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Hydrophobic Sites of the Mitochondrial Electron Transfer System

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Summary

Various classes of hydrophobic organic compounds of the aliphatic and aromatic series specifically inhibit electron transfer in the respiratory chain between NAD and cytochrome *b*. In liver mitochondria this effect can be reversed by vitamin K_3. There is a quantitative correlation between the inhibitory activity of these compounds and the value of their partition coefficients in octanol-water.

By measuring the effect of the inhibitors on the surface tension at the heptane-water interphase, it has been shown that the concentrations of hydrophobic inhibitors in the water phase required to fill 50% of the interphase boundary area are very close to the concentrations of these substances which decrease the rate of mitochondrial respiration by 50%. The conclusion is drawn that the region of the NADH dehydrogenase which binds these inhibitors resembles, in its adsorbtional properties, the hydrocarbon-water interphase.

Anions of carboxylic acids and phenols with $pK_a < 7.7$ inhibit the respiratory chain before, and not after, NADH. Their effect is not reversed by vitamin K_3. In this case, again, the efficiency of the inhibitor depends upon their lipid solubility.

The fact that electroneutral hydrophobic compounds inhibit the respiratory chain NADH dehydrogenase, whereas charged ones do not, is discussed in connection with the mechanism of action of this enzyme. It is proposed that, after reduction, the negatively charged carrier dissociates from the hydrophobic region of the enzyme.

Introduction

It is known that the segment of the respiratory chain between NAD and cytochrome *b* is sensitive to inhibitors of widely differing chemical

structure [1, 2, 3, 4]. The high affinity of these inhibitors to lipids indicated that the linkage between the substance and the enzyme was maintained via hydrophobic interactions [+]. In fact, Hansch and Anderson found a quantitative correlation between the inhibiting efficiency of barbiturates and the value of their partition coefficient in the octanol-water system [4]. However, these data did not allow us to answer the question of whether any functional groups are involved in the enzyme-inhibitor interaction.

Other authors have reported [5, 6] that purified NAD-linked dehydrogenase can be inhibited by lipid-soluble phenol derivatives. In the present paper, data are summarized to show that non-specific adsorption of these substances on the hydrophobic region of the enzyme, rather than interaction with a functional group, is responsible for the inhibition of NADH-dehydrogenase by hydrophobic agents.

Methods

Rat liver mitochondria and beef heart submitochondrial particles (SMP) were prepared according to the procedures described in refs. [7] and [8], respectively. The respiration rate was recorded polarographically, using a stationary platinum electrode. The incubation medium for mitochondria contained 0.2 M sucrose, 0.03 M Tris-HCl buffer, pH 7.5, 0.01 M KH_2PO_4, 0.005 M $MgSO_4$, 0.02 M KCl, 250 μM EDTA and respiration substrates (5 mM glutamate plus 5 mM malate). The protein content was 3-4 mg/ml. SMP incubation medium contained 0.03 M sucrose, 0.01 M Tris-HCl buffer, pH 7.5, 1 mM NADH. The protein content was 1-1.5 mg/ml.

Partition coefficients in the octanol-water and hexane-water systems were determined using a "Hitachi" differential spectrophotometer (see Table I).

Interphase tension (σ) was determined by measuring the weight and volume of the drops. Water, the heavier liquid, was pressed out through a teflon capillary into the lighter liquid, heptane. The lifetime of a drop was not less than two minutes before it reached an adsorption equilibrium [9]. The value of σ was calculated by taking into account drop shape corrections [10]. Before making the measurements, the water-heptane system was equilibrated for at least twenty-four hours. The deviation in the measurements was about 0.5 dynes. Figure 1 shows a plot of the interphase tension against the concentration of the substances.

