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# The Stoichiometry and Site Specificity of the Uncoupling of Mitochondrial Oxidative Phosphorylation by Salicylanilide Derivatives*

David F. Wilson

ABSTRACT: The compound 5-chloro-3-*t*-butyl-2′-nitrosalicylanilide is effective at concentrations lower than any other known compound which can uncouple mitochondrial electron transport from the energy conservation reactions. In rat liver mitochondria complete loss of respiratory control and maximal stimulation of adenosine triphosphatase activity requires 0.6 and 1.0 molecule of uncoupler per respiratory chain, respectively. The release of the inhibition of respiration by azide (which is specific for electron transport at cytochrome oxidase) requires 1.35 molecules of uncoupler/respiratory chain.

This titer is independent of the rate of electron flux and the rate of generation of high-energy intermediates. This salicylanilide derivative therefore binds stoichiometrically to a site which is approximately one per respiratory chain. The site is inferred (from the specificity of the azide inhibition) to be associated with the cytochrome oxidase portion of the respiratory chain.

A very large number of compounds have been discovered which uncouple mitochondrial oxidation from phosphorylation of ADP (Loomis and Lipman, 1948), and other energy-requiring reactions including divalent cation transport (Saris, 1963; Chappell *et al.*, 1963), antibiotic induced monovalent cation transport (Moore and Pressman, 1964), the energy-linked reduction of $NAD^+$ (Chance and Hollunger, 1957, 1960), and the energy-linked transhydrogenase (Klingenberg and Schollmeyer, 1961; Estabrook *et al.*, 1962). There is very little known about their actual mechanism(s) of action and several hypotheses have been proposed (Slater, 1953; Lardy and Wellman, 1953; Chance and Hollunger, 1957; Mitchell, 1961, 1966; Harris *et al.*, 1967; Slater and Colpa-Boonstra, 1961). These hypotheses include such diverse mechanisms as that the uncouplers increase the permeability of the mitochondrial membrane to protons (Mitchell, 1961, 1966), that they are substituting for substrate anions in an energy-requiring anion pump (van Dam and Slater, 1967), and they catalyze the hydrolysis of a chemical high-energy intermediate (Lardy and Wellman, 1953; Slater, 1953).

A new and very powerful class of uncouplers has recently been reported by Williamson and Metcalfe (1967). They reported that some of these salicylanilide dervatives (including 5-Cl-3-*t*-butyl-2′-Cl-4′-$NO_2$-salicylanilide) are effective at concentrations lower than the carbonyl cyanide derivatives prepared by Heytler (1963).

In the present communication the observations of Williamson and Metcalf (1957) are confirmed. It is also shown that the 5-chloro-3-*t*-butyl-2′chloro-4′-nitrosalicylanilide (S-13) is a stoichiometric uncoupler of mitochondrial oxidative phosphorylation and that it appears to be specific for the energy conservation site at the cytochrome oxidase of the electron transport chain. A preliminary report of this work has been published (Wilson and Azzi, 1968).

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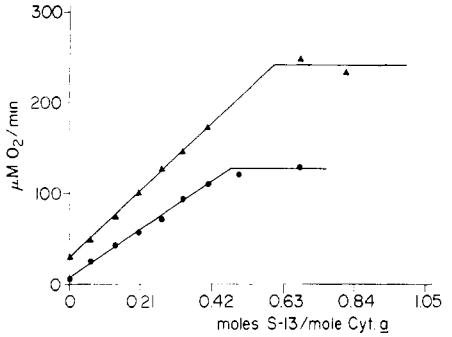


FIGURE 1: The titration of the release of mitochondrial respiratory control with the uncoupler S-13. Rat liver mitochondria were suspended at $2.1 \times 10^{-7}$ M cytochrome *a* in a 0.12 M KCl, 20 mM Tris, and 10 mM $KH_2PO_4$ medium with a final pH of 6.9. The substrate was 11.6 mM succinate in the presence of 3 μM rotenone (△) or 6.7 mM glutamate and 6.7 mM malate (○). The abscissa is the ratio of the molecules of S-13 added to the molecules of cytochrome *a* in the suspension.

