Biochemical Pharmacology, Vol. 30, pp. 427–432.

0006-2952/81/050427-06 $02.00/0

CATECHOLAMINES AND THE BRAIN MICROSOMAL Na, K-ADENOSINETRIPHOSPHATASE—I. PROTECTION AGAINST LIPOPEROXIDATIVE DAMAGE

PETR SVOBODA* and BEDŘICH MOSINGER†

*Institute of Physiology, Czechoslovak Academy of Sciences, Vídeňská 1083, Prague 4, and †Institute for Clinical and Experimental Medicine, Vídeňská 800, Prague 4, Czechoslovakia

(*Received* 12 *May* 1980; *accepted* 22 *September* 1980)

Abstract—The brain microsomal Na,K-ATPase is selectively inactivated by endogenous inhibitory system comprised from ascorbic acid and ferrous ions. The mechanism of this inhibition appears to be identical with the radical damage of unsaturated membrane phospholipids (lipoperoxidation) induced by these agents. Both adrenaline and EDTA block the manifestation of the lipoperoxidative capacity of brain subcellular fractions and simultaneously protect the Na,K-ATPase activity against inhibition. A possible biological importance of these data is discussed.

It is generally accepted that hormonal effects in intact cells are mediated via several steps of which the activation of adenylcyclase [1] is best known.

Previously, the lipolytic effect of catecholamines on white adipose tissue was found to be inhibited by omission of potassium from or by addition of ouabain to the incubation medium [2, 3]. As it was recognized that the adenylcyclase activity and lipolytic effect of methylxanthines and dibutyryl 3′–5′ cyclic adenosinemonophosphate were unaffected by ions or ouabain it was suggested that hormones interact with an ion-sensitive structure distinct from the adenylcyclase–cAMP chain reaction [4]. The hypothetical ionsensitive structure should obviously be localized in the plasma membrane and its properties should be similar to the ouabain-sensitive, sodium plus potassium activated adenosinetriphosphatase (Na,K-ATPase).

From this point of view several reports demonstrating a direct effect of hormones on the isolated Na, K-ATPase activity seemed of particular interest. Thus, it was found that catecholamines stimulate the Na, K-ATPase of brown adipose tissue [5, 6]. A number of authors showed that catecholamines increase activity of the ouabain-sensitive Na, K-ATPase in the rat brain homogenates [7, 8] and microsomal fractions [9, 10]. This increase was interpreted as result of direct action of catecholamines on the enzyme [6], consequence of adenylcyclase activation [11], suppression of the inhibition exerted by calcium ions on the Na, K-ATPase [8] or chelation of divalent iron ions [8, 9]. The present investigation was undertaken with the aim of discriminating among these controversial possibilities and of defining more closely the mechanism of action of catecholamines on the rat brain microsomal Na, K-ATPase. The microsomal membrane fraction from the rat cerebral cortex homogenate provided a suitable system for the study of the above mentioned problems.

MATERIALS AND METHODS

Preparation of the microsomal particles from the rat cerebral cortex. White rats of the Wistar strain weighing 140–220 g were killed by quick decapitation. The cerebral hemispheres were removed, separated from the white matter, weighed, washed and homogenised in ice cold 0.32 M sucrose buffered with 0.05 M Tris–HCl, pH 7.4. The 10% homogenate was prepared in a Elvehjm–Potter homogenizer (teflon–glass, clearance 0.03 mm, 2000 rpm). The brain subcellular fractions were prepared according to the cell fractionation method of De Robertis *et al.* [12] with the modification that the homogenisation solution did not contain any Ca^{2+} and the second centrifugation was carried out at 12,000 *g* instead of 11,500 *g*. The supernatant 12,000 *g* was centrifuged then for 60 min at 100,000 *g*. The resulting 100,000 *g* sediments were divided in the two identical portions (based on the same volume of supernatant 12,000 *g* and determination of the protein).

