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Enhanced oxidative phosphorylation in rat liver mitochondria following prolonged *in vivo* treatment with imipramine

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1 Effects of prolonged *in vivo* administration of the tricyclic antidepressant drug imipramine on oxidative energy metabolism in rat liver mitochondria were examined.

2 Imipramine treatment resulted in an increase in state 3 respiration rates with all the substrates tested as early as one week after treatment; this was sustained through the second week of treatment.

3 The changes in respiration rates were accompanied by a selective increase in the intramitochondrial cytochrome aa_3 and $c + c_1$ contents after both one and two weeks of treatment.

4 Administration of imipramine did not alter the total liver protein content per g tissue, the mitochondrial protein content per g tissue or the mitochondrial yield.

5 Kinetic analyses of succinoxidase activity in terms of Arrhenius plots indicated possible alterations in mitochondrial membrane lipid milieu and membrane fluidity after the drug treatment, especially in the second week.

Introduction

Imipramine is the most widely used tricyclic antidepressant drug for the treatment of various kinds of depressions (Bickel, 1981) and is believed to relieve depression by inhibiting the re-uptake of monoamines (Lidbrink *et al.*, 1971). However, the monoamine hypothesis has been questioned and modified in recent years (Barbaccia *et al.*, 1983). Our own studies have shown that under *in vitro* conditions, imipramine is able to bind with rat liver and brain mitochondria and inhibit respiration in a cooperative manner, thus pointing to specific interactions of this drug with mitochondrial membranes (Rajan & Katyare, 1985).

Beneficial effects of imipramine therapy, however, become apparent only one or two weeks after initiation of treatment (Bickel, 1981). It is therefore reasonable to assume that *in vivo* studies could give better insights into the mechanisms underlying imipramine action.

Our earlier studies had shown that prolonged *in vivo* treatment with imipramine resulted in increased respiration rates in brain mitochondria suggesting that imipramine may act through the mitochondrial route by making more energy available to overcome depression (Rajan & Katyare, unpublished observations).

The present studies were undertaken to determine whether the respiration-stimulating effects of prolonged *in vivo* imipramine treatment are specific for brain mitochondria or whether similar effects could be observed in mitochondria from other metabolically active tissues such as liver. Several other investigators have used a similar approach to examine the *in vivo* effects of tricyclic antidepressants on α- and β-adrenoceptors and GTPase and adenylate cyclase activities; a one to two week treatment period in their studies was considered as 'long-term' treatment (Lace & Antelman, 1983; Menkes *et al.*, 1983; Pilc & Enna, 1986).

The results of the present studies indicate that as in the case of brain mitochondria, administration of imipramine also stimulated respiration in liver mitochondria. The enhancement of respiration rates was accompanied by increase in intramitochondrial cytochromes aa_3 and $c + c_1$ contents.

Examination of temperature-dependent changes in the succinoxidase activity indicated that imipramine

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treatment also resulted in possible alterations in the lipid milieu of the mitochondrial membranes.

Methods

Management of the animals

Female rats of the Wistar strain weighing approximately 270 g were used. Imipramine solution was made up freshly in physiological saline before use and was administered (i.p.) to the rats twice daily at a dose of 10 mg kg^{-1} body weight; control animals received the vehicle alone.

Isolation of mitochondria

The animals were killed at the end of one week or two weeks of treatment (hereafter referred to as N_1, N_2 and I_1, I_2 respectively for the control and the imipramine-treated groups) by decapitation approximately 18 h after the last injection and their livers were quickly removed and processed for isolation of mitochondria as described previously (Satav & Katyare, 1982).

Briefly, tissue homogenates (10% w/v) were prepared in 0.25 M sucrose in a Potter-Elvehjem type homogenizer with a tight fitting Teflon pestle (wall clearance: 0.18 mm) and were centrifuged in a Sorvall RC5C centrifuge at 650 *g* for 10 min to sediment nuclei and unbroken cell debris. The supernatant was centrifuged at 6500 *g* for 10 min to sediment mitochondria. The mitochondrial pellets thus obtained were washed once by resuspending in the isolation medium and resedimenting at 6500 *g* for 10 min. Finally the mitochondrial pellets were suspended in the isolation medium to give ca. 25 mg mitochondrial proteins ml^{-1}. All operations were carried out at 0–4°C.

