Determination of the P/2e\(^{-}\) Stoichiometries at the Individual Coupling Sites in Mitochondrial Oxidative Phosphorylation

EVIDENCE FOR MAXIMUM VALUES OF 1.0, 0.5, AND 1.0 AT SITES 1, 2, AND 3\(^*\)

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P/2e\(^{-}\) stoichiometries in six assay systems spanning different portions of the respiratory chain were estimated by direct determinations of P\(_{i}\) uptake in suspensions of bovine heart mitochondria containing a hexokinase trap. The electron donors were malate + pyruvate, succinate, and ascorbate + N\(_{N,N,N',N'}\)-tetramethyl-p-phenylenediamine, and the electron acceptors were ferriyanide (Site 1, Site 2, and Sites 1 + 2) and O\(_{2}\) (Sites 1 + 2 + 3, Sites 2 + 3, and Site 3). A major objective was to find conditions in which the six systems yield results in sufficiently good agreement to allow confidence as to their reliability. This objective was achieved, and maximum values of 1.1, 0.5, and 1.0 were observed in the Sites 1, 2, and 3 systems, respectively. This required that the energy-conserving reactions be relatively nonlimiting and that the P/2e\(^{-}\) ratios be estimated from the slopes of plots of respiration rate versus phosphorylation rate obtained by inhibiting oxidative phosphorylation with respiratory chain inhibitors. The latter requirement allows avoidance of the effect of an apparent endogenous uncoupler and is based on the observation (Tsou, C. S., and Van Dam, K. (1969) 

**Biochim. Biophys. Acta** 172, 174–176) that uncoupling agents at low concentrations decrease the rate of phosphorylation nearly as much in absolute amount at low rates of respiration as at high rates. The maximum P/2e\(^{-}\) stoichiometry at Site 1 is considered to be 1.0, and the value observed in the Site 1 system is suggested to be higher as a result of H\(^{+}\) ejection at the transhydrogenase level. Respiratory control due to carboxyatractyloside inhibition was examined and found to differ greatly among the systems. It is pointed out that this observation is not consistent with the lack of complete control being due primarily to ion cycling and that, in view of this, the relatively meager control at Site 3 is not consistent with O\(_{2}\) being reduced on the matrix side of the coupling membrane.

A matter of considerable interest and importance in the study of mitochondrial oxidative phosphorylation has been the mechanistic stoichiometries in the coupling of respiration to the phosphorylation of extramitochondrial ADP. In consequence, many studies have been conducted to determine these stoichiometries employing a remarkable variety of techniques. Despite these efforts, uncertainties persist owing to variable and conflicting results. In fact, there now appears to be widespread skepticism concerning the possibility of determining mechanistic stoichiometries by direct methods (e.g. see Refs. 1 and 2), and theoretical models of oxidative phosphorylation now often include mechanisms whereby the stoichiometries might normally vary.

Early studies on the stoichiometry of oxidative phosphorylation involved primarily determinations of P/O ratios in mitochondria oxidizing succinate or NAD-linked substrates. The values obtained usually approached 2 with succinate and 3 with NAD-linked substrates, indicating a P/2e\(^{-}\) quotient of 1.0 at Site 1 and of 2 over the Sites 2 + 3 span. Although values lower than these were usually observed, it became somewhat of a tradition to consider the observed values to be low because of unavoidable losses of respiratory energy in other processes and of a belief that the true values should be whole numbers. This tradition was broken as a result of the rise of the chemiosmotic theory and of the relatively recent study of Hinkle and Yu (3). These workers reviewed much of the early work, and in determinations of their own, obtained P/O ratios close to 1.3 with succinate as the oxidizable substrate and close to 2 with 3-hydroxybutyrate. On the basis of these observations, Hinkle (4) later predicted maximum values of 1.5 and 2.5 for the Sites 2 + 3 and Sites 1 + 2 + 3 spans, respectively. Using the ADP/O method of Ogata et al. (5), Lemasters (6) recently observed values averaging 1.71 with succinate and 2.61 with 3-hydroxybutyrate. On the basis of thermodynamic data and considerations of and ADP/O estimates obtained by assuming that the nonphosphorylating respiration of State 4 persists during State 3, he considered these values to be low and predicted maximum values of 2 for succinate oxidation and 3.25 for 3-hydroxybutyrate. Beavis and Lehninger (7) subsequently reported values of 1.63 with succinate and 2.66 with 3-hydroxybutyrate, obtained by estimating P, fixation from the H\(^{+}\) consumed. Although they considered these values not to be significantly different from those of Hinkle and Yu (3), from extrapolations based on thermodynamic considerations and observations on the effects of inhibitors and uncouplers, they considered them to represent lower limits and suggested mechanistic P/O ratios of 1.75 for succinate oxidation and 2.75 for 3-hydroxybutyrate.