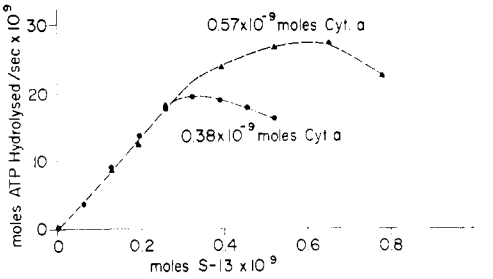


FIGURE 3: The activation of the mitochondrial ATPase activity by S-13. The rat liver mitochondria were suspended to a final volume of 4.7 ml in a 0.2 M mannitol, 0.05 M sucrose, 17 mM KCl, and 15 mM Tris-Cl medium (pH 7.1). The ATP was added to a final concentration of 2.0 mM and the rate of ATP hydrolysis was measured with a recording pH meter by the method of Nishimura *et al.*, (1962).

## Methods

Rat liver mitochondria were prepared in a 0.22 M mannitol, 0.07 sucrose, and 0.2 mM EDTA medium (pH 7.2) essentially as described by Schneider (1948). The quality of the mitochondrial preparation was judged by their respiratory control with 11 mM succinate as a substrate in the presence of 2 μM rotenone. Preparations were considered satisfactory if the respiratory control was greater than 5 in both MST-$P_i$ and KCIT-$P_i$ media (see below). Respiratory controls of between 6 and 10 were routinely obtained.

Submitochondrial particles from rat liver mitochondria were prepared by the method of Keilley and Bronk (1958).

Mitochondrial respiration was measured polarographically in either a 0.2 M mannitol, 0.05 M sucrose, 0.02 M Tris, and 0.01 M $KH_2PO_4$ medium (MST-$P_i$) or a 0.12 M KCl, 0.02 M Tris, and 0.01 M $KH_2PO_4$ medium (KCIT-$P_i$). The values are indicated in the figure and table legends.

Bromothymol blue color changes were measured in a Johnson Foundation dual-wavelength spectrophotometer at 618–700 mμ, the assay medium contained 0.25 M sucrose, and 20 mM Tris-Cl (pH 7.4). Submitochondrial particles (to give a cytochrome *a* concentration of approximately 0.2 μM), bromothymol blue (3.3 μM final concentration), and oligomycin (2–5 μg) were added and the reaction was started with either 6.7 mM succinate or 700 μM DPNH.

The uncoupler stimulated ATPase activity was measured by a pH electrode in either a 0.2 M mannitol, 0.05 M sucrose, 0.02 M Tris, and 0.015 M KCl medium or a 0.12 M KCl and 0.02 M Tris medium. In either case the final pH was 7.1 and the ATP hydrolysis rate was calculated assuming 0.8 $H^+$ ion was released per ATP molecule hydrolyzed (Nishimura *et al.*, 1962).

The ATP and DPNH were obtained from Sigma Chemical Co. and the uncoupler S-13 was the generous gift of the agricultural division of Monsanto Chemical Co., St. Louis, Mo. The other reagents were the same as previously described (Wilson and Gilmour, 1967).

The cytochrome content of the mitochondrial preparation was measured by adding 0.2 ml of 10% sodium deoxycholate to 1.5 ml of rat liver mitochondrial suspension and then diluting to 5.0 ml with 33 mM phosphate buffer (pH 7.2). The difference spectrum was then measured in a Coleman-Hitachi 124 double-beam spectrophotometer for the succinate reduced minus oxidized suspension and the cytochrome content was calculated. The millimolar extinction coefficients used were cytochrome *a* (605–630 mμ) = 24 (van Gelder, 1966), cytochrome *c* (550–540 mμ) = 19.1, cytochrome *b* (562–575 mμ) = 22 and cytochrome $a_3$ (445–465 mμ) = 164 (van Gelder, 1966). The ratios of $a:a_3:b:c + c_1$ are 1:1:1:2.7, respectively, for rat liver mitochondria.

The reported numbers are the mean of the experimental values plus and minus the range encompassing both the highest and lowest of the experimental values.

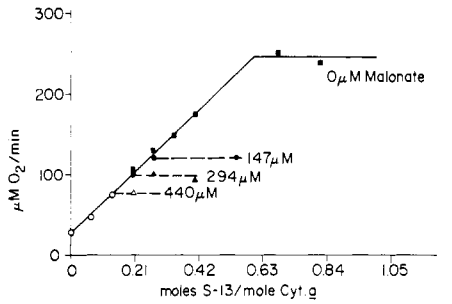


FIGURE 2: The effect of malonate inhibition of succinate oxidation on the S-13 required to release mitochondrial respiratory control. The conditions were the same as in Figure 1. The concentrations of malonate added are given at the appropriate line. The first three experimental points coincide for all malonate concentrations and are represented as open circles for simplicity.

