CHAPTER : 2

Insulin and sulfonylurea treatments of the human diabetics. Effects on erthrocyte membrane and serum enzymes.

Introduction

Increased blood glucose level or hyperglycemia is the established feature of diabetes (1). Hyperglycemia is known to result in increased content of glycosylated hemoglobin - Hb_{A1c} (2). Additionally, non-enzymatic glycosylation of E-amino group of lysine gives rise to a product called 'fructosamine' via Amadori rearrangement (2). Hb_{A1c} levels serve as useful index for monitoring the glucose control (2). However the clinical application of glycated hemoglobin data is hindered due to rigorous experimental procedures and the time required for determination of glycated hemoglobin (3). Measurement of fructosamine, on the other hand, is relatively simple and albumin contributes significantly to the final results. However, the concentration of albumin varies depending on pathophysiological conditions and hence the method has limited applicability (4). Hyperglycemia is also known to cause non-enzymatic glycation of membrane proteins - both integral and peripheral- in the erythrocytes (5). Post-translational non-enzymatic glycation of Na^+,K^+ ATPase results in the inactivation of the enzyme (6).

In diabetic condition the Na^+,K^+ ATPase activity is found to be decreased in kidney cortex and medulla, glomerulus, brain and red blood cell membranes (2, 7, 8). In the kidney the decrease is evident up to 3 weeks of induction of diabetes in the experimental animals, but the activity increases significantly by the sixth week (2). Decreased

acetylcholinesterase (AChE) activity in the erythrocyte membranes in alloxan-diabetic rats has also been reported (9). Similar findings have been noted for the type I human diabetic patients where the Km of erythrocyte AChE was unchanged but the Vmax decreased (9). By contrast, the butyrylcholinesterase (BChE) activity in the serum of streptozotocin-diabetic rats increased significantly (10 - 12). Similar findings in human type I and type II diabetic patients have also been reported (13). It has also been shown that incubation in vitro with insulin can significantly influence the Na^+,K^+ ATPase and AChE activities in the erythrocyte membranes from control as well as diabetic groups (9, 14).

For treatment of human diabetics two strategies are used : either insulin therapy or treatment with sulfonylurea type of drugs is given (15). The two treatments are known to have differential effect on lipoprotein metabolism at least in the non insulin dependent diabetes mellitus (NIDDM) patients, although the blood glucose control is similar (16).

In view of the above it was of interest to find out if the extent of glycosylation of erythrocyte membrane proteins and serum proteins, as well as the activities of erythrocyte membrane Na^+,K^+ ATPase and AChE and serum BChE are differentially affected under these treatment conditions. Studies were also extended to find out whether the kinetic properties of the three enzymes mentioned above were

influenced by diabetic state and subsequent insulin or sulfonylurea therapy. The results of these investigations are described in this chapter which confirm that the effects of the two therapies are indeed differential.

Materials Methods

Chemicals

Acetylthiocholine iodide (ACTI), butyrylthiocholine iodide (BCTI) and ethopropazine hydrochloride (ETPZ.HCl) were purchased from Sigma Chemical Co., USA. 5,5' dithio-bis (2-nitrobenzoic acid) (DTNB) and sodium salt of vanadium free adenosine 5' triphosphate (ATP) were purchased from SRL, India. Thiobarbituric acid (TBA) was purchased from LOBA Chemie, India, and NPH insulin from Knoll Pharmaceuticals Ltd., India. All other chemicals were of analytical reagent grade and were purchased locally.

Subjects

Total 39 subjects were chosen for the study. These included 10 healthy controls (4 males and 6 females; mean age 52 Yrs.), 11 untreated diabetic (UTD) (4 males and 7 females; mean age 53 yrs.), 10 insulin treated diabetics (ITD) (5 males and 5 females; mean age 55 yrs.) and 8 tablet (oral hypoglycemic drug sulfonylurea) treated diabetics (TTD) (6 males and 2 females; mean age 55 yrs.) as shown in Table 1. The insulin / tablet treatment was for variable durations of 2 to 8 years. All the subjects gave informed consent and the

study was approved by the local ethics committee.

Preparation of erythrocyte membranes

Preparation of erythrocyte membranes was essentially according to the method of Hanahan et al. (17). Briefly, blood was collected in heparinized vials and was centrifuged at 475 X g for 8 min. Supernatant and the buffy coat overlying the RBC pellet was discarded and the pellet was washed twice with 0.9 % NaCl. The washed RBCs were then subjected to hypotonic lysis in 14 mM Tris-HCl buffer pH 7.4 (18) and after centrifugation at 30,000 X g for 35 min the supernatant was discarded. The pellet was washed repeatedly with the same buffer to obtain the hemoglobin free membranes. The pellet was finally resuspended in the same buffer to give erythrocyte membrane suspension containing Ca. 2 mg protein / ml.

Serum

The blood was allowed to clot at room temperature and sera were collected after centrifugation in a clinical centrifuge.

Enzyme assay

Na^+,K^+ ATPase activity

Na^+,K^+ ATPase activity was measured by the method of Kumthekar and Katyare (18) in a medium (total volume 0.4 ml) containing 100 mM NaCl, 10 mM KCl, 4 mM $MgCl_2$, 50 mM Tris-HCl buffer pH 7.4 with 100 - 200 ug of erythrocyte membrane

protein used as the enzyme source. The reaction was initiated by adding ATP at the final concentration of 4 mM. The reaction was carried out at 37°C for 2 h. and at the end of the incubation period, the reaction was terminated with 0.1 ml of 5 % (w/v) sodium dodisyl sulphate (SDS) (18). The liberated inorganic phosphorous was estimated by following the procedure of Fiske and Subba Row (19). For kinetic studies, the substrate (ATP) concentration range used was from 0.1 to 4.0 mM.

The data were analyzed by the Lineweaver Burk, Eadie-Hofstee and Eisenthal and Cornish-Bowden methods for the determination of Km and Vmax (20). The values of Km and Vmax obtained by the three methods of analysis were found to be in close agreement and were averaged. The results are given as mean$\pm$S.E.M. of the averaged values. The data were computer analyzed employing Sigma Plot version 5.0.

Assay of AChE and BChE activities

The AChE / BChE activities were determined essentially according to the procedure of Ellman et al. (21) as described previously (22) with some modifications. Thus for the determination of AChE activity, the assay system contained in a total volume of 1 ml : 100 mM potassium phosphate buffer pH 8.0, 0.32 mM DTNB, 0.1 mM ETPZ.HCl and 10 to 50 ug erythrocyte membrane protein as the source of the enzyme. For substrate kinetics studies concentration of substrate i.e.

ACTI was varied from 0.05 to 10 mM. Linear rate of reaction recorded over a period of 60 to 90 seconds at 37°C was used for the calculation of the rate of the reaction. ETPZ.HCl was included in the assay as the inhibitor of BChE (24).

For determination of BChE activity the assay system was the same as above except that 0.05 M Tris-HCl buffer pH 8.0 replaced the potassium phosphate buffer, ETPZ.HCl was omitted and BCTI was the substrate. 20 ul of 1 : 10 diluted serum was used as the source of the enzyme (23).

