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The Potentiating Effect of Adenosine Diphosphate in the Uncoupling of Oxidative Phosphorylation in Potato Mitochondria†

George G. Laties

ABSTRACT: In the absence of added ADP the rate of oxygen utilization by isolated potato mitochondria oxidizing citrate, pyruvate, malate, or succinate is relatively unresponsive to uncouplers of oxidative phosphorylation, *viz.* carbonyl cyanide *m*-chlorophenylhydrazone (*m*-Cl-CCP). *m*-Cl-CCP causes marked respiratory stimulation when added after, or concomitantly with, ADP. The rate of oxygen uptake when *m*-Cl-CCP is added to mitochondria in state 4 is invariably less than the rate typical of state 3. Additional ADP added after *m*-Cl-CCP to state 4 mitochondria elicits the maximal rate of oxygen use characteristic of state 3. A modulator or effector role is imputed to ADP, distinct from its acceptor function in oxidative phosphorylation. ADP potentiation of uncoupler action is second order, with an $s_{0.5}$ of approximately 80 μM. The effector function of ADP is not fulfilled by guanosine nucleotides, nor by AMP or ATP.

Whether oxidative phosphorylation is effected by chemical coupling (see Lehninger, 1970) or by charge separation as per the chemoosmotic theory (Mitchell, 1966), conventional uncouplers are considered to act previous to any involvement of ADP. In fact a classical uncoupler, DnpOH,[1] inhibits not only the P_i–ATP exchange reaction, but also inhibits oxygen exchange between phosphate and water (Cohn, 1953; see Lehninger, 1970), further emphasizing that conventional uncouplers act well before the step in which ADP participates in ATP formation. Nevertheless, with potato mitochondria we have consistently failed to elicit marked respiratory stimulation with uncouplers in the absence of added ADP. By contrast, the respiration of potato mitochondria in state 4, *i.e.*, where added ADP has been largely converted to ATP (Chance and Williams, 1956), is sharply enhanced by uncouplers, and the stimulated respiration is resistant to oligomycin.

The foregoing observations suggest a dual role for ADP in potato mitochondrial electron transport: (1) as a conventional phosphate acceptor in oxidative phosphorylation and (2) as a modulator in the implementation of uncoupler activity. A dual role was suggested some time ago (Laties, 1953), but since the observations leading to the deduction centered largely on α-ketoglutarate oxidation, and coincided with the discovery of substrate-level phosphorylation, the need for ADP to implement uncoupler effectiveness was subsequently erroneously attributed solely to its role in substrate-linked phosphorylation. In this vein, two subsequent examples of the dependence of uncoupler effectiveness on ADP both dealt with pyruvate oxidation—by horsefly sarcosomes in one instance (Van den Bergh, 1964) and by insect mitochondria in the other (Gregg *et al.*, 1964). In each case pyruvate oxidation was complete, and the dependence on ADP was shown to be due to the substrate-level phosphorylation attending α-ketoglutarate oxidation. However, the experiments set out below demonstrate the dual role of ADP with a variety of substrates where substrate-level phosphorylation is not at issue.

Isolated plant mitochondria frequently show an attenuated state 3 on the first presentation of ADP. With several cycles of alternating state 3–state 4, state 3 rises to a maximum, the phenomenon having been called "conditioning" (Raison *et al.*, 1973a). Conditioning apparently characterizes isolated mammalian mitochondria as well (Raison and Lyons, 1971; Raison *et al.*, 1973a). We have subsequently discovered that state 4 brings about conditioning as effectively as alternating cycles, and that in fact continuous state 3 is inimical to maximal conditioning (Raison *et al.*, 1973b). Nevertheless, conditioning is abetted by ADP in the absence of oxidative phosphorylation, a fact which can be demonstrated by incubating mitochondria with ADP in the absence of inorganic phosphate, and subsequently initiating state 3 by addition of the latter. ADP is consequently implicated in conditioning, and apparently for reasons other than its role as phosphate acceptor in oxidative phosphorylation. Thus we must ask

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[1] Abbreviations used are: DnpOH, 2,4-dinitrophenol; Mercap, 2-mercaptobenzothiazole, Na salt; *m*-Cl-CCP, carbonyl cyanide *m*-chlorophenylhydrazone; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

whether ADP is an intrinsic effector of uncoupler action, or whether it acts solely by dint of its role in conditioning. The evidence favors the direct influence of ADP on uncoupler effectiveness.

