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FACTORS AFFECTING THE STABILITY OF CHLOROPLAST MEMBRANES, *IN VITRO*

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SUMMARY

Factors which affect the stability of light-induced atebrin fluorescence quenching activity in chloroplast membranes, a measure of the electron transport dependent formation of energy-linked H^+ gradients, were investigated *in vitro*. Class II spinach chloroplast membranes were isolated and stored at 0-4°C and aliquots were subsequently tested for their retention of energizing capacity. The main factors which increase the stability of this activity were found to be a) isolation in a potassium-containing medium but storage in a sucrose medium containing a low concentration of electrolytes; b) the presence of butylated hydroxytoluene (an antioxidant), and a protein such as bovine serum albumin to remove free fatty acids in the medium during storage. Under these conditions, the energization capacity of chloroplasts is retained for more than 40 days.

INTRODUCTION

Biomembranes are relatively unstable *in vitro*. Instability is particularly evident in functionally complex membranes such as the inner chloroplast membrane which possesses a photochemical system for electron transport and an energy-generating system for ion transport and ATP synthesis. It is known that chloroplasts are more stable in the dark[1,2] and that high rates of CO_2 fixation are manifested by illuminated chloroplasts only shortly after isolation[3]. In this investigation we study the factors which affect the stability of the chloroplast thylakoid membrane system devoid of the soluble components of the stroma which are largely removed during preparation (Class II chloroplasts). The capacity of these membranes to develop H^+ gradients by means of their photochemical electron transport system provides a means of assessing the efficiency of this membrane system with respect to energization.

The quenching of atebrin fluorescence in illuminated chloroplasts[4-8] is a sensitive and rapid measure of their capacity to develop H^+ gradients. We have analyzed atebrin fluorescence levels, light-induced quenching and dark recovery of fluorescence to assess the factors which are involved in the preservation and stabilization of chloroplast thylakoid membranes *in vitro*. We have evaluated the influence of various isolation and storage media, the presence of an antioxidant, and the removal of free fatty acids on the retention of energization capacity.

MATERIALS AND METHODS

Isolation and Storage

Class II chloroplasts were isolated from *Spinacia oleracea* L. leaves in several media as described elsewhere[9] and the final pellets were resuspended in various storage media and stored in the dark at 0-4°C (Fig. 1). Chlorophyll concentrations were determined in various stored chloroplast preparations according to the method of Kirk[10] and were found in all cases to be similar to those obtained immediately following isolation. To prevent inactivation by galactolipases and phospholipase D all procedures were carried out at pH 8.0[11,12].

The isolation and storage media were I) 175 mM NaCl plus 50 mM Tris-HCl, pH 8.0; II) 175 mM KCl plus 50 mM Tris-HCl, pH 8.0; and III) 200 mM sucrose, 20 mM NaCl, 3 mM $MgCl_2$ and 10 mM TES (*N*-tris(hydroxymethyl-2-aminoethane sulfonic acid)), pH 8.0.

In some cases additions of bovine serum albumin or butylated hydroxytoluene were made as indicated in the figures. Prior to use, bovine serum albumin was defatted by acetone extraction. Butylated hydroxytoluene was dissolved in ethanol and when diluted into the storage media the ethanol concentration was always less than 1%. At this concentration ethanol had no detectable effect on atebrin fluorescence parameters.

Assay of Light-induced Energization

Atebrin fluorescence changes were assayed as described previously[7,13] at 20°C. The reaction mixture was medium III to which pyocyanine, atebrin, and chloroplasts were added as indicated in the figure legends.

RESULTS

Upon addition of atebrin to a chloroplast suspension in the dark, there is a large increase in the relative fluorescence intensity (Fig. 2, insert). When chloroplasts are illuminated with red actinic light, fluorescence is quenched. The rate of quenching is exponential and reaches a steady-state level. When the activating light is turned off an exponential passive recovery of the original fluorescence level is observed. From such data the time constants with light on (τ_{on}) and off (τ_{off}), and the percentage of the light-induced atebrin fluorescence quenching relative to the dark stead-state level can be determined. Fig. 2 shows that the percentage of quenching and the values of τ_{off} are only dependent on chloroplast concentration below the equivalent of 20 μg chlorophyll/ml. Therefore, experiments were performed at chloroplast concentrations below the equivalent of 20 μg chlorophyll/ml, i.e., in the range of atebrin/chlorophyll ratios > 0.1 μmoles/mg.