## Results

*The Release of Respiratory Control.* When rat liver mitochondria in state 4 are titrated with the uncoupler S-13 there is a linear relationship between the increase in respiration rate and the number of molecules of S-13 added (Figure 1). This linearity is observed whether succinate or glutamate and malate are used as the substrate. The slope of the titration curve can be directly related to the number of ATP equivalent high-energy intermediates (~) hydrolyzed per uncoupler molecule per second by assuming the generation of two or three high energy intermediates per oxygen atom consumed with succinate or with glutamate and malate as substrates, respectively.

The effective "turnover number" of the S-13 (~/sec mole-

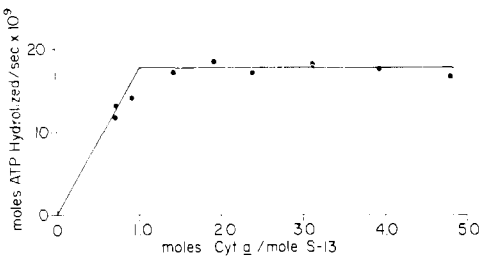


FIGURE 4: The dependence of the S-13-activated ATPase upon the mitochondrial concentration. The assay medium contained 0.12 M KCl and 20 mM Tris-Cl (pH 7.1). The other conditions were the same as in Figure 3. The abscissa is the ratio of the molecules of cytochrome *a* present to the molecules of S-13 added. It is the reciprocal of the ratio used in the other figures.

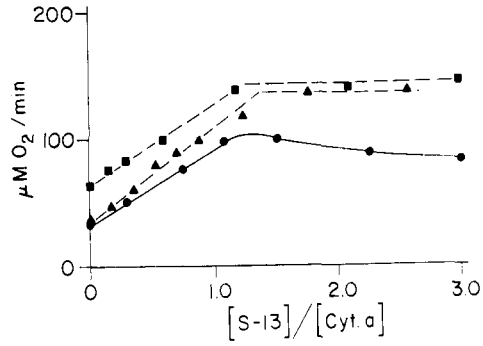


FIGURE 5: The release of the azide inhibition of state 3 mitochondrial respiration by S-13. The assay medium was 0.12 M KCl, 20 mM Tris, 10 mM $KH_2PO_4$, and 600 $\mu$M ADP; final pH 7.1. (●) 11.6 mM glutamate, 11.6 mM malate, and 590 $\mu$M $KN_3$: (▲) 11.6 mM succinate, 2 $\mu$M rotenone, and 290 $\mu$M $KN_3$: (■) 17.6 mM ascorbate, 2 $\mu$M rotenone, 590 $\mu$M *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, and 237 $\mu$M $KN_3$.

cule of S-13) is not only independent of the substrate used but is also independent of the rate of oxidation for a single substrate. When the rate of oxidation of succinate is limited by the malonate, an inhibitor which is competitive with respect to succinate (Figure 2), the slopes of the titration curves are identical. Although the maximal rate of succinate oxidation is less in the presence of malonate, the changes in the rate of succinate oxidation per uncoupler molecule added is constant. The "turnover number" of the S-13 for the single mitochondrial preparation is remarkably constant at ±5% whether the substrate is glutamate plus malate or succinate plus rotenone. When different rat liver mitochondrial preparations were compared, the turnover numbers were 81 ± 9 ~/sec molecule of S-13. This compares favorably in reproducibility with the observed turnover number for cytochrome *a* for state 3 (11 mM succinate, 600 mM ADP, and 10 mM orthophosphate) succinate oxidation of 51 ± 5 electrons/sec molecule of cytochrome *a*.

The number of uncoupler molecules per cytochrome *a* molecule required for complete release of the respiratory control is dependent only upon the maximal rate at which the system can generate high-energy intermediates (Figure 2).

*The Uncoupler Activated ATPase.* The S-13 activates a latent ATPase activity in rat liver mitochondria (Figure 3) as does each of the compounds classed as uncouplers. In the original mitochondria the ATPase activity is effectively zero when compared to the fully uncoupled system. As S-13 is added, the ATPase activity appears and increases as a completely linear function of the number of added uncoupler molecules. Above a certain uncoupler concentration, ATPase activity maximized and then is progressively inhibited by further additions of uncoupler.

