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BIPHASIC CHANGES IN RAT BRAIN MITOCHONDRIAL MEMBRANE STRUCTURE AND ENZYME ACTIVITY AFTER ACUTE OPIATE ADMINISTRATION TO RATS

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Abstract—Results from experiments performed previously in our laboratory have shown that, subsequent to the administration of 25 mg of morphine/kg of rat, the phase transition of brain mitochondrial membrane lipids is altered. In the present work, Arrhenius plots were generated from the activity of three brain mitochondrial inner membrane-bound enzyme systems, in order to determine what changes in activity and activation energy (*Ea*) occurred after administration of a similar acute dose of morphine. Similar studies were carried out with mitochondrial matrix enzymes. Brain mitochondria were selected because the low membrane cholesterol content enables demonstration of an endothermic phase transition in the membrane lipids. Only the membrane-bound enzymes from mitochondria harvested 0.5 hr after morphine treatment *in vivo* had decreased activities. Simultaneously, the temperature of discontinuity of Arrhenius plots of state 4 succinoxidase and cytochrome oxidase was abolished, as was the higher of the two discontinuities in state 3 succinoxidase; that of Mg^{2+} ATPase was decreased. When Arrhenius plots were generated with mitochondria harvested 48 hr after morphine administration, there was an increase in the activity of the succinoxidase and cytochrome oxidase systems, and their respective normal temperatures of discontinuity were abolished. With Mg^{2+}-stimulated ATPase, there was (1) an increase in the temperature of discontinuity and (2) the slopes of the activity curves differed markedly from the controls. Such effects were not obtained with the matrix enzymes 0.5 hr post opiate. When brain mitochondrial membranes harvested from rats 0.5 hr after opiate administration were incubated with 1-anilino-8-naphthalene sulfonic acid (ANS), there was a 15 per cent decrease ($P < 0.05$) in the fluorescence produced, compared with controls. This effect was abolished on treating the animals with either of the narcotic antagonists, oxilorphan (BC 2605A) or naloxone, 15 min after opiate administration. Brain mitochondria harvested 72 hr after acute morphine had a 22 per cent increase in fluorescnece on incubation with ANS ($P < 0.005$). These biphasic changes in both the activity and *Ea* of the membrane-bound enzymes, as well as the observed alteration in ANS fluorescence, were probably brought about by some type of morphine-induced change in the inner membrane of the mitochondria; they were entirely dependent on the time of sampling after opiate treatment. The probable role of concomitant changes in membrane lipid fluidity in these biphasic effects is advanced.

Previous work in our laboratory has shown that, subsequent to *in vivo* administration of an acute dose of 25 mg morphine/kg of rat, the melting profile of brain mitochondrial lipids, as assessed with the differential scanning calorimeter (DSC), is altered. This effect on the DSC scan was dependent on the dose of morphine, it was reversible by naloxone or oxilorphan administration to the animal 15 min after the opiate, and there was evidence of a stereoselective requirement. Further investigation has shown that this effect can be reproduced on interaction of an ether-precipitable complex from such morphine-treated animals with membrane lipids [1]. Loh *et al.* [2] and Lowney *et al.* [3] have suggested that such ether-precipitable material from lipids isolated from crude brain mitochondria might be identified with the opiate receptor. Because this phase transition is completed before the physiological temperature range is attained, the effects of morphine at such temperatures cannot be determined by the aforementioned method. Since there is considerable evidence in the literature that the activity of many mitochondrial membrane-bound enzymes is influenced by the state of the membrane lipids [4–6], it was decided to use these enzymes to determine whether or not a morphine-induced change in the lipid state could alter any of their functions at the physiological temperature.

Several authors [7, 8] have shown that Arrhenius plots of membrane-bound enzymes may be used to assess alterations in their structure through changes in the temperatures of discontinuities. Such discontinuities for enzymatic reactions occur at specific temperatures for a particular membrane when the lipids undergo a phase transition. Alterations in kinetic and regulatory properties of such enzymes may also be a reflection of such changes in membrane structure.

Vessey and Zakim [9] have suggested that changes in the lipid state resulting from a phase transition may also induce changes in the lipid bilayer structure. Because Arrhenius plots measure only a specific site (the enzyme environment), it was decided to use the non-specific fluorescence probe 1-anilino-8-naphthalene sulfonic acid (ANS) to determine if any general alteration in the membrane occurred after morphine treatment [10, 11]. Recently, it has been observed in our laboratory that the effect of the *in vivo* administration of opiates on the lipid phase transition

becomes biphasic in time (E. A. Hoscin and M. Lapalme, unpublished results). Accordingly, in the present paper, it was decided to study the effect of acute opiate administration *in vivo* on mitochondrial structure and function 48 hr after an acute injection of the drug.

