On the Functional Aspects of a Preparation of an Inner Membrane Fraction of Liver Mitochondria. II*

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ABSTRACT: An inner membrane fraction prepared from liver mitochondria, M_w, has been found to cause significant stimulation of mitochondrial respiration. This stimulation is severalfold greater than can be accounted for by the very low respiratory rates obtained with the membranes alone. The stimulation seems to involve the opening up of an electron transport pathway separate from that which is directly coupled to phosphorylation since (a) the synthesis of adenosine triphosphate occurs concurrently with this accelerated rate, (b) if allowance is made for the oxygen consumption brought about by the presence of inner membrane fraction (which may or may not be justified), then the adenosine diphosphate:oxygen ratio is identical with that obtained in the absence of inner membrane fraction, (c) the inner membrane fraction is incapable of respiration-linked phosphorylation. The possible participation of free fatty acid in this reaction is eliminated by the use of defatted bovine serum albumin. The stimulation of mitochondrial respiration by inhibitor-treated inner membrane fraction is indicative of a possible bypass of the inhibitor site, with the connotation that electrons are transferred back and forth between mitochondria and inner membrane fraction. The similarities between dinitrophenol and inner membrane fraction relative to their possible role as "uncouplers" are discussed.

The question of interparticle electron transfer in mitochondria is to us a very important one, and can be approached by obtaining mitochondria or mitochondrial particles which transport electrons inefficiently or not at all from substrate to oxygen. If such particles are obtained, one can examine their ability to use parts of the existing electron-transfer pathways of functionally normal mitochondria, as well as the ability of normal mitochondria to utilize the available systems of the improperly or nonfunctioning mitochondrial particles (Strasberg and Moore, 1969). If the particle–particle interaction of mitochondria and submitochondrial particles (in this case inner membranes) were to point to a stimulation of respiration, then one would have to answer the question posed by Chance *et al.* (1967) in dealing with oligomycin-supplemented submitochondrial particles. That is whether coupled and uncoupled chains coexist in the same vesicular structure or whether some vesicles are tightly coupled and others uncoupled. In that case, it was stated that if the collision of these oligomycin-treated particles were necessary for interaction, then upon dilution, the reaction characteristics should be altered and if they were not, then the mechanism would fall in the category of the reaction taking place in one particle.

This last idea was the basis for this present study since it was observed that the reaction kinetics of mitochondrial oxidative phosphorylation were altered by dilution in a nonlinear manner; that is, dilute suspensions of mitochondria respired, but the respiratory control ratio was nonexistent or very low and was improved somewhat by increasing the concentration of bovine serum albumin. Increasing mitochondrial concentration on the other hand disproportionately increased respiration and the respiratory control ratio (Chance and Williams, 1955). This illustrates a point of view antipodal to that observed with oligomycintreated particles.

In order to evaluate intraparticle electron transport, an inner membrane fraction of mitochondria was prepared which in most instances had very little activity with glutamate and malate, somewhat more activity with succinate, and an unimpaired activity with ascorbate–*N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine. The ability of these membranes to stimulate the respiratory activity of mitochondria was tested with the necessary precaution of checking for the presence of free fatty acid. The effects of inhibitors of electron transport, and of uncouplers of oxidative phosphorylation were also examined.

Materials and Methods

Preparation of Mitochondria and Mitochondrial Inner Membrane Fractions. Rat liver mitochondria were prepared as described previously (Moore, 1968). Mitochondria prepared by this procedure were well coupled, exhibiting respiratory control ratio values of greater than six with glutamate and malate and greater than three with succinate in the presence of rotenone.

Inner membrane fractions were prepared by a modification of the method by Parsons *et al.* (1966). Mitochondria were centrifuged through 0.8 M sucrose solution and the pellet was suspended in 20–40 volumes of 1 mM Tris-PO_4 (pH 6.9). The suspension was incubated at 30° for 5 min in a shaking water bath. After incubation, enough sucrose was added to adjust the sucrose concentration to 0.25 M, and the suspension was homogenized, using a Ten-Broeck hand homogenizer. The homogenate was centrifuged 15,000*g* for 20 min at 0° and the resulting pellet was resuspended in a small volume of ST (0.25 M sucrose–0.005 M Tris-HCl, pH 7.4) and layered on

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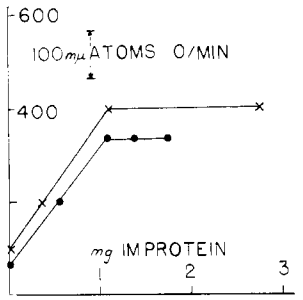

FIGURE 1: The stimulation of respiration of M_w preparations by IM_N. The rate of oxygen consumption is plotted against the amount of IM_N protein added to M_w. $T = 26°$. (×) 10 μmoles each of glutamate and malate in 1.7 ml of basic respiration medium (see Table I), and 13.2 mg of M_w protein. (●) Glutamate and malate, 8.4 mg of M_w protein.

top of a discontinuous density gradient composed of 1.5, 1.3, 1.2, 1.1, 1.0, and 0.8 M sucrose and centrifuged at 90,000*g* for 120 min at 0° in the SW25.1 rotor of the Spinco Model L centrifuge. The inner membrane fraction sedimented below the 1.5 M sucrose layer. Inner membrane fraction contained no spectrophotometrically demonstrable cytochrome b_5. The preparations differed slightly in oxidative ability; most inner membrane fractions could not utilize NAD^+-linked substrates or NADH, whereas others oxidized these slowly. On the other hand, all inner membrane fractions oxidized succinate and ascorbate–*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine to a limited extent.

Cytochrome Estimation. Cytochromes were estimated according to Chance and Williams (1956) using the Phoenix precision dual-wavelength-scanning spectrophotometer.

Inhibitor-Treated Mitochondria, M_{wI}. Mitochondria were titrated with rotenone or antimycin A, M_{wR} or M_{wA}, until inhibition of respiration was complete. Respiration studies were carried out using a Clark oxygen electrode (Chappell, 1961). These inhibitor poisoned mitochondria were centrifuged three times at 15,000*g* for 3 min at 0° and the pellet was resuspended twice in 20 volumes of ST (containing 1 mg of bovine serum albumin/ml) and then in ST alone, and recentrifuged. After each wash, a small portion of the pellet was resuspended in a minimal volume of ST and this concentrated mitochondrial solution was recentrifuged. The concentrated supernatant solution so obtained was added to a suspension of normal mitochondria respiring in a Clark oxygen electrode chamber to test for its inhibitory effect. It was found that no more than three washes were necessary to remove the excess rotenone (a minimum of four washes being used) or antimycin A (a minimum of four washes being used). Normal mitochondria were carried through a similar washing procedure when a comparison was to be made.

Inhibitor-Treated Inner Membrane, IM_I. Inner membrane fractions were treated with rotenone or antimycin A, IM_R or IM_A, as described for M_{wI}.

Respiration Studies. Oxygen utilization was measured using a modified Clark oxygen electrode (Chappell, 1961). For low-temperature studies, the temperature of the electrode chamber was thermostatically maintained.

Protein Determination. Protein was determined by a modification of the method of Lowry *et al.* (1951).

ATP Assays. ATP was assayed enzymatically using the glucose–ATP–hexokinase–G-6-P dehydrogenase $NADP^+$ method outlined by Greengard (1965). Reduced $NADP^+$ was determined fluorometrically in the Eppendorf fluorometer.

AMP Assays. AMP was assayed enzymatically according to the method of Adam (1965) using the fluorometric assay for NADH.

