CHAPTER : 3

Insulin and sulfonylurea treatments of the human diabetics. Effects on erthrocyte membrane structure-function relationship.

Introduction

Functions of plasma membrane such as enzyme activities and ligand receptor interactions depend on membrane fluidity,a concept related to the movements of lipids and proteins in the plane of the membrane (1). Certain membrane fluidity seems to be necessary for the membrane proteins to function appropriately (2). Shifts in membrane phospholipid content / composition may be important in regulating the activity of a variety of membrane-bound enzymes (3). Likewise fatty acid composition of membrane phospholipids can also influence the membrane functions (4).

Glomerular basement membrane and rat kidney basolateral membrane are found to be affected during diabetes (5, 6). It has been suggested that changes in the dynamic properties of the cell membrane i.e. membrane fluidity could be one of the events resulting in the altered insulin action (7).

The erythrocyte is most easily available and simplest human cell plasma membrane (8). Altered rheological properties of erythrocytes in diabetes have been reported (9 - 11). Phospholipid composition alterations were observed in diabetic condition in platelet membranes, polymorphonuclear leukocyte membrane, rat synaptosomal membrane and rat brain mitochondrial membrane and erythrocyte membrane (9, 12 - 14). The reports on the phospholipid composition and the membrane fluidity of erythrocyte membrane in diabetic patients are

however, controversial (15 - 19).

For management of diabetes either insulin therapy or treatment with sulfonylurea type of drugs are employed (20). The two treatments are known to control the blood sugar levels but are not able to rectify all the membrane defects (21). Besides, the effects of the two treatments could be differential (21).

In view of the above, it was of interest to look at the comprehensive picture of erythrocyte membrane properties as influenced by the diabetic state and the two treatment regimens. To this end the lipid / phospholipid profiles and the membrane fluidity together with the Arrhenius kinetics of the membrane-bound enzyme acetylcholinesterase (AChE) was examined. In simultaneous studies the temperature dependence of serum enzyme butyrylcholinesterase (BChE) was also determined. These findings are summarized in this chapter.

Materials and methods

Subjects

The details of the human volunteers used in these studies are as given in the Chapter 2 (Table 1), of the thesis.

Chemicals

Acetylthiocholine iodide (ACTI), butyrylthiocholine iodide

(BCTI), ethopropazine hydrochloride (ETPZ.HCl) and 1,6-diphenyl 1,3,5 hexatriene (DPH) were purchased from Sigma Chemical Co. USA. 5,5' dithio-bis (2-nitrobenzoic acid) (DTNB) was purchased from SRL, India. Silica gel G (Kieselgel 60G) was from Merck, Germany. NPH insulin was from Knoll Pharmaceuticals Ltd. India. All other chemicals were of analytical reagent grade and were purchased locally.

Preparative methods and enzyme assay procedures

Preparation of erythrocyte membranes was essentially according to the method of Hanahan et al. (22) as modified by Kumthekar and Katyare (23). The details of the membranes are already described in Chapter 2 of the thesis.

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

The AChE and BChE activities were determined essentially according to the procedure of Ellman et al. (24) as described previously with some modifications (25). For the assay of AChE activity, 5 mM ACTI was used as the substrate and 0.1 mM ETPZ.HCl was included as the inhibitor of BChE. In the BChE assay, ETPZ.HCl was omitted and BCTI (5 mM) was the substrate. Detailed procedures are already described in Chapter 2 of the thesis.

Serum glucose was estimated by the GOD-POD method.

Lipid analysis

Lipid extraction

Erythrocyte membrane lipids were extracted essentially according to the procedures described earlier (26,27). Appropriate aliquots of erythrocyte membrane protein (4 to 5mg) were taken in a final aqueous volume of 1.5 ml made up with 14 mM Tris-HCl buffer pH 7.4. To this 4.0 ml of freshly prepared 2:1 (v/v) chloroform : methanol mixture was added and the tube was vortexed vigorously and were centrifuged in a table top centrifuge at 2,000 rpm for 10 min. The lower phase was transferred to another tube using a graduated syringe and the volume was noted. To the first tube once again 2.0 ml of 2:1 chloroform : methanol mixture (2:1 v/v) was added and after re-extraction, the lower phase was once again measured and transferred to the tube containing previous chloroform : methanol extract. The volume of the pooled extract was noted and to this 0.2 volumes of 0.017 % $MgCl_2$ was added and after vortexing, the tubes were centrifuged at 2,000 rpm for 10 min. Once again the lower phase was transferred to another tube and the solvents were evaporated finally under stream of nitrogen (N_2). Volume was made up to 4.0 ml with the 2:1 chloroform : methanol mixture. From this aliquots were taken in separate tubes for estimating the total phospholipid (TPL), cholesterol (CHL) as well as for separation of the phospholipid classes by thin

layer chromatography (TLC).

Separation of phospholipids by TLC

preparation of plates

Glass plates (20 X 20 cm) were soaked overnight in chromic acid and washed repeatedly with tap water. The plates were finally rinsed with distilled waster and dried. Before coating the plates with the silica gel slurry they were thoroughly rinsed with acetone.