The microsomal or microsomal plus cytosol fraction were prepared by suspending the same amount of microsomal membrane particles (sediment 100,000 *g*) in the equal volume of 0.32 M sucrose or the cytosol fraction (supernatant 100,000 *g*). The microsomal fraction (microsomes) represented a suspension of the microsomal membranes in 0.32 M sucrose, whereas the microsomal plus cytosol fraction contained the microsomal particles together with soluble components of the cell. The amount of microsomal protein was the same in both fractions.

Adenosinetriphosphatases assay. The ATPase activities were determined in two reaction media:

(A) 100 mM NaCl, 20 mM KCl, 100 mM Tris–HCl pH 7.4 and 5 mM $MgCl_2$;

(B) 120 mM NaCl, 100 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$ and 2×10^{-4} M ouabain.

The complete reaction mixture (0.04–0.1 mg of

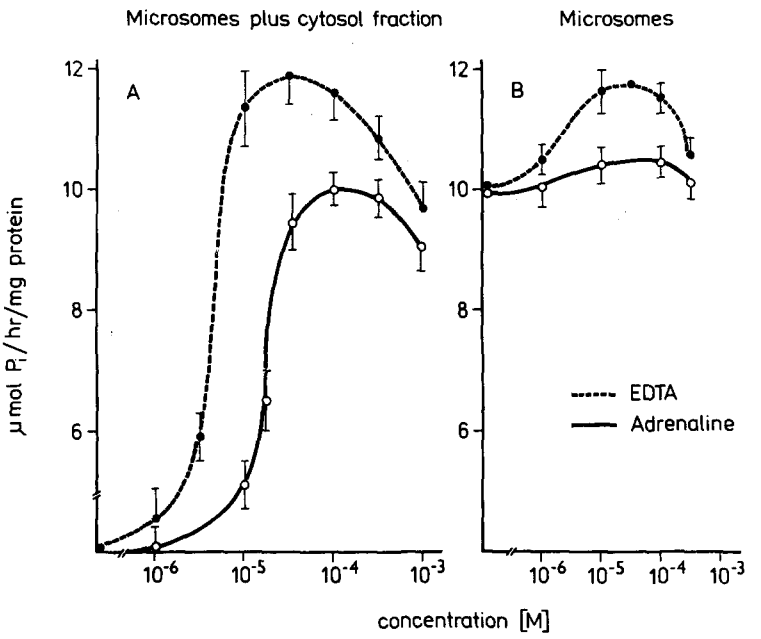

Fig. 1. Effect of adrenaline and EDTA on microsomal Na, K-ATPase. (A) Microsomes plus cytosol fraction. Rat brain microsomal membranes prepared according to De Robertis *et al.* [12] were suspended in the cytosol fraction (supernatant 100,000 *g*) at 1–3 mg membrane protein per ml. (B) Microsomes. Microsomal membranes were suspended in pure 0.32 sucrose at 1–3 mg/ml. For determination of ATPase activities, both subcellular fractions were diluted 10–20 times in the reaction media and enzyme activities were determined as described in Materials and Methods. The ATPase activities were expressed as μmoles P_i liberated per hour per mg membrane protein (= μmoles P_i/hr/mg protein). Each point represents the mean of 4–7 determinations and bars represent ± S.D.

the membrane protein in a total volume of 2 ml of media A and B) was preincubated for 5 min before starting the reaction with ATP. The final concentration of ATP was 2.5 mM. The ATPase reaction was terminated after 15 min at 37° by addition of 0.5 ml of 1M $HClO_4$. After sedimentation of the precipitated protein, an inorganic phosphate produced in the sample was assayed according to the method of Taussky and Shorr [13]. It should be stressed that all chemicals tested (adrenaline, EDTA, $FeSO_4$, ascorbate) were present in incubation media during the assay of ATPase activities. The Na, K-ATPase activity was expressed as the inorganic phosphate produced per hour per mg of the membrane protein in medium A minus medium B. The ouabain independent Mg-ATPase was defined as inorganic phosphate released per hour per mg of the membrane protein in medium B. The protein amount present in the cytosol fraction (see preparation of the microsomal plus cytosol fraction) was not considered for expression of the enzyme activity because there is not measureable Na, K-ATPase activity in the cytosol fraction.

