CHAPTER : 5

Effect of alloxan-diabetes on the kinetics attributes of erythrocyte membrane enzymes in male and female rats.

Introduction

Constitutionally increased Na-Li counter-transport activity has been implicated in the predisposition of some diabetic patients to nephropathy (1, 2). A pathogenetic mechanism for diabetic polyneuropathy involving alterations of the Na/K-ATPase activity has been proposed (3, 4). This enzyme activity is present in all eukaryotic cell membranes and can also be measured in the red cell membranes (5). Earlier reporter show that erythrocytes from streptozotocin (STZ)-induced diabetic rats exhibit a lesion in their sodium-potassium adenosine tri-phosphatase (ATPase) (Sodium pump) activity. (6). Similar diabetes-associated reductions in sodium pump activity have been reported by others in erythrocytes (7-9), glomerulus (10), lens (11) retina (12) and autonomic ganglion (13, 14).

There are two possible routes by which Na^+,K^+ ATPase inhibition could occur : 1) inactivation by post-translational modification of Na^+,K^+ ATPase (protein phosphorylation (15-18), oxidation (19) or non-enzymatic glycation (19)) and 2) altered synthesis and/or degradation of Na^+,K^+ ATPase (20-21). According to one report inactivation by post translational modification is the primary cause of inhibited Na^+,K^+ ATPase activity in the diabetic erythrocyte (22). In diabetes number of studies show alterations in membrane phospholipid content in different systems i.e. human RBC membranes (23), microsomal membranes

(24) etc. Shifts in membrane phospholipid content may be important in regulating the activity of a variety of membrane enzymes (25, 26, 27). and changes in membrane phospholipid content/composition would be expected to affect the enzyme activity (25).

The earlier studies (Chapter 4) had shown that phospholipid composition of the erythrocyte membranes was differentially influenced by alloxan-induced diabetes and subsequent treatment with insulin in the male and the female rats. In the light of the above observations, in the present study effect of insulin status on the Na^+,K^+ ATPase activity in erythrocyte membranes from rats belonging to both the sexes was examined. Studies were also carried out to check the substrate kinetics of this enzyme. Since AChE is also present in erythrocyte membranes (28), the studies were extended also to check activities and substrate kinetics of erythrocyte membrane acetylcholinesterase (AChE) in rats of both the sexes.

Materials and methods

Animals were made diabetic as described in chapter 4 of the thesis (29). Insulin treatment (30) was given to the diabetic rats as described in Chapter 4 of the thesis. Details of preparation of RBC membranes (31, 32) and Na^+,K^+ ATPase assay (32, 33) and AChE assay (34, 35) are as described in chapter 2 of the thesis. Procedures for Na^+,K^+ ATPase substrate kinetics (36) and AChE substrate and

temperature kinetics (36,37) are as described in chapter 3 of the thesis.

Results

Data in table 1 show Na^+,K^+ ATPase and AChE activities in the rat erythrocyte membranes. Thus in the control male rats the Na^+,K^+ ATPase activity was 1185 n mole Pi/h/mg protein; in the female rats the activity was somewhat high i.e. 1486 nmoles Pi/h/mg protein. In male diabetic rats the Na^+,K^+ ATPase activity increased by 81 % as compared to the controls and after insulin treatment the activity showed a further marginal increase of about 4 %. In contrast, in the diabetic female rats,the activity decreased by 33 %. After insulin treatment the enzyme activity increased by about 3 fold and was about 96 % higher as compared with the controls. Both the differences were statistically significant.

In male control rats the AChE activity was 234.0 nmoles/min/mg protein; the activity in the control females was comparable. AChE activity in diabetic and insulin treated diabetic group of both the sexes were very low and undetectable.

Typical substrate saturation curve for the Na^+,K^+ ATPase from erythrocyte membrane from rats belonging to both the sexes are shown in Figure 1. The corresponding Eadie-Hofstee plots

Table 1. Na^+,K^+ ATPase and AChE activities in rat erythrocyte membranes.

