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Binding of Propranolol and Chlorpromazine by Mitochondrial Membranes

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The characteristics of propranolol and chlorpromazine binding by rat liver mitochondria, digitonin particles, and submitochondrial particles prepared by sonication were studied. The mitochondrial membranes appear to have two independent sets of binding sites for these drugs. Chlorpromazine, promethazine, and laurylamine decreased the binding of propranolol to mitochondria, while butacaine, spermine, and Polybrene were less potent in this respect. Inorganic cations (Ca^{2+} and K^{+}) at concentrations of 10 to 1000 μmol/l were largely ineffective in decreasing drug binding. The lipophilic anions bromthymol blue (BTB) and 8-anilino-1-naphthalenesulfonic acid (ANS) increased the extent of binding. The order of addition influenced binding: more propranolol was bound when BTB was added before the drug than when propranolol was added first. Similarly chlorpromazine inhibited the binding of propranolol more when it was added prior to propranolol. Binding of the positively charged drugs increased with increasing pH over the range 6.0 – 8.5. An increase in pH caused diminished binding of the negatively charged substance BTB. These results are interpreted as reflecting mainly a change with pH of the state of membrane protonation. Temperature changes from 23°C to 45°C did not affect the binding of propranolol to mitochondria, indicating the importance of hydrophobic interaction.

Energization of mitochondria increased the binding of propranolol and chlorpromazine, the opposite being true of submitochondrial particles. It is suggested that a redistribution of charged groups occurs in the hydrophobic membrane core upon energizing the mitochondria. The role of mitochondrial phospholipids as the most important binding sites for these drugs is discussed as well as the role of the changed distribution and density of membrane negative charges in the structure and permeability properties of the mitochondrial membranes.

There have been few reports on the binding of local anaesthetics by subcellular organelles.[1–4] Owing to the numerous advantages over nerve tissues erythrocyte membranes have been used as a model for studying the mechanism of local, and even general, anaesthesia.[1,2,5–8] As pointed out by Kwant and Seeman,[8] it is difficult to obtain sufficient amounts of plasma membranes

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of neurones devoid of the contaminant glial membranes. Local anaesthetics have an antihemolytic effect on erythrocyte membranes at concentrations almost identical to those anaesthetizing nerve fibers.[8] Furthermore, all the compounds known to stabilize erythrocytes against hemolysis act as local anaesthetics,[8] and more generally, all lipid-soluble anaesthetics protect red cells.[9] The most important advantage of the erythrocyte membrane is that when using isolated cells the effects of local anaesthetics on the ion movements across membranes can be characterized rather accurately. The sites of action *in vivo* of local anaesthetics really seem to be the membranes of various cells and subcellular organisms. Thus when added to tissue homogenates chlorpromazine [2-chloro-10-(3-diaminopropyl)-phenothiazine] was, after fractional centrifugation, found almost entirely in particulate fractions and only little in supernatant ones (see Ref. 2). Kwant and Seeman [8] have also shown that the membrane concentration of chlorpromazine in erythrocyte ghosts is approximately the same as it should be according to the classical partition theory of anaesthesia.

Mitochondrial ion transport is also strongly affected by local anaesthetics and related agents.[10–14] It has previously been suggested that these agents affect the membranes by combining with the negatively charged groups in the mitochondrial membranes,[14–16] in addition to solubilizing in the hydrophobic phospholipid membrane phase. This would lead to an altered conformation and physical properties of the mitochondrial membranes, which is seen in the changed membrane function. This paper further elucidates the mode of action of chlorpromazine and propranolol [1-(isopropylamino)-3-(naphthyloxy)-2-propanol] by studying the binding characteristics of these drugs with mitochondrial membranes in various experimental conditions. The study was also undertaken in order to gain a clearer understanding of the important action of mitochondria in cellular respiration and ion transport, both of which may be affected by local anaesthetics and related agents *in vivo*.[2,12]

MATERIALS AND METHODS

Liver mitochondria were prepared from Sprague-Dawley rats (weighing 150 – 200 g) as described by Wikström and Saris.[17] Digitonin particles (DP) derived from rat liver mitochondria (RLM) were prepared as described by Scheitman *et al.*[18] These particles were fully devoid of outer limiting membranes, as was controlled by electron microscope studies, and were capable of taking up calcium ions in an energy-dependent fashion, indicating that the membranes of these particles were "inside-in". Submitochondrial particles (SMP) were prepared by sonication of rat liver mitochondria as described by Kielley and Bronk.[19] The protein concentrations of the particles mentioned were estimated by the Folin method according to Lowry *et al.*[20]

The incubation medium (final volume of 3.0 ml) in binding studies of the drugs contained 225 mmol mannitol, 75 mmol sucrose, and 10 mmol Tris, pH 7.0, per litre. In order to block the endogenous respiration 5 μmol rotenone per litre were present unless otherwise indicated. In some instances the pH of the incubation medium was 7.9. The binding of chlorpromazine and propranolol was measured by determining the amount of ^{35}S-chlorpromazine and ^{14}C-propranolol, respectively, in supernatant after centrifugation. Rat liver mitochondria and digitonin particles were incubated with the drugs for about 10 min at room temperature (24°C) and centrifuged for 10 min at 9500 *g*. Sub-

Abbreviations. ATP = adenosine triphosphate; ANS = 8-anilino-1-naphthalenesulfonic acid; BTB = bromthymol blue; DNP = 2,4-dinitrophenol; DP = digitonin particles; RLM = rat liver mitochondria; SMP = submitochondrial particles.

mitochondrial particles were separated from the incubation medium by centrifugation for 1 h at 100 000 *g*. Control studies in which the fluorescence of chlorpromazine in the supernatant after centrifugation was measured gave identical results. The binding of propranolol to RLM was also measured by determining the removal of ^{14}C-propranolol from the incubation medium after rapid filtration through Millipore membrane filters with a pore size of 0.45 μm. These results also gave results identical to those obtained by the centrifugation technique, the results of which are given unless otherwise indicated. Aliquots of the supernatant medium were counted in a liquid scintillation system (Wallac, model Decem-NTL ^{314}, Wallac, Turku, Finland).

