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Phospholipid-dependent interaction between dibromothymoquinone and iron-sulfur protein in mitochondrial ubiquinol-cytochrome *c* reductase

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(1) Dibromothymoquinone (DBMIB) inhibits antimycin A-sensitive ubiquinol-cytochrome *c* reductase activity; the maximal inhibition is 90%. (2) DBMIB alters the EPR spectra of reduced iron-sulfur protein in intact ubiquinol-cytochrome *c* reductase. The maximal spectral change occurs with 60 mol inhibitor per mol cytochrome c_1 in the reductase. (3) DBMIB causes little alteration in the EPR characteristics of iron-sulfur protein when ubiquinol-cytochrome *c* reductase is delipidated. (4) When delipidated ubiquinol-cytochrome *c* reductase is replenished with phospholipid, the effect of DBMIB reappears. However, when DBMIB is added to delipidated protein prior to replenishment with phospholipid, very little spectral alteration is observed. (5) DBMIB does not alter the EPR spectra of purified iron-sulfur protein, with or without phospholipid in the preparation. (6) Reduced DBMIB does not alter the EPR characteristics of iron-sulfur protein in intact or delipidated ubiquinol-cytochrome *c* reductase. (7) Cysteine and other thiol compounds can reverse the spectral alternation caused by DBMIB. This reversal probably results from the reduction of DBMIB.

Introduction

Dibromothymoquinone (DBMIB) (2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone) was first introduced as a specific inhibitor in the cytochrome b_6-f complex of chloroplasts by Trebst et al. [1]. This inhibitor was reported to block electron transfer from plastoquinol to plastocyanin and cause the alteration of EPR characteristics of the iron sulfur protein in spinach chloroplast [2,3]. Similar DBMIB effects were observed in the chromatophores and isolated cytochrome b-c_1 complexes of photosynthetic bacteria [4,5]. When DBMIB was used in the mitochondrial system, effects similar to those observed in the photosynthetic system were detected in the intact organelle and in the isolated Cyt b-c_1 complex [6,7]. However, these effects were less than those observed in the photosynthetic system.

The chemical structure similarity between DBMIB and ubiquinone or plastoquinone, and the ability of DBMIB to alter the EPR characteristics of iron-sulfur protein in chromatophores, chloroplasts, mitochondria, and isolated Cyt b-c_1 complexes, support the idea that there exists a specific interaction between quinone and iron

Abbreviations: DBMIB, dibromothymoquinone; Q_2, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone; Cyt, cytochrome.

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sulfur protein [8]. However, the inability of DBMIB to alter EPR characteristics of isolated Rieske's iron sulfur protein [3] and the lack of incorporation of arylazido-Q [9] or azido-Q [10] into the iron sulfur protein of mitochondrial Cyt b-c_1 complex after photolysis do not support a direct interaction between DBMIB and iron sulfur protein. Therefore, the binding site for DBMIB and the mode of binding remain unclear and need further investigation.

Recently we carefully examined the effect of this quinone-like inhibitor on highly purified mitochondrial ubiquinol-cytochrome c reductase under various conditions. Herein we report phospholipid-dependent inhibition of and alteration of EPR characteristics of iron-sulfur protein in isolated ubiquinol-cytochrome c reductase by DBMIB.

TABLE I

INHIBITION OF UBIQUINOL-CYTOCHROME c REDUCTASE ACTIVITY BY DBMIB

Concentration used (DBMIB/Cyt c_1)	Activity remained, %		
	Reported in Ref. 7		This work
	mito	SCR	
0	100 [a]	100 [b]	100 [c]
7	48	40	80
72	9	24	50
330	0	0	22

[a] Submitochondrial particles preparation with a turnover number of 70 s^{-1} using Q_3H_2 as a substrate.

[b] Crude succinate-Cyt c reductase with a turnover number of 225 s^{-1} using Q_2H_2 as a substrate and 20 μM Cyt c as an acceptor. Data extracted from Fig. 2 of Ref. 7.

[c] Ubiquinol-cytochrome c reductase with a turnover number of 477 s^{-1} using Q_2H_2 as a substrate, 100 μM Cyt c as an acceptor.

