Chapter 8

Effects of Alloxan-Diabetes and Subsequent Treatment with Insulin on Kinetic Properties of Na^+, K^+-ATPase and Glucose-6-Phosphatase from Rat Liver Microsomes

Introduction

Diabetic state significantly alters the membrane enzymes Na^+, K^+-ATPase and glucose-6-phosphatase (G6Pase) in different tissues/organelles (1-7). Na^+, K^+-ATPase activity is decreased in many tissues of streptozotocin-induced diabetic animals (1-3, 8). The enzyme G6Pase is a key enzyme of gluconeogenasis (7). Expression of G6Pase is known to increase in diabetes (4).

The microsomal membrane enzymes are known to have requirement of specific phospholipids (9, 10). As pointed out earlier (Chapter 2-7 of the Thesis), alloxan-diabetes differentially altered mitochondrial membrane structure-function relationship. Hence, it is of interest to find out if the microsomal membrane composition and properties of membrane-bound enzymes were also affected by alloxan-diabetes. Thus, effects of alloxan-diabetes and subsequent treatment with insulin on liver microsomes have been evaluated. To achieve this goal, the kinetic properties of Na^+, K^+-ATPase and G6Pase from rat liver microsomes were examined. The lipid/phospholipid profiles were also examined. The results are summarized below.

Materials and Methods

Chemicals

Details of chemicals required, procedure of induction of diabetes, insulin treatment, isolation of microsomes, extraction of lipid/phospholipids, estimation of cholesterol, determination of phospholipid profile and membrane fluidity are as described in Chapter 2 of the Thesis.

ATPase assay

ATPase activity was measured in the assay medium (total volume 0.1 ml) containing 100 mM Tris-HCl buffer pH 7.4, 20 mM KCl, 240 mM NaCl and 8 mM $MgCl_2$. After pre-incubating the microsomal protein (30-50 µg) in the assay medium at 37 °C for 1 min, the reaction was initiated by the addition of ATP at a final concentration of 5 mM (11). The reaction was terminated after 10 min by addition of 0.1 ml of 5% (w/v) sodium dodecyl sulfate (SDS) solution and the amount of librated inorganic phosphorus was estimated by the method of Katewa and Katyare (11).

G6Pase assay

G6Pase activity was measured in the assay medium (total volume 0.1 ml) containing 100 mM Na-acetate, pH 6.5 containing 2 mM EDTA. After pre-incubating the microsomal protein (30-50 µg) in the assay medium at 37 °C for 1 min, the reaction was initiated by the addition of G6P at a final concentration of 5 mM (11). The reaction was terminated after 10 min by addition of 0.1 ml of 5% (w/v) sodium dodecyl sulfate (SDS) solution and the amount of librated inorganic phosphorus was estimated by the method of Katewa and Katyare (11).

For the substrate kinetics studies concentration of ATP was varied in the range from 0.1 mM to 5 mM while concentration of G6P was varied in the range from 0.1 mM to 20 mM.

Temperature kinetics studies were carried out as detailed (Chapter 2 of the Thesis) using fixed ATP and G6P concentrations (5 mM).

The substrate and temperature kinetics data were analyzed as detailed (Chapter 2 of the Thesis).

Results

The results on body and liver weight, diabetes parameters etc are detailed in Chapter 2 of the Thesis.

Effects on ATPase

In the preliminary studies ATPase activity was determined at room temperature (25 °C) and at the physiological temperature (37 °C). The data are given in Table 1. As can be noted at early stage of diabetes ATPase activity increased by 41 and 24 % at two temperatures whereas at late stage the effect was differential. The activity increased by 37 % at 25 °C whereas it registered decrease of 28 % at 37 °C. Insulin treatment in one week diabetic animals partially restored the activity at 25 °C whereas the activity at 37 °C was unchanged. The opposite effect was noted in one month diabetic animals following insulin treatment. The activity at 25 °C was unchanged while the activity at 37 °C completely restored. Compared to the control, the activity ratios were low under all the experimental conditions. (Table 1), which is suggestive of differential temperature response.

Table 1. Effect of alloxan-diabetes and insulin treatment on Na^+, K^+ ATPase activity in rat liver microsomes

Group	Treatment	Activity (μmole Pi liberated/hr/mg protein)		Activity Ratio
		25 °C	37 °C	
One week	Control	2.73 ± 0.14	10.60 ± 0.39	3.88 ± 0.18
	Diabetic	5.23 ± 0.17[c]	12.52 ± 0.23[b]	2.39 ± 0.12[c]
	Diabetic + Insulin	4.40 ± 0.19[c,ψ]	13.11 ± 0.53[c]	2.98 ± 0.13[b,ψ]
One month	Control	2.79 ± 0.13	10.56 ± 0.42	3.78 ± 0.16
	Diabetic	3.84 ± 0.21[b]	7.56 ± 0.29[c]	1.97 ± 0.07[c]
	Diabetic + Insulin	4.45 ± 0.19[c,]	10.54 ± 0.18[§]	2.37 ± 0.11[c,§]

The experimental details are given in the text.

Activity ratio was calculated as: activity at 37 °C/activity at °C

The results are given as mean ± SEM of 6-8 independent experiments.

a, $p<0.01$; b, $p<0.002$ and c, $p<0.001$ compared to the corresponding control.
ψ, $p< 0.01$ and §, $p<0.001$ compared to the corresponding diabetic.

In view of the differential changes in one week and one month groups further experiments were carried out to examine the kinetic behavior of ATPase as a response to change in the substrate i.e. ATP concentration. The data on substrate kinetics was analyzed as detailed (Chapter 2 of the Thesis). The typical substrate saturation and corresponding Eadie-Hofstee plots and Hill plots for ATPase are shown in Fig. 1 and 2 Panels A-C and D-F respectively. As can be noted, in all experimental conditions the enzyme activity resolved in two kinetically distinguishable components (Fig. 1 and 2 Panels D-F).

Data on Km and Vmax are given in Table 2. Thus, in the control group the Km value of the two components were 0.14 and 0.47 mM respectively with corresponding values of Vmax of 7.6 and 12.4 units. The diabetic state resulted in general progressive increase in Km values for both the components. The Vmax of component I decreased by 47 % at late stage of diabetes. For component II the picture was different. The Vmax increased at early stage and showed a marginal decrease at late stage. Insulin treatment in one week diabetic group was ineffective in restoring the Km values to normality whereas in one month diabetic animals, insulin treatment completely restored the Km for component I whereas partial restoration was noted in Km value for component II. Vmax for component II increased in one week diabetic group and insulin treatment had no restorative effect. In one month diabetic group Vmax for both components decreased; insulin treatment more or less restored to normality (Table 2).

Figure 1

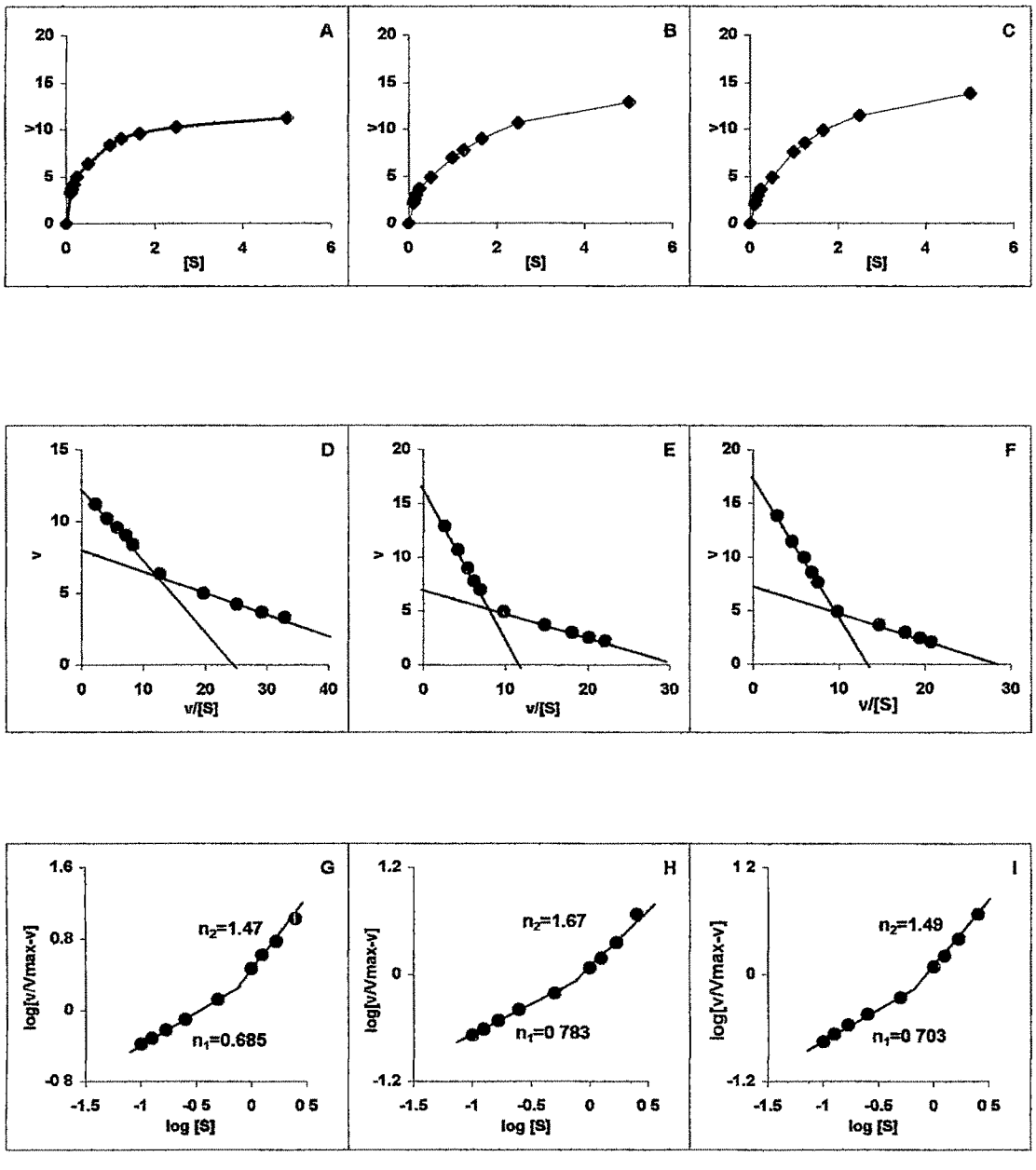
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Figure 2

