Equilibrium and Kinetic Effects of Sixteen Compounds on the Forms of Horse Heart Ferricytochrome *c*†

George H. Czerlinski* and Rita A. Galuszka

Department of Biochemistry, Northwestern University Medical School, Chicago, Illinois 60611. *Received July 1, 1971*

Equilibria and kinetics of the forms of horse heart ferricytochrome *c* were investigated in the pH range 7–10 in the presence of a variety of substances with the intent to find effectors which bind preferentially to one of the several forms. The absorption band at 695 mμ was used as indicator and is attributed to the form of ferricytochrome *c* which participates in electron transfer. Preferential binding would then be indicated either by an increase or a decrease in the absorption. Of a total of 16 tested compds, 4 showed an absorption increase of more than 20% (ADP, NaN_3, pyrazole, thiazole), while 2 showed an absorption decrease of more than 20% (aniline, imidazole), 5 compds were within 5% of the value of ferricytochrome *c* in buffer (AMP, benzimidazole, pyrimidine, thiazolidine-4-carboxylic acid, and thiophene-3-carboxylic acid), 3 compounds showed an intermediate increase in absorption (ATP, barbituric acid, 2,4-dinitrophenol) and 2 compds an intermediate decrease (hydantoin-5-acetic acid, pyrrolidine). These changes were observed at pH 7.4 within a few seconds after injection of cytochrome *c* into the reaction mixture. If the pH of horse heart ferricytochrome *c* in buffer is quickly changed from 7.4 to 10, 75% of the 695 band disappears with a time constant of about 1 sec. In presence of the various test substances, the same quick pH change reduced the 695 band to an extent and with a time constant, which is characteristic of the specific compound. The changes were smallest with imidazole and largest with thiazolidine-4-carboxylic acid. The time constant in presence of hydantoin-5-carboxylic acid probably exceeds the time resolution of the experimental arrangement (0.2 sec). The time constant for the disappearance of the 695 band is increased by about a factor of 10 in presence of AMP. The values in presence of all other compds lie in between. If the pH of horse heart ferricytochrome *c* in buffer is quickly changed back from 10 to about 7.4, the 695 band reappears with a time constant of close to 8 sec. The absolute value of the change back to pH 7.4 is generally somewhat smaller than the original absolute change in absorption (for pH 7.4 → 10), in some cases (*e. g.*, in presence of benzimidazole) substantially smaller, but larger in the presence of imidazole. The 695 band reappears generally slower in the presence of a test substance (slowest for thiophene-3-carboxylic acid) than in its absence; exceptions are imidazole (fastest reappearance), hydantoin-5-acetic acid, and thiazolidine-4-carboxylic acid.

Brandt, *et al.*,[1] demonstrated that the pH dependence of the redox potential of horse heart cytochrome *c* is due to a rather slow interconversion of different structural forms of the protein. This interconversion is proton coupled and only the form(s) stable at pH 7 are quickly reducible by ferrohexacyanide. For this specific electron transfer Brandt, *et al.*,[1] obtained rate constants from temperature jump experiments, which are in reasonable agreement with results of Sutin and Christman[2] and of Havsteen.[3] The proton release upon oxidation of ferrocytochrome *c* by ferrihexacyanide was subsequently investigated in detail by Czerlinski, *et al.*[4,5] Subsequent "pH-switching" experiments (see type IV experiments below) led to two apparent rate constants, which suggested the reaction cycle shown in Figure 1, Scheme A.

The equilibrium constant between components 1 and 4 of this scheme of Figure 1 is 20, a value which can be derived from the earlier data of Schejter and George.[6] The equilibrium constant between components 2 and 3 favors component 3 and the interconversion constant was estimated[4] at 3. With these numbers for the 2 interconversion constants and the apparent protonic dissociation constant of the overall reaction of $pK_1 = 9.1$, one obtains $pK_H' = 9.6$, and $pK_H'' = 7.8$ (see Figure 1, Scheme A, for definitions).