Materials and Methods

Materials. Asolectin was obtained from Associate Concentrate, Inc. Sodium cholate and horse Cyt c, type III, were from Sigma. DBMIB was from Aldrich. 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone (Q_2) and Q_2H_2 were synthesized in this laboratory as previously reported [11]. Other chemicals were of the highest purity commercially available.

Enzyme preparations. Purified bovine-heart mitochondrial ubiquinol-cytochrome c reductase [12] and its ubiquinone (Q)- and phospholipid-depleted form [13] were prepared as reported. Rieske's iron sulfur protein was prepared from purified ubiquinol-cytochrome c reductase according to Shimomura et al. [14].

Ubiquinol-cytochrome c reductase activity was assayed in a Cary spectrophotometer, model 219, at room temperature, using Q_2H_2 [12] as substrate. EPR measurements were performed in a Bruker ER200D spectrometer at 77 K. The detailed instrument settings are given in the legend of figures.

Results and Discussion

Inhibition of ubiquinol-cytochrome c reductase activity by DBMIB

Table I shows the effect of DBMIB concentrations on the activity of highly purified ubiquinol-cytochrome c reductase. A 50% inhibition required 72 mol DBMIB per mol protein, a value much higher than that reported [6,7] for submitochondrial particles and crude succinate-cytochrome c reductase. The reason for this discrepancy is unknown. One possible explanation is the different state of purity of the enzyme system used. Ubiquinol-cytochrome c reductase activity is much lower in the crude enzyme system. The lower acceptor concentration used in the activity assay also enhances the inhibition potency of DBMIB. DBMIB is very soluble in aqueous solutions. Therefore, the DBMIB which is bound to ubiquinol-cytochrome c reductase under the incubation conditions, may partially dissociate from the protein complex under activity-assay conditions. Since the amount of enzyme used in the activity assay is less for the enzyme preparation having higher specific molecular activity, compared to the preparation having lower specific molecular activity, the absolute concentration of DBMIB in the assay system is lower in the more active preparations than in the less active. In other words, the inhibitory effect of DBMIB may not depend solely on the inhibitor-to-protein ratio, but also on the absolute concentration of the inhibitor in the assay mixture.

The fact that the inhibitory effect of DBMIB is greater when Q_1H_2 is used as a substrate than

when Q_2H_2 is used [5] indicates that the binding between enzyme and DBMIB is quite weak. Q_2H_2 is better than Q_1H_2 in competing for the binding site with DBMIB, and thus a higher DBMIB concentration is needed to produce the same inhibition. In experiments here reported we used Q_2H_2 as substrate, because it is a better substrate for ubiquinol-cytochrome *c* reductase than any of the other Q analogues tested.

EPR spectra of iron-sulfur protein in intact and phospholipid-depleted ubiquinol-cytochrome c reductases

The EPR spectra of the iron sulfur protein in ubiquinol-cytochrome *c* reductase has been reported by several investigators [15–19]. While there is little dispute on the g_y (1.89) and g_z (2.03) of iron sulfur protein, the g_x (1.78) of iron-sulfur protein has been a subject of discussion. The shape of the g_x signal and the g value varies slightly with the preparation and measuring conditions used. Some investigators observe two g_x signals of iron-sulfur protein, interpreted as due to the existence of two different types of iron-sulfur proteins in the reductase [15,19], or the presence of one type of iron-sulfur protein in different environments [18].

