Inhibitory Effects of Synthetic Lanthanum-Crown Ether at the Reducing Side of Photosystem II

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Inhibitory effects of lanthanum-crown [La-$(Pic)_3$ (15-crown-6) $3H_2O$].was investigated on the O_2 evolution activity of photsystem II particles. Lanthanum (La)-crown inhibited the electron flow at the reducing side of PS II complex. Short duration (1–2 min) treatment of PS II membranes with trypsin partly developed resistance to La-crown inhibition. However, longer proteolytic treatment (>2 min) appeared to expose newer site(s) for La-crown inhibition. The inhibitory constant (K_i) for La-crown was nearly 0.17 μM. This inhibitory capacity is about 4 to 5 times less than the potent PS II inhibitor diuron which also binds at the acceptor side of PS II. The number of binding sites for La-crown was found to be 1 per 20 chlorophyll molecules. The Hill plot analysis showed the presence of three distinct straight lines suggesting that the compound acts at least at three sites. Furthermore, from the slope value (Hill coefficient) it is suggested that two of these sites provide minimum of two binding domains for the inhibitor.

Introduction

Crown ethers, a group of cyclic polyethers are known to have metal complexing properties (Frensdorff, 1971). They are highly potent in discriminating between and within the group of alkali and alkaline earth cations and exhibit preferential binding with associated anionic species (Poonia, 1974; Khan *et al.,* 1987). The crown ethers have been used to study the selective transport of cations across synthetic model membranes (Vijayavergiya and Mookerjee, 1989).

The structural, functional and the regulatory roles of cations like magnesium, calcium and manganese or anions such as chloride and bicarbonate in photosynthetic electron transport and O_2 evolution activity of chloroplast membranes have been established (Coleman and Govindjee, 1987; Govindjee, 1991; Debus, 1992). In view of crown ether interaction with anions/cations, an attempt was made earlier to characterize the effect of K-crown (K-picrate 18-crown-6) on the electron transport activity of thylakoids from spinach, wherein it was shown that K-crown could reversibly inhibit the PS II catalyzed electron flow (Sabat *et al.,* 1991). A recent publication (Kovacs *et al.,* 1996) on the inhibitory activity of K-crown indicated that depending on concentration, the complex selectively inhibits the PS II catalyzed electron flow either at donor (Tyr_D and Tyr_Z) or acceptor (between Q_A and Q_B) side of PS II complex.

In this report, we have studied the effect of lanthanum (La)-crown ether [tris 2,4,6-trinitrophenolato triquo benzo-15-crown-6 Lanthanum (III), $C_{32}H_{32}O_{29}N_9La$] on the electron transport activity of PS II O_2 evolving particles, prepared from spinach chloroplasts. The investigation showed that La-crown ether can disrupt the photosystem (PS) II catalyzed electron flow by interacting at the acceptor side of the complex and the inhibition is partially reversible.

*Abbreviations:*Chl, chlorophyll; D_1, the herbicide-binding 32 kDa protein of the photosystem II reaction center core; DCBQ, 2,5-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMSO, dimethylsulfoxide; DNOC, 4,6-dinitro-*o*-cresol; MES, 4-morpholinoethanesulfonic acid; PpBQ, phenyl-*p*-benzoquinone; PS, photosystem