Differences between the oxidized and reduced forms of cytochrome *c* have been known for some time, the oxidized protein having a more open and flexible conformation than the reduced form.[7] Among recent reports in this connection are studies on the optical rotary dispersion[8-11] and on circular dichroism[12,13] of the protein. The above mentioned isomerization of ferricytochrome *c* at alkaline pH to form a species with altered electron-transfer properties was also discussed by Greenwood and Palmer,[14] Urry,[15] and Myer and Harbury.[10] Watt and Sturtevant[16] determined the enthalpy of oxidation of ferricytochrome *c* over the pH range 6.0–10.9 and were also able to derive thermodynamic parameters for the interconversion between component 1 and 3 of Scheme A of Figure 1. Since these were equilibrium experiments, they had no information as to intermediate steps betwen components 1 and 3.

The original intent of the present investigation was to verify Scheme A of Figure 1 by using "effectors", which would hopefully exclusively bind to either form 1 or form 3. No such *exclusive* binding was revealed upon studying the following possible complexing agents: AMP, ADP, ATP, hydantoin-5-acetic acid, thiophene-3-carboxylic acid, 2,4-dinitrophenol, benzimidazole, imidazole, pyrazole, thiazole, thiazolidine-4-carboxylic acid, pyrrolidine, pyrimidine, $PhNH_2$, NaN_3, barbituric acid, amytal, and antimycin A (the last 2 in 40% EtOH). Many of the listed substances are known to have medicinal effects. It was therefore also of interest to look at the effects of these substances upon the most thoroughly characterized memeber of the electron transfer chain of the inner mitochondrial membrane: ferricytochrome *c*. Some of the substances developed demonstrated considerable ligand-induced conformational changes (either enhancing or counteracting the previously shown pH-induced conformational change).

Experimental Section

Horse heart cytochrome *c* Type III was obtd from Sigma Chemical Co. and further purified according to Margoliash and Lustgarten,[17] adding initially an equimolar amt of sodium ferrihexacyanide. All experiments involved the simultaneous observation of transmission changes at 633 mμ (a high shoulder of the 695 band) characteristic of protein conformation[6] and of pH changes, using a thermostated Pyrex vessel with N_2 blown over the soln to diminish CO_2 pickup ($7.0 \leq pH \leq 10.1$). The spectral change was observed

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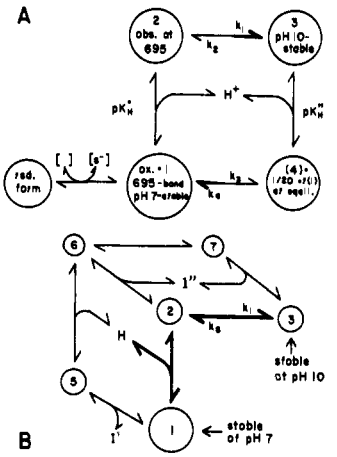


Figure 1. A. Reaction scheme of Czerlinski, *et al.*,[4] depicting 4 different forms of oxidized cytochrome *c*. The oxidation of reduced cytochrome *c* was performed with sodium ferrihexacynanide. This scheme is based on both equilibrium and kinetic experiments. B. A reduced scheme, implying only oxidized cytochrome *c* and inhibitor I reacting with any 1 of the 3 presented forms of cytochrome *c*. I' and I'' are one and the same inhibitor I, but differently labeled here for later discussions.