Assay of lipid peroxide. Lipid peroxidation was measured using the thiobarbituric acid color reaction [14]. A 1 ml aliquot of the incubation mixture (0.2 M phosphate buffer, pH 5.9) was added to 1 ml of 10% TCA, the color reaction was developed after treating the solution with the thiobarbituric acid reagent and boiling the sample for 10 min. The protein precipitate was centrifuged and the color was read at 530 nm. The amount of malonyldialdehyde formed in the color reaction was determined by using $e_{530} = 1.56 \times 10^5$ cm/nmole [14]. The lipoperoxidative capacity of the brain subcellular fraction was expressed as nmol of malonyldialdehyde (MDA) produced per min per mg of the membrane protein.

Analytical procedures. Calcium and iron content of the brain subcellular fraction were determined by means of atomic absorbtion spectrometry (Varian—Techtrom). Iron concentration was also determined colorimetrically using bathophenatroline.

Protein was determined according to Lowry *et al.* [15]. All experiments were performed at least four times and the results are presented as average ± S.D.

RESULTS

Adrenaline and EDTA effect on the rat brain microsomal Na, K-*ATPase*

Adrenaline was found to stimulate the Na, K-ATPase activity (see Fig. 1(A)) in the rat brain microsomal plus cytosol fraction, beginning as low as at 5×10^{-6} M and plateauing at about 5×10^{-5} M. Similarly, EDTA was found to stimulate the Na, K-ATPase activity in the microsomal plus cytosol fraction. However, the affinity and the maximum response of the EDTA effect were higher than those of adrenaline. The stimulatory effect of both agents seemed to be specific for the ouabain-dependent, sodium and potassium activated ATPase. The ouabain-independent, Mg-ATPase was influenced to a negligible degree (not shown).

On the contrary to the results presented in Fig.

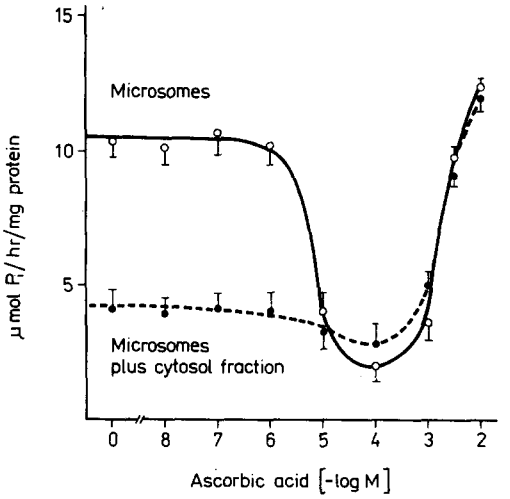

Fig. 2. Effect of ascorbic acid on the microsomal Na, K-ATPase. For definition of microsomal or microsomal plus cytosol fractions see Methods and legend to Fig. 1. Na, K-ATPase activity was determined after 5 min preincubation of the enzyme with increasing concentrations of ascorbate (see Methods). Ascorbate was present in incubation media during the enzyme assay. The values given are means ± S.D. of at least 4 independent determinations.

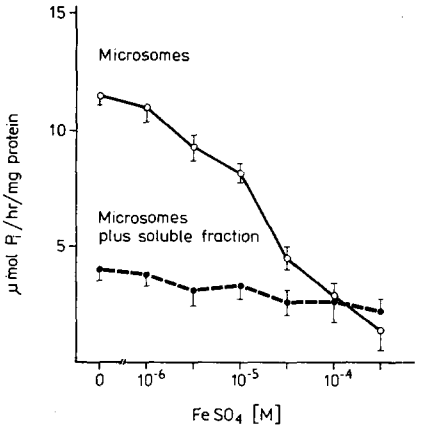

Fig. 3. Inhibition of microsomal N, K-ATPase by ferrous cations. For definition of microsomal resp. microsomal plus cytosol fractions see Methods and legend to Fig. 1. Na, K-ATPase activity was determined after 5 minutes of preincubation of the enzyme with increasing concentrations of $FeSO_4$ (see Methods). The points represent mean values of 4–5 determinations ± S.D.