Kieselgel 60 H was used to coat the plates. The slurry contained 7.0 g of the silica gel powder mixed in 15 ml distilled water per plate. This slurry was transferred to a de saga applicator and spread evenly on the plates to get 0.25 mm coat thickness. The plates were allowed to dry overnight and only those plates with an even and uniform coating were selected for further work.

Spotting of the sample

Before spotting the samples the plates were activated by placing them in an oven set at 110^{o}C for 30 min.

The aliquots of lipid extracts taken out for TLC were first brought to zero volume under stream of N_2 after which a fixed small volume (50 to 60 ul) of chloroform : methanol (2:1) mixture was added. The sample was then spotted on to the

plates, and the plates were placed in the solvent system for phospholipid separation.

Thin layer chromatography for phospholipid separation

This was essentially according to the method described earlier (28). The solvent system used was as follows :
Chloroform : methanol : glacial acetic acid : distilled water (25 : 15 : 4 : 2) (v/v)
The solvent jar was saturated with this solvent system before the plates are kept in it. After the run was over the plate was removed and air-dried to remove the solvent present on the plates. The plate was then placed in a jar containing iodine for visualizing the spots. Once all the spots develop the yellowish brown color, the plate was removed and the individual spots were marked and identified as per the pattern reported earlier (28).

Estimation of separated phospholipids

The gel in the marked and identified spots was scraped off into numbered tubes. These tubes along with the aliquots for TPL measurement taken from the lipid extract were then subjected to digestion.

To all the tubes 0.5 ml 10 N H_2SO_4 was added and the tubes were then placed in a sand-bath and heated for 2-3 h. At the end of this digestion period the tubes were cooled and a drop

of 70 % perchloric acid was added. The tubes were once again heated in the sand-bath till the odor of chlorine was no longer detectable and the contents of tubes were optically clear. The tubes were then allowed to cool down and the phosphorous content was estimated essentially according to the method described by Bartlett (29).

Cholesterol estimation

The sample tubes were made to zero volume and then 0.5 ml of isopropanol was added. The sample tubes were then taken for cholesterol estimation. This was carried out essentially as described previously (30).

Measurement of membrane fluidity

This was carried out essentially as described previously (27, 31). Freshly prepared erythrocyte membranes were used for the measurements.

The fluorescence polarization measurements were carried out in Tris-HCl buffer pH 7.4. DPH (2 mM) used as the probe was dissolved in tetrahydrofuran and stored in amber colored bottle in refrigerator.

For the actual measurement, membrane protein 0.2 mg / ml final concentration was taken in buffered sucrose and to this 15 ul of the stock probe (DPH) solution was added and the

tubes were vigorously vortexed and left in dark for 30 min to permit equilibration of the probe into the membranes. The probe to lipid ratio was maintained around 1:200 to 1:300 (32).

The fluorescence polarization measurement was carried out in a Shimadzu RF-5000 spectrophotofluorimeter with a polarizer attachment. instrument has a resident program for calculating and printing fluorescence polarization (P) values. The excitation and emission wavelengths used were 360 nm and 430 nm respectively and the band width were 5 and 10 nm respectively for the two wavelengths. Data were accumulated for 5 seconds each for both vertical (parallel P 0^{o}) and horizontal (perpendicular P 90^{o}) setting of the polarizer.

From the P values other parameters i.e. fluorescence anisotropy (r), limited hindered anisotropy (r_{∞}) and order parameter (S) were calculated from the following formulae (33, 34) :

$r = 2P/(3-P)$,

$r_o = (4r/3)-0.1$ and

$S = (r_{\infty}/r)^{1/2}$

Temperature kinetics studies

The enzyme activities were determined over a range of $5^{o}C$ to $53^{o}C$ with optimum substrate concentration (5 mM). The analysis of the data for determination of energies of

activation for the high and the low temperature ranges (E1 and E2 respectively) and phase transition temperature (Tt) was according to Raison (35).

All analyses were performed on computer employing Sigma Plot version 5.0.

Results

The Data in Table 1 show that in the erythrocyte membranes form the control group the total phospholipid (TPL) content was about 380 µg / mg protein, while the cholesterol (CHL) content was 194 µg / mg protein. As a result the TPL / CHL molar ratio was 1.0. This is in close agreement with the previously reported values (36). In the untreatetd diabetic (UTD) group the TPL content was somewhat low but the change was not statistically significant. Similarly the CHL content increased by 40 % but the change was not statistically significant. However the TPL / CHL molar ratio decreased and the value was statistically highly significant. In the insulin-treated diabetic (ITD) group the TPL content decreased significantly (20 % decrease) with the CHL content becoming almost double. Consequently the molar ratio of TPL / CHL became 0.42. In tablet treated diabetic (TTD) group also the TPL content decreased significantly (21 % decrease) without any change in the CHL content and the TPL / CHL molar ratio was comparable to that in the UTD group.