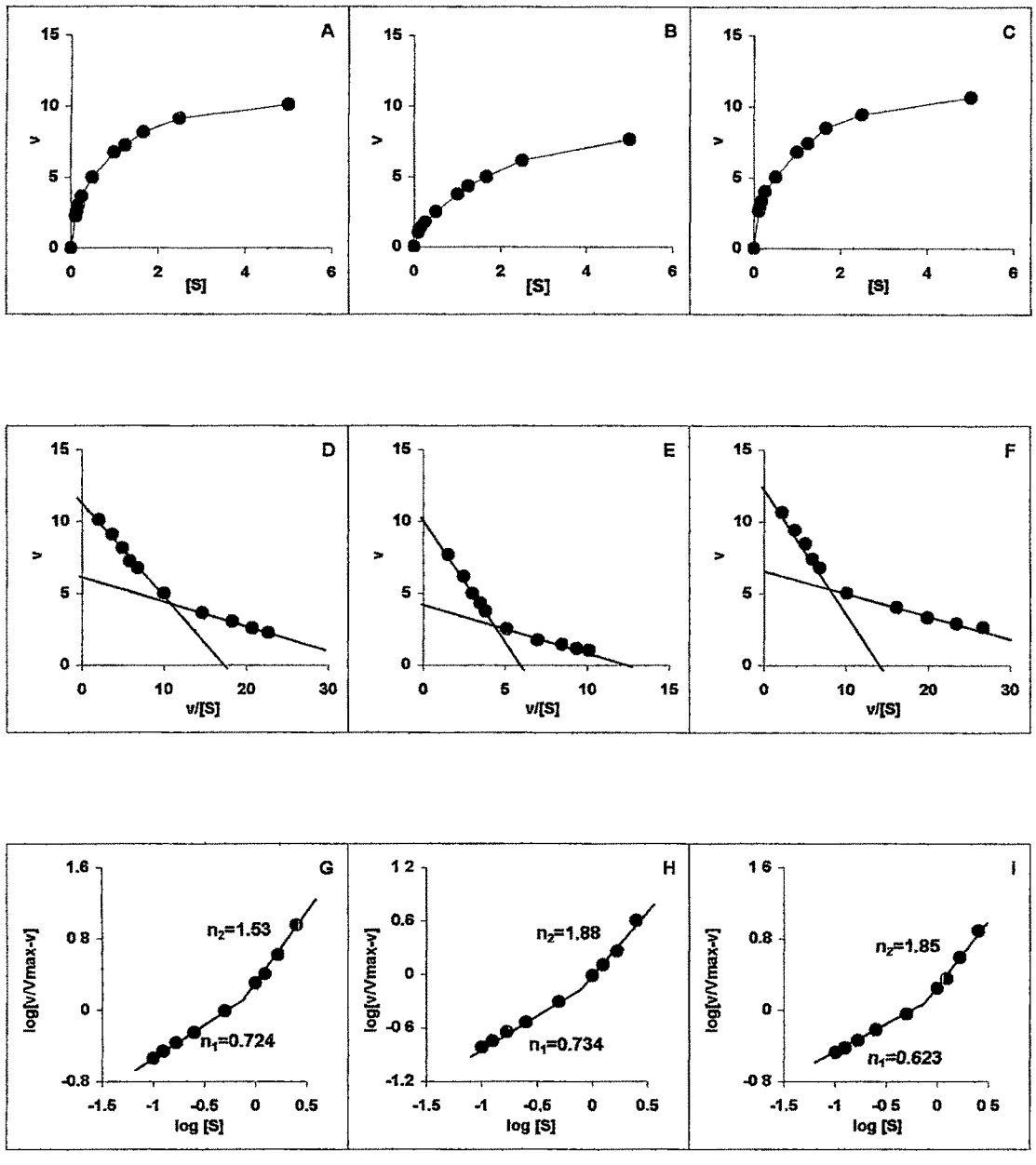
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Figure 1. Typical substrate saturation curve (Panels A, B, C), corresponding Eadie-Hofstee (Panels D, E, F) and Hill (Panels G, H, I) plots for Na^+, K^+-ATPase from liver microsomes for one week group. In substrate saturation curve the enzyme activity v on abscissa is plotted versus [S] on ordinate v is the enzyme activity at the given ATP concentration [S]. A, B and C represent to the control, diabetic and insulin-treated diabetic groups. In Eadie-Hofstee plot the enzyme activity v on abscissa is plotted versus v/[S] on ordinate. D, E and F represent to the control, diabetic and insulin-treated diabetic groups. In Hill plot log (v/Vmax-v) on abscissa is plotted versus log [S] on ordinate. The Hill coefficients n_1 and n_2 depict number of ATP molecules bound for the given concentration range of ATP. G, H and I represent to the control, diabetic and insulin-treated diabetic groups. The plots are typical of 6-8 independent experiments in each group.

Figure 2. Typical substrate saturation curve (Panels A, B, C), corresponding Eadie-Hofstee (Panels D, E, F) and Hill (Panels G, H, I) plots for Na^+, K^+-ATPase from liver microsomes for one week group. In substrate saturation curve the enzyme activity v on abscissa is plotted versus [S] on ordinate v is the enzyme activity at the given ATP concentration [S]. A, B and C represent to the control, diabetic and insulin-treated diabetic groups. In Eadie-Hofstee plot the enzyme activity v on abscissa is plotted versus v/[S] on ordinate. D, E and F represent to the control, diabetic and insulin-treated diabetic groups. In Hill plot log (v/Vmax-v) on abscissa is plotted versus log [S] on ordinate. The Hill coefficients n_1 and n_2 depict number of ATP molecules bound for the given concentration range of ATP. G, H and I represent to the control, diabetic and insulin-treated diabetic groups. The plots are typical of 6-8 independent experiments in each group.

Table 2. Effect of alloxan-diabetes and insulin treatment on substrate kinetics properties of Na^+, K^+ ATPase in rat liver microsomes

Group	Treatment	Component I		Component II	
		Km	Vmax	Km	Vmax
One week	Control	0.14 ± 0.01	7.59 ± 0.16	0.47 ± 0.02	12.35 ± 0.65
	Diabetic	0.23 ± 0.01[d]	7.07 ± 0.39	1.34 ± 0.09[d]	16.47 ± 0.84[c]
	Diabetic + Insulin	0.24 ± 0.01[d]	7.06 ± 0.47	1.26 ± 0.92[d]	17.41 ± 0.59[d]
One month	Control	0.16 ± 0.01	7.31 ± 0.19	0.43 ± 0.02	12.28 ± 0.59
	Diabetic	0.28 ± 0.01[d]	3.88 ± 0.14[d]	1.72 ± 0.03[d]	10.41 ± 0.42[a]
	Diabetic + Insulin	0.14 ± 0.01[§]	6.35 ± 0.27[b,§]	0.74 ± 0.04[d,§]	12.17 ± 0.89

The Km (mM) and Vmax (μmole of Pi liberated / hr / mg protein) values were calculated as described in the text.