Chlorpromazine was kindly supplied by Orion Oy, Helsinki and ^{35}S-chlorpromazine (specific activity 1.4 mCi/mmol) was obtained from the Radiochemical Centre, Amersham. Propranolol and ^{14}C-propranolol (specific activity 7.6 μCi/mg) were generously donated by Dr. V. Manninen, Helsinki. Even at the highest concentration used the amount of chlorpromazine and propranolol spun down in the absence of mitochondrial membranes never exceeded 5 %. All the binding experiments were performed in duplicate, most of them 5 – 10 times. Where indicated, statistical methods for evaluating the results were used. Otherwise the number in the tables represents the duplicate results, since the amount of mitochondrial protein differed from day to day (the protein concentration largely influences the amount of drugs bound by membranes).

Bromthymol blue (BTB) was purchased from British Drug Houses Ltd., Poole, and was purified by recrystallization from ethyl acetate until homogeneous in silica gel thin-layer chromatography.[21] The amount of BTB bound by the individual particles was determined spectrophotometrically measuring the amount of BTB in the supernatant after centrifugation.[21]

All the other reagents used in this study were obtained commercially and were of analytical grade.

The mitochondrial phospholipids were extracted as described in a previous paper.[22] Protein was removed from the extract by filtration. The extracts were then evaporated under nitrogen and the layer of phospholipids on the bottom of the acid-washed glass bottles was dissolved in 0.3 ml chloroform-methanol (2:1 v/v), to which the drugs were added. Aliquots of these suspensions were pipetted on DC-Fertigplatten, Kieselgel F 254, Merck AG, Darmstadt. The chromatography was performed with the solvent chloroform-methanol-water solution system (70:30:5, v/v) for 90 min. The individual phospholipids were visualized by immersing the plate in a tank with iodine vapours. Phospholipids were extracted and the radioactivity and inorganic phosphorous were measured, the latter by the Fiske-SubbaRow [23] method.

RESULTS

1. Binding of chlorpromazine and propranolol. Fig. 1, a and b, shows that propranolol and chlorpromazine are bound by rat liver mitochondria (RLM), digitonin particles (DP), and submitochondrial particles (SMP) in considerable amounts. Fig. 1 a shows that propranolol is bound to various preparations in similar amounts on a protein basis. The binding of chlorpromazine is, however, less homogeneous, and is shown in Fig. 1 b. Two straight lines can be drawn in these binding isotherms, which is in accordance with the results obtained using sarcoplasmic vesicles or phospholipids derived from them.[24]

To determine any reversibility in drug binding the pellets of sedimented rat liver mitochondria were resuspended in the incubation medium, free of the drugs, and sedimented again. The results are shown in Table 1. In one washing about 50 % of the drug primarily bound by RLM is released. This value roughly corresponds to the 60 % obtained with prenylamine and sarcoplasmic vesicles.[24] After the third washing no more propranolol was released. This table also strengthens the hypothesis that RLM have a tightly and a loosely bound fraction of chlorpromazine (see Ref. 1).

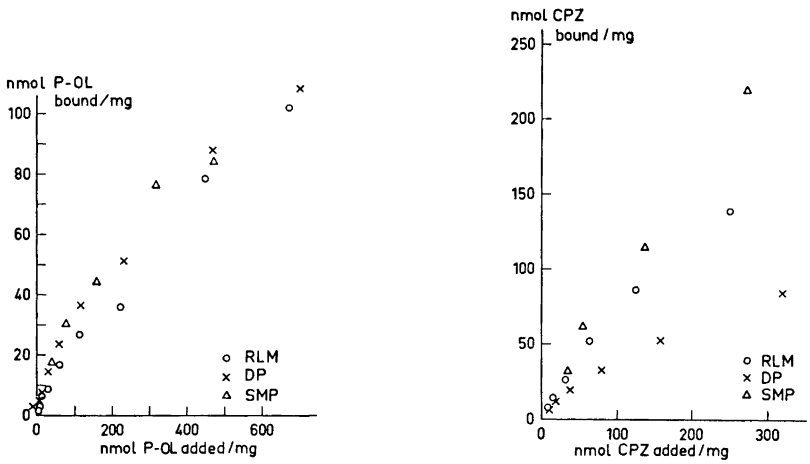

Fig. 1. Binding of propranolol (a) and chlorpromazine (b) by rat liver mitochondria, digitonin particles and submitochondrial particles. Conditions as described in Materials and methods. The final volume 3.0 ml. RLM = rat liver mitochondria, DP = digitonin particles, SMP = submitochondrial particles and mg = milligram of particle protein.

2. Scatchard plots of drug binding data. Linearization of binding data is possible in the case of a single binding system and may lead to a considerable loss of information.[25] In the Scatchard plot [26] the ligand bound divided by the free amount of the ligand is plotted against the ligand bound per mitochondrial protein. If this plot was linear all the binding sites for the drugs would have equal affinities and function independently. The nonlinearity of this plot may be used to indicate the presence of two or more binding sites differing in affinity. Reynafarje and Lehninger [27] have, by means of a Scatchard plot, reported on the presence of high and low affinity binding sites for Ca^{2+} in

Table 1. Effect of washing on the binding of propranolol and chlorpromazine by rat liver mitochondria. The effect of washing on the amount of free propranolol in the supernatant was measured by the isotope technique, while the amount of free chlorpromazine was estimated by its fluorescence (see Materials and methods). Mitochondrial protein, 1.1 mg/ml; final volume 3.0 ml, 100 μmol/l propranolol or 67 μmol/l chlorpromazine was added.