It is generally accepted that the opiate receptor is a membrane-bound entity [12, 13] and that in brain about 50 per cent of it resides in the crude mitochondrial fraction [13]. Smith and Loh [13] have shown that the receptor density in the highly purified lysed synaptosomal SPM fraction (0.097 pmole/mg) was much greater than that in either the light membranes (0.049 pmole/mg) or mitochondria (0.023 pmole/mg). These authors affirmed that the opiate receptor was distributed diffusely over the entire surface of nerve cells and not restricted to the synaptic region. In the present work, the rat brain mitochondrial membrane has been selected over other membranes as a model system to study the possible nature of morphine effects on membrane lipid fluidity because of its low cholesterol content [14]. Ladbrooke *et al.* [15] have shown that a high cholesterol content in a membrane can, depending on local factors, abolish endothermic phase transitions. Mitochondria were also used because we wanted to correlate the effects found here with phenomena observed previously in the same model system [1]. It is not assumed that these effects occur on the primary target of opiate action, but clearly they indicate effects of morphine on a particular biological membrane which contains the opiate receptor.

METHODS

*Preparation of brain mitochondria from rats**

Sprague–Dawley rats weighing about 200 g were used exclusively in these experiments. The saline- or opiate-treated animals were decapitated and the excised brain was placed in 10 vol. of ice-cold medium containing 0.25 M sucrose, 5 mM ethylene diamine tetra-acetate (EDTA) and 5 mM Tris–hydrochloride buffer, pH 7.4 (SET). The subsequent steps were carried out at 0–4°. The brain was homogenized with eight up and down strokes in a Tri-R-glass–Teflon homogenizer. The homogenate was centrifuged for 10 min at 750 *g* in a Sorval RC2-B refrigerated centrifuge. Supernatant fraction (i) was centrifuged at 16,000 *g* for 5 min. The pellet formed was resuspended in 20 vol. of a medium containing 0.25 M sucrose, 5 mM Tris–HCl, pH 7.4 (ST), and centrifuged for 5 min at 750 *g*. Supernatant fraction (ii) was then centrifuged for 5 min at 16,000 *g*. The pellet was suspended in ST (generally 0.5 ml), layered on a discontinuous Ficoll gradient (7.5 per cent/16 per cent) and centrifuged for 60 min at 53,000 *g* in the SW 27.1 rotor of a Beckman model L5-50 ultracentrifuge. The mitochondrial pellet formed was washed twice in approximately 20 vol. ST. The washed pellet was resuspended in a small volume of ST and subsequently used in the following studies.

*See Ref. 16.

†Gift from Endo Drugs, N.Y.

‡Gift from Bristol Laboratories, Syracuse, N.Y.

Fluorescence with ANS

Aliquots (0.2 ml) of the final mitochondrial suspension in ST, containing about 5 mg protein, were added in duplicate to 4 ml of 0.1 M phosphate buffer (pH 7.4); this served as the blank. To 2 ml of this suspension, 0.1 ml ANS (25 μg ANS/ml of 0.1 ml PO_4 buffer, pH 7.4) was added and thoroughly mixed at 22 . Blanks and samples with ANS were read in a Turner spectrofluorometer 430 at excitation 385 nm and emission at 470 nm. Corrected fluorescence per mg of protein for each sample was calculated and the values obtained from opiate-treated rats were expressed as a percentage of the value obtained from saline-treated (control) rats. Fluorescence values were comparable only when the protein content of the experimentals and controls were within 10 per cent of each other.

ATPase assay

Incubations were carried out in duplicate using individual 3-ml incubation cups with circulating water jackets to maintain constant temperature. Each cup contained a small magnetic stirring bar to allow constant mixing of the reactants throughout the incubation period.

To each reaction vessel containing 0.7 ml of incubation medium (1.25 μmoles $MgCl_2$, 1.1 μmoles NaCl, 50 μmoles Tris–HCl, pH 8, and 0.6 μmole 2-mercaptoethanol), 0.2 ml of a lysed (osmotic shock) [17] mitochondrial preparation was added. The samples were preincubated for 2 min to attain temperature equilibrium. A 0.1-ml solution containing 1.25 μmoles ATP was added and after 10 min the reaction was terminated by the addition of 1 ml of ice-cold 10% trichloroacetic acid. Inorganic phosphate was determined by the method of Chen *et al.* [18].

