Demonstration of a Na^+-K^+-Mg^{2+} Dependent ATPase in a Preparation from Hindgut and Malpighian Tubules of two Species of Insect

The physiological characteristics of ion transport in a variety of tissues, from many animal species, is now well documented, and has led to the identification and isolation of a membrane enzyme, an ATPase, which has been implicated in the active transport of sodium and potassium ions[1,2]. The biochemical characteristics of this enzyme are that it requires ATP as a substrate together with magnesium ions, and the simultaneous presence of Na^+ and K^+ for maximal activity. Furthermore the activity due to Na^+ and K^+ is abolished by ouabain[2].

Malpighian tubules and insect rectum have long been known to be involved in the transport of monovalent cations, the physiological characteristics of these transport systems have recently been reviewed[3-6]. In summary a net transfer of K^+ occurs from the haemolymph to the lumen of the Malpighian tubules[3,4], and an active resorption of these ions[4,7] and possibly water[6,8], takes place from the rectum. By comparison with other tissues one would therefore expect these tissues also to have cation pumps associated with their plasma membranes. BERRIDGE[9] has indeed suggested the basal membranes of Malpighian tubule cells possess a Na^+-K^+-Mg^{2+} ATPase and the apical membranes a potassium stimulated electrogenic ATPase. However, in the same paper he reports that ouabain had no effect on urine formation by Malpighian tubules, which would suggest that the basal membranes do not possess a coupled Na^+-K^+-Mg^{2+} ATPase. The only demonstration of membrane ATPases in these tissues is from electron histochemical techniques, and a Mg^{2+} ATPase was found in both basal and apical plasma membranes in the rectal papillae of the blowfly[10]. The purpose of this communication is to show that a characteristic coupled Na^+-K^+-Mg^{2+} ATPase (E.C. 3.6.1.3) is present in a membrane preparation obtained from hindgut and Malpighian tubule cells of 2 species of insect, *Schistocerca gregaria* and the tettigoniid *Jamaicana flava*.

The hindguts and Malpighian tubules were quickly dissected out from about 12 animals and placed in homogenization medium, which was either 250 m*M* mannitol, 5 m*M* $MgCl_2$, 10 m*M* EDTA, 0.1% sodium deoxycholate in 30 m*M* histidine-HCl, pH 7.2; or 0.25 *M* sucrose, 2 m*M* EDTA in 30 m*M* histidine-HCl, pH 7.2. The guts were cut open longitudinally and washed free from gut contents. They were transferred to a fresh 10 ml of the appropriate homogenization medium. Homogenization was carried out in a Potter-Elvehjem homogenizer with a tephlon pestle (clearance 0.1–0.15 mm) with 10 passes of the plunger at 1,000 rpm, the homogenizer was surrounded by ice throughout this procedure. A microsomal pellet was obtained from the sucrose medium homogenate following the techniques which have been used for mammalian brain[11,12] and crayfish muscle[13] in our laboratory. The deoxycholate medium homogenate was treated differently, it was extracted with sodium iodide following the method of NAKAO, TASHIMA, NAGANO and NAKAO[14]. This extract was then spun at 50,000 *g* for 30 min at 0°C using a MSE Automatic Superspeed 40 head No 2409. The pellet was discarded and the supernatant centrifuged at 100,000 *g* for 60 min. The resulting pellet was resuspended in 10 ml of a washing medium (5 m*M* NaCl + 1 m*M* EDTA) and recentrifuged at 100,000 *g* for 60 min. This washing procedure was repeated twice more. The resulting pellet (from both extractions) was resuspended in about 10 ml of neutralized deionized water.