Table 1. Effect of diabetes on erythrocyte membrane lipid content.

	Control	UTD	ITD	TTD
TPL (µg/mg protein)	376.8±27.27	327.3±25.26	270.4±18.99 a	257.2±19.68 a@
CHL (µg/mg protein)	193.9±10.20	273.7±39.58	369.5±46.90 b	211.0±26.17
TPL/CHL molar ratio	0.976±0.06	0.747±0.017	0.417±0.049 c#	0.670±0.068 a

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

a, $p<0.005$; b, $p<0.002$ and c, $p<0.001$ compared with control.

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

The data in Table 2 show phospholipid composition of erythrocyte membranes. Thus in the control group phosphatidylcholine (PC), sphingomyelin (SPM) and phosphatidylethenolamine (PE) were the major components with their contribution to TPL content amounting to 25, 24 and 22 % respectively. The content of phosphatidylinositol (PI), phosphatidylserine (PS), lysophosphatidic acid (Lyso) and phosphatidic acid (PA) ranged from 3.5 to 7.8 %. In the UTD the PC content decreased by 12 %, which was statistically significant. Similarly content of acidic phospholipids, PS and PI about doubled, which was statistically significant.

In the ITD group the PC content decreased further (34 % decrease), so also the contents of PI and Lyso decreased significantly. SPM component increased by 16 % but the change was not statistically significant. PA content increased by 30%.

In the TTD group also the PC content had decreased by 20 %. However the content of PI and PS became comparable to the UTD group. Interestingly, the SPM content had increased by 20 %, which was statistically significant.

In view of the dramatic change in phospholipid composition it was of interest to find out if erythrocyte membrane fluidity was altered under these conditions. The data (Table 3) on the fluidity of the erythrocyte membranes in the control group

Table 2. Effect of diabetes on erythrocyte membrane phospholipid composition.

	Phospholipid composition (% of total)			
	Control	UTD	ITD	TTD
Lyso	4.1±0.82	4.7±0.34	2.7±0.47 #	4.3±0.30
SPM	23.6±1.37	23.6±1.33	27.4±2.47	28.3±1.36 a@
PC	34.8±0.60	30.7±1.17 c	28.1±1.31 e	27.5±1.91 e
PI	3.5±0.51	6.1±0.96 a	3.0±0.47 @	6.5±0.67 d
PS	3.7±0.53	6.3±0.78 b	5.6±0.51 a	7.5±0.68 e
PE	22.3±1.15	21.8±1.07	23.1±1.38	21.0±1.16
PA	7.8±0.56	6.8±0.55	10.1±0.83 a@	6.8±0.35

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

a, $p<0.05$; b, $p<0.02$; c, $p<0.01$; d, $p<0.005$ and e, $p<0.001$ compared with control.

@, $p<0.01$ and #, $p<0.005$ compared with diabetic.

Table 3. Effect of diabetes on erythrocyte membrane fluidity.

Fluorescence parameters	Control	UTD	ITD	TTD
Fluorescence polarization (P)	0.312±0.006	0.375±0.013 c	0.179±0.003 c@	0.304±0.005 @
Fluorescence anisotropy (r)	0.240±0.005	0.286±0.011 b	0.127±0.002 c@	0.225±0.004 a@
Limited hindered anisotropy (r_{∞})	0.220±0.007	0.282±0.015 b	0.069±0.003 c@	0.200±0.005 a@
Order parameter (S)	0.956±0.005	0.965±0.027	0.733±0.010 c@	0.942±0.004 a

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

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

@, $p<0.001$ compared with diabetic.

are in close agreement with the earlier reported values (37). It can be noted from the data in Table 3 that in the UTD group the membrane fluidity decreased significantly. Insulin treatment brought about significant increase in the fluidity; even in the TTD group the fluidity increased but the magnitude of the increase was much lesser.

To check the effects of altered membrane composition and fluidity on the AChE activity the temperature kinetics of AChE was studied. Typical temperature curves are shown in Figure 1. These results are depicted in the form of typical Arrhenius plots in Figure 2. Thus it can be noted that in the control and the UTD groups the Arrhenius plot was biphasic. By contrast, in the ITD and TTD groups about 50 % of the population showed phase transition while in the case of the remaining 50 % the phase transition was abolished.

The values of energies of activation in the high and low temperature ranges (E1 and E2 respectively) and the phase transition temperature (Tt) are given in Table 4. Thus in the control group E1 and E2 values were 55.1 and 30.8 KJ / mole with phase transition occuring around 35-36^{o}C; the pattern did not change in the UTD group. The observations on control are in agreement with earlier reported values (38). In ITD group which showed phase transition, Tt did not change but the value of E1 decreased significantly, whereas in those showing no phase transition the energy of activation was 40

Figure 1.