Several studies have been done to determine the P/2e\(^{-}\) stoichiometries also in the Site 1, Sites 1 + 2, Site 2, and Site 3 spans of the respiratory chain. The Site 1 span of intact mitochondria apparently has been examined previously only by a thermodynamic approach involving the ATP-dependent reduction of acetocetate by succinate and yielding a value of 1.18 (8). In the case of the Sites 1 + 2 span, average values of 1.2 (9), 1.8 (10), 1.6 (11), 1.0 (12), and 1.38 (13) have been obtained in assays with NAD-linked substrates as the electron donor and with either ferricyanide or cytochrome c as the...
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acceptor. Using a thermodynamic approach, Forman and Wilson (14) obtained a value of 1.97 for this span. Site 2 has been examined in a number of studies using succinate as the electron donor and ferricyanide as the acceptor. Copenhaver and Lardy (9) reported a value of 0.6, Jacobs and Sanadi (15) values ranging from 0.3 to 0.45, Lee et al. (16) a value of 0.99, and Pozzan et al. (13) values averaging 0.42. Pozzan et al. (13) interpreted their results to mean that the maximum P/2e- stoichiometries at Sites 1 and 2 are 1.0 and 0.5, respectively. In the case of Site 3, early studies (11, 17) in which reduced cytochrome c was the electron donor yielded P/2e- ratios up to 0.86. Higher values approaching 1 or slightly more than 1 were subsequently observed using, in most cases, either ferricyanide or ascorbate plus a mediator to facilitate electron transfer to cytochrome c (see Refs. 15 and 18–22). The results have generally been assumed to indicate a maximum P/2e- stoichiometry of 1 at Site 3.

Owing to differences in technique, the numerous discrepancies evident in the above previous studies would be difficult to assess. In some cases, however, incomplete coupling of respiration to phosphorylation was likely a problem. In the present study, techniques were employed whereby this problem can be largely avoided. Other important features of the present study are the determination of P, fixation by a very reliable direct method and the determination of the P/2e- stoichiometries of the three coupling sites individually and in three combinations. Such a comprehensive investigation has not been done heretofore and has the important consequence that the reliability of the assays may be assessed by noting the degree to which they are consistent with one another in regard to results. A high degree of consistency has been achieved, and it is concluded from the results of this and other studies that the maximum P/2e- stoichiometries at Sites 1, 2, and 3 are 1.0, 0.5, and 1.0, respectively.

Experimental Procedures

Results

Preliminary Studies—A number of studies of this sort were conducted to assess the reliability of the assays. Most are described in Ref. 37, where it is indicated that the inhibitors used in the individual coupling site assays adequately isolate the individual coupling segments and that electron transport in the Site 1 assay is entirely coenzyme Q1-dependent and rotenone-sensitive and thus entirely through the Site 1 phosphorylation pathway. As shown originally by Ernster and Nordenbrand (38), it is very important in attempting to determine maximum P/2e- ratios to maintain conditions during the assays such that the energy-conserving reactions of the overall oxidative phosphorylation process (i.e., the P, transport, adenine nucleotide transport, ATP synthetase, and hexokinase reactions) are relatively nonlinear. Therefore, in setting up the assays, the tendency was to select saturating concentrations of hexokinase and extramitochondrial substrates of these reactions. An exception was P1. In this case, the concentration was made as low as was feasible because small changes in concentration could be detected with greater accuracy when the concentration was low than when it was high. In deciding how low a P1 concentration to use in the standard medium, the appropriate system to consider was the Sites 1 + 2 + 3 assay because it is the one in which P1 uptake was most rapid and extensive, the P1 concentration being decreased to about 0.85 mM during the assay. Under the conditions of this assay, the apparent Km for P1 is about 0.3 mM (39). Therefore, even at the conclusion of the assay, the concentration of P1 was still nearly 3 times that required for a half-maximal rate of the overall process and thus only slightly inhibitory relative to the initial concentration (2 mM).

It will be shown presently that such a slight inhibition of the overall reaction due to limitation at the energy conservation level has little or no effect on the P/2e- ratio, particularly in the cases of the assays involving electron transport through Site 1.

Determination of the Maximum P/2e- Stoichiometries—This objective was pursued initially simply by determining the P/2e- ratios in the various assay systems and observing the extent to which the results were consistent. It invariably turned out that the sum of the P/2e- ratios observed in the single-site system was lower than the sums of the values obtained in the two double-site + single-site combinations and that all of these sums were lower than the value obtained in the Sites 1 + 2 + 3 system. The results could be brought into near concordance by adding a certain value to the observed P/2e- ratios of all of the systems, suggesting the operation of a process serving to decrease the P/2e- ratio by a mechanism which was not greatly dependent on the differences among the systems. This suggested the presence of an endogenous uncoupler in the mitochondria, so a procedure was adopted whereby the effects of uncouplers can be largely avoided. This procedure derives from the observations of Tsou and Van Dam (40) concerning the effects of low concentrations of uncoupling agents on the relationship between the rate of phosphorylation and the rate of respiration when the rates are varied with respiratory chain inhibitors. These workers noted that uncoupling agents at low concentrations decrease the rate of phosphorylation nearly the same absolute amount at low rates of respiration as at high rates, with the result that the uncoupling has little effect on the slope of the linear relationship between the rate of respiration and the rate of phosphorylation and that the effect of uncoupling agents can be largely avoided by determining the P/2e- stoichiometry from the slope of this relationship. This observation was confirmed and used in the present study as well as in the recent study of Beavis and Lehninger (7).