Experimental Section

Isolation of Mitochondria. Mitochondria were isolated from potato tubers (var. Russet Burbank) as follows. Chilled washed tubers were cut in half along the short axis and cores of tissue approximately 1 in. in diameter were removed from within the vascular cylinder with a stainless steel borer; 100 g of tissue at a time was dropped into the port of a vegetable juicer (Oster Mfg. Co., Milwaukee) followed at once by 200 ml of extraction mixture. The juicer consisted of an aluminum-perforated basket centrifuge with a toothed plate in its base, all contained in a plastic housing. The plate and basket were spun at approximately 3000 rpm (*ca.* 750*g*), and the basket was lined with a strip of Miracloth (Chicopee Mills, New York, N. Y.) which filters cell wall material and intact cells. The extract was collected in an iced beaker, transferred to 50-ml plastic centrifuge tubes, and spun at 4000*g* for 3 min in a Sorvall RC-2B to remove starch. The supernatant solution was filtered through Miracloth into fresh centrifuge tubes and promptly centrifuged at 39,000*g* for 5 min. The supernatant solution was now discarded, the pellet was resuspended in extraction medium, and the suspension was centrifuged at 39,000*g* for 5 min. The pellet of this centrifugation was resuspended in 2 ml of suspension medium by gently drawing the pellet and medium into a Pasteur pipet and ejecting same until suspension was uniform. The final suspension contained approximately 4.5 mg of mitochondrial protein/ml.

Extraction, Suspension, and Reaction Media. EXTRACTION AND WASH MEDIUM: 0.35 M mannitol, 0.25 M sucrose, 0.025 M Tes buffer (pH 7.8), 1 mg/ml of bovine serum albumin; and 0.1 mg/ml of sodium mercaptobenzothiazole (Mercap; see Laties and Treffry, 1969; Laties, 1973a).

FINAL SUSPENSION MEDIUM: 0.35 M mannitol, 0.25 M sucrose, 0.025 M Tes buffer (pH 7.8), and 1 mg/ml of bovine serum albumin.

REACTION MEDIUM: 0.4 M mannitol, 0.025 M Tes buffer (pH 7.4), 5 mM Mg^{2+} (SO_4 or Cl), 5 mM potassium phosphate (pH 7.4), and 1 mg/ml of bovine serum albumin. Substrate, ADP, *m*-Cl-CCP, and oligomycin were added as indicated in the figures.

Respiratory Measurement. Mitochondrial suspension (0.2 ml; approximately 0.9 mg of mitochondrial protein) was added to approximately 3.2 ml of reaction mixture in a jacketed temperature-controlled plastic chamber. Oxygen concentration was measured with a Clark oxygen electrode, the chamber contents being stirred magnetically, and oxygen utilization recorded with a potentiometric recorder with 10-mV input. Addenda were made with a microsyringe; chamber temperature, 25°. Rates were expressed as nanomoles of O_2 per minute per milliliter of reaction mixture. Initial oxygen concentration was taken as 240 μM.

NITROGEN DETERMINATION. The final pellet was first suspended without bovine serum albumin. Aliquots were taken for nitrogen determination and bovine serum albumin thereupon was added at once to a concentration of 1 mg/ml. Nitrogen was determined by digestion and Nesslerization, with due correction being made for buffer nitrogen.

REAGENTS. Oligomycin was added in 5 μl of ethyl alcohol to a final concentration of 1 μg/reaction vessel (3.4 ml). An equal quantity of alcohol added to controls was without effect.