	Control	Diabetic	Insulin treated diabetic
Na^+,K^+ ATPase			
Male	1185.3±136.0(7)	2145.6±310.4(6)[b]	2226.1±188.6(4)[c]
Female	1486.0± 71.5(4)	797.8± 57.9(5)[d]	2329.8±292.3(6)[a,#]
AChE			
Male	239.6± 11.5(8)	N.D.	N.D.
Female	229.5± 9.2(8)	N.D.	N.D.

Results are expressed as mean±S.E.M. of the number of independent observations indicated in the parentheses.

Na^+,K^+ ATPase activity = n mole Pi liberated / h /mg protein

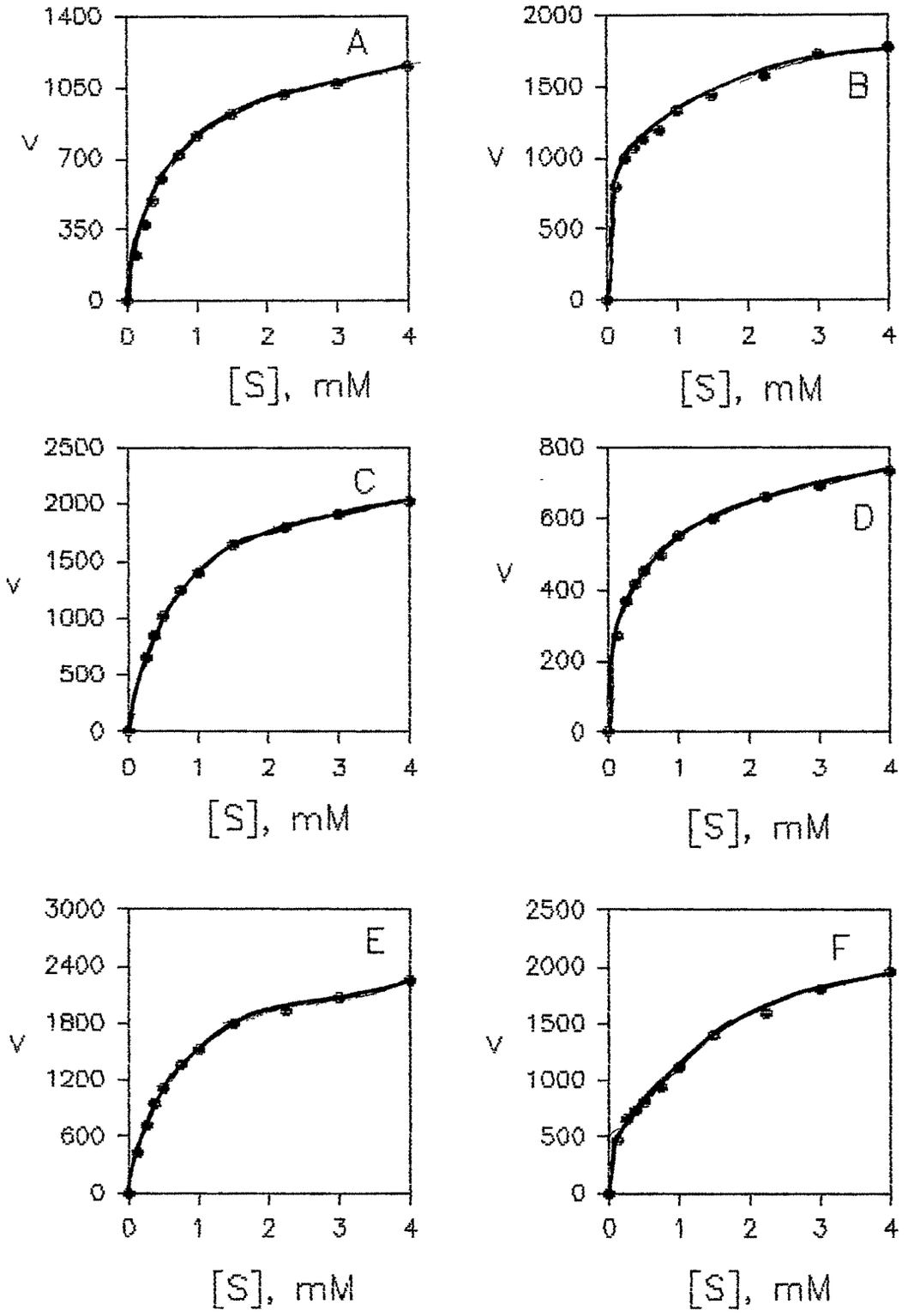
AChE activity = n moles / min / mg protein