The results are given as mean ± SEM of 6-8 independent experiments. As indicated in the text, the kinetic components represent the potential and the response of the enzyme to increasing concentrations of the substrate.

a, $p< 0.05$; b, $p< 0.02$; c, $p< 0.01$ and d, $p<0.001$ compared to the corresponding control.
§, $p<0.001$ compared to the corresponding diabetic.

Analysis of the data by Hill plots indicated that up to 0.6 mM ATP concentration one ATP molecule was bound to the enzyme while beyond this concentration of substrate two molecules of ATP were bound under all experimental conditions (data not given) Typical Hill plots are shown in Fig. 1 and 2 Panels G-I.

In the next set of experiment the temperature dependence of the enzyme activity was examined. The temperature kinetics data were analyzed as detailed in Chapter 2 of the Thesis. The typical activity versus temperature plots and corresponding Arrhenius plots for early and late effects are given in the Fig. 3 and 4 Panels A-C and D-F. As can be seen, in one week as well as one month groups the activity versus temperature plots in the experimental groups differed considerably from the corresponding controls (Fig. 3 and 4). The optimum temperature in the control group was 45 °C. In one week diabetic group the optimum temperature shifted to 49 °C; insulin treatment reversed the effect (Fig. 3 Panels A-C). Interestingly, in one month group the optimum temperature was unchanged in the experimental groups (Fig. 4, Panels A-C). This was also reflected in terms of corresponding Arrhenius plots (Fig. 3 and 4, Panels D-F). In the control group Arrhenius plots followed typical biphasic pattern i.e. the energies of activation in high temperature ranges (E_H) was lower than that of in low temperature ranges (E_L). In one week diabetic group the Arrhenius pattern was unchanged (Fig. 3 Panel E). However, following insulin treatment, the pattern reversed i.e. energies of activation in high temperature ranges (E_H) was higher than that of in low temperature ranges (E_L). (Fig. 3 Panel F). The data on energies of activation and phase transition temperature are given in Table 3. In control group the values of E_H and E_L were about 80 and 125 KJ/mole with phase transition at

Figure 3

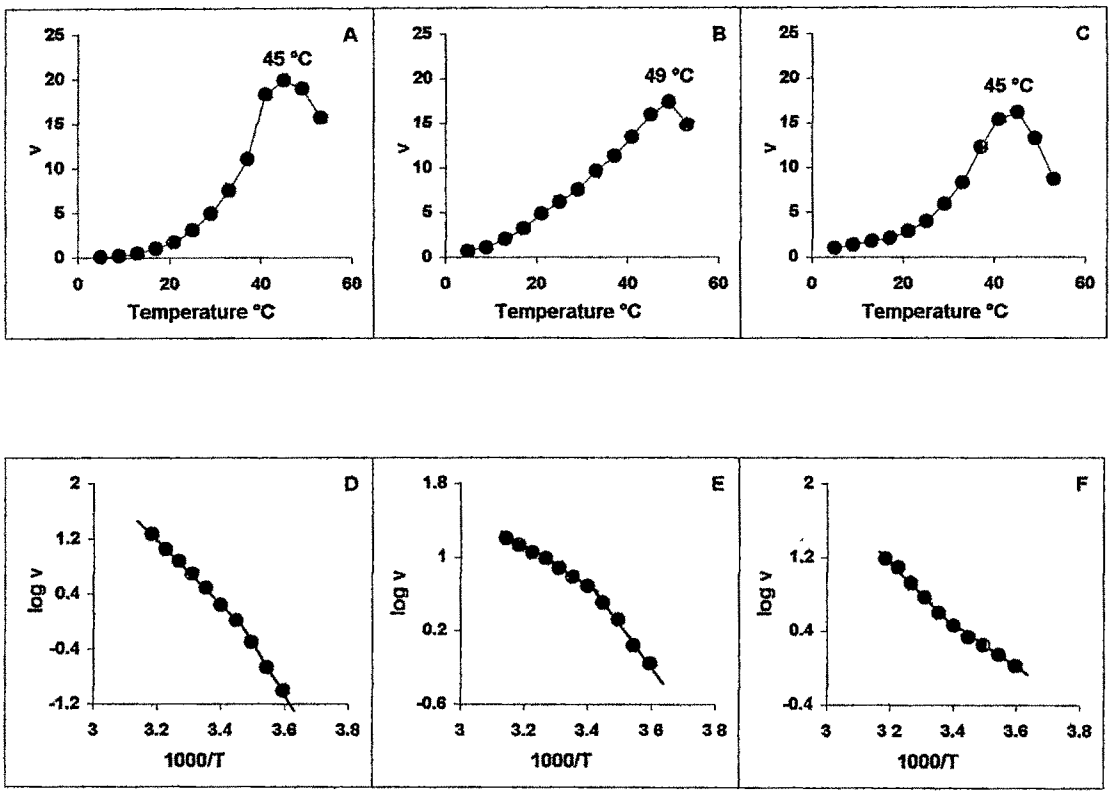
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Figure 4

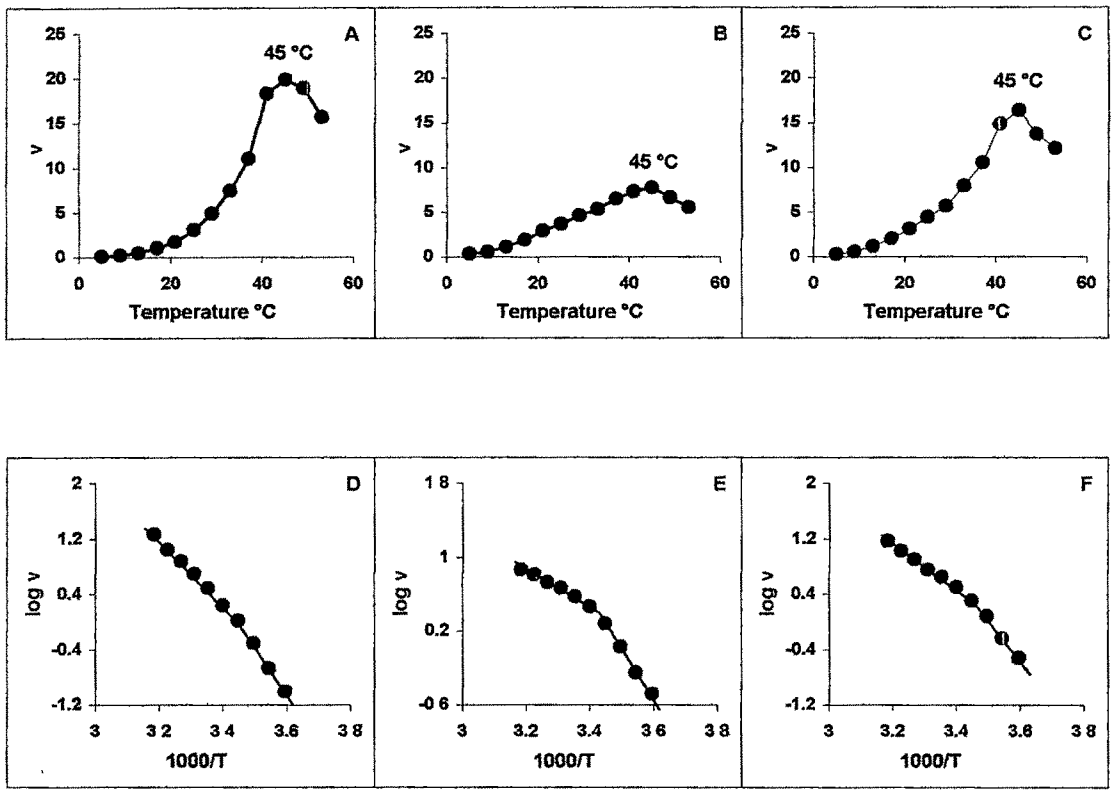
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Figure 3. Typical plots depicting dependence of enzyme activity on the temperature and corresponding Arrhenius plots for Na^+, K^+-ATPase from liver microsomes for one week groups. In temperature curves, enzyme activity v on abscissa is plotted verses temperature (°C) on ordinate. A, B and C represent to the control, diabetic and insulin-treated diabetic groups. In Arrhenius plots log of v on ordinate is plotted against 1000/T on abscissa where v and T represent respectively, the activity at corresponding absolute temperature T (temperature in ° Celcius + 273.2). D, E and F represent to the control, diabetic and insulin treated diabetic groups. The plots are typical of 6-8 independent experiments in each group.

Figure 4. Typical plots depicting dependence of enzyme activity on the temperature and corresponding Arrhenius plots for Na^+, K^+-ATPase from liver microsomes for one month groups. In temperature curves, enzyme activity v on abscissa is plotted verses temperature (°C) on ordinate. A, B and C represent to the control, diabetic and insulin-treated diabetic groups. In Arrhenius plots log of v on ordinate is plotted against 1000/T on abscissa where v and T represent respectively, the activity at corresponding absolute temperature T (temperature in ° Celcius + 273.2). D, E and F represent to the control, diabetic and insulin treated diabetic groups. The plots are typical of 6-8 independent experiments in each group.