1(A), adrenaline and EDTA had only a minimal stimulatory effect on the highly active Na, K-ATPase measured in the microsomes without cytosol suspended in 0.32 M sucrose (see Fig. 1(B)). Comparison of the results presented in Fig. 1(A) and 1(B) shows that the addition of the cytosol fraction (supernatant 100,000 *g*) to the brain microsomes results in a decrease of the Na, K-ATPase activity which can be prevented by adding increasing amounts of adrenaline or EDTA to the media. The results presented in Fig. 1(A) and (B) are in agreement with those of Schaeffer *et al.* [9], who described an inhibitor of the Na, K-ATPase activity localized in the cytosol fraction (supernatant 100,000 *g*). Later, the same authors showed that the inhibitor appears to be a complex system consisting of the membrane bound iron and ascorbic acid localized in the cytosol fraction.

Effect of ascorbic acid and Fe^{2+} *on the* Na, K-*ATPase activity*

When testing the effect of ascorbic acid on Na, K-ATPase, inhibition was found only within the range of 10^{-5}–10^{-3} M (Fig. 2). The inhibition was not observed in microsomes containing the cytosol fraction due probably to the fact that low concentrations of the endogenous ascorbate were already present and higher concentrations were stimulatory (Fig. 2).

In further experiments, the effect of ferrous cations on the Na, K-ATPase activity in the brain microsomes or microsomes plus the cytosol fraction was investigated (Fig. 3) following the hypothesis of Schaeffer [9, 10]. Increasing concentrations of fer-

Table 1. Total iron and calcium content of 0.32 M sucrose and rat brain subcellular fractions*

	Iron	Calcium
0.32 M sucrose	10^{-7} M	1.45×10^{-5} M
Cytosol fraction (supernatant 100,000 *g*)	1.43×10^{-6} M	1.8×10^{-5} M
Microsomes (sediment 100,000 *g* + 0.32 M sucrose)	3.12×10^{-6} M	2.92×10^{-5} M
Microsomes plus cytosol fraction (sediment 100,000 *g* + cytosol fraction)	4.55×10^{-6} M	3.27×10^{-5} M

* Concentration of total iron and calcium in 0.32 M sucrose and cytosol fraction was determined directly in aqueous media by atomic absorbtion spectrometry. The concentration of iron and calcium in microsomes or microsomes plus the cytosol fraction was calculated as the sum of the amount of these metals in the microsomes (sediment 100,000 *g*) and concentration in 0.32 M sucrose or the cytosol fraction. The amount of metals in microsomes was determined after mineralization of the 100,000 *g* sediment accomplished by heating the membranes for 2 hr at 180° in concentrated $HNO_3 + H_2O_2$ mixture.

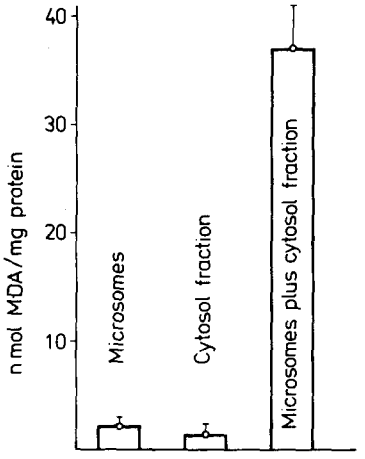

Fig. 4. Lipoperoxidative capacity of the rat brain subcellular fractions. Lipoproxidative capacity of the rat brain subcellular fractions was measured as malonyaldialdehyde production (MDA) as described in Materials and Methods. MDA production was related to the total protein concentration in each sample. Each point is the mean of 4 measurements with S.D. (vertical bars).

rous sulphate gradually inhibited only the Na, K-ATPase of the microsomal fraction and a concentration as high as 5×10^{-5} M was required to achieve the inhibition comparable to that observed in the microsomes plus soluble fraction (see Fig. 3).

