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DIFFERENTIAL SCANNING CALORIMETRY AND ENZYMIC ACTIVITY OF RAT LIVER MICROSOMES IN THE PRESENCE AND ABSENCE OF Δ^1-TETRAHYDROCANNABINOL

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Summary

The thermal transitions of rat liver microsomes and isolated lipids were investigated by using differential scanning calorimetry. Endothermic transitions at ≈—5°C and between ≈18° and 40°C were detected in the membranes and at ≈—10°C and between ≈10° and 20°C in the extracted lipids.

Interaction with Δ^1-tetrahydrocannabinol of microsomal membranes and of extracted lipids influences the thermotrophic behaviour as revealed by differential scanning calorimetry and eliminates the break in the Arrhenius plot of the enzymic activity of *O*-demethylase.

Introduction

Recently evidence was presented that Arrhenius plots of liver-microsomal enzyme activities show breaks in the curves. Different techniques were employed in these studies: spin labels as enzyme substrates [1] gave a break around 32°C, enzymic activities as measured by conventional means displayed discontinuities around 20° and 32°C [2—4] ESR [3] and NMR [5] spectroscopy revealed a phase transition around 20°C and 36°C. It was assumed that phospholipid phase transition from gel to liquid crystalline state is responsible for the above mentioned phenomena, but no direct proof was presented. In 1972 Blazyk and Steim [6] published a preliminary study of microsomal membranes using differential scanning calorimetry, showing only one lipid phase transition centered around 0°C. By employing a very sensitive differential scanning calorimeter we were able to show that microsomal membranes have also a second phase transition in the region ≈18°—40°C. Recently we have reported [7] that cannabinoids influence the thermotrophic behaviour of dipalmitoyl phosphatidylcholine.

The hepatic endoplasmic reticulum contains drug metabolizing enzymes [8].

It was also shown that liver concentrates hashish components in vivo [9] and the spectra of the microsomes are influenced by interaction with different cannabinoids [8,10]. It was therefore of interest to study the effect of Δ^1-tetrahydrocannabinol on the phase transition of microsomes and microsomal lipids.

We now present evidence that Δ^1-tetrahydrocannabinol affects the thermotrophic behaviour of liver microsomes and microsomal lipids, as revealed by differential scanning calorimetry and enzymic activity measurements.

Materials and Methods

Δ^1-Tetrahydrocannabinol was obtained from Makor Chemicals, Jerusalem, Israel, stock solution of the drug (0.1 M and 1 M in spectroscopic grade ethanol) were kept at −20° C. Sucrose was a BDH analytical reagent (BDH Chemicals, Poole, England). Nicotinamide adenine dinucleotide phosphate, reduced form type I (NADPH) and Trizma Base were purchased from Sigma, St. Louis, Mo. USA. *p*-Nitroanisol was an Eastman Kodak product, chloroform and methanol of analytical grade were freshly distilled. Male rats (CR type) were starved overnight. The livers were removed, homogenized in 0.25 M sucrose buffered with 0.05 M Tris · HCl pH 7.5. The homogenate was centrifuged twice at 9000 × *g* (20 min), once at 16 000 × *g* (20 min) and finally the microsomal pellet was obtained by centrifugation at 100 000 × *g* for 1 h [11]. The microsomes were stored at −20° C. The pellet contained about 70% water as found after freeze drying for 3 h.

The enzymic activity was determined essentially according to Duppel and Ullrich [2] using *p*-nitroanisol as a substrate. In brief the microsomal suspensions 0.5 ml (obtained by resuspension of the pellets in 0.1 M Tris · HCl, pH 7.5 at concentrations of about 2 mg membrane protein/ml) were incubated with the substrate at the desired temperature for 20 min, the reaction was stopped by addition of 0.1 ml Tris 1 M pH 9.5, centrifuged at 15 000 rev./min (20 min Sorvall Centrifuge) and the absorbance was read at λ = 405 nm in a Zeiss spectrophotometer at room temperature against the blanks without NADPH.

In the experiments with Δ^1-tetrahydrocannabinol 5 μl of alcoholic solution of Δ^1-tetrahydrocannabinol (5 μmol or 0.5 μmol) were added to ≈450 mg microsomes (in 20 ml buffer). The microsomes were incubated for 1 h at 37° C prior to the enzymic assay. Control experiments were performed by incubating the microsomes with similar amounts of ethyl alcohol.