Number of washings	Free propranolol μmol/l	Free chlorpromazine μmol/l
0	79.5	20.0
1	8.0	14.0
2	4.0	9.0
3	2.0	7.0
4	0.0	7.0
5	0.0	4.0

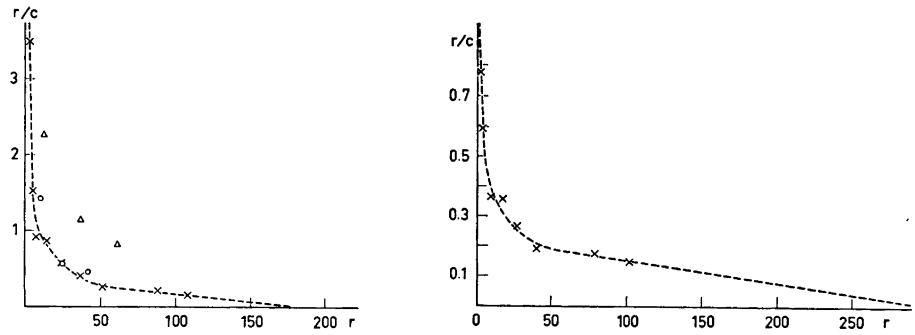

Fig. 2. Scatchard plots for binding of propranolol by rat liver mitochondria (a) and digitonin particles (b). Conditions as described in Materials and methods. r = nmol drug bound/mg protein, c = μmol free drug/l. In Fig. 2 (a) ○ is in the presence of 50 μmol/l DNP, and △ in the presence of 50 μmol/l BTB. The intercept on the abscissa is r: the intercept on the ordinate is rC, where C is the stability constant (or formation constant) and r is the number of binding sites per particle.

mitochondria. Fig. 2 shows Scatchard plots for propranolol binding by rat liver mitochondria (a) and digitonin particles (b). The number of binding sites and value for K (K = the concentration of the drug at which the binding sites are half saturated) are given in Table 2. It is evident that there exist at least two sets of binding sites in mitochondrial membranes for propranolol and chlorpromazine. In other words the membranes have two different re-

Table 2. The number and affinity of drug-binding sites as evaluated from Scatchard plots data. Conditions as described in Materials and methods. K is the concentration of the drugs at which the binding sites are half saturated. RLM = rat liver mitochondria, DP = digitonin particles and SMP = submitochondrial particles.

Drug	Particles	High or low affinity sites	Number of binding sites nmol/mg	K μmol/l
Propranolol	RLM	high	6	5
"	"	low	180	540
"	DP	high	8	9
"	"	low	310	1400
"	SMP	high	75	20
"	"	low	160	100
Chlorpromazine	RLM	high	60	40
"	"	low	220	135
"	DP	high	35	35
"	"	low	145	250
"	SMP	high	160	20
"	"	low	700	135

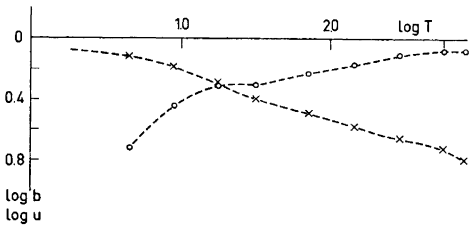

Fig. 3. A proportion graph for the binding of propranolol by rat liver mitochondria. Conditions as described in Materials and methods. T is the total ligand concentration, b is the value obtained by dividing the amount of bound ligand by T, and u is the value obtained by dividing the amount of unbound ligand by T (μmol/l).

ceptors for these drugs. The presence of an uncoupler DNP (2,4-dinitrophenol) was found by Reynafarje and Lehninger to abolish the biphasicity of the Scatchard plot for the binding of Ca^{2+}.[27] In their experiments DNP abolished the high affinity binding sites. Fig. 2 a shows that neither the presence of DNP nor the other lipophilic anion bromthymol blue (BTB) affected the biphasicity of this Scatchard plot.

The proportion graph method of Baulieu and Raynaud[25] has some advantages in the presentation of the experimental binding data of biological systems, since this method uses a log plot. Fig. 3 shows a proportion graph for the binding of propranolol by RLM. The more precise evaluation of the data obtained using this method is omitted here, mainly because of the considerable number of possible binding sites in mitochondrial membranes (see Ref. 25). However, Fig. 3 shows that in our conditions RLM have at least two specific binding systems for propranolol, and they may have two specific systems and a nonspecific binding system.

Sips' plot,[28] where log $(r/n-r)$ is plotted against log c (n is the number of binding sites, c is the concentration of free drug and r is the quantity of drug bound per mg protein) is used to obtain information about the heterogeneity of the binding energies. In the case of propranolol we have to use n_1 and n_2 for the high and low affinity binding sites, respectively. As the slope decreases in this plot the heterogeneity increases; a slope of 1 indicates a single dissociation constant. The slope for the binding data for propranolol by RLM is less than 1 with both values of n, thus indicating increased heterogeneity in binding, which is in accordance with the other results given in this paper (not shown).

3. Effects of various cationic and anionic substances on drug binding. It has been shown that local anaesthetics inhibit the binding of various cations in mitochondria and submitochondrial particles.[29,30] We have previously suggested that propranolol and chlorpromazine complex with membrane negative sites.[13,22] In this connection it was of interest to study the effects of various cations and anions on drug binding. Table 3 shows the effects of various cations and anions on this binding. Inorganic cations are unable to affect drug binding to any noticeable degree. On the other hand, other local anaesthetics can

strongly compete for the binding. The results obtained with RLM, DP, and SMP did not differ to any large degree. Table 3 also shows that the lipophilic anions, especially BTB, increase the binding of propranolol considerably.

Table 3. Effect of various cationic and anionic agents on the binding of propranolol. Conditions as described in Materials and methods. The calculations were made with an Olivetti Programa 101 computer. In (a) and (b) 100 μmol/l propranolol was added, and in (c), (d), (e) and (f) 67 μmol/l. CPZ = chlorpromazine, BUT = butacaine, LAU = laurylamine, PROM = promethazine, PBRE = Polybrene and SE = standard error of the mean.

Cation or anion added μmol/l	Bound propranolol nmol/mg	per cent of total
(a) Mitochondria, 1.6 mg protein/ml.		
None	18.5	31
100 Ca^{2+}	21.4	35
100 K^+	18.7	31
100 BUT	17.5	29
100 LAU	14.2	23
100 CPZ	7.5	12
100 PROM	4.6	8
100 BTB	38.2	62
100 ANS	28.0	46
(b) Mitochondria, 1.2 mg protein/ml.		
	Arithmetical mean of the per cent of total amount of bound propranolol	
None	29.1 (SE 0.2)	
10 Ca^{2+}	27.5 (SE 0.5)	
100 Ca^{2+}	28.9 (SE 1.7)	
1000 Ca^{2+}	25.9 (SE 1.0)	
10 K^+	27.8 (SE 0.8)	
100 K^+	29.9 (SE 0.6)	
1000 K^+	29.9 (SE 0.8)	
(c) Mitochondria, 1.3 mg protein/ml.		
	nmol/mg	per cent of total
None	14.5	29
10 Ca^{2+}	17.3	34
100 Ca^{2+}	15.5	30
1000 Ca^{2+}	12.3	24
10 K^+	14.7	29
100 K^+	16.2	31
1000 K^+	14.8	29
0.08 μg/ml PBRE	14.8	29
0.8 μg/ml PBRE	14.2	27
8.0 μg/ml PBRE	11.6	23
10 BTB	18.9	37
100 BTB	27.3	53

Table 3. Continued

(d) Digitonin particles, 1.1 mg protein/ml.