As the stock enzyme suspension in the reaction media used to determine the Na, K-ATPase activity is diluted 10–20 times the initial concentration of ferrous ions in the microsomes plus the soluble fraction should be even 10–20 times higher than 5×10^{-5} M. However, the measurement of the iron content of the rat brain subcellular fractions revealed that the total iron content of microsomes plus the soluble fraction does not exceed 10^{-6} M (see Table 1). This amount is obviously not sufficient to account for the pronounced inhibition of the Na, K-ATPase measured in this fraction.

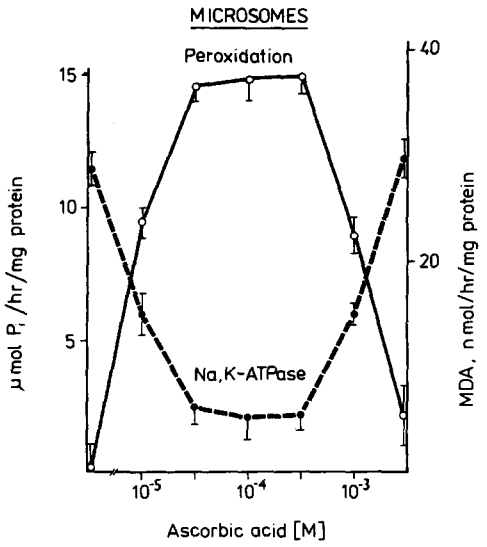

Fig. 5. Relationship between the Na, K-ATPase activity and lipoperoxidation as revealed by dependency of both reactions on ascorbate. Malonyldialdehyde production in the microsomal fraction was measured during incubation in 0.2 M phosphate buffer at pH 5.9 in the presence of increasing concentrations of ascorbic. In a separate set of experiments the influence of ascorbic acid on the Na, K-ATPase activity of the same microsomal preparation was determined as described in legend to Fig. 2. The values given are means ± S.D. of 5 experiments.

Activity of lipoperoxidation

The above mentioned data suggest that the mechanism involved in the cytosol induced inhibition of the Na, K-ATPase activity requires catalytic amounts of iron. A system comprised of ascorbic acid and traces of a heavy metal is known to induce the lipid peroxidation of the polyunsaturated fatty acids of the microsomal membrane phospholipids [16, 17]. Thus, the inhibition of the microsomal Na, K-ATPase caused by Fe^{2+} plus ascorbic acid could be a consequence of the lipoperoxidative degradation of its essential unsaturated phospholipids. Following this working hypotheses (Fig. 4), we found a low lipoperoxidative capacity in the rat brain microsomal or cytosol fractions alone. However, a combination of both fractions (i.e. microsomes plus the cytosol fraction) creates a highly active lipoperoxidative system.

An inverse relation between the Na, K-ATPase activity and the lipoperoxidation rate is demonstrated in Fig. 5. The addition of increasing amounts of ascorbate to the microsomes without the cytosol fraction resulted in a biphasic, dose-dependent effect on both the Na, K-ATPase activity and peroxidation intensity. The maximum inhibition of the Na, K-ATPase and activation of lipoperoxidation is reached within the concentration range of 5×10^{-5}–5×10^{-4} M ascorbate. A further increase of ascorbate concentrations above 10^{-3} reestablished the initial Na, K-ATPase activity and minimized the peroxidation.

In agreement with our proposal, adrenaline was found to inhibit the malonyldialdehyde production in the microsomal plus cytosol fraction in a concentration-dependent manner (Fig. 6). This inhibition was pH dependent. The highly active lipoperoxidation determined at pH 5.9 (0.2 M phosphate buffer) was only partially prevented by adrenaline, even at the concentrations as high at 10^{-4} M. On the contrary, the lower peroxidation intensity measured at pH 7.4 (0.2 M phosphate) was completely abolished at 5×10^{-5} M adrenaline. In this respect, it should be mentioned that the same adrenaline concentration fully recovers the Na, K-ATPase in the microsomes plus cytosol fraction (Fig. 1(A)).