The calorimetric measurements were performed on a Du Pont 990 differential scanning calorimeter with a cell base II. The calibrated model was used, sensitivity of 0.02 mcal/s per inch and heating rates of 2 or 5 deg/min. Hermetically sealed aluminium pans were used. The microsomal membranes were either transferred directly into the pans or after treatment with ethylene glycol or Δ^1-tetrahydrocannabinol. The microsomal pellets were resuspended in 0.1 M or 0.05 M Tris · HCl buffer pH 7.4, Δ^1-tetrahydrocannabinol, alcohol or ethylene glycol: H_2O (40 : 60 v/v) were added, as specified. The suspensions were incubated at 37° C for 1 h and the pellet recovered by centrifugation at 100 000 × *g* for 30 min. The microsomal lipids were extracted according to Folch et al. [12]. To approx. 10 mg lipids dissolved in 0.2 ml chloroform/methanol (2 : 1

v/v) 10 µl of alcoholic solution of Δ^1-tetrahydrocannabinol were added. The samples were left for 1 h at room temperature, subsequently the solvents were evaporated by a stream of N_2 and high vacuum (2 h). The lipids were transferred into the pans, appropriate amounts of buffer added and the pans were sealed. They were left for equilibration at room temperature for at least 12 h.

Pure lipids or the controls in the presence of alcohol underwent similar treatment.

Results and Discussion

Fig. 1 represents differential scanning calorimetry thermograms of rat liver microsomes, microsomal lipids and microsomal lipids interacting with Δ^1-tetrahydrocannabinol. (The structure of Δ^1-tetrahydrocannabinol is given in Fig. 4.) As shown in Fig. 1A the microsomal membranes undergo a melting in the range of ≈18°—40° C, with enthalpy of melting ≈0.21 mcal/mg dry membranes. Fig. 1A represents the thermogram of a first scan of the membranes (heating from 5°—80° C) during which the proteins are denatured. The denaturation process of the membranes proteins appears as a peak centered at 65° C. Subsequent cycles of cooling and heating do not show the protein peak denaturation, while the peak in the range 18°—40° C and its enthalpy of melting are unchanged. In Fig. 2B, when the membranes were dispersed in (40 : 60, v/v) ethylene glycol/H_2O and the heating started at —20° C, an additional endothermic transition with a maximum at ≈—5° C was observed. This transition was

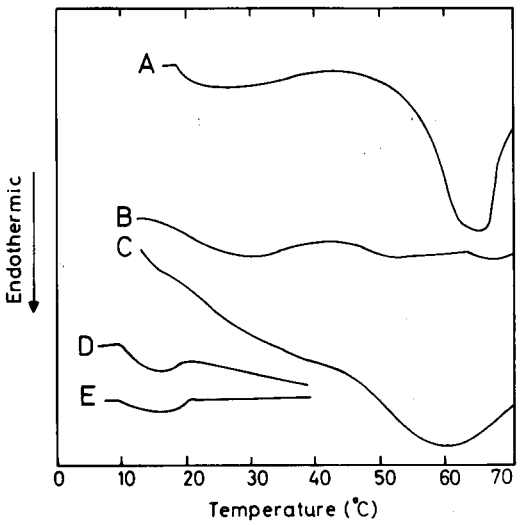


Fig. 1. The differential scanning calorimetry thermograms of microsomal membranes, extracted lipids and of microsomal membranes and extracted lipids after interaction with Δ^1-tetrahydrocannabinol (Δ^1-THC) dispersed in water. A, 20.7 mg wet microsomes, heating rate 5° C min, sensitivity 0.02 mcal/s per inch, first scan. B, second scan of microsomes presented in A, heating rate and sensitivity as above. C, 26.8 mg wet microsomes after interaction with Δ^1-tetrahydrocannabinol, heating rate 5° C min, sensitivity 0..02 mcal/s per inch; first scan. D, 6.2 mg lipids + 2.3 µl Tris · HCl 0.05 M heating rate 2° C min, sensitivity 0.02 mcal/s per inch. E, 11 mg lipids were mixed with 0.1 µmol Δ^1-tetrahydrocannabinol, 6.7 mg of the lipids + 3.6 µl Tris · HCl 0.05 M were inserted into the pan, heating rate 2°C/min sensitivity 0.02 mcal/s per inch. Experiments were performed at least in triplicate employing different microsomal preparations.