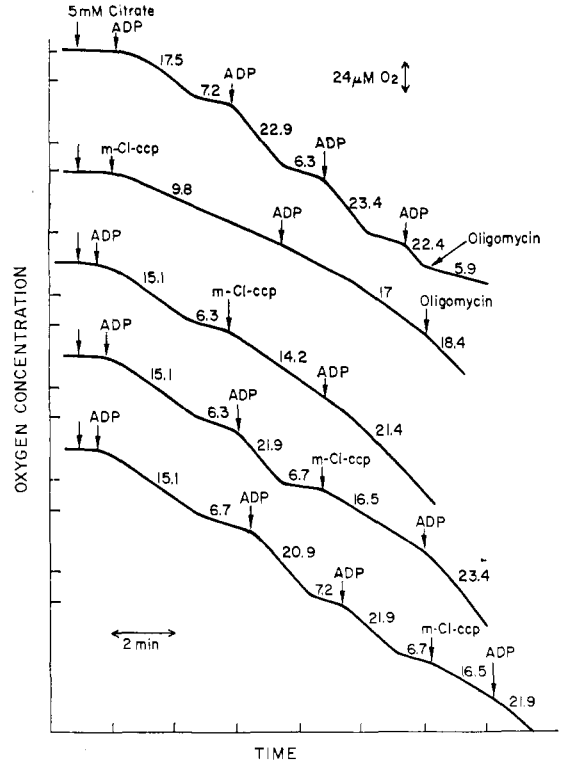

FIGURE 1: The effect of ADP on the uncoupling action of *m*-Cl-CCP during citrate oxidation. Experimental conditions as per Methods. Numbers indicate nmoles of O_2 per minute per ml of reaction medium. Total volume, 3.4 ml; temperature 25. Approximately 0.9–1.35 mg of total mitochondrial protein/reaction vessel.

m-Cl-CCP final concentration was 10^{-5} M. ADP final concentration was 0.17 mM unless otherwise specified.

Results

The genesis of this study stemmed from a wish to determine the isotherm for citrate (hence isocitrate) oxidation unobscured by possible kinetic limitations imposed by the electron transport chain. To this end one or another uncoupler (*i.e.*, DnpOH, *m*-Cl-CCP) was added immediately after the substrate in the absence of ADP. Respiratory rates invariably proved very low compared with rates elicited by ADP. Figure 1 exemplifies a case in point, and reveals several noteworthy features. Conditioning is indicated by the increasing state 3 rate with successive state 3–state 4 cycles in the upper trace. The uncoupler *m*-Cl-CCP added initially evokes a rate of oxygen utilization well below that elicited by ADP. When uncoupler is added after at least one state 4 its effect is greater than when added initially in the absence of ADP. Further, the addition of ADP *after m*-Cl-CCP invariably causes marked respiratory stimulation, and the respiration is totally resistant to oligomycin (*cf.* Figure 2). Thus it may be concluded that the response to ADP presented after uncoupler is not attributable to suboptimal uncoupler effectiveness. At 5 mM citrate there is little or no respiratory inhibition by arsenite. Oxygen utilization consequently reflects the oxidation of citrate (*i.e.*, isocitrate) with little or no contribution by α-ketoglutarate oxidation. The observations made with *m*-Cl-CCP apply as well to DnpOH, albeit with potato, *m*-Cl-CCP is the more effective uncoupler.

Figure 2 examines the influence of relative times of addition of *m*-Cl-CCP and ADP on the effectiveness of the latter in

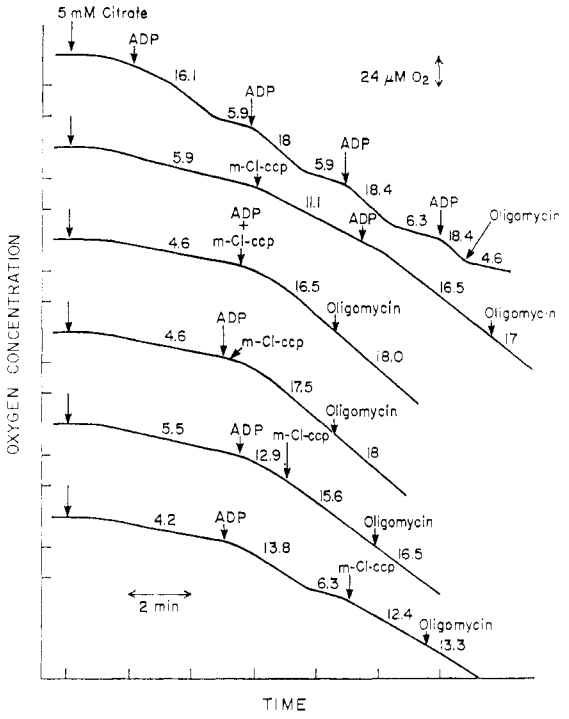

FIGURE 2: The effect of the relative time of addition of *m*-Cl-CCP and ADP, respectively, on the uncoupler action of *m*-Cl-CCP. Substrate, 5 mM citrate.