Fig. 1 shows the EPR spectra of iron-sulfur protein in intact and in phospholipid- and Q-depleted ubiquinol-cytochrome *c* reductases. The intact ubiquinol-cytochrome *c* reductase contains approx. 40 mol phospholipids per mol of the enzyme complex. The phospholipid and Q in the complex can be mostly removed by repeated ammonium sulfate precipitation in the presence of cholate without losing any essential redox components. The phospholipid- and Q-depleted reductase obtained is reconstitutively active. The preparation contains little Q (less than 0.1 mol per mol Cyt c_1) and phospholipids (less than 2 mol phospholipids per mol of complex, based on Cyt c_1), and shows practically no ubiquinol-cytochrome *c* reductase activity (less than 5%). Addition of Q prior to the addition of phospholipid restores more than 90% of the original activity to the depleted preparation. As shown in Fig. 1, there are no apparent differences in the g_y and g_z signals of iron-sulfur protein in intact and delipidated preparations. The concentrations of iron-sulfur cluster are the same in both preparations, 0.6 mol per mol Cyt c_1. The g_x signal of iron-sulfur protein, however, shifts slightly toward the higher field ($g = 1.76$) in the phospholipid- and Q-depleted ubiquinol-cytochrome *c* reductase. The addition of phospholipid (regardless of the presence or absence of Q) to the Q- and phospholipid-depleted reductase restores the g_x signal of iron sulfur protein to 1.78, indicating that the change in the g_x of iron sulfur protein is due to phospholipid and not to Q. This differs from the report [19] that the g_x signal of iron-sulfur protein in submitochondrial particles broadened and shifted to a higher field when Q was extracted from the particles. It should be mentioned that the g_x signal of iron-sulfur cluster of the intact ubiquinol-cytochrome *c* reductase differs slightly from the reported g_x of the iron-sulfur cluster in the submitochondrial particles. In the submitochondrial particles preparation a sharper g_x signal was observed when the preparation was reduced by ascorbate, and the signal broadened and shifted to the higher field when the preparation was reduced with the lower potential reductants [20]. The sharper g_x signal was not very apparent in the present study. The discrepancy between these two observations can be attributed at least partly to the different preparations used.

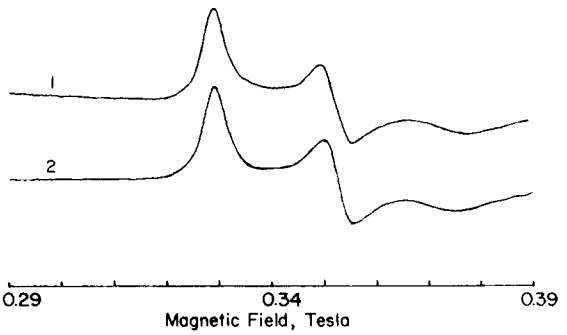

Fig. 1. EPR spectra of iron-sulfur protein in ubiquinol-cytochrome *c* reductases. The intact (2) and phospholipid- and Q-depleted (1) ubiquinol-cytochrome *c* reductases, 17 mg/ml, in 50 mM Tris-HCl buffer (pH 7.8) were reduced by sodium ascorbate. The EPR spectra were recorded in a Bruker ER-200D spectrometer at 77K. Instrument settings were: field modulation frequency, 100 kHz; modulation amplitude, 5 gauss; microwave frequency, 9.35 GHz; microwave power, 5 mW; time constant, 0.5 s; scan range, 1000 gauss; scan rate, 200 gauss/min.

The highly purified and active ubiquinol-cytochrome *c* reductase preparation used in the current study contains only 0.6 mol iron-sulfur cluster (2Fe–2S) per mole Cyt c_1, whereas in the submitochondrial particles, 2 mol iron-sulfur clusters per mol Cyt c_1 was reported [21]. It is possible that the iron-sulfur cluster with a sharper g_x signal is absent in the purified ubiquinol-cytochrome *c* reductase.

Effect of DBMIB on EPR characteristics of iron-sulfur protein in intact, delipidated and reconstituted ubiquinol-cytochrome c reductase preparations

When intact ubiquinol-cytochrome *c* reductase is treated with a 60-fold molar excess of DBMIB (based on Cyt c_1), a drastic change in the EPR characteristics of iron-sulfur protein is observed (see spectra 1 of Fig. 2). The g_y shifts from 1.89 to 1.94, and a small free radical-like signal appears at $g = 2.0$. Fig. 3 shows a titration curve of the signal intensity at $g = 1.94$ vs. DBMIB concentrations. Maximum signal alteration is observed when a 60-fold molar excess of DBMIB to reductase (based on Cyt c_1) is used.