DTNB solution was prepared by dissolving 13 mg DTNB + 5 mg $NaHCO_3$ in 10 ml potassium phosphate buffer or in Tris-HCl buffer for AChE and BChE assays respectively (21).

The kinetic data were analyzed as described above for Na^+,K^+ ATPase.

In vitro insulin effect on enzyme activities

For studying the in vitro effect of insulin on Na^+,K^+ ATPase, AChE and BChE activities, the enzyme (i.e. either erythrocyte membranes or serum) were pre-incubated in the respective assay system for 30 min at 37°C using three different insulin concentrations : 10^{-9}, 10^{-8} and 10^{-7} M (14). The enzyme activities were then determined as described above.

Serum glucose was estimated by GOD-POD method. Glycosylation of erythrocyte membrane and serum proteins was determined by the thiobarbituric acid method (24).

The extent of glycosylation of protein was determined by the thiobarbituric acid method (24)

The range of the method was 2 ug to 16 ug of fructose.

Reagent used were :

1) Fructose stock solution : 1 mg of fructose was dissolved in 1 ml of saline (stock solution). The working standard is 20 ug of fructose in 2 ml of 0.9 % NaCl.

2) Oxalic acid (0.7 moles / liter) : 6.3 gm of oxalic acid was dissolved in 100 ml of distilled water.

3) Thiobarbituric acid (TBA) (0.05 moles / l) : 0.721 gm of TBA is dissolved in distilled water, pH of the solution was adjusted to 6.0 using 5 M of NaOH and volume made up to 100 ml with distilled water.

Prepare fresh as it is unstable.

4) Saline : 8.78 gm sodium chloride dissolved in 1000 ml distilled water.

5) TCA : 40 gms of TCA crystals are dissolved in 100 ml of distilled water and volume made o 100 ml.

6) Acidic acetone : 2.5 gm oxalic acid was dissolved in 100 ml of acetone.

Protocol :

For standard different aliquots of fructose standard are taken (0.2 to 1.6 ml) and the volume is made up to 1.6 ml

with saline. Then 1.6 ml oxalic acid is added and tubes are autoclaved at 15 lb. / Sq. inch for 1 hr. After cooling the tubes to room temperature, 0.8 ml of TCA is added to all the tubes and the tubes are centrifuged at 2,000 - 3,000 rpm for 10 min. 2.1 ml of aliquots are removed to which 0.7 ml of TBA is added and the tubes were kept at 80°C for 15 mins. The O.D. readings were taken on ERMA using filter 420.
1 ug of fructose gives 0.060 O.D. reading.

For sample :

0.050 ml (50 ul) of plasma and 200 ug erythrocyte membrane proteins were taken to which 2 ml of acid acetone was added and after mixing the tubes were kept on ice for 10 min. The tubes were then centrifuged in a clinical centrifuge at 2,000 rpm for 10 min and the supernatant was discarded. Pellet obtained was then washed twice with 2 ml of acid acetone. Then the pellet was suspended in 1.6 ml of saline after which the protocol remains the same as given for the standard.

Protein estimations were carried out according to the procedure of Lowry et al. (25) with bovine serum albumin used as the standard.

Results

The data in Table 1 show that the mean age of all groups were well matched.

The data on serum glucose concentrations are given in Table

Table 1. Distribution of male and female volunteers.

	Male	Female	Mean
Control	52.0±1.41 (4)	51.2±0.83 (6)	51.5±0.72 (10)
UTD	55.3±1.25 (4)	51.7±1.40 (7)	53.0±1.01 (11)
ITD	50.8±3.48 (5)	57.6±1.29 (5)	54.2±2.09 (10)
TTD	54.8±1.78 (6)	53.5±3.50 (2)	54.5±1.48 (8)

Values are given as mean ± S.E.M. The numbers of volunteers are indicated in the parentheses.

2. Thus it can be note that the blood sugar level in the UTD group was about double compared to the controls. In the ITD and TTD groups the blood sugar levels decreased somewhat but were still higher than in the control group (Table 2). The extent of glycosylation of serum proteins was somewhat higher (+23 %) in diabetic group, although this value was not statistically significant. The value decreased in ITD group. However within the four groups listed in Table 2, the overall changes did not present any clear picture. Compared to the serum, the effect on the glycosylation of erythrocyte membrane proteins were dramatic. Thus in the UTD group the extent of glycosylation was 3.4 fold higher and insulin treatment did not provide any relief; in fact the level of glycosylation increased to 5.4 fold. In TTD group also the value was comparable to the diabetics (Table 2).

The data on erythrocyte membrane Na^+,K^+ ATPase and AChE and serum BChE in the four groups are given in Table 3. Observations on effect of pre-incubation on the enzyme activities are also included. Thus consistent with the observations of earlier researchers (18) the Na^+,K^+ ATPase activity decreased significantly in the diabetic group (65 % decrease). After insulin treatment the activity was restored considerably. However in the TTD group there was no improvement.

The AChE activity in the erythrocyte membranes from the controls was 0.95 μ moles / min / mg protein. In the UTD and

Table 2. Serum glucose concentration and protein glycosylation in diabetes.

	Serum glucose (mM)	Glycosylation	
		Erythrocyte membrane protein (ng / mg protein)	Serum protein (ng / ml serum)
Control (10)	5.5±0.18	44.2± 9.0 c	7.48±0.84
UTD (11)	11.1±1.00 d*	150.0±26.5 d@	9.16±1.96
ITD (10)	7.8±0.41 a#	240.5±29.1 b	7.75±0.70
TTD (8)	7.2±0.65	111.3±19.2	8.14±0.25

Methods for serum glucose estimation and glycosylation were as described in the text. Results are given as mean ± S.E.M. for the number of volunteers indicated in the parentheses.

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

@, $p<0.05$; *, $p<0.01$ and #, $p<0.005$ compared to the UTD group.

ITD groups the activity remained more or less the same but in the TTD group the activity decreased drastically by 43 % (Table 3).

The BChE activity in the serum of the controls was 10 μ moles / min / ml serum. In the diabetics there was a small but reproducible decrease (28 % decrease) in the activity which did not improve in the ITD group, while in the TTD group the activity increased and became comparable to the controls (Table 3).

In view of the above observations (Table 3) it was of the interest to find out if the observed changes could be related with the kinetic properties of the enzymes. With a view to illustrating this possibility the substrate kinetics parameters for the three enzymes were studied and the Km and Vmax values were calculated. Three different methods of analysis were employed as described earlier in the Materials and Methods section. The typical substrate saturation curves for the three enzymes are shown in Figures 1, 3 and 5. The typical Eadie-Hofstee plots for the three enzymes are shown in Figures 2, 4 and 6.

As can be seen from the figure (Figure 2) the erythrocyte membrane Na^+,K^+ ATPase showed presence of two components : one with high affinity (low Km) and the other with low affinity (high Km), in all the groups. A comparative account

Table 3. Erythrocyte membrane Na+,K+ ATPase and AChE, and serum BChE activities, and the effect of pre-incubation.

