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Increment in Sodium and Potassium Dependent Adenosine Triphosphatase of Brain Microsomal Fraction from Rats Treated with the Cholesterol Biosynthesis Inhibitor AY 9944

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SUMMARY

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Na^+, K^+-ATPase activity of brain microsomal fractions prepared from rats treated with the cholesterol biosynthesis inhibitor AY 9944 was found to be markedly increased when compared with nontreated control fractions. Both control and AY 9944-treated preparations increase their Na^+, K^+-ATPase activity with increasing temperatures; such increments were higher in the microsomal fractions prepared from the AY 9944-treated rats than those from the untreated controls. The apparent K_m calculated for Na^+, K^+ and Mg^{2+}, as well as the optimal ratio Mg^{2+}/ATP of the Na^+, K^+-ATPase from both microsomal fractions, were found to be the same in each case. The binding of [^{3}H]-ouabain as a function of time, as well as a function of the incubation temperature, was also the same in both preparations. If it is considered that treatment with AY 9944 results in the partial substitution of cholesterol by 7-dehydrocholesterol without modifying any other constituent in the microsomal membranes, the observed increment in the Na^+, K^+-ATPase activity could be interpreted as the result of an increased membrane "fluidity" and shows that Na^+, K^+-ATPase activity is dependent upon the environmental sterol composition.

INTRODUCTION

In mature mammalian brain, sterols are chiefly represented by nonesterified cholesterol (5-cholestan-3β-ol). The function of cholesterol as a prominent lipid constituent of many biological membranes is being intensively studied in several laboratories; however, this function has not yet been clarified (1). It is known that cholesterol forms a complex with phospholipids, having a substantial influence on the permeability properties of lipid bilayers (2, 3) and cell membranes (4).

The experimental models of administering such drugs as triparanol, 20,25-diazacholesterol and AY 9944, which block cholesterol biosynthesis, are being increasingly used to assess the role of cholesterol in the functional and morphological aspects of biological membranes. The compound AY 9944 is known to be an inhibitor of the Δ^7-reductase resulting in an accumulation of 7-dehydrocholesterol (5, 7-cholestadien-3β-ol) (5, 6). AY 9944 given to rats has been

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reported to cause a substantial accumulation of 7-dehydrocholesterol in all brain and spinal cord membrane fractions including myelin (7). These, as well as other experiments carried out in erythrocyte ghosts (8), indicate that sterols other than cholesterol can be incorporated into cellular membranes in place of cholesterol. Furthermore, erythrocyte ghosts do not discriminate between cholesterol and 7-dehydrocholesterol. From these observations, it has been suggested that 7-dehydrocholesterol can substitute for cholesterol in membranes without causing obvious damage or functional alterations in the membrane properties (8).

At the electron microscope level, however, myelin sheaths as well as endoplasmic reticulum of young animals treated with AY 9944 show morphological differences when compared with nontreated control animals. These inhibitors also retard the rate of nervous system development and result in the proliferation of abnormal membranous intracytoplasmic inclusions *in vivo* and *in vitro* (9–15). Brain and spinal cord slices from rats pretreated with AY 9944 showed decreased uptake of ^{14}C, from [*U*-^{14}C]glucose, into lipids and proteins obtained from both myelin and nonmyelin fractions (16). Therefore, although 7-dehydrocholesterol is able to substitute for cholesterol, it does not seem as efficient as cholesterol for the formation and maintenance of normal function in biological membranes. Thus, a membrane with decreased cholesterol or altered sterols within its structure may be functionally different from a normal membrane.

The present study deals with the *in vivo* effect of AY 9944 on the specific Na^+, K^+-ATPase of brain microsomal fraction. It was found that this enzyme system, which is involved in the active transport of Na^+ and K^+ across the cell membranes (17), shows an increased specific activity (amount of hydrolysis/mg prot/unit time) when compared with control animals. To our knowledge, this communication constitutes the first report that altered cholesterol biosynthesis induces an increment in the activity (amount) of the brain microsomal Na^+, K^+-ATPase complex.