Various combinations of the isolation and storage media employed were investigated in order to improve chloroplast stability during and after isolation (Fig. 3). Isolation followed by storage in medium I does not preserve

atebrin fluorescence quenching activity as well as isolation and storage in medium II (Fig. 3A). The addition of 250 μM butylated hydroxytoluene during storage in medium II improves retention of atebrin fluorescence quenching activity. Isolation in medium II followed by storage in medium III led to better preservation. In the presence of butylated hydroxytoluene, chloroplasts in medium III maintained high levels of atebrin fluorescence quenching activity for 20 days (Fig. 3B).

As shown in Fig. 3C, improvement in the preservation of activity was attained by the further addition of 0.1% bovine serum albumin to both the isolation medium II and storage medium III containing 500 μM butylated hydroxytoluene. With this procedure, significant levels of light-induced atebrin fluorescence quenching activity were maintained for 36 days. Isolation in medium II and storage in medium III supplemented with hydroxytoluene leads to retention of energization for 40 days. However, the combination of isolation in medium II plus bovine serum albumine and storage in medium III plus bovine serum albumin and butylated hydroxytoluene, allows chloroplast to maintain lower atebrin fluorescence quenching levels for 28 days after isolation. If butylated hydroxytoluene is absent during storage, energization capacity is lost after 19 days but the presence of bovine serum albumin still improves stability in the early phases of incubation, i.e. during the first 14-15 days.

The effect of butylated hydroxytoluene on atebrin fluorescence is shown in Fig. 4. During the first phases of storage in the presence of butylated hydroxytoluene the values of τ_{off} are progressively decreased by butylated hydroxytoluene (Fig. 4A). Fig. 4B demonstrates that the efficiency of chloroplasts in reaching maximum light-induced atebrin fluorescnece quenching levels, i.e. energization, decreases with storage, as detected by longer τ_{on} values with increasing in vitro storage. The initial percentage of atebrin fluorescence quenching (Fig. 4C) is also progressively decreased by butylated hydroxytoluene. Fig. 4C also shows that although the initial percentage of atebrin fluorescence quenching is lower after storage in the presence of 1 mM butylated hydroxytoluene, higher levels of this parameter are maintained after 38 days of storage.

In other experiments we have found that the loss of light-induced pH gradients closely parallels that of atebrin fluorescence, and pH gradients are present even after fluorescence can no longer be quenched upon illumination. We have also found that valinomycin has no effect on light-induced quenching but accelerates the rate of the passive recovery of fluorescence after the light is removed. These results indicate that changes in the potassium permeability do not account for the decline in atebrin fluorescence quenching activity.

Both results are consistent with previous studies[4-8] that quenching of atebrin fluorescence occurs as a result of the development of a pH gradient across the membrane causing the uptake of the amine into the membrane phase.

Photophosphorylation (assessed by the pH assay method) is also lost considerably earlier than atebrin fluorescence and pH gradients.

DISCUSSION

The findings of this investigation are consistent with previous studies from Kraayehof's[4-7] and Avron's[8] laboratories that light-dependent atebrin fluorescence quenching in chloroplasts is a fairly accurate measure of the development of a pH gradient that depends upon the ability of chloroplast membranes to couple light energy. Other activities as K^+ gradients, the development of membrane potentials, or photophosphorylation, are not sensed by the atebrin fluorescence assay. The main factors which appear to affect the stability of the chloroplast thylakoid membrane with respect to its capacity to be energized by light as judged by the atebrin fluorescence quenching assay are: a. isolation of the chloroplasts in the KCl-containing medium but subsequent storage in a sucrose medium containing low concentrations of electrolytes; b. the presence of an antioxidant and the removal of free fatty acids by defatted bovine serum albumin.

a) Influence of isolation and storage media

Nobel[14] and Dilley and Vernon[15] have demonstrated that K^+ is the major cation which is transported in illuminated chloroplasts. The presence of K^+ in the isolation but not in the storage medium may be an important factor in the maintenance of atebrin fluorescence quenching activity because this activity depends upon H^+ transport which requires the presence of a counter-ion to maintain charge neutrality[16]. Hence, the presence of high concentrations of internal K^+ favors retention of light-induced energization as assayed by the system which detects H^+ gradients. The beneficial effect of subsequent storage in sucrose (rather than a KCl-containing) medium may be due to prevention of denaturation of membrane proteins by prolonged contact with high salt concentration.

b) Antioxidant effects and removal of free fatty acids

Dark and photoinduced lipid peroxidation has been reported to destroy photochemical-mediated activities in Class II spinach chloroplasts[1,2]. Stabilization by butylated hydroxytoluene, a widely used antioxidant[17], is therefore not surprising. The action of butylated hydroxytoluene on atebrin fluorescence parameters may be explained by a dual action of this antioxidant. The molecule would readily partition in the hydrophobic membrane phase and at high concentrations would be expected to perturb membrane structure. It inhibits fluorescence quenching when

added to the storage medium in the concentration range of 250-1000 μM. Hence, its concentration in the chloroplast preparations must be critically poised in order to exert its beneficial effect at high concentration during storage, but it is reduced in concentration by at least 60-fold in the test medium when the stock chloroplast suspension is diluted.