implementing the response to *m*-Cl-CCP. *m*-Cl-CCP is added before ADP, together with ADP, directly following the addition of ADP or at the end of the first state 3. The experiment represented by Figure 2 addresses itself to the question of whether conditioning is the fundamental requirement for adequate uncoupler effectiveness or whether it is the presence of ADP *per se* which facilitates uncoupler responsiveness. In connection with the conditioning phenomenon attenuation is usually estimated from the ratio of the initial steady state 3 to the final maximum state 3, there being little change in state 4 during the course of the measurement. However, an attenuated initial state 3 is regularly associated with a gradual attainment of the first steady state 3 rate. That is, following ADP addition there is a "roll-off" to the first steady state 3 rate. By contrast, ADP addition following state 4 elicits a prompt response. It is an open question whether the roll-off itself is part of the conditioning process. In Figure 2 addition of ADP or *m*-Cl-CCP was withheld until a constant state 2 rate had been attained to avoid any ambiguity with respect to the explicit influence of the addenda of interest. Again, *m*-Cl-CCP exerts a major influence only in the presence of ADP. Most important, the potentiating effect of ADP occurs at once, that is, when given together with, or immediately following *m*-Cl-CCP, before any conditioning has taken place. When *m*-Cl-CCP is given alone the response is immediate, if not large. There is no roll-off period, and again it is evident that uncoupler can act without conditioning. Conditioning determines the ultimate extent of the respiratory ceiling. While ADP evokes maximal state 3 rates in fully conditioned mitochondria, lesser rates are attained when uncoupler is added to conditioned mitochondria in state 4. In the latter instance maximal rates are achieved only on further addition of ADP (Figures 1 and 4). Thus the affinity for ADP as acceptor in oxidative phosphorylation is higher than the affinity for ADP as effector. More importantly, however, involvement of ADP in the potentiation of uncoupler action is second order (Table I; Figure 3), as is the case in its conditioning role (Raison *et al.*, 1973b). The $S_{0.5}$ for ADP as potentiator of uncoupling activity is approximately 80 μM. The K_m^{ADP} for oxidative phosphorylation is considerably lower (see Discussion). None of the guanidine nucleotides substitute for ADP in its potentiating role. A minimal effect of ATP or AMP may be noted when the latter are given with *m*-Cl-CCP initially, the result of a low level of adenylate kinase activity. However neither ATP nor AMP is stimulatory following *m*-Cl-CCP addition to state 4 mitochondria (as is ADP, see

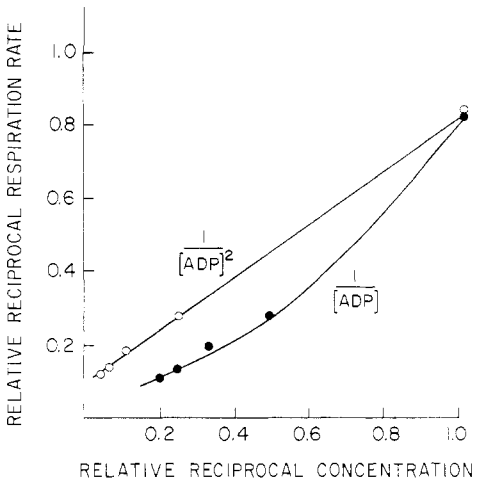

FIGURE 3: The kinetic order of ADP involvement in uncoupler potentiation. Double-reciprocal plot. Respiratory *increment* due to ADP taken for plotting. Closed circles, 1/[ADP]; open circles, 1/[ADP]² plotted against reciprocal of incremental respiration. Rates from Table I.

