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STUDY OF THE CHLOROPHYLL FLUORESCENCE IN CHLOROPLASTS AND ALGAE WITH THE PLASTOQUINONE ANTAGONIST DIBROMOTHYMOQUINONE

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SUMMARY

The effect of the plastoquinone antagonist dibromothymoquinone on chlorophyll fluorescence in vitro and in vivo was investigated.

1. With chlorophyll *a* in solution quenching is observed, more efficient than that of *p*-benzoquinone (the Stern-Volmer constant $K = 200\ M^{-1}$); ascorbate removes this effect.

2. With isolated chloroplasts, a dramatic enhancement of quenching occurs (also abolished by ascorbate) in the following order of importance: thermal step, photochemical step (of variable fluorescence), and constant fluorescence; K for the total variable fluorescence $\approx 480\,000\ M^{-1}$, for constant $\approx 190\,000\ M^{-1}$. Parallel to the quenching effect, an enlargement of the complementary area of the variable fluorescence is observed. Addition of ascorbate suppresses the quenching and this enlargement. If this area in the presence of reduced dibromothymoquinone is similar to that of the control, this means that A, the pool of oxidant next to Q, remains connected to it, a result contradicted by the O_2-burst measurement. However, when the excess of unbound dibromothymoquinone is washed out, which removes the quenching effect, a significant decrease of the area is seen, in agreement then with the burst results. Therefore, the meaning of the complementary area and the nature of the controlling factors may be questioned.

3. With whole cells (*Chlorella*), no quenching is observed; instead, an enhancement of the thermal step is noticed, together with an even larger increase of the complementary area. Dibromothymoquinone being in an oxidized form at the thylakoid level, it is proposed that in intact membranes, the chlorophyll molecules are not readily accessible to it: thus, no quenching is observable, and the predominant effect is on the redox chain. The specific effect of dibromothymoquinone on the thermal step supports the hypothesis that it is normally controlled by a secondary quencher, related to plastoquinone.

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone (dibromothymoquinone); DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea.

INTRODUCTION

The in vivo chlorophyll fluorescence (see insert in Fig. 6 for symbols) is a good indicator of the electron transfer chain activity. More precisely, Duysens and Sweers [1] showed that the "variable" fluorescence, involved in the resulting "complementary law" of photochemistry [2, 3], is controlled by the redox state of the primary electron acceptor of Photoreaction II, the "quencher" Q, which, once photoreduced, is reoxidized by a 10-times larger pool of oxidant A [4–7]. Delosme [8] has given evidence that the thermal part of the fluorescence rise involves, in addition to Q, a "secondary, non-photodestructible" quencher R, related to A without being necessarily identical to it (this R is different from the newly introduced quencher of Velthuys and Amesz [9]). The pool A could represent at least a part of the endogenous plastoquinone [10, 11]. Recently, a new inhibitor of the electron transfer chain was introduced [12, 13], DBMIB, which acts between the DCMU site and the Photoreaction I, most probably on the plastoquinone itself [14], as could be expected from its chemical nature [13]. With isolated chloroplasts, Lozier and Butler and others reported that oxidized DBMIB quenches the fluorescence [15–17] and slows down the increase of yield from F_0 to F_P [15]. With unicellular red or green algae, however, Gimmler [18, 19] presented figures where DBMIB had no quenching property and speeded up the rise of the variable fluorescence.

In a previous work [14], the action of DBMIB on some electron transfer reactions, particularly on the O_2 initial burst, which is a measure of A [4–7], was studied. It was concluded that DBMIB, in isolated chloroplasts and in whole cells, disconnects the plastoquinone pool A from Photoreaction II. To complete these results and to clarify the above mentioned contradictions, the DBMIB effect on the chlorophyll fluorescence of both isolated and in situ chloroplasts was therefore also investigated. Special attention was paid to that part of the variable fluorescence which is related to A: the thermal stage and the so-called "complementary area" [20, 21], which is a measure of the total pool Q+A

$$= \int_{t_0}^{t_P} (F_P - F_t)\mathrm{d}t, \text{ hereafter shortened to } \int F.$$

METHODOLOGY

Daily harvested *Chlorella* sp., grown under continuous white-light illumination in a purely mineral solution, were resuspended before the experiments in a fresh culture medium ($\approx$ 0.044 M in different salts, pH $\approx$ 6). Spinach chloroplasts were prepared as previously described [14]. Chlorophyll *a* was kindly provided by Dr J.-G. Villar, from this laboratory.