The reaction media used were as follows: 1. 4 m*M* Mg^{2+}; 2. 4 m*M* Mg^{2+} + 100 m*M* Na^+; 3. 4 m*M* Mg^{2+} + 20 m*M* K^+; 4. 4 m*M* Mg^{2+} + 100 m*M* Na^+ + 20 m*M* K^+, all buffered in 50 m*M* histidine-HCl, pH 7.2. ATP (*tris* salt) was added to the reaction medium to give a final concentration of 2 m*M*. The reaction was started by adding 0.5 ml of homogenate. The inhibitors ouabain, ethacrynic acid and Rontyl (hydroflumethiazide) when used were added to the appropriate salts solution to give a final concentration of 10^{-3} *M*. The reactions were carried out at 30°C, and were stopped by the addition of 4 ml of a mixture of 1% lubrol and 1% ammonium molybdate in

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ATPase activity of microsomal preparations obtained from Malpighian tubules and hindgut from the locust *Schistocerca gregaria* (Expts. I–III) and the tettigoniid *Jamaicana flava* (Expts. IV–V)

Experiment	4 m*M* Mg^{2+}		4 m*M* Mg^{2+} + 100 m*M* Na^+		4 m*M* Mg^{2+} + 20 m*M* K^+		4 m*M* Mg^{2+} + 100 m*M* Na^+ + 20 m*M* K^+				Activity in presence of Mg^{2+} + Na^+ + K^+ minus Mg^{2+}	Activity in presence of Mg^{2+} + Na^+ + K^+ minus activity in Mg^{2+} + Na^+ + K^+ + ouabain
No.	Ouabain		Ouabain		Ouabain		Ouabain		Rontyl	Ethacrynic acid		
	−	+	−	+	−	+	−	+				
I	29.3	29.0	21.3	21.7	29.2	27.9	25.7	20.4	—	—	− 3.6	+ 5.3
II	5.1	—	—	—	—	—	71.9	4.6	58.8	49.1	+66.8	+67.3
III	8.3	—	6.2	—	8.3	—	71.5	7.6	70.9	—	+63.2	+63.9
IV	11.1	—	13.4	—	11.3	—	26.0	11.2	—	—	+14.9	+14.8
V	15.1	—	13.3	—	15.5	—	33.9	15.2	—	25.9	+18.8	+18.7

Experiment I isolated in sucrose medium; experiments II–V isolated in mannitol and extracted with NaI. Activities expressed in nmoles P liberated/mg protein/min. Ouabain, ethacrynic acid and Rontyl are present at 10^{-3} *M*.

1.8N H_2SO_4[15]; any protein which precipitated was removed by centrifugation. The tubes were then left at room temperature for 10 min to allow the yellow colour to develop. The colour which developed was read at 390 nm. Protein determinations were made by the method of LOWRY, ROSEBROUGH, FARR and RANDALL[16], using bovine serum albumen Fraction V as a standard.

All solutions were made up in glass distilled water. All inorganic salts were present as chlorides and were AnalaR grade. Histidine, ATP, BSA and ouabain were obtained from Sigma Chemical Co. It is a pleasure to acknowledge the gifts of Rontyl (E. R. Squibb and Sons), ethacrynic acid (Merck, Sharpe and Dohme) and Lubrol (I.C.I. Dyestuffs Division).

The results are shown in the Table. Considering first the preparation obtained by the extraction method developed for nervous tissue (Expt I). These results show that the preparation was stimulated by Mg^{2+} alone, being unaffected by the further addition of K^+ and slightly inhibited by the addition of Na^+ (about 25%). This basic Mg^{2+} ATPase activity is also reduced (about 10%) when both sodium and potassium ions are present. It would seem that no synergystically stimulated Na^+-K^+-Mg^{2+} ATPase is present in this preparation; however that addition of ouabain further inhibits the enzymic splitting of ATP when both Na^+ and K^+ are present, although it did not affect enzyme activity when only one of the monovalent cations is present. Thus it can therefore be inferred that a ouabain sensitive component is present (i.e. a Na^+-K^+-Mg^{2+} ATPase). It has a fairly low activity of 5.3 nmoles Pi/mg protein/min in comparison with the Mg^{2+} ATPase which was 29.3 nmoles Pi/mg protein/min. Similar results have been obtained in our laboratory from studies on insect muscle (Wareham, A.C., unpublished) and mammalian muscle (Radcliffe, M.A., and Parkin, A.C., unpublished) and can be explained by sodium ion inhibition of the basic Mg^{2+} ATPase activity. Such inhibition would mask the coupled stimulation when both Na^+ and K^+ are present if the Na^+-K^+-Mg^{2+} ATPase proportion of the total ATPase activity is relatively small.