Butylated hydroxytoluene lowers the percentage of atebrin fluorescence quenching and hastens the light off reaction. This action is similar to that of uncouplers like S-13[6]. Since the restoration of atebrin fluorescence intensity in the dark is due to its release from the membrane, the "uncoupling" action of butylated hydroxytoluene may be explained by promoting the loss of membrane-bound atebrin. Thus a combination of its antioxidant action and membrane perturbing effect would seem to afford a satisfactory explanation for its effect to lower the initial level of atebrin fluorescence but, on the other hand, to preserve the capacity of chloroplasts to retain light-induced atebrin fluorescence quenching activity during storage.

The mechanism whereby peroxidation of membrane lipids leads to structural alterations is complex[1,2,18-20]. One aspect of their action on membrane lipid is to cleave glycolipids and phospholipids to release free fatty acids. Previous studies have demonstrated that free fatty acids, particularly unsaturated fatty acids, exert marked

deleterious effects on structure-function parameters in chloroplasts[1,2,20-22]. The release of free fatty acids is mostly due to the action of galactolipases, enzymes normally latent in chloroplasts but which, during isolation and storage, can be activated. Thus, McCarty and Jagendorf[11] found large increases in linolenic acid in homogenates of bean chloroplasts ground at pH 6.0 which led to a loss of Hill reaction activity. Since galactolipases and phospholipase D are normally inactive at alkaline pH[11,12], we performed experiments at pH 8.0 where these enzymes are relatively inactive.

According to Heise and Jacobi[23] as much as 30% of phospho- and sulfolipids are released from spinach thylakoid membranes under alkaline conditions, but only if incubated in salt media. The beneficial effect on preservation of energization capacity of thylakoid membranes stored in sucrose in the presence of defatted bovine serum albumin is most likely explained by the removal of low levels of fatty acids released by the action of galacto- or phospholipases and dark peroxidation processes[24-27] combined with the suppression of lipid release by sucrose rather than salt medium.

Since energy dependent maintenance of ion gradients in other membrane systems, such as erythrocytes and mitochondria[7], can also be assessed by atebrin fluorescence quenching, it suggests that this probe may be generally useful for studying the factors that affect the stability of biomembranes.

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FOOTNOTES

* On leave from the Botanical Institute, Faculty of Science, Hiroshima University, Hiroshima, Japan.

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FIGURE LEGENDS

Fig. 1 - Diagram summarizing chloroplast isolation and storage procedures.

Fig. 2 - Dependence of atebrin fluorescence on chloroplast concentration. Chloroplasts were isolated in medium II and resuspended in medium III. The reaction mixture was medium III plus 2 μM pyocyanine, and 2 μM atebrin. The insert shows a typical example of the kinetics of light-induced atebrin fluorescence quenching and dark recovery. The solid arrow indicates the point of addition of atebrin.

Fig. 3 - Dependence of atebrin fluorescence quenching activity on the conditions of chloroplast isolation and storage. For composition of media see Materials and Methods.

A - Comparison between isolation and storage media I and II. Reaction mixtures as in Fig. 2 plus chloroplasts equivalent to 15 μg chlorophyll/ml.

B - Comparison between the isolation medium II and III. Reaction mixtures as in 3A.

C - Effects of the addition of bovine serum albumin and butylated hydroxytoluene on retention of atebrin fluorescence. Reaction mixtures as in Fig. 2 plus chloroplasts equivalent to 10 μg chlorophyll/ml.

FIGURE LEGENDS (Continued)

Fig. 4 - Effect of butylated hydroxytoluene on the kinetics and steady-state levels of atebrin fluorescence parameters.