N.D. = Not detectable

a, $p<0.05$; b, $p<0.02$; c, $p<0.002$ and d, $p<0.001$ compared with corresponding control.

#, $p<0.001$ compared with corresponding diabetic.

Figure 1.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are shown in Figure 2. The data on Km and Vmax values derived from substrate kinetics of Na^+,K^+ ATPase from erythrocyte membrane of male rats are given in Table 2. It is evident that in all the groups only one component of Na^+,K^+ ATPase was present (eg. see Figure 2). The Km value was 0.56 mM for the control and did not change much in the diabetic or the insulin-treated diabetic group. Compared to the controls, the Vmax values were high in the diabetic as well as the insulin treated diabetic group.

Typical substrate saturation curve for the Na^+,K^+ ATPase activity in RBC membranes from the female rats are shown in Figure 1. The corresponding Eadie-Hofstee are shown in Figure 2. Data in Table 3 summarize the Km and Vmax values derived from substrate kinetics of Na^+,K^+ ATPase. In contrast with the males (eg. Table 3) in the females two component of the enzyme were present. In control group, component I had low Km (0.1 mM) and low Vmax (1100 n moles/h/mg protein) and component II had high Km (0.61 mM) and high Vmax (1648 n mole/h/mg protein). In diabetic female rats, Km of both the components were comparable with the control female rats, while 45 % and 48 % decrease was observed in Vmax of components I and II respectively. After insulin treatment the Km of both the components increased by was 2 and 4 fold compared to the control and diabetic female rats respectively. Both the increases were statistically significant. Vmax value of component I increased by 126 % after insulin treatment as compared to the diabetic group

Figure 2.