Succinate Assay. Electron transport particles prepared by sonicating repeatedly frozen and thawed mitochondria were used as the enzyme source. The assay system consisted of 0.3 mg of electron transport particle protein, 20 mμmoles of ferricytochrome *c*, 4.0 μg of rotenone, 100 mμmoles of KCN, and enough ST to make a total volume of 1 ml. The initial rate of reduction of cytochrome *c*, measured as an increased absorbancy @ 417 *vs.* 405 mμ, was an index of the succinate concentration.

Materials. The enzymes used for the various assays as well as rotenone, antimycin A, ATP, AMP, ADP, glutamic, malic, and succinic acids were purchased from Sigma Chemical Corp., St. Louis, Mo. Cytochrome C was type V cytochrome *c*, oxidized, obtained from Sigma.

TABLE I: Respiratory Stimulation and the Cytochrome Content of M_w and IM_N.[a]

mg	Cytochrome (mμmoles) $a + a_3$	b	c	mμatoms of O/min	% Stimulation
M_w					
8.1	2.97	2.49	4.95	77	
IM_N					
0.27	0.11	0.05	0.15	198	160
0.54	0.22	0.09	0.30	342	350
0.81	0.34	0.14	0.45	486	535
1.08	0.45	0.19	0.60	702	815
1.48	0.62	0.26	0.82	702	815
1.88	0.78	0.33	1.04	657	760
2.15	0.89	0.38	1.19	540	605
b	0.15	0.08	0.12		

[a] Increasing amount of IM_N protein added to 8.1 mg of M_w protein respiring in a Clark oxygen electrode chamber containing 1.7 ml of a basic medium composed of 0.25 M sucrose–5 mM Tris-Cl (pH 7.4), 10 mM Tris-PO_4, (pH 7.4), 5 mM KCl, 5 μmoles of glutamate, and 5 μmoles of malate; $T = 26°$. [b] IM cytochrome/M_w cytochrome at maximal stimulation.

Results

Inner Membrane–Mitochondrial Interaction. When normal inner membrane fraction, IM_N, was added to mitochondria, the respiratory stimulation displayed a linear dependence on the concentration of IM_N. This relationship is seen in Figure 1, where two examples are presented. For example, mitochondria, respiring at a rate of 7.66 mμatoms of O/mg of protein were stimulated to a maximum of 30.7 mμatoms of O/mg

TABLE II: IM_N Stimulation of M_w in the Presence and in the Absence of Defatted Bovine Serum Albumin.[a]

Additions in Order	Rate of Respiration (mμatoms of O/min)					
	Defatted Bovine Serum Albumin (μg)					
	0		945		1700	
M_w						
5.6 mg		27		27		27
IM						
176 μg	0	45	0	36	0	45
176 μg	0	63	0	57	0	50
352 μg	13	72	13	81	13	72
352 μg	13	99	13	99	13	120
528 μg	18	152	18	144	18	189

[a] Increasing amounts of IM protein added to 5.6 mg of M_w respiring in a Clark oxygen electrode chamber containing the basic medium described in Table I, and the amount of defatted bovine serum albumin indicated on the table; T = 26°.

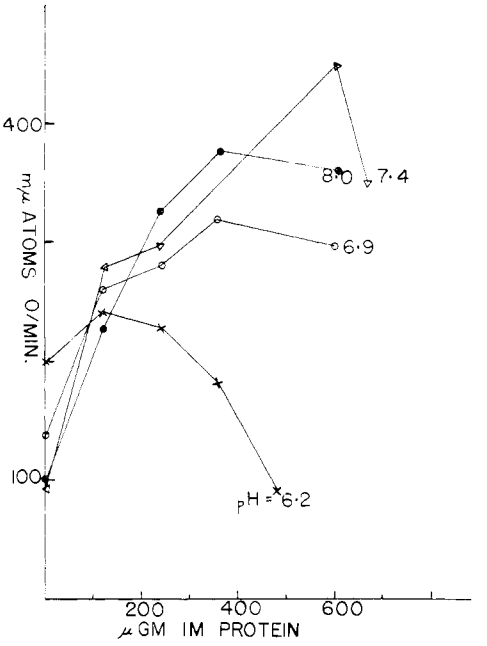

FIGURE 2: The effect of pH on the stimulation of M_w respiration by IM_N. The system consisted of 1.5 ml of respiration medium containing 0.25 M sucrose–5 mM Tris–Cl, pH 6.2 (6.9, 7.4, or 8.0), 10 mM Tris-PO_4, 5 mM KCl, 5 μmoles of glutamate, 5 μmoles of malate, 30 μg of bovine serum albumin, and 7.34 mg of M_w protein. T = 26°.

of protein in the presence of increasing amounts of IM_N protein. The ratio of IM_N/mitochondria (in terms of protein) was 0.10. Other preparations of IM_N gave stimulations as high as eightfold with a ratio of IM_N/mitochondria of 0.12. When this ratio was calculated on the basis of the cytochrome content, the ratios at which maximum stimulation was observed varied as seen in Table I from 0.16 for cytochromes *a* and a_3 to 0.07 for cytochrome *b* with an average value of 0.12 for cytochrome *c* with an eightfold stimulation of mitochondria respiration.

The concentrated preparations of mitochondria and inner membrane fraction were centrifuged at 15,000*g* for 3 min at 0° and the resultant supernatant solutions were tested to determine if the supernatant of mitochondria had any stimulatory effect on the inner membrane fraction and *vice versa*. The volume of supernatant solution used was equal to or greater than the volume of concentrated mitochondria or inner membrane fraction used in the experiments. The supernatant fraction of inner membrane fraction caused no respiratory stimulation of mitochondria, nor did that of mitochondria cause an increase in the respiration of inner membrane fraction. When the mitochondria and inner membrane fraction were mixed together and then centrifuged, the combined supernatant fraction likewise was inactive on mitochondria or inner membrane fraction. Thus the stimulation of mitochondria respiration by inner membrane fraction seems to depend upon direct particle interaction, and not to any substance(s) such as fatty acids released by the mixing of particles.

Results of Figure 2 indicate that the stimulation of mitochondrial respiration by inner membrane fraction exhibits a pH dependence, with maximal stimulation at pH 7.4.

Dialysis of inner membrane fraction in the presence or absence of EDTA did not alter its stimulatory activity. This minimizes the possibility of the stimulation as being due to divalent cations or other small ions. Addition of NAD^+, FAD, FMN, or coenzyme Q_{10} to inner membrane fraction did not cause any increase in the rate of respiration with any of the substrates tried.

It was also found that inner membrane fraction which was frozen three times in an acetone–Dry-Ice mixture was as active as IM_N (on a protein basis) whereas inner membrane fraction which had been boiled for 5 min lost its ability to stimulate the respiration of mitochondria (Figure 3).

Free Fatty Acids. To eliminate the possible contribution of fatty acids to the effect of inner membrane fraction on mitochondria, defatted bovine serum albumin was added to the preparation without any significant change in the stimulatory rate (Table II).

To check this further, a mixture of free fatty acid (50 μM)

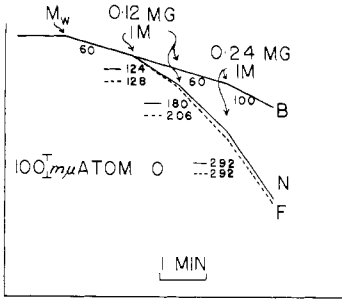

FIGURE 3: The stimulation of M_w respiration by frozen, boiled, or normal IM fractions. The system contained 10 μmoles each of glutamate and malate and 7.74 mg of M_w protein in 1.5 ml of basic respiration medium (see Table I). T = 26°. N = addition of normal IM fraction. F = addition of IM fraction which had been frozen three times in an acetone–Dry-Ice mixture. B = addition of IM fraction which had been boiled for 5 min. Number on slopes refer to millimicroatoms of oxygen utilized per minute.

