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The Effect of α-*N*-Substituted Histidine Derivatives on Bimolecular Lipid Membranes as Related to Their Ability to Uncouple Oxidative Phosphorylation

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A series of α-*N*-alkyl and α-*N*-aryl histidines was synthesized. Several of the more lipophilic derivatives were shown to be uncouplers of oxidative phosphorylation. A direct relationship was noted for the α-*N*-alkyl series between carbon chain length on the α-nitrogen of histidine, organic/water partition coefficient, efflux rate from liposomes, ability to lower electrical resistance of bimolecular lipid membranes, ability to increase respiration in coupled mitochondria, and ability to lower P/O ratios in coupled mitochondria. The aromatic derivative α-*N*-salicyl histidine was the best uncoupler in the series but was still not as effective an uncoupler as 2,4-dinitrophenol.

INTRODUCTION

Mitchell (*1*, *2*) first proposed that uncouplers of oxidative phosphorylation were lipid-soluble proton carriers which discharged an electrochemical gradient across the membrane. Lehninger and co-workers (*3*, *4*) showed that 2,4-dinitrophenol and several other uncouplers increased the electrical conductance of a synthetic bimolecular lipid membrane (BLM) by a sufficient amount to account for uncoupling in mitochondria according to Mitchell's chemiosmotic hypothesis. They further showed that the uncouplers were specific for proton conduction and had a maximum effect on the electrical conductance of BLM at a pH corresponding to the pK_a of the uncoupler (*4*), and they concluded that there was a direct relationship between an uncoupler's ability to disrupt respiratory control in mitochondria and its ability to increase electrical conductance across BLM. Several other workers later showed similar correlations for a number of uncouplers (*5*, *6*). However, Ting et al. (*7*), using 11 different uncouplers, showed that although most of the uncouplers increase electrical conductance across BLM, there was poor quantitative correlation between these parameters. Recently, Bakker et al. (*8*) have also shown a poor quantitative correlation between uncoupling in mitochondria and increasing of electrical conductance across BLM, but showed a good correlation between the ability to uncouple mitochondria and the ability to stimulate valinomycin-induced swelling, and ferrocene-mediated reduction of internal ferricyanide by external ascorbate in liposomes.

The uncouplers used in previous studies vary greatly in their chemical structures. Ting et al. (*7*) selected such a variety in order to determine if proton conductance alone

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were sufficient to account for all observed uncoupling. However, since enzymatic formation of the high energy bond must occur, it is not inconsistent with the Mitchell hypothesis for an uncoupler to act directly at the membrane-bound enzyme level as well as, or instead of, upon the proton gradient. Furthermore, as Bakker et al. assert (*8*), use of a single parameter (such as membrane conductance) may introduce artifacts due to the experimental conditions. Based on these considerations, we felt that the effect of a homologous series of potential proton carriers upon several membrane-related phenomena should be investigated.

We have synthesized a series of *N*-alkyl and *N*-aryl glycines and have shown that the diffusion rate of these derivatives across BLM is directly related to the organic/water partition of the derivative and hence, for the alkyl series, to the length of the carbon chain on the nitrogen (*9*). Since histidine contains a proton which is dissociable at physiological pH (imidazole p*K* is 6.0 (*10*)) its α-*N*-substituted derivatives should have the ability to shuttle protons across a membrane and to serve as uncouplers of oxidative phosphorylation. Benzimidazoles, in which the proton is carried on the imidazole ring, are known uncouplers of oxidative phosphorylation (*11*).

This paper reports the synthesis of a series of α-*N*-substituted histidines and compares: (i) the organic/water partition coefficients for the derivatives; (ii) the rate of efflux of the derivatives from liposomes; (iii) their effect on electrical resistance of BLM; and (iv) their ability to function as uncouplers of oxidative phosphorylation.