MATERIALS AND METHODS

Sprague-Dawley rats were given by intraperitoneal injection 20 mg/kg of AY 9944: |trans-1,4-bis (2-chlorobenzylaminomethyl)| cyclohexane dehydrochloride, dissolved in water. AY 9944 was administered every other day for a total of 5 doses, starting at 25 days of age. Rats of the same age, weight, and sex were used as controls. The animals were sacrificed at 34 days of age by decapitation and the brains collected in normal Ringer's solution at 4°C.

Preparation of Microsomal Fractions. The gray matter from the brains was separated from most of the white matter. The material was immediately homogenized at 0°C in 5 volumes of a solution of 0.25 M sucrose, 5 mM histidine, 5 mM EDTA and 0.2% deoxycholate (pH 6.8). The homogenate was centrifuged for 30 min at 12,000 × *g*. The pellet (P_1) was discarded and the supernatant recentrifuged for 30 min at 48,000 × *g*. The sediment from this centrifugation was suspended in a solution of 0.25 M sucrose, 5 mM histidine, and 1 mM Tris-EDTA (pH 7). This suspension was centrifuged for 30 min at 48,000 × *g*, and the sediment from this last centrifugation (P_2) was resuspended in the same sucrose, histidine, Tris-solution, and used for the study of the Na^+, K^+-ATPase. A small portion of this fraction (P_2) was fixed and processed to be analyzed at the electron microscope level.

Assay of Na^+, K^+-ATPase. Twenty microliters of the microsomal suspension (containing about 2 mg protein/ml) were preincubated for 5 minutes at the prescribed incubation temperature in a medium containing (final concentrations) 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$ and, when required, 100 mM NaCl, 20 mM KCl and 1 mM ouabain. The final volume was 1 ml. The reaction was started by adding ATP to the medium (2 mM final concentration). After 10 minutes the reaction was terminated by the addition of 1 ml of ice cold 6% $HClO_4$ to the incubation tubes. The samples were chilled and centrifuged and the liberated orthophosphate (Pi) was determined in the deproteinized solution (18). Under our incubation conditions there were

no differences in the initial rates of the enzyme activities. All samples were run in quadruplicate. Na^+, K^+ stimulated and Mg^{2+} dependent ATPase activities are expressed as nmoles of Pi produced per mg of protein per min after subtraction of a blank run in parallel without the 20 μl of microsomal suspension, which was added after the $HClO_4$. The same procedure was followed for the 5′ Nucleotidase assays, with 5 mM AMP (final concentration) used as substrate rather than ATP.

Ouabain Binding. The method described by Chipperfield and Whittam (19) was followed. Aliquots of the microsomal suspensions were added to a medium containing 20 mM imidazole-HCl (pH 7.6), 100 mM NaCl, 3 mM ATP, 5 mM $MgCl_2$ and 5 $\times$ 10^{-8} M [^{3}H]ouabain. The reaction was carried out at different times of incubation at the indicated temperatures. After incubation, the samples were cooled to 0°C and then centrifuged at 48,000 $\times$ g for 30 min. The pellets were washed once by recentrifugation after resuspension in the same incubation medium but without ouabain or ATP. The pellets were then resuspended in 5% aqueous "Triton X-100", and the bound ouabain was assayed by liquid scintillation counting.

Succinic dehydrogenase activity was determined by the method described by King (20) and glucose 6-phosphatase activity was carried out in the presence of 4 mM EDTA and 2 mM potassium fluoride to inhibit the nonspecific phosphatases.

The protein concentration was determined by means of Folin reagent (21). All experiments were carried out at least twice. The sterols (cholesterol and 7-dehydrocholesterol) were determined by gas-liquid chromatography following the method of Fumagalli *et al.* (7).

Ouabain (Strophanthin-G), EGTA, ATP, AMP, were purchased from the Sigma Chemical Company, St. Louis, Mo. U.S.A., [^{3}H]-ouabain (1 mg/ml, 1 mC/ml) from New England Nuclear, Boston, Mass., U.S.A.; AY 9944 was kindly supplied by Dr. Dvornik from Ayerst Laboratories, Montreal.