The drastic effect of DBMIB on the EPR characteristics of iron-sulfur protein in intact ubiquinol-cytochrome *c* reductase is not observed when Q- and phospholipid-depleted reductase are used (see spectra 2 of Fig. 2), indicating that the DBMIB effect requires lipid in the preparation. The small signal at $g = 1.94$ observed in the delipidated reductase resulted from residual phospholipid in the preparation. Fig. 4 shows the correlation between the DBMIB effect on EPR characteristics and the phospholipid content of ubiquinol-cytochrome *c* reductase. When DBMIB, at a 60-fold molar excess, is added to ubiquinol-cytochrome *c* reductases containing different amounts of phospholipids, but having the same amount of iron-sulfur cluster, the signal intensity at $g = 1.94$ decreases as the phospholipid content in the reductase decreases. The effect is not due to the decrease of the EPR detectable iron sulfur cluster because the signal intensities of the samples are the same before the DBMIB treatment, and the amount non-heme iron present in the samples have been determined chemistrically to be identical.

Although the cholate-ammonium sulfate treatment removes both phospholipid and ubiquinone from ubiquinol-cytochrome *c* reductase, the lack of a DBMIB effect on iron-sulfur protein in the delipidated reductase is not due to the absence of ubiquinone, because replenishment with Q_2 alone

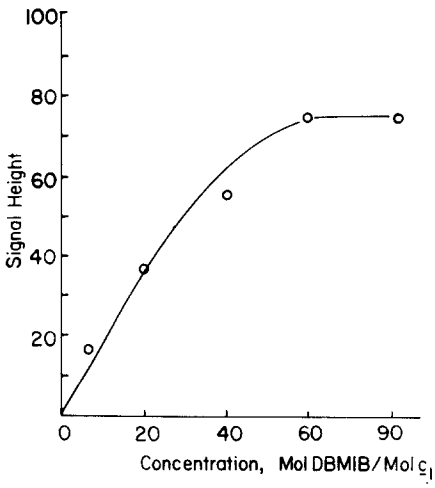

Fig. 3. Effect of DBMIB concentrations on the EPR spectra of iron-sulfur protein in ubiquinol-cytochrome *c* reductase. Intact ubiquinol-cytochrome *c* reductase, 15 mg/ml, in Tris-HCl buffer 9pH 7.8), were incubated with varying amounts of DBMIB for 10 min at 0°C before EPR spectra were taken under conditions given in Fig. 1.

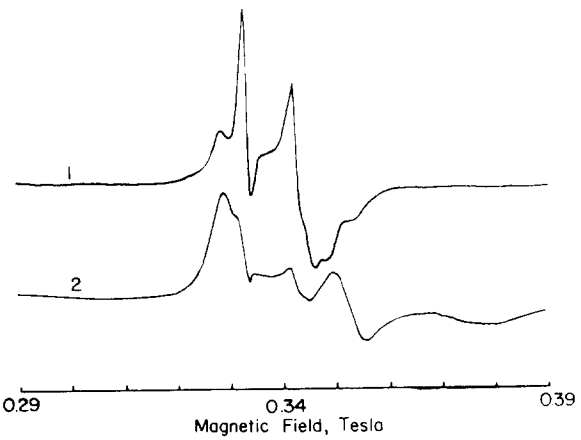

Fig. 2. Effect of DBMIB on the EPR spectra of iron-sulfur protein in intact, and in phospholipid- and Q-depleted reductases. The intact (1), and the phospholipid- and Q-depleted (2) ubiquinol-cytochrome *c* reductases were incubated with a 60-fold molar excess of DBMIB to Cyt c_1 at 0°C for 10 min before the EPR spectra were taken under conditions described for Fig. 1.