MATERIALS AND METHODS

The α-*N*-substituted histidines were synthesized by the procedure reported for *N*-substituted glycine (*9*). Histidine (Nutritional Biochemicals) was mixed with an excess of aldehyde and the resultant imine was reduced at pH 6.0 with sodium cyanoborohydride (Ventron). Cyanoborohydride was used because it has the ability to reduce imines faster than aldehydes (*12*). The progress of the reaction was followed by paper chromatography of aliquots in the solvent methanol/ethanol/water 9/9/2, which easily separates histidine ($R_f = 0.26$) from the α-*N*-substituted derivatives (R_f's range from 0.6 to 0.9). The histidine derivatives were visualized on paper by the Pauly spray which is specific for the imidazole ring (*13*). The derivatives were purified by paper chromatography followed by elution from Dowex 50-X8-200 (Sigma) by dilute aqueous ammonia. The water/chloroform partition coefficient of each derivative was determined by vigorously mixing 5.0 ml of chloroform with 5.0 ml of a 0.1 *M* KCl–0.05 *M* phosphate buffer, pH 7.0, containing various quantities (50–200 mg) of the histidine derivatives. After 24 hr, the phases were separated and the amount of α-*N*-substituted histidine in each phase determined by the Pauly color reaction (*13*). In all cases, the phases separated cleanly, and the solute recovery in the bulk phases was nearly 100% of the total added, indicating that no significant amounts accumulated at the interface.

Planar BLMs of phosphatidyl ethanolamine (7.52 mg, Nutritional Biochemicals) and cholesterol (7.74 mg, General Biochemicals, recrystalized five times from 96% ethanol) in 1.0 ml of decane were formed on a 2-mm-diameter orifice separating two chambers containing 0.1 *M* KCl in 0.05 *M* phosphate buffer at pH 7.0. Resistances were measured as previously described (*9*).

Liposomes of egg lecithin (Nutritional Biochemicals) and cholesterol were prepared

as described previously (*9*). Various amounts of the α-*N*-substituted histidines dissolved in the KCl–phosphate buffer were trapped inside the forming liposomes. After liposome formation, the unsequestered histidines were separated from those trapped inside the liposomes by column chromatography on Sephadex G-25 (Pharmacia) followed by dialysis for 3 hr against the KCl–phosphate buffer. The liposome suspension (2 ml) was then transferred to a fresh dialysis bag and dialyzed against 40 ml of the KCl–phosphate buffer for 24 hr. The amount of α-*N*-substituted histidine appearing in the dialysate after 24 hr of dialysis was determined colorimetrically. Diffusion rates are expressed as the percentage of α-*N*-substituted histidine originally trapped inside the liposome after the preliminary dialysis which diffused out after 24 hr. Since the volume of liposomes was less than 5% of the total dialysate volume, and a maximum 40% of the trapped solute was found in the dialysate, equilibrium was not approached in any of the diffusion rate experiments.

Coupled mitochondria were isolated from rat liver by the method of Johnson and Lardy (*15*). To eliminate possible metal ion contaminents, EDTA was added to all solutions and doubly glass-distilled water was used. Respiration was measured on a YSI Model 53 biological oxygen monitor using Clark-type polarographic electrodes. Tracings were made on a Sargent-Welch SRLG recorder. To 5.0 ml of a buffer containing 0.25 *M* sucrose, 10 m*M* KH_2PO_4, 6 m*M* $MgCl_2$, 20 m*M* KCl, 20 m*M* triethanolamine, and 1.0 m*M* EDTA at pH 7.4 was added 0.3 ml of the mitochondrial suspension at 26°C. This volume of mitochondrial suspension contained approximately 5 mg of protein, but the amount of each assay was not determined. However, each set of measurements comparing the uncoupling action of the series of compounds was done with a single preparation, so that the concentration was identical throughout the experiment. Although previous studies on the planar BLM and liposomes were done at pH 7.0, pH 7.4 was used in these experiments because it is the optimum for the mitochondrial oxidative phosphorylation. Succinate was used as the substrate and P/O ratios were determined as reported by Estabrook (*16*). The initial P/O ratio for succinate was approximately 1.8 and decreased as the mitochondria aged. All measurements were made within 4 hr of the mitochondrial isolation, and every 15 min the P/O ratio of the mitochondria in the absence of any uncoupler was determined.

Various amounts of α-*N*-substituted histidine in either absolute ethanol or water were added after a steady state rate of O_2 uptake in the presence of succinate was established. Ethanol alone, in the amount added (50 μl), had no effect. After a new respiration rate was established, the P/O ratio was determined by addition of limiting ADP. Results are reported both as the increase in respiration at 1 m*M* uncoupler and as the concentration of uncoupler required to give 50% uncoupling. The latter was determined by measuring P/O ratios at various uncoupler concentrations. All results are compared with the standard uncoupler 2,4-dinitrophenol.

RESULTS

Efflux of α-N-Substituted Histidines from Liposomes

The rate of diffusion of the α-*N*-substituted histidines from liposomes was determined and compared with the water/chloroform partition coefficient (Table 1 and Fig. 1).