TABLE I: Respiratory Stimulation by *m*-Cl-CCP as a Function of ADP Concentration.[a]

Initial ADP Concn (mM)		Oxygen Uptake (nmol of O_2 min^{-1} ml^{-1})			
		"State 3"	"State 4"	*m*-Cl-CCP	*m*-Cl-CCP + ADP
0		(6.7)	(6.7)	8.9	12.4
0.01		5.0	5.0	12.0	15.6
0.017		5.5	5.5	11.5	15.6
0.03		6.3	6.3	12.9	17.0
0.1		12.0	5.9	15.6	18.0
0.17	First	16.1	7.6		
	Second	21.4	7.2		
	Third	22.4	7.2	16.5	19.9

[a] Citrate 5 mM in all cases. Initial ADP added 1 min after substrate. State 3–State 4 headings are in quotation marks because there is no control below 0.1 mM ADP. Since states 3 and 4 are defined in relation to initial addition of ADP, values in the absence of added ADP technically represent state 2 and are in parentheses. At ADP levels of 0.1 mM and higher state 3 and state 4 have conventional meaning (Chance and Williams, 1956). *m*-Cl-CCP, 10^{-5} M; 0.025 M Tes, pH 7.4; where *m*-Cl-CCP and ADP are present together, ADP added after steady rate achieved in *m*-Cl-CCP. Final addition of ADP to concentration of 0.17 mM.

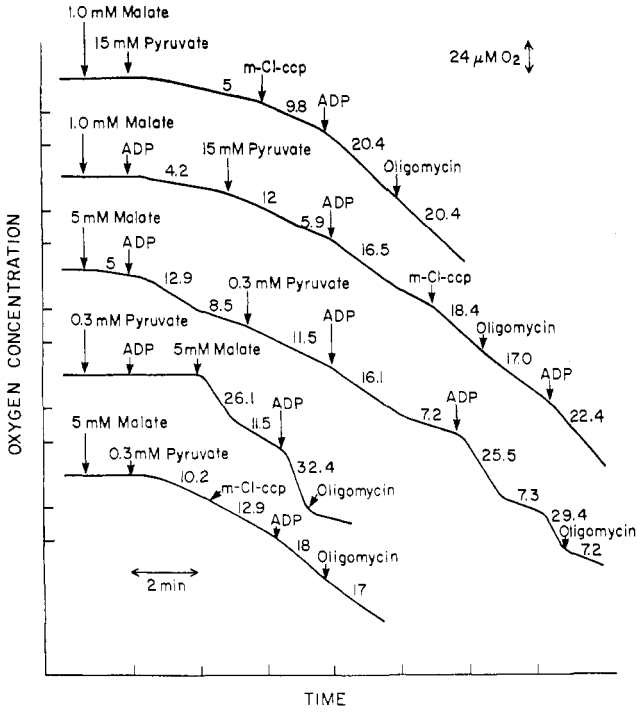

FIGURE 4: The effect of ADP on the uncoupling action of *m*-Cl-CCP during pyruvate and malate oxidation.

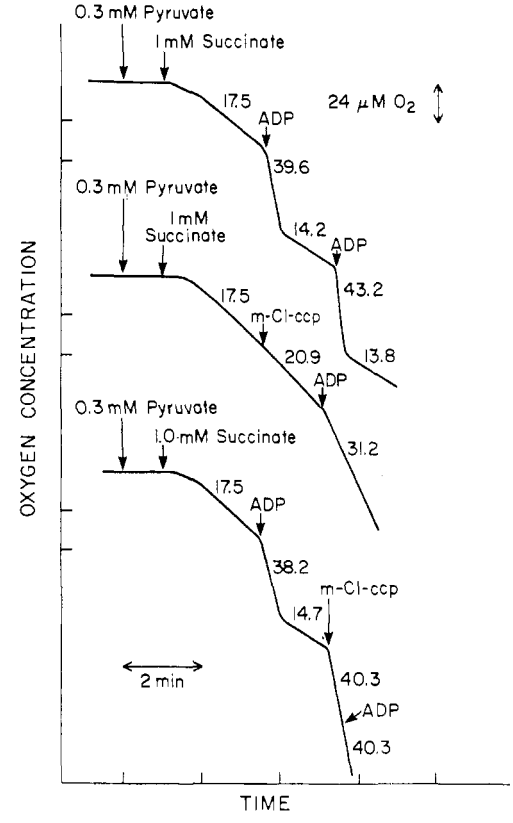

FIGURE 5: The effect of ADP on the uncoupling action of *m*-Cl-CCP during succinate oxidation.