The isotherms were calculated from the $\sigma = f[c]$ curves. Figure 2 shows adsorption isotherms of the substances expressed in the relative coordinates $\theta = C_\theta / C_\infty$ and $y = C/C_\theta = 0.5$, where $C_\theta = 0.5$ is the

TABLE I. Inhibitory activity and physicochemical parameters of various compounds

No.	Compound	log p[a] octanol-water system	log p[a] hexane-water system	pK_a	log[b] 1/c
1	Phenol	1.46	−0.85	9.90	2.00
2	4-Chlorophenol	2.39	−0.40	9.44	2.85
3	2-Methyl-4-chlorophenol	2.78	0.25	9.60	3.50
4	2,4-Dichlorophenol	3.08	0.40	7.75	3.65
5	2,4,6-Trichlorophenol	4.05	0.90	7.00	3.70
6	2,4-Dibromophenol	3.48	0.35	7.60	2.80
7	2,4,6-Tribromophenol	4.23		6.80	3.45
8	Pentachlorophenol	5.86		4.80	4.40
9	Aniline	0.90	0.01		1.72
10	4-Methoxyaniline	0.78			1.70
11	4-Methylaniline	1.39			2.20
12	4-Bromoaniline	2.05	0.50		3.00
13	N,N-Dimethylaniline	2.29			2.82
14	Methanol	−0.68			−0.30
15	Ethanol	−0.15			0.30
16	Butanol	0.32			0.65
17	Ethylbenzoate	2.20			2.80
18	Cinnamic acid ethyl ester	2.91			3.52
19	N-Ethyl-N-(2-acetoxyethyl)aniline	2.04			2.92
20	Diacetoxytoluol	1.62			3.00
21	1-Acetoxynaphthalene	2.78			3.50
22	1-Phenyl-2-Acetamidocyclopropane	1.43			2.66
23	Phenylacetic acid	1.51			2.14
24	Cinnamic acid	2.08			2.35
25	p-(N-2-Chloroethyl-N-acetyl)-aminophenylacetic acid	1.20			2.05
26	Benzamide	0.66	−2.30		2.00

[a] Some data were taken from papers [12, 13].
[b] C, the concentrations of the inhibitor decreasing the rate of mitochondrial respiration by 50%; the incubation medium contained 500 μM ADP and 0.01 M KH_2PO_4, glutamate (5 mM) and malate (5 mM).

concentration of the substances in the water phase when the interphase boundary is one-half filled.

All of the isotherms may be described with sufficient approximation by the Frumkin adsorption equation:

$$BC = \frac{I-\theta}{\theta} \exp(-2a\theta)$$

where B is the adsorption equilibrium constant describing the adsorbing ability of the molecules interacting with the surface; "a" is an attraction constant accounting for the interaction of the adsorbed molecules. The B and "a" constants were determined for all the substances.

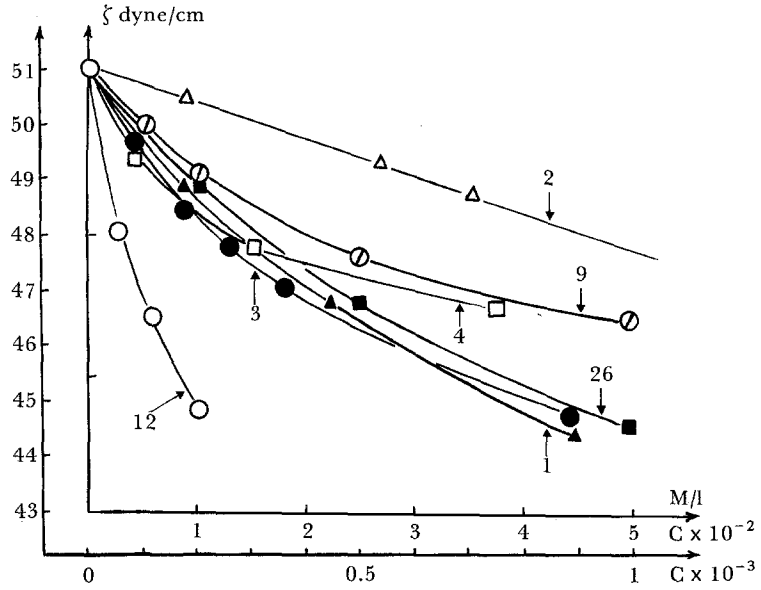

Figure 1. Dependence of the interphase tension of the aromatic compounds upon their concentration in the aqueous phase. Inner coordinates: phenol (1), aniline (9), 4-bromoaniline (12), benzamide (26); outer coordinates: 4-chlorophenol (2), 2-methyl-4-chorophenol (3), 2,4-dichlorophenol (4).