For measurement of succinate dehydrogenase activity, mitochondria were isolated essentially as described above except that known measured volumes (35 ml) of homogenate were used and the resulting mitochondrial pellets were suspended in 3.5 ml of the isolation medium as described above, care being taken to perform all operations in a quantitative manner. Yield of mitochondrial protein g^{-1} tissue in these experiments was recorded.

Determination of oxidative phosphorylation

Measurements of oxidative phosphorylation were carried out as described earlier (Satav & Katyare, 1982) at 25°C with a Clark-type oxygen electrode (Chance & Williams, 1955) in a medium (total volume: 1.3 ml) containing 225 mM sucrose, 10 mM potassium phosphate buffer, pH 7.4, 10 mM Tris-HCl pH 7.4 and 5 mM $MgCl_2$. Glutamate (10 mM), β-hydroxybutyrate (10 mM), succinate (10 mM), pyruvate (10 mM) + malate (1 mM) and ascorbate (10 mM) + TMPD (0.1 mM) were used as substrates. With succinate and ascorbate + TMPD, 1.0 μM rotenone was included in the reaction medium. Approximately 2–4 mg of mitochondrial proteins were used per experiment depending on the substrate employed. The respiration rates in the presence of ADP (state 3) and after its depletion (state 4) were recorded. Two responses with added ADP (100–150 nmol in 10–15 μl) were recorded in each experiment with all the substrates except those with ascorbate + TMPD; these were run in duplicate. Calculations of ADP/O ratios and respiratory control ratio (RCR) were as described previously (Katyare *et al.*, 1977), RCR being defined as the ratio of state 3/state 4 respiration rates.

Succinoxidase activity and Arrhenius plots

Temperature-dependent changes in succinate oxidation rates were measured polarographically in a medium (total volume: 1.3 ml) consisting of 67 mM potassium phosphate buffer, pH 7.4 containing 0.4 mM $CaCl_2$ and 0.4 mM $AlCl_3$ (Potter, 1959; Katyare *et al.*, 1971) in a water-jacketed cell over a temperature range of 10°C to 46°C with 4°C temperature increment at every step. Kinetic analyses for determinations of energies of activation were according to Raison *et al.* (1971), Raison, (1972).

Quantification of cytochrome contents

Contents of cytochromes in mitochondria were determined by following the procedure described earlier (Satav & Katyare, 1982). Mitochondria in phosphate-buffered isolation medium, pH 7.4 were solubilized with suitable aliquots of freshly prepared 10% (v/v) Triton X-100 at a final concentration of 2–4 mg protein ml^{-1} and the difference between spectra of dithionite-reduced minus ferricyanide-oxidized cytochromes were recorded in a Hitachi double-beam spectrophotometer Model 150–20. Calculations of cytochrome content were as described previously (Satav & Katyare, 1982).

Mitochondrial content of the tissue

For quantitating the mitochondrial protein contents in the tissues, the succinate dehydrogenase activity in whole homogenates and three-times washed mitochondria was determined as described by Caplan & Greenawalt (1968). The mitochondrial protein content in the tissue was calculated, based on the

Table 1 Effect of prolonged *in vivo* imipramine treatment on body and liver weight in rats

Animal group	*Body weight* (g)	*Liver weight* (g)	*Liver weight* (% body weight)
N_1 (20)	282.9 ± 2.8	9.7 ± 0.2	3.4 ± 0.1
N_2 (20)	298.1 ± 3.4	9.6 ± 0.2	3.2 ± 0.1
I_1 (20)	262.6 ± 2.9***	8.7 ± 0.2**	3.3 ± 0.1 NS
I_2 (20)	261.0 ± 4.9***	9.0 ± 0.2*	3.5 ± 0.1*

The animals were injected with saline or imipramine for one or two weeks as described in Methods. Values are given as mean ± s.e.mean of the number of observations indicated in parentheses.
* $P < 0.05$; ** $P < 0.002$; *** $P < 0.001$; NS not significant.

succinate dehydrogenase activity in the whole homogenate and the specific activity of the enzyme in three-times washed mitochondria as described earlier (Rajwade *et al.*, 1975).