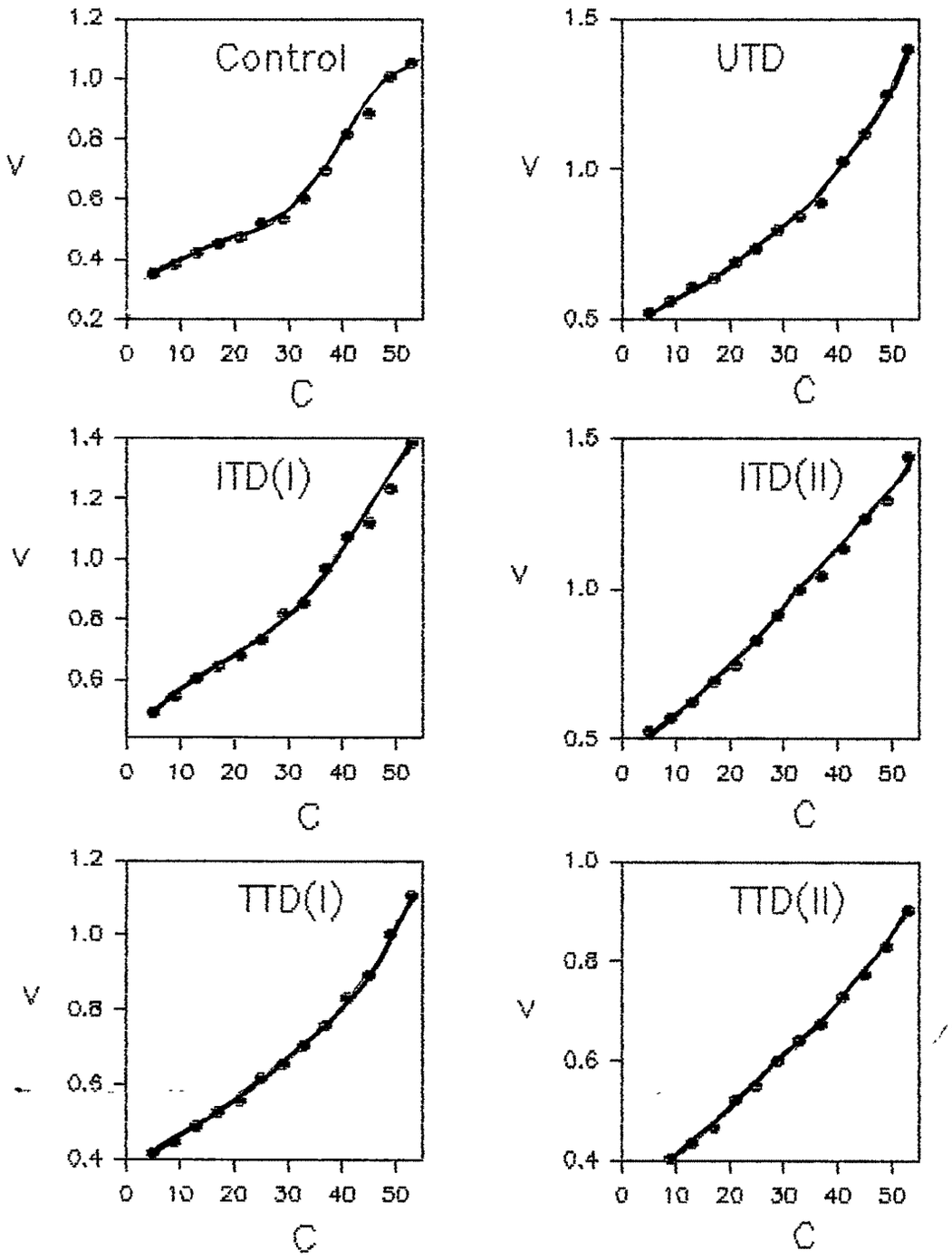
Control
UTD
ITD(I)
ITD(II)
TTD(I)
TTD(II)
V
C
0 10 20 30 40 50
1.2 1.0 0.8 0.6 0.4 0.2
1.5 1.0 0.5
1.4 1.2 1.0 0.8 0.6
1.5 1.0 0.5
1.2 1.0 0.8 0.6 0.4
1.0 0.8 0.6 0.4

Figure 2.