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Materials and Methods

Preparation of PS II O_2 evolving particle

Photosystem II O_2 evolving particles were prepared from spinach chloroplasts following Van Leeuwen *et al.* (1991). In brief, the isolated chloroplasts (5 mg Chl ml^{-1}) were incubated with Triton X-100 [Chl:Triton ratio 1:25 (w/w)] for 25 min at room temperature (25 °C). The membranes were then centrifuged at 40,000×*g* for 30 min. The pellet was suspended in a buffer containing 400 mM sucrose, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM NaCl and 20 mM MES-NaOH (pH 6.5) and centrifuged at 6000×*g* for 5 min to remove the undigested thylakoids. Photosystem II particles from the supernatant were pelleted again at 40,000×*g* for 30 min. The particles were frozen in liquid nitrogen (77 K) and kept at −80 °C until use. The preservation was done in presence of 5 percent DMSO as cryoprotectant. The relative ratios of PS II to PS I catalyzed electron transport activity were 0.40 in thylakoids and 6.32 in PS II particles (data not shown). The O_2 evolving PS II particles prepared following the above procedure had Chl *a/b* ratio 2.5 as against 4.7 in thylakoids. Chlorophyll was estimated following Porra *et al.* (1989). The PS I fluorescence emission peak at 735 nm (F_{735}) was missing in the particles (data not shown).

Trypsin treatment

For trypsin incubation, the particles were suspended in the suspension medium (see above) to a concentration of 100 μg Chl ml^{-1}. Trypsin (from Bovine Pancreas, Type I, 12,000 units mg $protein^{-1}$, Sigma Chemical Co. USA) was added to a final concentration of 20 μg/100 μg Chl. The reaction was stopped at various time intervals (see Results and Discussion) by addition of a 20 fold excess of trypsin inhibitor (from Serva Chemicals, Feinbiochemica, Germany). The suspension was centrifuged at 40,000×*g* for 30 min to pellet the PS II particles. The particles were resuspended in the suspension medium.

Electron transport measurement

The electron transport activity was measured in terms of O_2 evolution in a polarographic assembly at 25 °C. The incident light intensity was 1500 μE m^{-2} s^{-1}. To check the reversibility of inhibition of electron flow, PS II particles were washed with buffer after appropriate incubation with the inhibitor (Sabat *et al.*, 1991). Electron transport activity of PS II particle was also measured in presence of La^{3+} and 15-crown-6 (the constituents of the complex). Other details of the measurements are given in respective figure and table legends.

Chemicals

For details of preparation of La-picrate-crown [La $(Pic)_3$ (Crown) $3H_2O$] see references Nakagawa *et al.* (1988). In brief, $LaCl_3$ was used for preparation of La-picrate (by replacing Na^+ from Na-picrate). The La-picrate was then reacted with crown ether (15-crown-6) to obtain [La-crown] picrate (refereed here after as La-crown). The complex was dissolved in distilled water and appropriately diluted. DCMU (Sigma Chemical Co., St. Louis, USA) was re-crystallized from benzene at −20 °C.

Results and Discussion

The DCBQ and PpBQ supported O_2 evolution rate in PS II particles was about 350–360 μmol O_2 evolved $mgChl^{-1}$ h^{-1} (Figs. 1 a and b, solid lines). Compared to PpBQ, some 20–25 percent of DCBQ dependent O_2 evolution activity remained insensitive to inhibitory action of DCMU (Fig. 1 a, dotted lines); obviously because DCBQ in addition to Q_B site of electron acceptance (the secondary quinone electron acceptor), can also intercept electrons prior to the DCMU inhibition site (i.e, largely from the stable primary quinone electron acceptor, Q_A). Therefore, the electron transport assay from H_2O to DCBQ in presence of DCMU can be used to locate the effect(s) of crown ethers (if any) on the span of electron flow from H_2O to Q_A. The DCBQ supported electron transport activity was promptly inhibited upon addition of La-crown ether to an aliquot of PS II membranes (Fig. 2, solid lines). The decay in activity was exponential and tended to reach a steady state with a substantial low concentration (~9–10 μM) of La-crown. As expected, La-crown inhibited PpBQ supported electron flow completely (Fig. 2, dotted line).