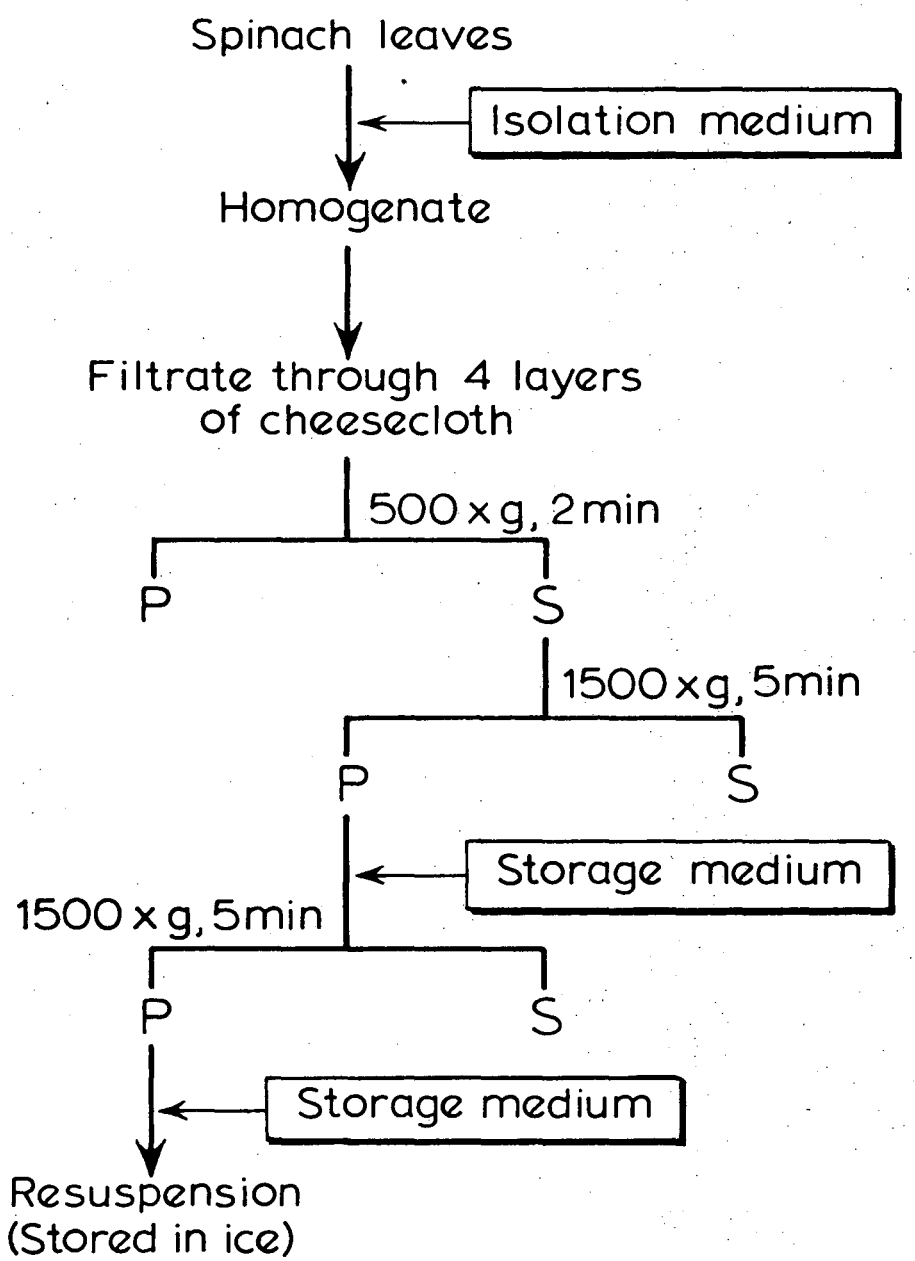
The determination of τ values is accurate only when the fluorescence quenching levels are >20%; thus τ values below these levels were omitted. Reaction mixtures as in Fig. 3A.

A - Time constant for recovery of fluorescence.

B - Time constant for fluorescence quenching.