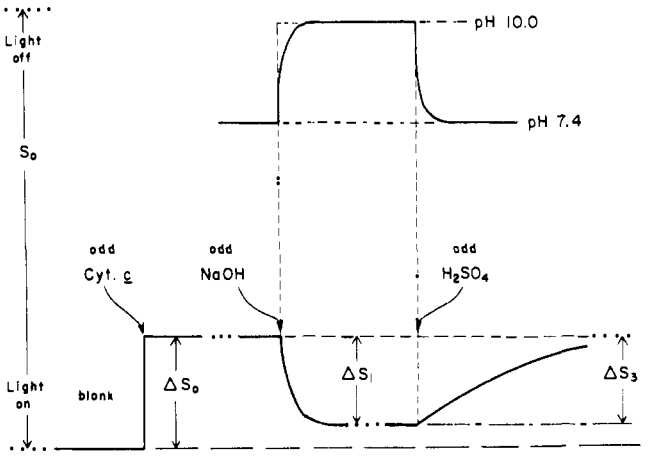


Figure 2. Schematic demonstration of an experimental sequence, indicating 2 recorder traces and defining the various signal changes. S_0 represents the signal before addition of cytochrome *c* and obtained such that the light beam through the test vessel was shut off by a piece of black paper. "Add cyt *c*" represents the quick injection of 500 μl of (nominally) 2 m*M* cytochrome *c* stock into 4.5 ml of a solution of the test substance (type II experiments). ΔS_0 represents thus the equilibrium signal change, associated with this injection (measured in mV, as all signals). "Add NaOH" represents the quick injection of enough 1 *N* NaOH, to reach (or exceed) pH 10 (type IV experiments); in some cases, more and/or stronger NaOH was needed to reach pH 10 (compare discussion of Table II). ΔS_1 represents the observed equilibrium signal change, associated with this injection. "Add H_2SO_4" represents the quick injection of enough 1 *N* H_2SO_4, to return the pH to 7.4 (type IV experiments); the equivalents of H_2SO_4 added generally equaled the equivalents of NaOH added before. ΔS_3 represents the observed equilibrium signal change associated with this last injection.

with a small He-Ne laser, University Laboratories Model 200, utilizing a beam splitter and 2 Ge photodiodes. The pH change was observed with a combination glass electrode, Type 14073 of Instrumentation Laboratories, and Radiometer pH-meter Model 26. The system was weakly buffered by 10 m*M* sodium phosphate and 4 m*M* glycine in all solns, except for those where the test substance has a pK_H in the range under investigation. The signals were fed into different channels of a Beckman Type RB Dynograph (with Type 9853 plug-ins). The 4 types of experiments are briefly described in the next paragraphs.

Type I. Oxidized cytochrome *c* (5.00 ml, 0.2 m*M*) in the above buffer and 0.030 *M* Na_2SO_4 were adjusted to pH 7.40 (or 10.00) within the thermostated vessel (25°). Fifty μl of 22 m*M* test substance in buffer, adjusted to pH 7.40 (or to 10.00), was then injected quickly. Spectral and pH changes were recorded simultaneously. Fifty μl of 10 m*M* H_2SO_4 were injected after equilibration to calibrate the pH changes. Spectral changes were calibrated by a "blank" of type IV (below).

Type II. Test substance (4.50 ml, 0.11 *M*) (for deviating concns, see Table II) in the above buffer and enough Na_2SO_4 to maintain constant ionic strength (0.1 *M*) thorughout all types of experiments, was adjusted to pH 7.40 (or 10.00) within the thermostated vessel (25°). Oxidized cytochrome *c* (500 μl, 2 m*M*) in buffer (ionic strength adjusted to 0.1 with Na_2SO_4), adjusted to pH 7.4 (or 10.00), was then quickly injected and changes recorded. In the control, Na_2SO_4 was substituted for the test substance (compare type I experiments).

Type III. Cytochrome *c* was reduced by the addition of powdered sodium dithionite (purified, low in Fe, of Fisher Scientific Co.) to its soln in such an amt that twice the molarity of cytochrome *c* could have been reduced. The soln was then dialyzed 3 times against the buffer mixt and spectrophotometrically tested for purity (98% or more in the reduced form). Reduced cytochrome *c* (5 ml, 0.2 m*M*), in 0.10 *M* test substance and the above buffer with Na_2SO_4 to adjust the ionic strength to 0.1, if necessary, was adjusted to pH 7.40 (or 10.00) at 25°. $Na_3Fe(CN)_6$ (22 m*M*, 50 ml) in the above buffer (pH 7.40 or 10.00) was quickly injected, and signal changes were recorded. The control contd Na_2SO_4 in place of the test substance. Injection of H_2SO_4 followed after equilibration (as for type I experiments above).