None	14.7	24
10 Ca^{2+}	15.2	25
100 Ca^{2+}	15.6	26
1000 Ca^{2+}	16.2	26
10 K^+	14.4	24
100 K^+	16.0	26
1000 K^+	17.7	29
67 BUT	15.8	26
67 CPZ	6.3	10
10 BTB	19.3	32
100 BTB	25.0	42

(e) Submitochondrial particles, 0.21 mg protein/ml.

None	67.0	21
10 Ca^{2+}	73.0	23
100 Ca^{2+}	64.0	20
1000 Ca^{2+}	67.0	21
10 K^+	66.8	21
100 K^+	62.2	20

(f) Submitochondrial particles, 0.17 mg protein/ml.

100 K^+	66.1	16
100 Ca^{2+}	72.0	18
100 BUT	49.1	12
100 CPZ	21.1	5
10 BTB	83.0	21
100 BTB	131.0	33

Table 4 shows similar measurements with chlorpromazine. The results are not different from those obtained using propranolol except that chlorpromazine is bound more than propranolol to various particles. It is worth noting that the potency of the various substances in inhibiting the binding of these drugs to membranes roughly correlates with their potencies in damping mitochondrial volume oscillations,[13] in inhibiting the action of phospholipase A on mitochondria [22] and in changing the net surface charge of mitochondria towards positive.[16] Spermine had some potency in displacing the bound chlorpromazine or propranolol, but Polybrene was potent only at a rather high concentration. This further strengthens the hypothesis previously put forward that Polybrene, and to a lesser degree spermine, are not as capable of penetrating the same membrane phase as local anaesthetics.[14] The Lineweaver-Burk plots indicated that the competition between Ca^{2+}, K^+, and spermine with propranolol was clearly of a non-competitive type. The inhibition of propranolol binding in RLM by chlorpromazine is also of a non-competitive type, but not nearly as clearly as that by inorganic cations. These results suggest that in binding with mitochondrial membranes Ca^{2+}, K^+, and probably spermine and Polybrene, do not *in toto* bind to the same sites with local anaesthetics although they may partially have the same binding sites. Quantitatively the common binding sites would

Table 4. Effect of various cationic and anionic agents on the binding of chlorpromazine. Conditions as in Table 3. In (a), (b) and (d) 67 μmol/l chlorpromazine was added, and in (c) and (e) 100 μmol/l. BUT = butacaine, P-OL = propranolol and SPINE = spermine.

Cation or anion added μmol/l	Bound chlorpromazine nmol/mg	per cent of total
(a) Mitochondria, 0.97 mg protein/ml.		
None	47.8	70
10 Ca^{2+}	47.5	69
100 Ca^{2+}	41.6	60
1000 Ca^{2+}	34.0	49
10 K^+	43.5	63
100 K^+	44.9	65
17 P-OL	42.8	62
67 P-OL	37.5	54
17 BUT	48.0	70
67 BUT	43.5	63
10 BTB	54.8	63
100 BTB	61.0	88
(b) Mitochondria, 1.2 mg protein/ml.	Arithmetical mean of the per cent of total amount of bound chlorpromazine	
None	79.1 (SE 0.9)	
10 Ca^{2+}	78.4 (SE 0.8)	
100 Ca^{2+}	75.8 (SE 0.9)	
1000 Ca^{2+}	76.8 (SE 0.9)	
Mitochondria, 1.1 mg protein/ml.		
None	70.1 (SE 0.3)	
10 K^+	68.9 (SE 0.7)	
100 K^+	67.5 (SE 0.3)	
1000 K^+	69.7 (SE 0.6)	
100 ANS	79.2 (SE 0.3)	
100 BTB	82.5 (SE 0.6)	
(c) Digitonin particles, 1.24 mg protein/ml.	nmol/mg	per cent of total
None	43.3	53
10 Ca^{2+}	44.6	55
100 Ca^{2+}	44.9	56
1000 Ca^{2+}	42.8	53
10 K^+	42.8	53
100 K^+	40.6	50
1000 K^+	43.8	54
67 P-OL	41.0	50
67 BUT	43.1	53
10 BTB	50.2	62
100 BTB	62.3	77

Table 4. Continued

(d) Submitochondrial particles, 0.12 mg protein/ml.		
None	308	50
10 Ca^{2+}	299	50
100 Ca^{2+}	283	47
10 K^+	286	47
100 K^+	249	41
10 BTB	384	62
100 BTB	675	100
(e) Submitochondrial particles, 0.11 mg protein/ml.		
None	577	64
67 P-OL	538	60
67 BUT	540	60
67 SPINE	538	60

in any case be scarce. This theory was further strengthened when the binding studies were so arranged that the agents to be bound were added at different times. All the results reported above were obtained when propranolol or chlorpromazine were added before or at the same time as the other agents studied. Table 5 shows the effect of order of addition on the binding of propranolol by RLM. It can be seen that if the agent to be bound is lipophilic in nature, *i.e.* capable of penetrating the hydrophobic membrane phase, the order of addition is of great importance as in the case of chlorpromazine, propranol and BTB. The same is not true of Ca^{2+} or K^+, which do not greatly affect the degree of drug binding even when the order of addition is altered. Thus these inorganic cations can be expected to have at least partially different binding sites. It may well be that the common binding sites for local anaesthetics and inorganic cations are in the region of the low affinity binding sites.

Table 5. Effect of order of additions on the binding of propranolol by rat liver mitochondria. Conditions as described in Materials and methods. Mitochondrial protein 1.63 mg/ml. CPZ = chlorpromazine and P-OL = propranolol.