TABLE III: Uptake of Succinate by M_w in the Presence and in the Absence of IM or DNP.[a]

Expt	Time (min after succinate)	Succinate Concn (μmoles/ml of medium)				
I				561 μg of M_w Plus		
		3.31 mM succinate	3.31 mM succinate 1.28×10^{-5} M DNP	3.31 mM succinate 6.4×10^{-4} M DNP		
	0 0	0.238	0.337	0.289		
	1 0	0.977	0.873	0.703		
	5 0	0.770	0.607	0.722		
				2100 μg of M_w Plus		
II		6 mM succinate	6 mM succinate 9.4×10^{-5} M DNP	6 mM succinate 1.6×10^{-3} M DNP	6 mM succinate 540 μg of IM	6 mM succinate 700 μg of IM
	2 0	6.370	5.330	5.600	3.440	3.990
	8 0				5.780	5.890

[a] Succinate was added to 1.56 (I) and 2.046 ml (II) of a basic respiration medium (see Table I) containing 561 μg M_w (I) and 2100 μg of M_w (II), 5 μg of rotenone and the amount of DNP or IM indicated. Samples were withdrawn and layered over 0.5 M sucrose and centrifugation begun at the times indicated on the table. In zero-time experiments M_w were layered directly over the medium (adjusted to 0.32 M with sucrose) containing all the necessary components and this was centrifuged immediately. Samples were withdrawn from the tops of tubes and assayed as described in the methods. The lower concentrations of DNP or IM were those that gave a fast (stimulated) rate of M_w respiration; at the higher concentrations M_w respiration had begun to slow down (inhibited).

was added to 10.8 mg of mitochondria. A concentration of 50 μM was found necessary to give a stimulation approximating twice that of inner membrane fraction. When this system was titrated with defatted bovine serum albumin, 500 μg/ml was required to return the state 4 respiration rate to an unstimulated value. Twice this amount of defatted bovine serum albumin had no effect on the stimulation brought about by inner membrane fraction.

Boiling of inner membrane fraction which would not be expected to destroy fatty acid (but might however alter its binding characteristics) resulted in an inactivated preparation, also indicating that fatty acids might not be the source of the stimulation.

2,4-Dinitrophenol. If the concentration dependence were plotted to include extreme concentrations of inner membrane fraction, one observed a decrease in the stimulated rate (Figure 4). The shape of this curve greatly mimics that obtained with DNP. From the data of Van Dam and Slater (1967) and Van

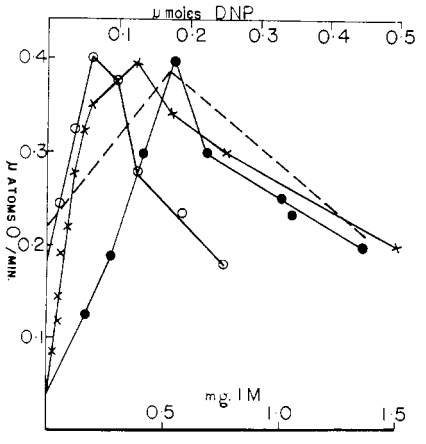

FIGURE 4: A simple comparison of IM_N and DNP on M_w. Basic respiration medium (1.7 ml) with 10 μmoles each of glutamate and malate and 4 mg of M_w protein. (●) Increasing amounts of IM_N protein. (×) Increasing concentration of DNP. (○) Increasing concentration of DNP in the presence of 0.27 mg of IM_N protein. (– – –) Increasing amounts of IM_N protein in the presence of 0.025 μmole of DNP. $T = 26°$.

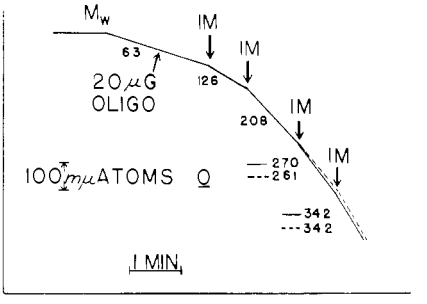

FIGURE 5: The stimulation of M_w respiration by IM_N in the presence of oligomycin. The system contained 10.8 mg of M_w protein in 1.6 ml of basic respiration medium. IM refers to the addition of 35 μg of IM_N protein. The numbers on the slopes represent millimicroatoms of oxygen utilized per minute. $T = 26°$.

TABLE IV: ATPase Activity of M_w and IM.[a]

Expt	Time (min)	ATP Added (μmoles)	IM Protein (mg)	M_w Protein (mg)	Residual ATP (μmoles)
I	8	3.12			3.50
	8	3.12	1.82		3.16
	8	6.24	1.82		5.75
	8	3.12		3.80	3.61
	8	6.24		3.80	7.10
	8	6.24	1.82	3.80	4.55
	8	6.24	1.82	3.80	4.06
II	8	3.75			3.20
	8	3.75	1.82		3.29
	8	3.75		2.88	3.43
	0	3.75	1.82	2.88	3.43
	1	3.75	1.82	2.88	3.35
	3	3.75	1.82	2.88	3.35
	5	3.75	1.82	2.88	2.70
	8	3.75	1.82	2.88	2.61

[a] Experiments I and II were performed in 1.5 ml of a basic respiration medium (see Table I) at 26°. At the times indicated 1 ml of incubation medium was added to 0.5 ml of cold 10% trichloroacetic acid and the suspensions were centrifuged. ATP in the supernatant was assayed as described in Methods. Experiments I and II were performed with separate preparations of M_w and IM.

Dam (1967), it would appear that high concentrations of DNP are capable of inhibiting respiration by interfering with the substrate accumulation. In our own experiments the uptake of succinate was determined according to the protocal outlined. We believe that if the succinate uptake is truly inhibited by the presence of DNP or inner membrane fraction, then, the difference between systems oxidizing succinate in the presence and absence of DNP or inner membrane fraction should be more evident in long-term experiments, and not as in the short-term experiments of Harris *et al.* (1967a,b). However, we have found that the accumulation of succinate is similar in the presence of high or low concentrations of DNP or inner membrane fraction (Table III).

ATPase. Experiments were done to determine whether the inner membrane fraction was stimulating respiration by exhibiting ATPase activity, and thus in actual fact changing the state 4 rate to a state 3 rate. Mitochondria which phosphorylated ADP in the presence of glutamate and malate were treated with oligomycin. Added ADP was not phosphorylated, whereas subsequent addition of inner membrane fraction caused an increased rate of respiration equal to that in the absence of oligomycin (Figure 5). DNP has also been reported to overcome the oligomycin inhibition of state 3 respiration.

Mitochondria, in an acetate rather than a phosphate buffer, were treated with calcium to remove any endogenous P_i. Under these conditions, only in the presence of added P_i was ADP phosphorylated, but again inner membrane fraction (or DNP) stimulated the respiratory rate (Figure 6).