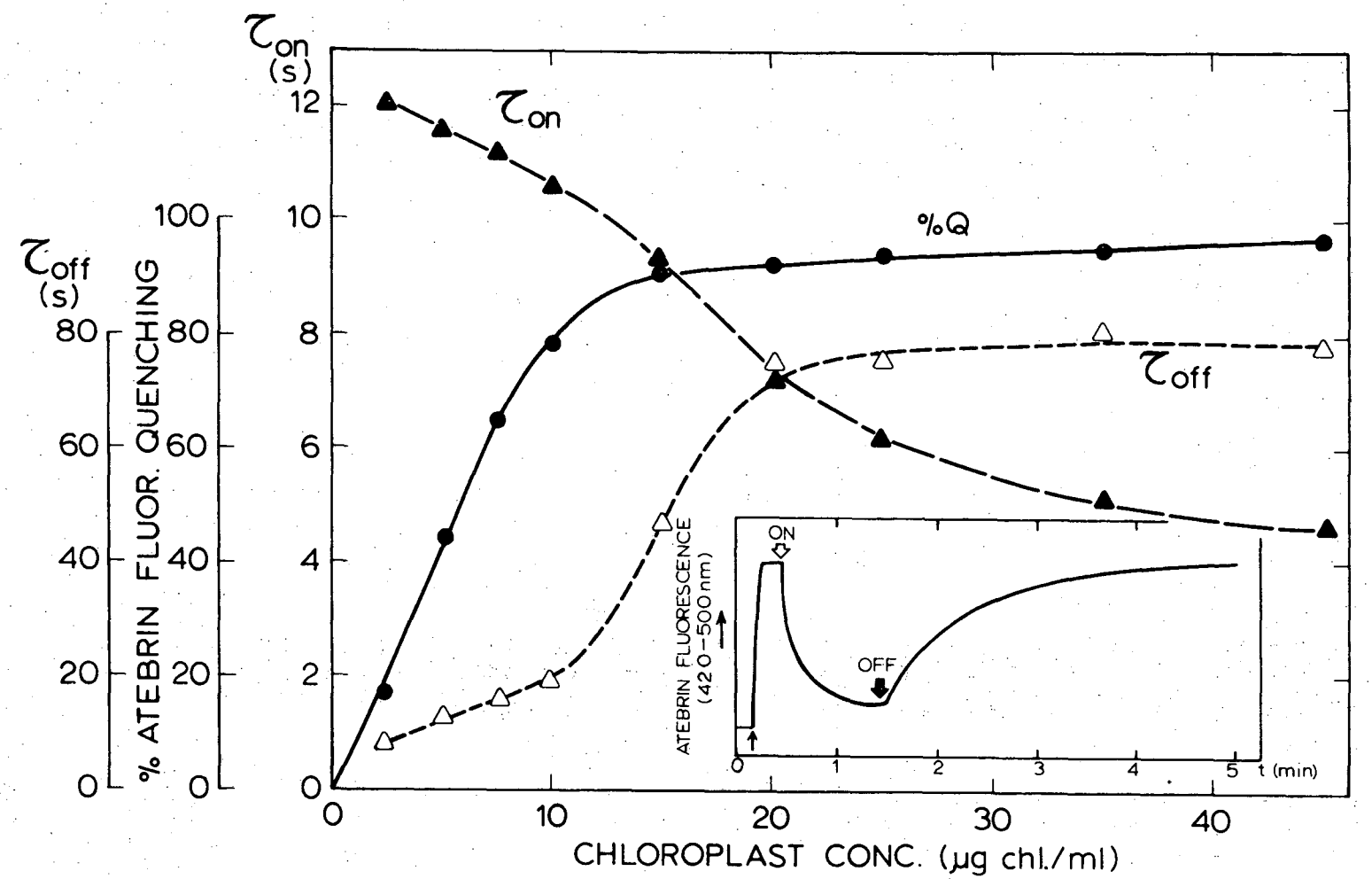
C - Relative fluorescence quenching.