Figs. 1 and 2 present the results of the P/2e- determinations by the procedure indicated above. As may be seen (Fig. 2), excellent agreement among the six systems is indicated. Consistent with the presence of an endogenous uncoupler in the mitochondria, inhibiting the rate of oxidative phosphorylation to a particular extent with a respiratory chain inhibitor decreases the P/2e- ratio approximately the same absolute amount in all of the systems (Fig. 1, A–F), and linear plots of respiration rate versus phosphorylation rate intersect the respiration axis at values greater than zero (Fig. 1, G–I). As can be expected from the relatively low P/2e- stoichiometry in the Site 2 system, the linear relationship for this system intersects the respiration axis at a relatively high level. In the case of the Site 1 system, the respiration axis is intersected at a higher level than might be expected from the results obtained with the other systems. This can be attributed to
uncoupling by the coenzyme Q₁ present in the Site 1 system. The uncoupling action of coenzyme Q₁ at the concentration used in the Site 1 system was verified in a Sites 1 + 2 + 3 system, and it was determined also with this system that bovine serum albumin at the concentration used in the Site 1 system (0.5 mg/ml) decreases the uncoupling activity of the coenzyme but does not eliminate it. Bovine serum albumin alone was tested in all of the other systems and appeared to have no effect on either respiration or its coupling to phosphorylation. However, the bovine serum albumin was not of the defatted variety, and the tests were carried out only under conditions of maximum respiratory activity, where uncoupling is least noticeable.

Extrapolation of plots of P/2e⁻ versus the reciprocal of the respiration rate to the P/2e⁻ axis gives the same maximum values as those in Fig. 2. This occurs because the nearly constant uncoupler-induced absolute decrease in the phosphorylation rate becomes less significant as the respiration rate increases. Extrapolation of plots of P/2e⁻ versus incubation time to the P/2e⁻ axis (i.e. to zero incubation time)
also yields the values of Fig. 2. This occurs because the respiratory rates are average rates and the assays were carried out such that the average rate of respiration was inversely proportional to the incubation time (i.e. the amount of O₂ or ferricyanide reduced was constant among determinations within individual experiments). From these observations, it is evident that the procedure employed here also avoids errors due to time-dependent changes in O₂ concentration such as those due to backflow of O₂ into the incubation mixture and to O₂ consumption by the O₂ electrode.

Using site-specific respiratory chain inhibitors other than those indicated appeared to have no effect on the maximum P/2ₑ⁻ ratios of Fig. 2. However, this was checked thoroughly only in the case of the Sites 1 + 2 system by using rotenone rather than 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT). In any case, the inhibitors themselves appeared not to be involved in the apparent uncoupling. This is evident in the case of cyanide from the fact that it was present at relatively high concentration in the Site 1, Site 2, and Sites 1 + 2 incubation mixtures and in the case of rotenone from the fact that it was present at relatively high concentration in the Site 2, Site 3, and Sites 2 + 3 incubation mixtures.

Effect on the P/2ₑ⁻ Stoichiometry of Inhibiting the Energy-Conserving Reactions—Ernst and Nordenbrand (38) showed that if the energy-conserving reactions are made more limiting by decreasing the concentration of ADP or hexokinase or by inhibiting the ATP synthetase reaction with oligomycin, the degree of coupling can be markedly decreased. These findings indicate that uncoupling occurs whether the overall reaction is inhibited thermodynamically by a high extramitochondrial ΔGₚ or kinetically by decreasing the effective concentration of an enzyme of the energy-conserving reactions. The present investigation included a number of studies which strongly support this indication. Thus, it was observed that, for a particular degree of respiratory inhibition, virtually the same degree of uncoupling occurs whether respiration is inhibited by decreasing the concentration of hexokinase, decreasing the concentration of ADP, inhibiting the ATP synthetase reaction with aurovertin, or inhibiting the adenine nucleotide transport reaction with carboxyatractyloside. All the systems were examined only in the cases of aurovertin and carboxyatractyloside inhibition. The carboxyatractyloside results are presented in Fig. 3. Previous studies in this laboratory (24, 39) have indicated that carboxyatractyloside binds to the adenine nucleotide carrier nearly stoichiometrically up to a concentration of about 0.8 nmol/mg of mitochondrial protein under conditions similar to those employed here and that the concentration of binding sites in heart mitochondria is approximately 1.2 nmol/mg of protein. According to this, the experiments of Fig. 3 indicate that in all the systems other than half of the adenine nucleotide carriers can be inactivated with only a slight decrease in the degree of coupling under the conditions employed.