Type IV. Oxidized cytochrome *c* (5.00 ml of 0.2 m*M*) in the above buffer and 0.10 *M* test substance (or 0.030 m*M* Na_2SO_4 for the control) were adjusted to pH 7.40 (or 10.00) at 25°. NaOH (1 *N*, 40 μl) was injected quickly, to bring the pH to about 10. Upon equilibration, 40 μl of 1 *N* H_2SO_4 was quickly injected, to bring the pH back to 7.4. To observe slow changes of pH after the rapid change in pH, proper biasing of the channel measuring pH changes is necessary. These are calibrated again by injections of 50 μl of 10 m*N* H_2SO_4.

Experiments of type IV could easily follow the other 3 types of experiments. Most frequently, we carried out first type II, followed by type IV experiments. To illustrate the sequence of these experiments, Figure 2 is presented. Figure 2 introduces also the various symbols used in the evaluations.

Evaluation of Kinetic Data. As the observed changes in pH and in optical transmission are quite small, the relevant differential equations may easily be linearized. The change of the trace on the graph is given by

$$dx/dt = -\lambda x \quad (1)$$

with x = particular ordinate value on the graph (in mm, convertible to mV). As the abscissa is directly recorded in seconds, the apparent rate constant λ is given in sec^{-1}. One obtains in zero approximation

$$x = \bar{x} \exp(-\lambda t) + a \quad (2)$$

with $\bar{x}$ equal to the equilibrium change, extrapolated back to $t = 0$. The constant term a (with $a \ll \bar{x}$) takes care of any (initial) *rapid* change. A semilog arithmic plot results in a straight line with a slope of λ. Most of our kinetic evaluations, however, were performed by using a computer program of Berman.[18]

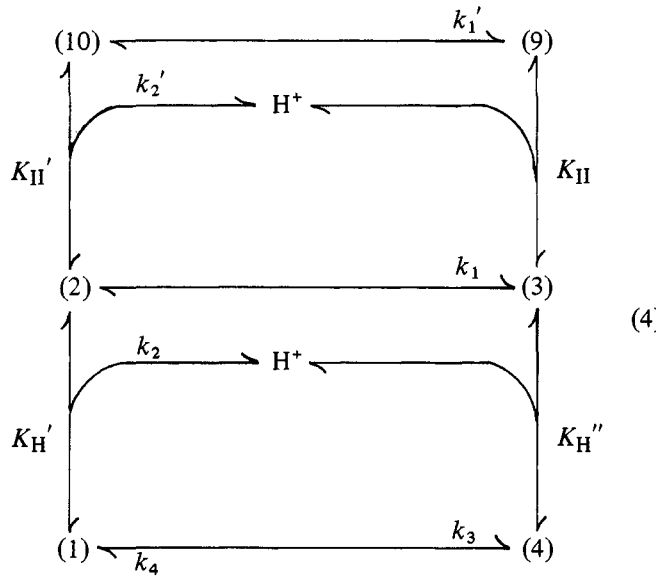
The above equations imply that the time-dependent change can be properly represented by a single exponential decay curve. This is not necessarily so, but in these surveying experiments this was assumed in first approximation. If Scheme B of Figure 1 applies, a stepwise pH change from 10 to 7 cytochrome *c* alone (that is without test substance present) should directly lead to the rate constant k_2, as the protonic step proceeds much faster than the isomerization step.[4,5] However, for pH switching from component 1 to 3, in the absence of inhibitor, one could only expect to obtain a limiting value for the rate constant k_1. Nevertheless, this lower limit is of value in further discussions of the applicability of this scheme.

The rate constants, derived from a stepwise pH change for the cytochrome *c* system are dependent upon the final pH value. The magnitude of the total signal change upon a stepwise pH change is a function of the initial and the final pH. The signal change for the linearizable case (relatively small transmission changes) is given by

$$\Delta S = \eta(c_1 + c_2) \quad (3)$$

It is assumed in this equation that a signal derives only from components 1 and 2 and both components have the same coeffi-cient η (in mV/μ*M*).

The reaction scheme may be written in the general form (not considering effectors).



(4)

One may then consider two cases.