Protein estimation was according to the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as the standard.

Student's *t* test was used to determine statistical significance of differences between means.

Chemicals

Sodium salts of succinic acid, L-glutamic acid, pyruvic acid, L-malic acid and ascorbic acid as well as ADP, rotenone, Triton X-100, imipramine hydrochloride and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. N,N,N',N'-tetramethyl-*p*-phenylene diamine (TMPD) was from British Drug Houses, Poole, Dorset. The sodium salt of β-hydroxybutyric acid was from Fluka AG, Switzerland.

Results

In preliminary experiments it was observed that the control animals gained weight steadily. Treatment of animals with imipramine resulted in a decrease in body weight which was evident as early as on the 3rd day of the treatment with maximum decrease (10–15 g) being seen on day 4; subsequent to this the animals did not gain or lose any weight and thus their body weight remained more or less constant throughout the entire experimental period (data not given). Thus practically at all stages the animals in the experimental group weighed less than their corresponding controls. This is typified by data in Table 1. It can be noted that at the end of one week, the imipramine-treated animals recorded a weight loss of about 20 g; at the end of the second week their body weights decreased further by about 37 g (7% and 12% decrease respectively compared to the corresponding controls). The liver weights also decreased by 6–10% under these conditions (Table 1). The reasons for the decrease in body and liver weights after imipramine treatment are unclear at this stage; normally a xenobiotic would be expected to result in

Table 2 Effect of prolonged *in vivo* treatment with imipramine on mitochondrial oxidative phosphorylation using glutamate, β-hydroxybutyrate and pyruvate + malate as the respiratory substrates

Substrate	*Animal group*	*ADP/O ratio*	*Respiration rate* (nmol O_2 mg^{-1} protein min^{-1}) + ADP	− ADP
Glutamate	N_1 (8)	2.7 ± 0.1	34.5 ± 1.0	1.3 ± 0.3
	N_2 (9)	2.5 ± 0.1	34.4 ± 0.9	1.2 ± 0.2
	I_1 (8)	2.4 ± 0.1	39.2 ± 1.6*	3.3 ± 0.8*
	I_2 (11)	2.2 ± 0.1	40.3 ± 1.2††	3.5 ± 0.5††
β-Hydroxybutyrate	N_1 (6)	2.8 ± 0.1	26.3 ± 1.5	1.6 ± 0.3
	N_2 (14)	2.8 ± 0.1	26.2 ± 1.0	1.0 ± 0.3
	I_1 (7)	2.3 ± 0.1	34.2 ± 1.2†	2.8 ± 0.7 NS
	I_2 (6)	2.2 ± 0.1	35.4 ± 2.1††	4.8 ± 1.1***
Pyruvate + malate	N_1 (8)	2.8 ± 0.1	17.3 ± 1.0	1.9 ± 0.4
	N_2 (14)	3.1 ± 0.1	17.7 ± 0.6	1.2 ± 0.3
	I_1 (12)	2.5 ± 0.1	20.6 ± 0.8**	2.4 ± 0.5 NS
	I_2 (7)	2.2 ± 0.2	20.6 ± 1.0*	3.1 ± 0.6**

The animals were injected (i.p.) with saline or imipramine as described in Methods and killed at the end of 1 week or 2 weeks of the treatment period. Results are given as mean ± s.e.mean of the number of observations indicated in parentheses.
* $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$; † $P < 0.002$; †† $P < 0.001$; NS not significant.

Table 3 Effect of prolonged *in vivo* treatment with imipramine on mitochondrial oxidative phosphorylation using succinate and ascorbate + N,N,N',N'-tetramethyl-*p*-phenylene diamine (TMPD) as the respiratory substrates