First addition	Second addition (After 10 min incubation)	Bound propranolol nmol/mg	per cent of total
100 μmol/l P-OL	100 μmol/l CPZ	8.7	14
100 μmol/l CPZ	100 μmol/l P-OL	5.6	9
100 μmol/l P-OL	100 μmol/l BTB	27.0	44
100 μmol/l BTB	100 μmol/l P-OL	38.8	62
100 μmol/l P-OL	100 μmol/l K^+	16.8	27
100 μmol/l K^+	100 μmol/l P-OL	16.6	27
100 μmol/l P-OL	100 μmol/l Ca^{2+}	16.0	26
100 μmol/l Ca^{2+}	100 μmol/l P-OL	15.9	26

4. Binding of BTB by mitochondrial membranes. BTB was found to be very potent in increasing the binding of propranolol and chlorpromazine to mitochondrial membranes. In SMP the membrane consisting of inner mitochondrial membranes is "inside-out", and these particles still bind local anaesthetics and BTB in a way similar to the other particles used. This indicates that the binding is probably at the hydrophobic membrane phase. BTB has been used to measure the pH of this inner membrane phase, whatever the pH is in lipid phase, and often in the presence of local anaesthetics.[10] The same is true in measuring the fluorescence of ANS.[31] It was thus of interest to measure the binding of BTB by mitochondrial membranes, too, and to see whether the drugs used could affect it. Table 6 shows that local anaesthetics and related agents strongly increase the binding of BTB. In other words this change is in the same direction as that caused by BTB in the binding of propranolol or chlorpromazine. These results remind us how careful we must be in interpreting results obtained in the presence of local anaesthetics and BTB (possibly also ANS). The binding of BTB is also greatly influenced by changing the pH of the incubation medium, Table 6, the binding increasing at a lower pH.

Table 6. Binding of BTB by rat liver mitochondria (a), digitonin particles (b) and submitochondrial particles (c). Conditions as described in Materials and methods. 10 μmol/l BTB was added. CPZ = chlorpromazine, P-OL = propranolol, SPINE = spermine and PBRE = Polybrene.

Cation added μmol/l	Bound BTB nmol/mg	per cent of total
(a) Mitochondria, 1.23 mg protein/ml.		
None	5.4	66
17 CP^+	6.1	75
34 CP^+	8.1	100
34 P-OL	7.3	90
167 P-OL	8.1	100
17 SPINE	5.9	73
167 SPINE	8.1	100
0.08 μg/ml PBRE	5.6	68
8.0 μg/ml PBRE	7.8	95
pH		
6.0	8.1	100
7.0	5.9	73
8.5	1.5	18
(b) Digitonin particles, 0.5 mg protein/ml.		
None	11.4	57
34 CPZ	16.3	82
100 P-OL	16.3	82
10 Ca^{2+}	12.3	62
100 Ca^{2+}	14.2	71
10 K^+	13.3	62
100 K^+	13.3	62
pH		
6.0	18.1	90
7.0	11.4	57
8.5	5.6	28

Table 6. Continued.

(c) Submitochondrial particles, 0.37 mg protein/ml.

None	15.4	57
34 CPZ	12.7	47
67 CPZ	16.6	62
100 CP^+	17.2	64
100 Ca^{2+}	18.8	66
pH		
6.0	20.5	76
7.0	15.4	57
8.5	0.0	0

(d) Submitochondrial particles, 0.15 mg protein/ml; pH of the medium 6.9.

None	62
100 P-OL	76

5. Effect of pH on drug binding. With an increasing pH of the incubation medium the binding of propranolol and chlorpromazine increases, Table 7. The double-reciprocal plots (bound drug against the amount of drug added at different pH's) indicated that protons competitively inhibited the binding of propranolol to RLM (not shown). This probably reflects changes in the state of membrane protonation rather than changes in the dissociation of the drug since the latter is small due to the high pK_a of propranolol (9.45).

Table 7. Effect of pH on the binding of propranolol and chlorpromazine. Conditions as described in Materials and methods. RLM = rat liver mitochondria, DP = digitonin particles, SMP = submitochondrial particles, CPZ = chlorpromazine and P-OL = propranolol.

pH	Added drug	Bound drug		Particles	Protein concentration
	μmol/l	nmol/mg	per cent of total		mg/ml
6.0	67 P-OL	16.8	23	RLM	0.87
7.0	"	18.9	25	"	"
8.5	"	25.7	35	"	"
6.0	"	14.6	24	DP	1.1
7.0	"	14.7	24	"	"
8.5	"	16.5	27	"	"
6.0	"	57.3	18	SMP	0.21
7.0	"	67.0	21	"	"
8.5	"	95.0	30	"	"
6.0	67 CPZ	39.0	56	RLM	0.97
7.0	"	46.3	67	"	"
8.5	"	60.9	88	"	"
6.0	"	37.1	46	DP	1.24
7.0	"	43.3	53	"	"
8.5	"	63.0	78	"	"
6.0	"	326	52	SMP	0.12
7.0	"	308	50	"	"
8.5	"	678	100	"	"

6. Effect of temperature on the binding of propranolol. No significant effect of temperature on the binding of propranolol to RLM could be found. Thus of 100 μmol propranolol/l (the mitochondrial protein concentration was 1.0 mg/ml) added 32.5 % was bound at 23°C, 32.0 % at 37°C, and 33.5 % at 45°C (not shown).

7. Effect of energization on drug binding. It has been shown by Azzi [32] that the binding of ANS and auramine, or another positively charged substance, ethydium bromide,[33] can be altered by changing the energy state of mitochondria. When energized they bind less ANS, which is negatively charged, and more auramine.[32] On the other hand SMP were shown to bind more ANS and less auramine upon energizing.[32] These findings indicate that the outer phase of mitochondrial membrane displays a change towards the negative when the mitochondria are energized, this change being reversed by de-energization. In accordance with the results of Azzi [32] on the binding of auramine RLM and digitonin particles bind more propranolol and chlorpromazine in the energized state than in the de-energized state, Table 8. It can also be seen from this table that in this respect submitochondrial particles behave in the opposite way.