Case I. $K_{II}' \ll K_{II}$. This condition allows one to neglect K_{II}', k_1' and k_2'. For this reduced scheme one may easily derive for the relative signal change

$$1 - A = \frac{\bar{\bar{c}}_1 + \bar{\bar{c}}_2}{c_1^\circ} = \frac{1 + \bar{\bar{c}}_H/K_H'}{(1 + 1/K_{4,3})\bar{\bar{c}}_H/K_H' + (1 + K_H/\bar{\bar{c}}_H)K_H'/\bar{\bar{c}}_H} \quad (5)$$

One obtains for the apparent rate constant $\lambda = 1/\tau$

$$\tau - 1 = \frac{k_1}{1 + \bar{\bar{c}}_H/K_H'} + \frac{k_2}{1 + \bar{\bar{c}}_H/K_H'' + K_{II}/\bar{\bar{c}}_H} + \frac{k_3}{1 + K_H'/\bar{\bar{c}}_H} + \frac{k_4}{1 + (1 + K_{II}/\bar{\bar{c}}_H)K_H''/\bar{\bar{c}}_H} \quad (6)$$

Case II. $K_{II}' = K_{II}$. This condition leads automatically to the fact that $k_2'/k_1' = K_{2,1}$. If also $k_1' = k_1$, the system of eq 4 may be treated as if the upper cycle is not present at all (assuming that component 10 has the same η as components 1 and 2). The equations for the reduced cycle are easily obtained by setting $K_{II} = 0$ in eq 5 and 6. For further details see the Appendix.

Results

The experimental results are listed below in two tables.

Table I summarizes the results from the control (line 1) and 3 representative inhibitors. Controls were measured on every day on which materials were tested for preferential binding. Two to six tests of the type shown in Figure 2 were generally conducted on each test substance. Only those results were used for further evaluation, where the curves indicating the pH change most closely approximated a stepwise change.

Table II summarizes the results obtained on all materials tested for preferential binding in an abbreviated manner. $\Delta S_1'/\Delta S_0'$ and $\Delta S_3'/\Delta S_0'$ should be compared with the corresponding values for the controls, $\Delta S_1/\Delta S_0 = 0.44$ and $\Delta S_3/\Delta S_0 = 0.38$. The larger the deviations from these control values are, the stronger is the effect by the test substance. Deviations within ±10% cannot be considered significant. The limiting overall response time of the experimental arrangement is close to 0.2 sec, which is in good agreement with the largest λ_1* found experimentally. One derives, with λ_1* 1.04 sec^{-1} for the control from Table I together with $\lambda_1'/\lambda_1 = 4.7$ from Table II for the hydantoin-derivative, λ_{max} 4.9 sec^{-1}. Any λ_1'/λ_1 between 1 and 5 may be corrected for this overall response time (omitted here).

All the compounds in Table I were tested for equivalent proton release upon injection of cytochrome *c*. Unfortunately, the experimental error could not be reduced below 0.2 equiv. Upon the injection of cytochrome *c*, almost all inhibitors showed a change of pH which corresponded to not more than the given error. There were 3 exceptions. Thiophen-3-carboxylic acid, dinitrophenol, and $PhNH_2$ showed a proton release of 1 equiv within the experimental error. However, *no* proton change was detectable with imidazole and pyrimidine, apparently due to the buffering capacity of these compounds since upon injection of the calibrating acid, no pH change could be detected.

The data in Tables I and II do not reveal that the vol of injected base (or acid) was not always the same. Generally, around 30 μl of 1 *N* acid or base was used, but twice this amount was needed for the 3 adenosine phosphates and for benzimidazole; 1.5 times this minimum amount was needed for pyrrolidine and 2.5 times this amount for aniline. NaOH (12 *N*, 15 μl) was needed for imidazole and twice this amount for hydantoin-5-acetic acid. These larger amounts of acids and bases are needed for those compounds, which have protonic dissociation constants at or near the experimental range.