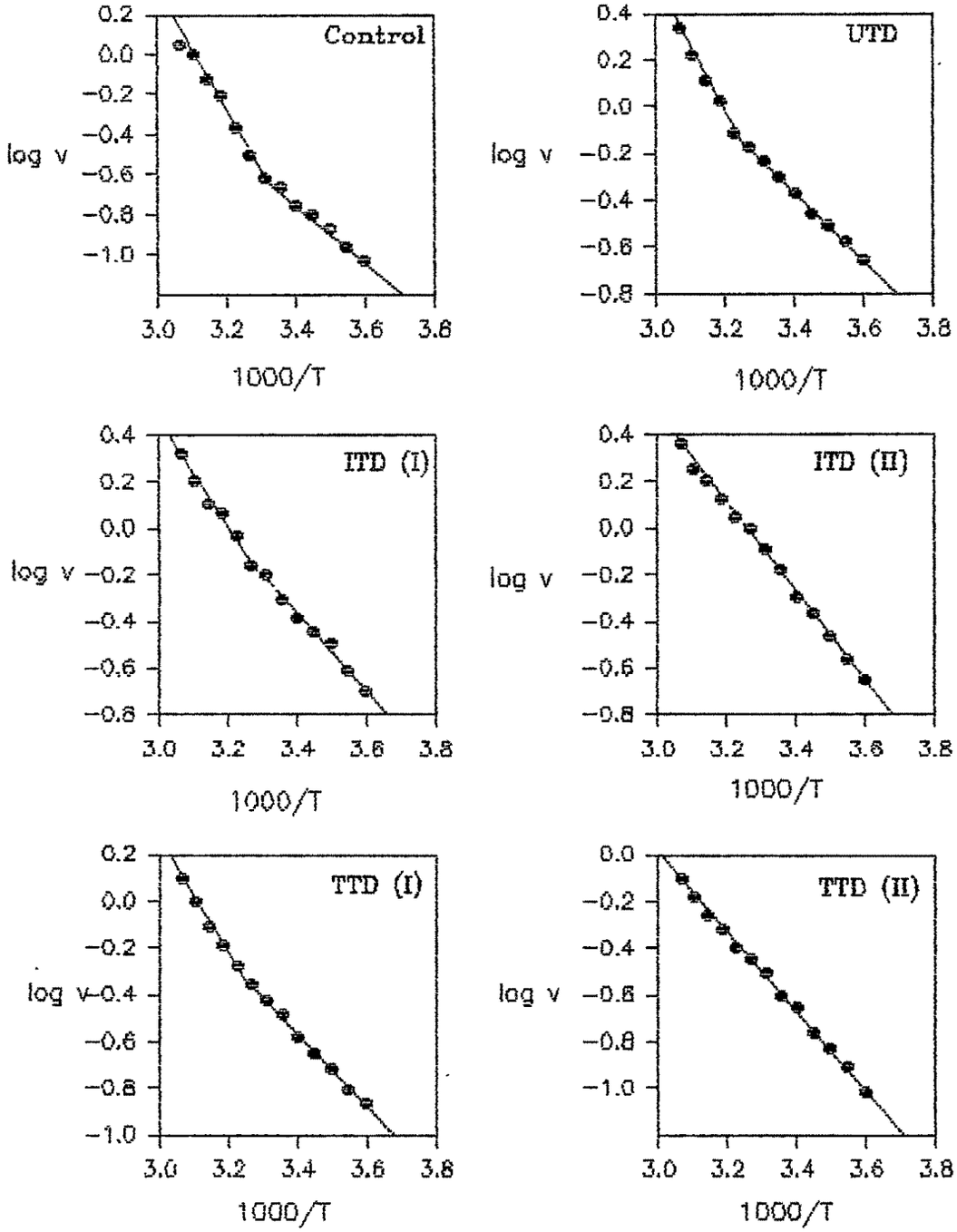

Table 4. Effect of diabetes on temperature kinetics of erythrocyte membrane AChE.

	Energy of activation (KJ / mole)		Phase transition temperature (Tt), ^{o}C
	E_1	E_2	
Control	55.1±5.00	30.8±1.85	34.8±1.29
UTD	51.4±2.66	30.0±1.10	36.5±1.51
ITD - I	48.0±1.62^{c}	31.1±2.07	34.5±2.49
ITD - II	40.0±2.90$^{a\#}$	___	___
TTD - I	41.3±2.54$^{a@}$	27.9±2.30	35.9±2.80
TTD - II	35.7±2.88^{b*}	___	___

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

a, $p<0.05$; b, $p<0.005$ and c, $p<0.001$ compared with control.

@, $p<0.05$; #, $p<0.02$ and *, $p<0.002$ compared with diabetic.

I and II refer to subtype in ITD and TTD group.

KJ / mole. In the TTD group with phase transition, E1 decreased significantly without any change in the values of E2 and Tt. In the remainder of the TTD group the value of energy of activation had decreased further to 35.7 KJ / mole.

Studies were also extended to check the temperature dependent kinetics of serum BChE. The typical temperature curves are shown in the Figure 3. The typical Arrhenius plots are shown in Figure 4, from which it can be noted that the enzyme from the controls did not show phase transition but that from the UTD group did. In the ITD group 50 % of the population showed presence of phase transition. In TTD group phase transition was evident in all the cases. The values of energy of activation and phase transition temperature are given in Table 5. from which it can be noted that in the control group the energy of activation was about 24 KJ / mole. In the UTD the values of E1 and E2 was 15.3 and 31.2 KJ / mole respectively with phase transition occuring around 31^{o}C. In ITD group showing phase transition the pattern was comparable with UTD but in the case of the remainder of the ITD population the value of energy of activation was intermediate i.e. 21 KJ / mole. In the TTD group also the values of E1 and E2 did not change, however phase transition temperature decreased by 3.8^{o}C, which was statistically significant.

Discussion

From the data presented (Table 1), it is clear that there is a generalized tendency of decreased TPL content and increased

Figure 3.