ISOLATION PROCEDURE



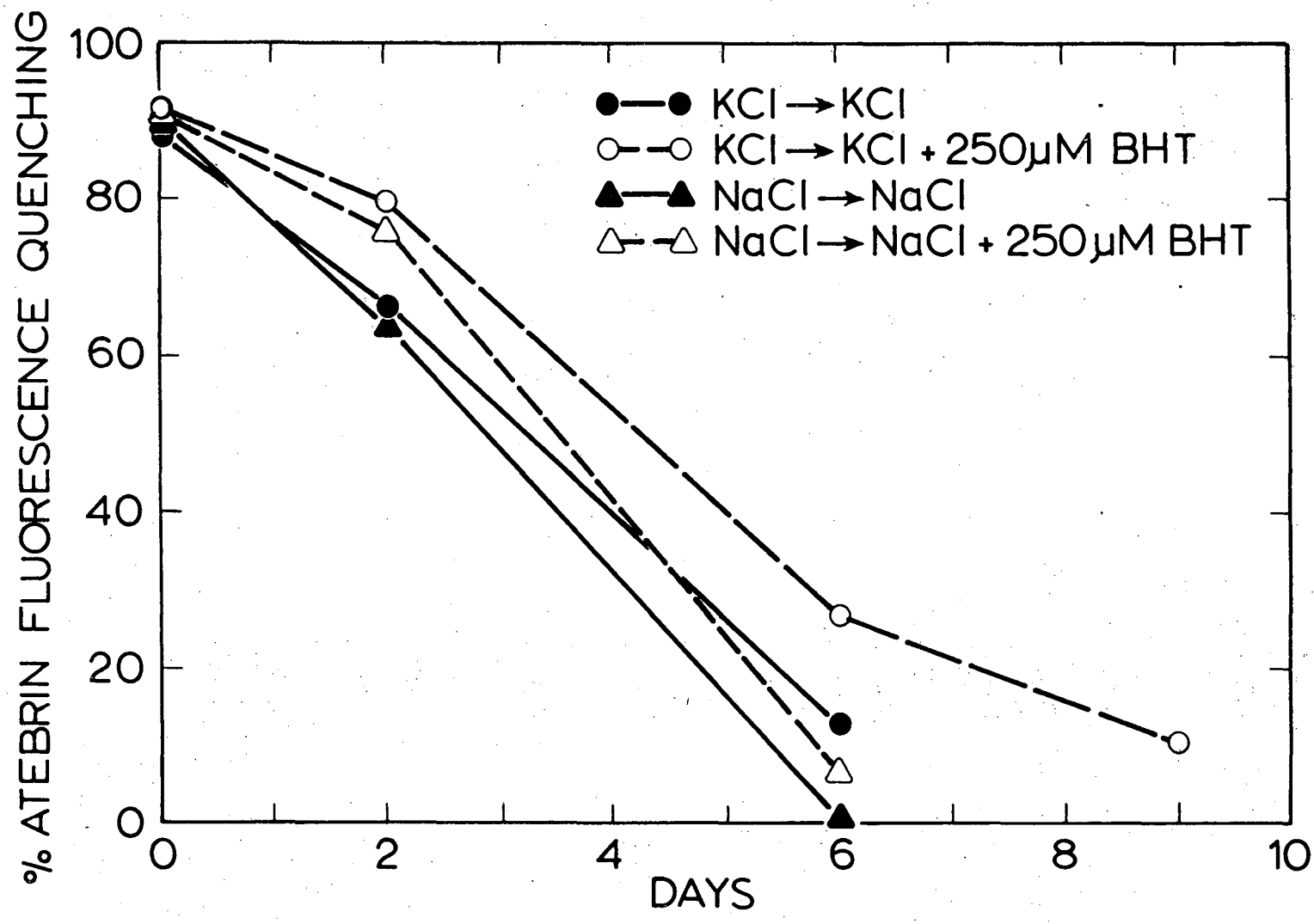
XBL 745-837

Fig. 1.



XBL 745-836

Fig. 2.



XBL 745-838

Fig. 3a.