Table II does not reveal that the systems with the thiazolidine-4-carboxylic acid undergo a slow change, which was the principal reason for our use of 0.02 *M* of this test substance in the experiments listed in Table II. The apparent rate constant of this slow change was found to be a function of the analytical concn of the thiazolidine derivative and of pH. When the reaction mixture of the thiazolidine derivative with ferricytochrome *c* was kept long enough, the color of the reduced cytochrome *c* appeared. We suspected that the S of thiazolidine might have replaced the S

Table I. Summary of Representative Results[a]

No.	Effector (0.1 *M*)	S_0, mV	ΔS_0, mV	pH_0	ΔS_1, mV	ΔS_1*, mV	λ_1*, sec^{-1}	pH_1	ΔS_3, mV	ΔS_3*, mV	λ_3*, sec^{-1}	pH_3
1	None	1535	63	7.425	28	29 ± 1	1.04 ± 0.03	10.049	24	22 ± 0.6	0.120 ± 0.007	7.460
2	Azide (Na)	1550	81	7.440	46	44 ± 0.6	0.63 ± 0.02	9.960	42	37 ± 0.7	0.070 ± 0.003	7.440
3	Benzimidazole	1550	62	7.469	28	30	0.42 ± 0.02	11.150	10	11 ± 0.5	0.067 ± 0.007	7.635
4	Imidazole	1525	43	7.371	9	8.3 ± 0.2	1.93 ± 0.07	10.015	11	5 ± 0.2	0.31 ± 0.02	7.469

[a] The initial signal S_0 is defined in Figure 2, as well as the ΔS_i; however, the ΔS_i in this table are normalized for identical initial signal (reference S_0 = 1500 mV) such that the experimentally obtained $(\Delta S_i)_{obsd}$ is multiplied with the factor $(1500/S_0)$. The three pH values refer to the pH measured after the new equilibrium value is reached and are thus subscripted like the preceding ΔS. The pH before injection of cytochrome *c* is generally within 0.05 unit of pH_0. Four parameters in the table are superscripted by an asterisk to indicate that the listed values have been obtained by an iterative computer program; the standard error is also listed together with these values. The subscript refers to the same (equilibrium) signal change as for ΔS_i; λ_i* represents a pH-dependent (apparent) rate constant and appears as parameter in $\Delta S_i(t) = \Delta S_i^* \exp(-\lambda_i^* t) + \alpha^*$, where α* represents a computer-generated constant to optimize the fit of the theoretical curve to the data. These results are needed for Table II.

Table II. Summary of Evaluation Results[a]

Effector	pH_1	$\Delta S_1'/\Delta S_0'$	$\Delta S_3'/\Delta S_0'$	$\Delta S_0'/\Delta S_0$	λ_1'/λ_1	λ_3'/λ_3
AMP	10.001	0.46	0.50	0.97	0.10	0.35
ADP	10.140	0.44	0.37	1.21	0.28	0.46
ATP	10.158	0.45	0.38	1.17	2.9	0.76
Aniline	11.228	0.39	0.35	0.79	2.1	0.42
Azide(Na)	9.960	0.57	0.54	1.29	0.61	0.58
Barbituric acid	10.110	0.44	0.50	1.16	0.48	0.37
Benzimidazole	11.150	0.45	0.16	0.99	0.40	0.56
2,4-Dinitrophenol	9.970	0.60	0.34	1.1	0.43	0.27
Hydantoin-5-acetic acid	10.921	0.49	0.41	0.81	4.7	1.67
Imidazole	10.015	0.21	0.26	0.69	1.8	2.58
Pyrazole	10.234	0.62	0.47	1.28	0.67	0.39
Pyrimidine	10.205	0.50	0.28	0.96	1.2	0.38
Pyrrolidine	10.110	0.46	0.34	0.91	0.33	0.57
Thiazole	9.949	0.49	0.39	1.26	0.38	0.33
Thiazolidine-4-carbox acid	10.020	0.64	0.21	1.0	0.3	1.2
Thiophene-3-carbox acid	10.120	0.52	0.47	1.0	0.26	0.13