TABLE 1

DIFFUSION OF HISTIDINE DERIVATIVES FROM LIPOSOMES COMPARED TO THE PARTITION COEFFICIENT ($H_2O/CHCl_3$) OF THE COMPOUND[a]

Compound	Amount diffused (%)	Partition coefficient
1. Histidine	0.8	15900
2. Imidazole	9.9	296
3. α-*N*-methyl histidine	0.95	2210
4. α-*N*-ethyl histidine	17.3	185
5. α-*N*-propyl histidine	19.0	152
6. α-*N*-butyl histidine	23.5	109
7. α-*N*-pentyl histidine	38.4	9.0
8. α-*N*-hexyl histidine	14.0	0.49
9. α-*N*-heptyl histidine	11.0	0.036
10. α-*N*-benzyl histidine	15.0	520
11. α-*N*-salicyl histidine	11.5	11.5

[a] The amount diffused indicates the percentage of compound originally sequestered inside egg lecithin–cholesterol liposome which diffused out after 24 hr.

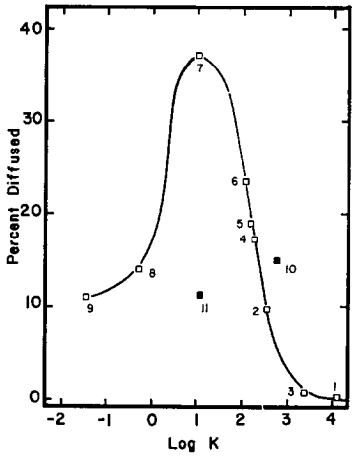

FIG. 1. Diffusion of histidine derivatives as a function of partition coefficient $K_{(H_2O/CHCl_3)}$. Data are those from Table 1. Point numbers correspond to the number of the compound in Table 1.

Each methylene added to the α-nitrogen resulted in a large increase in the lipophilicity of the derivative and also resulted in an increase in diffusion rate from α-*N*-methyl through α-*N*-pentyl, but then a decrease for the α-*N*-hexyl and α-*N*-heptyl derivatives. This correlation is similar to that reported for *N*-substituted glycines (*9*) where the diffusion rate increased from *N*-methyl to *N*-hexyl but then decreased for *N*-nonylglycine, probably due to the more lipophilic derivatives remaining in the membrane rather than diffusing across it. To show whether the liposomes were becoming disrupted (i.e., "leaky") in the presence of the α-*N*-substituted histidines, [^{14}C]glucose or

[^{14}C]glycine, both of which are known to diffuse very slowly across BLM (*17*), were cosequestered with the α-*N*-alkyl histidine in the liposomes. Since neither the α-*N*-butyl, α-*N*-pentyl nor the α-*N*-hexyl histidines greatly affected the rate of efflux of [^{14}C]glucose or [^{14}C]glycine, we assumed that the measured rate of α-*N*-alkyl histidine diffusion was in fact the true rate of diffusion of the compound and not the result of damaging effect of the derivatives on the membrane integrity. As shown in Fig. 1, the diffusion of the α-*N*-aryl derivatives (α-*N*-salicyl histidine and α-*N*-benzyl histidine) did not follow the same correlation with distribution coefficient as did the α-*N*-alkyl series.

Effect of α-N-Substituted Histidines on Electrical Resistance of BLM

Since the α-*N*-substituted histidines were shown to diffuse across BLM and had pK_a's between 5.0 and 7.0,[3] they should affect the electrical resistance as does 2,4-dinitrophenol, as shown by Lehninger (*3*, *4*). The electrical resistance across the membrane bathed in solutions of α-*N*-substituted histidines in concentrations from 0.1 to 10 m*M* in the KCl–phosphate buffer was measured. Figure 2 shows the decrease in membrane resistance as a function of concentration of *N*-salicyl histidine. All com-

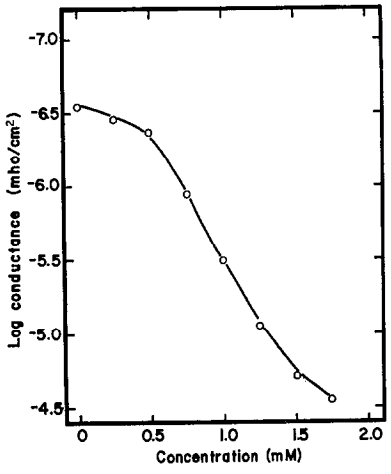

FIG. 2. The effect of concentration of *N*-salicyl histidine on electrical conductance on a planar BLM at pH 7.2. Membrane and measurements were as described in the text.