Succinoxidase assay (crude mitochondrial fraction, without Ficoll separation)

Oxygen uptake was measured polarographically on a Fisher series 5000 recorder using standard Clark-type electrodes, assuming a constant pressure of 1 atm. The activity of the succinoxidase system (state 4) was determined in an incubation mixture of 1 ml containing 0.25 M sucrose, 0.01 M Tris, 0.01 M K_2HPO_4, 50 mM $MgCl_2$, 50 mM succinate and 0.5 mg/ml of bovine serum albumin, adjusted to pH 7.4.

State 3 oxygen uptake was determined in separate experiments with the addition of 0.25 ml ADP (1.63 mg/ml) to the state 4 system.

The ratios of oxygen uptake were calculated from activity slopes obtained in the initial 60 sec.

Cytochrome oxidase assay

Cytochrome oxidase was assayed by the method of Wharton and Tzagoloff [19].

Protein concentrations for all experiments were determined by the method of Lowry *et al.* [20].

Rats received an acute dose of 5, 10, 15 or 25 mg morphine/kg to produce analgesia. In some experiments, either 3 mg naloxone†/kg or oxilorphan (BC 2605 A)‡ was administered i.p. to antagonize the opiate action. Times and dosages of agonists, antagonists, and of brain mitochondrial harvest were variable and

dependent on the particular experiment described in the text. All the experiments were repeated at least five times and the mean with S. D. and levels of significance calculated.

RESULTS

ANS fluorescence

Brain mitochondria harvested from rats 0.5 hr after acute (25 mg/kg) morphine administration were incubated with the fluorescent probe ANS. As shown in Fig. 1, compared with the controls ($\bar{X}_{12}$; 85.6 ± 4, fluorescence units/mg of protein) there was a 15 per cent decrease in ANS fluorescence (eleven experiments; 85 per cent ± 2.3; $P < 0.005$) at that time of sampling. What is probably most significant is that it was not of a transient nature since such mitochondria stored at 4° had identical (72 units/mg of protein) fluorescence when tested daily over a period of 1 week. Mitochondria from naloxone (3 mg/kg)- or oxilorphan (3 mg/kg)-treated rats, killed 0.5 hr after opiate treatment, had ($\bar{X}_{11}$) 84.3 ± 2.7 and ($\bar{X}_{7}$) 88.7 ± 1.3 fluorescence units/mg of protein, respectively, indicative of complete reversal of a specific opiate-induced alteration in the membrane.

Sampling of brain mitochondria at different times subsequent to a single injection of the opiate indicated normal fluorescence values 2 hr post morphine administration. However, as shown in Fig. 1, there was a 16 per cent increase in ANS fluorescence (six experiments; 116 per cent ± 1.7, $P < 0.001$) at 3 hr which gradually diminished to normal values by 18 hr and persisted until 24 hr. Thereafter, there was a sustained increase in ANS fluorescence at 48 hr with a maximum (seven experiments; 122 per cent ± 1.1, $P < 0.005$) occurring at 72 hr. By 96 hr near normal values were obtained.

Arrhenius plots

Since ANS is a non-specific fluorescent probe which interacts with membrane-bound proteins and/or lipids, it seems reasonable to assume that one explanation for the observed biphasic change in fluorescence might be an induced perturbation of the membrane structure. In an attempt to study this possibility further, Arrhenius plots of brain mitochondrial inner membrane-bound enzymes from control and morphine-treated rats were generated by studying their activities over a range of temperatures.

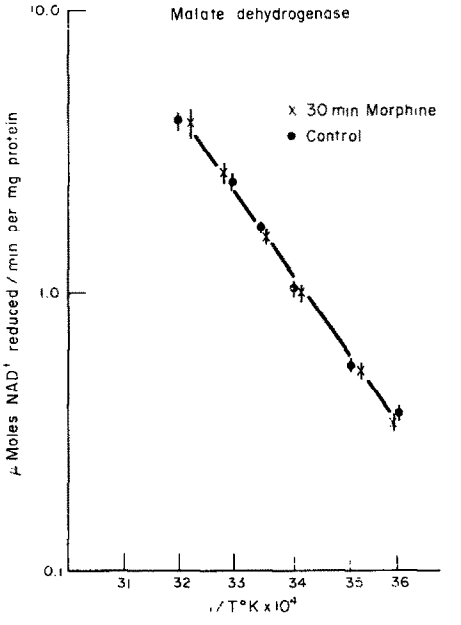

Fig. 2. Rats were injected i.p. with 25 mg morphine/kg. The brain mitochondria were harvested at 0.5 hr after drug administration and the malate dehydrogenase activity of control (●) and morphine (×) = treated brain mitochondria was determined.