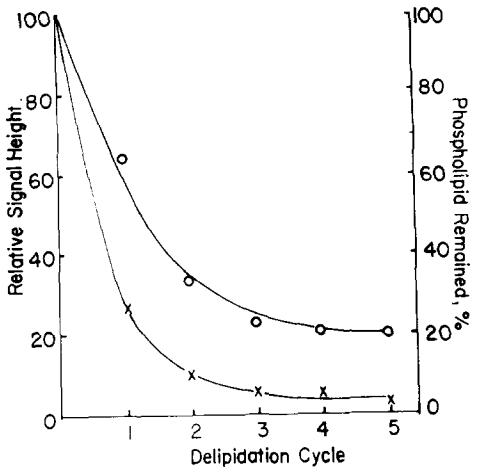

Fig. 4. Correlation between the effect of DBMIB on iron-sulfur protein and phospholipid content in ubiquinol-cytochrome *c* reductase. The ubiquinol-cytochrome *c* reductase was dissolved in 50 mM phosphate buffer (pH 7.8) containing 0.5% sodium cholate to a protein concentration of approx. 10 mg/ml. Neutralized, saturated ammonium sulfate solution was added slowly and the delipidated protein was recovered by centrifugation. The cholate-ammonium sulfate treatment was repeated five times. Precipitates obtained at each cycle of treatment were dissolved in 50 mM Tris-HCl buffer (pH 7.8) and incubated with DBMIB at a 60-fold molar excess to Cyt c_1 for 10 min before the EPR spectra were taken under conditions described in Fig. 1. Curves with circles (O——O) and crosses (×——×) represent relative signal height at $g = 1.94$ and amount of phospholipid remaining in ubiquinol-cytochrome *c* reductase, respectively.

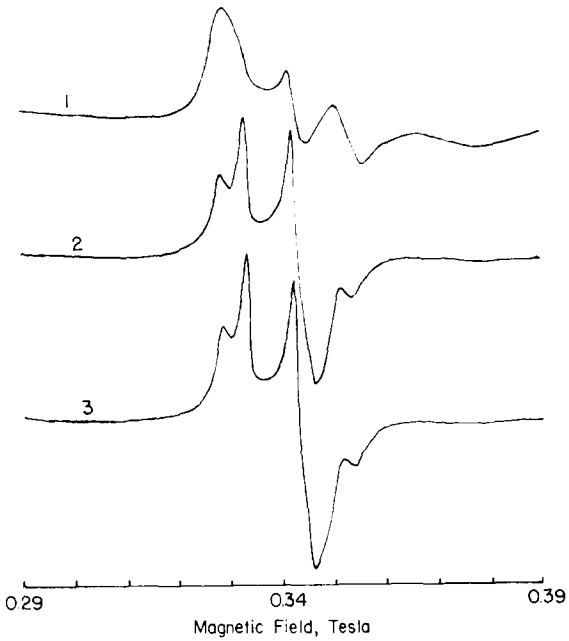

Fig. 5. The effect of DBMIB on the EPR spectra of iron-sulfur protein in the delipidated ubiquinol-cytochrome *c* reductase after reconstituting with Q, with phospholipid, and with Q- and phospholipid. Aliquots of delipidated reductase, 5 mg/ml in 20 mM Tris-HCl buffer (pH 7.8) were added (1) Q_2 at a 10-fold molar excess to Cyt *b* in the preparation; (2) asolectin micellar solution at a 1:1 weight ratio to protein; (3) Q_2 and asolectin micellar solution. Concentrations of Q_2 and aoslectin in (3) were the same as those in (1) and (2). After incubation for 30 min at 0°C DBMIB was added at a 60-fold molar ratio to Cyt c_1 in the preparation. The DBMIB-treated preparations were then concentrated with centricon-30 to a protein concentration of approx. 20 mg per ml and the EPR spectra were taken as described in Fig. 1.

does not restore this effect. Replenishing phospholipid in the Q- and phospholipid depleted reductase prior to addition of DBMIB, restores the DBMIB effect (spectra 2 of Fig. 5). Surprisingly, when DBMIB is added to the delipidated reductase prior to the replenishment of phospholipid, little DBMIB effect is observed (spectra 1 of Fig. 5). Addition of DBMIB to the phospholipid- and Q-reconstituted reductase (spectra 3 of Fig. 5) shows effects similar to that observed with preparations reconstituted only with phospholipid. These results indicate that the binding site of DBMIB in ubiquinol-cytochrome *c* reductase requires phospholipid; in the absence of phospholipid, DBMIB may be bound to other parts of the reductase molecule, and such binding is not easily reversed by the addition of phospholipid.

It should be noted that although the effect of DBMIB on the EPR signal of iron-sulfur protein in the phospholipid- and Q-reconstituted ubiquinol-cytochrome *c* reductase is identical to that observed with intact reductase, no semiquinone-like signal at $g = 2.00$ is observed. The reason for this is unknown.