DBMIB (dissolved in methanol) and DCMU (dissolved in ethanol) were gifts of Drs Trebst and Drs Heytler, respectively. The final alcohol concentration was around 1 %.

Two sets of apparatus were used for measuring the chlorophyll fluorescence. One [22] had a 5 mm light-path cuvette, illuminated with broad-band blue light (Corning filter 4.96: half-transmission bandwidth, $\Delta\lambda\frac{1}{2}$, from 390 to 550 nm); the total band of chlorophyll fluorescence ($>$ 675 nm) was analyzed in the axis, through

two cut-off filters (Corning 2.64 + Kodak, Wratten 70). The resulting signal was displayed on an oscilloscope screen and photographed, and also fed into a multichannel analyser. The other set-up consisted of a thermostated spectroscopic cuvette placed on top of a magnetic stirrer. The narrow or broad-band blue exciting light was obtained with an interference filter (432 nm, $\Delta\lambda\frac{1}{2} = 7$ nm) or with the Corning 4.96 filter, respectively; the fluorescence emission was analyzed at right angles, at 685 nm (interference filter of $\Delta\lambda\frac{1}{2} = 12$ nm). The opening times of the shutters were between 1 and 3 ms.

RESULTS

Quenching of chlorophyll a in solution by DBMIB

The quenching of chlorophyll solutions by various organic chemicals is well documented, and quinones are especially efficient for that purpose [23, 24].

Fig. 1 indicates that DBMIB is even more powerful than the classical *p*-benzoquinone and that this effect is abolished by the addition of a reductant such as ascorbate. The Stern-Volmer's representation [23] gives a straight line with a slope equal to the quenching constant K. Fig. 1 shows that for DBMIB, $K = 200\ M^{-1}$ and for benzoquinone, $K = 125\ M^{-1}$ (slightly above the value found by Livingston and Ke [23], but in pure acetone: $123.5\ M^{-1}$).

DBMIB effect on chlorophyll fluorescence of isolated chloroplasts

As shown in Fig. 2, DBMIB strongly inhibits the variable fluorescence, and prolonges the induction time: the complementary area is enlarged, meaning that the concentration of Photoreaction II electron acceptor was increased by the amount of added oxidized quinone.

In Fig. 3, the behaviour of the different parameters is depicted more precisely. As stated above, and noticed with numerous quinones [24] and with *m*-dinitrobenzene

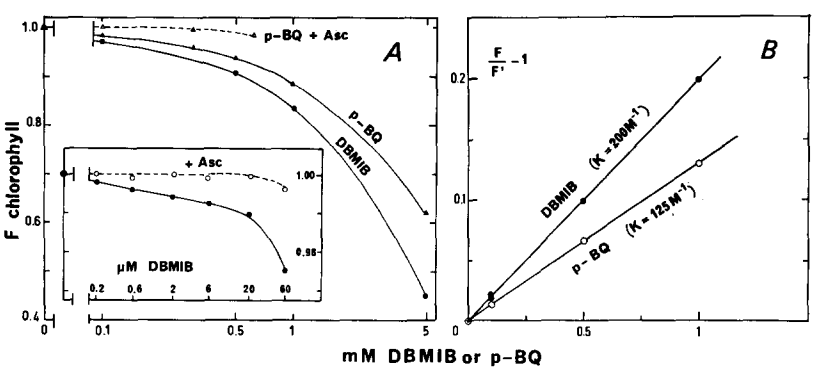

Fig. 1. Quenching of chlorophyll fluorescence in solution by DBMIB and *p*-benzoquinone (p-BQ). Purified chlorophyll *a* (2 μM) in acetone 70 %+water 30 %. Ascorbate (Asc): 1 mM final concentration. Air, 20 °C. Excitation: 432 nm, $\approx 20\ \mu W \cdot cm^{-2}$; analysis: 685 nm. A: direct results. (The apparently resistant quenching by high concentrations of ascorbate-reduced quinones is due to the formation of a transient component of brownish color (a charge-transfer complex?) which absorbs the blue exciting light.) B: Stern-Volmer's representation (F = control and F' = quenched fluorescence).