pounds gave similar curves. Histidine, imidazole, α-*N*-methyl histidine, and α-*N*-ethyl histidine did not affect the normal electrical resistance (4×10^6 ohm·cm^2) of a phosphatidyl ethanolamine–cholesterol–decane membrane. Table 2 shows the increase in conductance (decrease in resistance) relative to the unmodified membrane due to the addition of α-*N*-substituted histidines at 1.5 m*M* and also compares the ability of these compounds with that of an equal concentration of 2,4-dinitrophenol to increase electrical conductance. The ability to increase electrical conductance increases as the

[3] The p*K* of the imidazole ring was assumed to be little affected by the substitution of the amino group. Titration curves for histidine, *N*-hexylhistidine, and *N*-formyl histidine indicated imidazole pK_a's between 6.0 and 6.5. *N*-salicyl histidine did not yield a sharp titration curve but did appear to have pK_a's at about 5 and 7.5 but not in the region of 6–7.

TABLE 2

ELECTRICAL CONDUCTANCE INCREASE (RESISTANCE DECREASE) IN THE PRESENCE OF THE α-*N*-SUBSTITUTED HISTIDINES[a]

Compound	Increase in electrical conductance	Percentage increase in electrical conductance relative to 2,4-dinitrophenol
None	[1]	
2,4-Dinitrophenol	330	[100.0]
α-*N*-propyl histidine	3	0.92
α-*N*-butyl histidine	8	2.45
α-*N*-pentyl histidine	18	5.5
α-*N*-hexyl histidine	37	11.3
α-*N*-heptyl histidine	74	22.3
α-*N*-benzyl histidine	2	0.60
α-*N*-salicyl histidine	111	34.0

[a] Each compound was present in the buffer in which the membrane was formed in a concentration of 1.5 m*M*.

chain length increases from α-*N*-propyl to α-*N*-heptyl histidine. α-*N*-salicyl histidine had by far the greatest effect on electrical conductance, being 34% as effective as 2,4-dinitrophenol. The presence of the *ortho* hydroxyl group on the benzene ring greatly affects both the partition coefficient and the ability to increase conductance.

Effect of α-N-Substituted Histidines on Oxidative Phosphorylation

Since several of the α-*N*-substituted histidines were shown to increase electrical conductance across BLM, the effect of α-*N*-butyl, α-*N*-pentyl, α-*N*-hexyl, α-*N*-heptyl, α-*N*-benzyl and α-*N*-salicyl histidine on coupled mitochondria was measured. A characteristic of all uncouplers of oxidative phosphorylation is that they increase the respiration rate of mitochondria when phosphate acceptor is limiting (state 4) (*18*). The α-*N*-substituted histidines all were shown to increase the respiration rate of mitochondria in the absence of ADP, although the increase was not as large as with 2,4-dinitrophenol (Table 3). The ability to release respiratory control was directly related to the chain length on the α-nitrogen and hence to the organic/water partition coefficient. Again, α-*N*-salicyl histidine was shown to be the most effective derivative and was far better than α-*N*-benzyl histidine.

The α-*N*-substituted histidines were also shown to lower the P/O ratio of coupled mitochondria and so were true uncouplers (Table 3). The concentrations of an α-*N*-substituted histidine required to reduce the P/O ratio by 50% was estimated from measured P/O ratios at several concentrations of the derivative. The ability to uncouple increased from α-*N*-butyl through α-*N*-heptyl histidine in direct relation to the partition coefficient. α-*N*-salicyl histidine again proved to be the best uncoupler and was much better than α-*N*-benzyl histidine. Table 3 shows the ability of these derivatives compared

TABLE 3

ABILITIES OF THE α-*N*-SUBSTITUTED HISTIDINES TO INCREASE RESPIRATION AND DECREASE P/O RATIOS IN COUPLED MITOCHONDRIA, COMPARED WITH 2,4-DINITROPHENOL

Uncoupler	Increase in state 4 respiration rate caused by 1 m*M* uncoupler	Relative efficacy in increasing respiration[a]	Uncoupler conc. (m*M*) resulting in 50% uncoupling	Relative efficacy in lowering P/O ratios
None	[1.0]	—	—	—
2,4-Dinitrophenol (0.01 m*M*)	1.57	[100]	0.029	[100]
α-*N*-butyl histidine	1.13	0.17	4.50	0.65
α-*N*-pentyl histidine	1.26	0.81	3.36	0.87
α-*N*-hexyl histidine	1.60	1.02	1.77	1.65
α-*N*-heptyl histidine	3.22	2.06	0.764	3.84
α-*N*-benzyl histidine	1.04	0.67	6.89	0.43
α-*N*-salicyl histidine	3.41	2.18	0.185	15.80

[a] Based on extrapolation from 1.57-fold increase at 0.01 m*M* dinitrophenol.

with 2,4-dinitrophenol to uncouple. Once again 2,4-dinitrophenol was a more effective uncoupler than any of the histidine derivatives.