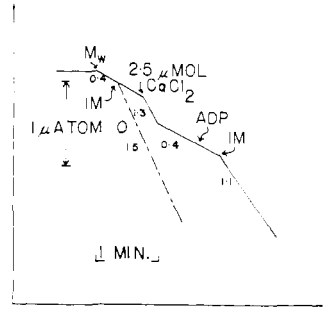

FIGURE 6: The stimulation of M_w respiration by IM_N in the absence of phosphate. The system contained 0.25 M sucrose, 5 mM Tris-Cl (pH 7.4), 10 mM Tris-acetate (pH 7.4), 5 mM KCl, 50 μmoles each of glutamate and malate, 31.5 mg of M_w protein, 2.475 mg of IM_N protein (where added), and 500 of mμmoles of ADP (where added) in 5 ml. Number on slopes refer to microatoms of oxygen utilized per minute. $T = 26°$.

Thus under conditions where ATP could not be formed, the inner membrane fraction was still capable of stimulating the rate of respiration of mitochondria, and it could therefore be concluded that any ATPase activity of inner membrane fraction was not responsible for their stimulatory effect.

To test for ATPase activity, inner membrane fraction, mitochondria, and inner membrane fraction plus mitochondria were incubated at 26° for 0–8 min in a medium containing ATP. Inner membrane fraction or mitochondria alone exhibited no ATPase activity for up to 8 min; however, approximately 30% of the ATP was broken down in the presence of the mixture of mitochondria and inner membrane fraction after 5–8 min (Table IV).

ATP Synthesis and Breakdown. It has been shown that the

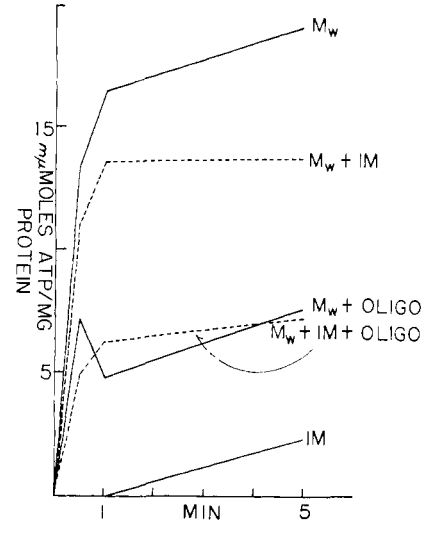

FIGURE 7: ATP synthesis by M_w in the presence and absence of IM_N. Incubations were carried out at 26° in 3.24 ml of a basic respiration medium (see Table I) containing 5 mM $MgCl_2$, 0.065 mM ADP, 15 μmoles each of glutamate and malate, 10.8 mg of M_w, 2.85 mg of IM (no state 4–3 transition is apparent when this much IM is added to the M_w), and 40 μg of oligomycin. Samples (1 ml) were withdrawn after 30-sec, 60-sec, and 5-min incubation and added to 0.5 ml of cold 10% trichloroacetic acid. After centrifugation the supernatant fractions were neutralized to pH 6.8 with KOH and ATP was assayed as described in the methods.

TABLE V: Respiratory Control Ratio and ADP:O Values of M_w in the Presence and in the Absence of IM_N.[a]

Substrate	mμatoms of O/min: State 4 −	State 4 + IM_N	State 3 −	State 3 + IM_N	Respiratory Control Ratio −	Respiratory Control Ratio + IM_N	ADP:O −	ADP:O + IM_N	ADP:O[b] + IM_N
1. Glutamate and malate	55	170	220	375	4.00	2.20	3.01	2.01	2.72
2. Glutamate and malate	55	170	220	270	4.00	1.58	3.00	1.82	2.80
3. Succinate	120	210	330	450	2.80	2.10	2.00	1.58	2.04
4. Succinate	100	225	295	420	2.95	1.90	1.98	1.57	2.02
5. Ascorbate *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine	180	185	190	190	1.06	1.02	0.92	0.71	0.93

[a] Basic medium as in Table I. Substrates: glutamate and malate = 5 μmoles each of glutamate and malate; succinate = 10 μmoles of succinate (in the presence of 10 μg of rotenone); ascorbate–*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine = 1.5 μmoles of ascorbate and 85 mμmoles of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (in the presence of 5 μg of antimycin A). M_w protein (8.4 mg) was used in 1–5, 390 μg of IM_N protein was used in 1 and 2, and 130 μg in 3, 4, and 5. [b] Corrected by the following formula used to account for the extra oxygen consumed in the presence of IM_N:

[state 3 O used by IM_N + M_w] − [(state 4 O/min by IM_N + M_w − state 4 O/min by M_w alone) × (min of state 3)] = true state 3 O consumed

ADP:O ratio of mitochondria was not significantly altered if corrections were made for that amount of oxygen accounted for by the stimulatory effect of inner membrane fraction (Strasberg and Moore, 1969). This was taken as evidence for a lack of true uncoupling by the inner membrane fraction. Addition of low concentrations of inner membrane fraction to mitochondria showed the classical respiratory stimulation but still allowed the state 3–4 transition to be seen (Table V). Thus at less than maximal respiratory capacity, one is able to apply the correction for extra respiratory activity and derive the corrected ADP:O ratio. At higher inner membrane fraction concentration when respiration is maximized, and the state 4–3 transition is not evident, the system must be analyzed for ATP using the hexokinase–glucose trap, and subsequently determining the glucose 6-phosphate formed. As seen in Figure 7, inner membrane fraction alone has very little or no initial phosphorylative ability. The formation of a small quantity of ATP by inner membrane fraction is due to myokinase activity, since AMP (formation) accompanied the ATP found in the extracts. The amount of ATP synthesized by mitochondria in the presence of inner membrane fraction was

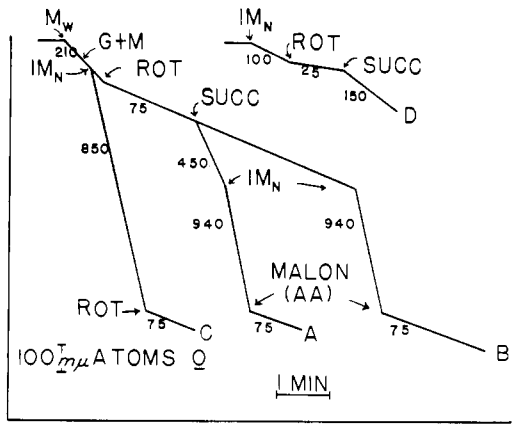

FIGURE 8: The effect of inhibitors on the stimulation of M_w respiration by IM_N. The system contained 21.6 mg of M_w protein in 5 ml of basic respiration medium. G&M = 10 μmoles each of glutamate and malate; succ = 5 μmoles of succinate; ROT = 2 μg of rotenone; AA = 10 μg of antimycin A; MALON = 5 μmoles of malonate; IM_N = addition of 2.4 mg of IM_N protein. Additions were made where indicated in each of the three expt A–C. Numbers on slopes represent millimicroatoms of oxygen utilized per minute. T = 26°.

TABLE VI: Respiratory Stimulation of M_w by IM in the Presence of Antimycin A.[a]

Addn in Order	A[b] a −	A[b] a +	A[b] b −	A[b] b +	B[b] a −	B[b] a +	B[b] b −	B[b] b +
Succinate (*b* only)			0	126			0	126
2 μg of Antimycin A	0	0	0	0	0	0	0	0
N,N,N′,N′-tetramethyl-*p*-phenylenediamine	0	57	0	90	0	13	0	18
IM (54 μg)	1	63	31	126	18	13	13	81
IM (36 μg)	1	63	49	180	27	13	18	
IM (36 μg)	1	62	57	234	27	13	18	216
Succinate	1	243			27	252		

[a] Rate of respiration is given in microatoms of O per minute. Additions were made to a Clark oxygen electrode chamber containing 1.65 ml of a medium composed of 0.25 M sucrose, 5 mM Tris-Cl (pH 7.4), 10 mM Tris-PO_4 (pH 7.4), 5 mM KCl, 5 μg of rotenone, 15 μmoles of succinate (where added); T = 26°. Experiments in columns a and b were initiated in the absence (a) and presence (b) of succinate, respectively. [b] *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine, 60 μM; 3.8 mg of M_w.