RESULTS

Table 1-A shows the Mg^{2+}-dependent and the Na^+, K^+-stimulated, ouabain sensitive ATPase activity (EC 3.6.1.3) of the different fractions from the control and the AY 9944-treated rat brains of one experiment. We can see in both cases a similar distribution of the Mg^{2+}-dependent activity as well as of the Na^+, K^+-activity for the different fractions. However, while the

TABLE 1-A

ATPase activity of different fractions prepared from control and AY 9944-treated rat brains

The different fractions were incubated for ATPase activity. Requisite amounts of the different fractions (to make a final concentration of about 50 μg protein/ml) were added to 1 ml of the incubation medium containing a final concentration in mM: Tris-HCl (pH 7.2), 50; $MgCl_2$, 5; ATP-Na, 2; NaCl, 100; KCl, 20 and ouabain, when required, 1. After 5 minutes of preincubation at 37°C, the reaction was started by adding ATP to the medium and it was stopped after 10 min by addition of 1 ml of ice cold 6% perchloric acid to the incubation tubes. The samples were chilled, centrifuged and assayed for the presence of Pi. The homogenates and subfractions were prepared by pooling four rats in each group before fractionation. Each of the subfractions was then assayed in quadruplicate. The values are expressed as the mean of the four determinations ± SE. The same experiment was repeated with three different preparations, with equivalent results. P1 = Pellet of 12,000 $\times$ g; S = Supernatants after 48,000 $\times$ g centrifugations. P2 = Pellet of 48,000 $\times$ g.

Fraction	ATP hydrolyzed (nmole/mg protein/min)					
	Control			AY 9944-treated		
	$Na^+ + K^+$	$Na^+ + K^+$ + Ouab.	Δ ($Na^+ + K^+$)	$Na^+ + K^+$	$Na^+ + K^+$ + Ouab.	Δ ($Na^+ + K^+$)
P_1	465 ± 3	158 ± 2	+ 307 ± 4	516 ± 3	132 ± 4	384 ± 5
P_2	2,304 ± 27	371 ± 4	+1,933 ± 27*	2,729 ± 23	387 ± 7	+2,342 ± 24*
S	111 ± 4	41 ± 2	+70 ± 4	142 ± 3	57 ± 2	+85 ± 4

* $p < 0.001$.

Mg^{2+}-dependent activity is similar for both, control and AY 9944-treated animals, the Na^+, K^+-activity is higher for the AY 9944-treated animals. Thus, the P_2 fraction (the microsomal fraction), shows a Na^+, K^+-stimulation of 2,342 nmole of P_i/mg prot/min compared with the control animals of 1,933 nmole of P_i/mg prot/min. In both cases, the ATPase activity of the supernatants (S) is very low compared with the microsomal fractions (P_2). Since the ATPase activity may vary due to preparative procedures and to the effects of aging, causing significant differences between one preparation and another, the brains from each group of experimental animals were prepared and run for ATPase activity at the same time as each group of control animals. One such experiment is shown in Table 1-A. In addition, a comparison was done with the values from the P_2 fractions of six different preparations obtained at different times of the year. Each preparation was obtained from brains pooled from four rats and the assays were done in quadruplicate. The results, shown in Table 1-B, are expressed for each preparation as the mean of the four determinations. The mean values for the Na^+, K^+-stimulated ATPase activity of the six experimental groups is significantly higher ($0.01 > p > 0.005$) than that of the control groups. Comparable results were obtained when the experiment was carried out with membranes extracted in the absence of deoxycholate. Larger quantities of AY 9944 did not induce a further increment of the enzyme activity. The effect of smaller doses is currently under study.

TABLE 1-B

Na^+ + K^+-stimulated ATPase activity of P_2 fractions from control and AY 9944-treated rat brains measured in six different experiments

Values, calculated as the total activity in the presence of Mg^{2+} + Na^+ + K^+ minus the activity in the presence of Mg^{2+}, were done for each preparation. Each preparation was done with four pooled rats (in each group) and the assays were done in quadruplicate. The results are expressed for each preparation as the mean of the four determinations.

Experiment No.	ATP hydrolyzed (nmol/mg protein/min)	
	Control	AY 9944-treated
1	1,847	2,284
2	1,479	1,836
3	1,522	1,888
4	1,951	2,537
5	1,781	2,197
6	1,594	2,139
	1,696 ± 78	2,147 ± 106

($0.01 > p > 0.005$).

