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The energy-linked nature of respiration-dependent bromothymol blue color decrease in submitochondrial particles[1]

CHANCE AND MELA[1] showed that the blue color of the indicator dye, bromothymol blue, bound to submitochondrial particles was partially discharged on the initiation of respiratory activity or on the addition of ATP. When NADH or succinate was the electron donor the color decrease of the dye was inhibited by respiratory poisons as well as uncouplers of oxidative phosphorylation. A similar color response, linked to the generation of energy at the third site of phosphorylation, was produced on the aerobic oxidation of ascorbate in the presence of the red-ox dye, toluylene blue[2]. The results presented in this communication indicate that the extent of the color change obtained with NADH, succinate and ascorbate–toluylene blue is dependent on the number of sites of phosphorylation being energized by the respiratory activity.

Two types of submitochondrial particles were used in these experiments. The "ammonia" particles were prepared by sonic disruption of heavy layer bovine heart mitochondria in the presence of EDTA and ammonia[3], and phosphorylating electron transport particles prepared from heavy layer beef heart mitochondria (ETPH) in the presence of $MgCl_2$ and ATP[4]. The reaction system for the measurement of the color response contained 20 mM Tris–HCl buffer (pH 7.4), 0.25 M sucrose, bromothymol blue as indicated and 0.5 mg of particle protein in a total reaction volume of 3 ml. The reaction was initiated by stirring in 0.05 ml of 20 mM NADH, 0.03 ml of 0.2 M succinate or 0.04 ml of 0.5 M ascorbate followed by 0.02 ml of a solution containing 1.5 mM toluylene blue and 50 mM ascorbate. The toluylene blue was reduced with ascorbate before addition to the reaction system in order to discharge its color. The decrease in the blue color of the membrane-bound bromothymol blue was measured by the decrease in absorbance at 618–700 mμ in the Aminco-Chance dual wavelength spectrophotometer. When ascorbate–toluylene blue was used as the electron donor, 1 μg each of antimycin A and rotenone were added to the reaction system to prevent electron flow in reverse. Wherever indicated, oligomycin (0.5 μg in 0.01 ml ethanol), dithiothreitol (0.5 mM) and EDTA (1 mM) were added before the addition of the electron donor. All aqueous solutions were adjusted to pH 7.4 with KOH before use.

The results presented in Table I indicate that the magnitude of the absorbance decrease depended on the electron donor as well as on the presence of dithiothreitol or EDTA in the reaction medium. When ammonia particles were used, no significant color response could be observed unless EDTA or dithiothreitol was added to the reaction medium "prior" to the addition of ascorbate–toluylene blue. The stimulatory effect of EDTA and dithiothreitol was seen with the other electron donors and also with ETPH. The increased response of the dye in the presence of these two activating agents was not due to increased respiratory activity because neither the cytochrome oxidase nor the NADH oxidase activity of these particles was stimulated by dithiothreitol or EDTA under the same experimental conditions (data are not shown). The

Abbreviations: ETPH, phosphorylating electron transport particles prepared from heavy layer beef heart mitochondria; TMPD, tetramethyl-*p*-phenylenediamine.

TABLE I

BROMOTHYMOL BLUE COLOR RESPONSE PRODUCED BY DIFFERENT ELECTRON DONORS IN SUBMITOCHONDRIAL PARTICLES

The reaction system and conditions of assay have been described in the text. The assay medium did not contain oligomycin.

Addition	*Bromothymol blue* (μM)	*Ammonia particles* ($\Delta A \times 10^3$)			*ETPH* ($\Delta A \times 10^3$)		
		Electron donor			*Electron donor*		
		NADH	*Succinate*	*Ascorbate–toluylene blue*	*NADH*	*Succinate*	*Ascorbate–toluylene blue*
None	6.7	18	7	0.0	27	12	3.5
EDTA	6.7	25	13	2.0	32	24	7.0
Dithiothreitol	6.7	23	14	1.5	30	24	7.0
None	13.3	25	10	0.0	29	12	4.0
EDTA	13.3	37	23	3.5	36	24	7.0
Dithiothreitol	13.3	32	25	4.5	35	27	7.5

TABLE II

EFFECT OF OLIGOMYCIN ON BROMOTHYMOL BLUE COLOR RESPONSE PRODUCED BY DIFFERENT ELECTRON DONORS

The values given are the % increase obtained over those given in Table I on the addition of 0.5 μg of oligomycin.