DISCUSSION

It was found that adrenaline increases the activity of the brain microsomal Na, K-ATPase measured in the presence of the cytosol cell fraction. The high Na, K-ATPase activities of the microsomes without cytosol were only slightly stimulated. A similar effect was exhibited by EDTA. Therefore, the effect of adrenaline and EDTA on the brain Na, K-ATPase should be interpreted in terms of protection against the inhibition caused by the cytosol fraction.

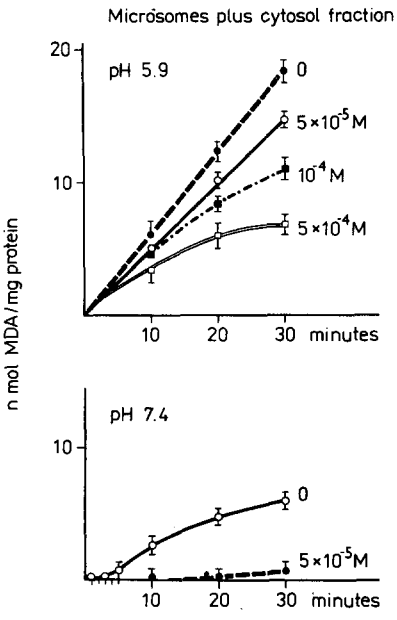

Fig. 6. Inhibition of lipoperoxidative activity of the microsomes plus cytosol fraction by adrenaline. Time course of malonyldialdehyde production in the rat brain microsomal plus cytosol fraction was measured in the presence of various concentrations of adrenaline in 0.2 M phosphate buffer at pH 5.9 and 7.4. At various time intervals, aliquots of 0.5 ml were removed from the incubation mixture (5 ml total volume) and analysed for malonyaldialdehyde produced (see Methods). Each value is the mean of 5–7 measurements ± S.D.

Schaeffer *et al.* [9, 10] identified this inhibitory system with ascorbic acid localized in the cytosol fraction plus membrane bound ferrous ions. The experimental data were interpreted suggesting that the inhibition of the Na, K-ATPhase is due to the oxidoreduction properties of ascorbate: Fe^{3+} is reduced to Fe^{2+}, the latter cation being known as a potent inhibitor of the Na, K-ATPase activity [18]. The favorable effect of catecholamines on Na, K-ATPase was explained by chelation of Fe^{2+} ions by the ortho-catechol structure of catecholamine [9, 10].

Therefore, in the present work, the effect of ascorbic acid and ferrous cation on the Na, K-ATPase was critically evaluated (see Figs. 2 and 3). The inhibition of the microsomal Na, K-ATPase activity at 10^{-5}–10^{-4} M ascorbate may be explained by the redox reaction with Fe^{2+} [9]. On the other hand, the reactivation of the enzyme at concentrations higher than 10^{-3} M is probably due to the chelating ability of ascorbate [19] with respect to Fe^{2+} ions.

This type of the effect of ascorbate appears to be similar to that of catecholamines [20, 21] and reflects probably the situation *in vivo* as the rat brain contains as much as 35 mg of ascorbate per 100 *g* tissue, which is equivalent to a concentration of 2.5 mM [20, 22].

So far Schaeffer [9, 10] and our data were in agreement. However, the quantitative aspects of the putative Na, K-ATPase inhibitor, Fe^{2+} cations are contradictory (see Table 1). The total iron concentrations of the rat brain subcellular fractions were less than 10^{-6} M, i.e., not sufficient to account for the pronounced inhibition of the Na, K-ATPase activities measured in the microsomes plus cytosol fraction.

Similar objections can be raised against the interpretation of Godfraind [8], that the action of catecholamines on the brain Na, K-ATPase is due to suppression of the inhibition exerted by Ca^{2+} cations. According to Godfraind [8], a pronounced 60–70 per cent inhibition of the Na, K-ATPase activity is observed at 5×10^{-5} M Ca^{2+}. The comparable inhibition occurs when the microsomes fraction is suspended in the cytosol fraction instead of in 0.32 M sucrose (see Fig. 1).