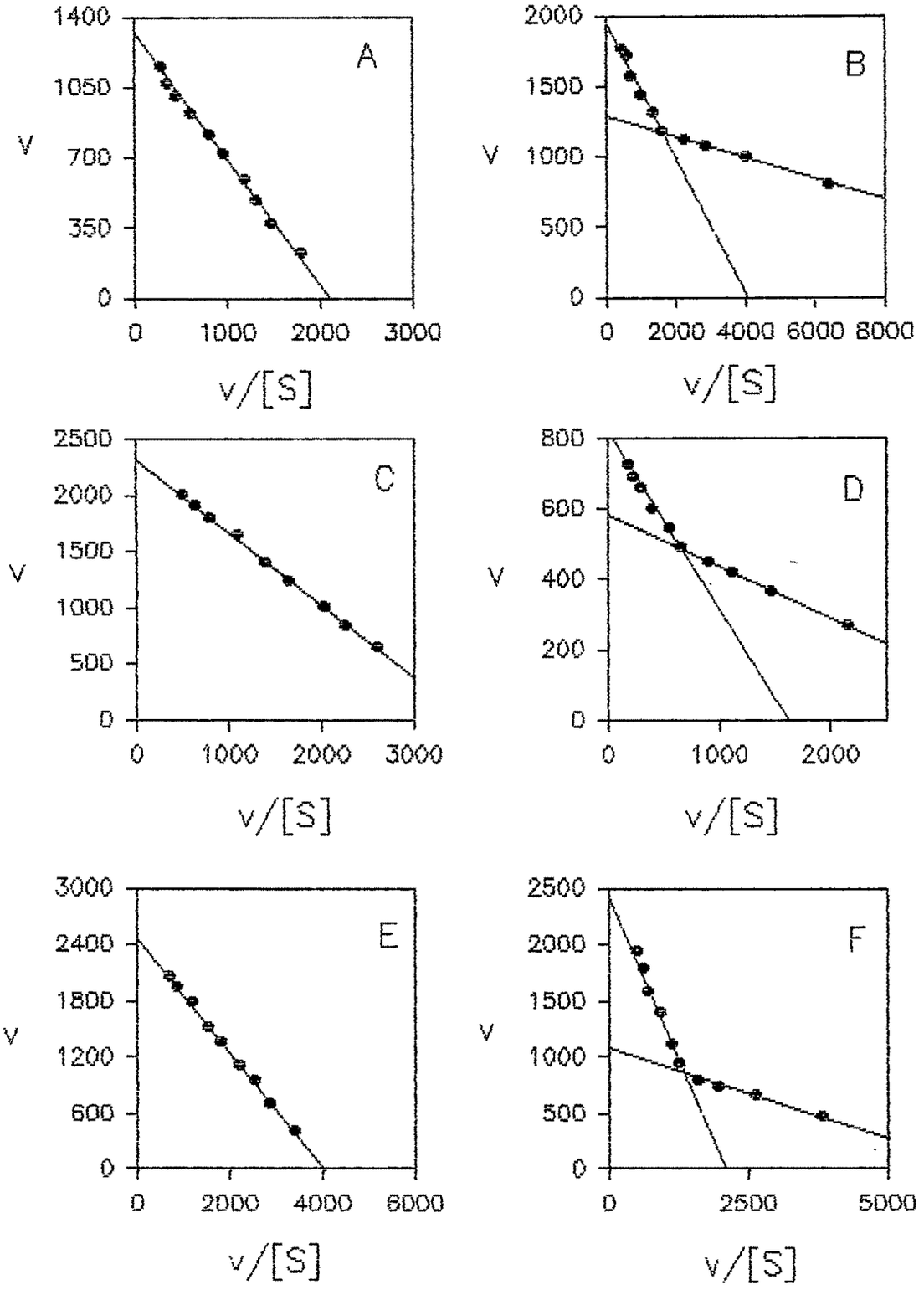
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Table 2. Substrate kinetics of erythrocyte membrane Na^+,K^+ ATPase from male rats.

Animals	Km	Vmax
Control (7)	0.56±0.069	1569.37±169.38
Diabetic (6)	0.60±0.078	2273.30±351.71
Insulin treated diabetic (4)	0.72±0.074	2518.60± 66.83[a]

Results are expressed as mean±S.E.M. of the number of observations indicated in the parentheses.

Units : Km = mM; Vmax = n moles Pi liberated / h / mg protein

a, $p<0.001$ compared with corresponding control.

Table 3. Substrate kinetics of erythrocyte membrane Na^+,K^+ ATPase from female rats.

Animals	Component I		Component II	
	Km	Vmax	Km	Vmax
Control (4)	0.10±0.030	1099.8± 25.8	0.61±0.042	1647.9± 75.0
Diabetic (5)	0.13±0.034	604.6± 59.2 c	0.59±0.065	864.0± 75.2 c
Insulin treated diabetic (6)	0.37±0.089 a,@	1368.6±127.8 #	1.25±0.254 b,#	2385.6±235.6 a,#

Results are expressed as mean±S.E.M. of the number of independent observations indicated in the parentheses.

Units : Km = mM; Vmax = n mole Pi liberated / h / mg protein.

a, $p<0.02$; b, $p<0.05$ and c, $p<0.001$ compared with corresponding control.