Table 3. Effect of alloxan-diabetes and insulin treatment on Arrhenius kinetics properties of Na^+, K^+ ATPase in rat liver microsomes

		Energy of activation (KJ/mole)		Phase transition temperature T_t (°C)
Group	Treatment	E_H	E_L	T_t
One week	Control	70.58 ± 6.26	111.92 ± 9.63	17.49 ± 1.16
	Diabetic	44.30 ± 1.90^{c}	86.46 ± 3.97^{a}	19.77 ± 0.56
	Diabetic + Insulin	65.76 ± 0.56$^{\psi}$	43.18 ± 0.85$^{b,\psi}$	17.46 ± 1.00
One month	Control	82.92 ± 1.61	128.42 ± 4.35	19.61 ± 0.91
	Diabetic	37.62 ± 2.97^{d}	87.37 ± 1.94^{c}	21.41 ± 1.64
	Diabetic + Insulin	56.19 ± 1.01$^{d,\psi}$	110.01 ± 2.17^{b}	16.99 ± 1.28§

The experimental details are given in the text. The results are given as mean ± SEM of 6-8 independent experiments.

a, $p< 0.05$; b, $p< 0.01$; c, $p<0.002$ and c, $p<0.001$ compared to the corresponding control.

§, $p< 0.01$ and ψ, $p<0.001$ compared to the corresponding diabetic.

around 18 °C. Both early and late diabetic states significantly decreased the values of E_L and E_H. Insulin treatment in one week animals restored only E_H to normality whereas in one month diabetic animals insulin treatment had partial restorative effects on both E_L and E_H values (Table 3). Phase transition temperature (T_t) in general, did not change in any experimental condition.

Effects on G6Pase

Data on effects of alloxan-diabetes and subsequent insulin treatment on G6Pase activity are given in Table 4. Thus, in one week diabetic group, measurements of G6Pase activity at 25 °C increased by 28 % while in one month diabetic group, phenomenal increase (3.8 fold) was noted in the activity. Almost similar picture was noted when the activity measurements was carried out at 37 °C. In early stage of diabetes activity increased by 43 % and at late stage 3 fold increased was noted. Insulin treatment was not effective at early stage but had partial restorative effects at late stage of diabetes. In early diabetic state, the activity ratio was unchanged while decreased at late stage. Insulin in one month diabetic group restored the activity ratio to normality (Table 4).

In the next set of experiment the kinetic behavior of G6Pase in response to change in substrate concentration i.e. G6P concentration. Typical substrate saturation curve and corresponding Eadie-Hofstee plots and Hill plots for G6Pase are shown in Fig. 5 and 6. The Eadie-Hofstee plots revealed that the enzyme activity resolved in two kinetically distinguishable components in all the groups (Fig. 5 and 6, Panels D- F).

Table 4. Effect of alloxan-diabetes and insulin treatment on glucose-6-phosphatase activity from rat liver microsomes

Group	Treatment	Activity (μmole Pi liberated/hr/mg protein)		Activity Ratio
		25 °C	37 °C	
One week	Control	2.56 ± 0.08	8.53 ± 0.23	3.33 ± 0.12
	Diabetic	3.27 ± 0.15[b]	12.23 ± 0.38[c]	3.74 ± 0.19
	Diabetic + Insulin	4.94 ± 0.23[c,§]	15.87 ± 0.49[c,§]	3.21 ± 0.14[Ψ]
One month	Control	2.78 ± 0.17	8.85 ± 0.36	3.18 ± 0.15
	Diabetic	10.48 ± 0.50[c]	26.41 ± 0.49[c]	2.52 ± 0.11[c]
	Diabetic + Insulin	5.21 ± 0.18[c,§]	17.61 ± 0.51[c,§]	3.38 ± 0.11[§]

The experimental details are given in the text.

Activity ratio was calculated as: activity at 37 °C/activity at °C

The results are given as mean ± SEM of 6-8 independent experiments.

a, $p<0.002$ and a, $p<0.001$ compared to the corresponding control.
Ψ, $p< 0.05$ and §, $p<0.001$ compared to the corresponding diabetic.

Figure 5

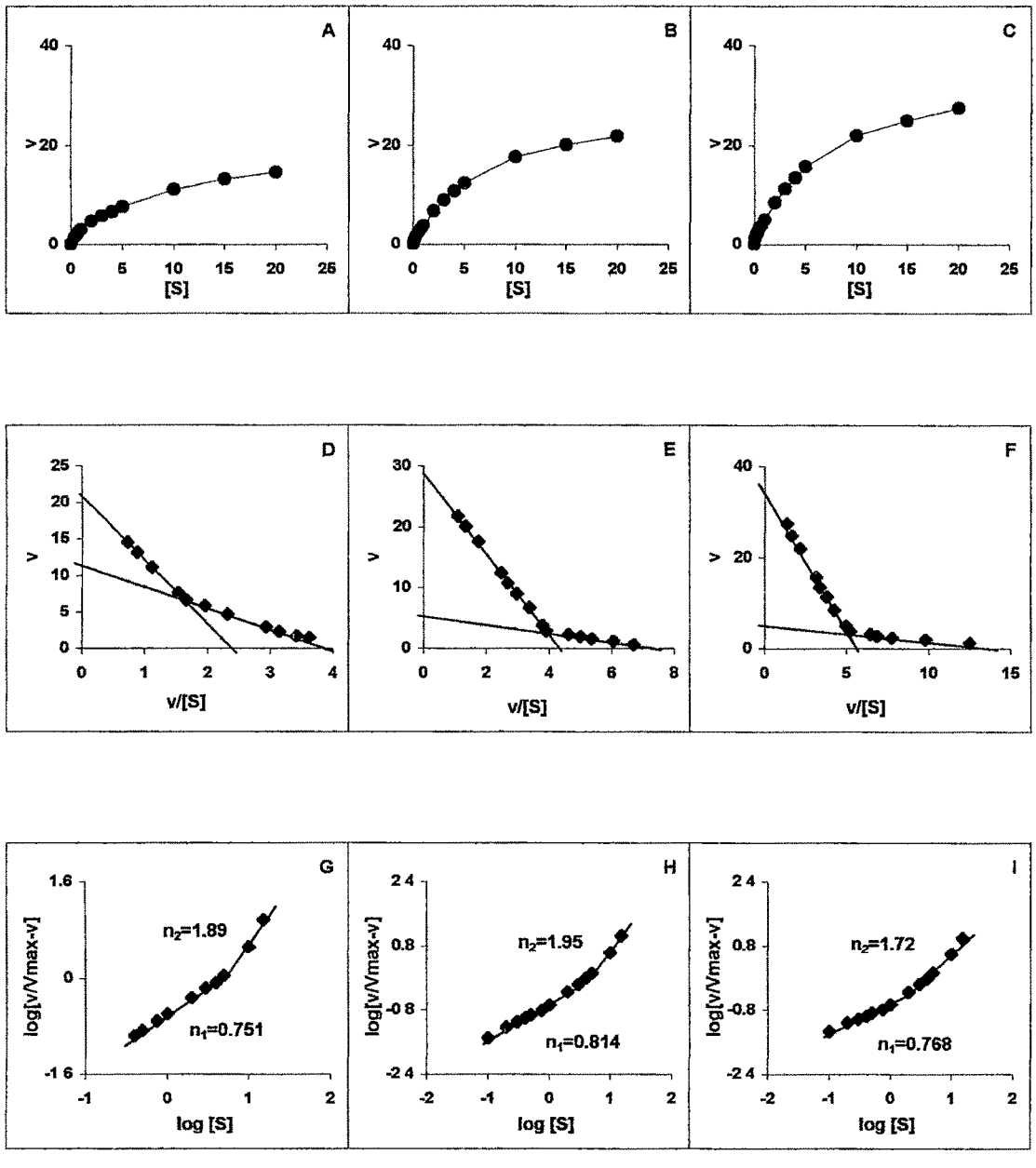
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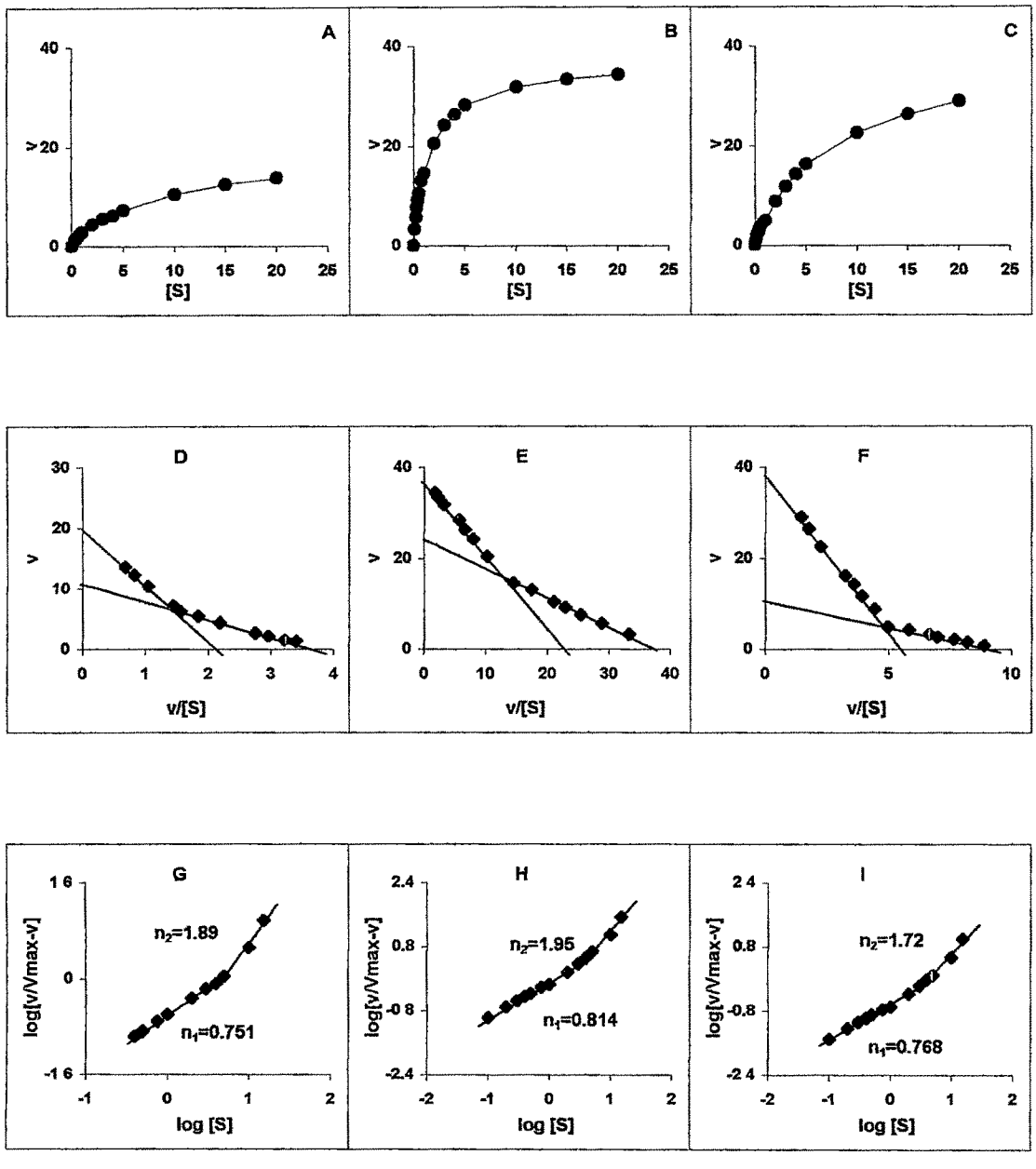
Figure 6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n1=0.751
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n1=0.814
n2=1.72
n1=0.768