Figures 1 and 2), presumably because the ADP arising from ATP or AMP addition is limited.

Figure 4 demonstrates the potentiating effect of ADP on *m*-Cl-CCP responsiveness in the oxidation of malate and pyruvate, while Figure 5 affirms the same phenomenon for succinate oxidation. With succinate as substrate additional ADP does not cause rate enhancement beyond that evoked by *m*-Cl-CCP when uncoupler is added during state 4. In both cases, however, there is no effect of oligomycin in the presence of uncoupler. Low levels of malate are required for steady pyruvate oxidation, while low levels of pyruvate stimulate malate oxidation. In the absence of low levels of pyruvate, succinate oxidation drops with time—presumably owing to the presence of oxalacetate and the consequent inhibition of succinic dehydrogenase.

Potato mitochondria, as plant mitochondria in general, readily oxidize exogenous NADH, and oxidation is usually inhibited by antimycin A (Hackett *et al.*, 1960; Carmeli and Biale, 1970). While there is general agreement on antimycin sensitivity, results have been disparate with respect to respiratory control (*cf.* Carmeli and Biale, 1970; Hackett, 1961). The difficulty seems to lie in the fact that NADH may be oxidized by more than one path in plant mitochondria, the prevalent path depending upon the levels of Mg^{2+}, ADP, and cytochrome *c* (Hackett, 1956). The various paths may share a common terminal oxidase, and perhaps part of a common electron-transfer chain. In connection with the experiments reported herein, the oxidation of NADH by potato mitochondria was sharply increased by ADP, and the increase was inhibited by oligomycin. Nevertheless there was no respiratory control, and *m*-Cl-CCP proved fully effective with or without ADP. Consistent with the latter observation, *m*-Cl-CCP produced no further stimulation in ADP-treated mitochondria.

Finally it is of interest to examine the behavior of mitochondria from potato slices aged for a day, for the latter differ drastically both physiologically and biochemically from freshly prepared slices (Laties, 1963). Mitochondria from the two types of tissue display markedly different characteristics as well. For example, the isotherm for citrate oxidation by mitochondria from fresh slices is sigmoidal, or positively cooperative, while that for citrate oxidation by mitochondria from day-old slices is hyperbolic, or noncooperative (Laties, 1973b). Nevertheless the same dependence on ADP for the potentiation of *m*-Cl-CCP action is evident in mitochondria from aged slices. In a typical example a rate of 10 nmol of O_2/min with *m*-Cl-CCP alone was raised to 25 nmol/min with ADP.

Discussion

Examples of adenine nucleotides as modulators of allosteric enzymes are legion, but in this context attention has been focused primarily on substrate metabolism. With the observation herein that ADP potentiates uncoupler action, a possible modulating role of ADP is indicated in connection with the electron transport chain. There have been a few reports of the influence of ADP on mitochondrial metabolism in a role other than phosphate acceptor in oxidative phosphorylation. In one instance ADP *per se* has been shown to prevent phosphate induced swelling in rat liver mitochondria under conditions precluding phosphorylation (Connelly and Hallstrom, 1967). In another series of investigations, Kun *et al.* (1969) have demonstrated that ADP implements the loss of mitochondrial Mg^{2+} when mitochondria are preincubated with uncoupler (*e.g.*, DnpOH) and ADP together in the absence of substrate. The subsequent oxidation of glutamate or α-ketoglutarate in the continuing presence of DnpOH and ADP is normally impaired by the indicated pretreatment, but may be fully sustained by Kun's "cytoplasmic metabolic factor (CMF)." The effect of ADP and DnpOH on Mg^{2+} availability, and the effectiveness of CMF in counteracting the loss of mitochondrial Mg^{2+}, have been attributed to the influence of

the substances in question on intramitochondrial compartmentation, that is, on the conformation of one or more mitochondrial components (Kearney *et al.*, 1969).