[a] The concns of effector were 0.1 *M*, except for 2,4-dinitrophenol (0.01 *M*) and thiazolidine-4-carboxylic acid (0.02 *M*). The ionic strength was 0.1, except for the highly charged adenosine phosphates. The initial pH_0 (compare Table I for definition) was 7.40 within 0.07 units in all cases, the final pH_3 was 7.4 within 0.1 units except for amiline (7.56), benzimidazole (7.64), hydantoin-5-acetic acid (8.53), pyrimidine (7.63), and thiophene-3-carboxylic acid (7.54). pH_1 is the intermediate pH, as defined in Table I. The equilibrium signal changes in presence of effector are denoted by a prime for distinction from those in absence of effector. The subscripts are otherwise those of Table I; for simplicity, a star is omitted for the λ parameters. One derives easily from line 1 of Table I that for the control $\Delta S_1/\Delta S_0 = 0.44$ and $\Delta S_3/\Delta S_0 = 0.38$. The ratio $\Delta S_0'/\Delta S_0$ would reflect any differences in cytochrome *c* concentrations. However, the analytically detd concns of cytochrome *c* varied only from 0.167 to 0.170 m*M*, well within the error of the detns of ΔS.

of methionine 80 in the sixth ligand position. To test this idea, we conducted a series of experiments on the Cary recording spectrophotometer. The spectrum of our compound with thiazolidine (high concentrations) was identical with that of reduced cytochrome *c*.

Results from experiments of types I and III are not explicitly tabulated, as their presentation would not provide much *new* information. Most ideal would have been a type I experiment as the perturbation of the system by the minute injection could certainly be neglected. However, this experiment was only comparatively successful with the thiazolidine derivative, as indicated below. Otherwise, the observable effects for type I experiments were quite small and merely gave marginal confirmations of the results already listed, namely that the binding for the duration of the experiments is generally weak. Some type III experiments were conducted at pH 7 and also at pH 10. In most cases, essentially the same apparent rate constants were obtained except for imidazole and dinitrophenol, suggesting that the process of oxidizing reduced cytochrome *c* in the presence of these compounds does not proceed along a single path, as depicted thus far.

Discussion

The various materials tested can be classified into those which have practically no effect and those which do effect the red band (695–633 mμ) of cytochrome *c*. Those which do effect the red band of cytochrome *c* may be divided into those which enhance the absorption band and those which result in a decrease of the absorption band. The former ones may also be labeled activators, the latter ones inhibitors, although their specific action regarding electron transfer is not quantitatively established in this paper.

It is by now well established that the 695 band is caused by the S atom of methionine 80, located in the sixth ligand position of Fe^{+3}. Activation may thus be considered as strengthening the binding of this group to the heme Fe; inhibition may be considered as removing this S to a smaller or larger extent from this ligand position. There is also the trivial solution that the red absorption band is diminished by the reductive action of the test substance upon cytochrome *c*, producing the reduced form.

With respect to the results summarized in Table II, one may distinguish compounds which seem not to effect cytochrome *c* from those which either enhance the red absorption band or diminish it. The equilibrium results of the 3 adenosine phosphates are rather similar to those of the control, although $\Delta S_3'/\Delta S_0'$ is somewhat large for AMP and $\Delta S_0'/\Delta S_0$ is somewhat large for ADP. On the other hand, the kinetic parameters for these 3 adenosine phosphates differ considerably. In fact, AMP shows the slowest apparent rate constant in Table II, if the pH is changed stepwise from 7.4 to 10.0. Next to this slowest value follow the thiophene derivatives, ADP and the thiazolidine derivatives, in that sequence (but all rather close together). The thiophene derivative is also associated with the slowest apparent rate constant for a stepwise pH change from 10 to 7.4, giving $\lambda_3'/\lambda_3 = 0.13$. Fastest for this latter change are imidazole and hydantion-5-acetic acid, the 2 compounds which also needed the largest amount of base for obtaining the proper change in pH. A secondary reaction of imidazole is reflected in the rather small values for $\Delta S_1'/\Delta S_0'$ and $\Delta S_3'/\Delta S_0'$; however, this slow reaction was not further investigated.