The inhibition does not depend on the time of incubation of the inhibitor with PS II particle (Table I). The inhibition was however partially re-

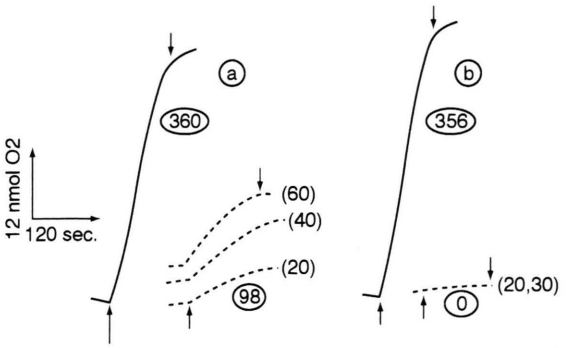

Fig. 1. Polarographic traces showing PS II catalyzed O_2 evolution activity in PS II particles in presence (– – –) and absence (——) of 10 μM DCMU. The electron acceptor used was 400 μM DCBQ (a) or PpBQ (b) with combination of 1.25 mM potassium ferricyanide. The numbers in circle represent the rate of electron transport (μmol O_2 evolved $mgChl^{-1}$ h^{-1}).The bracketed numbers represent the μg Chl used in the assay of electron transport in presence of DCMU. The reaction mixture for measurement of control activity, in 1 ml contained: sucrose 100 mM, $MgCl_2$ 5 mM, $CaCl_2$ 5 mM, MES-NaOH 20 mM (pH 6.5) and 20 μg Chl. The up- and down-ward arrows indicate, respectively, the switch on and off of light.

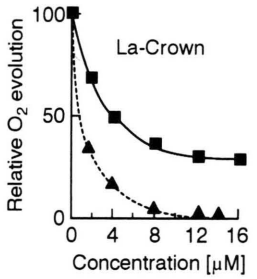

Fig. 2. Crown ether concentration dependent decline in O_2 evolution activity of PS II particles in presence of electron acceptors DCBQ (■——■) and PpBQ (▲– – –▲). Reaction details and the 100 percent activity were as mentioned in Fig. 1.

versible (Table I). Upon washing, the inhibition was reduced by nearly 45–50 percent. Since La-crown has two major chemical components, La-picrate and 15-crown-6, the La-crown mediated inhibition of electron transport was examined for these two specific chemical components. Furthermore, La^{3+} has also been shown to impair the photoelectron transport activity by replacing Ca^{2+} (Bakou *et al.,* 1992) specifically at the donor side of PS II. Our results indicated that the La^{3+} ion (added as $LaCl_3$) does not effect the electron

Table I. Inhibition of O_2 evolution activity upon incubation of PS II particle with La-crown for different time duration (min). The La-crown concentration was 3 μM. The table also includes the relative extent of remaining inhibition of activity upon washing the La-crown incubated PS II particles. The 100 percent activity of PpBQ supported activity was 353 μmol O_2 evolved $mgChl^{-1}$ h^{-1}. '00' refers to the assay done just after addition of the inhibitor.

Incubation time [min]	Percent inhibition of control	Percent inhibition of control after washing
Control	100	100
0	82	–
5	80	–
10	80	45
20	82	47
30	80	46

transport activity when added to PS II suspension in micro molar range; a concentration range of La-crown which brings significant inhibition of electron transport reaction. Furthermore, the crown ether (15-crown-6) also had no effect on the electron flow (data not shown) This suggests that the picrate constituent of the La-crown complex is the potent candidate to exhibit inhibitory effects on the electron transport reaction.

Since DCBQ can support the partial electron flow from H_2O to Q_A in presence of DCMU (Fig. 1 a), the effect of La-crown was investigated in this DCMU insensitive electron transport activity (data omitted). La-crown, however, had no effect on the DCMU insensitive electron flow. La-crown inhibition of electron transport at the reducing side of PS II (Q_A/Q_B complex) has been further verified from room temperature (25 °C) Chl *a* fluorescence emission studies. The results were compared with the fluorescence emission characteristics obtained in presence of DCMU. Addition of La-crown induced nearly 1.5 fold increase in Chl *a* fluorescence emission over control (in absence of La-crown) samples. The extent of increase in fluorescence emission is comparable to DCMU treated samples (data not shown). Under saturating light intensity, the steady state fluorescence emission intensity of Chl *a* represents the intermediate reduction of Q_A. Addition of DCMU increases the rate constant of Q_A reduction and results in higher accumulation of reduced Q_A [electron flow to Q_B from Q_A is inhibited by DCMU which is further translated into high Chl *a*

fluorescence emission (yield/intensity)]. Therefore, observations obtained both from electron transport activity and steady state fluorescence emission intensity suggest that La-crown inhibits electron transport at the reducing side of PS II.