Similar studies carried out with malate and glutamate dehydrogenases, and citrate synthetase, which are mitochondrial matrix enzymes, showed that morphine administration *in vivo* was without effect on the activities of these enzymes. The results with malate dehydrogenase are shown in Fig. 2.

To show that the morphine-induced effects on membrane-bound enzymes were not due to anoxia, rats were anesthetized with ether, the mitochondria harvested and Arrhenius plots of the succinoxidase system generated. As shown in Fig. 3, the curve obtained was not significantly different from that prepared from the control animals shown in Fig. 5.

Succinoxidase (intrinsic membrane-bound enzymes) (1) *State 4 respiration*: The state 4 succinoxidase

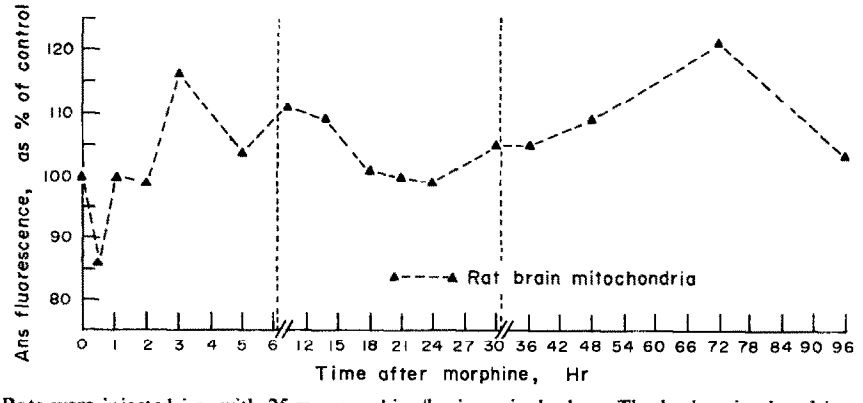

Fig. 1. Rats were injected i.p. with 25 mg morphine/kg in a single dose. The brain mitochondria were harvested at different times over the subsequent 4-day period and treated with the fluorescence probe ANS.

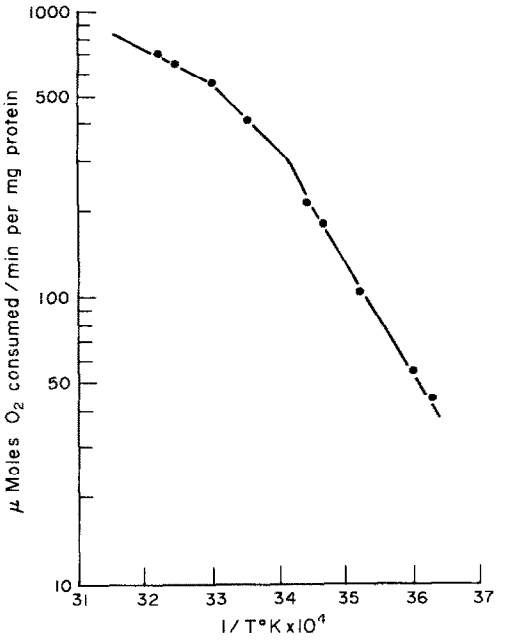

Fig. 3. Rats were anesthetized with ether for 5 min and the crude rat brain mitochondria were isolated. State 3 succinoxidase activity of control( )and anesthetized (●) animals was determined.

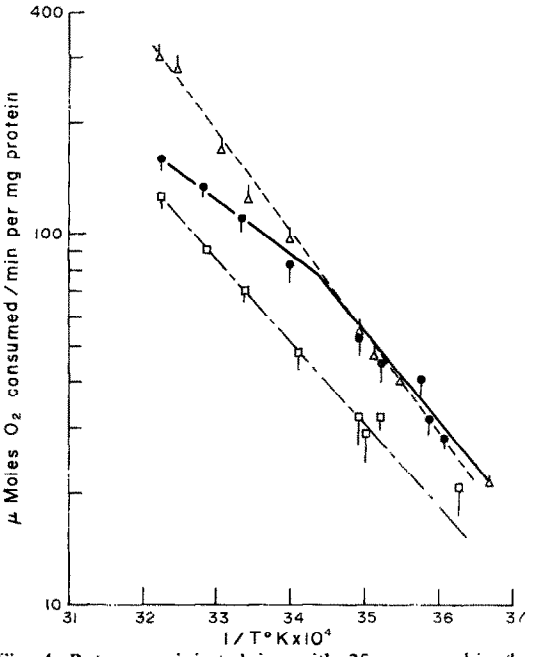

Fig. 4. Rats were injected i.p. with 25 mg morphine/kg. The brain mitochondria were harvested at 0.5 and 48 hr after morphine administration. State 4 respiration of the crude rat brain mitochondria from control (●), 0.5 hr (□) and 48 hr (△) was determined.