DISCUSSION

The α-*N*-alkyl and α-*N*-aryl histidines which have a sufficiently high organic/water partition coefficient function to increase conductance of BLMs and as uncouplers of oxidative phosphorylation. Within the α-*N*-alkyl series the partition coefficient, the ability to increase electrical conductance, the ability to release respiratory control in coupled mitochondria, and the ability to lower P/O ratios in coupled mitochondria increase with increasing chain length. There is an understandable discrepancy with the diffusion rate across BLM. As noted in Table 1 and Fig. 1, the diffusion rate was much lower for the α-*N*-hexyl and α-*N*-heptyl derivatives, but of the alkyl derivatives, these two were the most effective at increasing the electrical conductance and uncoupling oxidative phosphorylation. This discrepancy is probably due to the more lipophilic nature of the two derivatives. The more hydrophilic derivatives can diffuse away from the membrane where they are detected in the dialysate. The α-*N*-hexyl and α-*N*-heptyl histidines, on the other hand, probably diffuse into and cross the BLM more readily than do the other derivatives but they tend to remain associated with the membrane and so do not appear in the dialysate and so give a lower apparent transport rate. The fact that they affect the electrical properties of BLM and P/O ratios of coupled mitochondria to the extent that they do is probably because of their facile diffusion into and mobility within the membrane. Thus, they can readily shuttle protons through the membrane, resulting in increased membrane conductance, and discharging of proton gradients.

As seen in Table 4, there is a close correlation between the relative effect on membrane conductance and the relative effect on P/O ratio of coupled mitochondria within the alkyl series of derivatives. If increase in state 4 respiration is considered, the correlation is not quite as good, the factor varying by threefold. This variation is small, however, compared to the large variations seen by Ting et al. (*7*), and may not be significant because of the small change in respiration observed at 1 m*M* concentration of some of the derivatives.

TABLE 4

COMPARISON OF THE EFFECT OF HISTIDINE DERIVATIVES ON MEMBRANE CONDUCTANCE AND P/O RATIO OR RESPIRATION OR COUPLED MITOCHONDRIA[a]

Compound	Relative effect on			Correlation factor	
	Conductance	P/O	Respiration	Cond/(P/O)	Cond/Resp
Butyl histidine	2.45	0.65	0.71	3.8	3.5
Pentyl histidine	5.5	0.87	0.81	6.3	6.8
Hexyl histidine	11	1.65	1.02	6.7	10.8
Heptyl histidine	22	3.84	2.06	5.7	10.7
Benzyl histidine	0.6	0.43	0.67	1.4	0.9
Salicyl histidine	34	15.8	2.18	15.6	15.7

[a] Data are those from Tables 2 and 3. The correlation factor is the ratio of relative effect on conductance to relative effect on P/O or respiration.

α-*N*-salicyl histidine was far more lipophilic and had a far greater effect on both the electrical properties of BLM and coupled mitochondria than did α-*N*-benzyl histidine. The presence of the ortho hydroxy group greatly affected the properties of the molecule. The effects of α-*N*-aryl histidines do not appear to correspond well with the α-*N*-alkyl histidines. Thus, factors other than distribution across the water–lipid interface must also influence a particular compound's ability to diffuse across a membrane or to affect other membrane properties. It is not surprising, therefore, that poor correlation between uncoupling and altering of electrical conductance by various uncouplers of diverse chemical structure is observed (*7*, *8*). Our approach of correlating such properties among an homologous series of compound appears to offer greater promise in elucidating mechanisms of membrane-related phenomena.

We have not considered the effect of pH or alteration of the imidazole pK_a upon the membrane phenomena. A detailed study of pH effects was beyond the scope of this present work, but in view of the fact that *N*-salicyl histidine does appear to have a significantly altered p*K* of the imidazole group and also shows other anomalous properties when compared to the alkyl-substituted histidines, such a study should be undertaken.

It has been shown here for a homologous series of uncouplers, all carrying protons by the same mechanism, there is a definite relationship between partition coefficient, diffusion rate, ability to lower electrical resistance, ability to release respiratory control in coupled mitochondria, and ability to lower P/O ratios in coupled mitochondria.

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