One more example of uncoupler–ADP interaction must be mentioned if only to set it aside to avoid confusion. Fritz and Beyer (1969) reported ostensible respiratory control (*i.e.*, control of respiration rate by ADP) in the presence of uncoupler in bovine heart mitochondria oxidizing acetate or acetylcarnitine. However, the explanation hinged on acetate activation by ATP and formation of the latter from ADP by endogenous adenylate kinase.

ADP has been implicated in the conditioning of plant mitochondria (see above) in experiments in which mitochondria were incubated with substrate in the absence of inorganic phosphate (Raison *et al.*, 1973b). While experiments herein lead to the view that conditioning is not a prerequisite for the synergistic effect of ADP on *m*-Cl-CCP effectiveness, the involvement of ADP in both the conditioning phenomenon and in the potentiation of uncoupler action point to an effector role of ADP distinct from that of acceptor in oxidative phosphorylation. The distinction is emphasized by the second-order involvement of ADP both in conditioning and in the potentiation of uncoupler action. In the latter role the $S_{0.5}$ is approximately 80 μM. In potato mitochondria which exhibit good control maximal rates of oxidative phosphorylation persist until ADP is virtually totally converted to ATP, *i.e.*, where the concentration is 20 μM or less (*cf.* Lehninger, 1970). In the implementation of *m*-Cl-CCP action the effect of ADP increases with concentration to at least 170 μM (Table I), and, as has been said, ADP involvement is second order (Figure 3). At the end of state 3, or in state 4, the level of ADP is normally apparently too low to maximally potentiate uncoupler action, and additional ADP added with or after uncoupler raises the respiratory rate above that with uncoupler alone (Figures 1, 2, and 4). The absence of oligomycin inhibition in the presence of uncoupler speaks to the full effectiveness of the latter. In the cited study of Raison *et al.* (1973b) substrate transport (and a role of ADP therein) was ruled out as an explanation of mitochondrial conditioning. The participation of ADP in the transport or permeation of tricarboxylic acid cycle substrates may similarly be rejected in explanation of the potentiating effect of ADP on uncoupler action. Chappell and coworkers (see Chappell and Haarhoff, 1967) have made a thorough study of the passage of tricarboxylic acid cycle acids into several types of mammalian mitochondria (see also Meijer *et al.*, 1969). While di- and tricarboxylic acid uptake is carrier mediated, uptake proceeds in the absence of ADP and persists in the presence of inhibitors of electron transport. Citrate absorption by potato mitochondria is entirely akin to that of rat liver mitochondria (Ribereau-Gayon and Laties, 1969). Further, since levels of ADP too low to stimulate respiration in the presence of uncoupler nevertheless sustain maximal state 3 in the absence of uncoupler, the effector role of ADP cannot be on substrate absorption.

It is uncertain whether ADP influences uncoupler effectiveness at each phosphorylation site. The fact that *m*-Cl-CCP fully stimulates NADH oxidation by potato mitochondria without ADP suggests uncoupler responsive loci may differ with respect to dependence on ADP. P/O ratios for NADH oxidation by sweet potato mitochondria may be quite low (Hackett, 1961; *cf.* Wiskich and Bonner, 1963; Carmeli and Biale, 1970), and it seems that depending on the circumstances electrons from NADH may enter the conventional mitochondrial electron-transport chain at more than one place (Hackett, 1956; Hackett *et al.*, 1960).

Finally, the fact that both uncoupler effectiveness and the ability of state 4 to bring about conditioning (Raison *et al.*, 1973b) depend upon ADP raises once again the question of the meaning of state 4 and whether it should or should not be subtracted from state 3 rates in calculating P/O ratios. To the extent that state 4 is a more effective conditioner than state 3 in plant mitochondria, and to the extent that P/O ratios for tightly coupled plant mitochondria are more nearly theoretical when state 4 is subtracted, it would seem the events of state 4 at least partially underlie state 3, and that state 4 should therefore be subtracted. The foregoing presumption is entirely at odds with the accepted convention (Chance and Williams, 1955; Estabrook, 1967).