Table 1. Activation energy (*Ea*) calculated from Arrhenius plots of matrix and membrane-bound mitochondrial enzymes after morphine administration *in vivo*

Enzyme	Treatment	Temperature range	Arrhenius activation energy (*Ea*) (kcal/mole)
Intrinsic			
Succinoxidase state 3 (Fig. 5)	Control	Lower	17.8
		Middle	13
		Upper	5.6
	Morphine (0.5 hr)	Lower	14.5
		Upper	4.5
	Morphine (48 hr)	Entire	16.5
Succinoxidase state 4 (Fig. 4)	Control	Lower	11.4
		Upper	6.7
	Morphine (0.5 hr)	Entire	10
	Morphine (48 hr)	Entire	11.9
Cytochrome oxidase (Fig. 7)	Control	Lower	3
		Upper	9
	Morphine (0.5 hr)	Entire	8
	Morphine (48 hr)	Entire	9
Extrinsic			
Mg^{2+}-stimulated ATPase (Fig. 8)	Control	Lower	21
		Upper	5.4
	Morphine (0.5 hr)	Lower	15.4
		Upper	5.4
	Morphine (48 hr)	Lower	11
		Upper	3.4
Soluble (Fig. 2)			
Malate dehydrogenase	Control		13.2
	Morphine (0.5 hr)		13.2
Citrate synthetase	Control		6.4
	Morphine (0.5 hr)		6.4
Glutamate dehydrogenase	Control		4.6
	Morphine (0.5 hr)		4.6

activity of the brain mitochondria from the control rats in Fig. 4 shows a discontinuity temperature at 17°, in agreement with Kemp *et al.* [21]. The pH of this system fell from 8.27 at 16° to 8.17 at 22°. Brain mitochondria from the rats killed 0.5 hr after morphine treatment showed abolition of that temperature of discontinuity and decreased succinoxidase activity. The state 4 succinoxidase activity of brain mitochondria from rats treated first with opiate, then 15 min later with oxilorphan, and then killed 0.5 hr after opiate injection was normal. The succinoxidase activity of brain mitochondria harvested from rats 48 hr after opiate administration was higher than normal in the physiological range of temperature and there was no indication of a discontinuity at 17°. Values of activation energy (*Ea*) for controls and opiate-treated animals are given in Table 1.

The respiratory control ratio assayed at 37° for control mitochondria was 4.24, while that from morphine-treated animals killed 0.5 hr post opiate was 2.6, indicative of uncoupling of oxidative phosphorylation.

(2) *State 3 respiration*: Mitochondria harvested from rats killed 0.5 hr after acute (25 mg/kg) morphine treatment had decreased succinoxidase state 3 activity and a discontinuity at 24°, as shown in Fig. 5. This value is intermediate between the transition temperatures of 17 and 29° obtained for succinoxidase activity with control brain mitochondria. Similar discontinuities have been found with liver mitochondria using this system [21, 22]. Values for *Ea* of the different slopes of these curves are shown in Table 1. When rats were given naloxone 15 min after morphine and killed 15 min later, the brain mitochondria harvested showed a complete reversal of the opiate effect. As shown in Fig. 6, the values obtained were identical to those of the controls over the entire temperature range.

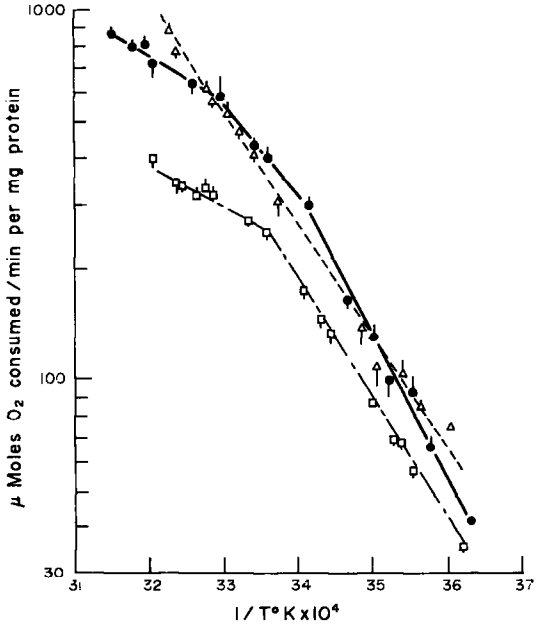

Fig. 5. Rats were injected i.p. with 25 mg morphine/kg. The brain mitochondria were harvested at 0.5 and 48 hr after morphine administration. State 3 respiration of the crude rat brain mitochondria from control (●), 0.5 hr (□) and 48 hr (△) was determined.