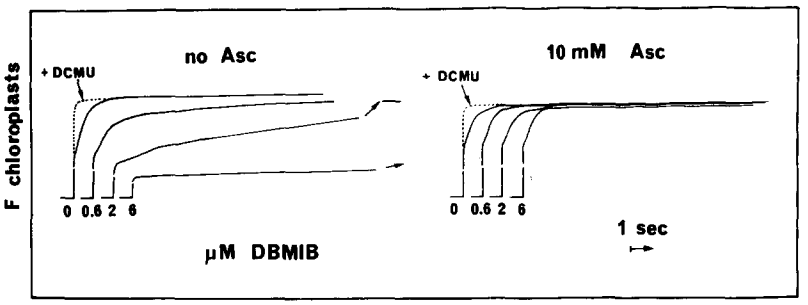

Fig. 2. Oscilloscope traces of chlorophyll fluorescence of isolated chloroplasts at increasing DBMIB concentrations. Spinach chloroplasts in Tris–NaCl–sucrose buffer pH 8. Air, 22 °C. Ascorbate (Asc): 10 mM final concentration; DCMU: 100 μM final concentration. Excitation: broad-band blue light, $\approx$ 1.6 mW · cm^{-2}; analysis: total chlorophyll emission band.

[24–26], the most sensitive part of the fluorescence is the variable part, more precisely its thermal stage [25, 26]. The Stern-Volmer's representation was reasonably linear only for F_0 and, but less, for F_P; for F_I, a strong deviation was seen, and the quadratic equation of Livingston and Ke [23] seemed applicable to this case. In the experiments of Figs 2 and 3, $K_0 \approx 190\,000\ M^{-1}$, $K_{I-0} \approx 250\,000\ M^{-1}$ (a speculative value, computed below 6 μM), and $K_{P-I} \approx 830\,000\ M^{-1}$. The variable/constant ratio is an evaluation of the importance of the photochemical energy conversion yield ρ relative to the sum of all other yields of deactivation (fluorescence, heat, etc.): $(F_{P-0})/F_0 = \rho/(1-\rho)$. Its decrease with the DBMIB concentration increase is mainly due to the F_{P-I} fraction. Finally, it may be noticed that the complementary area rises only up to 6 μM in DBMIB: the lowering of the curve seen above this concentration is certainly due to the strong initial quenching, which lasts for a long time, because of

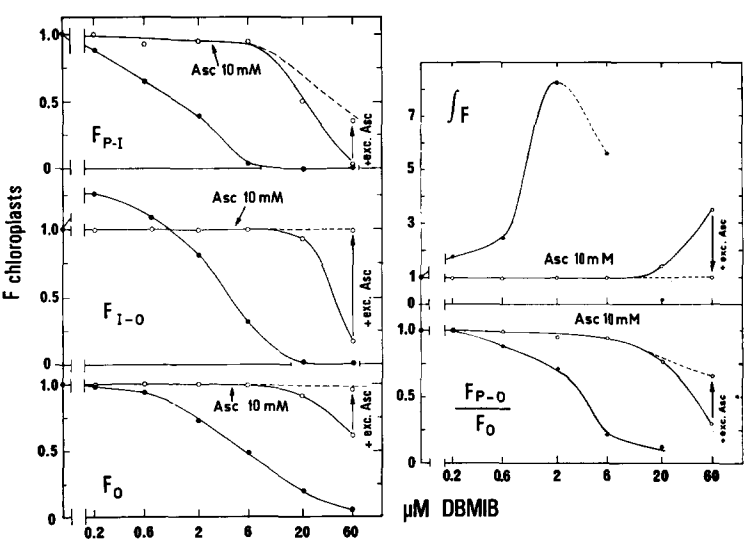

Fig. 3. Effect of DBMIB on the various parameters of chlorophyll fluorescence of isolated chloroplasts. See Fig. 2 for experimental conditions, insert in Fig. 6 for symbols, and the text for details.