Earlier experiments of Czerlinski, *et al.*,[4,5] had shown that exactly 1 H^+ is released at pH 10, at which point we measured a signal change of 30 mV (compared to the signal, which was present at pH 7.4, observing at 633 mμ in our thermostated vessel). We changed the pH stepwise to 11, leading to decreasing increments of voltage changes, totaling 10 mV. This change is due to the second dissociation constant, labeled pK_H^{II}, which was 10.4. In crude approximation, the given ratio of voltage changes should result in the equilibrium constant between components 2 and 3. Certainly these are crude approximations as the protonic dissociation constants have to be taken into account quantitatively. However, the possibility of measuring such a substantial additional change suggests that a protonic dissociation is not associated with component 2 of scheme A of Figure 1, but only with component 3. This result eliminates case II, listed above under eq 6. The pH dependence of the observables are therefore presumably given by eq 5 and 6. Effectors would influence these pH dependencies, as outlined in scheme B of Figure 1.

Scheme B of Figure 1 in fact represents a simplified mech-

anism for the combination of a ligand-induced (represented by I, I′, and I″) with a pH-induced conformational change. Detailed measurements aiming at exact values for individual constants, are now in progress.

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Appendix

Equation 6 with $K_{II} = 0$ was previously derived.[19] Eq 6 presents thus an expression of the former derivations. More details on the derivation of eq 5 and 6 are also obtainable from a monograph on chemical relaxation[20] (see especially Tables 8.2 and 8.3, as well as Chapter 7). Expressions for apparent rate constants for the two-step scheme of Figure 1, heavy lines of part B (and K_H' defined as in part A) are easily obtained from eq 6 by setting $k_3 = k_4 = 0$ (as well as $K_{II} = 0$).

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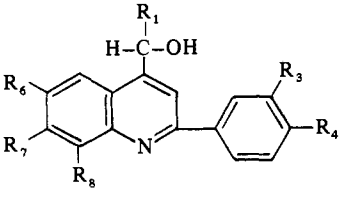
Structure-Activity Correlations of Antimalarial Compounds. 1. Free-Wilson Analysis of 2-Phenylquinoline-4-carbinols

Paul N. Craig*

Smith Kline & French Laboratories, Philadelphia, Pennsylvania 19101. *Received April 23, 1971*

Sixty-nine 2-phenylquinoline-4-carbinols which had been tested in the mouse for antimalarial activity were studied by the Free-Wilson method for structure-activity correlation. The results for this study significantly support the additivity concept assumed by the Free-Wilson approach. The substituent constants for groups at the para position of the 2-Ph ring were found to correlate significantly with both Hammett's meta σ constants and Hansch's π values for those substituents. Substituents on the 7 position of the quinoline ring correlate well with para σ and π values. Substituent constants for groups at position 8 of the quinoline ring correlate with π values for these substituents. Substituent constants for groups at position 6 and on the meta position of the 2-Ph ring failed to correlate with σ or π values; the substituent constants for the 16 different aminoalkyl side chains failed to correlate with π, or π and π^2. The significance of these results is discussed.

The Free-Wilson method of structure-activity correlation[1] has been applied with varying degrees of success in recent years.[2-5] This paper reports a successful application of the technique to compare the antimalarial test results in mice for 69 substituted 2-phenylquinoline carbinols of general structure I.



I

The biological test reports, obtained from the Walter Reed computer record system, gave data obtained by Rane and coworkers by the reported method.[6] The dose in milligrams which cured 50% of the animals was obtained by extrapolation of the number of cures found for each of the doses tested, and was converted to the customary log $1/C$ value, where C = moles/kg test animal. For every compd, at least 3 graded doses were given to 5 animals per dose.

Results

Following the reported method[1] the matrix listed in Table II was used as input to be solved by a matrix inversion program developed by Free and coworkers.

The substituent constants (*sc*) which resulted from the regression analysis are listed in Table I. The correlation coefficient for the analysis is 0.905; the standard deviation is 0.359, and the overall "average" log $1/C$ value is 3.39.† The

*Address correspondence to Craig Chemical Consulting Services, Inc., 120 Stout Rd., Ambler, Pa. 19002.

†This is the theoretical value for a hypothetical molecule in which none of the 6 substitutable positions contains any substituent group, including H. Substituent constants at R_3, R_4, R_6, R_7, and R_8 could be related to H as 0 by arbitrarily setting the matrix so that columns Q, U, AB, AF and AK in Table II are set equal to 0, rather than 1.