The linear portion of the titration curve is characterized by a "turnover number" of 58 ± 10 ATP molecules hydrolyzed/sec molecule of S-13. The number of S-13 molecules required to activate the ATPase may be calculated by back-extrapolation of the inhibitory part of the curve to give a corrected maximal ATPase activity. This extrapolation gives a value about 15% greater than the measured maximum. The number of S-13 molecules required for maximum stimulation is then 1.05 ± 0.1/cytochrome *a* molecule.

The S-13 stimulated ATPase activity is a direct function of the S-13 concentration in the range for which it is limiting and is almost completely independent of the amount of mitochondrial protein present (Figure 4). The amount of nonspecific binding of the S-13 by mitochondrial proteins and lipids is apparently very small in this range of S-13 concentrations.

*The Release of Azide Inhibition by the Uncoupler.* Uncouplers partially release the azide inhibition of mitochondrial respiration by causing an increase in the observed inhibitor constant of more than tenfold (at least for the carbonyl cyanide derivatives, benzimidazole derivatives and S-13, some of the other uncouplers may be less effective (Wilson and Chance, 1967) ). Azide inhibition is maximal for mitochondria in state 3 (Wilson and Chance, 1967) and for this experiment the mitochondria were suspended in either a KCIT-$P_i$ or a MST-$P_i$ medium containing 660 $\mu$M ADP and the indicated azide concentration. The respiration was then started with substrate and the rate of oxygen consumption measured as a function of added S-13 (Figure 5).

The increase in respiration rate is again a linear function of the added S-13 but unlike the ATPase activity there is no detectable inhibition by excess uncoupler except when the substrate is glutamate and malate. The S-13 does inhibit succinate oxidation at very high S-13 concentrations and low succinate concentrations but it is less inhibitory per unit of uncoupler activity than are the carbonylcyanide derivative uncouplers (Wilson and Merz, 1967), and the inhibition is therefore insignificant under the conditions of this experiment. The release of azide inhibition also differs from the release of respiratory control in that the number of uncoupler molecules required for maximal release of the azide inhibition is independent of the number of phosphorylation sites through which the electrons pass. Thus the number of uncoupler molecules required for maximal release of the inhibition is the same whether the substrate is glutamate and malate, succinate, or ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, although the electrons pass through three, two, and one phosphorylation sites, respectively.

*The Electron Flux Dependence of the Uncoupler Induced Release of Azide Inhibition.* The azide inhibits at a site common to both electron transport and energy conservation and is specific for the terminal phosphorylation site (Wilson and Chance, 1967). It was therefore interesting to determine if the release of the azide inhibition of the ascorbate–*N,N,N',N'*-tetramethylparaphenylenediamine oxidation is a linear function of the S-13 concentration. The maximal respiration rate

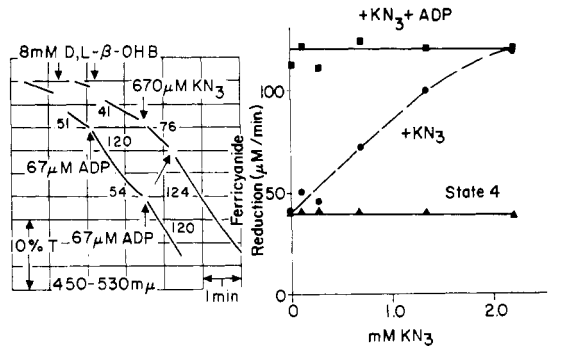


FIGURE 6: The uncoupling effect of azide on the reduction of ferricyanide by β-hydroxy butyrate. Rat liver mitochondria were suspended in MST-$P_i$ medium; pH 7.2 at 2 mg of protein/ml of suspension. The assay medium contained 667 μM NaCN and 667 μM potassium ferricyanide. Additions were made as shown in part A (left) and the ferricyanide reduction measured with a filter dual-wavelength spectrophotometer at 450 mμ minus 530 mμ. In a parallel experiment one-half inhibition of state 3 (700 μM ADP) succinate (11 mM) plus rotenone (2 μM) respiration required 64 μM $KN_3$.