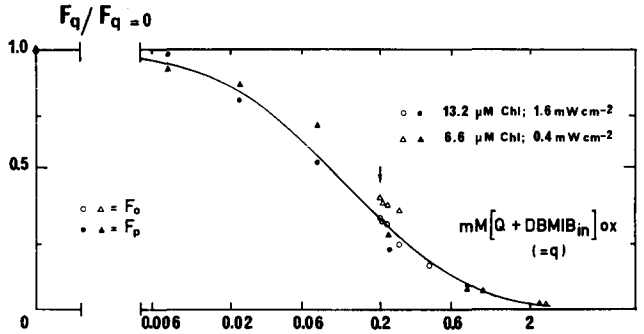

Fig. 4. Combined variations of the minimum (F_0) and the maximum (F_p) fluorescence levels with respect to the total concentration of quenchers (endogenous Q+internal DBMIB). Experimental conditions similar to that of Figs 2 and 5 (spinach chloroplasts). The arrow indicates the F_0 values of the control samples.

the relative slowness of DBMIB reduction, which releases this quenching. Thus, even a qualitative estimation of $\int F$ is impossible under these conditions.

Addition of ascorbate abolishes the quenching effect and seems to bring the area back to the control value, a result not expected from the O_2 burst experiments (ref. 14, but see below).

The differential sensitivity of the variable and the constant fluorescence towards quenching is consistent with the concept of their heterogenous origin [27]. However, the constant fluorescence is already chemically quenched by oxidized Q ($= Q_0$), whereas the peak fluorescence is not ($Q = 0$). Thus, when using an external quencher D such as DBMIB, it might be necessary to consider the total concentration Q+D $=$ q (cf. ref. 25). If this were true, a unique quenching function should control both O and P levels. As will be shown, this is only approximate.

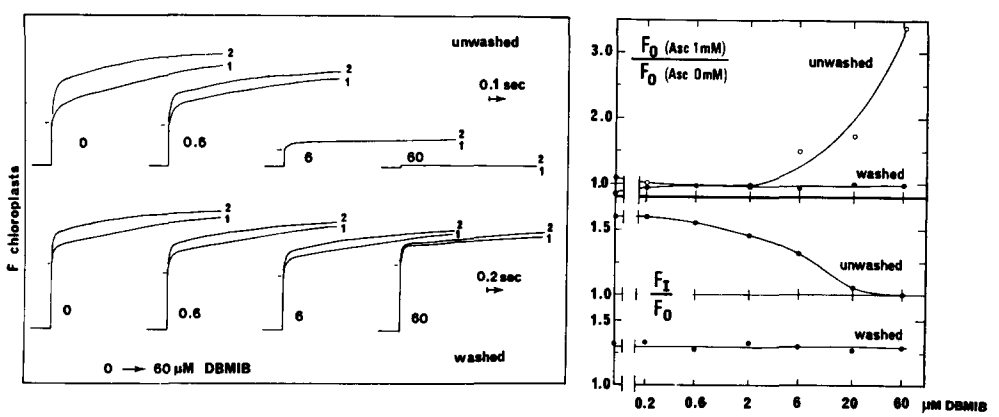

Fig. 5. Effect of washing on the DBMIB action on chlorophyll fluorescence of isolated chloroplasts. Spinach chloroplasts at 6.6 μM chlorophyll in Tris–NaCl–sucrose buffer pH 8. Air, 15 °C. Ascorbate (Asc): 10 mM final concentration. Excitation: broad-band blue light, 0.4 mW · cm^{-2}; analysis: 685 nm. Trace 1: after few minutes of darkness, Trace 2: 15 s after a 2 s preillumination with the excitation light.