Figure 5. Typical substrate saturation curve (Panels A, B, C), corresponding Eadie-Hofstee (Panels D, E, F) and Hill (Panels G, H, I) plots for G6Pase from liver microsomes for one week group. In substrate saturation curve the enzyme activity v on abscissa is plotted versus [S] on ordinate v is the enzyme activity at the given ATP concentration [S]. A, B and C represent to the control, diabetic and insulin-treated diabetic groups. In Eadie-Hofstee plot the enzyme activity v on abscissa is plotted versus v/[S] on ordinate. D, E and F represent to the control, diabetic and insulin-treated diabetic groups. In Hill plot log (v/Vmax-v) on abscissa is plotted versus log [S] on ordinate. The Hill coefficients n_1 and n_2 depict number of ATP molecules bound for the given concentration range of ATP. G, H and I represent to the control, diabetic and insulin-treated diabetic groups. The plots are typical of 6-8 independent experiments in each group.

Figure 6. Typical substrate saturation curve (Panels A, B, C), corresponding Eadie-Hofstee (Panels D, E, F) and Hill (Panels G, H, I) plots for G6Pase from liver microsomes for one week group. In substrate saturation curve the enzyme activity v on abscissa is plotted versus [S] on ordinate v is the enzyme activity at the given ATP concentration [S]. A, B and C represent to the control, diabetic and insulin-treated diabetic groups. In Eadie-Hofstee plot the enzyme activity v on abscissa is plotted versus v/[S] on ordinate. D, E and F represent to the control, diabetic and insulin-treated diabetic groups. In Hill plot log (v/Vmax-v) on abscissa is plotted versus log [S] on ordinate. The Hill coefficients n_1 and n_2 depict number of ATP molecules bound for the given concentration range of ATP. G, H and I represent to the control, diabetic and insulin-treated diabetic groups. The plots are typical of 6-8 independent experiments in each group.

Data on Km and Vmax values of the two components as affected by insulin status are given in Table 5. Thus, in the control group the Km value of the two components were 2.22 and 8 mM respectively with corresponding values of Vmax of 9.5 and 20 units. In one week diabetic animals the Km value for component I decrease to less than half of the control value without any change being seen for component II. Late stage of diabetes resulted in substantial 4.6-5.2 fold reduction in Km value of the two components. Insulin treatment was ineffective at early stage; at late stage insulin treatment partially restored the Km value for component I with complete restoration of Km of component II. In early stage of diabetes Vmax of component I decreased (32 % decrease) whereas the opposite effect was seen for Vmax of component II (52 % increase). At late stage Vmax of both the components show phenomenal increased (2.3 and 1.93 fold increase). Insulin treatment only checked the Vmax value component I in one month diabetic animals.

Analysis of the data by Hill plots indicated that up to 4.7 mM G6P concentration one G6P molecule was bound to the enzyme while beyond this concentration of substrate two molecules of G6P were bound under all experimental conditions (data not given) Typical Hill plots are shown in Fig. 5 and 6 Panels G-I.

Table 5. Effect of alloxan-diabetes and insulin treatment on substrate kinetics properties of rat liver microsomal glucose-6-phosphatase

Group	Treatment	Component I		Component II	
		Km	Vmax	Km	Vmax
One week	Control	2.22 ± 0.12	9.80 ± 0.25	7.98 ± 0.13	20.44 ± 0.49
	Diabetic	0.80 ± 0.02[b]	6.64 ± 0.19[b]	7.56 ± 0.25	31.21 ± 0.25[b]
	Diabetic + Insulin	0.35 ± 0.02[b,§]	5.05 ± 0.20[b,§]	8.40 ± 0.29	38.68 ± 0.60[b,§]
One month	Control	1.92 ± 0.12	9.14 ± 0.26	8.13 ± 0.13	19.94 ± 0.45
	Diabetic	0.37 ± 0.02[b]	21.91 ± 0.55[b]	1.77 ± 0.07[b]	38.53 ± 0.46[b]
	Diabetic + Insulin	0.98 ± 0.03[b,§]	9.10 ± 0.25[§]	8.69 ± 0.18[a,§]	40.78 ± 0.60[b,ψ]

The Km (mM) and Vmax (μmole of Pi liberated / hr / mg protein) values were calculated as described in the text.

The results are given as mean ± SEM of 6-8 independent experiments.

a, $p< 0.05$ and b, $p<0.001$ compared to the corresponding control.
ψ, $p< 0.05$ and §, $p<0.001$ compared to the corresponding diabetic.