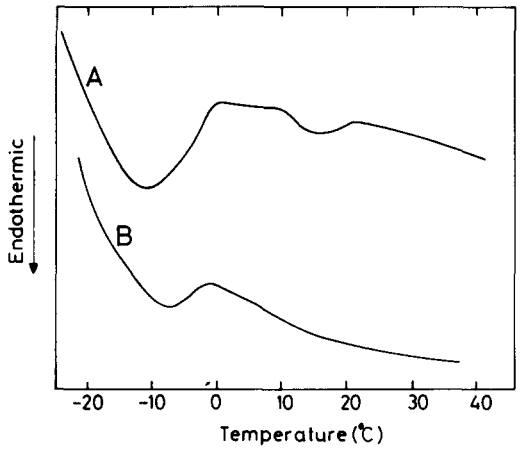


Fig. 2. The differential scanning calorimetry thermograms of microsomal membranes and extracted lipids dispersed in water/ethylene glycol (40 : 60 v/v). A, 6.3 mg lipids + 2.5 µl Tris · HCl 0.05 M/ethylene glycol (40 : 60, v/v), heating rate 2°C/min, sensitivity 0.02 mcal/s per inch, second scan. B, 27.3 mg wet microsomes, heating rate 2°C/min, sensitivity 0.02 mcal/s per inch, first scan.

found previously by Blazyk and Steim [6].

When the membranes were incubated with Δ^1-tetrahydrocannabinol (0.7 or 0.07 µmol Δ^1-tetrahydrocannabinol per ≈80 mg microsomes) the peak between 18° and 40°C markedly diminished (Fig. 1C). The residual peak was about 25% of that of the untreated microsomes. The low temperature transition was unchanged.

Fig. 1D shows the thermogram of the microsomal lipids. During the first scan, the equilibration with water continues. After that the enthalpy of melting did not change any further with subsequent heating and cooling, therefore the second scan is presented. The region of transition of lipids moved to lower temperatures (≈10°—20°C) and the enthalpy of melting decreased (≈0.24 mcal/mg lipid) as compared to the enthalpy of the whole microsomes. When the heating of the lipids was started at —20°C the peak centered around —10°C was also seen (Fig. 2A).

A difference in the melting range and the enthalpy of melting of the lipids and the whole microsomes (assuming 40% lipids in the microsomal membranes) points towards the importance of lipid : protein interactions on the thermotrophic behaviour, suggesting that the lipids associated with the proteins are more difficult to melt.

The lipid protein interactions were studied extensively on model biomembranes by applying differential scanning calorimetry [13—15]. Different proteins, peptides and lipids were employed in these studies.

The interacting proteins can be grouped into three classes, according to their influence on the thermotrophic behaviour of the lipids: (1) those that cause a decrease in ΔH and T_c of the lipid (cytochrome *c*); (2) those that do not influence T_c but decrease the ΔH (proteolipid apoprotein, gramicidin A, copolymer lysine-phenylalanine); (3) those that cause an increase in ΔH with or without increase in T_c (ribonuclease, polylysine).

The thermotrophic behaviour of the microsomal membranes, as compared to the microsomal lipids, agrees qualitatively with the behaviour of type three model membranes, but probably the kind of interactions are different in both cases.

In the model membranes, the interaction is mainly an electrostatic one with surface adsorption, whereas in the microsomal membranes other specific interactions are involved resulting in immobilization of lipid hydrocarbon chains. This notion agrees with a model proposed by Stier and Sackmann [1] in which they suggest that the microsomal enzymes are embedded within a mosaic-like structure of the membrane, the enzymes being enclosed by a rather rigid phospholipid halo.

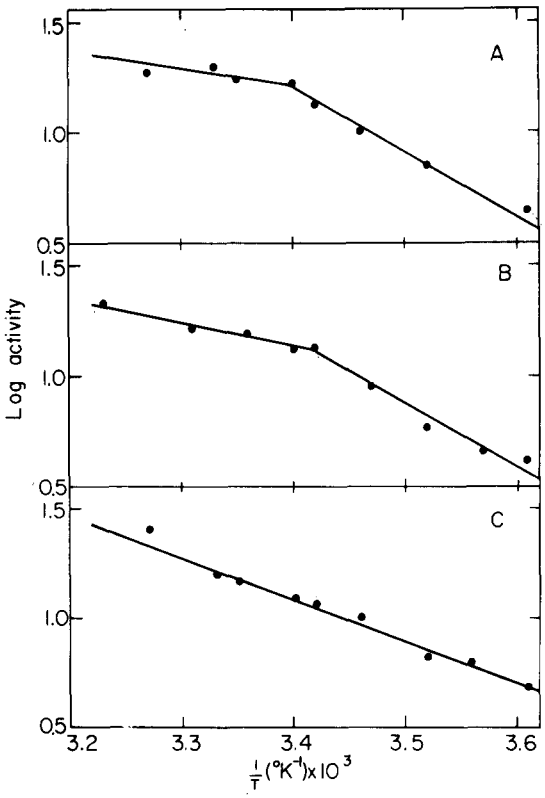
Transitions centered at 15° and 35°C were also detected by Martonosi [16] in sarcoplasmic reticulum membranes, but only when limited amounts of water were present. She found that the enthalpy in the second scan (after denaturation of proteins) and of the extracted lipids was higher than that of the intact membranes (first scan).