However, the results presented in Table 1 show unequivocally that the total calcium content in 0.32 M sucrose and the cytosol fraction is nearly equal. The difference between these values is apparently not sufficient to account for the 60–70 per cent inhibition of the Na, K-ATPase activity.

Therefore the role of metals in the ascorbate-induced inhibition of the Na, K-ATPase should be considered in terms of a catalytic rather than a stoichiometric relationship. It is known from the literature [16, 17] that ascorbic acid plus traces of heavy metals serve as potent inducers of the radical-chain degradation of membrane phospholipids, i.e. lipoperoxidation. At the same time, the enzyme activity of the membrane bound microsomal ATPase was shown to depend closely on the structural integrity of the membrane. Results of Sunn [23], as well as other data [24–26] showed that synaptosomal or microsomal phospholipids are essential for the Na, K-ATPase activity and that their degradation leads to the irreversible inhibition of the enzyme. In addition, microsomal membranes contain phospholipids with a high degree of unsaturation [27]. These double bounds play a critical role in determining the functional activity [28] and allosteric properties [29] of the Na, K-ATPase. The results presented in Figs. 4 and 5 demonstrate a close inverse correlation between the lipoperoxidative capacity and Na, K-ATPase activity. The high Na, K-ATPase activity can be measured only when a minimum lipoperoxidation is detected and vice versa. Both adrenaline and EDTA exhibited the strong antilipoperoxidative effect. Peroxidation was completely blocked at 5×10^{-5} M adrenaline and 2×10^{-5} M EDTA.

According to the results obtained the adrenaline and EDTA effect on the brain Na, K-ATPase activities *in vitro* may be visualized as follows: an intrinsic heavy metal, namely iron, is bound to a storage site in the microsomal membrane. Dilution of the concentrated stock microsomal preparation in the ATPase incubation media may change the equilibrium between the bound and free forms of this heavy metal, thus it is eluted to the media. When chelators like catecholamine or EDTA are present, they bind the iron cations forming a complex molecule and no inhibition occurs. If a chelator is not present in the incubation media, the iron cations combine with ascorbic acid and both compounds together induce the lipoperoxidative capacity of the tissue. The terminal step of this reaction sequence may be represented by a radical damage of the unsaturated fatty

acids in the membrane phospholipids, which are essential for the Na, K-ATPase activity. This step may explain the relative specificity of the ouabain-dependent part of the ATPase activities to the inhibition. As shown by Sunn [23], an artificially induced peroxidation of unsaturated brain phospholipids leads preferentially to degradation of oubain-sensitive Na, K-ATPase. This type of enzyme inhibition resembles that of the Fe^{2+} and Ca^{2+} inhibition of ATPase activity described before [8–10].

The last question, which should be apparently clarified in the future regards a possible physiological importance of these data. The catecholamine-like lipomobilizing effect of EDTA on the both intact and homogenized white adipose tissue was described by Mosinger [31]. EDTA potentiated the adrenaline-induced lipolysis. The maximum response was observed when both agents were present simultaneously [31]. These data indicate that the catecholamine effect on the plasma membrane of the target tissue may share some common steps with EDTA. The results of the present work suggest that this step could be represented as stabilization of the plasma membrane Na, K-ATPase by the antilipoperoxidative effect of both agents. This effect may be explained by chelating properties of EDTA and the ortho-catechol moiety of the catecholamine molecule.

A number of attempts were made at directly identifying adrenergic receptors by means of binding studies with [^{3}H]epinephrine and [^{3}H]-norepinephrine. More than 99 per cent binding was represented by the nonspecific binding related to the ortho-catechol structure of catecholamine [32, 33] whose biological importance remained undetermined. It may be visualized that these binding sites may function in stabilization of the phospholipid moiety of the Na, K-ATPase. This conclusion is similar to a certain degree to the results of Okuda *et al.* [34, 35] who suggested that phospholipids are essential for the adrenaline induced lipolysis in white adipocytes.