In regard to respiratory control, Fig. 3 shows that large differences occur among the coupling sites. As might be expected, respiratory control is closely related to the degree of coupling. This can be seen from Table I, which presents the respiratory control ratios observed in the experiments of Fig. 3 and indicates the extents to which the P/2ₑ⁻ ratios are decreased at various degrees of respiratory inhibition by carboxyatractyloside. It can be seen also from Table I that respiratory control at the individual sites is largely additive. Correction of the State 4 rates for uncoupling by coenzyme Q₁₀ and the apparent endogenous uncoupler using the respiratory axis intercepts of Fig. 1 (G-I) as indicators of the contributions of the uncoupling yields respiratory control ratios that suggest complete additivity.

**DISCUSSION**

**On the Maximum P/2ₑ⁻ Stoichiometries**—As indicated above, values very close to 1.1, 0.5, and 1.0 in the Sites 1, 2, and 3 systems, respectively, were observed. A question that needs consideration is how these stoichiometries come about. In this regard, it is now generally accepted that respiration results in H⁺ ejection and that phosphorylation is driven by the gradient thus formed. In recent years, interest in stoichiometry has been focused primarily on this process. Owing to the many technical difficulties in conducting such studies, uncertainties persist here also. However, it is now generally agreed that the net charge separated across the inner membrane as a result of H⁺ ejection is the activity that is relevant to the coupling of respiration to phosphorylation and that the q+/2ₑ⁻ stoichiometry at Site 2 is 2 for the oxidation of succinate by cytochrome c or ferricyanide. As noted recently by Wikström (41, 42), if one knows the charge stoichiometry at one site, one can deduce the charge stoichiometries at the others if the site/site ratios are known. Since the present study gives these ratios, something can be said here concerning the charge stoichiometries at the three coupling sites assuming, of course, that the q+/2ₑ⁻ stoichiometry at Site 2 is in fact 2. Proceeding on this assumption and the assumption that the observed maximum P/2ₑ⁻ ratios came about as a result of H⁺ transport only at the level of the respiratory chain, one arrives at q+/2ₑ⁻ stoichiometries of 4.4, 2.0, and 4.0 for Sites 1, 2, and 3, respectively. Since only integral stoichiometries are acceptable in this case and since a q+/2ₑ⁻ stoichiometry of 4 is in the integral value that is most consistent with that deduced for Site 1, a P/2ₑ⁻ stoichiometry of 1.0 will be assumed for this site.

The question now is how to explain the nonintegral q+/2ₑ⁻ stoichiometry of the Site 1 system indicated in the above considerations. That the Site 1 P/2ₑ⁻ stoichiometry is in fact 1.0 rather than 1.1 is suggested by preliminary determinations of P/2ₑ⁻ quotients with 3-hydroxybutyrate as the oxidizable substrate and parapryuvate, a potent and highly specific inhibitor of the 2-ketogluatrate dehydrogenase reaction (43), to block acetocetate oxidation, which occurs in heart mitochondria and is absolutely dependent on the operation of the Krebs cycle. The values observed in systems of this sort were invariably lower by approximately 0.1 as compared to those observed with malate + pyruvate under similar conditions. 3-Hydroxybutyrate was not used in the main investigation.
FIG. 3. Effects of carboxyatractyloside inhibition on the respiratory activities and P/2e\(^{-}\) stoichiometries in the six systems examined in this study. The data of A–C were obtained in one experiment, and the data of D–F in another. The points in E represent means of three determinations, and those in the others represent means of two determinations.

TABLE I

| Experiment | System | RCR* | Decrease in P/2e\(^{-}\) ratio at x% inhibition of respiration |
|------------|--------|------|---------------------------------------------------------------|
| 1          | Site 1 | 8.4  | 0.4 0.8 1.2 1.9 2.3 |
|            | Site 2 | 2.0  | 19 33 45 62 |
|            | Site 3 | 1.5  | 13 39 63 |
| 2          | Sites 1 + 2 + 3 | 15.8 | 0.4 0.8 1.2 1.9 2.3 |
|            | Sites 1 + 2 | 12.7 | 1.3 2.6 4.0 6.0 7.9 |
|            | Sites 2 + 3 | 4.6  | 5.8 8.6 10.8 13.7 20.1 |

*Rate of respiration in the absence of carboxyatractyloside divided by the rate under conditions of maximum inhibition by carboxyatractyloside.

because of the unfavorable equilibrium of the 3-hydroxybutyrate dehydrogenase reaction (44) and of a consequent marked decrease in the rate of the reaction in the course of achieving enough O\(_2\) or ferricyanide reduction to obtain an accurate estimate of P\(_i\) consumption.