When our work was in progress an abstract by Narasimhulu [17] appeared where he reports that bovine adrenocortical microsomes studies by fluorescence polarization display two phase transistions at ≈21° and ≈32°C, but the extracted lipids have only the lower transition. These values are within the range of the phase transitions observed by us (Fig. 1).

Fig. 1E represents the differential scanning calorimetry thermogram of the microsomal lipids interacting with Δ^1-tetrahydrocannabinol. At low ratio of Δ^1-tetrahydrocannabinol to lipid (approx. 1 : 100 mol : mol) the melting range did not differ from that of the pure lipid, but a small decrease in the enthalpy of melting was observed. The decrease amounted to about 30%, reducing the enthalpy of melting to ≈0.16 mcal/mg lipids. The same effect was observed at a higher ratio of cannabinoids to lipids (about 1 : 10 mol : mol). The fact that a 10-fold increase in the cannabinoid to lipid ratio did not have any additional effect on the thermotrophic behaviour of the lipid may be due to the limited solubility of Δ^1-tetracannabinol in the lipid phase [7].

Fig. 3 shows the Arrhenius plots of the dealkylation of *p*-nitroanisol by microsomal enzymes. These enzymes are membrane bound [2] and a simple enzymatic assay developed by Duppel and Ullrich [2] is available. Therefore, this assay was chosen for studying the influence of Δ^1-tetrahydrocannabinol on the Arrhenius plots of the enzymic activity of microsomal enzymes. Plots 3A and 3B (3B is a control in the presence of ethanol) show breaks at around 20°C consistent with the findings of Duppel and Ullrich [2]. The breaks occur at the temperature of the onset of the transition of microsomal membranes or the end of the transition of lipids as revealed by differential scanning calorimetry (Fig. 1).

De Kruyff et al. [18] reported also that the break in the Arrhenius plot of bacterial ATPase activity occurs at the temperature of the beginning of the phase transition of the membranes as detected by differential scanning calorimetry. Similar correspondence between breaks in Arrhenius plots and differential scanning calorimetry measurements of lipids was found by Kimelberg and Papahadjopoulos [19] in the reactivation of delipidated (Na^+ + K^+)-ATPase from rabbit kidney by different lipids.



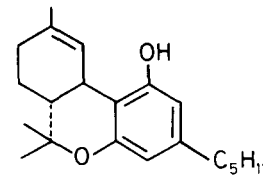


Fig. 3. Arrhenius plots of microsomal enzyme activity. The incubation mixture for the enzymic assay consisted of: ≈11 mg wet membranes in 0.5 ml 0.1 M Tris · HCl pH 7.5 + 0.5 ml *p*-nitroanisol 0.001 M in 0.1 M Tris · HCl pH 7.5 and 10 μl (0.025 M) NADPH in 0.1 M Tris · HCl. A, microsomes with no additives. B, microsomes to which 5 μl of ethyl alcohol per 20 ml suspension was added. C, microsomes incubated with 5 μl Δ^1-THC (0.1 M in ethyl alcohol per 20 ml membrane suspension. Results are expressed as log activity in *A* units X 100. Experiments were performed in triplicate employing different microsomal preparations.

Fig. 4. The structure of Δ^1-tetrahydrocannabinol.