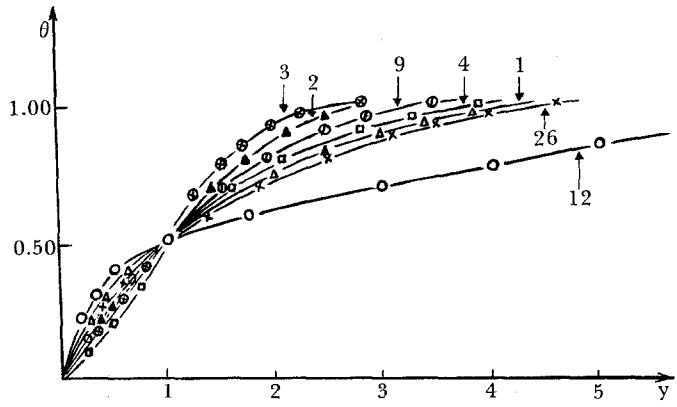

Figure 2. Isotherms of adsorption of aromatic compounds at the water-heptane interface in the relative coordinates: $\theta = C_0/C_\infty$ and $y = C/C_{\theta=0.5}$

Figure 3 shows the degree of approximation with which the resulting isotherms can be described by the Frumkin adsorption equation. The free energy of adsorption was calculated from equation:

$$\Delta G = -RT \ln 55.5\ B$$

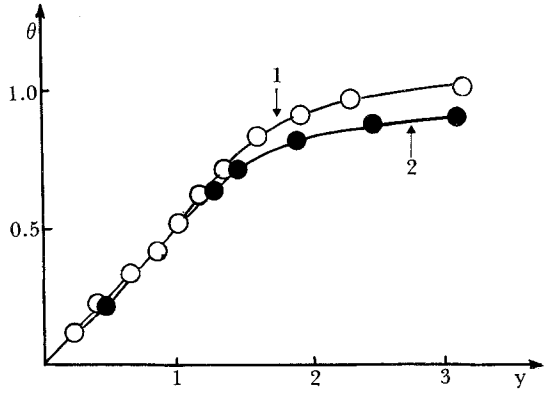

Figure 3. Experimental isotherm (1) of 2-methyl-4-chlorophenol and the theoretical isotherm (2) in the relative coordinates $\theta = C_0/C_\infty$ and $y = C/C_\theta = 0.5$; the latter was drawn using the calculated values of "a" and B.

Results

Evidence for the Existence of the Mitochondrial NADH Dehydrogenase Hydrophobic Site

We have found that different classes of aromatic compounds (Table I), i.e. esters. amides (17-22, 26), aniline derivatives (9-13) and phenols (1-8) with a low dissociation constant ($pK > 7.7$) [11], specifically inhibit electron transfer at the NADH-cytochrome *b* segment in the respiratory chain. The inhibiting effect of these compounds can be reversed partly by vitamin K_3 (Fig. 4).

The efficiency of the above substances does not depend upon the reactivity of the functional groups but, rather, is determined by the degree of their affinity to lipids. One can see in Fig. 5 that there is a correlation between the efficiency of the inhibitors and their partition coefficients (P) in the octanol-water system.

Since all of these substances belong to an aromatic series of compounds, two types of enzyme-inhibitor linkage can be considered: complex stabilization either at the expense of hydrophobic interactions, or as a result of π-complex formation between the aromatic nuclei of the substance and the enzyme.