One possibility for explaining the higher value obtained with malate + pyruvate is that these substrates were oxidized beyond 2-ketogluarate, resulting in phosphorylation at the succinate thiokinase level. This investigation included several studies which indicate this possibility to be very unlikely, the most convincing of which show that parapyruvate has no effect whatever on the P/2e\(^{-}\) ratios observed in the malate + pyruvate systems. The only other viable possibility seems to be that some phosphorylation occurred as a result of transhydrogenase activity. In fact, there has been much speculation as to the involvement of the transhydrogenase reaction in oxidative phosphorylation, and it has been demonstrated that reduction of NAD by NADPH via the mitochondrial transhydrogenase can drive ATP synthesis (45). Furthermore, heart mitochondria contain a relatively large amount of this enzyme (46–48) as well as a relatively large amount of NADP-specific isocitrate dehydrogenase and the transhydrogenase. In addition, Moyle and Mitchell (51) have presented evidence indicating that isocitrate oxidation occurs exclusively by way of the NADP-specific enzyme. However, this has been disputed (see Ref. 52).

The observed deviation of the maximum P/2e\(^{-}\) stoichiometry from 1.0 in the Site 1 system can be accounted for very well by assuming involvement of the NADP-specific isocitrate dehydrogenase and the transhydrogenase. This can be seen from Fig. 4, which presents a reaction scheme for each of the single-site systems examined in this study. In the case of the Site 1 scheme, it is assumed that malate and pyruvate are oxidized to 2-ketogluarate, that pyruvate enters the mitochondrial via the monocarboxylate carrier in exchange for intramitochondrial OH\(^-\), that malate enters via the 2-ketogluarate carrier in exchange for 2-ketogluarate, and that only the NADP-specific isocitrate dehydrogenase is active. The oxidation of 1 molecule each of malate and pyruvate would thus be accompanied by the formation of 2 molecules of NADH and 1 of NADPH. The NADPH is assumed to reduce NAD via the transhydrogenase reaction with an H\(^+\)/2e\(^{-}\) stoichiometry of 1 in accordance with Earle and Fisher (53), the overall process resulting in the conversion of 3 molecules of NAD to 3 of NADH in the matrix and the separation of 1 net
FIG. 4. Scheme giving the stoichiometries for proton transport that are most consistent with the information in the literature and the maximum P/2e\(^{-}\) stoichiometries observed in this study. Complete reaction schemes for each of the three single-site systems examined here are presented assuming the ejection of four protons per molecule of extramitochondrial ATP formed from extramitochondrial ADP and Pi. In the case of the Site 1 system, it is assumed that malate and pyruvate are oxidized to 2-ketoglutarate, that only the NADP-specific isocitrate dehydrogenase is active, and that NADPH reduces NAD via the transhydrogenase reaction with an H\(^{+}\)/2e\(^{-}\) stoichiometry of 1. With these assumptions, maximum P/2e\(^{-}\) stoichiometries of 1.08, 0.50, and 1.00 in the Sites 1, 2, and 3 systems, respectively, are predicted. Catalytic components are indicated only in the cases of those that are membrane-bound. MCC, monocarboxylate carrier; KGC, 2-ketoglutarate carrier; TH, transhydrogenase; NDH, NADH dehydrogenase; bc, cytochrome bc complex; a\(_{2}\), cytochrome a\(_{2}\) complex; SDH, succinate dehydrogenase; PC, phosphate carrier; AS, ATP synthetase; ANC, adenine nucleotide carrier; DCC, dicarboxylate carrier; DHAA, dehydroascorbic acid; TMPD, N,N,N,N-tetramethyl-p-phenylenediamine.

electrical charge equivalent across the coupling membrane due to H\(^{+}\) ejection. Assuming, as above, that the q\(^{-}\)/2e\(^{-}\) stoichiometry at Site 1 is 4 and that 4 of the charges are consumed per molecule of extramitochondrial ATP formed from extramitochondrial ADP and Pi (Fig. 4), the expected P/2e\(^{-}\) quotient would be 1.08, a value in good agreement with the maximum value observed with the Site 1 system.