	Na+,K+ ATPase		AChE		BChE	
	Pre-incubation time		Pre-incubation time		Pre-incubation time	
	(0 min	30 min)	(0 min	30 min)	(0 min	30 min)
Control (10)	170.4±10.1	195.0± 8.21 a	0.98±.019	1.00±0.10 c	9.99±0.22 d	7.67±0.35 d
UTD (11)	58.9±11.5 c*	102.6±12.04	1.10±.010	0.75±0.03	7.19±0.63 d	9.30±0.98 d#
ITD (10)	113.4±11.8 d	90.6±10.98 b	0.92±0.10 #	0.95±0.10	7.19±0.63 @	6.16±0.51 @
TTD (8)	47.1± 4.2	35.5± 3.10	0.56±0.06	0.44±0.04	9.43±0.63	8.25±0.50

The enzyme assays were carried out as described in the text with or without pre-incubation of the enzyme with the buffer for 30 min as indicated. The results are given as mean ± S.E.M. for the number of individual volunteers as indicated in the parentheses.
Na^+,K^+ ATPase activity = n moles of Pi liberated / hr / mg protein
AChE activity = μ moles / min / mg protein
BChE activity = μ moles / min / ml serum
a, $p<0.05$; b, $p<0.02$; c, $p<0.005$ and d, $p<0.001$ compared to the control group.
@, $p<0.05$; *, $p<0.01$ and #, $p<0.001$ compared to the respective unincubated sample.

Figure 1.

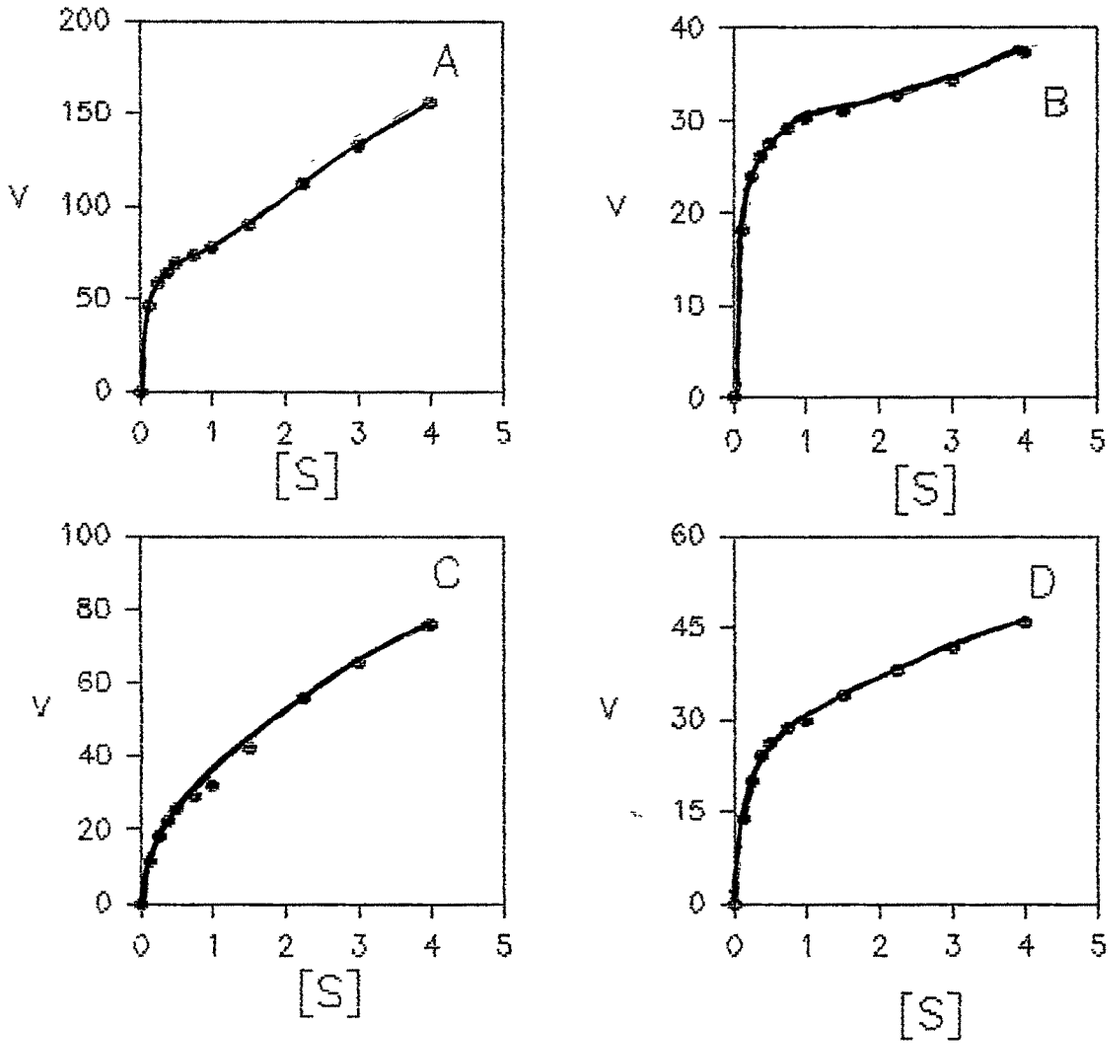

Figure 2.

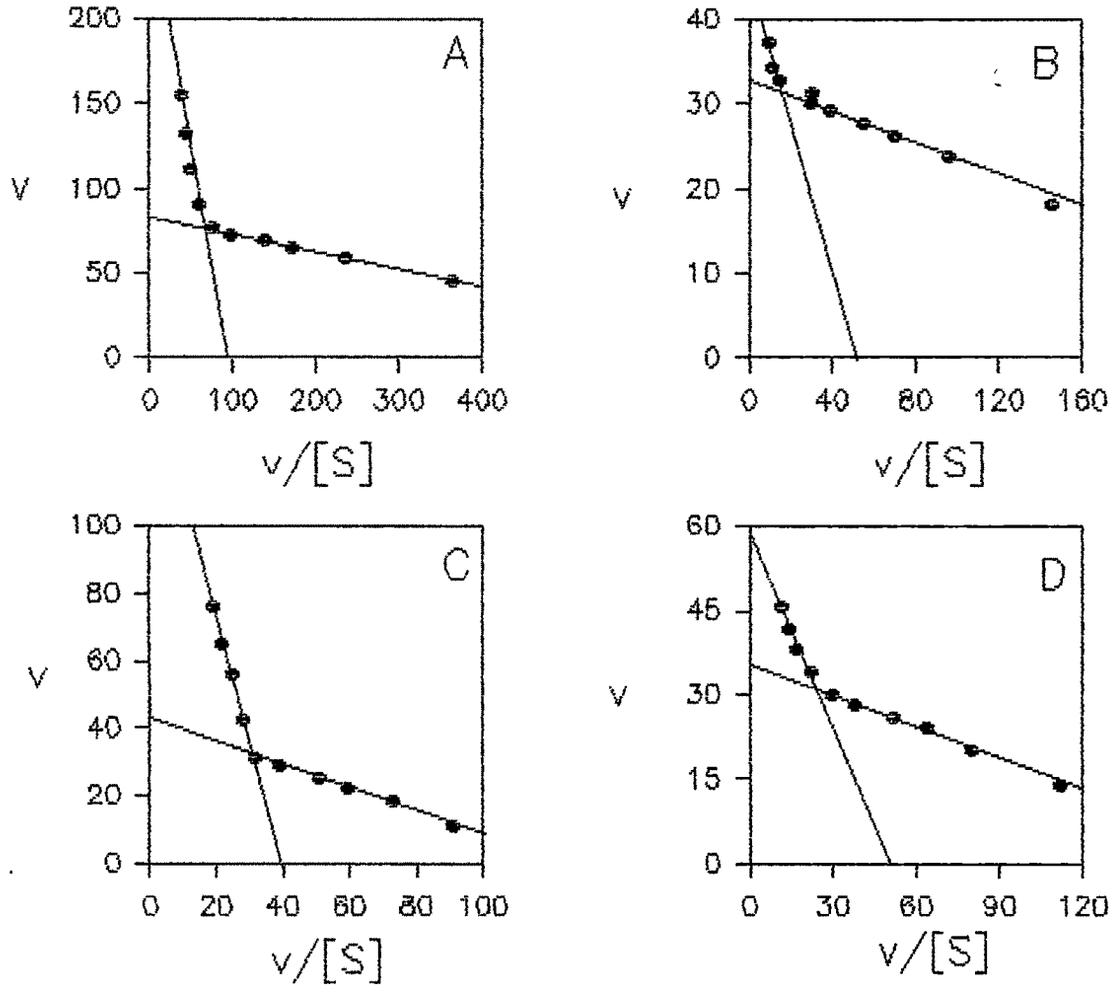
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Figure 3.