Figure 7

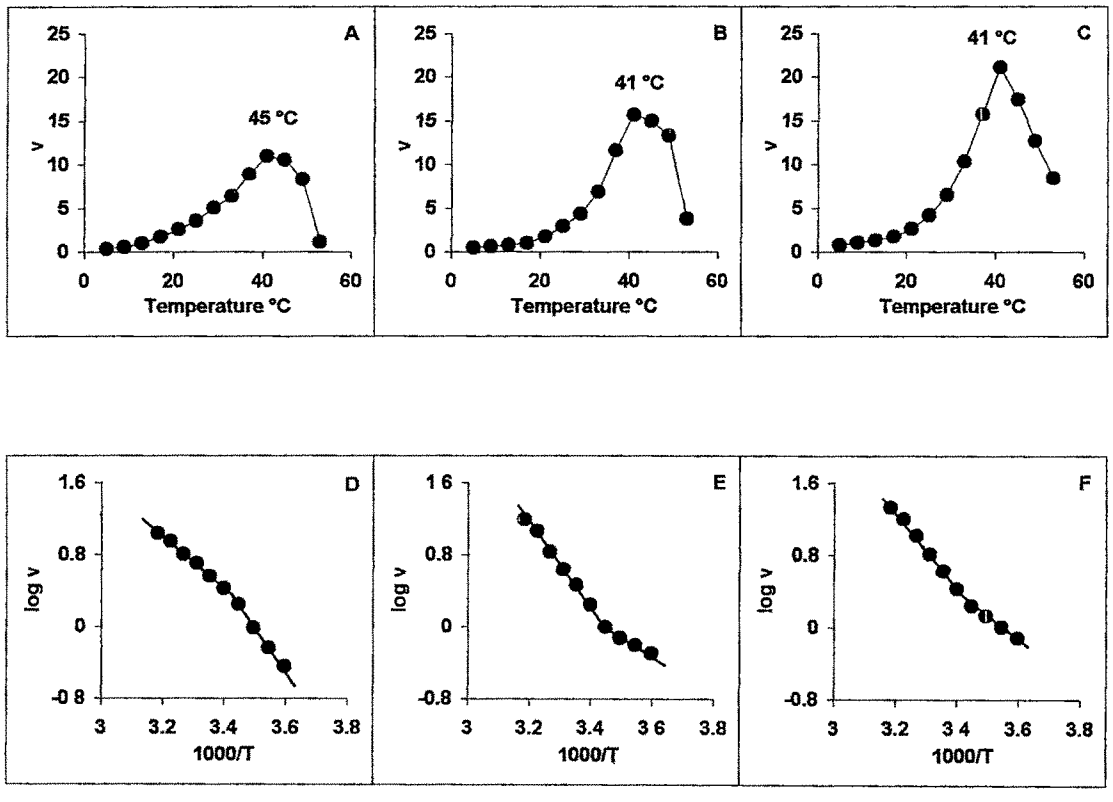
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Figure 8

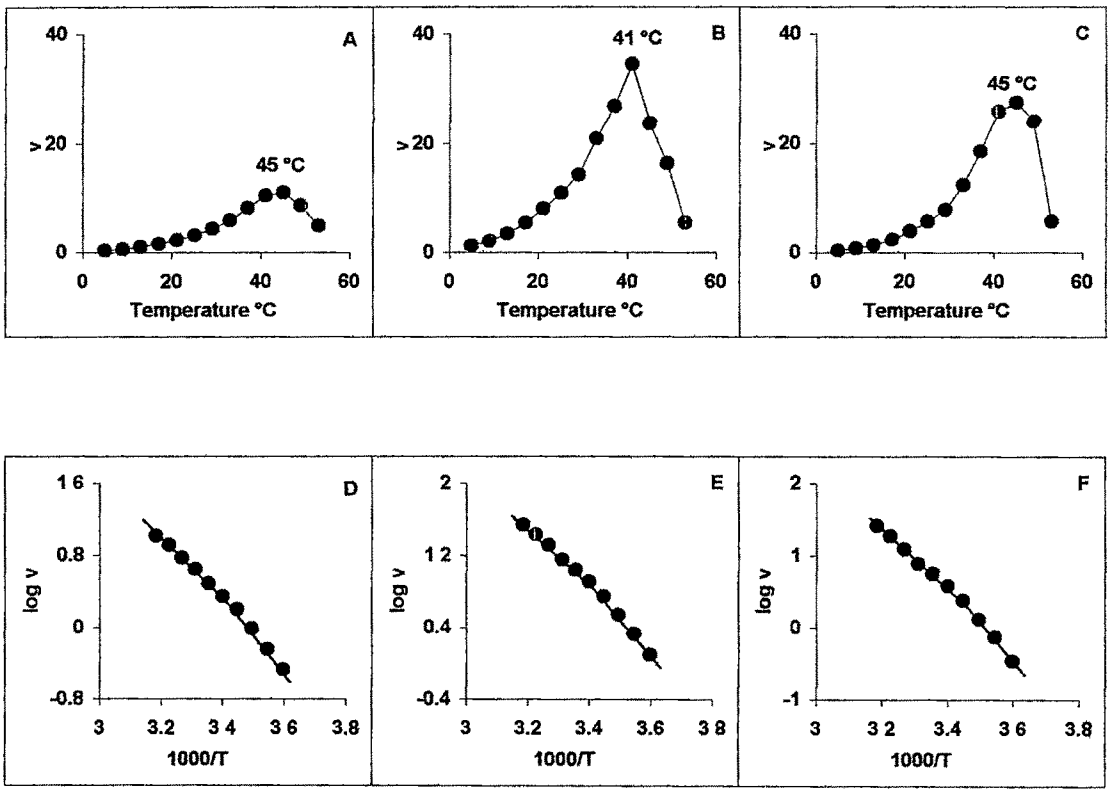

Figure 7. Typical plots depicting dependence of enzyme activity on the temperature and corresponding Arrhenius plots for G6Pase from liver microsomes for one week groups. In temperature curves, enzyme activity v on abscissa is plotted verses temperature (°C) on ordinate. A, B and C represent to the control, diabetic and insulin-treated diabetic groups. In Arrhenius plots log of v on ordinate is plotted against 1000/T on abscissa where v and T represent respectively, the activity at corresponding absolute temperature T (temperature in ° Celcius + 273.2). D, E and F represent to the control, diabetic and insulin treated diabetic groups. The plots are typical of 6-8 independent experiments in each group.

Figure 8. Typical plots depicting dependence of enzyme activity on the temperature and corresponding Arrhenius plots for G6Pase from liver microsomes for one month groups. In temperature curves, enzyme activity v on abscissa is plotted verses temperature (°C) on ordinate. A, B and C represent to the control, diabetic and insulin-treated diabetic groups. In Arrhenius plots log of v on ordinate is plotted against 1000/T on abscissa where v and T represent respectively, the activity at corresponding absolute temperature T (temperature in ° Celcius + 273.2). D, E and F represent to the control, diabetic and insulin treated diabetic groups. The plots are typical of 6-8 independent experiments in each group.

shifted to 41 °C. Insulin treatment at late stage restored the optimum temperature to normality. The differences were reflected in terms of corresponding Arrhenius plots (Fig. 7 and 8 Panels D-F). In control animals typical biphasic pattern was noted for Arrhenius plots. In one week diabetic group the pattern reversed i.e. E_H is higher than E_L; insulin treatment was ineffective (Fig. 7, Panel D-F). In one month experimental groups, the Arrhenius pattern resembled the control group (Fig. 8, Panel D-F). The data on energies of activation and phase transition temperature are given in Table 6. In the control group the values of E_H and E_L was 40 and 75 KJ/mole respectively. In early diabetic state the E_H was significantly high and about double of the control while the E_L decreased significantly and became almost half. At late stage, there was small but reproducible increase was noted in E_H and E_L. Effect persisted even after insulin treatment. Insulin status had marginal effect on the phase transition temperature (Table 6).

In view of the observed changes in the substrate and temperature kinetics properties of ATPase and G6Pase, the effects of insulin status on lipid/phospholipid profiles of the microsomal membrane were examined. The results are given in Tables 7-9. In one week diabetic group the content of Total phospholipids (TPL) and cholesterol (CHL) increased by about 24 and 36 % respectively. Insulin treatment restored only the TPL content. In the one month diabetic group TPL did not change whereas 2.5 fold increase was noted for CHL content. Insulin treatment caused 28 % decreased in TPL and CHL was partially restored. These changes were reflected in the molar ratio of TPL/CHL which decreased in late diabetic state and also following insulin treatment in both the diabetic states (Table 7).

Table 6. Effect of alloxan-diabetes and insulin treatment on Arrhenius kinetics properties of rat liver mitochondrial glucose-6-phosphatase

		Energy of activation (KJ/mole)		Phase transition temperature T_t (°C)
Group	Treatment	E_H	E_L	T_t
One week	Control	40.98 ± 2.92	74.71 ± 1.05	16.55 ± 0.21
	Diabetic	88.28 ± 3.07[d]	38.56 ± 3.15[d]	17.68 ± 0.64[a]
	Diabetic + Insulin	77.11 ± 1.00[d,§]	43.92 ± 1.62[d]	17.92 ± 0.76[d]
One month	Control	41.90 ± 0.72	76.18 ± 1.80	17.39 ± 0.42
	Diabetic	59.03 ± 2.65[d]	88.23 ± 3.19[b]	17.63 ± 0.80
	Diabetic + Insulin	70.72 ± 3.24[d,ψ]	92.43 ± 3.26[c]	16.97 ± 0.80

The experimental details are given in the text. The results are given as mean ± SEM of 6-8 independent experiments.

a, $p< 0.02$; b, $p< 0.01$; c, $p< 0.001$ and c, $p< 0.001$ compared to the corresponding control.
ψ, $p< 0.02$ and §, $p<0.01$ compared to the corresponding diabetic.