A dual role for ADP in potato mitochondria electron transport is indicated by the evidence. In this connection it is of exceptional interest that the exchange of isotopic oxygen between inorganic phosphate and water which occurs during mitochondrial electron transport independently of ATP formation was reported by Cohn (1953) not only to be sensitive to the uncoupler DnpOH, but to require adenylic acid (sic). It may be anticipated that the extent to which a dual role is demonstrable in mitochondria from other sources may depend on the level of endogenous mitochondrial ADP and the avidity of its retention upon mitochondrial isolation.

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A Technique for the Detection of Deleted Immunoglobulin Heavy Chains†

Blas Frangione‡

ABSTRACT: A method, "carboxymethylcysteine diagonal map," has been developed to detect immunoglobulins with structural defects. It is based on the comparison of all cysteine-containing peptides present in the molecule with their normal counterparts. Immunoglobulin molecules have a high content of cysteine residues which are strategically spread along the chains and can be used as markers for different domains and interdomain regions. Since they occupy a distinct position on the map, the absence of one or more peptides defines rather accurately the nature of the defect.

Amino acid sequence studies of several γ heavy-chain disease (γHCD)[1] proteins (Frangione and Milstein, 1969; Franklin and Frangione, 1971; Cooper *et al.*, 1972) and the heavy (H) chain of a myeloma protein (Fett *et al.*, 1973) have shown internal deletions ranging in size from 15 to 240 residues. These results have indicated (1) that these smaller synthetic products resulted from abnormalities of gene expression rather than degradation of intact molecules and (2) that codons GAA–GAG specifying glutamic acid at position 216 (γ1 numbering) (Edelman *et al.*, 1969) have special significance since in three instances reinitiation of normal synthesis after an internal deletion started at position 216 (Frangione and Milstein, 1969; Franklin and Frangione, 1971; Cooper *et al.*, 1972) and in one case the gap commenced at the same position (Fett *et al.*, 1973). Since the interpretation of these data is not yet clear and since studies of such proteins promise to be of great value in developing theories to explain the genetic mechanisms involved in the control of immunoglobulin synthesis, a method has been developed to detect deleted immunoglobulins. It is based on the comparison of all cysteine-containing peptides in the molecule with those in its normal counterpart. The method used employs "carboxymethylcysteine diagonal maps" and differs from classical diagonal maps (Brown and Hartley, 1966) in that the proteins are subjected to mild thiol reduction and labeling with iodo[^{14}C]-acetic acid prior to the performance of the diagonal map. This results in the selective labeling of the labile interchain disulfide bridges.

Materials and Methods

As previously described, 5 mg of purified protein (Frangione *et al.*, 1969a) was dissolved in 0.5 ml of 0.27 M Tris-HCl buffer (pH 8.2) and mildly reduced with 0.005 M dithiothreitol at room temperature under N_2. After incubation for 60 min at 37°, reduction was terminated by the addition of iodo[^{14}C]-acetic acid (0.01 M, specific activity 0.7 Ci/mol) and incubated at room temperature for another 60 min. The solution was then dialyzed overnight in 5% formic acid and digested with pepsin (Worthington, twice crystallized), enzyme–substrate ratio 1:50 (w/w) for 14 hr at 37°, freeze-dried, dissolved in 0.2 M ammonium bicarbonate (pH 8.3), and digested with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone trypsin (Worthington), enzyme–substrate ratio 1:50 (w/w) for 6 hr at 37°. The digest was dried, dissolved in 0.1 ml of water, and applied as a 3-cm band on 3MM Whatman paper and subjected to high-voltage paper electrophoresis in solvent-cooled tanks (pyridine–acetic acid–water, 1:10:190, v/v, pH 3.5) for 1 hr, 60 V/cm. A mixture of aspartic acid, glutamic acid, ϵ-Dnp-lysine, and glycylalanine was applied at the sides of the paper as markers. Peptides containing *S*-carboxymethylcysteine were detected by autoradiography using Kodak Royal Blue Medical X-Ray film.

The strip from this ionogram (without the markers) was incubated for 2 hr at room temperature in a desiccator con-

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[1] Nomenclature of immunoglobulin and their chains follow the recommendation of the World Health Organization (*W.H.O. Bull. 33*, 721 (1965); *35*, 953 (1966); *38*, 151 (1968); *41*, 975 (1969)). Myeloma proteins are designated by the first three letters of the patient's name.