@, $p<0.05$ and #, $p<0.001$ compared with corresponding diabetic.

and was 24 % higher than in the control female rats. After insulin treatment Km of component II increased and 2 and 2.2 fold higher than the control and diabetic female rats respectively. Both the increases were statistically significant. After insulin treatment Vmax of component II increased by 2.8 fold as compared to the diabetic female group and was 45 % higher than the control females.

Activity of AChE in rat erythrocyte membrane even in the control group was low and it was not possible to detect AChE activity in diabetic and insulin treated diabetic groups of both the sexes. Hence data on substrate kinetics only of the control group are presented. Typical substrate saturation curves and Eadie-Hofstee plots for this enzyme are shown in Figure 3. From which presence of three components can be noted. Component I had low Km (0.056 mM) and low Vmax (100 nmole/min/mg protein), component II had intermediate Km (0.24 mM) and intermediate Vmax (189 nmole/min/mg protein) and component III had high Km (1.37 mM) and high Vmax (302 n mole/min/mg protein). In females the Km and Vmax values of all the components were comparable to those of males except for the Vmax of component I. Which was 30 % lower.

Typical temperature curves and Arrhenius plots of rat erythrocyte membrane AChE are given in Figure 4, which show presence of phase transition in animals of both the sexes. Data in Table 5 summarize temperature kinetics of rat

Figure 3.

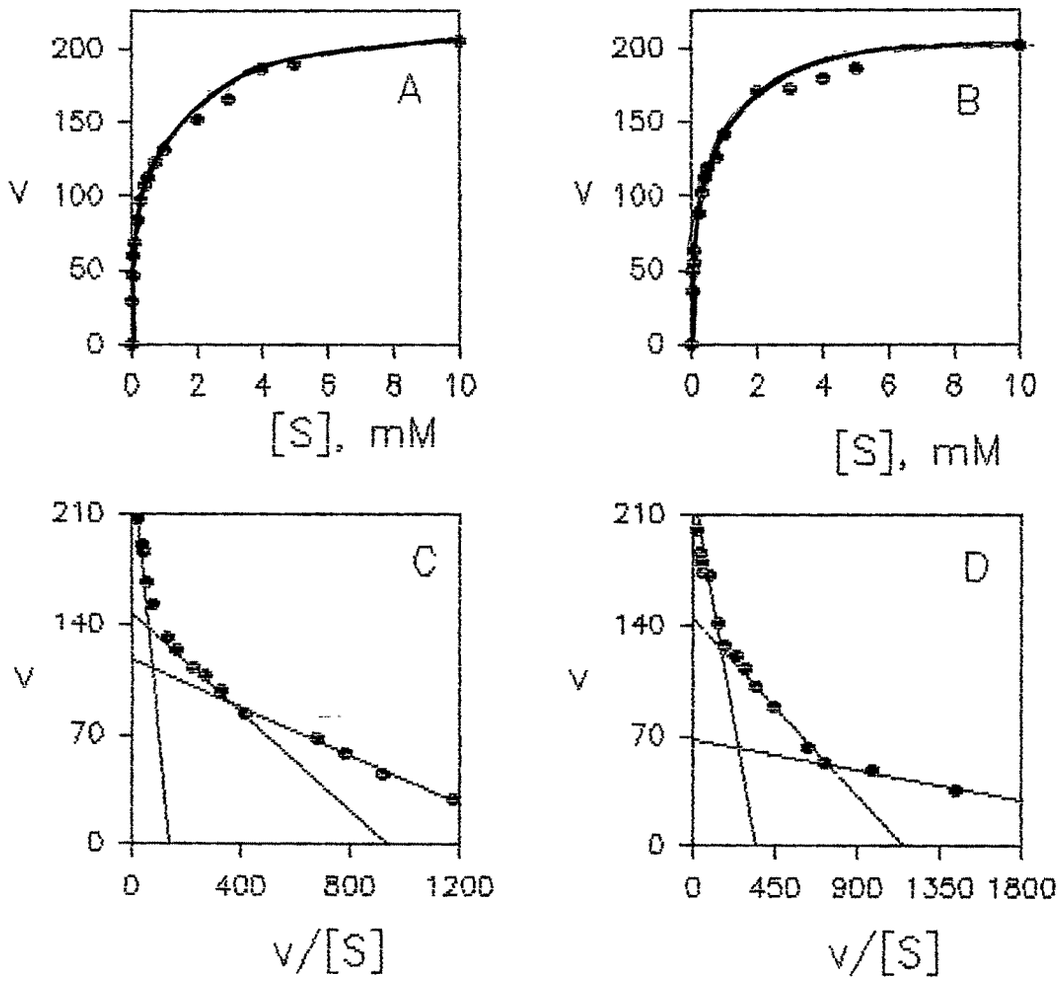
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Figure 4.