Using a less direct method than that employed by Earle and Fisher (53), Moyle and Mitchell (51) estimated the H\(^{+}\)/2e\(^{-}\) stoichiometry of the transhydrogenase reaction to be 2. If this value were assumed, a P/2e\(^{-}\) stoichiometry of 1.17 would be predicted for the Site 1 system. For a number of reasons, this higher value for the H\(^{+}\)/2e\(^{-}\) stoichiometry at the transhydrogenase level cannot be considered to be inconsistent with the observations of this study. For one, it seems likely that some of the tricarboxylates escaped to the medium via the tricarboxylate carrier in exchange for malate and thus were not oxidized to 2-ketoglutarate. Heart mitochondria have been observed to carry out malate/tricarboxylate exchange, although only at very low rates relative to the rates of the exchanges catalyzed by the dicarboxylate and 2-ketoglutarate carriers (54-56). For another, it is likely that some of the isocitrate oxidation is catalyzed by the NAD-specific dehydrogenase. This enzyme is present in heart mitochondria but apparently at very low levels relative to the NADP-specific enzyme (49, 50). Also, the NAD-specific enzyme has a much lower affinity for substrates than the NADP-specific enzyme (57, 58) and is subject to fairly strong inhibition by ATP (59) and to moderate inhibition by cyanide (52, 58), which was present at a concentration of 2 mM in the Site 1 and Sites 1 + 2 incubation mixtures. Still another consideration in the above matter is that the maximum P/2e\(^{-}\) estimate obtained with the Site 1 system may be slightly low because of the presence of uncouplers (coenzyme Q\(_{1}\) and the apparent endogenous uncoupler) and of the fact that the procedure used here to avoid the effects of uncouplers in estimating the maximum P/2e\(^{-}\) ratios is not entirely successful. This lack of complete success can be seen from the data reported recently
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by Beavis and Lehninger (7), who extended the procedure in an effort to eliminate this deficiency. Thus, they determined the slope and intercept changes that occur in plots of respiration rate versus phosphorylation rate as the concentration of an uncoupler is increased and then extrapolated the slope values to zero intercept assuming a linear relationship between the change in slope and the change in intercept. This extension is likely valid and probably should be used in P/2e- determinations with mitochondria having a high level of the apparent endogenous uncoupler. In the present study, the slope changes due to the uncoupler S-13 (5-chloro-3-t-butyl-2'-chloro-4'-nitrosalicylanilide) at concentrations that result in intercept values comparable to those shown in Fig. 1 (G-I) were too small to detect by the methods employed, and consequently the slope changes due to uncoupling by coenzyme Q and the apparent endogenous uncoupler were judged to be negligible.

In regard to H+ ejection at Site 1, there appears to be little disagreement at present. Consistent with Fig. 4, an H+/2e- stoichiometry of 4 has been indicated in recent measurements on Sites 1 + 2 systems (e.g. see Refs. 13, 42, and 60). In contrast, the H+/2e- stoichiometry at Site 3 is currently a matter of controversy (e.g. see Refs. 2, 61, and 62). Values of 0 (63, 64), 2 (41, 65-69), and 4 (22, 70) have been suggested from various kinds of measurements in recent years. Fig. 4 indicates an H+/2e- quotient of 2 at Site 3 and is consistent throughout with the recent observations of Wikström (41, 42) on intramitochondrial pH changes associated with respiratory activity at the three coupling sites indicating that the Site 1/2/Site 3 q+/2e- stoichiometry is a multiple of 2/1/2.

On the Mechanisms of Respiratory Control—It was pointed out in Ref. 37 that in regard to respiratory control a distinction should be made between kinetic and thermodynamic control. Kinetic control occurs as a result of limitation of the physical needs of a reaction to proceed (e.g. substrates, active catalysts), whereas thermodynamic control occurs as a result of a reaction approaching thermodynamic equilibrium. It was pointed out also that this distinction should be made at the level of the energy-yielding respiratory chain reactions as well as at the level of the energy-conserving reactions. Here we are concerned primarily with respiratory control of the kinetic kind at the level of the energy-conserving reactions, in particular that brought about by inhibition of the adenine nucleotide transport reaction by carboxyatractysolide.

The results of this study (Fig. 3 and Table 1) show that the maximum degree of respiratory control differs greatly among the three coupling sites and that the control exerted at the individual sites appears to be additive in the multisite systems. In addition, a good correlation between the degree of respiratory inhibition and the degree of coupling is shown (Table 1). Assuming that ΔP is delocalized within individual mitochondria, as previous studies in this laboratory have indicated (37), these observations suggest that the incompleteness of respiratory control is not due primarily to cycling of protons or other ions as a result of their leaking or being electrophoresed across the coupling membrane. If such were the case, one could expect the uncontrolled portion of the respiratory activity to be highest in the system capable of developing the highest ΔP.

Before looking into this expectation, it is important first to normalize the uncontrolled respiratory activities to take into account the fact that the q+/2e- stoichiometry differs among the systems. This will be done by multiplying the observed uncontrolled (State 4) respiratory activities by the observed maximum P/2e- stoichiometries of Fig. 2 to give "apparent leakage" rates (μeq of charge/min/mg of mitochondrial pro-tein) that might be expected if the uncontrolled respiratory activity were in fact due to ion cycling and each of the systems were to have a P/2e- stoichiometry of unity rather than what was observed. Doing this in the case of the single-site experiment of Fig. 3 yields values of 0.11, 0.19, and 0.59 for the Sites 1, 2, and 3 systems, respectively. Correcting these values for leakage due to the apparent endogenous uncoupler and coenzyme Q, by subtracting the products of the maximum P/2e- ratios and the respiratory axis intercepts of Fig. 1 (G-I) yields values of 0.05, 0.15, and 0.56 for the Sites 1, 2, and 3 systems, respectively. These values indicate that the apparent leakage rate due to inhibition at the energy conservation level differs among the single-site systems approximately in the ratio 1/3/11. This ratio differs greatly from the ratio of the proton motive forces that might be expected to develop in the single-site systems. The ratio of the proton motive forces is likely to be similar to the ratio of the redox potential spans, which is similar to the ratio of the P/2e- stoichiometries (i.e. 2/1/2).