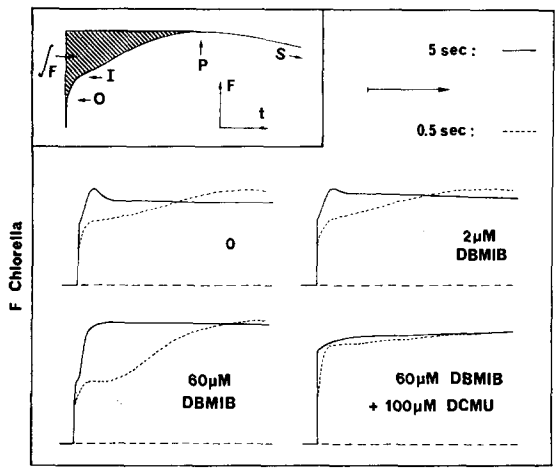

Fig. 6. Oscilloscope traces of chlorophyll fluorescence of algae with and without DBMIB. *Chlorella* cells in the fresh culture medium at ≈ 13.2 μM chlorophyll. Air, 22 °C. Excitation: broad-band blue light, ≈ 1.6 mW · cm^{-2}; analysis: total chlorophyll emission band. The traces corresponding to the different time scales were obtained with different fresh samples. Insert: schematic diagram of in vivo chlorophyll fluorescence induction curve. 0 = minimum ("constant") yield, F_0; P = maximum fluorescence yield, F_P ("variable" fluorescence = F_{P-0}); I = intermediate level (F_{I-0} = photochemical step, F_{P-I} = thermal step); S = steady-state fluorescence yield, F_S (= F_P in isolated chloroplasts). ∫ F is the complementary area [20, 21] of the variable fluorescence.

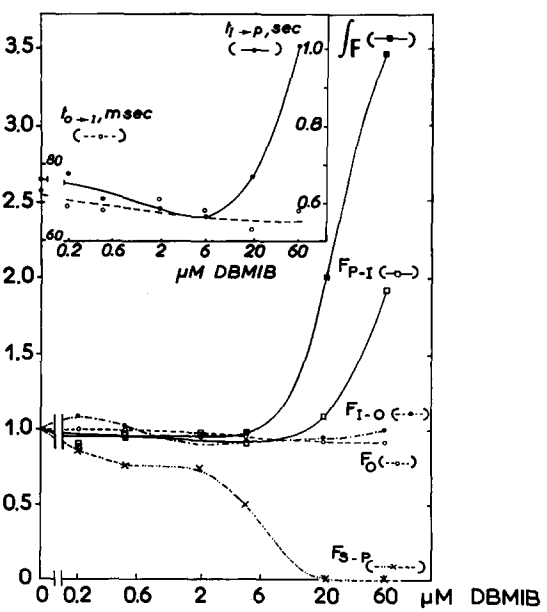

Fig. 7. Effect of DBMIB on the various parameters of chlorophyll fluorescence of *Chlorella* cells. See Fig. 6 for experimental conditions and symbols.

The Stern-Volmer's equation for F_0 may be written: $(F_0/F'_0)-1 = Q_0^{-1} \cdot D_{in}$, if DBMIB internal concentration is expressed in equivalents of Q (F' = externally quenched fluorescence). Since there is 1 Q for approx. 600 chlorophylls [6, 7] and the chlorophyll concentration on the thylakoid membrane space, calculated from ref. 28, is about 0.12 M, $Q_0 \approx 2 \cdot 10^{-4}$ M in situ. Thus, when $F'_0 = 0.5\, F_0$, a situation obtained for an external concentration of DBMIB $(D_{ex}) = 5.2 \cdot 10^{-6}$ M, $D_{in} = Q_0 = 2 \cdot 10^{-4}$ M. The corresponding ratio of "bound" over total DBMIB may then be calculated: $D_{in}/D_{ex} = 38.5$. This factor applied to each value of D_{ex} gives D_{in}, which is added to Q to obtain q. Fig. 4, in which two completely different experiments were combined, shows that a unique curve for F_0 and F_P vs q may be drawn at first sight. However, for low DBMIB concentrations, F_0 values are consistently above what could be expected, suggesting that only a fraction of it has the same origin as F_P, and this supports the hypothesis that part of F_0 is emitted by System I [26, 27, 29].

The quenching effect of DBMIB is completely reversed by simple washing: see Fig. 5. The lack of ascorbate effect is an indirect confirmation of that. Nevertheless, the inhibitory property of DBMIB is still maintained [14]. Thus, as shown in control traces, the fluorescence rise is faster after a preillumination than after a long dark-period [30]. With chloroplasts treated by DBMIB (especially at high concentration) then washed, the traces become comparable without or with a preillumination and, moreover, the complementary area is diminished, two facts indicating a disconnection between Q and A.