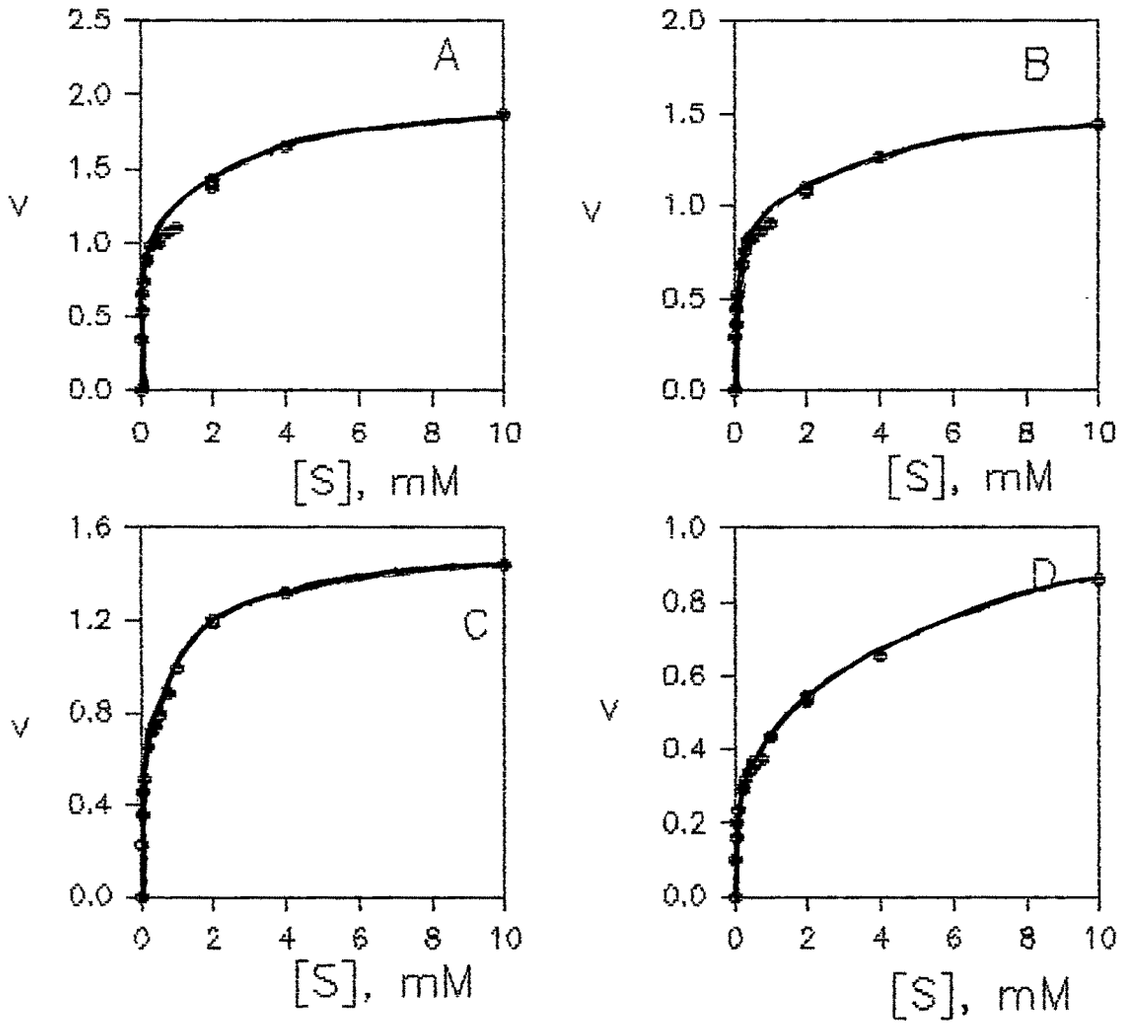
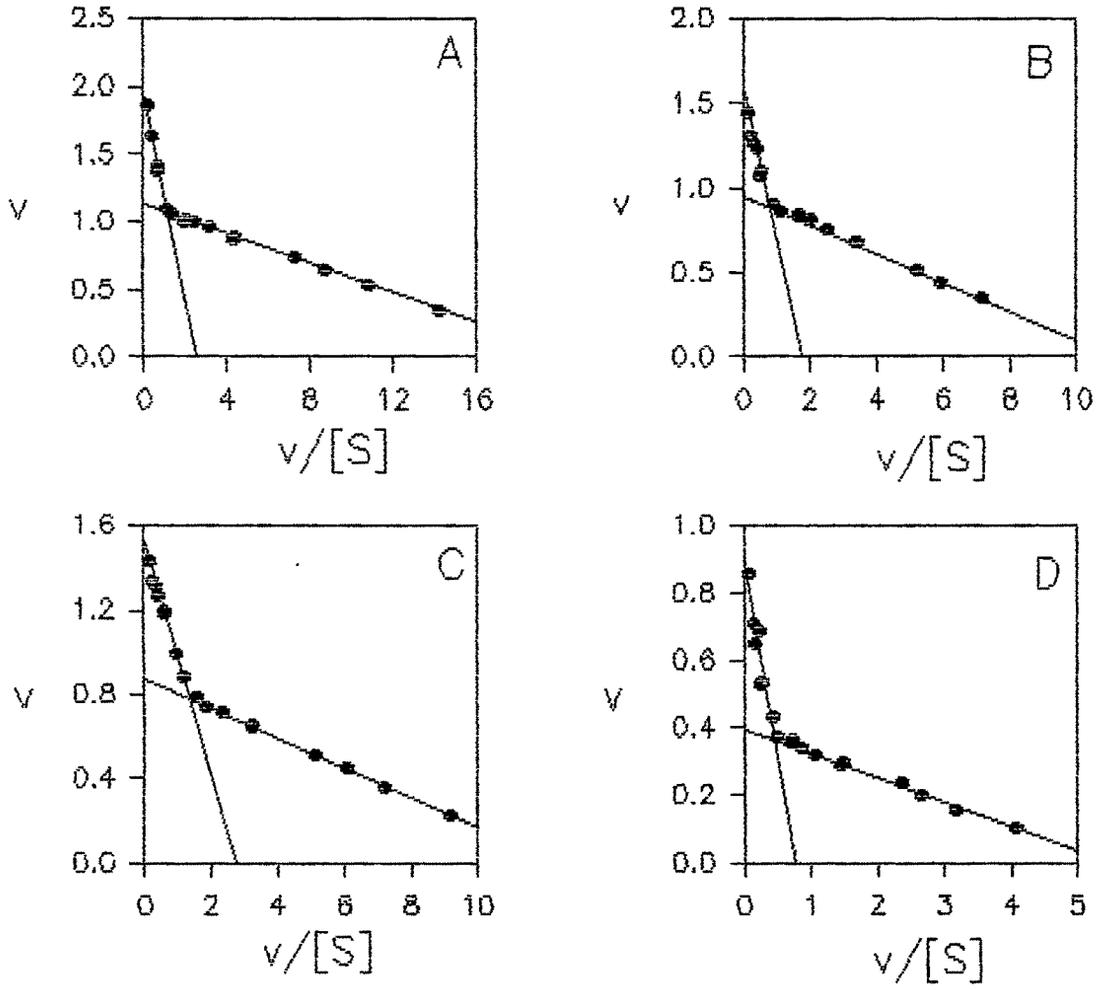