TABLE VII: The Effect of M_{WR} on M_w Respiration and Phosphorylation (mμatoms of O/min).[a]

Rotenone (μg)/Protein (mg)								Expt Temp (°C)
		I. 3.54 mg of M_w Protein						
	Additions	None	0.6 mg of $M_{WR,0°}$	0° supernatant of $M_{WR,0°}$	26° supernatant of $M_{WR,0°}$			
	State							
0.50	4	36	0 (0)	31	27			26
	3	135	0	135	18			
		II. 8.08 mg of M_w Protein						
	Additions	None	1.52 mg of $M_{WR,0°}$			1.48 mg of $M_{WR,26°}$	0° supernatant of $M_{WR,26°}$	
	State							
0.15	4	22	22 (4)			54 (11)	24	26
	3	162	22			36	24	
		III. 5.68 mg of M_w Protein						
	Additions	None	2.26 mg of $M_{WR,0°}$	0° supernatant of $M_{WR,0°}$	26° supernatant of $M_{WR,0°}$			
	State							
0.10	4	63	63 (0)	45	54			26
	3	387	72	252	369			
		IV. 11.36 mg of M_w Protein						
	Additions	None	4.52 mg of $M_{WR,0°}$					
	State							
0.10	4	27	36 (0)					
	3	54	54					

[a] M_{WR} prepared at 0 ($M_{WR,0°}$) or 26° ($M_{WR,26°}$) or their supernatants prepared by spinning down M_{WR} at 0 or 26° (hence 0° or 26° supernatant) were added (20 μl in I and II; 40 μl in III; 80 μl in IV) to M_{WN} respiring in a Clark oxygen electrode chamber in 1.65 ml of basic respiration medium (see Table I) (state 4). To initiate state 3, 200 mμmoles of ADP was added to the medium. Experiments III and IV were performed with the same preparations of M_W and $M_{WR,0°}$. The numbers in parentheses in columns 4 and 7 refer to the respiration of M_{WR} alone.

almost as much as in the absence of inner membrane fraction up to about 1 min. However, after 5 min, the mitochondrial system alone contained 28% more ATP than mitochondria and inner membrane fraction. This would be expected considering the 30% hydrolysis of ATP by mitochondria and inner membrane fraction in 5–8 min (Table IV). Under these conditions the lack of true uncoupling activity of these membranes becomes evident.

Inhibitors. When rotenone is added to mitochondria respiring in the presence of glutamate and malate and under the stimulus of inner membrane fraction, respiration is inhibited and the inhibition is overcome by succinate to a new stimulated rate subject to inhibition by antimycin A or malonate (Figure 8).

If the *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine bypass of the antimycin A inhibition is functioning in the inner membrane fraction, then the stimulation of mitochondrial respiration by inner membrane fraction should also be exhibited. One of the peculiarities of most preparations of the inner membrane fraction is a high degree of oxidative activity with ascorbate–*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine, while with *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine alone, succinate is necessary to see the respiratory stimulation of mitochondria by inner membrane fraction in the presence of antimycin A (Table VI). This is in agreement with the work of Lee *et al.* (1965) on the bypass of the antimycin A site by *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine in intact mitochondria. In cases where mitochondria are treated with rotenone or antimycin A and washed before use, some data are indicative of a bypass of the rotenone or antimycin A inhibited site in the presence of inner membrane, unaccompanied by phosphorylation, while others give evidence of an inhibition of inner membrane fraction respiration by inhibitor poisoned mitochondria. These results are believed to have been due to the release of the inhibitors into the medium and an uptake by the unpoisoned inner membrane fraction or mitochondria.

TABLE VIII: The Effect of M_{WA} on Respiration and Phosphorylation of M_W.[a]

Antimycin A (μg)/Protein (mg)						Exptl Temp (°C)
				I. 3.54 mg of M_w Protein		
	Additions	None	0.7 mg of $M_{WA,0°}$	0° supernatant of $M_{WA,0°}$	26° supernatant $M_{WA,0°}$	
	State					
0.20	4	36	9 (0)	45	9	26
	3	135	9	144	9	
				II. 9.30 mg of M_w Protein		
	Additions	None	2.01 mg of $M_{WA,0°}$		1.34 mg of $M_{WA,26°}$	
	State					
0.14	4	45	54 (7)		54 (2)	26
	3	243	216		216	

[a] M_{WA} (20 μl) prepared at 0° ($M_{WA,0°}$) or at 26° ($M_{WA,26°}$) or 20 μl of their supernatants prepared by spinning down M_{WA} either at 0 or 26° (hence 0 or 26° supernatant) were added to M_{WN} as described in Table VII for M_{WR}. The numbers in parentheses refer to the respiratory rate of the added M_{WA} alone.

Table VII (which shows effects at three different concentrations of rotenone) indicates that when mitochondria are poisoned with an excess of inhibitor (0.5 μg of rotenone/mg of protein) (lines 1 and 2) and then washed repeatedly with bovine serum albumin (see Methods) the M_{WR} still retained enough inhibitor to block both states 4 and 3 respiration of normal mitochondria. The concentrated supernatant solutions of M_{WR} (prepared by centrifuging concentrated preparations of M_{RW}) when obtained at 0° were without effect of M_{WN}, but when such supernatant solutions were obtained by centrifuging M_{WR} at 26°, they inhibited the state 3 respiration with very little effect on the state 4 rate, and indeed the state 3 rate was always less than the state 4 rate. While similar observations were made for supernatant solutions of M_{WR} poisoned with 0.15 μg of rotenone/mg of protein, the M_{WR} then obtained did not inhibit state 4 respiration but did inhibit state 3 respiration of normal mitochondria.

M_{WR} poisoned and washed at 26° stimulated the state 4 rate but depressed the state 3 rate. Supernatant solutions prepared at 0° from these $M_{WR,26°}$ had no effect on state 4, but allowed no state 4–3 transition (Table VII, lines 3 and 4, column 7 and 8). Since it appeared that a factor was being released from M_{WR} during the respiration studies at 26°, the respiration experiments tested on lines 7 and 8 were carried out at 5°. At 0.10 μg of rotenone/mg of mitochondrial protein and at high M_{WN} concentration one observes a lack of state 3 inhibition and indeed there was a stimulation of the state 4 rate.

Similar experiments were carried out with antimycin A (Table VIII). Mitochondria were poisoned with 0.14 and 0.20 μg of antimycin A per mg of mitochondrial protein and the procedure of washing with bovine serum albumin carried out at

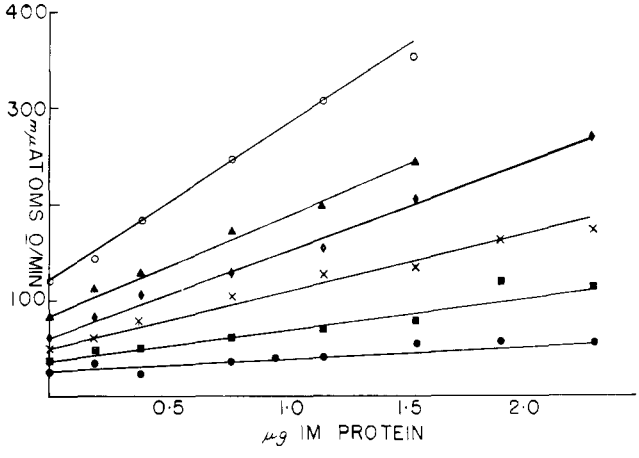

FIGURE 9: The effect of temperature on IM_N stimulation of respiration of M_w. Experiments were carried out in a Clark oxygen electrode chamber, the temperature of which was thermostatically controlled. Basic respiration medium (1.8 ml) containing 10 μmoles each of glutamate and malate and 12.8 mg of M_w was used. (●) 5.5, (■) 11, (×) 16, (◆) 20, (▲) 23, (○) 29°.