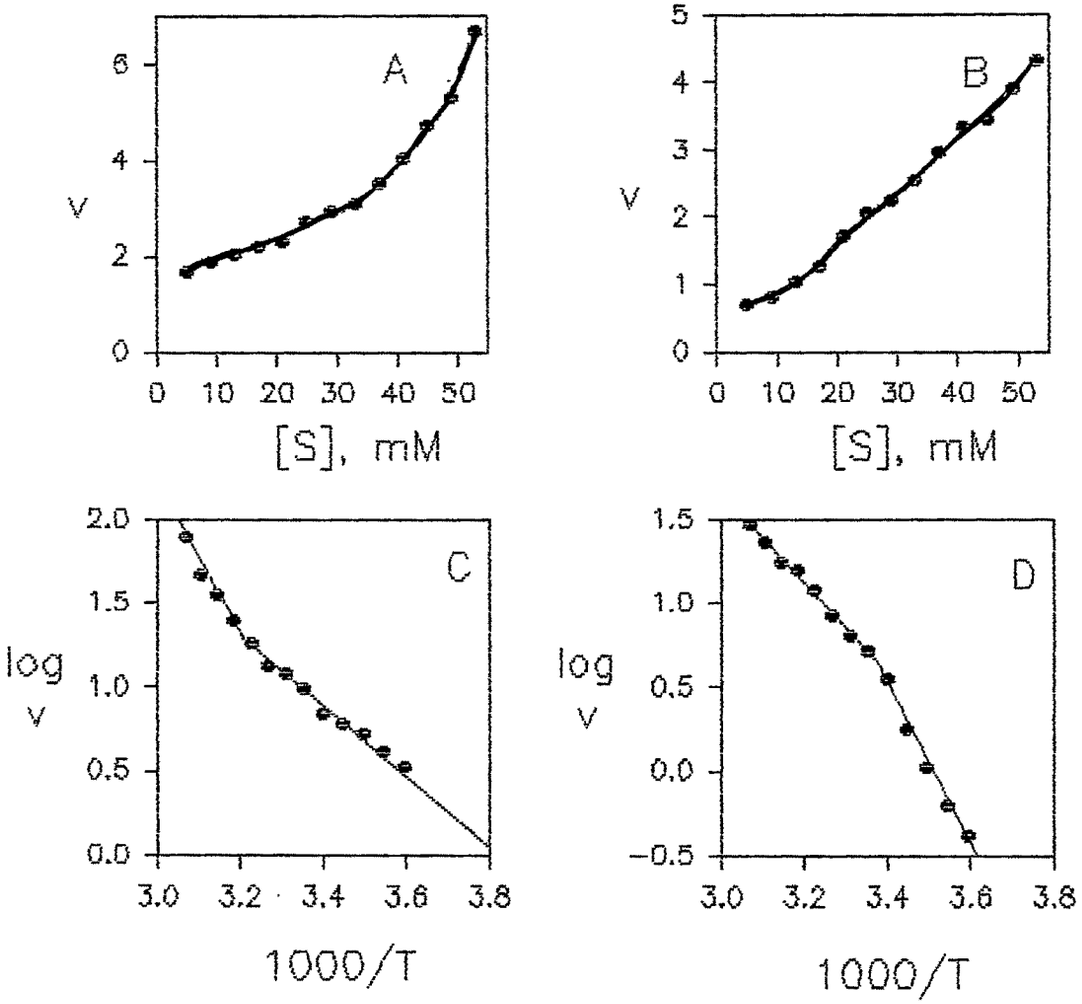

Table 4. Substrate kinetic of erythrocyte membrane AChE from male and female rats.