TABLE I: The Titration of the Uncoupler-Dependent Release of Azide Inhibition by S-13.[a]

| *N,N,N',N'*-Tetramethyl-*p*-phenylenediamine (mM) | $KN_3$ (μM) | Oxygen Uptake (μM $O_2$/min) | | Moles of S-13/mole of Cytochrome *a* |
|---|---|---|---|---|
| | | No S-13 | Excess S-13 | |
| 1.14 | 0 | 350 | 350 | |
| 1.14 | 28 | 160 | 250 | 1.58 |
| 1.14 | 57 | 105 | 200 | 1.32 |
| 1.14 | 114 | 60 | 160 | 1.39 |
| 0.57 | 0 | 245 | 245 | |
| 0.57 | 57 | 105 | 163 | 1.24 |
| 0.57 | 114 | 65 | 115 | 1.27 |
| 0.28 | 0 | 145 | 145 | |
| 0.28 | 57 | 90 | 120 | 1.27 |
| 0.28 | 114 | 62 | 97 | 1.35 |

[a] Rat liver mitochondria were suspended at $6.3 \times 10^{-10}$ mole of cytochrome *a* in a final volume of 3.5 ml. The assay medium was MST-$P_i$ medium; pH 7.1 containing 2 μM rotenone, 17 mM ascorbate, 280 μM ADP, and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine and azide as indicated. The respiration was started with *N,N,N',N'*-tetramethyl-*p*-phenylenediamine and titrated with S-13 as shown in Figure 5. The uncoupler required for 100% effect is given as the ratio of uncoupler molecules to cytochrome *a* molecules (column 5).

is achieved at 1.3 molecules of S-13/cytochrome *a* molecule, the same as for succinate and for glutamate and malate oxidation. As shown in Figure 5, azide inhibition of the ascorbate–*N,N,N',N'*-tetramethyl-*p*-phenylenediamine oxidation is a linear function of the S-13 concentration. As was true for the inhibition of succinate oxidation, the release of azide inhibition is independent of the azide concentration and with ascorbate–*N,N,N',N'*-tetramethyl-*p*-phenylenediamine as substrate the uncoupler required is also independent of the *N,N,N',N'*-tetramethyl-*p*-phenylenediamine concentration (Table I).

The number of uncoupler molecules required for maximal release of azide inhibition was not changed when the uninhibited electron flux was increased by more than twofold by increasing the *N,N,N',N'*-tetramethyl-*p*-phenylenediamine concentration from 0.28 to 1.14 mM. The uncoupler requirement was also unchanged when the *N,N,N',N'*-tetramethyl-*p*-phenylenediamine concentration was held constant and the electron flux was increased by decreasing the azide concentration.

The uncoupler-dependent release of azide inhibition is therefore unaffected by either the number of phosphorylation sites through which the electrons pass or the rate of generation of high energy intermediates at the terminal phosphorylation site. Since the release of the azide inhibition by S-13 is dependent upon the number of mitochondrial respiratory units and not the rate of electron transport, it appears to be a true titration of an uncoupler binding site.

*The Specificity of the Azide Inhibition.* Azide has been shown to be a highly specific inhibitor of electron transfer between reduced cytochrome *a* and oxidized cytochrome $a_3$ (Keilin and Hartree, 1939, Wilson, 1967). Azide has also been reported to be an uncoupler of oxidative phosphorylation (Loomis and Lipman, 1948; Judah, 1951), and although evidence has already been represented that it is an uncoupler only at very high (millimolar) concentrations (Wilson and Chance, 1967), it is useful to know the quantitative relationship between the inhibitory activity and uncoupling activity. We therefore measured the uncoupling activity of azide on the β-hydroxy butyrate reduction of ferricyanide, a reaction having a respiratory control of 3–5 and an ADP/2e of approximately 2. As shown in Figure 6A, B, the concentration of azide required to release 50% of the respiratory control of this reaction was about 850 μM while 50% inhibition of succinate oxidation required only 64 μM. The uncoupling activity of azide is therefore insignificant in experiments such as that shown in Figure 5.

The rate of reduction of ferricyanide by β-hydroxy butyrate is not inhibited by azide.