REFERENCES

1. E. W. Sutherland, *Science* **177**, 401 (1972).
2. B. Mosinger, in *Adipose Tissue, Regulation and Metabolic Functions* (Eds B. Jeanrenaud and D. Hepp), pp. 71–75. Academic Press, N.Y. (1970).
3. B. Mosinger and M. Vaughan, *Biochim. biophys. Acta* **144**, 556 (1967).
4. B. Mosinger and M. Vaughan, *Biochim. biophys. Acta* **144**, 569 (1967).
5. P. A. Herd, B. A. Horwitz and R. E. Smith, *Experientia* **26**, 825 (1970).
6. B. A. Horwitz, *Eur. J. Pharmac.* **34**, 241 (1975).
7. K. Yoshimura, *J. Biochem.* **74**, 389 (1973).
8. T. Godfraind, M.-Ch. Koch and N. Vebeke, *Biochem. Pharmac.* **23**, 3505 (1974).
9. A. Schaeffer, G. Unyi and A. K. Pfeifer, *Biochem. Pharmac.* **21**, 2289 (1972).
10. A. Schaeffer, A. Sergi and M. Komlos, *Biochem. Pharmac.* **23** 2257 (1974).
11. J. W. Phillis, *Life Sci.* **14**, 1189 (1974).
12. E. De Robertis, A. P. De Iraldi, G. R. De Lorez and A. L. Salganicoff, *J. Neurochem.* **9**, 23 (1962).
13. H. H. Taussky and E. Shorr, *J. biol. Chem.* **202**, 675 (1953).
14. Z. A. Placer, L. L. Cushmann and B. C. Johnson, *Analyt. Biochem.* **16**, 359 (1966).
15. O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randal, *J. biol. Chem.* **193**, 265 (1951).
16. F. Bernheim, *Radiation Res. Suppl.* **3**, 17 (1963).
17. A. A. Barber, *Radiation Res. Suppl.* **3**, 33 (1963).
18. J. Donaldson, R. St-Pierre, J. Minnich and A. Barbeau, *Can. J. Biochem.* **49**, 1217 (1971).
19. S. Lewin, *Comp. Gen. Physiol.* **47B**, 681 (1974).
20. R. R. Boxal and P. J. R. Phizackerley, *Biochem. Soc. Trans.* **1**, 5 (1973).
21. R. J. Flannery, B. Ke, M. W. Grieb and D. J. Trivich, *J. Am. Chem. Soc.* **77**, 2996 (1955).
22. R. Ralajakshimi and A. J. Palet, *J. Neurochem.* **15**, 195 (1968).
23. A. Y. Sun, *Biochim. biophys. Acta* **266**, 350 (1972).
24. J. Charnock, D. A. Cook, A. I. Almeida and R. To, *Archs Biochem. Biophys.* **159**, 393 (1973).
25. K. P. Wheeler, J. A. Walker and D. M. Barker, Biochem. J. **146**, 713 (1975).
26. K. P. Wheeler and J. A. Walker, *Biochem. J.* **146**, 723 (1975).
27. C. Cotman, M. L. Blank, A. Moehl and F. Snyder, *Biochemistry* **8**, 4606 (1969).
28. R. E. Barnett and J. Palazzozzo, *Ann. N.Y. Acad Sci.* **242**, 69 (1970).
29. R. N. Farias, B. Bloj, R. D. Morero, F. Sineriz and R. E. Trucco, *Biochim. biophys. Acta* **415**, 231 (1975).
30. S. Bishayee and A. S. Balasubramanian, *J. Neurochem.* **18**, 909 (1971).
31. B. Mosinger, *Life Sci.* **8**, (II), 137 (1969).
32. P. Cuatrecacas, *A. Rev. Biochem.* **43**, 169 (1974).
33. R. J. Lefkowitz, *Fedn. Proc.* **37**, 123 (1978).
34. H. Okuda, Y. Saito, N. Matsuoka, E. Takeda and A. Kumai, *J. Biochem.* **83**, 887 (1978).
35. Y. Saito, N. Matsuoka, H. Okuda and S. Fujii, *J. Biochem.* **80**, 929 (1976).