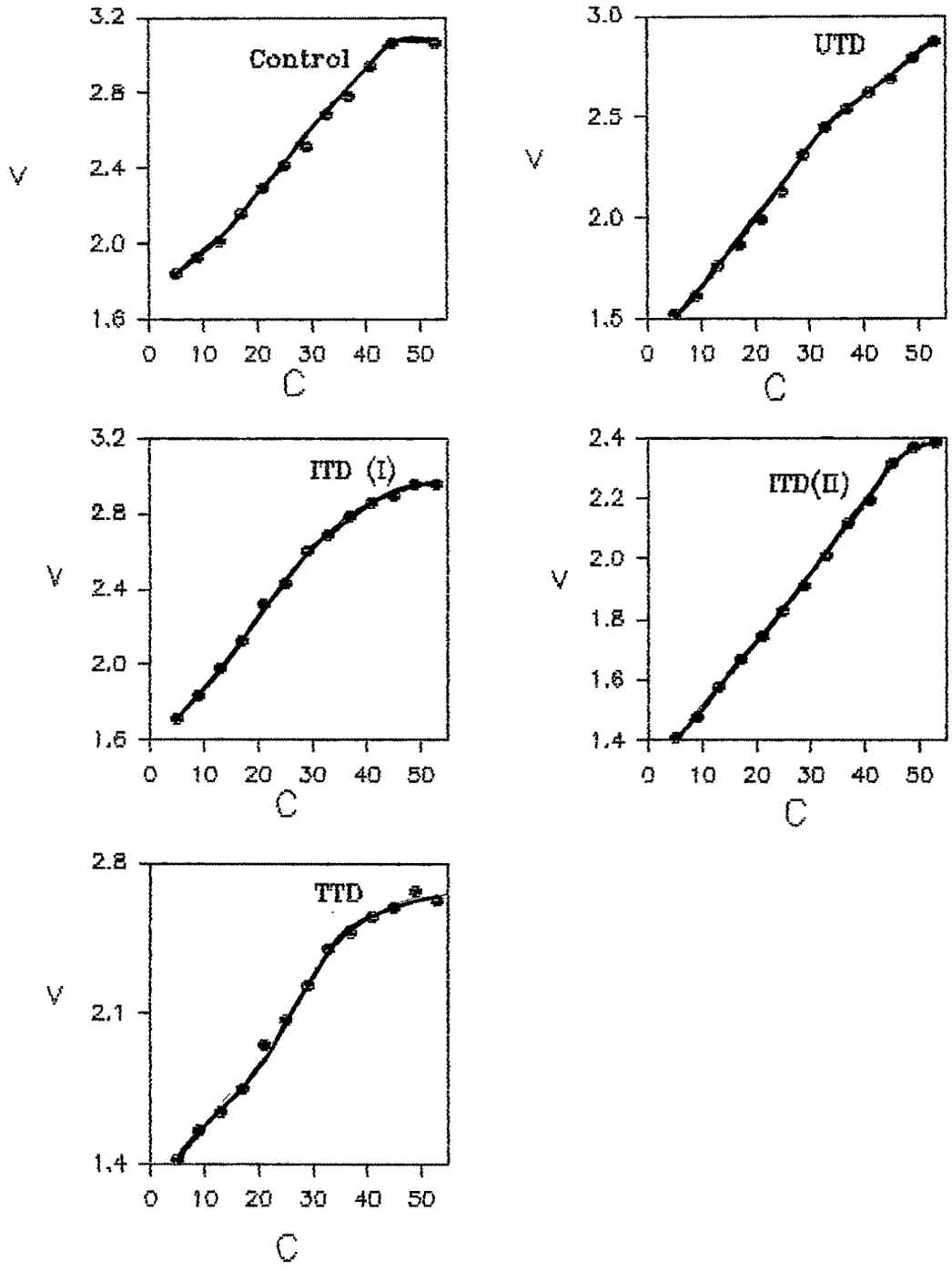
Control
UTD
ITD (I)
ITD(II)
TTD
V
C
3.2
2.8
2.4
2.0
1.6
3.0
2.5
1.5
2.2
1.8
1.4
2.1
0
10
20
30
40
50

Figure 4.