*The Titration of the Respiration-Dependent Bromothymol Blue Color Change in Submitochondrial Particles by Uncouplers.* Chance and Mela (1967) observed that when the pH indicator bromothymol blue is added to submitochondrial particles there is a respiration dependent acidification of the bromothymol blue. They demonstrated that this acidification is reversed by uncouplers without a corresponding change in the respiration rate and is probably related to the energy level of the particles. Sanadi and coworkers have titrated this bromothymol blue color change with the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and concluded that there is one uncoupler molecule bound at site III, one bound at site II and 14–16 (Kurup and Sanadi, 1968) or 2-4 (Sanadi, 1968) bound at site I per cytochrome *b*.

The respiration dependent bromothymol blue change in submitochondrial particles from rat liver is reversed by uncouplers (Figure 7) including S-13. The titration of this reversal with uncouplers gives titration curves very similar to those reported by Kurup and Sanadi (1968).

TABLE II: The Extrapolated Number of Uncoupler Molecules Required per Respiratory Chain for 100% Release of the Respiration-Dependent Bromothymol Color Change in Submitochondrial Particles.[a]

| Substrate | Carbonyl Cyanide *m*-Chloro-phenyl-hydrazone | Carbonyl Cyanide *p*-Trifluoro-methoxy-phenyl-hydrazone[b] | S-13 |
|---|---|---|---|
| Succinate + rotenone | 6.5 ± 2.5 | 2.4 | 0.6 ± 0.15 |
| DPNH | 10.3 ± 2 | 4.6 | 1.4 ± 0.3 |

[a] The assay conditions are given in Methods. The values for 100% effect were determined by extrapolation of the initial linear part of the titration curve to 100% loss of the bromothymol blue color change. In rat liver mitochondria the cytochrome *a* content is equal to the cytochrome *b* content (as determined in Methods) and the expressed values are uncoupler molecules per cytochrome *a* molecule. [b] Taken from Kurup and Sanadi (1968) and Sanadi (1968).

Extrapolation of the initial linear portion of these curves to 100% gives a hypothetical number of uncoupler molecules required for 100% effect. As shown in Table II these numbers are different from the uncouplers with S-13 requiring only about one-eighth as many molecules as carbonyl cyanide *m*-chlorophenylhydrazone and one-third as many molecules as carbonyl cyanide *m*-chlorophenylhydrazone according to Sanadi (1968). These values accurately reflect the uncoupling activity of the individual uncouplers but cannot represent the number of uncoupler binding sites particularly since S-13 requires less than one uncoupler molecule per respiratory chain with succinate as a substrate.

## Discussion

The chemical (Lardy and Wellman, 1953; Slater, 1953; Chance and Williams, 1956) and the chemiosmotic (Mitchell, 1961, 1966) hypotheses for the coupling of electron transport to the energy conservation reactions incorporate very different mechanisms of uncoupler action. In the chemical coupling hypothesis the energy is conserved by a series of reactions which may be depicted as

$$AH_2 + B + I \longleftrightarrow \begin{matrix} A\sim I + BH_2 \\ \text{or} \\ BH_2\sim I + A \end{matrix} \quad (C\sim I) \qquad (1)$$

$$C\sim I_{1,2,3} + X \longleftrightarrow C + X\sim I_{1,2,3} \qquad (2)$$

$$X\sim I + ADP + P_i \longleftrightarrow ATP + X + I \qquad (3)$$

where C represents either A or $BH_2$ and the subscripts 1, 2, and 3 refer to the three energy conservation sites. A, B, $AH_2$, and $BH_2$ represent electron carriers in the oxidized and reduced forms. I represents a hypothetical endogenous inhibitor, and

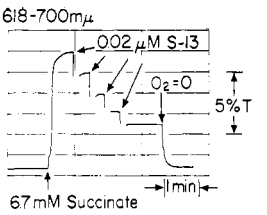


FIGURE 7: The S-13 induced loss of the respiration-dependent bromothymol blue acidification in submitochondrial particles. Particles to give a cytochrome *a* concentration of 0.20 μM, bromothymol blue at 3.3 μM and 2 μg of oligomycin were added before the succinate. An upward deflection represents a decrease in absorption at 618 mμ relative to 700 mμ. The light path in the sample was 1 cm.

X represents hypothetical endogenous compound capable of forming a high-energy compound common to all three phosphorylation sites. Equation 3 summarizes the oligomycin sensitive reactions which give rise to ATP formation.