The microsomal fractions (P_2) from both control and AY 9944-treated brains appeared similar at the electron microscope level. These fractions were formed by membranes with no sign of contamination with mitochondria, lysosomes or ribosomes. The distribution of the activity of several enzymes for the different fractions from control and AY 9944-treated animals were determined in order to ascertain whether or not we were dealing with similar fractions in both cases.

Table 2 shows the distribution of 5′ nucleotidase, glucose 6-phosphatase, and succinate dehydrogenase, which are widely accepted marker enzymes for plasma membrane (22–24), endoplasmic reticulum (25, 26) and mitochondria (27), respectively. All these enzymes show similar values for each fraction in both the control and the AY 9944-treated brains; we may conclude that the fractionation method gives similar preparations for both cases.

The fact that there is very low succinate dehydrogenase activity in P_2 fractions compared with P_1 fractions (mitochondrial fractions) for both control and AY 9944-treated preparations indicates a very low mitochondrial contamination in P_2 fractions, corroborating the conclusions drawn from the electron microscope study. This is further indicated by the fact that oligomycin (0.12 μg/ml), a known inhibitor of the mitochondrial Mg^{2+}-ATPase activity (28), was able to inhibit approximately 35% of the Mg^{2+}-ATPase of the P_1 fractions in both cases, but showed no appreciable effect on the activity of P_2 fractions.

Figure 1 demonstrates the effect of incubation temperature on the activity of the microsomal fractions (P_2) from control and AY 9944-treated brains. The Mg^{2+}-dependent activity has a similar behavior in the two cases, with a peak at 47°C. The Na^+, K^+-activity of the AY 9944-treated brains

TABLE 2

Distribution of several enzymes in different fractions prepared from control and AY 9944-treated rat brains

The homogenates and subfractions were prepared by pooling four rats in each group before fractionation. Each of the subfractions was then assayed in quadruplicate. The values are expressed as the mean of the four determinations ± SE. The same experiment was repeated with three different preparations, with equivalent results. P_1 = Pellet of 12,000 × *g*; P_2 = Pellet of 48,000 × *g*; S = pooled supernatants.

Fraction	5'Nucleotidase[a]		Glucose 6-phosphatase[b]		Succinic dehydrogenase[c]	
	Control	AY 9944 treated	Control	AY 9944 treated	Control	AY 9944 treated
P_1	2.00 ± 0.15	3.10 ± 0.23	8.1 ± 0.1	8.3 ± 0.3	5.1 ± 0.4	5.8 ± 0.1
P_2	10.00 ± 0.9	10.00 ± 1.00	15.5 ± 0.5	15.0 ± 0.2	0.7 ± 0.1	0.7 ± 0.1
S_3	2.94 ± 0.3	2.87 ± 0.12	16.1 ± 0.1	15.3 ± 0.1	undetectable	undetectable

[a] AMP hydrolysed: nM/mg protein/min.

[b] Glucose 6-Phosphate hydrolysed: nM/mg prot/min.

[c] Succinic Dehydrogenase activity: ΔUA/mg prot/min.

is not only higher than the control for a given temperature but also more stimulated with increasing temperatures. Thus, while the activity of the preparation from the AY 9944-treated brains is 15% higher at 27°C, it is 26% higher at 37°C and 31% higher at 47°C. The Na^+, K^+-ATPase reaches its maximum at 47°C, being strongly inhibited when the reaction is carried out at 57°C.

In order to determine whether the observed increment in Na^+, K^+-ATPase of the treated animals compared with the controls was due to the presence of a higher number of enzymes or ATPase systems, the uptake of [^{3}H]-ouabain in control and AY 9944-treated microsomal fractions was measured as a function of the incubation time for a given temperature, as well as of the temperature of incubation for a given time. As shown in Fig. 2, the uptake of [^{3}H]-ouabain as a function of time at 37°C follows a similar pattern in both cases, reaching maximal values after 15 minutes of incubation. Control and AY 9944-treated samples were incubated for 60 minutes at 27°C, 37°C and 47°C for the study of [^{3}H]-ouabain uptake; the results appear in Table 3. In both cases the [^{3}H]-ouabain incorporated is about the same at any of the tested temperatures.