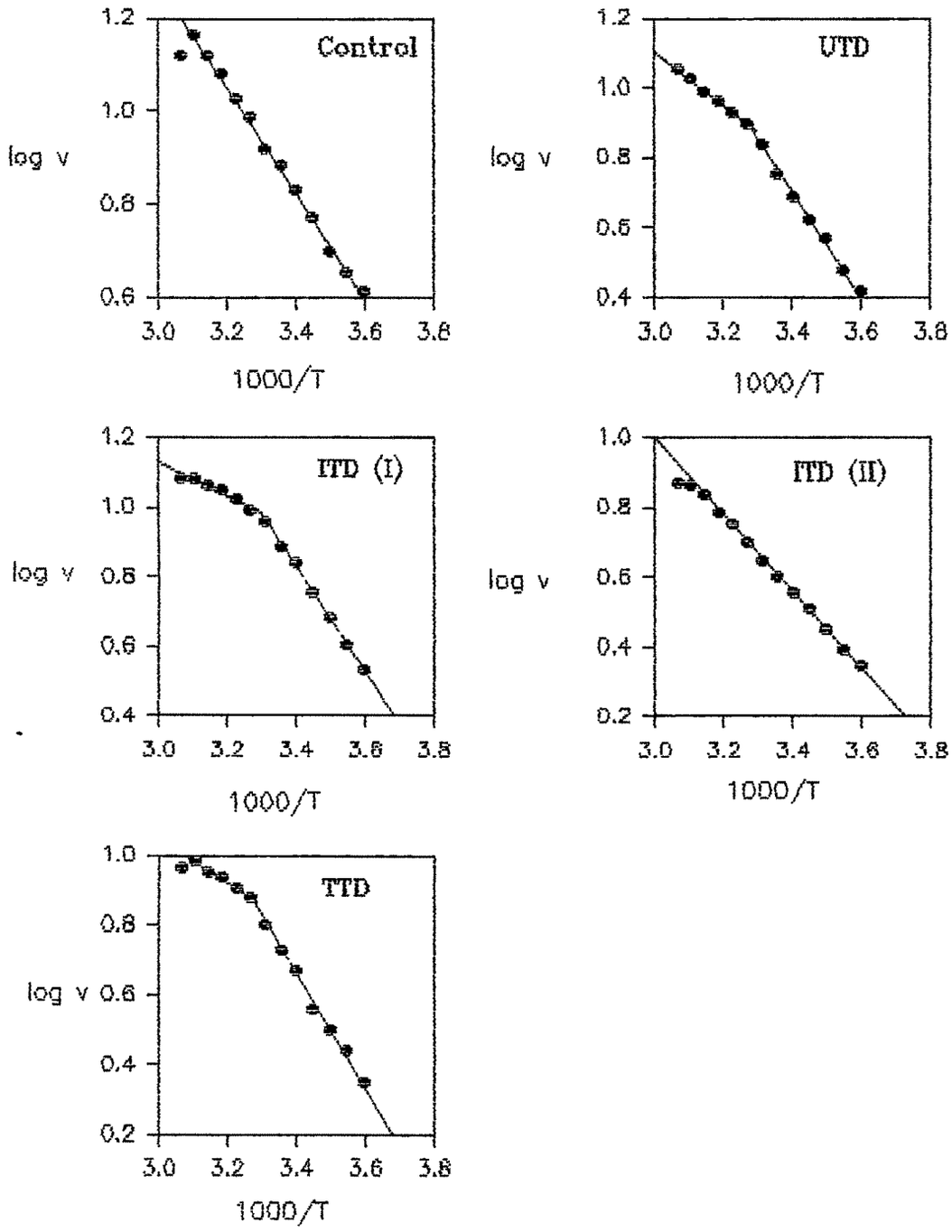
Control
UTD
ITD (I)
ITD (II)
TTD
log v
1000/T
1.2
1.0
0.8
0.6
0.4
0.2
3.0
3.2
3.4
3.6
3.8

Table 5. Effect of diabetes on temperature kinetics of serum BChE.

	Energy of activation (KJ / mole)		Phase transition temperature (Tt), ^{o}C
	E_1	E_2	
Control	23.9±0.58	---	---
UTD	15.3±0.75 b	31.2±1.33	31.2±1.20
ITD - I	13.2±1.61 b	32.1±2.67	31.3±2.39
ITD - II	20.9±0.64 a#	___	___
TTD	14.3±1.63 b	32.9±1.61	27.4±1.15 @

The results are given as mean±S.E.M. of observations indicated in the parentheses.

a, $p<0.005$ and b, $p<0.001$ compared with control.

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

I and II refers to subtypes in ITD group.

CHL content in the erythrocyte membranes from the diabetics. Insulin or sulfonylurea treatments were in general ineffective in arresting the process. Consequently compared to the control, the TPL / CHL molar ratios were always low.

The data on phospholipid analysis of the erythrocyte membranes in the control, are in general agreement with the values reported by the others (9, 19, 36). In the UTD group there was a small but reproducible decrease in PC content while PI and PS components almost doubled. Other researcher however failed to find any change in erythrocyte membrane phospholipids in the diabetic / obese groups (9, 19, 36). It may be pointed out that these authors performed the analysis by HPLC and were able to resolve the phospholipids only in four major groups viz. PC, PE, SPM and PI. In the present studies the separation was achieved by TLC and it was possible that the phospholipids could be resolved in seven components (Table 2). It is thus possible that in the case of the other researchers (9, 36) the effect of diabetic state might have been masked because the phospholipids separated in only four groups and there might have been cross contamination amongst the phospholipid classes. The trend of decrease in PC in diabetes was not arrested either by insulin or by sulfonylurea treatment; the acidic phospholipid content also remained elevated. Besides, in the ITD and TTD groups the SPM content increased significantly, which may be a compensatory mechanism for decreased PC.