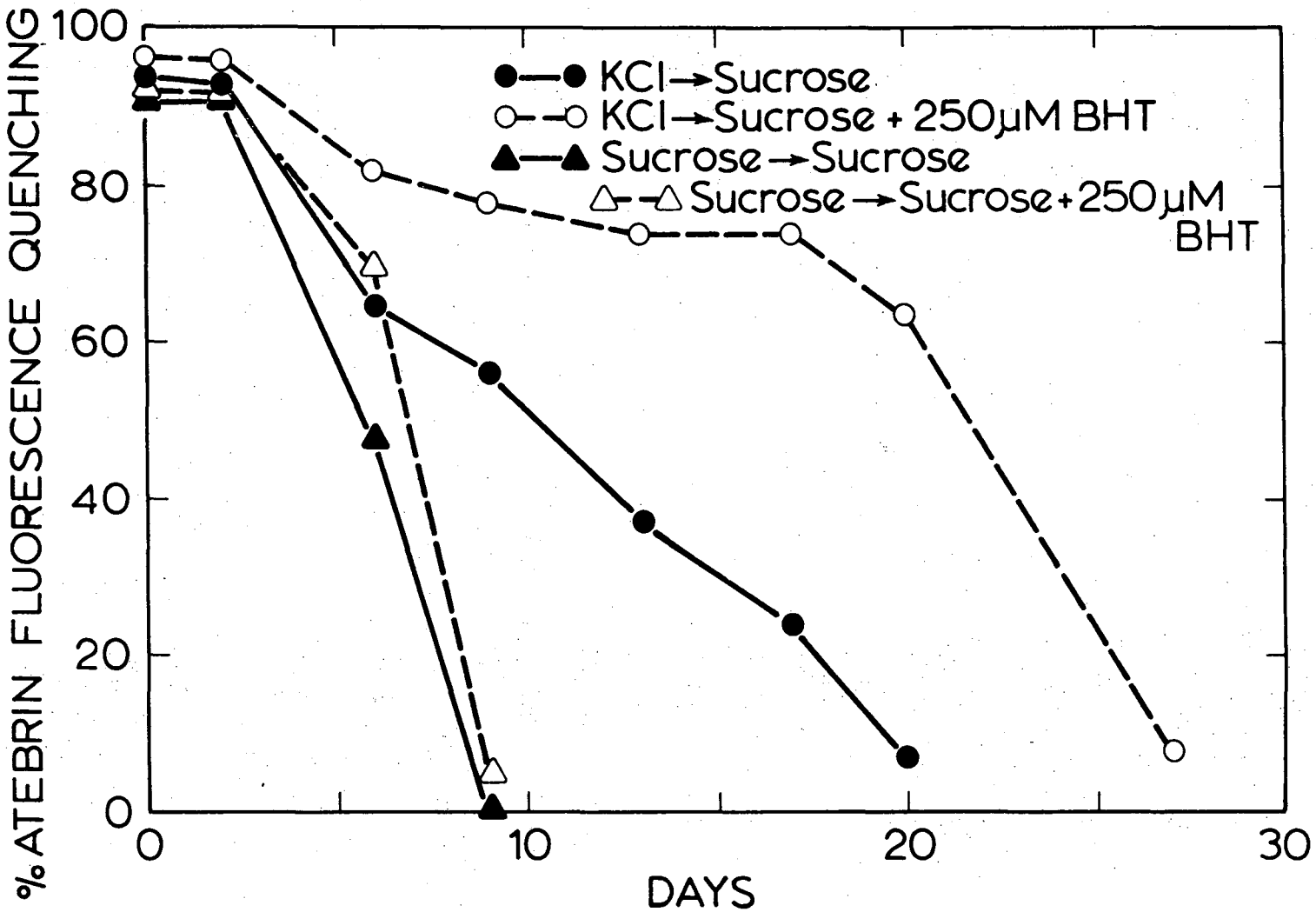


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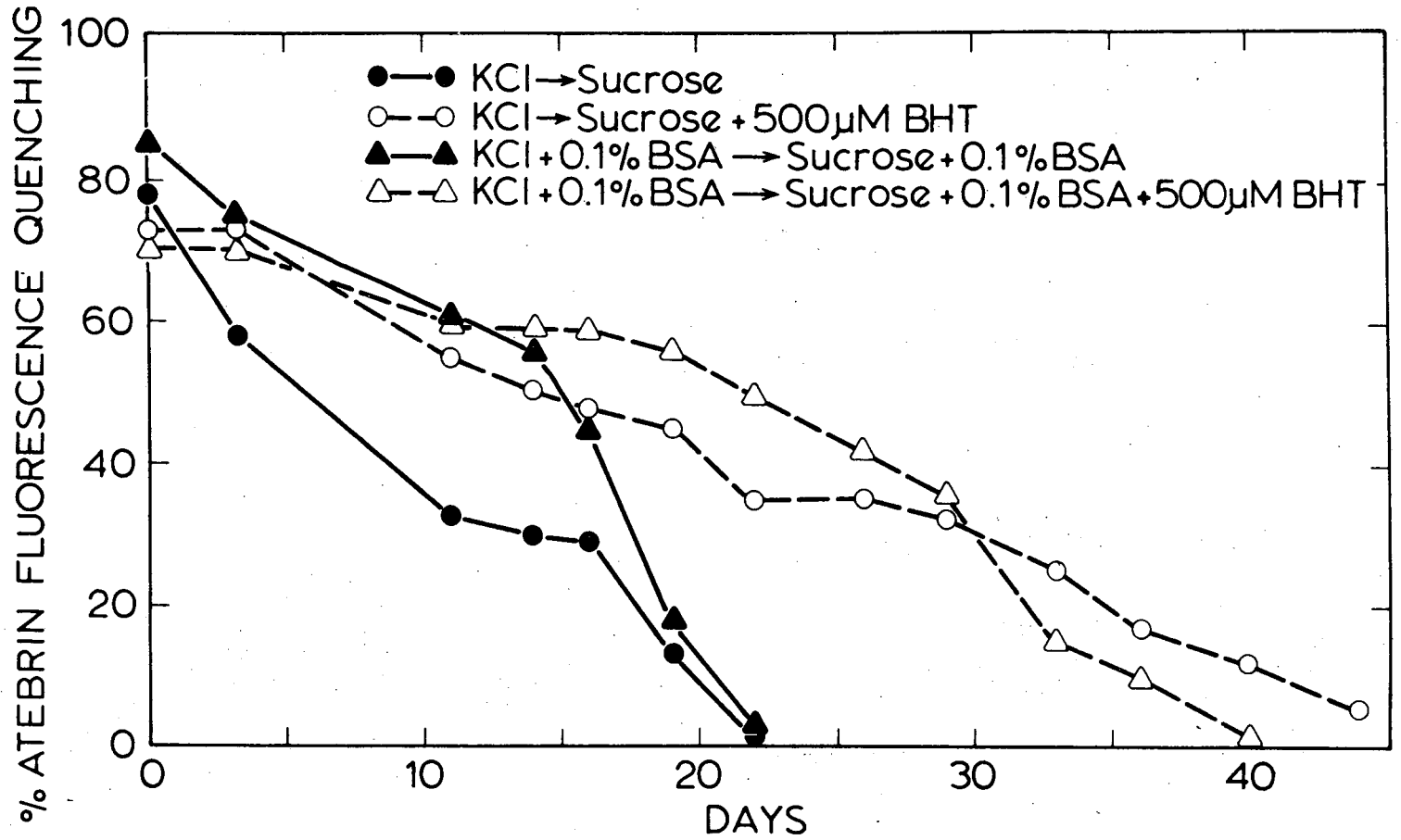