Although it is clear, so far, that the effect of DBMIB on the EPR characteristics of iron-sulfur protein in ubiquinol-cytochrome *c* reductase requires phospholipid, it is not known whether the observed effect results from the direct binding of DBMIB to iron sulfur protein in the presence of phospholipid or whether other components in the Cyt b-c_1 complex are needed. To test this, isolated, reconstitutively active iron-sulfur protein was used. Iron-sulfur protein obtained from ubiquinol-cytochrome *c* reductase by the method of Shimomura et al. [14] has EPR characteristics

($g_z = 2.03$, $g_y = 1.89$ and $g_x = 1.78$) identical to those of iron-sulfur protein in ubiquinol-cytochrome *c* reductase. No EPR spectra alterations are observed upon treatment with DBMIB. Moreover, the addition of phospholipid to isolated iron-sulfur protein prior to the addition of DBMIB does not cause modification of the EPR signals. These results strongly suggest that the effect of DBMIB on the EPR characteristics of iron-sulfur protein in ubiquinol-cytochrome *c* reductase might be secondary and that DBMIB might be bound to component(s) (possibly Cyt *b*) other than iron sulfur protein in a phospholipid-sufficient environment. This protein-DBMIB adduct then indirectly affects the EPR spectra of iron-sulfur protein.

Effect of redox state of DBMIB on the EPR characteristics of iron-sulfur protein in ubiquinol-cytochrome c reductase

During the course of this investigation it was observed that the effect of DBMIB on the EPR characteristics of iron-sulfur protein in ubiquinol-cytochrome *c* reductase depends on the extent that ubiquinol-cytochrome *c* reductase is reduced. Maximum EPR signal alteration is observed when the Cyt c_1 in ubiquinol-cytochrome *c* reductase is completely reduced with a limited amount of ascorbate. Under this condition, all the iron-sulfur protein in the reductase is reduced. Reduction of ubiquinol-cytochrome *c* reductase with sodium dithionite decreases the effect of DBMIB on iron-sulfur protein. However, when ubiquinol-cytochrome *c* reductase is reduced with excess dithionite, the effect of DBMIB on the EPR spectra of iron-sulfur protein is completely lost. The loss of DBMIB effect may result from the reduction of a certain component in the enzyme complex that interacts with the inhibitor or, alternatively, results from the reduction of the inhibitor itself.

When DBMIB is chemically reduced by sodium dithionite or sodium borohydride prior to addition to ascorbate reduced ubiquinol-cytochrome *c* reductase, no alteration in the EPR spectra of iron-sulfur protein is observed. Similarly, when DBMIB is treated with cysteine or other thiol compounds prior to the addition to the ascorbate reduced ubiquinol-cytochrome *c* reductase, no effect on the EPR spectra is observed. These results suggest that fully reduced DBMIB is not the active inhibitor. It has been reported [6] that DBMIB's effects on the EPR characteristics of iron-sulfur protein can be reversed by the addition of excess cysteine. This phenomenon was explained as being the result of modification of ubiquinol-cytochrome *c* reductase by cysteine, not of a change in the redox state of the inhibitor.

Since results of the DBMIB inhibition study [7] ruled out the possibility that the oxidized form of DBMIB is the active inhibitor, perhaps the active form is its semiquinone. Since the redox potential of DBMIB (180 mV) is higher than that of ubiquinone, it is possible that the semiquinone of DBMIB is formed under conditions in which both iron-sulfur protein and Cyt c_1 in the reductase are reduced by ascorbate. The E_m for the two halves of the redox reaction of DBMIB (QH_2/Q^- and Q^-/Q) were estimated to be 290 and 70 mV, respectively [22]. Thus formation of the DBMIB semiquinone radical is likely under conditions when both Cyt c_1 and iron sulfur protein are reduced. The E_m for Cyt c_1 is 223 mV. It has been shown that the formation of the ubisemiquinone radical [23,24] in ubiquinol-cytochrome *c* reductase requires phospholipid. If we assume that binding of DBMIB to the reductase is similar to that of Q, then the requirement of phospholipid for formation of the semiquinone of DBMIB is logical. Since DBMIB does not react with iron-sulfur protein directly in the presence of phospholipid, the requirement of phospholipid may be for the component that binds and stabilizes the DBMIB semiquinone radical.

Acknowledgements

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