ON THE USE OF BROMTHYMOL BLUE AS AN INDICATOR OF INTERNAL pH CHANGES IN CHROMATOPHORES FROM RHODOSPIRILLUM RUBRUM

Z. Gromet-Elhanan and S. Briller
Department of Biochemistry, Weizmann Institute of Science
Rehovoth, Israel

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Chance and Mela (1966) first proposed the use of BTB* as an indicator of pH changes inside intact mitochondria. Their proposition was based on two characteristic features of BTB, namely that it was tightly bound to the particles and that when bound, it underwent spectral changes which indicated pH shifts in a direction opposite to that recorded in the outer medium. Following these observations BTB has also been used as an indicator of internal pH changes in R. rubrum chromatophores (Chance et al., 1966; Nishimura et al., 1968; Nishimura and Pressman, 1969).

While testing the effect of nonactin on photophosphorylation, external pH changes and BTB changes in the PMS + HOQNO system of R. rubrum chromatophores, we have observed that although in the presence of nonactin the degree of binding of BTB to the chromatophores remained unchanged, the mirror image correlation of the BTB change with the external pH change disappeared. It is therefore concluded that BTB can not be used in general as a reliable indicator of internal pH changes in chromatophores.

METHODS - R. rubrum cells were grown and chromatophores isolated as previously described (Gromet-Elhanan, 1969), except that the chromatophores were washed and finally suspended in 0.25 M sucrose instead of Tris-sucrose. Bacteriochlorophyll was determined using the in vivo extinction coefficient given by Clayton (1963).

Absorption changes of BTB were measured at 590 nm with a Cary Model 14 spectrophotometer equipped with a scatter attachment. At this wavelength the chromatophores gave a minimal light minus dark change in the absence of BTB. The samples were illuminated by a 500W slide projector (without its heat filter) through a combination of CS 2-59 and CS 7-69 corning filters and 9 cm of water. The photomultiplier was protected from

*The abbreviations used are: BTB, bromthymo blue; HOQNO, 2-heptyl-4-hydroxy-quiniline N-oxide; PMS, N-methyl phenazonium methosulfate.

scattered actinic light by 1 cm of a solution of 15% $CuSO_4$. External pH changes were recorded simultaneously by a combination of a Radiometer GK 2024C pH electrode, a Radiometer PHM 22 pH meter, and a Photovolt Model 43 recorder.

Binding experiments were performed by incubating the reaction mixture containing chromatophores and BTB in a 5 ml automatic glass syringe attached via a Luer-Lok to a micro-syringe holder for millipore filters. A G.S. 0.22 μ millipore filter which was pre-washed in 0.03 M tricine pH 8.5 was placed in the micro-syringe holder. After a 1 min incubation in the light or dark the suspension was squeezed through the millipore filter. The unbound BTB was estimated by the absorbance at 620 nm of the filtrate. The amount of bacteriochlorophyll which passed to the filtrate was checked in each experiment and never exceeded 0.2% of the original bacteriochlorophyll concentration.

Nonactin was generously provided by Dr. W. Simon of the Eidg. Technische Hochschule, Zurich. HOQNO was obtained from the Sigma Chemical Co., St. Louis, Missouri.

RESULTS AND DISCUSSION - In order to compare external pH changes with BTB changes the pH of the reaction mixture should be as close as possible to the pK of BTB. In the experiments described here this pK was found to be 7.5 in the presence of chromatophores. At this pH the endogenous chromatophore system showed light-induced pH changes which were neither completely reversible nor reproducible.

Von Stedingk (1967) has reported that at pH 6.2 the PMS + HOQNO system gave a much more reversible pH effect. However, at the high intensity of white light that he used, a lag period which was attributed to heating artifacts preceeded the rise in pH. As can be seen from Fig. 1A a reversible and fully reproducible pH effect was obtained with the PMS + HOQNO system at pH 7.35. Using the far-red illumination setup described under METHODS there was no lag in the pH rise, provided that enough ascorbate was added to reduce the PMS before the beginning of illumination. In this system the BTB absorption changes were indeed found to follow the external pH changes closely, but in an opposite direction (Fig. 1A). However, this mirror image correlation between the BTB absorption changes and external pH changes, which forms the basis for accepting the BTB changes as indicating internal pH changes, has not been observed under all circumstances. Two examples of conditions in which this correlation did not hold are illustrated in Fig. 1A and B, viz. in the presence of nonactin and/or when the external buffer capacity was increased.

Nonactin was found by Thore et al., (1968) to stimulate the light-induced pH change without affecting photophosphorylation in the succinate system of R. rubrum chromatophores. In the PMS + HOQNO system, the external pH change was also stimulated (Fig. 1A)