Figure 4.



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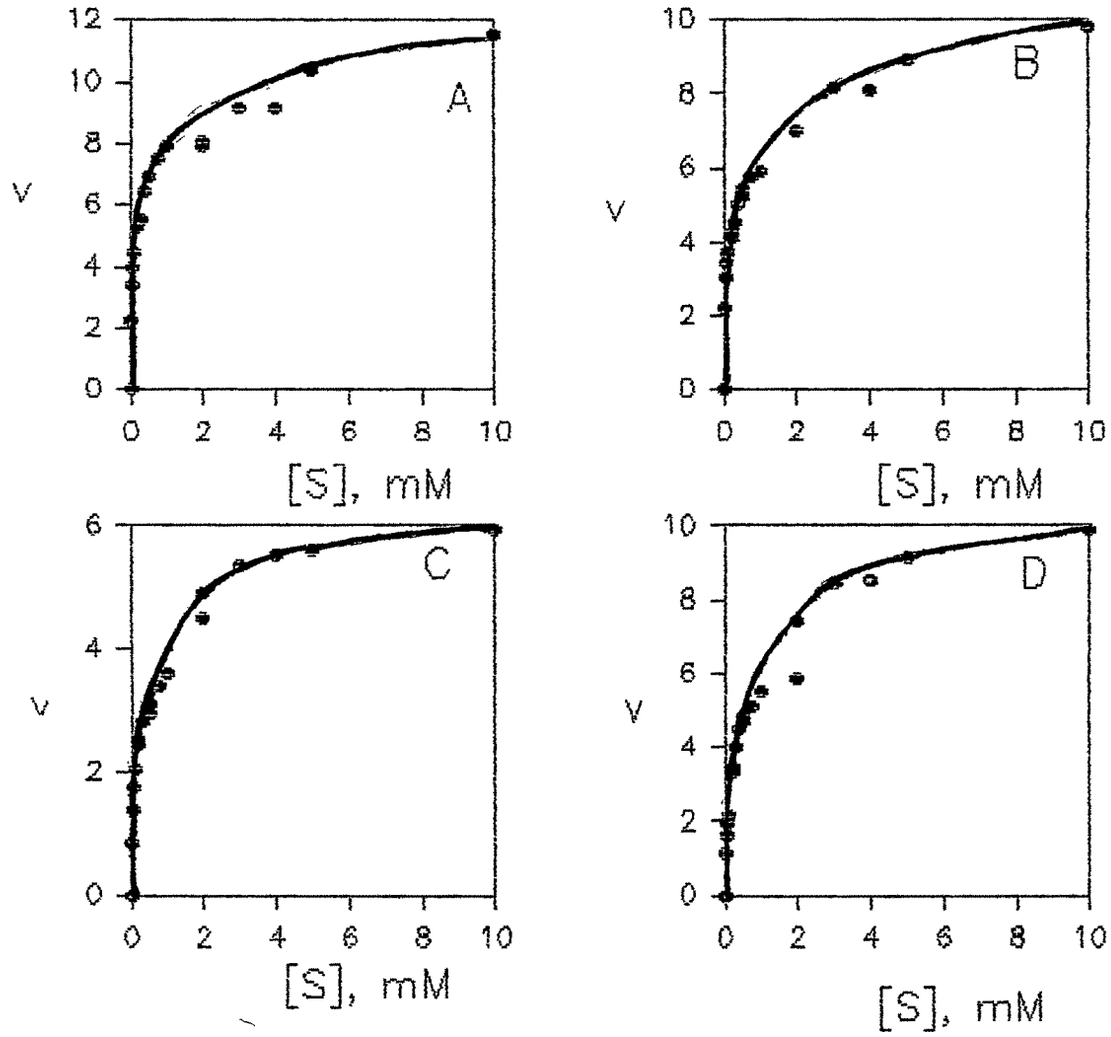
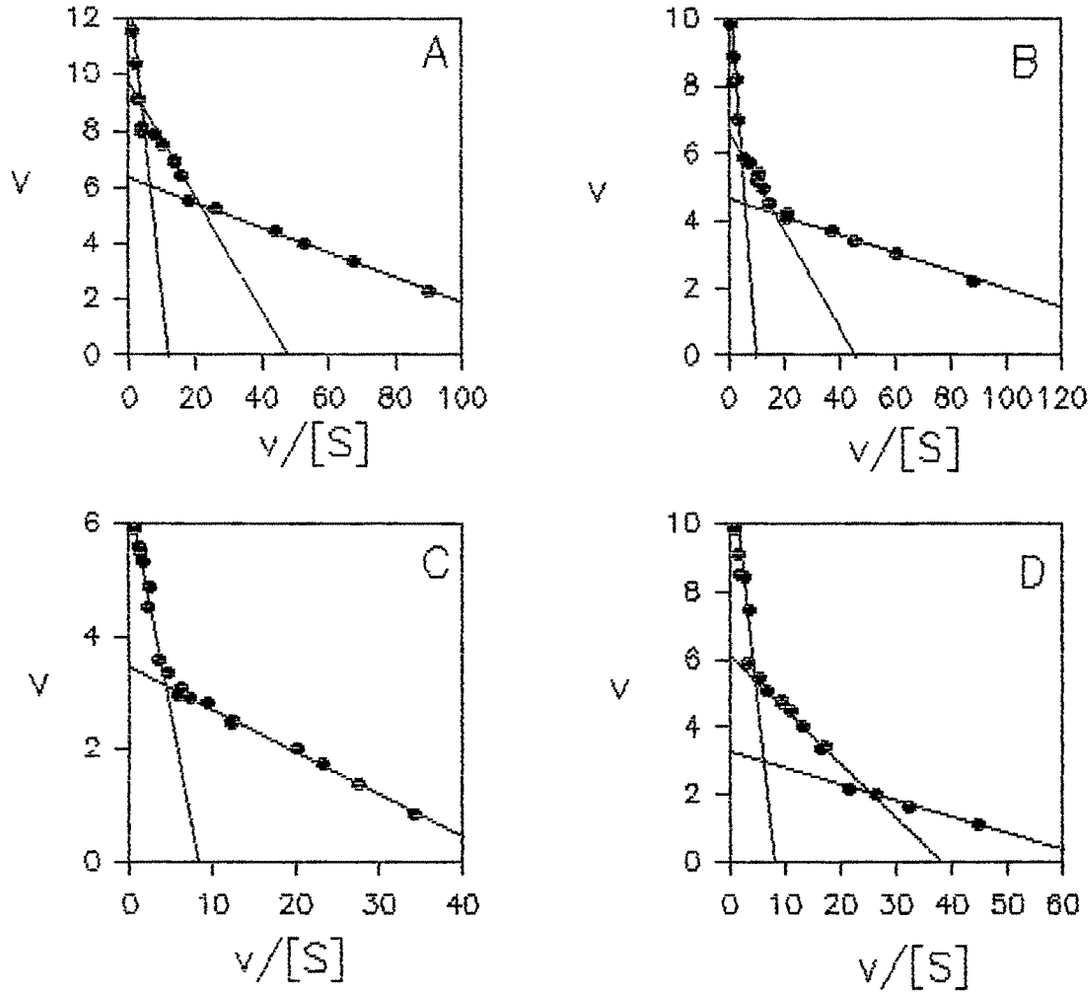