Substrate	*Animal group*	*ADP/O ratio*	*Respiration rate* (nmol O_2 mg^{-1} protein min^{-1}) + ADP	− ADP
Succinate	N_1 (8)	1.6 ± 0.1	61.3 ± 1.8	8.8 ± 2.0
	N_2 (9)	1.7 ± 0.1	63.3 ± 2.4	7.3 ± 1.6
	I_1 (12)	1.6 ± 0.1	70.6 ± 3.4*	17.8 ± 3.5*
	I_2 (9)	1.5 ± 0.1	83.6 ± 4.3†	14.6 ± 5.4 NS
Ascorbate + TMPD	N_1 (7)	0.4 ± 0.1	34.3 ± 1.7	10.0 ± 0.6
	N_2 (13)	0.4 ± 0.1	35.8 ± 2.5	9.7 ± 0.7
	I_1 (7)	0.5 ± 0.1	40.5 ± 1.3**	8.7 ± 0.2 NS
	I_2 (7)	0.4 ± 0.1	59.8 ± 3.3†	12.9 ± 0.6***

Experimental details are as given in Methods and in Table 2. Results are expressed as mean ± s.e.mean of the number of observations indicated in parentheses.
* $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$; † $P < 0.001$; NS not significant.

proliferation of liver endoplasmic reticulum (Hutson, 1970). Interestingly, it has been reported that imipramine and desimipramine when given twice daily for 7–14 days resulted in increased cytochrome P-450 contents in livers of male Wistar rats (Daniel *et al.*, 1984).

The results on oxidative energy metabolism in liver mitochondria as influenced by *in vivo* imipramine treatment are summarized in Tables 2 and 3.

The data in Table 2 show that state 3 respiration in liver mitochondria with glutamate, β-hydroxybutyrate and pyruvate + malate as substrates increased by 14–35% following treatment with imipramine. The stimulation was evident in the first week of treatment and was sustained through the second week. Interestingly, the highest stimulation (30% and 35% increase respectively in the first and in the second week) was seen with β-hydroxybutyrate as the substrate.

State 4 respiration rates showed a tendency towards increase (26–380% increase). However, statistically significant elevation in state 4 respiration rates was observed only in the case of glutamate and at the end of the second week in case of β-hydroxybutyrate and pyruvate + malate (Table 2).

The mitochondria in the control group were tightly coupled as judged by their high RCR values (although not given in the table, these values can be easily calculated from the information on the respiration rates therein). These values were somewhat low in the experimental groups but were still within acceptable range for tightly coupled mitochondria (Johnson & Lardy, 1967; Katyare, 1986). Imipramine treatment *in vivo*, therefore, did not result in uncoupling of mitochondria.

ADP/O ratios in all the groups were in the expected theoretical range for all the substrates (Johnson & Lardy, 1967; Katyare, 1986).

When succinate was employed as the substrate the extent of stimulation of respiration was about 15% in the first week which was enhanced further in the second week to 32% increase (Table 3). The state 4 respiration rate was enhanced in the first week and though it remained elevated in the second week, it was not statistically significant. With ascorbate + TMPD as substrate (Table 3), state 3 respiration was stimulated by 18% but in the second week the stimulation was as high as 67%. State 4 respiration rate increased by 32% in the second week only. The ADP/O ratios agreed well with theoretical values and the respiratory control ratios were within acceptable limits for tightly coupled mitochondria for both the above substrates (Johnson & Lardy, 1967; Katyare, 1986).

Since prolonged imipramine treatment resulted in significant changes in the respiratory activity of liver mitochondria, we tried to quantify the contents of intramitochondrial cytochromes; these results are given in Table 4. It is clear that imipramine administration resulted in an initial 17% increase in cytochrome aa_3 content, followed by a further increase to the extent of 32% in the second week. The content of cytochrome $c + c_1$ also increased significantly by approximately 27% during the treatment period. Imipramine administration, however, did not have any effect on cytochrome b content.

In view of the increased respiratory rates and cytochromes aa_3 and $c + c_1$ contents after imipramine treatment, it was of interest to see whether quantitative changes occurred in the mitochondrial protein content in liver tissue. These results are given in Table 5. It is evident that neither the total tissue protein content nor the mitochondrial protein content in the tissue changed significantly in the experimental groups. Also the activities of the marker enzyme, succinate dehydrogenase in the

Table 4 Cytochrome content in liver mitochondria from normal and imipramine-treated rats