8. Binding of the drugs with phospholipids. It is known that propranolol and chlorpromazine *in vitro* avidly bind with phospholipids.[1,3,5,35,36], Balzer *et al.*[24] have shown that the binding of chlorpromazine with phospholipids extracted from sacroplasmic vesicles shows characteristics similar to those seen with intact sacroplasmic vesicles, and that the phospholipids have an even higher

Table 8. Effect of energization on the binding of propranolol and chlorpromazine on mitochondrial membranes. Conditions as described in Materials and methods. The calculations were made with an Olivetti Programa 101 computer. On one occasion the binding of chlorpromazine (67 μmol/l added, mitochondrial protein 1.1 mg/ml) by rat liver mitochondria was calculated according to the method described by H. De Jonge [34] in order to discover whether the difference in drug binding between energized and non-energized state is significant. The estimations were made in each group: in the energized state the mean value of the amount of chlorpromazine bound to mitochondria was 40.7 μmol/l (SE 0.3) and in the non-energized state it was 38.5 μmol/l (SE 0.6). The method of De Jonge [34] gave a result of $p < 0.01$.

The concentration of ATP was 1.3 mmol/l, of KCN 1.7 mmol/l, and of oligomycin 0.83 mg/l. RLM = rat liver mitochondria, DP = digitonin particles, SMP = submitochondrial particles, P-OL = propranolol and CPZ = chlorpromazine.

Concentration of the particles	Added drug μmol/l	Bound drug nmol/mg	
		ATP + CN^-	ATP + CN^- + oligomycin
1.0 mg/ml, RLM	67 P-OL	17.3	16.0
1.1 mg/ml, DP	67 P-OL	16.4	15.8
0.17 mg/ml, SMP	67 P-OL	48.0	63.5
2.2 mg/ml, RLM	134 CPZ	50.2	48.2
0.84 mg/ml, DP	100 CPZ	62.4	55.0
0.12 mg/ml, SMP	100 CPZ	251	286

capacity for binding chlorpromazine than the vesicles themselves. We incubated propranolol and chlorpromazine with phospholipids extracted from RLM and pipetted aliquots of this mixture on DC-Fertigplatten. Table 9 shows quantitatively to which phospholipids these drugs are bound and also the phosphorus content in the various phospholipid spots. The results of propranolol and chlorpromazine are not comparable since the drugs have different R_F-values on thin-layer chromatography and they probably have different affinities in binding with Kieselgel. The results do, however, show that much of the drugs is moved together with cardiolipin if calculated on phospholipid present in the spot (seen from the amount of P_i). The region of phosphatidyl ethanolamine is also affected to a considerable degree, which may in part be due to the fact that propranolol changes the mobility of cardiolipin

Table 9. Movement of propranolol and chlorpromazine on thin layer chromatography with various fractions of phospholipids isolated from rat liver mitochondria. Abbreviations: LESI = phosphatidyl choline (lecithine), CAR = diphosphatidyl glycerol (cardiolipin), PEA = phosphatidyl ethanolamine, P-OL = propranolol, CPZ = chlorpromazine and P_i = inorganic phosphate.

In the case of propranolol the spots of P-OL, PEA, and CAR did not separate in three isolated spots, as in the presence of chlorpromazine, but in three spots touching one another so that the borders between them were not clear enough to extract them as pure. This was due to the changed mobility of CAR in the presence of propranolol. This area was therefore divided into three parts that together consist of propranolol, PEA and CAR. These three parts are expressed in the table as "P-OL COMPLEX I" and "II" and "ABOVE P-OL". The expression "ABOVE ALL" is used to mean that this part consists of all the activity in the area above the visible spot on the chromatogram. "SUB-CPZ I" and "II" mean that the area below the spot of CPZ was divided into two, from which the amount of CPZ was estimated (no visible spot was seen in this area); the same was done with propranolol. The conditions were as described in Materials and methods.

The spot on the plate	Control		In the presence of CPZ			In the presence of P-OL		
	μg P_i	per cent	μg P_i	per cent	Bound CPZ (%):	μg P_i	per cent	Bound P-OL (%)
LESI	2.3	40.0	1.95	36.0	0.39	1.95	36.5	1.55
CAR	1.1	19.2	0.58	10.7	0.57	0.16	3.0	5.6
PEA	2.0	35.0	1.75	32.5	0.69			
CPZ-COMPLEX			0.3	3.2	98.0			
P-OL-COMPLEX I						2.12	38.0	68.0
P-OL-COMPLEX II						0.97	18.0	13.7
ABOVE P-OL						0.07	1.3	9.9
ABOVE ALL	0.1	1.75	0.09	1.7	1.85	0.11	2.05	1.25
		99.25 (%)		99.90 (%)	101.50 (%)		98.85 (%)	100.0 (%)
Without phospholipids:								
Spot of CPZ					98.0			
SUB-CPZ I					1.6			
SUB-CPZ II					0.43			
					100.03 (%)			
Spot of P-OL								83.0
SUB-P-OL I								14.2
SUB-P-OL II								3.3
								100.5 (%)

Table 10. Effect of propranolol on the movement of cardiolipin on thin layer chromatogram. 40 µl of commercially obtained cardiolipin (8.9 mmol/l P_i) was mixed with 10 µl of propranolol (100 mmol/l). 20 µl of this mixture was pipetted on the plate. The DC-Fertigplatte (see Materials and Methods) prepared in this way was allowed to develope for 90 min, after which the R_F-values were calculated.

Substance	R_F-values (× 100)
Propranolol	58
Cardiolipin	61
Cardiolipin + propranolol	72

on the plate, Table 10. It is thus impossible to calculate the exact amounts of the drugs bound to individual phospholipids. The results given in Table 9 agree with our previous results,[22] which demonstrated that propranolol and chlorpromazine, among other similar substances, inhibit the action of phospholipase A on mitochondria. Similar results have also been obtained using DP instead of RLM (not published). Decreasing the pH from 7.5 to 6.5 also inhibits the action of exogenous phospholipase A on mitochondria by about 30 – 49 % (not published), which further arouses interest in the protonable charged groups in mitochondria.