Figure 6.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of Km and Vmax values of the two components is given in Table 4. Thus in the control group, component I has a Km of 0.1 mM, while the component II has a Km of 2.24 mM, the difference in the Km values being about 20 fold. The Vmax values of the two components were 81 and 239 n moles / hr / mg protein respectively. The Km of component I was not influenced by diabetic state but was elevated 3 folds in ITD groups; even in the TTD group the Km was about 1.6 times higher. For Vmax of component I the pattern was compatible with activities given in Table 3 above. For component II the pattern was somewhat different i.e. Km in the UTD group decreased significantly which was restored in ITD group; in the TTD group Km remained comparable to UTD. The pattern for Vmax values was compatible with the data given in Table 3.

The typical Eadie-Hofstee plots for the AChE activity of the erythrocyte membranes from the four groups are shown in Figure 4. It is evident that in all the groups the AChE activity resolved in two components. The Km and Vmax values of the two components for the four groups are given in Table 5. It can be noted that Km of component I did not change under any of the condition; Vmax value decreased in the TTD group significantly (52 % decrease). The Km of component II was somewhat high in the ITD and the TTD groups and once again the Vmax had decreased significantly in the TTD group.

Figure 6 shows typical Eadie-Hofstee plots for serum BChE.

Table 4. Substrate kinetics analysis of erythrocyte membrane Na^+,K^+ ATPase.

	Component I		Component II	
	Km	Vmax	Km	Vmax
Control (10)	0.101±0.024	80.5± 2.94	2.24±0.19	239.2±11.95
		b	b	b
UTD (11)	0.106±0.008	30.0± 3.24	0.71±0.05	45.7± 2.54
	b@		a*	*
ITD (10)	0.290±0.049	57.1±13.34	3.49±0.33	193.3±29.55
		b	b	b
TTD (8)	0.160±0.050	37.6± 2.97	0.87±0.10	61.8± 6.94

The results are given as mean±S.E.M. for the number of individual volunteers as indicated in the parentheses.

Unit : Km = mM; Vmax = n moles / hr / mg protein

a, $p<0.005$ and b, $p<0.001$ compared to the control group.

@, $p<0.002$ and *, $p<0.001$ compared to the UTD group.

Table 5. Substrate kinetics analysis of erythrocyte membrane AChE.

	Component I		Component II	
	Km	Vmax	Km	Vmax
Control (10)	0.059±0.007	0.91±0.18	0.90±0.12	1.45±0.28
UTD (11)	0.052±0.013	0.89±0.07	1.01±0.16	1.54±0.09
ITD (10)	0.058±0.009	0.73±0.03 a@	1.18±0.16	1.39±0.08 a@
TTD (8)	0.059±0.012	0.44±0.03	1.24±0.24	0.77±0.07

The results are given as mean±S.E.M. for the number of individual volunteers as indicated in the parentheses.

Units : Km = mM; Vmax = μ moles / min / ml serum

a, $p<0.05$ compared to the control group.

@, $p<0.001$ compared to the UTD group.

As can be seen, in the control group three components of BChE differing in their kinetic properties were present. Similar pattern was seen for UTD and TTD group; ITD group showed variation in that only two components were seen. The kinetic properties of individual components in the four groups are given in Table 6. Thus it can be noted that the Km of component I decreased significantly in TTD group while the Vmax decreased from 26 to 39 % in all the groups; maximum effect was seen in the TTD group. For component II the Km as well as Vmax were lower by about 31 to 41 % in the UTD and TTD groups; the ITD group proved to be unique in that component II was altogether absent. The Km for component III decreased by 25 to 43 % in the TTD and UTD groups while Vmax was lower by 16 to 32 %. The maximum effect on Km was noted for component III.

It has been reported earlier that the in vitro addition of insulin significantly influences erythrocyte membrane Na^+,K^+ ATPase and AChE activities (4, 14). In the light of this it was of interest to find out the in vitro effects of insulin on the three enzymes systems described above in the four different groups as shown in Table 1. These results are summarized in Table 3 and Figures 7 and 8.

From the data in Table 3 it is evident that 30 min pre-incubation at room temperature only marginally influenced Na^+,K^+ ATPase activity in the erythrocyte membrane from the control group, while in the UTD the activity increased by 74

Table 6. Substrate kinetics analysis of serum BChE.

	Component I		Component II		Component III	
	Km	Vmax	Km	Vmax	Km	Vmax
Control (10)	0.056±0.006	6.29±0.16	0.259±0.018	9.97±0.23	1.41±0.14	13.34±0.31
UTD (11)	0.046±0.005	4.65±0.48 b	0.171±0.019 c	6.72±0.60 d	1.31±0.12	11.22±1.07
ITD (10)	0.047±0.004	4.26±0.55 c	--- d	--- c	1.06±0.13 d@	8.81±0.75 a
TTD (8)	0.038±0.005 a	3.83±0.69	0.154±0.010	6.88±0.88	0.81±0.05	10.79±0.99

The results are given as mean±S.E.M. for number of individual volunteers as indicated in the parentheses.
Unit : Km = mM; Vmax = μ moles / min / ml serum
a, $p<0.05$; b, $p<0.01$; c, $p<0.005$ and d, $p<0.001$ compared to the control.
@, $p<0.002$ compared to the UTD group.