TABLE IX: Q_{10} of M_w Respiration with and without IM.[a]

System	Q_{10} at T_2/T_1 (°C) of 16/5.5	20/11	29/20
M_w (alone)	1.97	2.12	1.53
+ IM (385 μg)	2.70	2.16	1.72
+ IM (770 μg)	2.33	2.02	1.89
+ IM (1150 μg)	2.37	2.33	1.94
+ IM (1550 μg)	1.86	2.52	1.72

[a] M_w protein (12.8 mg) was used in each experiment. Experimental conditions and rates of respiration are as described for Figure 9.

TABLE X: The Effect of IM_N on M_{WR} Respiration (mμatoms of O/min).[a]

Addns in Order	M_{WR}, 5.25 mg, 26°		M_{WR}, 7.9 mg	
	18	18	18	18
Glutamate and malate	18	18	18	18
IM (135 μg)		45 (0)		
Rotenone	0	0	0	0
Succinate	81	108 (13)	162	36
IM (135 μg)			207 (13)	
IM (135 μg)				45 (0)
Antimycin A	0	0		0
Ascorbate–*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine	153	234 (27)		99
IM (270 μg)				153 (18)

[a] M_{WR} was prepared by poisoning M_w with rotenone (0.36 μg/mg of M_w protein) as described in the methods. Respiration was studied in a Clark oxygen electrode chamber of total volume 1.7 ml of a medium consisting of 0.25 M sucrose, 5 mM Tris-Cl (pH 7.4), 10 mM Tris-PO_4 (pH 7.4), and 5 mM KCl. Additions to the chamber were made in the order indicated in column 1. Numbers in parentheses refer to the rates of respiration of the added IM fractions alone under similar conditions. Glutamate and malate = 0.25 μmole each of glutamate plus malate; rotenone = 5 μg of rotenone; succinate = 10 μmoles of succinate, antimycin A = 5 μg of antimycin A; ascorbate–*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine = 15 μmoles of ascorbate and 100 mμmoles of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

TABLE XI: The Effect of IM_N on M_{WA} Respiration (mμatoms of O/min).[a]

Addns in Order	M_{WA}, 7.28 mg, 26°		M_{WA}, 11 mg, 11°	
	9	9	16	16
IM (405 μg)	9 (0)		16 (0)	
Glutamate and malate	9	9	16	
Rotenone	9		16	
Succinate	9	9	16	
Antimycin A		9		
Ascorbate–*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine		243		207
IM (135 μg)		270 (18)		
IM (270 μg)		315 (51)		243 (18)
IM (135 μg)		315 (67)		279 (27)
IM (135 μg)				306 (54)

[a] M_{WA} were prepared by poisoning M_w with antimycin A (0.14 μg/mg of M_w protein). See Table X for explanation of this table. Numbers in parentheses refer to the respiration of the total amount of IM present in the absence of M_w.

0 and 26°. At the higher antimycin A concentration, M_{WA} at both temperatures inhibited both states 4 and 3 respiration of normal mitochondria, whereas at the lower concentration the state 4 rate was approximately an additive rate. The state 4–3 transition was essentially unchanged. Thus, it became clear that in order to study the effects of inner membrane fraction on M_{WI} or the reciprocal experiments, the level of inhibitor used was critical, and the temperature dependence was also of importance.

Temperature Dependence. In view of the above, the temperature dependence of the stimulation of mitochondrial respiration by inner membrane fraction was studied and the results are presented in Figure 9 and Table IX. From these data, it can be seen that inner membrane fractions can stimulate mitochondrial respiration at temperatures as low as 5.5°. The approximate Q_{10} between 5.5 and 29° is 2.0 both in the presence and absence of IM_N.

Inhibitor-Treated Mitochondria and IM_N. The interaction of inhibitor poisoned mitochondria with inner membrane fraction at both room (26°) and low temperature was studied. M_{WR} barely respired and did not phosphorylate added ADP with glutamate and malate as substrates (Table X) but illustrated good respiratory and phosphorylative ability with succinate and respired well with ascorbate–*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine. Addition of IM_N to M_{WR} at 26° increased the glutamate and malate supported respiration rate (indicating a bypass of the rotenone site in the presence of IM_N). The succinate- and ascorbate–*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine-supported respirations were stimulated, as expected. Similar results were reported earlier (Strasberg and Moore, 1969).

At 11°, the addition of IM_N to M_{WR} caused similar increases in respiratory rates of both succinate and ascorbate–*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine supported respirations. Thus no obvious difference between the effect of IM_N on M_{WR} respiration at 26 or 11° is apparent.

On the other hand, when IM_N were added to M_{WA} (Table XI) the increase in the ascorbate-*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine supported respiration was merely an addition of the IM_N and M_{WA} rates at 26°, but a stimulation of respiration was apparent at 11°. No bypass of M_{WA} antimycin A site by inner membrane fraction in the presence of succinate was evident.

Inhibitor-Treated Inner Membrane Fractions and M_{WA}. In reciprocal experiments, IM_N poisoned with antimycin A (0.55 μg of antimycin A/mg of protein) was used. Addition of increasing amounts of IM_A to mitochondria followed by ADP resulted first in a gradual decrease in the respiratory control ratio (at 26°) with no change in state 4 respiration and finally in inhibition of the state 4 respiration (Figure 10A) and cessation of phosphorylation. When a sample of antimycin A was diluted to the approximate concentration it would have been in the IM_A notwithstanding any concentrative binding of antimycin A to the membranes, mitochondrial respiration became inhibited when the amount of antimycin A added was 20 times that of IM_A (Figure 10B). This indicated that at 26°, antimycin A was probably released from the IM_A and interfered with mitochondrial function as indicated for M_{WI}. At 11°, however, addition of IM_A to mitochondria caused a

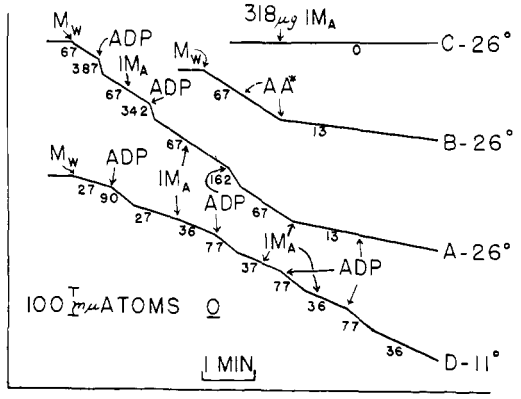

FIGURE 10: The effect of IM_A on M_N respiration and phosphorylation I. Experiments A–C were carried out at 26° and D at 11°. IM was poisoned as described in the methods with an initial concentration of 0.55 μg of antimycin A/mg of protein. The system consisted of 10.6 mg of M_w protein in A and B, and 15.9 mg in D, in a total volume of 1.8 ml of respiration medium. AA* denotes additions of antimycin A, each at a dilution equivalent to ten times the final concentration in the inner membrane fraction irrespective of binding. ADP was added in aliquots (200-mμmoles) wherever indicated. Numbers on slopes refer to millimicroatoms of oxygen utilized per minute.