Animals	Component I		Component II		Component III	
	Km	Vmax	Km	Vmax	Km	Vmax
Male (8)	0.06±0.010	100.5±7.57	0.24±0.028	189.3±11.06	1.37±0.18	302.1±13.57
Female (8)	0.04±0.012	70.2±3.30[a]	0.22±0.025	175.6± 8.74	1.11±0.10	283.0±12.02

Results are expressed as mean±S.E.M. of number of independent observations indicated in the parentheses.

Units : Km = mM, Vmax = n mole / min / mg protein

a, $p<0.02$ compared with corresponding male value.

Table 5. Temperature kinetics of erythrocyte membrane AChE from male and female rats.

Animals	Energy of activation (KJ / mole)		Phase transition temperature (Tt), ^{o}C
	E1	E2	
Male (11)	91.5±3.4	42.2±1.8	38.7±0.56
Female (8)	54.7±2.2[a]	95.7±3.3[a]	20.6±0.34[a]

Results are expressed as mean±S.E.M. of number of independent observations indicated in the parentheses.

a, $p<0.001$ compared with corresponding male value.

erythrocyte membrane AChE. In male rats for higher temperature range energy of activation E1 was high (91 KJ/mole), while for lower temperature range energy of activation E2 was low (42 KJ/mole) and phase transition temperature (Tt) was about 39^{o}C. In female rats the picture was reversed. For higher temperature range the energy of activation E1 was low (55 KJ/mole) and for lower temperature range energy of activation E2 was high (96 KJ/mole). The phase transition temperature (Tt) was about 18^{o}C lower than the control male rats.

Discussion

Decreased Na^{+},K^{+} ATPase activity in different membrane systems including RBC membranes in the diabetic rats is well established (7, 14). In contrast to this it was observed in the present studies that in the diabetic male rats the enzyme activity increased (Table 1). The reason for this discrepancy is not clear at the present. However, the observed difference may be attributed to strain difference i.e. the present studies were carried out using rats belonging to Charles-Foster strain. In contrast to the male rats, in the diabetic female rats the enzyme activity decreased. This agrees well with the observations of other researchers referred to above (7, 14).

Na^{+},K^{+} ATPase is and integral protein in erythrocyte membrane (24) and its activity can be influenced by phospholipids

(38). The erythrocyte membrane Na^+,K^+ ATPase differed in males and the females with respect to its kinetic attributes. Thus there was only one component of the enzyme in the males while in the females there were two kinetically distinguishable components. Response to insulin treatment was also differential in the male and female rats. The above differences could be explained at least partly on the basis of differential phospholipid composition/metabolism reported earlier (Chapter 4).

AChE activity in erythrocyte membranes from control male rats reported in the present studies is higher than the earlier reported value (39). However, in our Labs we are consistently getting high activity values (40). No sex-dependent difference in AChE activity in the erythrocyte membrane was observed. Decreased in erythrocyte membrane AChE activity in diabetic condition in humans and rats is reported by earlier workers (39, 41). But we were unable to measure AChE activity in both diabetic as well as insulin treated diabetic group of animals of both the sexes. AChE is loosely bound to outer surface of the erythrocyte membrane by glycolipid anchor (28, 42). It is possible that due alterations in membrane phospholipid composition (Chapter 4) AChE may not bind properly to the RBC membrane in the diabetic and the insulin-treated diabetic rats. Which could explain the negligible enzyme activity under these condition.