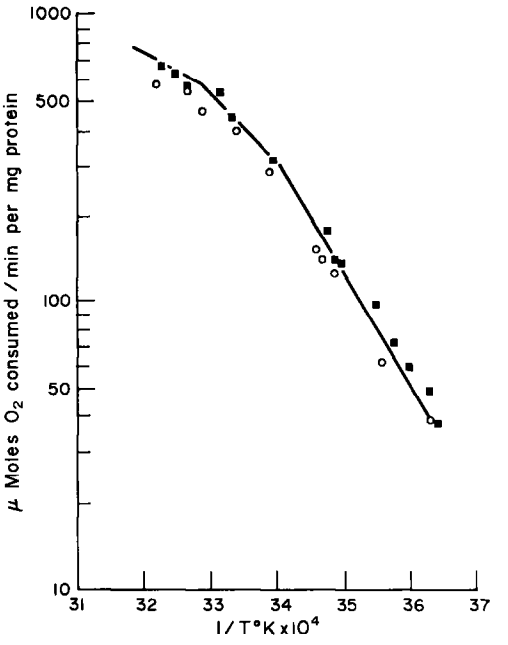

Fig. 6. Rats were injected i.p. with 25 mg morphine/kg. Fifteen min later the animals were treated with 3 mg/kg of naloxone and the animals were killed 15 min later. State 3 respiration in mitochondria harvested from control (○) and 0.5 hr after opiate and antagonist (■) was determined.

When rats were given an acute dose of morphine and killed 48 hr later, there were no discontinuities, but a linear plot with *Ea* and activity similar to the controls before the first transition temperature was obtained, as shown in Fig. 5. However, above the second transition temperature, there was a linear increase in activity. Rats given a similar dose of morphine followed by naloxone 15 min later and killed 48 hr after opiate administration showed normal succinoxidase activity over the entire range of temperatures, as shown in Fig. 6.

(3) *Cytochrome oxidase*: The cytochrome oxidase of brain mitochondria harvested from normal animals shown in Fig. 7 had a transition temperature at 17°. When rats were treated with morphine and the brain mitochondria were harvested 0.5 hr later, Arrhenius plots showed a decrease in enzyme activity with disappearance of the normal discontinuity temperature. Brain mitochondria harvested 48 hr after morphine administration showed an increase in the cytochrome oxidase activity in the physiological range with abolition of the normal phase transition temperature. The *Ea* values are shown in Table 1.

In all of these studies with the Arrhenius plots, it was observed that the activity of inner membrane-bound enzymes from mitochondria harvested 0.5 hr post opiate was decreased. When similar studies were performed with intrinsic membrane-bound enzymes harvested from animals killed 48 hr post opiate, there was a significant increase in enzymatic activity

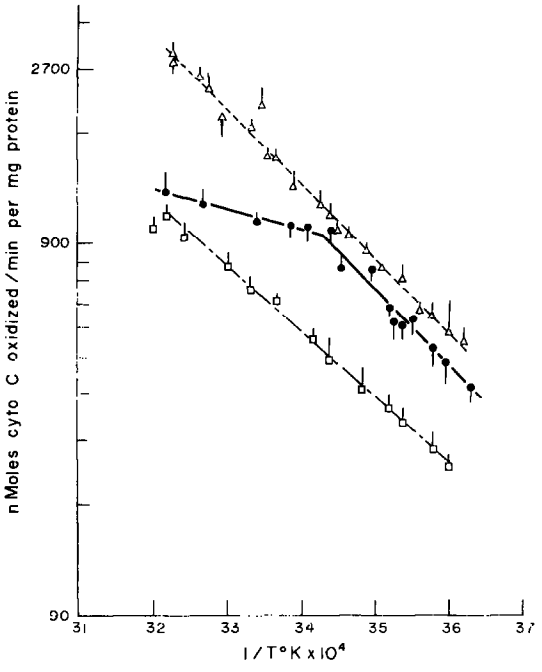

Fig. 7. Rats were injected i.p. with 25 mg morphine/kg. The brain mitochondria were harvested at 0.5 and 48 hr after morphine administration. The cytochrome oxidase activity of rat brain mitochondria from control (●), 0.5 hr (□) and 48 hr (△) was determined.

over controls at physiological temperatures. With cytochrome oxidase, the increase was absolute over the entire temperature range. Complete reversal of the opiate-induced inhibitory action on the enzyme was achieved when oxilorphan was administered 15 min post opiate and the mitochondria were harvested either 0.5 or 48 hr later.