Animal group	*Cytochrome content* (pmol mg^{-1} protein) aa_3	b	$c + c_1$
N_1 (10)	95.0 ± 4.3	177.0 ± 12.5	289.0 ± 15.0
N_2 (10)	99.0 ± 4.2	198.0 ± 10.0	301.0 ± 14.0
I_1 (7)	111.0 ± 3.0*	209.0 ± 12.6 NS	369.0 ± 22.5*
I_2 (10)	131.0 ± 3.6**	215.0 ± 07.2 NS	381.0 ± 15.2**

The experimental details are as given in Methods. Results are given as mean ± s.e.mean of the number of observations indicated in parentheses.
* $P < 0.01$; ** $P < 0.001$; NS not significant.

whole tissue and in the mitochondria did not change after imipramine treatment. In separate experiments we also attempted to quantitate yield of mitochondrial protein g^{-1} tissue as described in the Methods section. These values for the normal and imipramine-treated animals were comparable. They were: 23.8 ± 0.8 and 23.0 ± 0.7 mg g^{-1} tissue respectively for the 1 and 2 week controls and 23.5 ± 0.7 and 22.8 ± 1.1 mg g^{-1} tissue respectively for the 1 and 2 week imipramine-treated groups. It would therefore seem that the changes in mitochondria were more of a qualitative than of a quantitative type.

Since respiration rates with succinate as substrate had increased significantly (Table 3) without any change in the specific activity of the enzyme succinate dehydrogenase (Table 5) in the mitochondria, it was of interest to see whether any alterations in membrane characteristics had occurred following imipramine treatment. This was ascertained in terms of temperature-dependent changes in succinoxidase activity in mitochondria (Potter, 1959; Katyare *et al.*, 1971). The results are shown in Figure 1. It is clear that the profile of the enzyme activity is different for the two-week imipramine group (Figure 1d). In particular, the optimum temperature had increased by approximately 3°C compared to control. Also the activity of the enzyme at the optimum temperature (40°C) had increased. When the data were analyzed in terms of the Arrhenius equation by plotting log of specific activity against reciprocal of absolute temperature (Figure 2) biphasic plots were obtained for control and one-week imipramine-treated animals with energies of activation of 19.68 kJ mol^{-1} and 32.79 to 36.07 kJ mol^{-1} for the two portions corresponding to higher and lower reaction temperature ranges. The phase transition points were at 24°C and 19°C respectively for controls and one week imipramine-treated groups. On the other hand, the plot for the two-week imipramine-treated animals was a monophasic straight line with an energy of activation of 22.96 kJ mol^{-1} and no phase transition point was noticeable.

Table 5 Effect of prolonged *in vivo* treatment with imipramine on mitochondrial protein content in liver

Animal group	*Liver protein content* (mg g^{-1} tissue)	*Succinate dehydrogenase activity in liver* (μmol substrate transformed mg^{-1} protein min^{-1})	(μmol substrate transformed g^{-1} tissue min^{-1})	*Succinate dehydrogenase activity in mitochondria* (μmol substrate transformed mg^{-1} protein min^{-1})	*Mitochondrial protein content* (mg g^{-1} tissue)
N_1	160.7 ± 3.4	33.4 ± 4.2	5399 ± 640.0	145.7 ± 9.0	38.5 ± 2.6
N_2	161.2 ± 3.9	35.7 ± 1.4	5684 ± 326.2	150.5 ± 13.3	36.3 ± 2.3
I_1	159.4 ± 3.2 NS	36.4 ± 2.9 NS	5803 ± 403.2 NS	154.8 ± 9.5 NS	36.5 ± 4.4 NS
I_2	156.8 ± 4.1 NS	38.2 ± 1.6 NS	5896 ± 288.8 NS	165.7 ± 12.9 NS	37.0 ± 2.2 NS

Mitochondrial protein content in the tissue was determined on the basis of succinate dehydrogenase activities of whole liver homogenates and three-times washed mitochondria as described in the Methods section (Rajwade *et al.*, 1975). Values are given as mean ± s.e.mean of 12 independent observations.
NS not significant.