In the second series of experiments (II-V) in which the sodium iodide extraction method was used a very different picture emerges. This technique has been shown to proportionally increase the activity of the Na^+-K^+-Mg^{2+} ATPase as compared with the Mg^{2+} ATPase in other preparations[14], and has produced the same effect in our work. This is most obvious in the *Schistocerca* preparations, where the basic Mg^{2+} ATPase is about 5–10 nmoles Pi/mg protein/min and the activity stimulated by the addition of Na^+ and K^+ is about 65 nmoles Pi/mg protein/min. The basic Mg^{2+} ATPase activity is not stimulated by the addition of either monovalent cation alone. Similar results have also been obtained with *Jamaicana* although the stimulation due to Na^+ and K^+ is only about 100%. In both cases, however, this stimulated activity was completely abolished in the presence of $10^{-3}M$ ouabain. This preparation would therefore seem to fulfill the requirements of a classical Na^+-K^+-Mg^{2+} ATPase, that is it is synergystically stimulated by sodium and potassium and inhibited by ouabain. It is notable that ethacrynic acid, which is not such a potent inhibitor as ouabain[17–19], inhibits the stimulated activity by only about 30%, that being about the same as described for kidney cortex[19]. The addition of Rontyl, a carbonic anhydrase inhibitor, is even less potent, and in 1 of the 2 experiments where it was used it caused no inhibition.

It is tempting to speculate that the differences in the proportion of the Na^+-K^+-Mg^{2+} ATPase activity to the basic Mg^{2+} ATPase in the preparations from *Schistocerca* and *Jamaicana* may well reflect the known differences in the ability of the 2 species to retain water. The locust is suited to live in dry conditions and has a highly efficient means of water retention. By contrast *Jamaicana* requires a regular supply of water in the diet and presumably is less well able to resorb water.

Zusammenfassung. Die Präsenz einer charakteristischen Na^+-K^+-aktivierbaren ATPase (E.C. 3.6.1.3) wurde erstmals in einer mikrosomalen Fraktion des Enddarmes und der malpighischen Gefässe bei der Wüstenschrecke *Schistocerca gregaria* und der Laubheuschrecke *Jamaicana flava* nachgewiesen.

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Enzymic and Behavioural Changes Induced in Mice Fed Polychlorinated Biocides Followed by Starvation

Polychlorinated biocides are mainly stored in fat tissue[1], but a certain amount is metabolized by the microsomal cytochrome P-450 complex[2] which is sensitive to dietary factors, i.e. ethanol[3,4] and starvation[4]. These factors exert either a protracted or a more acute influence. Since most studies on the activity of the P-450 complex due to deficiency states have concerned permanent or substantial lack of one or more dietary factors[4,5], the present communication intends to relate previous intake of polychlorinated biocides to a syndrome following acute starvation of the animals.

Material and methods. Two-month-old female mice (33g) were divided into groups and fed ad libitum for 7 days on a diet optimal in all respects: group 1: without any additions, group 2: was fed the diet of group 1 containing 1500 ppm DDT and group 3: fed the diet of group 1 but containing 1500 ppm γ-hexachlorocyclohexane. After 7 days feeding half of the animals of group 1 and 2 were starved for 4 days. The hexachlorocyclohexane-fed mice were killed without starvation after 7 days feeding due to high mortality (40%).

Chemicals: Glucose-6-phosphate and NADP were from Boehringer, Germany. DDT (dichloro-diphenyltrichloroethane) was from Struers Co., Copenhagen, γ-hexachlorocyclohexane (Lindan) was from Ferrosan Ltd., Copenhagen. Before use, these 3 substances were recrystallized twice from ethanol. All other chemicals were of highest obtainable purity from British Drug Houses, Poole, U.K.

Methods: Animals were killed by a blow on the neck and cytoplasmic fraction of livers was obtained as previously described[3]. The activity of the cytochrome P-450