A possible cause of the enhanced ATPase activity could be a variation of the affinity of the ATPase system to the different ligands involved in the process. This possibility was tested by studying the effect of increasing concentrations of Na^+, K^+ and Mg^{2+}, as well as the variation of the Mg^{2+}/ATP ratio in both control and AY 9944-

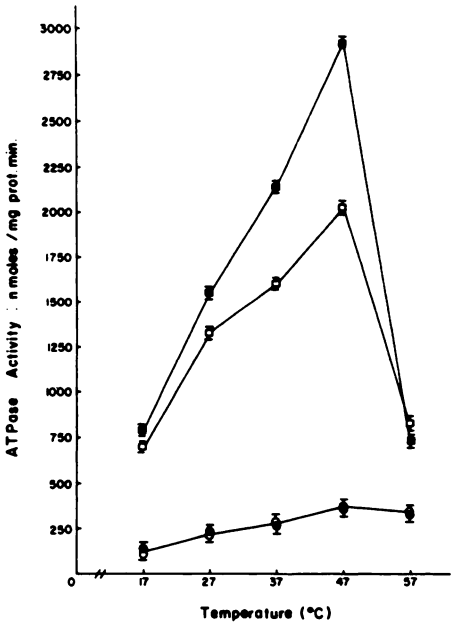

FIG. 1. *Effect of increasing temperature on the Mg^{2+}-dependent and on the Na^+, K^+-ATPase activity (total activity in the presence of Mg^{2+} + Na^+ + K^+, minus the Mg^{2+}-dependent activity) of microsomal fractions (P_2) from control and AY 9944-treated rat brains*

The assays carried out as indicated in METHODS and table 1. For Figs. 1, 2, 3, 4, 5, 6 the P_2 fractions were prepared by pooling four rats in each group before fractionation; the assays were done in quadruplicate; the values are expressed as the mean of the four determinations ± SE; the same experiment was repeated with two different preparations, with equivalent results. O-----O Mg^{2+}-ATPase, control, ●-----● Mg^{2+}-ATPase, AY 9944-treated, □-----□ Na^+, K^+-ATPase, control, ■-----■ Na^+, K^+-ATPase, AY 9944-treated.

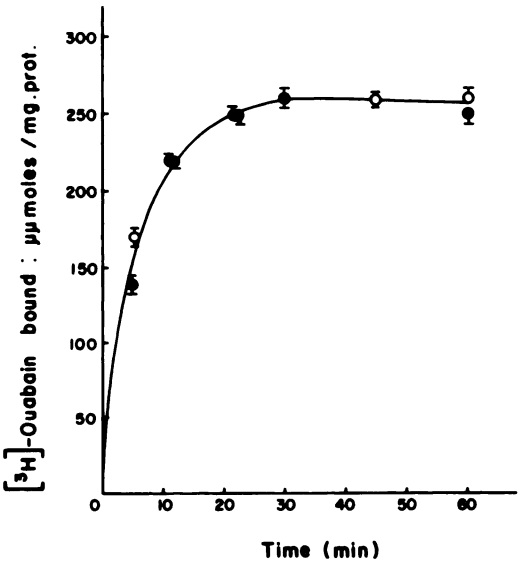

FIG. 2. *Ouabain binding of microsomal fractions (P_2) from control (O- - -O) and AY 9944-treated (●- - -●) rat brains as a function of the incubation time.*

The binding experiment was carried out as indicated in METHODS and Table 3. The incubation temperature was 37°C. At the indicated times the samples were cooled to 0°C, centrifuged, washed, resuspended in "TRITON X-100" and finally assayed by liquid scintillation counting.

TABLE 3

Ouabain binding of microsomal fractions (P_2) prepared from control and AY 9944-treated rat brains

The microsomal fractions were incubated at the indicated temperatures for 60 minutes in a medium containing, in mM: Imidazole-HCl (pH 7.6), 20; NaCl, 100; ATP-Na, 3; $MgCl_2$, 5 and 5×10^{-8} M [^{3}H]-ouabain. After incubation, the tubes were cooled to 0°C and then centrifuged at 48,000 × *g* for 30 minutes. The pellets were washed once with the same medium but without ouabain or ATP and then resuspended in 5% aqueous "TRITON X-100." The bound ouabain was assayed by liquid scintillation counting. The P_2 fractions were prepared by pooling four rats in each group before fractionation. The assays were done in quadruplicate. The values are expressed as the mean of the four determinations ± SE. The same experiment was repeated with three different preparations, with equivalent results.