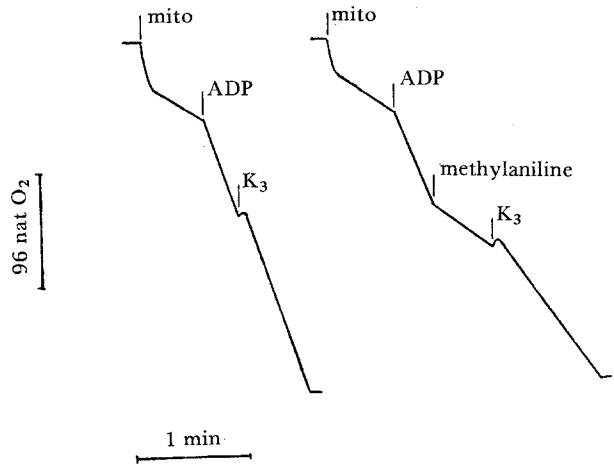

Figure 4. Vitamin K_3 reversal of the inhibition of glutamate-malate oxidation by electroneutral substances. ADP, Methylaniline and vitamin K_3 were added at the concentrations: 1 mM, 5 mM and 40 μM respectively.

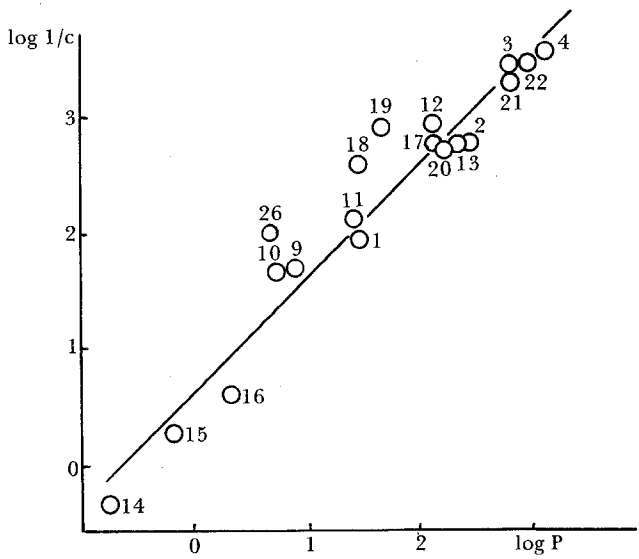

Figure 5. Inhibition of the glutamate and malate oxidation by rat liver mitochondria. Correlation between the inhibition effectivity (1/C) and the partition coefficient (P) in the octanol water system for electroneutral substances. C—the concentration of substances (M) required for 50% inhibition. The digits correspond to the numbers of the substances in Table I.

The latter possibility was ruled out after the action of aliphatic alcohols [14-15] on mitochondrial respiration had been studied. These compounds also specifically inhibited the respiratory chain at the site between NADH and cytochrome *b*. This effect is reversed by vitamin K_3 and the efficiency of the inhibitors depends upon their partition coefficients (P), as it did in the case of the aromatic series of substances (Fig. 5, Table I).

Vitamin K_3 is the electron translocator between NADH and cytochrome *b* [19, 20].

It is concluded from these data that in the NADH-cytochrome *b* site of the mitochondrial respiratory chain there is a functionally important hydrophobic region possessing a high affinity for various hydrophobic organic substances. Adsorption of the one of these substances on the surface of this site results in interruption electron transfer.

Nature of the hydrophobic site. The following properties of the hydrophobic region localized between NADH and cytochrome *b* have been revealed:

(1) Aromatic acids with relatively high dissociation constants cannot be adsorbed on this region. This is clearly seen when compounds in the phenol series with differing dissociation constants are compared (Fig. 6).