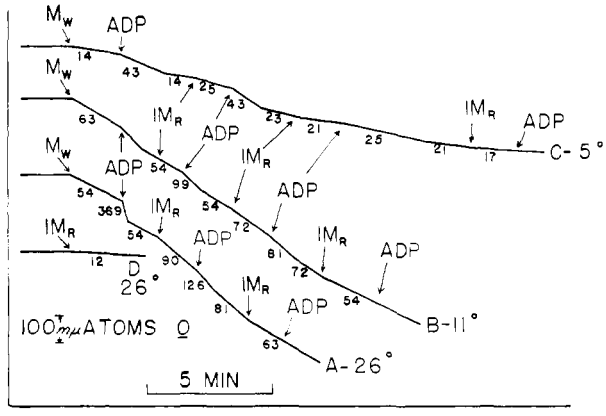

FIGURE 12: The effect of IM_R on M_w respiration and phosphorylation. Experiments A and D were carried out at 26°, B at 11°, and C at 5°. IM was poisoned as described in Methods with an initial concentration of 0.17 μg of rotenone per mg of protein. The system consisted of 6.28 mg of M_w protein in A and 12.48 mg in B and C in a total volume of 1.8 ml of respiration medium. IM_R denotes addition of 4.50 μg of IM_R protein and ADP represents addition of 200 mμmoles of ADP. Numbers on slopes refer to millimicroatoms of oxygen utilized per minute.

slight increase in state 4 respiration and no decrease in rate or extent of phosphorylation (Figure 10C).

Another preparation of IM_A (0.44 μg of antimycin A/mg of inner membrane fraction protein) stimulated the glutamate-plus-malate- and succinate-supported respirations at 26° and 11° (Figure 11) but did not by itself respire with these substrates. Thus stimulation of mitochondrial respiration by inner membrane fraction can take place even if the inner membrane fraction were poisoned with antimycin A. Excess inhibitor may mask this at 26° by being released into the medium and acting on the mitochondria (Strasberg and Moore, 1969).

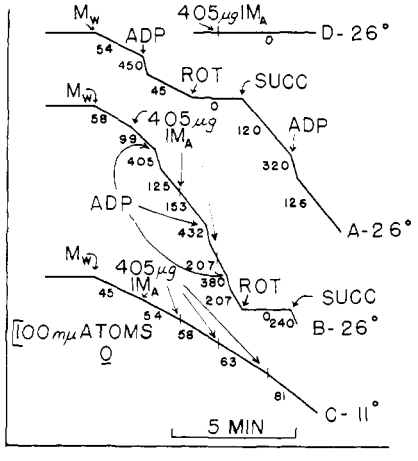

FIGURE 11: The effect of IM_A on M_w respiration and phosphorylation II. Experiments A, B, and D were carried out at 26° and C at 11°. IM was poisoned as described in the methods with an initial concentration of 0.44 μg of antimycin A/mg of protein. The system consisted of 6.24 mg of M_w protein in A and B, and 12.48 mg in C in a total volume of 1.6 ml of respiration medium. ADP was added in 200-mμmole aliquots whenever indicated. Numbers on slopes refer to millimicroatoms of oxygen utilized per minute.

We reported earlier (Strasberg and Moore, 1969) that the glutamate and malate or succinate supported respiration of mitochondria was stimulated upon addition of IM_R and that phosphorylation was not inhibited. Increasing amounts of IM_R however prevented phosphorylation with glutamate and malate as substrate. In our present experiments when inner membrane fractions were poisoned with rotenone, there was at first a stimulation of the state 4 rate with glutamate and malate, followed by a decrease in respiratory control ratio (at increased concentrations of IM_R) and finally an inhibition of state 4 respiration even at 5° (Figure 12). Thus with IM_R, rotenone still seemed to "be slowly released" at low temperature at the concentrations tested.

The fact that membranes poisoned with inhibitors can stimulate respiration of mitochondria, and that normal membranes can under certain circumstances cause slight stimulation of inhibited mitochondria (before the site of inhibition) suggests that as pointed earlier (Strasberg and Moore, 1969) the interactions of inner membrane fraction with mitochondria indicate the possibility of a bypass or a back and forth movement of reducing equivalents between inner membrane fraction and mitochondria.

The fact that M_{WR} also stimulated M_{WN} under the conditions described in Figure 6 is also indicative of the possibility of mitochondrial–mitochondrial interaction.

Discussion

The interaction of inner membrane fraction with mitochondria resulting in a stimulation of respiration is a phenomenon which cannot be accounted for by free fatty acid presence in or released from the inner membrane fraction. If such a release were indeed the cause of the respiratory stimulation, then it would have been possible to simulate the activity of inner membrane fraction on mitochondria by preincubating mitochondria with inner membrane fraction, so as to obtain a concentrated supernatant solution now capable of respir-

atory stimulation. This was found not to be the case. Defatted bovine serum albumin would also have been expected to overcome the respiratory stimulation. This only occurred at exorbitantly high concentrations of bovine serum albumin (4 mg/ml), far beyond that necessary to prevent stimulation by added free fatty acid. These extremely high concentrations of bovine serum albumin significantly inhibited normal mitochondrial respiration. In some way the effect resembles the inhibition of mitochondrial respiration by high concentrations of inner membrane fraction (Figure 4).

The increased electron transport is mediated *via* a pathway which is incompletely sensitive to rotenone and antimycin A as (when these are now added in excess of the number of binding sites on the particles being poisoned) some electrons can by-pass the inhibited sites of either mitochondria or inner membrane fraction and get to oxygen. One of the arguments that can be raised by this and the system in general is that it seems highly unlikely that electrons can escape from one particle and get to another. While the exact molecular organization of such a reaction is not at all clear, some ionic interaction is taking place between components of inner membrane fraction and the mitochondria as indicated by the pH dependency of this reaction. At acid pH values, the reaction is inhibited at lower concentrations of inner membrane fraction, and also has a low V_{max}, while at higher pH values, the inhibition is delayed and the V_{max} is higher.

The temperature sensitivity of the reaction indicates that not only is the flow of electrons of mitochondria decreased at lower temperature but the ability of inner membrane fraction to stimulate mitochondria is also substantially decreased. This is more evident in experiments with poisoned inner membrane fraction, where respiratory stimulation is still evident but the percentile effect is drastically reduced.

This latter experiment with poisoned membranes also indicates that inhibition occurs at lower concentrations of IM_I at the higher temperatures than at lower ones, pointing to an increase in frequency of collisions brought about by increasing temperatures. This is reminiscent of the thermal agitation and tunnelling described by Chance *et al.* (1967).

This factor of frequency of collision must be mentioned in relation to the dilution experiment described by Chance *et al.* (1967) for oligomycin-treated particles. From Figures 2 and 4 it can be seen that there are two factors involved, one of ionic species, as judged from the pH effect, and the other of interaction as judged from the temperature and concentration effects.

The data in Figure 4 resemble antibody–antigen titration curves. The values plotted on the extreme left resemble the zone of antigen excess, and on the extreme right that of antibody excess. It would also appear (if one were to move from right to left along the abscissa) that dilution of IM results in an activation possibly by the release or diminution of inactive overlap between membranes and mitochondria. Extreme dilution results in decrease in activity by a lack of complementary collisions or interactions. Thus it seems that this phenomenon could be related to the activity of the particles in collision with normal mitochondria, and different from the case described by Chance *et al.* (1967) where both coupled and uncoupled chains exist in the same particle.