The role of phospholipids in drug binding is also seen from the preliminary results shown in Table 11. The re-binding of cardiolipin and a smaller amount of phosphatidyl ethanolamine with lipid-depleted mitochondria restores the drug binding capacity of lipid-depleted mitochondria to the level obtained with intact mitochondria. We think that these results further suggest that phospholipids are indeed important binding sites for these drugs in mitochondria. This would also be in accordance with the results of Balzer *et al.*[24] on drug binding with sarcoplasmic vesicles and their phospholipids.

Table 11. Binding of chlorpromazine with lipid-depleted and lipid-restored rat liver mitochondria. The lipid-depleted mitochondria were prepared according to the method of Fleischer *et al.*[37] Commercially obtained phosphatidyl ethanolamine (PEA) and diphosphatidyl glycerol (CAR, cardiolipin) were mixed (5:45 w/w) in water (*plus* 0.5 mmol/l EDTA) and then sonicated for 10 min. After centrifugation for 30 min at 100 000 *g* the supernatant was used as a source of phospholipids. After 10 min incubation the lipid-depleted mitochondria were sedimentated by a centrifugation at 20 000 *g* for 10 min. The intact mitochondria were similarly treated but in the absence of any added phospholipids.

Particles	Bound chlorpromazine (per cent of total)
Lipid-depleted mitochondria	47
Lipid-depleted mitochondria + PEA – CAR	77

DISCUSSION

The present studies establish that rat liver mitochondria, digitonin particles, and submitochondrial particles have the capacity to bind considerable amounts of propranolol and chlorpromazine. All these particles apparently have two independent sets of binding sites, or receptors, for these drugs. Our previous suggestion in conjunction with the reports of Balzer *et al.*,[24] Feinstein,[35] and Nayler,[36] on the interaction of local anaesthetics and related agents with phospholipids in membranes now seems to be justified. In mitochondria the principal phospholipids involved in this binding may well be cardiolipin and phosphatidylethanolamine. This would be logical, since cardiolipin has two negative charges per molecule and is very abundant among mitochondrial phospholipids. These drugs may, however, have important protein binding sites on the membrane surfaces and in the structures of various enzymes in addition to their binding and solubilization in the hydrophobic phase of the membranes.[1,2,8,30] This idea is also favoured by Hasselbach *et al.*[38] and Balzer *et al.*[24], who have shown that the binding capacity of the sarcoplasmic vesicles for chlorpromazine is lost when the lipids are extracted from the vesicular membranes. The binding to the isolated phospholipids was found to correspond to that in intact sarcoplasmic vesicles.[24] For chlorpromazine there are many other binding sites in mitochondria, and maybe to a lesser degree for propranolol. This may in fact be the cause of the different binding characteristics of propranolol and chlorpromazine. These extra binding sites are flavorproteins, nucleic acids and other negative groups present in mitochondria.[2] It seems to us, however, that these extra binding sites are quantitatively of minor significance, mainly since the phospholipids isolated from sarcoplasmic vesicles bind chlorpromazine to such a large extent.[24] Neuraminic acid, which is reported to be essential for the binding of chlorpromazine with erythrocytes, does not participate in the binding of propranolol in mitochondria or digitonin particles (S. Nordling, A. Huunan-Seppälä, and N-E. L. Saris, not published).

Our results show that propranolol and chlorpromazine have at least partly the same binding sites in membranes. These drugs do inhibit each other's binding in a non-competitive way. The complexity of the interaction is evident from the fact that the order of addition strongly influences the binding. Thus the tightly bound fraction of the drug cannot be removed by the other "competing" drug added to the suspension much later, Table 5. Spermine and Polybrene are less potent inhibitors of binding, while the effect of inorganic cations like calcium and potassium was even less and might reflect interaction with surface binding sites only.

The very strong effect of BTB and ANS, and to a lesser degree of DNP, in increasing drug binding indicates that the binding of the drugs occurs in the same membrane phase as that of these lipophilic anions. The effect of BTB also suggests that negative charges in this phase, and maybe in the membranes generally are important in regulating the interplay of structure and function. It has been shown that iodide is very effective in inhibiting the binding of BTB to an isolated mitochondrial structural protein-phospholipid complex.[21] DNP was also found to be competent.[21] This again suggests that protein might also play some role in this kind of binding.

It is known that the conformation and function of mitochondrial membranes can be altered by changing the state of membrane protonation.[39] Jacobus and Brierley [30] have suggested that pH changes lead to a rearrangement of membrane components. Alterations in pH also significantly affect the potency of local anaesthetics. Correspondingly, changes in pH can affect the action of phospholipase A on mitochondria, presumably by altering the conformation of the substrate, *e.g.* the membranes.[22,40] A decrease in pH is expected to increase the amount of positive membrane sites that are protonable. Consequently, the negatively charged BTB is bound more extensively at lower pH's, the opposite being true of local anaesthetics. Manninen has reported that if the pH of the incubation medium is increased from 7.0 to 7.5 the amount of propranolol bound to erythrocytes increases.[41] Taking into account the high pK_a of propranolol (9.45) it seems evident that the differences in the amounts of propranolol and chlorpromazine bound to mitochondria at different pHs are mainly due to changes in the membrane receptors.

The binding of propranolol to mitochondrial membranes was found to be independent of temperature. This suggests that an entropy increase is the driving force for the binding of propranolol. Similar results have been obtained by Balter *et al.*[24] in the binding of reserpine, chlorpromazine, and prenylamine (the latter being similar to propranolol in clinical use), and by Kwant and Seeman [8] in measuring the effect of temperature on the concentration of chlorpromazine in red cell membranes. Thus it is to be expected that with an increasing temperature the enthalpy becomes more negative. These findings suggest that the interaction of chlorpromazine and propranolol with mitochondrial membranes might be mainly hydrophobic in nature, thus further strengthening the idea that phospholipids are the principal drug receptors.