	Ouabain binding: µµ M/mg protein	
Temperature	Control	AY 9944-treated
°C		
27	262 ± 35	249 ± 33
37	250 ± 23	260 ± 18
47	250 ± 20	250 ± 16

treated preparations. The results are shown in Figs. 3, 4 and 5. The calculated K_m for Na^+, K^+ and Mg^{2+} is exactly the same in both cases: 20 mM for Na^+ (Fig. 3), 2 mM for K^+ (Fig. 4), and 1.1 mM for Mg^{2+} (Fig. 5). In each case the V_{mx} was found to be higher for the AY 9944-treated brains than for the control brains. The effect of the ratio Mg^{2+}/ATP in both preparations was studied at a fixed Mg^{2+} concentration (5 mM) changing the ATP concentration from 0.5 to 5 mM. The results are shown in Table 4. Both control and AY 9944-treated preparations show very similar behavior, reaching optimal activity at a ratio of 5 mM Mg^{2+}/2 mM ATP.

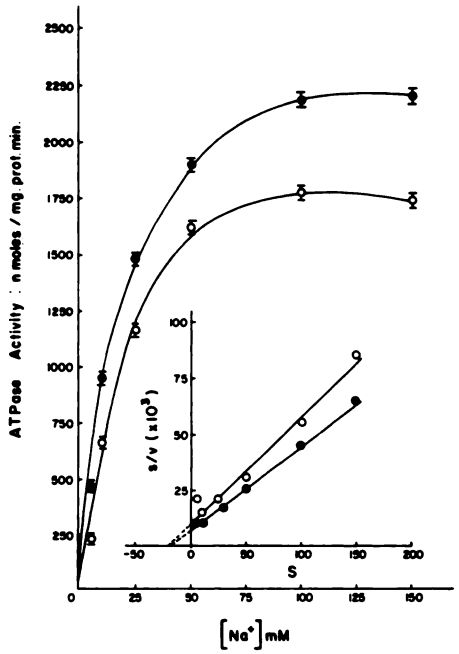

FIG. 3. *Effect of increasing Na^+ concentration on the Na^+, K^+-ATPase activity (total activity minus Mg^{2+}-dependent activity) of microsomal fractions (P_2) from control (O- - -O) and AY 9944-treated rat brains (●- - -●)*

The assays were carried out as indicated in METHODS and Table 1. For Figs. 3, 4, 5, the apparent K_m was calculated by means of a derivative of the Lineweaver-Burk transformation of the Michaelis equation of the form

$$\frac{S}{V} = \frac{Km}{Vmax} + \frac{S}{Vmax}$$

where Km = S on the S axis (see insert). Mg^{2+} concentration 5 mM. K^+ concentration 20 mM.

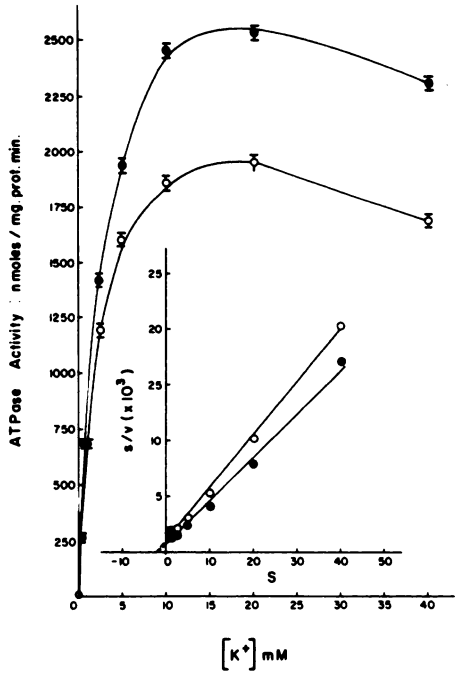

FIG. 4. *Effect of increasing K^+ concentration on the Na^+, K^+-ATPase activity (total activity minus Mg^{2+}-dependent activity) of microsomal fractions (P_2) from control* (O- - -O) *and AY 9944-treated rat brains* (●- - -●)

The assays were carried out as indicated in METHODS and Table 1. Mg^{2+} concentration 5 mM. Na^+ concentration 100 mM.