Figure 7

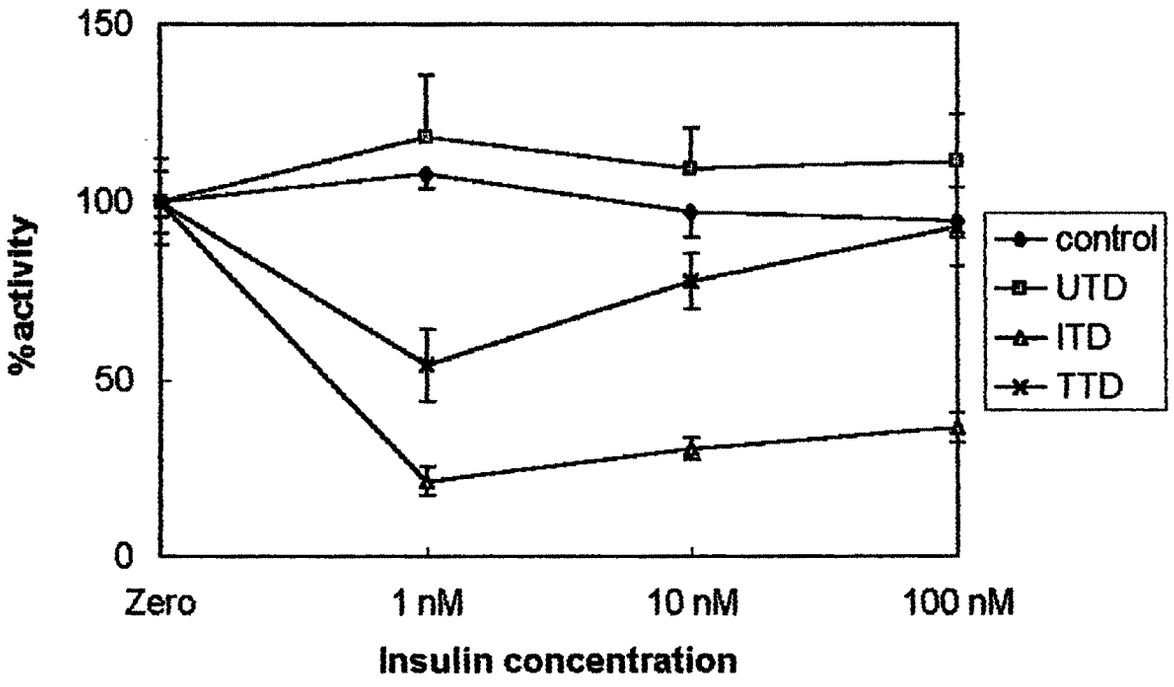
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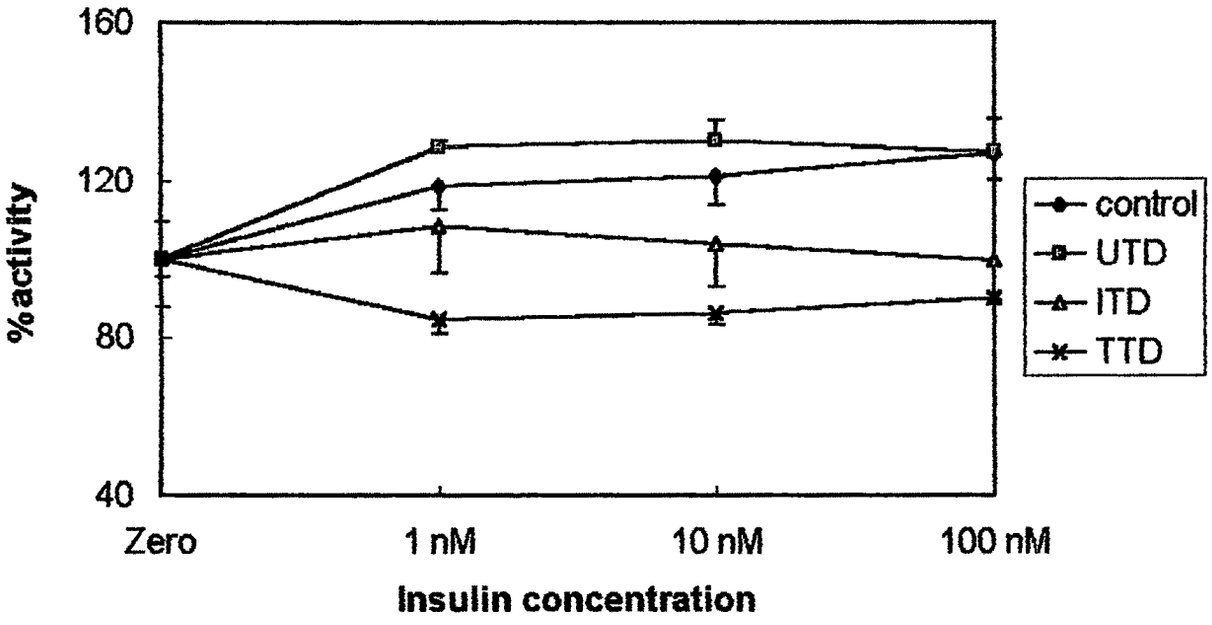
Figure 8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%. In the ITD group there was about 20 % decrease, which however, was not statistically significant. In the TTD group a statistically significant decrease (25 % decrease) could be noted. Pre-incubation resulted in 32 % decrease in the AChE activity in the UTD group; other groups were not affected. For BChE, pre-incubation caused 23 and 15 % decrease respectively in the control and TTD groups with no effect seen in the UTD group. Surprisingly, however, in the ITD group the activity increased by 80 % upon pre-incubation.

The effects of pre-incubation with insulin on the Na^+,K^+ ATPase activity are shown in Figure 7. It is evident that pre-incubation with insulin had only marginal effects in the control and UTD groups, whereas significant decrease was seen in the ITD and TTD groups with the effect being more pronounced in the former than in the latter group. Pre-incubation with insulin did not have much effect on the erythrocyte membrane AChE activity in any of the groups (data not shown). For BChE activity pre-incubation of serum from control and UTD groups resulted in 18 to 30 % stimulation of the activity, whereas no effect was seen in ITD group. In the TTD group the activity decreased by 10 - 15 % after incubation with insulin.