We further examined the nature and location of membrane component responsible for binding to La-crown. It is known that thylakoids when treated with trypsin (a proteolytic enzyme) makes the membrane insensitive to DCMU inhibition (Regitz and Ohad, 1975; Renger, 1976). These results have been interpreted as that DCMU inhibition is mediated through its binding to protein component located to the outer surface of the membrane. Treatment of membranes with trypsin for 2 to 6 min rendered the PS II particles partially insensitive to DCMU inhibition of PS II electron transport activity. The rate of electron transport in control membrane (in absence of added inhibitor) was marginally reduced (about 10 percent) upon trypsin treatment. In contrast to DCMU, the La-crown mediated inhibition of PS II electron transport activity in trypsin digested membranes, depending on the duration of treatment, showed a biphasic nature. During early period of incubation (1–2 min), a partial resistance to La-crown inhibition was noticed which was followed by an increased susceptibility to crown inhibition with increase in duration (>2 min) of incubation (Fig. 3).

Unlike the urea type of herbicide (DCMU), the phenolic herbicide, DNOC (4,6-dinitro-0-cresol) exert an enhanced inhibition on the PS II electron transport activity after mild digestion with trypsin (Böger and Kunert, 1979). Our observations suggest that La-crown, like DCMU, also interact with membrane proteins, but it has other inhibitory site(s), which is/are exposed to inhibitor molecule upon trypsin treatment. It may be pointed out that DCMU belongs to a group of urea-type inhibitors with =C–N structural group and which have preferential binding ability to D_1 protein while DNOC constitute the phenolic family of inhibitors (also known as inhibitor-uncouplers) having binding capacity not only to D_1 protein but to CP47 chlorophyll-protein-complex as well (Oettmeier *et al.*, 1980). Obviously crown compound with their trinitrophenolate (picrate) anionic species should belong to the latter group of inhibitors. However, from the trypsin digestion results it appears that La-crown possesses the characteristics properties of both urea (liopophilic/electronic binding) and phenolic (steric binding) type of herbicides (Trebst and Draber, 1979). The dual nature of herbicidal action of the compound needs further investigation.

Since La-crown showed inhibitory effect on the reducing side of PS II by interacting with membrane protein, we further studied the binding char-

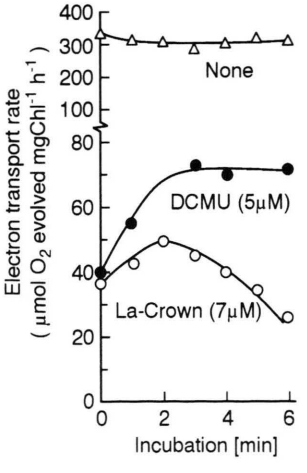

Fig. 3. Inhibition of electron transport activity in trypsin treated PS II particles against DCMU (●——●) and La-crown (○——○). Effect of trypsin treatment on control (△——△) activity has also been included. The electron transport measurements were done as mentioned in Fig. 1 using PpBQ as electron acceptor.