Table 7. Effects of alloxan diabetes on total phospholipids (TPL), cholesterol (CHL) and CHL ratio in rat liver microsomes

Groups	Treatment	TPL (μg/mg protein)	CHL (μg/mg protein)	TPL/CHL (mole:mole)
One week	Control	384.2 ± 15.6	107.8 ± 3.96	1.80 ± 0.09
	Diabetic	474.3 ± 14.7[b]	146.3 ± 9.99[a]	1.71 ± 0.13
	Diabetic + Insulin	390.4 ± 17.5[ψ]	158.2 ± 6.76[c]	1.24 ± 0.05[c,ψ]
One month	Control	393.6 ± 16.0	111.5 ± 4.09	1.78 ± 0.09
	Diabetic	380.9 ± 9.57	275.8 ± 8.76[c]	0.70 ± 0.02[c]
	Diabetic + Insulin	283.9 ± 15.4[c,§]	142.8 ± 5.83[b,§]	1.00 ± 0.04[c,§]

The experimental details are given in the text. The results are given as mean ± SEM of 6-8 independent experiments.

a, $p < 0.01$; b, $p < 0.002$ and c, $p < 0.001$ compared to the corresponding control.
ψ, $p < 0.05$ and §, $p < 0.001$ compared to the corresponding diabetic.

Table 8. Effects of alloxan-diabetes and insulin treatment on phospholipid composition in rat liver microsomes

Phospholipid Class	Composition (% of total)					
	One week			One month		
	Control	Diabetic	Diabetic + Insulin	Control	Diabetic	Diabetic + Insulin
Lyso	4.11 ± 0.08	4.89 ± 0.29	1.71 ± 0.05^{c,§}	4.21 ± 0.11	6.78 ± 0.18^{c}	2.43 ± 0.18^{c,§}
SPM	12.38 ± 0.25	7.46 ± 0.33^{c}	5.84 ± 0.21^{c,§}	12.70 ± 0.32	8.29 ± 0.24^{c}	8.42 ± 0.24^{c}
PC	49.18 ± 0.22	57.77 ± 0.58^{c}	60.41 ± 0.98^{c}	48.61 ± 0.40	52.66 ± 0.51^{c}	42.14 ± 0.61^{c,§}
PI	4.03 ± 0.06	5.06 ± 0.19^{c}	3.54 ± 0.24§	4.13 ± 0.09	5.56 ± 0.15^{c}	6.53 ± 0.21$^{c,\psi}$
PS	5.63 ± 0.14	3.16 ± 0.27^{c}	6.50 ± 0.25^{a,§}	5.71 ± 0.19	6.57 ± 0.15^{c}	7.28 ± 0.26^{c,¶}
PE	18.9 ± 0.21	18.36 ± 0.40	20.50 ± 0.87	18.06 ± 0.40	14.70 ± 0.41^{c}	24.67 ± 0.47^{c,§}
PA	5.74 ± 0.13	3.31 ± 0.14^{c}	1.51 ± 0.09^{c,§}	5.99 ± 0.11	5.44 ± 0.26	8.54 ± 0.23^{c,§}

The experimental details are given in the text. The results are given as mean ± SEM of 6-8 independent experiments.

Lyso: Lysophospholipid; SPM: sphinghomyelin; PC: phosphatidylcholine; PI: phosphatidylinositol; PS: phosphatidylserine; PE: phosphatidylethanolamine; PA: phosphatidicacid

a, $p< 0.02$; b, $p< 0.01$ and c, $p<0.001$ compared to the corresponding control.
¶, $p< 0.05$; ψ, $p<0.01$ and §, $p<0.001$ compared to the corresponding diabetic.

Table 9. Effects of alloxan-diabetes and insulin treatment on phospholipid content in rat liver microsomes

Phospholipid Class	Content, μg / mg protein					
	One week			One month		
	Control	Diabetic	Diabetic + Insulin	Control	Diabetic	Diabetic + Insulin
Lyso	15.89 ± 0.77	23.22 ± 1.61[b]	6.62 ± 0.26[c,§]	16.21 ± 0.78	25.86 ± 0.87[c]	6.36 ± 0.40[c,§]
SPM	47.50 ± 1.50	35.69 ± 2.36[b]	22.78 ± 1.23[c,§]	49.84 ± 1.51	31.74 ± 1.43[c]	23.62 ± 0.99[c,§]
PC	188.87 ± 5.31	273.66 ± 7.92[c]	237.16 ± 13.64[a,§]	190.24 ± 5.58	200.51 ± 3.42	120.37 ± 8.12[c,§]
PI	15.45 ± 0.46	23.93 ± 1.01[c]	14.04 ± 1.38[§]	15.75 ± 0.48	21.20 ± 0.82[c]	18.82 ± 1.59
PS	21.81 ± 1.14	15.25 ± 1.71[a]	25.23 ± 1.30[§]	21.36 ± 0.77	25.09 ± 0.84[a]	20.72 ± 1.41[ψ]
PE	72.64 ± 2.09	86.86 ± 2.66[b]	78.74 ± 2.48[ψ]	71.77 ± 1.99	56.16 ± 2.28[c]	69.26 ± 2.72[¶]
PA	22.25 ± 1.14	15.65 ± 0.76[c]	5.85 ± 0.38[c,§]	22.51 ± 1.21	20.86 ± 0.94	24.12 ± 1.26

The experimental details are given in the text. The results are given as mean ± SEM of 6-8 independent experiments.

a, $p< 0.01$; b, $p< 0.002$ and c, $p<0.001$ compared to the corresponding control.
ψ, $p< 0.05$; ¶, $p<0.01$ and §, $p<0.001$ compared to the corresponding diabetic.

Analysis of phospholipid profile (Table 8) revealed that early diabetic state resulted in increase in Lysophospholipid (Lyso), phosphatidylcholine (PC) and phosphatidylinositol (PI) components whereas sphinghomyelin (SPM), phosphatidylserine (PS) and phosphatidicacid (PA) components decreased. Insulin treatment restored the PI and caused further decreased in SPM and PA. Under these conditions the Lyso decreased substantially. In one month diabetic group, Lyso, PC, PI and PS increased whereas SPM and PE decreased. Insulin treatment lowered the Lyso and PC while PE and PA increased. The computed contents of the individual phospholipid classes were generally consistent with the compositional changes (Table 9).

Changes in the levels of cholesterol and altered phospholipid composition and contents could alter the fluidity of the membrane. This was ascertained by measuring the fluidity of the membranes. It can be noted from the data in Table 10 that the microsomal membrane fluidity decreased significantly in both the diabetic groups. Insulin treatment was effective only at early stage and membranes were more fluidized.

Discussion

It is clear from the data presented that insulin status affected the two enzyme systems differently, the important difference being that the Km for ATPase increased in diabetes whereas that for G6Pase decreased significantly.

The increase in Km for ATPase is suggestive of increased demand for ATP for the normal function of the microsomal enzyme. However, earlier studies from our laboratory

Table 10. Effects of alloxan-diabetes and insulin treatment on fluidity parameters of liver microsomes

Groups	Treatment	Fluidity Parameters			
		Fluorescence Polarization, p	Fluorescence anisotropy, r	Limited hindered anisotropy, rα	Order parameter, S
One week	Control	0.172 ± 0.006	0.121 ± 0.004	0.061 ± 0.003	0.315 ± 0.024
	Diabetic	0.257 ± 0.004[c]	0.188 ± 0.003[c]	0.151 ± 0.004[c]	0.612 ± 0.008[c]
	Diabetic + Insulin	0.122 ± 0.002[c,§]	0.085 ± 0.001[c,§]	0.013 ± 0.002[c,§]	0.175 ± 0.014[c,§]
One month	Control	0.170 ± 0.002	0.120 ± 0.002	0.060 ± 0.002	0.388 ± 0.008
	Diabetic	0.214 ± 0.002[c]	0.154 ± 0.002[c]	0.105 ± 0.002[c]	0.511 ± 0.006[c]
	Diabetic + Insulin	0.300 ± 0.003[c,§]	0.222 ± 0.002[c,§]	0.196 ± 0.003[c,§]	0.700 ± 0.006[c,§]

The experimental details are given in the text. The results are given as mean ± SEM of 6-8 independent experiments.

a, $p < 0.001$ compared to the corresponding control.
ψ, $p < 0.001$ compared to the corresponding diabetic.

showed that in streptozotocin-diabetes, the overall oxidative capacity of the liver mitochondria decreased significantly at the late stage of the disease (12).

It may be anticipated that component I of ATPase and G6Pase could be important in physiological context because of the low Km values which are compatible with the intracellular concentration of ATP and G6P. Such an assumption is also supported by the fact that up to the concentration of 0.6 mM, 1 ATP molecule is bound to the enzyme (Fig. 1 and 2 Panels G-I) and up to 4.5 mM, 1 G6P molecule is bound to the enzyme (Fig. 5 and 6 Panels G-I).