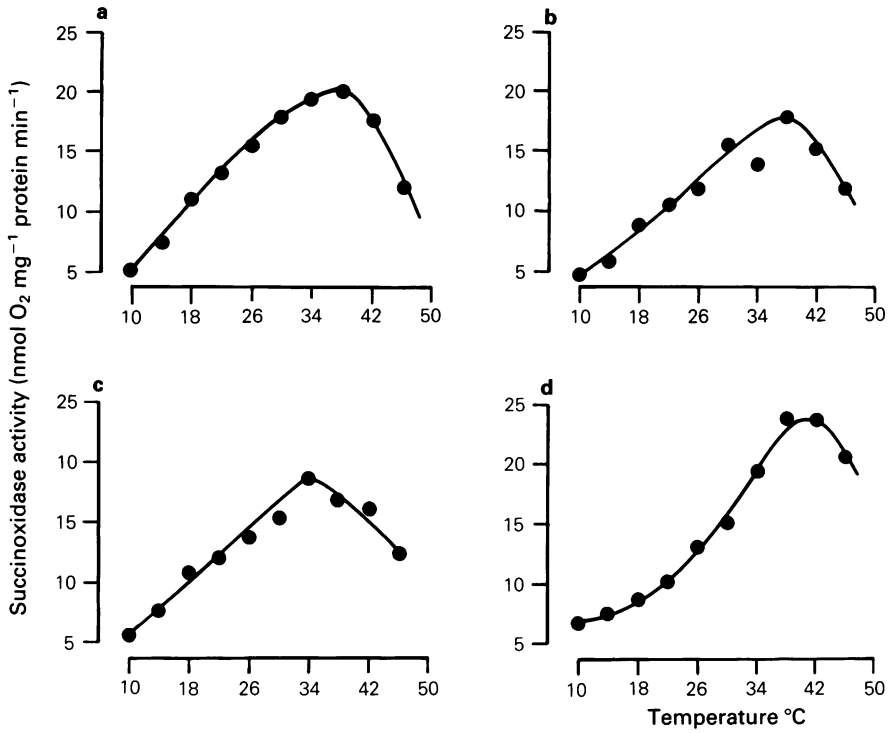

Figure 1 Temperature-dependent changes in succinoxidase activity in rat liver mitochondria: (a) 1 week-saline; (b) 2 weeks-saline; (c) 1 week-imipramine and (d) 2 weeks-imipramine. Experimental details are as described in Methods section. Each point represents mean of 8–14 independent observations. The value of s.e.mean did not exceed 10% of the mean.

Discussion

The results of the present study have clearly shown that in liver mitochondria, prolonged *in vivo* imipramine treatment resulted in a significant and sustained stimulation of respiratory activity with all substrates examined (Tables 2 and 3). The increased respiratory activity was accompanied by increased cytochromes aa_3 and $c + c_1$ content. Interestingly, the content of cytochrome aa_3 increased steadily up to the second week of treatment whereas that of cytochrome $c + c_1$ had already reached a steady state level by the end of the first week (Table 4). The correlation between the increased respiratory rates and the contents of cytochrome aa_3 and $c + c_1$ is thus self-evident. The content of cytochrome b, however, did not seem to be influenced by imipramine treatment nor was the level of succinate dehydrogenase altered under these conditions (Tables 4 and 5). In this connection it is interesting to note that while cytochrome c and c_1 are exclusively cytoplasmic gene products, cytochrome oxidase (aa_3) contains polypeptides synthesized both on the cytoribosomes as well as mitoribosomes (Schatz & Mason, 1974; Tzagoloff, 1982). By contrast, cytochrome b seems to be coded by the mitochondrial gene (Schatz & Mason, 1974; Tzagoloff, 1982). Succinate dehydrogenase, on the other hand, is synthesized on the cytoribosomes (Brown & Beattie, 1978). Thus imipramine would apparently seem to act by selectively enhancing synthesis only of some of the respiratory chain components of mitochondria which are of cytoplasmic origin (Schatz & Mason, 1974; Tzagoloff, 1982). Imipramine can, therefore, possibly be used as a tool to illustrate nuclear-mitochondrial interrelationships in mitochondrial biogenesis (Schatz & Mason, 1974; Tzagoloff, 1982).