DBMIB effect on chlorophyll fluorescence of whole cells (Chlorella)

The main features depicted in Figs 6 and 7 are: the stability of the constant and of the photochemical parts of fluorescence; the increase of the thermal stage and the decrease of the subsequent decay; and the augmentation of the complementary area. Also, if the time for reaching F_I is only slightly shortened, that for developing F_I to F_P is strongly prolonged, since DBMIB increases the pool size of System II-oxidants (like in chloroplasts). That is, DBMIB was not significantly reduced during its travelling through the cell. A direct proof of that is the existence of a DBMIB-dependent O_2 evolution [14], which incidently contradicts the conclusion of Gimmler and Avron that DBMIB is not an electron acceptor in algae [18]. Even "normalized" [27], $\int F$ still increases with the DBMIB concentration, showing that this is not simply due to the peak P heightening and confirming again that the DBMIB present was in the oxidized form. Thus, at 60 μM DBMIB and relative to the corresponding controls,

$$\int F = 3.6,\ F_P^{-1} \int F = 2.7 \text{ and } F_{P\text{-}0}^{-1} \int F = 2.1.$$

In the presence of DBMIB+DCMU (Fig. 6), a small rise above the initial level is observed, which suggests that DBMIB could take a few electrons from Q, bypassing the DCMU inhibition site. This may be compared to the partially DCMU-resistant, DBMIB-dependent O_2 evolution seen with chloroplasts in certain conditions [14].

DISCUSSION

The quenching of chlorophyll *a* fluorescence in solution, a necessary but often neglected control of the in vivo effect, needs no special comment, except that the

high efficiency of DBMIB is interesting to note. This property is dramatically enhanced with isolated chloroplasts and the quenching constants found here are greater than any other given in the literature [24]. The more the benzoquinone is substituted, the stronger seems the quenching: thus, at 120 μM, the inhibition of the total fluorescence was found to be $\approx$ 45 % with trimethylquinone, $\approx$ 60 % with tetramethylquinone and $\approx$ 100 % with DBMIB.

This makes the complete ineffectiveness of DBMIB as a quencher of the chlorophyll fluorescence in whole cells even more surprising. It may be that, with intact thylakoids of in situ chloroplasts, the chlorophyll molecules are not directly accessible to an external substance such as DBMIB. Thus, the only expected effect on fluorescence would be indirect, due to the blocking of the electron flow from Q to Photoreaction I, more precisely to the disconnection between Q and A, as is indicated by the O_2 evolution measurements [14]. This should decrease the complementary area: the increase actually seen is therefore accountable to the presence of oxidized DBMIB and also, as shown, but to a lesser extent, to the heightening of the peak P. The suppression of the P to S decay seems insufficient to explain this P heightening alone. If the thermal step is under the control of a "non photochemically destructible" additional quencher (Delosme's R [8]), the disconnection of plastoquinone from Q would probably remove that secondary quenching effect, resulting in the observed rise of the fluorescence yield.

With isolated chloroplasts, the membrane intactness is probably altered and the chlorophyll molecules become accessible to DBMIB, resulting in the quenching effect. The complementary area behavior observed with reduced DBMIB, shown in Fig. 3 and in ref. 15, would mean that A was still in contact with Q, which is contradicted by the O_2 burst measurements [14]. The burst test indicates precisely the DBMIB inhibition site and, as discussed in ref. 14, the suggestions of some authors that this site is between plastoquinone and cytochrome *f* do not contradict the present conclusion, since they are only a minimal estimate of the distance of this point of action from Photoreaction I. However, it should be noted that the DBMIB inefficiency in decreasing the complementary area is not always as marked: see, for instance, in Fig. 5, the traces obtained with chloroplasts treated with high concentrations of DBMIB, then washed to remove the quenching effect. It is therefore possible that in the presence of ascorbate, a sufficient amount of unbound DBMIB was maintained in the oxidized form, especially with the non-saturating light available here, since dibromothymohydroquinone is rather autooxidizable (cf., in Fig. 3 of ref. 14, the apparent "recovery" of the O_2 burst with high concentration of reduced DBMIB). Whatever the reasons are, these results may raise the question of the meaning of the complementary area and of the nature of the factors controlling it. In this respect, it is interesting to recall that some observations were published [25, 31], showing that fluorescence and photochemistry may behave relatively independently of each other.

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