If one considers the ratio of Vmax/Km as an apparent index of enzyme efficiency; it can be noted that in diabetes the apparent efficiency index of ATPase decreased progressively for the component I. At the early stage insulin treatment treatment failed in correcting the apparent index whereas at the late stage insulin checked the apparent index. For component II, similar picture can be noted in one week experimental group. However, at late stage even after insulin treatment the apparent efficiency index remained low (data not shown but can be calculated easily from values of Vmax and Km in Table 2).

Interestingly, as can be noted at the late stage the ATPase activity decreased significantly (Table 1).

Studies on temperature kinetics showed that the diabetic state resulted in significant decrease in the energies of activation in both high and low temperature ranges. This may

probably reflect a compensatory mechanism for efficient functioning of the enzyme in spite of the increase in Km.

Regulation of Na^+, K^+-ATPase is a complex process controlled by several factors which include the subunit composition, stoichiometry of the subunits and interaction of the enzyme with membrane proteins (13-15). As is well documented, the enzyme comprises α, β and γ subunits (13-15). α is the catalytic subunit while β functions as the regulatory subunit (13-15). Four isoforms of α (α 1-4) and 3 isoforms of β (β 1-3) subunits have been reported (13). The α1-isoform in association with β1- subunit is found in nearly every tissue (13). Interestingly, it has been reported that the microsomal enzymes from various tissues contain β3 subunit and that the stoichiometry of α and β3 subunits is tissue-specific (16). Thus the liver microsomal enzyme represents pure α1 β3 form (16). Tissue-specific expression of α and β subunits by thyroid hormones is well documented (17-19). However, at this stage it is not clear if insulin also regulates expression of the α and β subunits and if so whether in tissue-specific manner.

The second factor which regulates the Na^+, K^+-ATPase activity is the acidic phospholipids (9, 10). Data of the present study on phospholipid composition show that the diabetic state had reciprocal effect on SPM and PC components. The acidic phospholipid PI and PS were also affected reciprocally at early diabetic state whereas at the late stage both the phospholipid classes increased. Insulin treatment had also differential effects on the phospholipid classes. Attempts to seek correlation between different kinetic parameters and lipid/phospholipid classes by regression analysis

revealed that, paradoxically, the acidic phospholipid PS and PI did not seem to have any role in regulating either the activity or the substrate kinetics properties of the enzyme. This is in contrast with the known requirement of acidic phospholipids for the activity of the plasma membrane Na^+, K^+-ATPase (9, 10). Regression analysis also revealed PC and CHL to be the positive regulators of Km of both the components and SPM seemed to be the negative regulator. It may hence be suggested that phospholipid requirement of the plasma membrane and microsomal enzyme may be quite different. Interestingly, SPM positively correlated with the energies of activation (Table 11).

Increase in G6Pase activity in diabetes is well documented (4). Results of our studies are consistent with the reported observations (4). Besides, the results point out for the first time that the G6Pase activity resolved in two kinetically distinguishable components. Decrease in Km value of G6Pase in diabetes is a matter of concern since it suggests that the enzyme can function efficiently even at lower concentration of glucose-6-phosphate (G6P) (Table 5). Energy constraint in diabetes referred to above (4) could lower the rate of phosphorylation of glucose to G6P and thereby a net decrease in intracellular G6P concentration. On the other hand G6Pase can efficiently hydrolyze G6P due to low Km. this will only result in futile cycle hindering the process of glycolysis. If one analyzes the data in terms of apparent efficiency, at early stage of diabetes apparent efficiency of component I of G6Pase became almost double which increased dramatically more than 10 fold at the late stage. Insulin treatment resulted in partial restoration but the apparent efficiency indexes were significantly higher than those seen in the control groups. Almost similar effect was noted for component II (data not shown but can be calculated easily

Table 11. Correlation of kinetic parameters of Na^+, K^+-ATPase and glucose-6-phosphatase with membrane lipid/phospholipid composition

Parameter	Correlation with Phospholipid Class			
	Na^+, K^+-ATPase		Glucose-6-phosphatase	
	Positive	Negative	Positive	Negative
Substrate kinetics				
Km_1	PC (+ 0.775) CHL (+ 0.812)	SPM (- 0.650) TPL/CHL (- 0.528)	SPM (+ 0.937)	CHL (- 0.720)
Km_2	PC (+ 0.609) CHL (+ 0.855)	SPM (- 0.662) TPL/CHL (- 0.668)	PE (+ 0.769)	CHL (- 0.925)
$Vmax_1$	TPL/CHL (+ 0.872)	CHL (- 0.963)	CHL (+ 0.819)	PE (- 0.963)
$Vmax_2$	PC (+ 0.745)	SPM (- 0.586)	PI (+ 0.542) CHL (+ 0.617)	SPM (- 0.869)
Temperature kinetics				
E_H	SPM (+ 0.621)	PI (- 0.616) CHL (- 0.767)	—	SPM (- 0.898)
E_L	SPM (+ 0.862)	PC (- 0.807)	PI (+ 0.550) PS (+ 0.682)	PC (- 0.858) TPL (- 0.766)
Tt	CHL (+ 0.655)	PE (- 0.836)	PC (+ 0.754)	SPM (- 0.690)

The experimental details are given in the text.

Values given in the parentheses indicate r, regression coefficient which is based on 6-8 independent experiments in each group.

from values of Vmax and Km Table 2). It is well documented that insulin treatment suppresses the activity of G6Pase by decreasing the amount of messenger ribonucleic acid (m RNA) of the catalytic subunit (5, 6). Despite this, as is evident from the data presented, there was only partial control.

The enzyme G6Pase is deeply embedded in the microsomal membrane (20, 21). However, it is not known whether the enzyme has requirement for specific lipid/phospholipid for its activity. The regression analysis of kinetic parameters with phospholipid classes across the groups revealed that the CHL seem to be the negative regulator of Km and positive regulator of Vmax. The role CHL seemed to be the opposite of that in case of ATPase. PI and PS seem to be the positive regulator of energies of activation in low temperature ranges (Table 11). As far as we are aware regulation of kinetic parameters of G6Pase by specific lipids has not been reported earlier.

Summary

Effects on ATPase

At the early stage of diabetes the microsomal Na^+, K^+-ATPase activity increased whereas an opposite effect was noted at late stage. Insulin treatment restored the activity only in one month diabetic group.

Substrate kinetics studies revealed that the enzyme activity resolved in two kinetically distinguishable components.

Diabetic state in general increased the values for Km; insulin treatment was effective only at the late stage of diabetes. In one week diabetic animals the Vmax of component II increased. By contrast in one month diabetic group Vmax of both the components decreased; insulin treatment once again was effective only at the late stage.

The Hill plot analysis of substrate kinetics data revealed that up to 0.95 mM ATP concentration one ATP molecule was bound while beyond this concentration two ATP molecules were bound to the enzyme under all the experimental conditions.

The temperature kinetics data revealed that the E_H and E_L values decreased significantly in diabetic animals; insulin treatment restored the E_H and partially corrected the E_L value only at late stage. No change was noted in T_t under any experimental conditions.

Effects on ATPase

The G6Pase activity increased significantly with time in diabetic state; insulin treatment had marginal restorative effect only at late stage.

From the substrate kinetics studies it can be noted that the G6Pase activity displayed a two kinetic component systems under all the experimental conditions.

The Km of component I decreased significantly in one week diabetic animals; in one month group Km of both the components decreased. Insulin treatment was ineffective at early stage whereas it restored the Km of component II while partially correcting the Km of component I at the late stage.

Hill plots analysis of the substrate kinetics data revealed that up to 2 mM glucose-6-phosphate (G6P) concentration one G6P molecule was bound while beyond this concentration two G6P molecules were bound to the enzyme under all the experimental conditions.

The Arrhenus pattern was reversed in one week diabetic as well as insulin treated diabetic animals. E_H increased in diabetic groups; insulin treatment was ineffective. E_L decreased at early stage of diabetes whereas increased at the late stage; insulin treatment again was ineffective. No change was observed in T_t under all the experimental conditions.

In one week diabetic animals the TPL and CHL contents increased by 23 and 36 % respectively; insulin treatment restored only TPL content. In one month diabetic group the CHL content increased almost by 2.5 fold which was partially brought back by insulin treatment.

The diabetic states resulted in increased Lyso, PC and PI whereas SPM decreased. Phosphatidicacid (PA) and PS decreased only at early stage and PE at late stage of diabetes. Insulin treatment was effective only in restoring the PI and PS at early stage.

Diabetic states decreased the membrane fluidity; insulin treatment increased the fluidity beyond control in one week diabetic group whereas in one month diabetic group there was further decrease.

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