Fig. 3C shows that incubation of microsomal membranes with $\approx 2.5 \cdot 10^{-5}$ M Δ^1-tetrahydrocannabinol (≈450 mg wet membranes incubated with 0.5 μmol Δ^1-tetrahydrocannabinol) abolishes the break in the Arrhenius plot. The data do not exclude the possibility that the break was shifted to a much lower temperature, where the absolute values of optical density are very low and small changes could not be detected. The experiments were repeated with $\approx 2.5 \cdot 10^{-4}$ M Δ^1-tetrahydrocannabinol giving the same results as above. 5 μl alcohol used as a control had no effect on the break as seen from Fig. 3B. The summary of the differential scanning calorimetry and enzymic activity measurements is presented in Table I. The enzymic activity is very sensitive to a low concentration of Δ^1-tetrahydrocannabinol ($2.5 \cdot 10^{-5}$ M), a concentration at which the drug has initially no immediate lytic effect on lysosomes [20] or macrophages [21] and has a protective effect on red blood cells against hypotonic hemolysis [22].

Δ^1-Tetrahydrocannabinol abolished the break in Arrhenius plot and at the

TABLE I

SUMMARY OF THE DIFFERENTIAL SCANNING CALORIMETRY AND ENZYMATIC ACTIVITY DATA

	Transition midpoint temperature T_m (°C)	Width of transition (degrees)	Enthalpy of melting (mcal/mg)	Discontinuity temperature in Arrhenius Plot T_k (°C)	Activation energy (kcal/mol)	
					Below T_k	Above T_k
Microsomes	≈28	≈22	≈0.21	≈21	13.7	5.9
Microsomes + Δ^1-THC	≈26	≈20	≈0.05	—	8.6	—
Extracted lipids	≈16	≈11	≈0.24			
Extracted lipids + Δ^1-THC	≈16	≈11	≈0.16			

Δ^1-THC, Δ^1-tetrahydrocannabinol.

same time it did not influence the melting temperature of the microsomes or microsomal lipids but caused a big decrease in enthalpy of melting, especially of the membranes. This discrepancy can be explained as follows. A possibility exists that the range of melting of part of the lipids is spread over even wider range, and any lower temperature transitions could be too small to be detected by differential scanning calorimetry. On the other hand, Δ^1-tetrahydrocannabinol may have the same effect on these membranes as cholesterol has. The influence of Δ^1-tetrahydrocannabinol on the thermograms of microsomes and extracted lipids is similar to the influence of cholesterol on the thermograms of synthetic lipids. Also it was reported [2] that cholesterol removes the discontinuity in the Arrhenius plot of the microsomal enzymes. Moreover, the effect of Δ^1-tetrahydrocannabinol on the enzymic activity is probably not specific for the drug and is due to a perturbation in the lipid microenvironment of the enzyme.

It was claimed that drug-induced disorganization of the lipid environment of ($Na^+ + K^+$) ATPase [23,24] and of NADH oxidase from rat brain and heart mitochondria [25] has inhibitory effects on the corresponding enzymic activities.

Our observations on remarkable shift in the melting temperature of dipalmitoyl phosphatidylcholine by Δ^1-tetrahydrocannabinol (≈15°C) [7] strongly support the notion that the non-specific effect of Δ^1-tetrahydrocannabinol on enzymic activity is due to a fluidization in the lipid phase. Lawrence and Gill [26], by employing ESR, found also that Δ^1-tetrahydrocannabinol fluidized phosphatidylcholine/cholesterol liposomes.

It is pertinent to note that high concentrations of glycerol (30%) [2] or lipophylic substrates [2,4] result in a similar effect on the Arrhenius plot of microsomal enzyme activity as Δ^1-tetrahydrocannabinol.

High concentrations of butanol cause a removal of the break in the enzymic activity; temperature curves of mitochondrial ATPase, and diethyl ether shifts it to lower temperatures [27]. To our knowledge the effect of other drugs on the Arrhenius plots of membrane enzymes was not reported. However, several reports have appeared dealing with the effect of various drugs and different

organic materials on the transition temperature and enthalpy of transition of phospholipids [28—31].

Cater et al. [28] found that some derivatives of morphine and tricyclic antidepressants shifted the transition temperature of the phospholipids to lower values without appreciable change in the enthalpy of transition. Similar results were detected by Jain et al. [29] and Papahadjopoulos et al. [30] on the interaction of dipalmitoyl phosphatidylcholine with a wide range of drugs. Recently Eliasz et al. [31] investigated the influence of a series of alcohols, acids and quaternary ammonium salts on the thermotrophic behaviour of dipalmitoyl phosphatidylcholine. Various effects on the thermotrophic behaviour were detected and were correlated with the structure of the compounds.

The finding that some of the lipids of endoplasmic reticulum are in crystalline state around and above room temperature and can be fluidized by low concentrations of drugs may be of high significance since this suggests that different compounds and ions [32] can influence the native environment of the proteins modulating or controlling their activity.

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