Finally, the possibility of a direct enhancing effect of the amphiphilic AY 9944 molecule on the enzyme activity was tested. The results of a dose-response experiment are shown in Fig. 6. Both the control and the AY 9944-treated preparations are inhibited for the tested concentrations of the drug. In both cases, the Na^+-K^+-ATPase was inhibited 100% at a concentration of AY 9944 of 1 mg/ml. Concentrations lower than 0.3 mg/ml did not show any appreciable effect.

DISCUSSION

The present experiments indicate that treatment of rats with AY 9944, an inhibitor of the 7-dehydrocholesterol reductase, results in an increased Na^+, K^+-ATPase activity present in brain microsomal fractions (P_2).

There is increasing evidence that a close association exists between cholesterol and the Na^+, K^+-ATPase. Thus, reconstitution studies indicate that cholesterol is indispensable in order to see any activity of the enzyme complex isolated from rat brain (29). On the other hand, studies involving phospholipid vesicles and other model membranes indicate that variation of the phospholipid-cholesterol ratio results in a marked variation of the phospholipid-dependent Na^+, K^+-ATPase (30–32). Furthermore, in skeletal muscle sarcolemma from patients with Duchenne muscular dystrophy, characterized by a pronounced increase of the membrane cholesterol content, the activity of the Na^+, K^+-ATPase is lower than in controls (33).

The gas liquid chromatography analysis indicated that cholesterol was the only sterol present in the P_2 fraction from the control rats. On the other hand, the P_2 fraction from the AY 9944-treated rats had 23% of the total sterols in the form of 7-dehydrocholesterol, the remaining 77% being cholesterol. Furthermore, in a previous work we have found that brains from

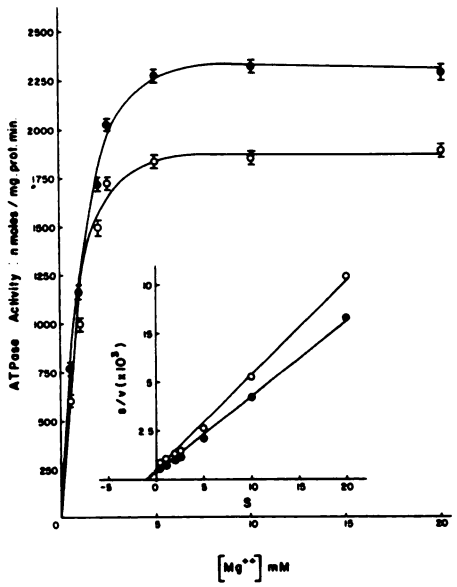

FIG. 5. *Effect of increasing Mg^{2+} concentration on the Na^+, K^+-ATPase activity (total activity minus Mg^{2+}-dependent activity) of microsomal fractions (P_2) from control* (O- - -O) *and AY 9944-treated rat brains* (●- - -●)

The assays were carried out as indicated in METHODS and Table 1. Na^+ concentration 100 mM. K^+ concentration 20 mM.

TABLE 4

Effect of increasing ATP concentration on the Mg^{2+}-dependent and the Na^+,K^+-stimulated ATPase activity in control and AY 9944-treated animals

1 mM ouabain, added when indicated. Mg^{2+} concentration, 5 mM. The P_2 fractions were prepared by pooling four rats in each group before fractionation. The assays were done in quadruplicate. The values are expressed as the mean of the four determinations × S.E. The same experiment was repeated with two different preparations, with equivalent results.

ATP (mM)	ATP hydrolysed (nM/mg prot/min) Incubation medium					
	Control			AY 9944-treated		
	$Na^+ + K^+$	$Na^+ + K^+$ + Ouab	$\Delta(Na^+ + K^+)$	$Na^+ + K^+$	$Na^+ + K^+$ + Ouab	$\Delta(Na^+ + K^+)$
0.5	1,477 ± 14	315 ± 7	1,162 ± 16*	2,017 ± 14	322 ± 6	1,695 ± 15*
1	1,940 ± 23	313 ± 2	1,627 ± 23*	2,536 ± 13	333 ± 3	2,203 ± 13*
2	2,104 ± 18	369 ± 3	1,735 ± 19*	2,742 ± 19	357 ± 5	2,385 ± 20*
5	1,842 ± 20	316 ± 5	1,526 ± 21*	2,398 ± 24	279 ± 4	2,119 ± 24*

*$p < 0.001$.