Phenols (1-3) with $pK > 7.7$ effectively inhibit SMP respiration. As shown in Fig. 6, the efficiency of these compounds increases with an increase in partition coefficient (P). Alternatively, the inhibiting capacity of phenols (4-8) with $pK < 7.7$ does not depend appreciably on the value of the partition coefficient. In liver mitochondria a sharp change in

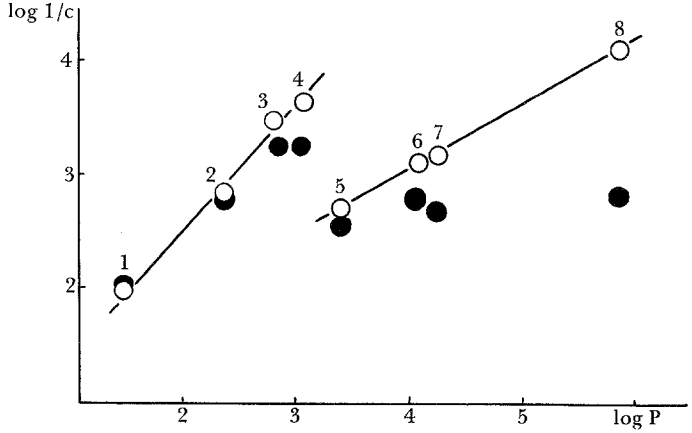

Figure 6. The phenol's action on the NADH oxidation by SMP (—●—) and on malate by rat liver mitochondria (—○—). C—the concentration of substances (M) required for 50% inhibition. The digits correspond to the members of the substances in Table I.

the inhibiting properties is also observed in the same pK range. Vitamin K_3 is capable of reversing the effect of only those phenols whose pK was > 7.7; it does not reverse the inhibiting effect of acidic phenols.

Aromatic acids (23-25) (pK ~ 5) have the same effect as phenols 4-8 and vitamin K_3 is unable to reverse this effect. The inhibiting efficiency of the acids increases as the distribution coefficient increases (Fig. 7).

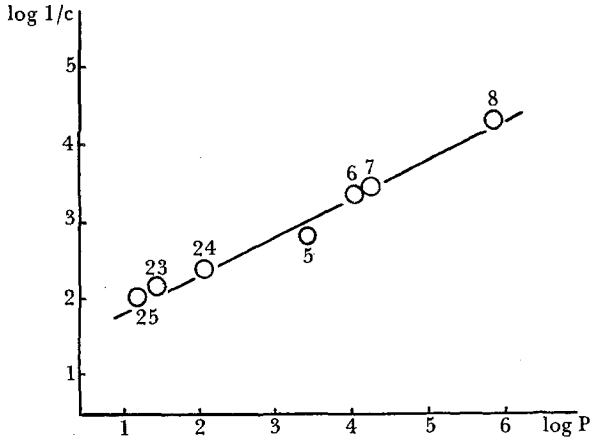

Figure 7. Inhibition of the glutamate and malate oxidation by rat liver mitochondria. Correlation between inhibition effectivity (1/C) and the partition coefficient (P) in the octanol-water system for strong acids.

Thus, it has been shown that there is a qualitative distinction between electroneutral compounds and strong acid which, under the experimental conditions employed, exist as anions. The first type of inhibitors preferentially suppress the enzyme which is localized between NADH and cytochrome *b*, the second type inhibits NAD-linked dehydrogenases [5, 6]. Binding of the inhibitors to the enzymes is apparently determined by two factors: the change of the molecule and the degree of its affinity for lipids. The fact that the inhibitors fall into two groups suggests that the hydrophobic sites on which they adsorb are also different.

(2) A study of electro-neutral molecules with low dipole moments (2-methyl-4-chlorophenol methyl ether and α-naphthol methyl ether) has shown that the properties of these compounds do not come under the general rule represented in Fig. 5. Their efficiency proved to be 10-100 times lower than could be expected on the basis of their partition coefficients. This means that the degree of inhibition of electron transport at the initial stages of the respiration chain depends not only

upon the value of the partition coefficient (P) of a given compound but also on some other parameter, such as the dipole moment.

