concentration. Shown are enzyme activities as determined between the 20th and 25th min of incubation, i.e. the 10th and 15th min after the addition of ouabain. Individual data. The broken and the solid line represents the fit according to equation 1 (negative cooperativity) and equation 2 (two independent receptors), respectively. Ordinate: Na, K, Mg ATPase activity as percent of the respective control, 100% = 0.34 ± 0.04 µmoles/min/mg protein (n = 7). Abscissa: ouabain concentration, µmoles/l, log scale.

by ouabain could be resolved into two phases. But in contrast to our results particularly the contribution of the 'high-affinity' site appeared to be reduced in the rat. Therefore, presumably, the 'two-independent-receptors' hypothesis proved statistically superior over the assumption of negative cooperativity in the analysis of Noel and Godfraind[15]. Evidence in favor of heterogeneous inhibition of Na,K,Mg ATPase by ouabain was also presented by Mansier and Lelievre[16]. Perfusion of rat hearts with Ca-containing or Ca-free solutions before homogenization revealed a differential effect of ouabain on enzyme activity. The findings suggested the existence of either two isozymes, in accordance with previous results on brain particles[17], or alternatively, of one class of enzyme molecules with a Ca-dependent, differential sensitivity to ouabain.

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Immobilization of chicken liver fructose 1,6-bisphosphatase on CNBr-activated Sepharose[1]

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Summary. Chicken liver fructose 1,6-bisphosphatase is readily immobilized on CNBr-activated Sepharose. The immobilization alters some enzymatic properties. They include broader pH activity curve, loss of activation by K^+ or NH_4^+, increased resistance to inactivation by trypsin, decreased sensitivity to AMP inhibition, and loss of cooperative interaction among AMP-binding sites. The immobilized enzyme retains about 38% or 19% of the specific activity of the native enzyme when the activity is measured in the absence or presence of K^+, resepctively.
Key words. Immobilization; chicken liver fructose 1,6-biphosphatase; CNBr-activated Sepharose.

Fructose 1,6-bisphosphatase (Fru-P_2ase, EC 3.1.3.11) was first discovered in rabbit liver by Gomori in 1943[2]. It has since then been shown to play a key role in gluconeogenesis. During the past two decades, Fru-P_2ases from many sources have been extensively investigated. However, research on immobilization of Fru-P_2ase has attracted little attention. In 1973, Falb et al.[3] reported the attachment of 'alkaline' form of rabbit liver Fru-P_2ase to aminoethyl cellulose via glutaraldehyde. This enzyme polymer adduct retained only about 1% of the specific activity of the native enzyme. These researchers gave no information about the properties of this immobilized enzyme except that the pH optimum was shifted from 9.3 to 8.5. In this communication, we report some properties of chicken liver Fru-P_2ase immobilized on CNBr-activated Sepharose.

Materials and methods. CNBr-activated Sepharose 4B and 6MB, trypsin from bovine pancreas, and other chemicals used in this study were all purchased from Sigma Chemical Company. Before use, activated Sepharose (1 g) was swollen in 100 ml of distilled water. It was then washed for 15 min on a sintered glass filter with 300 ml of 1 mM HCl. The gel was washed thoroughly with 0.1 M $NaHCO_3$ solution (pH 7.9) containing 0.5 M NaCl and 0.2 mM EDTA. Fru-P_2ase was purified from chicken liver by the procedure previously described[4]. The concentration of the purified enzyme was determined by the extinction coefficient at 280 nm ($E^{0.1\%}_{1\,cm} = 0.71$) or by the method of Lowry et al.[5] using purified Fru-P_2ase as standards.

The activity of Fru-P_2ase was assayed by measuring the release of P_i according to the method of Tashima and Yoshimura[6]. Unless otherwise indicated, the reaction mixture (1.0 ml) contained 25 mM triethanolamine/25 mM diethanolamine-HCl buffer (pH 7.5), 1.5 mM $MgCl_2$, 1 mM cysteine, 0.1 mM EDTA, 0.05 mM fructose 1,6-bisphosphate (Fru-1,6-P_2), and an appropriate amount of Fru-P_2ase (native or immobilized). The reaction was initiated by the addition of Fru-1,6-P_2 and was carried out with gentle shaking at 25 °C. It was terminated after 5 min by the addition of the color developing agent[6]. After 15 min, the absorbance at 650 nm was determined and compared to values obtained with P_i standards. In monitoring the Fru-P_2ase activity in the supernatant fluid during immobilization reaction (see below), a continuous spectrophotometric assay was used. The reaction mixture was the same as described above except that 0.2 mM NADP and 1 unit each of phosphoglucose isomerase and glucose-6-P dehydrogenase were additionally included. Fru-P_2ase activity was determined by following the rate of formation of NADPH at 340 nm.

Immobilization of Fru-P_2ase on CNBr-activated Sepharose 4B or 6MB was carried out as follows: moist Sepharose (equiv. 1 g dry wt) was added to 5 ml of 0.1 M $NaHCO_3$ solution (pH 7.9) containing 0.5 M NaCl, 0.2 mM EDTA, 4 mM Fru-1,6-P_2, and 2.0 mg of purified enzyme. The reaction mixture was incubated at 25 °C with gentle shaking for 30 min and the fluid was removed by vacuum filtration (filtrate I). To remove the trace of