Normalizing and correcting the uncontrolled portions of the respiratory activities in the multisite experiment of Fig. 3 yields values of 0.12, 0.07, and 0.25 for the Sites 1 + 2 + 3, Sites 1 + 2, and Sites 2 + 3 systems, respectively. From these and the single-site values, it appears that the apparent leakage rate in a multisite system is determined largely by the coupling site within the system having the lowest apparent leakage rate rather than by ΔP.

These observations indicate that under the conditions employed the degree of respiratory control is determined mostly by how tightly the transport of electrons through the coupling sites is linked to the ejection of protons rather than by cycling of protons or other ions through the coupling membrane. In other words, they indicate that, at least in the cases of the Sites 2 and 3 systems, the uncontrolled portions of the respiratory activities were due largely to molecular slipping (71, 72). Since the coupling sites differ in regard to mechanisms of electron transport, it is not surprising that they differ also in this regard. However, the above indication would be surprising if one were to assume that O2 is reduced to water on the matrix side of the coupling membrane as originally visualized in the chemiosmotic theory. If O2 were reduced on the matrix side, proton transport at Site 3 would be required for only half of the charges separated across the membrane per electron transported. Since separation of half of the charges would thus be completely coupled to electron transport and since the driving force for ion leakage in the Site 3 system is likely to be about the same as that in the Site 1 system, one could expect the magnitude of the uncontrolled respiratory activity in the Site 3 system to be at most only about twice that observed in the Site 1 system rather than the observed value of 11 times the Site 1 value. This observation might be taken to suggest that all of the redox components at Site 3 are located near the outer side of the coupling membrane and are linked to the matrix by a proton channel. A model of this sort has been favored in recent years by Wikström and co-workers on thermodynamic and topological grounds (e.g. see Ref. 73).

In view of the low degree of respiratory control at Site 3 and of the consequent sensitivity of this site to uncoupling of the type that occurs as a result of a high extramitochondrial ΔG0 and limitation at the energy conservation level, it might be supposed that the maximum P/2e- ratio observed with the Site 3 system does not closely approximate the mechanistic stoichiometry at this site. In view of the close agreement among the systems (Fig. 2), this seems unlikely. In any case, one could expect uncoupling of this nature to have been most
serious in the Sites 1 + 2 + 3 system, in which the rate and extent of phosphorylation were highest. Also, since inhibition at the respiratory chain level in the cases of the Sites 1 and 3 systems results in the energy-conserving reactions becoming less limiting (37), one could expect uncoupling of this nature to have resulted in a tendency for the P/2e− ratios in these systems to increase as the respiratory reactions were progressively inhibited (Fig. 1) and thereby to have resulted in a lowering of the maximum P/2e− estimates. In the Site 2 system, the opposite could be expected because the inhibition affected at the respiratory chain level in this case results in the energy-conserving reactions becoming more limiting (37). In the Sites 1 + 2 + 3 system, the two opposing tendencies could be expected to have balanced more or less exactly (37, 39), whereas in the Sites 1 + 2 and 2 + 3 systems, the tendency of the Site 2 system could be expected to have predominated (37).

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SUPPLEMENTAL MATERIAL TO
DETERMINATION OF THE P70+/2e STOICHIOMETRIES AT THE INDIVIDUAL COUPLING SITES IN MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION. EVIDENCE FOR MAXIMUM VALUES OF 1.4, 0.9, AND 1.6 SITES 1, 2, AND 3.

by
Clinton D. Stans

EXPERIMENTAL PROCEDURES

Mitochondria were isolated from slaughterhouse bovine hearts according to a slightly modified procedure (3). Homogenates were prepared in a medium containing 300 mM sucrose, 5.8 mM HEPES, 4.5 mM K$_2$HPO$_4$, 5.8 mM and 0.5 mM of bovine proteinase/g of tissue. The mitochondria were washed twice and resuspended finally at a concentration of 194 mg protein/ml in a medium containing 350 mM sucrose and 5.8 mM HEPES (pH 7.0). 400 mg of these were incubated for 20 min at 25°C. The reaction was stopped by the addition of 50 mg of trichloroacetic acid (TCA). The absorbance of the mixture was measured at 410 nm using a spectrophotometer from the wavelength difference. 440-490 nm using an absorbance coefficient of 0.595 $\pm$ 0.042. The values of the absorbance at 440 nm was calculated from the absorbance of the mixture with no added mitochondria. The absorbance of the mixture with no added mitochondria was subtracted from the absorbance of the mixture with added mitochondria.