In the chemiosmotic hypothesis in its most general sense the energy from electron transport is conserved as a membrane potential and/or ion gradient. This potential or gradient is generated by a directional ion pump (proton, cation, or anion).

It is not necessary in this discussion to consider in detail the mechanisms postulated by the chemical and chemiosmotic hypothesis to give rise to this stored energy because there is a fundamental difference in the role of uncouplers. In the chemical coupling hypothesis the uncouplers act either as

$$C_{1,2,3}\sim I + U \text{ - - - - - } C_{1,2,3} + I + U + \text{heat} \qquad (4)$$

or

$$X\sim I + U \text{ - - - - - } X + I + U + \text{heat} \qquad (5)$$

In each equation the uncoupler (U) is written on both sides of the equation to express its catalytic role in the breakdown of the high-energy intermediate. In the chemiosmotic hypothesis the uncouplers are postulated to increase the permeability of the mitochondrial membrane to protons (Mitchell, 1961, 1966) and thus discharge the membrane potential and ion gradient. In eq 4 the $C_{1,2,3}$ represents three chemically distinct species, each specific for the energy-coupling site designated by the subscript 1, 2, or 3. The uncoupler would therefore be expected to have three binding constants, one for each site, but these different binding constants could be observed only if the intercommunication through X~I were blocked by some means.

The specificity of the uncoupler S-13 for the energy conservation site at cytochrome oxidase as implied from the release of the azide inhibition is evidence for uncoupling by eq 4 of the chemical coupling hypothesis. The uncoupler would then bind specifically to $C_3\sim I$ and catalyze its breakdown to $C_3 + I$. The other high-energy intermediates ($C_1\sim I$), ($C_2\sim I$), and ($X\sim I$) would be hydrolyzed by a rapid equilibration with the $C_3\sim I$. The site specificity of S-13 is evidence against the chemiosmotic hypothesis in its present form (Mitchell, 1966) and with uncoupling as shown in eq 5 of the chemical coupling hypothesis.

Recently van Dam and Slater (1967) have proposed a mechanism for uncoupling in which the uncoupler anions substitute for substrate anions in an energy-dependent anion

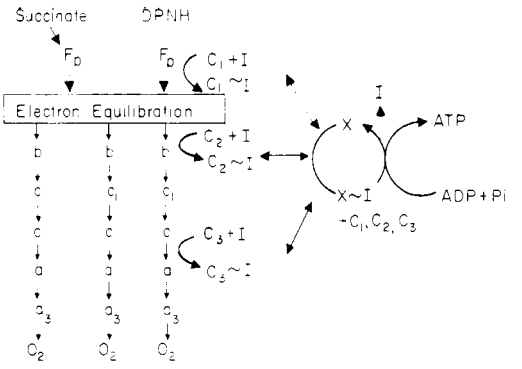


FIGURE 8: A schematic representation of mitochondrial electron transport and energy coupling.

pump. This uncoupling mechanism is analogous to that for the chemiosmotic hypothesis and neither stoichiometry nor site specificity would be expected.

One aspect of the uncoupling by S-13 which is of particular interest is the observation that when the succinate oxidation rate is limited with malonate the slope of the titration curve for the release of respiratory control is unchanged but the amount of S-13 required for 100% loss of control is decreased. Similar results were obtained by Margolis *et al.* (1967) for the uncoupling with carbonyl cyanide derivatives for which complete uncoupling (inhibition of ATP synthesis) could be achieved with 1 uncoupler molecule/20 respiratory units when the rate of substrate oxidation was limited by inhibitors to a small fraction of the uninhibited values. These data were interpreted by Margolis *et al.* to mean that the uncouplers have a high affinity only for actively functioning coupling sites. They further suggested that "following discharge of some high-energy intermediates(s) at these sites, the uncoupling molecules are released into a phase in the membrane where the molecules are so mobile that their rate of transfer to other functioning sites does not become rate limiting under the experimental conditions usually employed." This interpretation requires that the uncoupler titration be dependent upon the rate of generation of high-energy intermediates and is not possible for the uncoupler S-13 which has a titer for the release of the azide inhibition which is independent of the rate of generation of high-energy intermediates. The S-13 thus behaves in a manner analogous to the electron transport inhibitors rotenone (Ernster *et al.*, 1963) and antimycin A (Estabrook, 1962) which are tightly bound and specific for their respective sites of action.