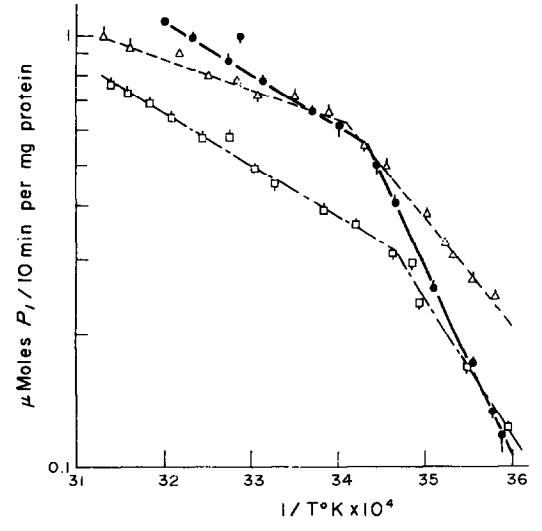

Fig. 8. Rats were injected i.p. with 25 mg morphine/kg. The brain mitochondria were harvested at 0.5 and 48 hr after morphine administration. The Mg^{2+}-stimulated ATPase activity of rat brain mitochondria (treated with osmotic shock) from controls (●), 0.5 hr (□) and 48 hr (△) was determined.

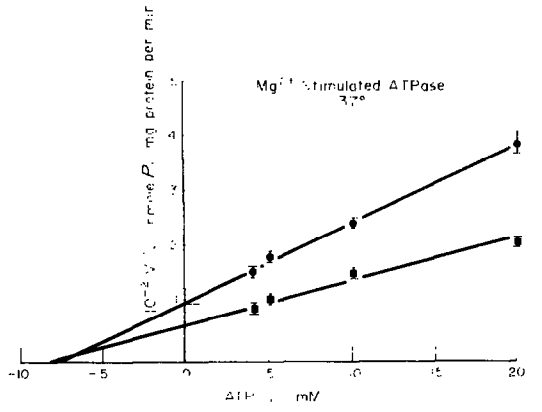

Fig. 9. Double reciprocal plot of rat brain mitochondrial Mg^{2+}-stimulated ATPase activity vs ATP concentration. Key: (■) control; (●) 0.5 hr after morphine (25 mg/kg) administration.

Magnesium-stimulated ATPase (extrinsic membrane-bound enzyme). The Arrhenius plots shown in Fig. 8 were generated from the inner mitochondrial membrane Mg^{2+} ATPase, over a temperature range from 4 to 40°. The temperature of discontinuity of this enzyme from lysed brain mitochondria, harvested at 0.5 hr after acute administration of 25 mg morphine/kg of rat, showed a decrease of 2.5° from the normal at 18.5° with a concomitant decrease in activity over most of the range of the incubation temperature studied. This effect is also entirely reversed by treating the morphinized animal with oxilorphan 15 min post opiate. As shown in Fig. 9, the K_m of this enzyme from the morphinized animal determined at 37° is virtually identical to the control value ($-7.4\ mM^{-1}$ as opposed to $-7.6\ mM^{-1}$). When the reaction was carried out with mitochondria harvested 48 hr after morphine administration, the discontinuity temperature increased to 20.5° (indicative of a 4.5° deviation between the two *in vitro* assayed values sampled 48 hr apart post opiate administration). There was a rapid increase in enzyme activity prior to the break in temperature of discontinuity (below 18.5°), as shown in Fig. 8. Compared with the control values, there was a decrease in enzyme activity in the physiological range of temperatures studied. The *Ea* values are shown in Table 1.

Table 2. Influence of the administration of various acute doses of morphine on the discontinuity temperature on rat brain mitochondrial Mg^{2+} ATPase

Morphine (mg/kg rat)	Time of mitochondrial harvest (hr)	Discontinuity temperature (°)
0 (Saline control)	0.5	18.5
5	0.5	17.7
10	0.5	17.1
15	0.5	15.9
25	0.5	16.0
	48.0	20.5

These experiments were repeated with brain mitochondria harvested 0.5 hr subsequent to the administration of 5, 10 or 15 mg morphine/kg of rat, and changes observed in the discontinuity temperature are shown in Table 2. As the dose of morphine was increased, there was a gradual decrease in the discontinuity temperature. It is interesting to note that 15 mg morphine/kg induced a decrease in the discontinuity temperature of the membrane lipids [1] in harmony with that found in Fig. 8 for brain Mg^{2+}-stimulated ATPase with 25 mg morphine/kg.