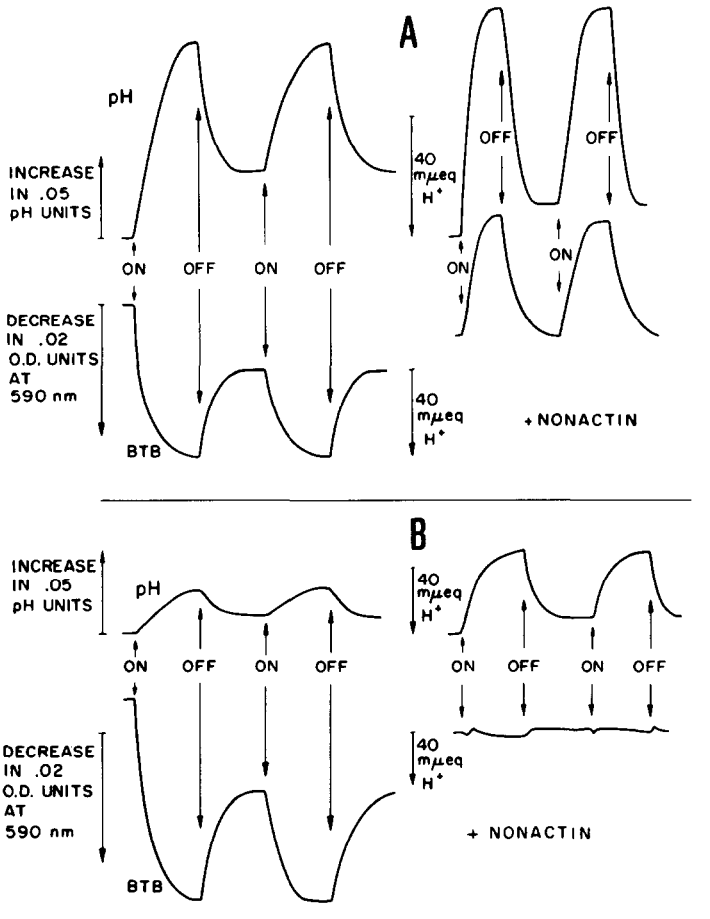

Figure 1: Effect of nonactin and buffer concentration on light-induced external pH and BTB changes. The reaction mixture contained in a total volume of 3 ml: 200 mM NaCl; 40 μM PMS; 60 μM Na ascorbate; 1 μM HOQNO; 6.6 μM BTB; 44 μg of bacteriochlorophyll and when indicated 13 μM nonactin. Final pH 7.35. A - no added buffer; B - with 1 mM tricine.

while photophosphorylation was hardly affected whether in the absence or presence of BTB (not shown here). In contrast to the increase in the external pH change the BTB absorption change in the light was completely reversed, following in the presence of nonactin the external rather than the internal pH change (Fig. 1A). The stimulatory effect of nonactin on the pH changes required the presence of an alkali metal. Either Na^+ (as shown here) or K^+ ions were effective confirming the observation of Graven et al. (1966) that at nonactin concentrations above 10^{-6}M both ions were active.

Cost and Frenkel (1967) observed light-induced BTB changes in R. rubrum chromatophores under conditions when no external pH changes could be detected. They, therefore,

attributed the BTB changes to conformational rather than internal pH changes. Since they used a relatively high buffer concentration (20 mM) it is conceivable that all the external pH changes were buffered out while internal changes could still be observed. Fig. 1B illustrates the effect of 1 mM buffer on both reactions. While the pH change measured by the pH electrode was only 30% of that observed in the absence of added buffer (Fig. 1A) the BTB change showed a two fold increase. Addition of nonactin in the presence of the buffer again stimulated the light induced pH rise, but completely eliminated the BTB change (Fig. 1B).

The effect of both nonactin and buffer could be explained by differences in the degree of binding of BTB to the chromatophores. If not all the BTB was bound, the unbound fraction would record external pH changes. In the presence of buffer only the internal pH changes would be recorded, and would therefore appear greater than the net change in the absence of buffer. Also, as has been reported by Saris and Seppala (1969), the degree of binding of BTB to mitochondrial structural protein varied with the pH, the anion concentration, and the anion species in the medium. Thus, nonactin which has been shown to induce active transport of alkali metal cations (Lardy et al., 1967; Pressman, 1965) might also affect the degree of binding of BTB to the chromatophores.

From the data of Table I it is however clear that no significant change in the binding of BTB was induced by illumination or by nonactin or by both. Also, since

Table I: Effect of light and nonactin on the binding of BTB to chromatophores.

Conditions		Unbound BTB in the Filtrate	
Light	nonactin	620 nm absorption	% of control
-	-	0.083 ± 0.011	22.5 ± 1.6
+	-	0.072 ± 0.014	19.5 ± 2.0
-	+	0.075 ± 0.018	20.5 ± 2.6
+	+	0.078 ± 0.018	21.5 ± 2.6

The reaction mixture contained in a total volume of 4 ml; 1 mM tricine buffer pH 7.5; 320 mM NaCl; 33 µM PMS; 50 µM Na ascorbate; 1 µM HOQNO; 6.6 µM BTB; 60 µg of bacteriochlorophyll and when indicated 13 µM nonactin. Experimental conditions are described under METHODS. The final pH of the filtrate was 7.8 and each number is an average of 5 experiments. In the control experiment which underwent the same treatment but was injected in the absence of the millipore filter the absorption of BTB at 620 nm was 0.370.

only 20% of the BTB remained unbound, the elimination in the presence of buffer of even all the external pH change recorded by the unbound BTB could not explain the two fold increase observed in the BTB change by the addition of 1 mM buffer (Fig. 1AB).

It is still possible, as has been pointed out by Mitchell et al. (1968), that the functional groups of the bound BTB might be distributed in the membrane in such a way as to face either the inner or the outer aqueous phase. Nonactin might cause a redistribution of these groups to face almost entirely the outer aqueous phase. The BTB change would thus follow the direction of the large external pH change in the absence of buffer(Fig. 1A) or be eliminated when the external pH change is 70% reduced in the presence of buffer (Fig. 1B).

Nishimura and Pressman (1969) have recently observed that valinomycin increased the external pH rise, but decreased the BTB change and nigericin decreased the external pH rise but increased the BTB change in the endogenous system of R. rubrum chromatophores. They explained these results by proposing that BTB may partially reflect alterations in membrane potential or other physical properties of the system, although they still consider BTB to be mainly an indicator of internal pH.

Our results with nonactin clearly indicate that in this case at least the BTB changes do not reflect at all internal pH changes. Since as yet no independent way is available to asses the fraction of BTB which records the internal pH changes, and which seems to vary widely with experimental conditions, BTB cannot be used as a reliable quantitative internal pH indicator.

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