The alarming fact is that there seems to be overlapping activity between chains of intact mitochondria and the inner membrane fraction particles. Whether the theory of super conductance is of relevance here must await further experimental tests.

The functional resemblance between the inner membrane fraction particles and 2,4-dinitrophenol is very striking. Just as DNP with increasing concentration becomes inhibitory, so do these particles.

The stimulation of latent ATPase by DNP is also mimicked by inner membrane fraction. Other partial reactions of oxidative phosphorylation have not as yet been examined.

One feature of the inner membrane fraction is that while it stimulates respiration severalfold, it does not interfere with the phosphorylation mechanism. This is deduced from the fact that in the presence of high concentrations of inner membrane fraction, there is approximately 30% less ATP synthesized by mitochondria than in the absence of inner membrane fraction. However, the ATPase activity of IM + M_w would indicate that during the same period of incubation, 30% of added ATP was hydrolyzed.

Similar results were obtained with DNP.

The findings that ATP synthesis if measured at high concentrations of DNP approaches zero can be considered from the point of view that the ATP synthesized by the mitochondria can be more rapidly hydrolyzed than added ATP especially if there is a transport process involved in the over-all enzymatic process. In any event, the high degree of hydrolysis of ATP could possibly negate the finding of newly synthesized ATP in the presence of high ($\sim 10^{-3}$ M) concentrations of DNP. At lower DNP concentration one is able to see the state 4–3 transition, and ATP synthesis is always measurable. If one were to take the liberty of proposing that DNP does its "uncoupling" at the level of the mitochondrial membrane, and the evidence for this is increasing (Carafolli and Rossi, 1967) it is quite plausible to take the point of view that it, like the inner membrane fraction, is stimulating or opening up pathways of electron flow which are separable from the coupled system and thus could in the case of DNP indeed be in agreement with the presence of one coupled and another uncoupled chain in the same particle as described by Chance *et al.* (1967). With inner membrane fraction however, the uncoupled chain belongs to an extramitochondrial system interacting with the mitochondrial system. Whether the high DNP concentration uncouples all the electron transport systems or whether ATPase activity and decreased transport of ADP can truly account for the lack of ATP is disputable and it is hoped that further studies on the inner membrane fraction system and DNP would allow for understanding of the problems of coupled oxidative phosphorylation.

References

Adam, H. (1965), *in* Methods of Enzymatic Analysis, Bergmeyer, H. U., Ed., New York, N. Y., Academic, pp 573–577.

Carafolli, E., and Rossi, C. S. (1967), *Biochem. Biophys. Res. Commun. 29*, 153.

Chance, B., Lee, C. P., and Mela, L. (1967), *Fed. Proc. 26*, 1341.

Chance, B., and Williams, G. R. (1955), *J. Biol. Chem. 217*, 383.

Chance, B., and Williams, G. R. (1956), *Advan. Enzymol. 17*, 65, 74.

Chappell, J. B. (1961), *Proc. 1st Intern. Symp., Stockholm*, 71–83.

Greengard, P. (1965), *in* Methods of Enzymatic Analysis,

Bergmeyer, H. U., Ed., New York, N. Y., Academic, pp 551–555.
Harris, E. J., Van Dam, K., and Pressman, B. (1967a), *Nature 213*, 1126.
Harris, E. J., Van Dam, K., and Pressman, B. (1967b), *Fed. Proc. 26*, 610.
Lee, C. P., Nordenbrand, K., and Ernster, L. (1965), *Proc. Intern. Symp. Oxidases Related Redox Systems, Amherst, Mass., 1964*, 960.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Moore, C. L. (1968), *Biochemistry 7*, 301.
Parsons, D. F., Williams, G. R., and Chance, B. (1966), *Ann. N. Y. Acad. Sci. 137*, 643.
Strasberg, P. M., and Moore, C. L. (1969), *Life Sci.* (in press).
Van Dam, K. (1967), *Biochim. Biophys. Acta 131*, 407.
Van Dam, K., and Slater, E. C. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 2015.

Isolation of Rat Liver Mitochondrial Membrane Fractions and Localization of the Phospholipase A*

Moseley Waite

ABSTRACT: Mitochondria were isolated by sucrose density gradient centrifugation from livers of rats previously injected with [^{14}C]ethanolamine, which was incorporated into the membrane phosphatidylethanolamine. Marker enzymes showed that these preparations were contaminated by no more than 1% of the microsomes and about 2% of the lysosomes present in the homogenate. Mitochondrial membranes were isolated either by osmotic shock or by incubation with Ca^{2+}, followed by density gradient centrifugation. Osmotic shock appeared to cause 75–85% of the outer membrane to be stripped from the mitochondria with little inner membrane contamination. Up to 40% of the matrix enzymes were solubilized under these conditions. Incubation of mitochondria with Ca^{2+} apparently caused cross-contamination of the membrane fractions and solubilized 85–95% of the matrix enzymes. Mitochondrial phospholipase A catalyzed the hydrolysis of the outer membrane phosphatidylethanolamine mainly. Comparison of the specific activity of the phospholipase A in the outer and inner membrane with that of outer membrane marker enzyme levels raises the possibility that some phospholipase A might be associated with the inner membrane however. The specific activity (disintegrations per minute per micromole of phosphorus) of the phosphatidylethanolamine only changed in the inner membrane during incubation which suggests that not all of the inner membrane phospholipid was hydrolyzed at the same rate.

Recent work (Bjørnstad, 1966; Waite *et al.*, 1969a) demonstrated that rat liver mitochondria contain a phospholipase A which hydrolyzes both endogeneous and added phospholipids. It was shown that there is a relation between the hydrolysis of mitochondrial phospholipid and certain types of mitochondrial swelling (Waite and van Golde, 1968; Waite *et al.*, 1969b), substantiating the earlier proposal of Wojtczak and Lehninger (1961). Addition of either fatty acid or Ca^{2+} to mitochondria stimulated phospholipase A activity and swelling. EDTA, however, inhibited both swelling and phospholipase A activity. A recent paper by Nachbaur and Vignais (1968) provided evidence that the outer membrane fraction of rat liver mitochondria contained a phospholipase A capable of hydrolyzing added phosphatidylethanolamine. The phospholipase A reported by them appears to be the same as that studied by Bjørnstad (1966) and Waite *et al.* (1969a,b) and that described here. In the present extension of these studies mitochondrial membranes were disrupted and separated in order to determine the phospholipase A activity in each membrane fraction.

Several conflicting reports have appeared regarding the character of mitochondrial fractions based on marker enzyme studies. Allmann *et al.* (1968) used snake venom phospholipase A or high levels of oleic acid to isolate membrane fractions. In contrast, Sottocase *et al.* (1967a,b), Parsons *et al.* (1966, 1967), and Schnaitman and Greenawalt (1968) have used either mechanical means such as osmotic shock and sonication or detergents to remove the outer membranes from mitochondria. In the present studies experiments using both these approaches for the separation and characterization of membranes were compared. This comparison has led to the conclusion that the distribution of the marker enzymes studied is drastically changed under conditions leading to phospholipid hydrolysis.

Experimental Procedures

Male Wistar rats were injected with 20 μCi of [^{14}C]ethanolamine (specific activity 3.7 mCi/mmole) in 50% ethanol (Bjørn-

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