A specific binding of S-13 to the energy conservation site at cytochrome oxidase would not prevent its uncoupling at the other energy conservation sites. It has been conclusively demonstrated (Packer *et al.*, 1963; Slater and Tager, 1963) that energy conserved at the cytochrome oxidase site may be utilized to drive the energy-linked reduction of $DPN^+$ by succinate and the energy-dependent transhydrogenase even in the presence of excess antimycin A and oligomycin. An uncoupler acting at one site could easily uncouple all three sites by equilibration through X~I. The electron transport and energy conservation mechanisms may be represented in a highly schematic form as shown in Figure 8.

The term "electron equilibration" is used here to indicate a region of the electron transport system in which the rate of interchain electron transport (branching) is equal to or greater than the rate of electron transport through the chain to oxygen. The effect of this is to randomize the selection of the linear electron transport chain utilized and permits superstoichiometry of uncouplers in the release of respiratory control. The electron chains from cytochrome *b* to oxygen have low rates of interchain electron transport relative to the rate electron transport from succinate to oxygen (Chance *et al.*, 1967). The randomizing process is also required by the observation that both succinate dehydrogenase and DPNH dehydrogenase are present in amounts less than one per cytochrome chain but each dehydrogenase donates electrons to all of the respiratory assembles without discrimination.

In the diagram three cytochrome chains are shown but from Figure 2 it is probable that at least eight to ten chains are in communication through the region of electron equilibration. When succinate oxidation is inhibited with malonate the number of uncoupler molecules required to release respiratory control is determined only by the number of respiratory chains required to conduct the available electrons to oxygen at the maximum rate. The schematic diagram in Figure 8 shows only one energy conservation system but it is intended that each respiratory chain have an associated energy conservation system.

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# Inhibition and Activation of Calcium Transport in Mitochondria. Effect of Lanthanides and Local Anesthetic Drugs*

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ABSTRACT: Using murexide to measure the $Ca^{2+}$ accumulation rates and bromothymol blue to measure the rates of the intramitochondrial pH changes, and by measuring the oxidation–reduction changes of the respiratory chain carriers during $Ca^{2+}$ accumulation, it was found that lanthanides at a concentration of about 0.05–0.07 mμmole/mg of protein specifically inhibit the reactions of $Ca^{2+}$ with the mitochondrial membranes and the accumulation of $Ca^{2+}$ into the mitochondria. No other functions of the mitochondria were found to be inhibited. It was also found that local anesthetics like butacaine enhance the accumulation of $Ca^{2+}$. Maximum activation was obtained at about 40 mμmoles/mg of protein. On the basis of these findings the nature of the mechanism of the mitochondrial divalent cation accumulation is discussed. It is suggested that in the mitochondria there exists a divalent cation carrier which can be identified and blocked by the lanthanides. This carrier is functionally specific and acts only as a divalent cation carrier. The functionally unspecific $Ca^{2+}$ binding sites can be titrated with butacaine. By blocking the unspecific sites the $Ca^{2+}$ accumulation can be enhanced.

Energy-dependent calcium accumulation in mitochondria has been found to be strongly inhibited by trivalent cations, lanthanum and praseodymium, which belong to the group of lanthanides (Mela, 1968a). The specific inhibition of calcium and manganese accumulation by lanthanides was shown by the diminished response of the respiratory carriers and the elimination of the membrane pH gradient induced by calcium accumulation. Recently the energy-independent binding of calcium to the mitochondrial membranes (Scarpa and Azzi, 1968) also has been shown to be inhibited by lanthanum ions (Rossi *et al.*, 1968).

It has also been shown that butacaine and a few other local anesthetic and antihistaminic drugs increase the intramitochondrial pH change which is concomitant with the calcium accumulation (Mela, 1968a; Chance *et al.*, 1968). Also Scarpa and Azzi (1968) have studied the effect of local anesthetics on the energy-independent calcium binding in submitochondrial particles and found an inhibition of this binding at higher concentrations of local anesthetics.

The present paper presents data, on the one hand, on the inhibition of mitochondrial calcium accumulation by various lanthanides (Cotton and Wilkinson, 1967) and, on the other hand, on stimulation of the accumulation by local anesthetics, as measured with a cation sensitive indicator murexide. The application of the use of murexide in mitochondria has been reported elsewhere (Mela and Chance, 1968). This technique enables one to follow the kinetics of the calcium accumulation

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