(3) *Model of the NADH-dehydrogenase Hydrophobic Site*

In principle, two types of binding of hydrophobic inhibitors with the enzyme are possible. Either the inhibition is due to the inhibitor being dissolved in the hydrophobic phase (i.e. the molecule of the inhibitor is entirely submerged in the hydrophobic region of the enzyme), or the inhibitor molecule is adsorbed at the interphase boundary between the hydrophobic region and the polar environment.

As shown above, the inhibitory activity of the polar compounds is relatively higher than that of the non-polar ones. This led us to the idea that the hydrophobic region of the enzyme might have properties resembling those of the polar-non-polar liquids interphase. To verify this suggestion, we have looked into the relationship between the inhibiting efficiency of substances (1-4, 9, 12, 26) and their ability to adsorb on the interphase boundary.

Concentrations of inhibitors ($C_{\theta=0.5}$) at which half the area of the interphase is occupied by inhibitor molecules, were determined by measuring the interphase tension in the heptane-water system. It is seen in Fig. 8 that the $C_{\theta=0.5}$ values for each compound are approximately equal to the concentrations that suppress electron transfer by 50%. This quantitative agreement between the inhibiting concentrations and the concentrations for 50% occupation of the interphase boundary show that the hydrophobic region of the enzyme studied is very similar in its nature to the hydrocarbon-water interphase.

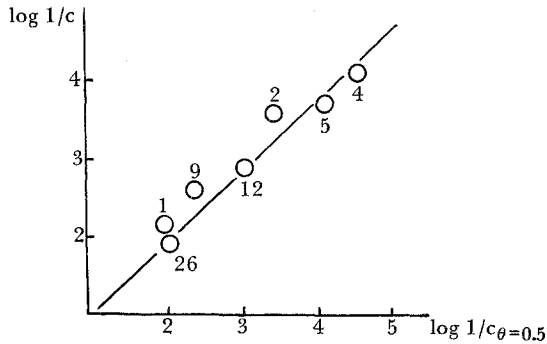

Figure 8. Correlations between concentrations of the hydrophobic inhibitors (1-4, 9, 12, 26) required to decrease the rate of mitcchondrial respiration by 50% (C_{50}) and the concentration of these substances in the water phase required for the surface tension of the heptane-water interphase to be filled to 50% ($C_{\theta=0.5}$), C–the concentration of substances (M).

It should be emphasized that with the interphase model there is a coincidence in the absolute values of the inhibitor concentrations occupying by 50% the interphase boundary and those inhibiting the enzyme by 50%. In contrast, the octanol-water system (Fig. 5) is merely a model of the relative changes in affinity to the hydrophobic site for the series of the inhibitors studied.

That the inhibitory properties of the compounds studied have to be correlated with two different parameters (Figs. 5 and 8) is explained by the fact that there is a quantitative correlation between ΔG of the passage from water to the interphase boundary and ΔG of the passage from water to octanol (Fig. 9). Conversely, no correlation was found between the biological efficiency of compounds (1-4, 9, 12, 26) and their partition coefficient in the hexane-water system.

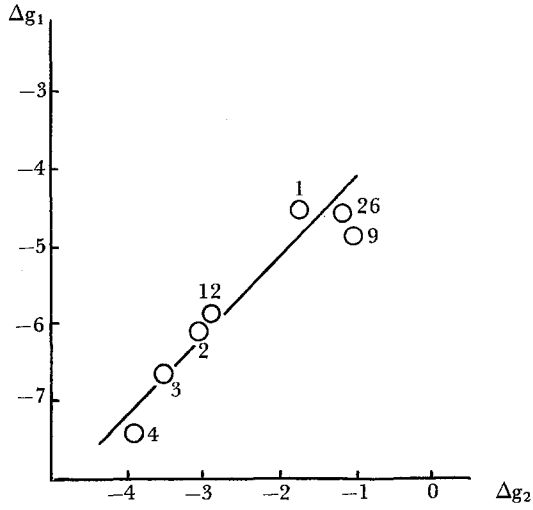

Figure 9. Dependence between the free energy of the transference of the substances from water to the water-heptane interphase (ΔG_1) and of the water-octonal transition (ΔG_2, kcal/M).