The results of the present studies taken together with those on brain mitochondria referred to earlier (Rajan & Katyare, unpublished observations), would thus suggest that imipramine may have a generalised respiration-stimulating effect on mitochondria. However, it is likely that in the brain it may have a more beneficial effect by making more energy avail-

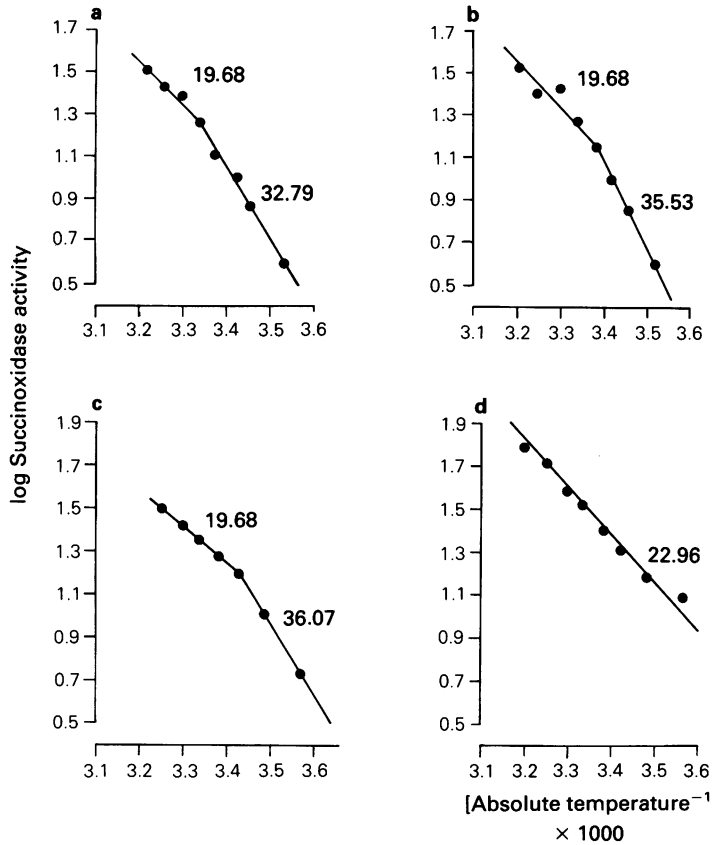

Figure 2 Arrhenius plots for temperature-dependent succinoxidase activity in liver mitochondria: (a) 1 week-saline; (b) 2 weeks-saline; (c) 1 week-imipramine and (d) 2 weeks-imipramine. The experimental details are as given in the text. Each point represents the mean of 8–14 independent measurements. The s.e.mean did not exceed 10% of the mean. Other details are as described for Figure 1.

able to overcome the depressed state (Rajan & Katyare, unpublished observations).

The observed changes relating to increased respiration rates, however, were not reflected in the total mitochondrial protein content or their yield (Table 5). It would therefore seem that imipramine-treatment caused a selective increase in the specific respiratory chain components without any change in the mitochondrial mass (Table 5). The observed changes therefore are more qualitative than quantitative in nature. The tissue and body weights of the animals also decreased after imipramine treatment (Table 1). Reasons for this are not clear at this stage but may perhaps relate to toxic effects of this drug. Although there was no apparent change in succinate dehydrogenase activity (Table 5), imipramine treatment did bring about significant changes in the lipid milieu and fluidity of the mitochondrial membrane as seen in terms of Arrhenius kinetics of succinoxidase activity (Figures 1 and 2). Thus the phase transition temperature in the one-week imipramine-treated group decreased, indicating increased levels of unsaturated fatty acids (Raison *et al.*, 1971; Raison, 1972; Watson *et al.*, 1975) in the two-week imipramine-treated group, the phase transition point was not discernible.

Changes in lipid metabolism viz. increased lipid content (Albouz *et al.*, 1982), enhanced [^{14}C]-glycerol incorporation (Fauster *et al.*, 1983) and inhibition of phospholipid degradation in rat C_6 glioma cells and fibroblasts by imipramine and desmethylimipramine have been reported (Fauster *et al.*, 1983). Since several mitochondrial enzymes, including succinate dehydrogenase (Grover *et al.*, 1975; Vik & Capaldi, 1977; Ashraf *et al.*, 1980; Fry & Green, 1980; Abuirmeileh & Elson, 1980) have a known

requirement for specific phospholipids, it would be of interest to examine further the effects of imipramine on membrane lipid metabolism and membrane function.

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