Addition	*Bromothymol blue* (μM)	*Ammonia particles*			*ETPH*		
		Electron donor			*Electron donor*		
		NADH	*Succinate*	*Ascorbate–toluylene blue*	*NADH*	*Succinate*	*Ascorbate–toluylene blue*
None	6.7	28	174	0	2	33	0
EDTA	6.7	32	100	275	2	8	0
Dithiothreitol	6.7	30	114	430	0	10	0
None	13.3	8	120	0	3	17	25
EDTA	13.3	16	61	185	8	21	0
Dithiothreitol	13.3	22	60	145	3	7	13

succinate oxidase activity was, however, increased 2-fold by EDTA and 3–4-fold by dithiothreitol. It may be mentioned in this context that other energy-linked activities like the nicotinamide nucleotide transhydrogenase and the succinate-dependent reduction of NAD^+ by reversed electron flow were also greatly stimulated by these two reagents when the energy was derived from aerobic oxidation[5]. The substrate (NADH, succinate or ascorbate–tetramethyl-*p*-phenylenediamine (TMPD)) concentration was optimal for maximum bromothymol blue response in these experiments. Increasing NADH or succinate did not change the oxidation rate. In the case of ascorbate–TMPD, the oxidation was increased by increasing TMPD, but the bromothymol blue absorbance change was not increased.

The magnitude of the color response seemed to depend on the number of phosphorylation sites activated by the electron transport activity. The response was

always maximum with NADH which energizes all the three sites of phosphorylation, was less with succinate which energizes two phosphorylation sites and was minimum with ascorbate–toluene blue which involves only the third site of phosphorylation. The relatively low absorbance change associated with the oxidation of ascorbate–toluylene blue by ammonia particles is stimulated to a great extent on the addition of oligomycin to the reaction system (Table II). In general, for energy-linked reactions these particles were more dependent on oligomycin than were ETPH. This is consistent with the suggestion[6] that poorly phosphorylating submitochondrial particles prepared in the presence of alkali and EDTA are characterized by a high rate of hydrolytic breakdown of high energy intermediates and that the energy leak is inhibited by oligomycin. It is interesting to note that as the number of sites generating energized intermediates increases from ascorbate–toluylene blue through succinate to NADH, the response to the addition of oligomycin decreases (Table II). This would suggest that as the high energy compounds are generated from more sites, the effect of loss due to hydrolysis becomes less serious.

The absorbance decrease at 618–700 mμ shown by bromothymol blue-supplemented submitochondrial particles upon the initiation of respiratory activity or the addition of ATP has been ascribed by CHANCE AND MELA[1] to intravesicular acidification caused by the energy-linked movement of bound cations from the inside of the vesicles to the outer medium. Differences with this interpretation as applied to whole mitochondria[7] have been expressed by GEAR *et al.*[8], and ROSSI AND AZZONE[9]. MITCHELL *et al.*[10] have questioned the usefulness of the bromothymol blue color as an efficient measure of the internal pH changes of whole mitochondria. From a study of the influence of physical factors on the binding of bromothymol blue with beef heart "structural protein", SARIS AND SEPPALA[11] concluded that intravesicular pH could be the main factor that governs the color response in submitochondrial particles. The fact that the color response is inhibited by respiratory poisons and uncoupling agents[1,2], and the results presented here clearly show that it is a sensitive index of the energized state of these particles and that the reaction responsible for the absorbance decrease is an energy-linked one.

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