It was not possible to perform temperature kinetics of Na^+,K^+ ATPase because the erythrocyte membrane preparations were not available under these condition.

For AChE, the substrate kinetics data were almost comparable for the males and females except that the Vmax of component I was 30 % lower in the females. Survey of the literature revealed that although data on AChE content of RBC membrane are available (41) no information on the substrate kinetics of this enzyme has been reported (28, 42). Based on the data presented it was possible to resolve the enzyme activity in three components. However no major sex-linked differences in the substrate kinetics of AChE could be noted although the Vmax of component I was somewhat low in the females. The sex-linked differences could be observed in the temperature kinetics of the enzyme. In the male rats Arrhenius plot was the reverse of what is conventionally expected (37) i.e. in the higher temperature range the energy of activation was high and for lower temperature range the energy of activation was low (37). The plot in female rats was opposite to that of the males and conformed with the conventional Arrhenius plots (37). Also in the females, the phase transition temperature was close to room temperature rather than near physiologic as in the case of the males (Table 5). It is possible that the observed differences could arise due to lipid/phospholipid content and compositional changes referred to above (Chapter 4).

In conclusion although the erythrocyte membrane Na^+,K^+ ATPase activity was almost comparable in both the sexes drastic difference was observed in their kinetic attributes i.e. in male erythrocyte membrane only one component of Na^+,K^+ ATPase was present while in females two components of this enzyme were present. In spite of differences in membrane phospholipid composition no sex-dependent differences were observed in the activity of erythrocyte membrane AChE and its substrate kinetic attributes. In diabetic condition AChE is possibly unable to bind erythrocyte membranes properly and the defect was not corrected by insulin treatment. Although substrate kinetic attributes of AChE from both the sexes were similar, drastic sex-dependent difference was observed in temperature kinetics which might be because of difference in membrane phospholipid composition.

Summary

The Na^+,K^+ ATPase activity of the erythrocyte membranes increased in the diabetic male rats. By contrast, in the diabetic female rats the Na^+,K^+ ATPase activity decreased.

There was only one component of Na^+,K^+ ATPase in the males while in the females there were two kinetically distinguishable components.

Component(s) of Na^+,K^+ ATPase were differentially affected after insulin treatment in the male and the female rats.

No sex-dependent difference in AChE activity in the erythrocyte membrane was observed in the control group and the enzyme activity was not detectable in the diabetic as well as insulin-treated diabetic group of animals of both the sexes.

Sex-linked differences were observed in temperature kinetics of the AChE. The Arrhenius plot for AChE in female rats was opposite to that of the males and was compatible with the conventional Arrhenius plots.

In the females, the phase transition temperature was close to room temperature rather than near physiologic as in the case of the males.

Figure legends

Figure 1 Typical substrate saturation curves for rat erythrocyte membrane Na^+,K^+ ATPase. (A) Control male (B) Control female (C) Diabetic male (D) Diabetic female (E) Insulin treated diabetic male (F) Insulin treated diabetic female rats. Other experimental details are as described in the text.

Figure 2 Typical Eadie-Hofstee plots for rat erythrocyte membrane Na^+,K^+ ATPase. (A) Control male (B) Control female (C) Diabetic male (D) Diabetic female (E) Insulin treated diabetic male (F) Insulin treated diabetic female rats. Other experimental details are as described in the text.

Figure 3 Typical substrate saturation curves and Eadie-Hofstee plots for rat erythrocyte membrane AChE. (A) substrate saturation curve for control male (B) substrate saturation curve for control female (C) Eadie-Hofstee plot for control male (D) Eadie-Hofstee plot for control female rats. Other experimental details are as described in the text.

Figure 4 Typical temperature curves and Arrhenius plots for rat erythrocyte membrane AChE. (A) Temperature curve for control male (B) Temperature curve for control female (C) Arrhenius plot for control male (D) Arrhenius plot for control female rats. Other experimental details are as described in the text.

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