Discussion

The present studies were initiated to find out if insulin or sulfonylurea treatment of diabetic patients differentially

affects the extent of glycosylation and the erythrocyte membrane and serum enzyme activities. As is clear from the data in Table 2, both insulin and sulfonylurea treatments were able to control the glucose level in the serum almost to the same level. However, the extent of serum proteins glycosylation was almost comparable amongst all the groups. As against this, the extent of erythrocyte membrane glycosylation increased by 3.4 fold in the UTD group. Increased erythrocyte membrane protein glycosylation from diabetic patients has also been reported by earlier workers (5). Insulin treatment, rather than alleviating, aggravated the situation and the extent of membrane glycosylation increased to 5.4 fold. Compared to this, in the TTD group the membrane protein glycosylation decreased to some extent. The results therefore emphasize the point that the two treatments while controlling glucose levels had differential effects on membrane protein glycosylation and possibly membrane functions. Indeed the data in Table 3 also validate this assumption. Thus the decreased Na^+,K^+ ATPase activity in UTD group could be restored partly only by insulin treatment. On the other hand, the AChE activity decreased significantly only in the TTD group. For BChE it was interesting to note that the activity of this enzyme was somewhat low in UTD and ITD group, but was restored in TTD group. Other researchers have reported that the serum BChE is elevated in the diabetic humans and in experimental animals (10 -13). But in the present study such differences were not found. The reason for

this remains unclear. It may, however, be pointed out that in the human studies referred to above (10), the activities were measured in 20 mM Tris-HCl buffer pH 7.6 containing 10 mM $CaCl_2$ (10). It is possible that the observed differences could be attributed to the lower pH and the presence of high Ca^{2+} concentration in their assay medium (10). Nevertheless, the results of the present study would suggest that the effects of exogenous and indigenous insulin even on liver metabolism were differential. The BChE in the serum is believed to originate from the liver (26). Hence the observed changes could be taken as reflecting the insulin-status-effect on the liver function.

The examination of the kinetic properties revealed that the Na^+,K^+ ATPase activity comprised of two components. This is in agreement with our earlier observations on rat liver microsomal ATPase (27). Consistent with the observations in Table 4 the Vmax of both the components decreased in the UTD group. Also the Km of the low affinity component decreased which may be a compensatory mechanism. Insulin treatment caused significant increase in the Km of both the components while partially restoring Vmax values. However, in the TTD group the pattern more or less resembled to that in the UTD. The results once again emphasize the differential effects of the two treatments. Consistent with our earlier reported observations, the AChE activity of the erythrocyte membrane comprised of two components (23) and only in the TTD group the Vmax of both the component decreased significantly.

It may be mentioned here that the Na^+,K^+ ATPase is the integral membrane protein (28) while the AChE is bound to the outer surface of the erythrocyte membrane via glycolipid anchor (26). The Na^+,K^+ ATPase activity is known to be dependent on the acidic phospholipids viz. phosphatidylserine and phosphatidylinositol (28). In parallel studies we have found that the content of these phospholipids in the erythrocyte membranes had in fact increased significantly in the UTD, ITD as well as TTD groups (Chapter 3 of the thesis). It would therefore appear that the observed decrease in the Na^+,K^+ ATPase activity may be attributed either to absolute decrease in the contents of the enzyme units and/or altered membrane phospholipid milieu.

AChE has no known requirement for phospholipids and the activity also does not seem to be dependent on the extent of glycosylation of the enzyme per se (26). However, the membrane glycosylation can influence the enzyme activity (6). It is possible that the decreased AChE activity in the TTD group may relate to the altered membrane glycosylation, which could also have resulted in the changes in the kinetic attributes. Alternatively, the absolute content of the enzyme itself might have decreased possibly through decreased membrane binding.

The serum BChE profile might be considered as an index of the

liver metabolism as influenced by the diabetic state and indeed the data in Table 6 show that the Vmax of component I and II decreased significantly in UTD group. Interestingly, in the ITD group the component II was totally absent and in TTD group peculiarly enough the Km of all three component decreased significantly together with decrease in the Vmax values. Taken together the results imply that the liver metabolism was also differentially influenced by diabetic state and subsequent treatment regimens.

The differential effects of insulin or sulfonylurea treatment are once again borne out by the in vitro incubation experiments (Table 3, Figures 7-8).

In conclusion, the results of the present studies have shown that diabetic state and subsequent treatments can differentially influence not only the activities but also the kinetic properties of the erythrocyte enzymes Na^+,K^+ ATPase, AChE and serum BChE. Besides, the present studies also suggest that erythrocyte membrane glycosylation could be a better index of the glucose control.

It may also be pointed out that neither of the treatments could completely rectify the observed enzyme defects i.e. Km or Vmax. This is consistent with the general observation that all the maladies of diabetes are not corrected by insulin treatment (29).

Summary

Investigations were carried out on erythrocyte membranes and serum samples from control, untreated diabetic (UTD), insulin treated diabetic (TTD) and sulfonylurea tablet treated diabetic (TTD) human subjects.

Erythrocyte membrane protein glycosylation increased by 3.4 fold in diabetes and insulin treatment caused further (5.4 fold) increase. In TTD group, the extent of glycosylation was comparable to the UTD group.

The serum protein glycosylation was comparable in all the groups including control.

Decreased erythrocyte membrane Na^+,K^+ ATPase activity in the diabetics could be restored partly by insulin treatment but not by tablet (sulfonylurea) treatment.

Erythrocyte membrane acetylcholinesterase (AChE) activity decreased only in TTD group.

Serum butyrylcholinesterase activity was relatively low in the diabetic and ITD groups, but could be restored by sulfonylurea treatment.

The Km and Vmax of the components of Na^+,K^+ ATPase from

erythrocyte membranes were differently affected in the diabetic and the two treatment groups, whereas for the AChE the effect was decreased Vmax of both the components only in the TTD group.

Diabetic state resulted in generalized decrease of Vmax of the serum BChE, especially for component I and II. In the ITD group, component II was absent. In the TTD group, the picture resembled to that of the diabetics. However, the Vmax decreased for all the components.

In vitro incubation with insulin had differential effects on erythrocyte membrane Na^+,K^+ ATPase and serum BChE; AChE activity was not significantly influenced.

Figure legend

Figure 1 Typical substrate saturation curves for erythrocyte membrane Na^+,K^+ ATPase. The experimental details are as described in the text. A) Control, B) UTD, C) ITD and D) TTD.

Figure 2 Typical Eadie-Hofstee plots for erythrocyte membrane Na^+,K^+ ATPase. The experimental details are as described in the text. A) Control, B) UTD, C) ITD and D) TTD.

Figure 3 Typical substrate saturation curves for erythrocyte membrane AChE. The experimental details are as described in the text. A) Control, B) UTD, C) ITD and D) TTD.

Figure 4 Typical Eadie-Hofstee plots for erythrocyte membrane AChE. The experimental details as are described in the text. A) Control, B) UTD, C) ITD and D) TTD.

Figure 5 Typical substrate saturation curves for serum BChE. The experimental details are as described in the text. A) Control, B) UTD, C) ITD and D) TTD.

Figure 6 Typical Eadie-Hofstee plots for serum BChE. The experimental details are as described in the text. A) Control, B) UTD, C) ITD and D) TTD.

Figure 7 Effect of <u>in vitro</u> insulin addition on erythrocyte membrane Na^+,K^+ ATPase activity.

Figure 8 Effect of <u>in vitro</u> insulin addition on serum BChE activity.

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