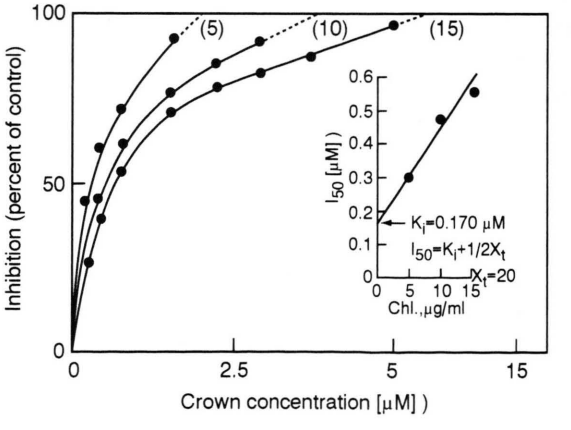

Fig. 4. I_{50} inhibition of O_2 evolution activity by La-crown at (5), (10), (15) μg Chl. I_{50} values were plotted to obtain the K_i (see inset). Details of the electron transport measurement are given in legend to Fig. 1. The each point represent the mean of 4 to 5 determinations. For clarity of the graph the standard bars have not been included.

acteristics of the compound. The I_{50} value of inhibition of La-crown was determined at varied concentrations of Chl (Fig. 4). When I_{50} values were plotted against the increasing concentrations of Chl, a straight line graph was obtained which on extrapolation to zero Chl concentration provided with the inhibition constant (K_i) of about 0.17 μM (Fig. 4 inset). The value of 0.17 μM is rather high compared to DCMU that has a K_i of about 10 to 40 nM (Van Rensen *et al.,* 1978) in isolated chloroplasts.

Using the K_i, the concentration of specific binding sites for La-crown was determined following the equation $I_{50} = K_i + 1/2\ X_t$ (Tischer and Strotmann, 1977). The X_t represents the concentration of specific binding site. This equation has been derived from a simple assumption that the inhibitor (La-crown in this investigation) binds to a specific component in the electron transport chain to form an inhibitory complex. For La-crown the X_t value obtained was about 1 per 20 Chl molecules (see inset Fig. 4) . On Chl basis it is high compared to DCMU which has 1 binding site for 300 Chl molecules, but it is rather low compared to PS II inhibitor like DNOC having one binding site per 2.3 Chl molecules (Van Rensen *et al.,* 1978).

More information for La-crown inhibition was obtained from Hill plot analysis of the inhibition curve. The inhibition curve for 15 μg Chl was constructed as Hill plot. The plotting was done with log (Inhibition/1-Inhibition) against log of inhibitor concentration (i.e. log $(\frac{v}{v_{max}-v})$ where v_{max} is the velocity without inhibitor and v is the velocity with inhibitor. The Hill plot showed three distinct straight lines with slope value (Hill coefficient) of 1.6, 0.7 and 1.7 (Fig. 5). The appearance of three straight lines suggest that La-crown has at least three different sites of inhibition. Since the La-crown does not effect the DCMU insensitive electron flow from H_2O to Q_A, it is assumed that all the three inhibitory sites are located at the reducing side of PS II. Furthermore, since the Hill coefficient (slope of the curve) can never be greater than the number of binding sites per molecule (Dahlquist, 1978) it is logical to deduce that two of the La-crown susceptible sites provide at least

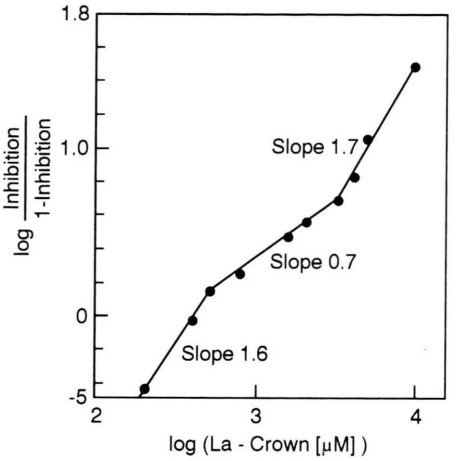

Fig. 5. Hill plot analysis of La-crown inhibition. The inhibition curve obtained from 15 μg Chl ml^{-1} was considered for Hill plot construction.
The notation log $\frac{\text{Inhibition}}{\text{1-Inhibition}}$ represents log $(\frac{v}{v_{max}-v})$.

two binding domains for the inhibitor (with Hill coefficients 1.6 and 1.7). It may be mentioned that DCMU has been shown to possess two binding sites (Van Rensen *et al.,* 1978).