DISCUSSION

In the present work it was observed that, 0.5 hr after opiate administration, the activity of the inner membrane-bound enzymes from brain mitochondria is decreased in the physiological temperature range while that of the soluble, matrix enzymes is unaffected. Since many authors [4–6] have shown that such membrane-bound enzymes depend on the membrane lipids for both structure and function, it is possible that the disruptive effects of morphine on the lipid-packing array observed previously [1] could account for the loss of the activity restricted to the membrane-bound enzymes.

Raison *et al.* [7, 8] have shown that the discontinuities in the Arrhenius activation energy (*Ea*), can be observed in the rat liver mitochondrial succinoxidase, cytochrome oxidase and Mg^{2+}-stimulated ATPase. The temperature at which all these enzymes show an *Ea* discontinuity corresponded to the final temperature (T_f) of the liver mitochondrial lipid phase transition.

Data presented in our previous work [1] and in this study indicate that the enzymes undergo a change in *Ea* at the final temperature of the brain mitochondrial lipid phase transition (17–18°) in control preparations. The morphine-induced abolition of the *Ea* discontinuity in these same enzymes 0.5 hr after opiate administration may, therefore, be due to the concomitant shift in the T_f of the lipid phase transition to −1° demonstrated earlier with the DSC [1]. The extrinsic enzyme, Mg^{2+}-stimulated ATPase, showed only a slight downward shift of the *Ea* discontinuity. Since this enzyme is not embedded in the membrane [23], unlike the other intrinsic systems, morphine would not be expected to exert as great an effect. All of these effects were abolished by naloxone administration to the morphinized animal, demonstrating a specific opiate action.

Silvius *et al.* [24] have suggested that discontinuities in some Arrhenius plots may well be due to variable factors such as changes in pH and K_m which are temperature dependent. In the present work our data suggest it is unlikely that the discontinuities observed in the membrane-bound enzymes from control and morphine-treated rats were caused by temperature induced changes in either pH or K_m. In addition, experimental scatter was very slight in these assays. It therefore seems unlikely that the biphasic Arrhenius plots are the result of an artefactual discontinuity due to a strongly curved plot, as suggested by Silvius *et al.* [24].

The decrease in ANS fluorescence observed with brain mitochondria harvested 0.5 hr after opiate administration is also abolished by the *in vivo* administration of naloxone, indicative of a specific opiate action. Since the binding of ANS to such membranes is non-specific, it is conceivable that, in our work, the bulk of the membrane from the morphine-treated animal could have been altered, since the majority of bound ANS was apparently in a more polar environment. In addition, this alteration persisted for over 1 week when the mitochondria were stored at 4°, indicative of a rather stable structural alteration induced by the drug.

When the same systems were studied 48 hr after a single injection of morphine, several effects opposite to those described above were observed.

In all the intrinsic membrane-bound enzyme systems studied, there was an increase in enzyme activity in the physiological range of temperatures. We cannot at this time explain the anomalous behavior of the extrinsic enzyme, Mg^{2+}-stimulated ATPase, which showed a decrease in activity. There was abolition of the intrinsic enzyme *Ea* discontinuities normally found between 16 and 17° unlike that observed at 0.5 hr post opiate. The *Ea* observed for the 48-hr samples from morphine-treated animals is similar to the appropriate control values in the lower temperature range of the respective enzymes. Since in other work (E. A. Hosein and M. Lapalme, unpublished observations) the T_f of the lipid phase transition has been found to be increased 48 hr after opiate administration, it may be that the enzymes were maintained in a low temperature conformation during the temperature range of the assay.

The results from the ANS studies 48 hr after opiate treatment showed that there was a significant increase in fluorescence with brain mitochondria at the time of sampling. This is suggestive of a less polar environment at the membrane-binding sites and is the reverse of the results found 0.5 hr after morphine administration.

In summary, we have seen that, 0.5 hr after opiate treatment, control *Ea* discontinuities were either abolished or decreased with concomitant decrease in activity of membrane-bound enzymes. On the other hand, when the assays were performed 48 hr after opiate administration, a reverse pattern was generally obtained. Such changes in enzyme activity, *Ea* and ANS fluorescence might, therefore, be reflections of changes in the microenvironment, possibly changes in the physical state of the membrane lipids. The fact that we obtained a biphasic response in each of these parameters indicates that important structural changes had taken place in the membrane during the 48-hr period of action of the drug *in vivo*.

Since all of the effects recorded in this paper can be correlated with the state of the membrane lipids [7, 8, 25] at the time of mitochondrial harvest, these data support the view presented earlier [1] that alteration of the membrane lipid phase transition may be fundamental to the action of morphine in this system.

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