Discussion

It has been established that a hydrophobic site plays an important role in the functioning of some enzymes of the mitochondrial respiratory system. This leads to a number of important consequences:

(1) Since the hydrophobic region possesses an ability to adsorb non-specifically molecules of widely different classes of organic compounds, there are grounds for believing that the absorption of hydrophobic compounds by mitochondria should inhibit utilization of

reduced pyridine nucleotides and oxygen, both of which are substrates for non-specific microsomal oxidases. This might lend to a competition between microsomal and mitochondrial oxidases. The hydrophobic region of NADH dehydrogenase might, therefore, play the role in regulating the microsomal oxidase system.

(2) The existence of the hydrophobic region clarifies the role of by-passes in the respiratory chain. In the mitochondria of organs which are often subjected to attack by foreign compounds (e.g. liver, kidney), there are pathways by which electrons may bypass the NADH dehydrogenase of the respiratory chain. The electrons may be transferred from flavin to cytochrome *b* by the menadione reductase bypass. The second by-pass, the so-called free oxidation pathway involving cytochrome b_5 reductase and cytochrome b_5 serves to carry electrons from NADH to cytochrome *c*. These pathways of oxidation, upon being activated, may allow the mitochondria to synthesize ATP in the cytochrome oxidase coupling site, even in the presence of high concentrations of inhibitors which suppress the initial part of the respiratory chain.

It is important to note that there is a competitive relationship between the non-specific oxidases and the bypassing systems, since the high activity of the latter decreases the level of reduced pyridine nucleotides and oxygen which are substrates of the oxidases.

(3) Another important fact established in the present investigation is that the respiratory chain possesses two qualitatively different types of hydrophobic sites. The NADH dehydrogenase in the respiratory chain can be inhibited by electro-neutral molecules but not by anions. On the other hand, the region localized near the NAD-linked dehydrogenases readily adsorbs negatively charged molecules.*

(4) The differences in the affinity of charged and electro-neutral molecules for the respiratory chain (Fig. 6) permit the suggestion that the electron carrier localized at the hydrophobic site of NADH-dehydrogenase should leave the binding site upon reduction and then should adsorb on the site after being oxidized. In this way, one could account for a shuttle movement of certain respiratory chain carriers.

An example of this kind of carrier may be coenzyme Q, a polar aromatic molecule possessing a high lipid solubility. The reduced coenzyme (QH_2) may have properties similar to those of phenols with low dissociation constants, which, as was shown above, effectively bind to the hydrophobic region of NADH dehydrogenase. In this hypothesis, it is essential that the partially reduced form of coenzyme Q (i.e. the semiquinone) has the properties of a relatively strong acid (pK = 6.4)

* In this case, it is possible that adsorption of the aromatic molecule on the enzyme proceeds via π-complex formation.

(18) which, as shown above (Fig. 6), seems incapable of adsorbing on the hydrophobic region. Partial reduction of coenzyme Q or oxidation of coenzyme QH_2 into the semiquinone ought to result in the latter being released into the polar environment. Thus, electron transfer may be accomplished via the shuttle of coenzyme Q at the interphase boundary.

(5) A comparison of the equation derived from the curve for the hydrophobic interaction of electroneutral substances (Fig. 5) ($\lg 1/c = 1.04 \lg P + 0.646$) with the equation of Hansch and Anderson [4] ($\lg 1/c = 1.1 \lg P + 1.2$), which establishes the relationship between the effectivity of barbiturates and the value of their partition coefficients, allows the conclusion to be made that barbiturates are also hydrophobic inhibitors.

A small difference in free member values of equations obtained by Anderson and by us may be due to the fact that the same source was not used. Anderson used beef heart mitochondria, whereas in this work liver mitochondria were studied.

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