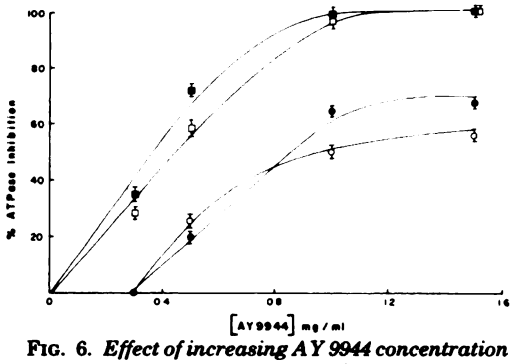

FIG. 6. *Effect of increasing AY 9944 concentration in the incubation medium on the Mg^{2+}-dependent and the Na^+, K^+-ATPase activities (total activity minus Mg^{2+}-dependent activity) of microsomal fractions (P_2) from control and AY 9944-treated rat brains.*

The assays carried out as indicated in METHODS and Table 1. O- - -O Mg^{2+}-ATPase, control, ●- - -● Mg^{2+}-ATPase, AY 9944-treated, □- - -□ Na^+, K^+-ATPase, control, ■- - -■ Na^+, K^+-ATPase, AY 9944-treated.

rats similarly treated with AY 9944 showed no difference in the content and composition of phospholipids or any other membrane component but cholesterol (34). The observed Na^+, K^+-ATPase change described in this paper is most likely a consequence of the modification in the sterol composition of the microsomal fraction (P_2).

Sarcolemma and erythrocyte ghosts from rats treated with the cholesterol biosynthesis inhibitor 20, 25-diazacholesterol also show an increased Na^+, K^+-ATPase (35, 36). The inhibitor 20, 25-diazacholesterol acts on the Δ^{24}-reductase inducing an accumulation of 24-dehydrocholesterol (desmosterol) instead of 7-dehydrocholesterol (37). Fiehn and Seiler (38) have suggested that the double bond in the side chain of desmosterol could be responsible for the altered enzyme activity. As shown in this work, however, an increment of the enzyme activity also occurs in the presence of 7-dehydrocholesterol which has an extra double bond in the B ring of the molecule. Therefore, the extra double bond in the side chain (24-dehydrocholesterol) or in the B ring (7-dehydrocholesterol) of the sterol molecule may be accomplishing similar roles in the interaction of these molecules with the other membrane components.

The observed increment in the Na^+, K^+-ATPase activity may indicate a) that there is an increased number of enzymes or active sites or b) that a change in the turnover rate of the enzyme has taken place. If we consider the binding of [^{3}H]ouabain as an indicator of the number of enzymes or active sites (39), the first possibility can be excluded since we have found that incorporation of [^{3}H]ouabain at different times and temperatures of incubation was similar for both the control and the AY 9944-treated microsomal fractions (P_2). Therefore, the turnover rate of the enzymes may have changed. This last possibility is currently being tested and the results will form part of a future paper.

Variation in the affinity of the system for different ligands involved in the reaction could be responsible for the observed increment in the Na^+, K^+-ATPase activity. This

is not the case here, since the control and the AY 9944-treated P_2 fractions showed similar affinities toward Na^+, K^+ and Mg^{2+} (Figs. 3, 4 and 5) and responded in similar ways to different Mg^{2+}/ATP ratios (Table 4).

We have found that both control and AY 9944-treated P_2 fractions increased their activities with increasing temperatures up to 47°C. However the increment produced by the increasing temperatures was always higher for the AY 9944-treated fraction than for the control. These observations suggest a change in the microenvironment of the Na^+, K^+-ATPase which is presumably due to its lower cholesterol content. By influencing the mobility of the phospholipids hydrocarbon chains, cholesterol could act as a regulator of membrane fluidity, thereby influencing the Na^+, K^+-ATPase activity (38, 40, 41). The physiological implications of the present observations must await further investigation.

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