The changes in the lipid / phospholipid profiles (Tables 1, 2) were also reflected in terms of membrane fluidity which decreased in UTD; and could be restored in the TTD group. In the ITD the membrane seems to be unusually fluidized. The ratios of PC / PE and TPL / CHL are accepted parameters of membrane fluidity (27, 39) and a positive correlation between these ratios and membrane fluidity was obtained. Also the membrane fluidity correlated negatively with CHL and the PA content, whereas it was positively correlated with the content of Lyso, PI and Lyso / PA ratio (data not shown). It may hence be suggested that in the erythrocyte membrane regulation of membrane fluidity is a complex process involving several phospholipid components and their ratios as outlined above.

The change in phospholipid composition did not affect the Arrhenius kinetics of erythrocyte membrane AChE in the UTD group. However in the ITD and UTD groups two different sub-classes were seen. One sub-class resembled the control; the other one differed from the control and UTD in that the phase transition was abolished. Similar trend was seen in the TTD group also. The results thus emphasize the fact that the two treatments could restores membrane function, in only about 40 % of the subjects.

The picture was somewhat different for serum BChE, where the control group did not show phase transition. However the UTD

group did. Similar pattern was also seen in TTD. In ITD group two sub-classes, one showing phase transition and the other one without phase transition could be noted. The results thus indicate that even restoration of liver metabolism is only partial after insulin treatment. BChE is known to originate from the liver (40) and hence could serve as an index of liver metabolism.

In conclusion the results of the present studies have brought into focus the alteration in membrane composition and function, which are insulin-status-dependent. Besides, these studies have also shown that all the maladies of diabetes can not be corrected by insulin treatment (41).

summary

In the human diabetic subjects (UTD) the TPL content decreased and CHL content increased in the erythrocyte membranes. Insulin or sulfonylurea treatment were in general ineffective in arresting this trend.

PC content in the erythrocyte membrane decreased in diabetics, while the PI and PS contents doubled. Insulin treatment resulted in decreased Lyso and PC contents, while PS and PA content increased; the TTD group was comparable to the diabetic.

The membrane fluidity decreased significantly in diabetes and insulin treatment resulted in significant fluidization of the membrane. The fluidity seems to be related in a complex manner to the phospholipid molar ratios, and TPL and CHL contents.

The temperature kinetics of AChE did not change in the untreated diabetics with the phase transition occuring near physiologic temperature. Insulin and sulfonylurea tablet treatments had dual effects. Two groups, one with and one without phase transition was seen.

Serum BChE in the control did not display phase transition but it became evident in the diabetic group. Insulin treatment had dual effect as in the case of AChE.

Sulfonylurea treated group was comparable to the diabetic group although the values of E1 and Tt decreased significantly.

Figure legends

Figure 1. Typical temperature curves for human erythrocyte membrane AChE. Control, Untreated diabetics (UTD), Insulin treated diabetics with phase transition temperature (ITD I), Insulin treated diabetics without phase transition temperature (ITD II), Tablet (oral hypoglycemic sulfonylurea) treated diabetics with phase transition temperature (TTD I) and Tablet (oral hypoglycemic sulfonylurea) treated diabetics without phase transition temperature (TTD II). Enzyme activities were monitored over the temperature range of 5^{o}C to 53^{o}C. Optimum substrate (ACTI) concentration, 5 mM was used. Other details are as described in the text.

Figure 2. Typical Arrhenius plots for human erythrocyte membrane AChE. Control, Untreated diabetics (UTD), Insulin treated diabetics with phase transition temperature (ITD I), Insulin treated diabetics without phase transition temperature (ITD II), Tablet (oral hypoglycemic sulfonylurea) treated diabetics with phase transition temperature (TTD I) and Tablet (oral hypoglycemic sulfonylurea) treated diabetics without phase transition temperature (TTD II). Enzyme activities were monitored over the temperature range of 5^{o}C to 53^{o}C. Optimum substrate (ACTI) concentration, 5 mM was used. Other details are as described in the text.

Figure 3. Typical temperature curves for serum BChE. Control, Untreated diabetics (UTD), Insulin treated diabetics with phase transition temperature (ITD I), Insulin treated diabetics

without phase transition temperature (ITD II), Tablet (oral hypoglycemic sulfonylurea) treated diabetics (TTD). Enzyme activities were monitored over the temperature range of 5^{o}C to 53^{o}C. Optimum substrate (BCTI) concentration, 5 mM was used. Other details are as described in the text.

Figure 4. Typical Arrhenius plots for serum BChE. Control, Untreated diabetics (UTD), Insulin treated diabetics with phase transition temperature (ITD I), Insulin treated diabetics without phase transition temperature (ITD II), Tablet (oral hypoglycemic sulfonylurea) treated diabetics (TTD). Enzyme activities were monitored over the temperature range of 5^{o}C to 53^{o}C. Optimum substrate (BCTI) concentration, 5 mM was used. Other details are as described in the text.

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