The effect of the energy state on drug binding in mitochondria strengthens the concept of an energy-induced change in the distribution of charged groups in mitochondrial membranes.[32] An increase in net negative charge of intact mitochondrial membrane and of digitonin particles (inner membrane preparations) occurs when these particles are energized, as measured by increased binding of propranolol and chlorpromazine upon energizing these membranes by ATP *plus* cyanide. A decrease in binding was found when these particles were de-energized by the addition of oligomycin. The opposite results were obtained in SMP, the membrane of which is known to be "inside-out" as a result of the sonication procedure. Azzi has shown [32] that energization of mitochondria increases the binding of the positively charged substance auramine and decreases the binding of negatively charged ANS. It is probable that the BTB used to measure the pH in the mitochondrial inner membrane phase behaves similarly to ANS.[42,43] It seems evident that the uneven charge distribution occurring in mitochondrial inner membrane upon energizing is not a plain surface phenomenon. We have previously shown that changing the energy state of intact mitochondria does not affect the electrophoretic mobility of mitochondria in a cell electrophoresis apparatus.[16] This indicates that there is no major change in the net surface charge of RLM upon energizing. It is also of note that changes in the binding of various agents can only be detected by means of agents that are all lipophilic, *i.e.* are able to penetrate the hydrophobic phase of the membrane. Thus the charge distribution is ex-

pected to occur across the membrane. Skulachev, in his recent review,[44] pointed out how well this kind of charge distribution agrees with the concepts of electric fields in coupling membranes. He also writes that probes like ANS and BTB may be used for detecting membrane potentials.[44]

Chlorpromazine has been extensively used in studies of the effects of local anaesthetics on membranes, especially red cell membranes. It is known that all compounds stabilizing red cell membranes (or protecting them against hemolysis) can act as local anaethetics.[8,9] There are other important types of binding to the membranes than those involving negatively charged binding sites, judging from the fact that alcohols and steroids can also act as local anaesthetics.[1,9] Polyamines have local anaesthetic properties mainly by combining with negative charges and thereby affecting ion transport, while propranolol and chlorpromazine may well affect by this mechanism, but also by solubilizing in the membranes in a way similar to steroids and alcohols. It has been shown that chlorpromazine expands the erythrocyte membranes.[5,8] We could not detect any significant effect of propranolol or chlorpromazine on the water content of mitochondria (as judged by the method of adding ^{14}C-sucrose and $^{3}H_2O$ in the incubation medium [45] and determining the amounts of these tracers spun down with mitochondria in the presence of local anaesthetics (not published). It may well be that in erythrocytes these agents expand the membrane by causing intermolecular repulsion. It is known, in fact, that local anaesthetics increase the surface tension of many phospholipid monolayers.[46] In mitochondria, by binding, *e.g.*, to cardiolipin, one of the main components of the inner membrane, the opposite would occur. This reaction might compensate the expanding effect of binding with other phospholipid constituents. In fact Shah and Schulman have proposed a similar kind of cardiolipin monolayer contraction in the presence of calcium ions.[47]

All biological particles carry a net negative surface charge. The binding of positively charged substances decreases the net surface charge of the particles, as shown for mitochondria.[16] According to the classical membrane model proposed by Danielli and Davson [48] the negative surface charges would be negative groups of the proteins covering the inner phospholipid core. It is not, according to this model, easy to see how phospholipases so easily attack the membrane phospholipids.[22,49] Furthermore, mitochondria can adsorb specific cardiolipin antibodies present in syphilitic sera (A. Huunan-Seppälä, N. E. L. Saris and K. Aho, not published). It is thus possible that there is a mosaic structure, or defects in the protein layer, allowing phospholipid molecules to reach the outer membrane boundary exposing their charges. Martonosi et al.[50] have used various phospholipases in studying the transport processes in sarcoplasmic reticulum membranes. According to these authors [50] the Danielli-Davson model may still be valid even though phospholipases can easily hydrolyze the membrane phospholipids. There might, however, be substantial differences in different membranes. We prefer a membrane model in which the inner core of the membrane is the hydrophobic phase, where in mitochondria the change in charge distribution takes place upon energizing. This would be the membrane phase in which BTB is bound.[51] The role of protein would be an organizing one. Binding to the protein sites may also cause changes in membrane conformation, for instance by means of an al-

losteric induction.[52] The hydrophobic inner core of the membrane may have some movable charges and also protonable groups. When energized, the outer face of the hydrophobic inner phase may become more negative, thus binding the positively charged lipophilic molecules such as propranolol and chlorpromazine more. The opposite would be true of the inner face, judging from the binding characteristics presented by SMP.

Liberman and Skulachev have proposed [43] that intact mitochondria accumulate cations by means of an electric field generated in the membrane, and SMP take up anions by an analogue mechanism, since in these particles the membrane polarity is reversed. Our model of a charged energized membrane is in agreement with the model of Liberman and Skulachev [43,44] with the modification that we assume the presence of mobile charged species capable of moving in the electric field. Likely candidates for these would be phospholipids, which could function in hydrophobic milieu and serve the reacting chemical groups in the outer face of the inner core in energized mitochondrial membrane. It is of interest that in phosphatidyl choline membranes it is possible to detect inside-outside transitions of the polar head groups, a mechanism that could account for an associated chloride translocation.[53] Similarly the complex between a cation and a phospholipid might be capable of transferring ions through hydrophobic membranes. As proposed by Saris [54] lipophilic complexes of acidic phospholipids with calcium ions may account for calcium translocation in a manner similar to that in which ionophorous antibiotics [55] increase the permeability towards monovalent cations.

The change in charge distribution may depend on the formation of an electric field in the membrane, or even generated by it. In intact mitochondria the electric field is so arranged that the 'minus' is inside,[43] which would cause the appearance of more positive groups near the inner membrane face, while the 'plus' being outside helps the appearance of negative groups in the outer membrane face. It is possible that the increase in the number of these groups is responsible for the increased binding of local anaesthetics to mitochondria upon energizing. This mechanism of charge distribution may, however, depend entirely on the presence of an appropriate protein (*e.g.* enzymes) or protein conformation and on the structural integrity of the membrane. The decreased binding to SMP upon energizing is probably also due to a changed charge distribution and not to a transport of these agents outside the inner vesicle space, where presumably no local anaesthetics are present. Furthermore, the strong influence of BTB upon drug binding leads us to think that local anaesthetics bind to the membranes in a similar way and for the most part in the same membrane phase as lipophilic anions BTB and ANS: This mechanism does not exclude the possibility that various ions are transported in mitochondria by the mechanism of the action of an electric field potentiated upon energizing.[43,44]

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