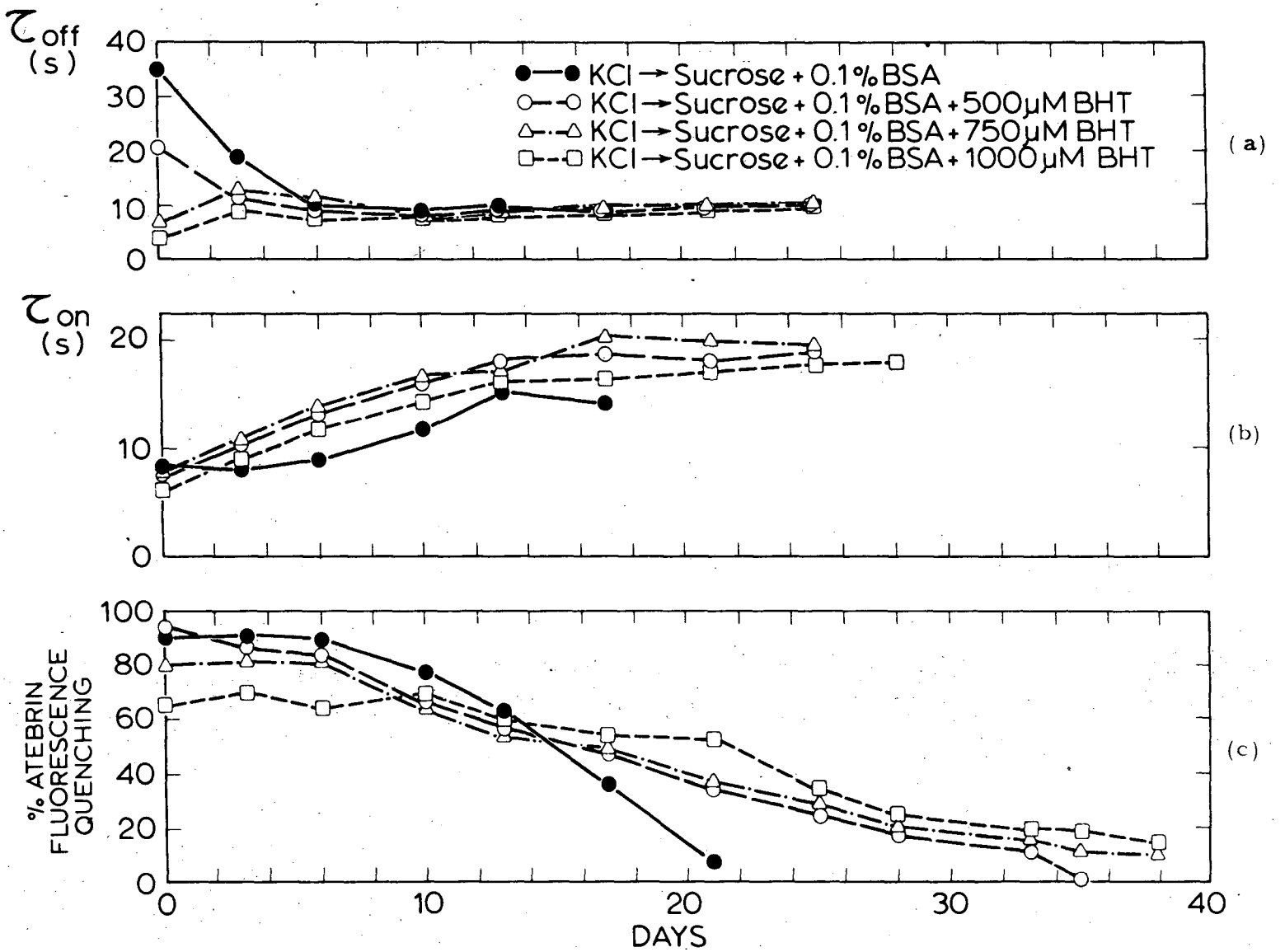
Fig. 3b.



XBL 745-840

Fig. 3c.



XBL 745-841

Fig. 4.

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