The standard incubation medium was the same for the two systems and contained 115 mM sucrose, 18 mM glucose, 10 mM K$_2$HPO$_4$ (pH 7.0), 1.5 mM K$_2$-EDTA, and 20 mM K$_2$-EGTA (pH 7.0). In each reaction, the temperature of incubation was 22°C and the concentration of K$_2$-EGTA was 2.5 mM. In some instances the concentration of K$_2$-EDTA used in the standard incubation medium varied from 0.05 mM to 2.5 mM and the order of addition of substrates to the incubation mixture differed among the experiments and are given in the descriptions of the individual assays.

The concentration of the standard incubation medium was determined with NADH and heart subchromatophores (4, 15). In the course of the 12 experiments reported in this communication, 15 determinations being made in each of the experiments. The medium was essentially similar but containing 0.1 M HEPES pH 7.0, 5 mM MgCl$_2$, and 0.5 mM EGTA. The absorbance at 465 nm was determined for each of the different concentrations. The absorbance of the mixture with no added mitochondria was subtracted from the absorbance of the mixture with added mitochondria.

The absorbance coefficient for NADH was determined on three occasions. The values obtained were 0.688, 0.594, and 0.680 $\pm$ 0.042. For absorbance in the Astron DN-2 at 440-490 nm. The determinations were made using the standard incubation medium and an absorption coefficient of 0.529 $\pm$ 0.032, which was determined with the concentration of NADH in the Astron DN-2 using 0.01 mg/ml of NADH in the Astron DN-2.

In the absence of Mg the terminal electron acceptor was monitored such that the concentration of O$_2$ in the assay medium could be easily established and then followed with the oxygen electrode. This was accomplished by adding the equilibrium standard medium to the incubation chamber and starting and adjusting the recording apparatus as described for the O$_2$ determinations before making additions to the medium. In general the order in which additions were made was chosen with the object of minimizing reoxidation and phosphorylation prior to initiating maximum activity upon making the final addition. In the figure S1 + 2, 2 assay was initiated by adding 0.2 mg of the standard medium to the mitochondria for 0.5 min, 2.0 mM K$_2$-EDTA and 0.1 mM pyruvate were added and after an additional 0.5 min, ADP and hexokinase in standard incubation medium. In the case of the middle 2 + 3 assay, rotenone at a 1:1000 dilution and any other inhibitors that were added were added initially and then the mitochondria. The mitochondria were preincubated for 1.5 min before initiating oxidative phosphorylation by adding in rapid succession ADP + hexokinase and 35 mM K$_2$-ATP. The preincubation period was rapid enough that in 2.5 s after the addition of the ADP + hexokinase in a total reaction volume of 0.5 ml, the absorbance of the reaction mixture was reduced to 0.25 $\pm$ 0.02 absorbance units. In the experiments involving the standard medium only, additions of ADP and hexokinase were made after the mitochondria. In the experiments involving the standard medium only, the mitochondria were preincubated for 1.5 min before adding 0.15 mM NADH, ADP, and hexokinase and 0.05 mM ADP + 0.5 mM FMN at 30 s intervals. When cytochrome c inhibition was involved, ADP + hexokinase was added before the mitochondria and NADH was added immediately after the mitochondria. 10 mM NADH was added to the assay medium in the absence of cytochrome c and in steady reaction order was initiated.

In the case of the S1 + 2, 2 assay, 2.5 mg protein each of rotenone and oxydysulfur were added before the mitochondria. In experiments involving cytochrome oxidase inhibition, the inhibitor was added initially and then the mitochondria were preincubated for 1 min before adding 0.15 mM NADH, ADP, and hexokinase. 0.25 mM NADH in 0.05 mM ADP + 0.1 mM FMN at 30 s intervals. When cytochrome c inhibition was involved, ADP + hexokinase was added before the mitochondria and NADH was added immediately after the mitochondria. 10 mM NADH was added to the assay medium in the absence of cytochrome c and in steady reaction order was initiated.

In the case of the S1 + 2, 2 assay, oxidative phosphorylation was allowed to proceed to the point where all or nearly all of the terminal electron acceptor was reduced. When this point was reached, 18.0 nmol absorbance change was completed in a total reaction volume of 0.5 ml. At this point, the reaction was stopped by the addition of 0.1 ml of 1:1000 dilution of myxothiazol. The absorbance was measured at 465 nm and then the absorbance of the reaction mixture was reduced to 0.25 $\pm$ 0.02 absorbance units. In the experiments involving the standard medium only, the mitochondria were preincubated for 1.5 min before adding 0.15 mM NADH, ADP, and hexokinase and 0.05 mM ADP + 0.5 mM FMN at 30 s intervals.

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The absorbance coefficient for S1 + 2, 2 assay was determined on three occasions. The values obtained were 0.688, 0.594, and 0.680 $\pm$ 0.042. For absorbance in the Astron DN-2 at 440-490 nm. The determinations were made using the standard incubation medium and an absorption coefficient of 0.529 $\pm$ 0.032, which was determined with the concentration of NADH in the Astron DN-2 using 0.01 mg/ml of NADH in the Astron DN-2.