In this report we have presented experiments which certify the inhibitory action of La-crown at the reducing side of PS II and the characteristic inhibition refers both to phenolic and urea type of inhibition. However, the exact molecular mechanism of interaction of inhibitor with the target site(s) still remains to be disclosed. Unlike K-crown (Sabat *et al.,* 1991), La-crown showed partial irreversible inhibition of electron transport activity. Since La-crown inhibits electron flow at the level of Q_B, at the acceptor side of PS II complex, it is of interest to study the nature of this permanent impairment.

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Bakou A., Buser C., Dandulakis G., Brudvig G. W. and Ghanotakis D. F. (1992), Calcium binding site(s) of photosystem II as probed by lanthanoides. Biochim. Biophys. Acta **1099,** 131–136.

Böger P. and Kunert K.-J. (1979), Differential effect of herbicides upon trypsin treated chloroplasts. Z. Naturforsch. **34c,** 1015–1020.

Coleman W. and Govindjee. (1987), A model for mechanism of chloride activation of O_2 evolution in photosystem II. Photosynth. Res. **13,** 199–233.

Debus R. J. (1992), The manganese and calcium ions of photosynthetic oxygen evolution. – Biochim. Biophys. Acta **1102**, 269–352.

Dahlquist F. W. (1978), The meaning of scatchard and Hill plots. In: Methods in Enzymology (C. H. W Hirs,. and S. N. Timasheff, eds.) **Vol.48**, Academic Press, New York. pp. 270–279.

Frensdorff H. K. (1971), Salt complexes of cyclic polyethers, Distribution equilibria. J. Am. Chem. Soc. **93,** 4684- 4688.

Govindjee (1991), A unique role of carbon dioxide in photosystem II. In: Impact of Global Climatic Changes on Photosynthesis and Plant Productivity (Y. Abrol, P. N. Wattal, A. Gnanam, Govindjee., D. R. Ort, and A. H. Teramura, eds.). Oxford & IBH Publ. Co. Pvt. Ltd., NewDelhi, Bombay, Calcutta, pp. 349–369.

Khan A. S., Baldwin W. G. and Chow A. (1987), Extraction of alkali metals into polyester – based polyurethane foam using crown ether and picric acid. Can J. Chem. **65,** 1103–1106.

Kovacs L., Hegde U., Padhye S., Bernat G. and Demeter S. (1996), Effect of Potassium- (picrate)-(18-crown-6) on the photosynthetic electron transport. Z. Naturforsch. **51c,** 539- 546.

Nakagawa K., Okada S., Inoue Y., Tai A. and Hakushi T. (1988), Solvent extraction of lanthanoid picrates with crown ethers, preferential sandwich complexation and unique cation selectivity. Anal. Chem. **60,** 2527–2532.

Oettmeier W., Masson K. and Johanningmeier U. (1980), Photoaffinity labeling of the photosystem II herbicide binding protein. FEBS Lett. **118,** 267–270.

Poonia N. S. (1974). Co-ordination chemistry of sodium potassium complexation with macrocyclic polyethers. J. Am. Chem. Soc. **96,** 1012–1019.

Porra R. J., Thompson W. A. and Kriedemann P. E. (1989), Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophyll *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim. Biophys. Acta **975,** 384–394.

Regitz G. and Ohad I. (1975), Changes in the protein organization in developing thylakoids of *Chlamydomonas reinhardtii* Y-1. In: Proc. IIIrd Int. Congress on Photosynthesis. (M. Avron,. ed.) Scientific, Elsevier, Amsterdam. pp. 1615–1625.

Renger G. 1976. Studies on the structural and functional organization of system II of photosynthesis; the use of trypsin as a structurally selective inhibitor at the outer surface of the thylakoid membrane. Biochim. Biophys. Acta **440,** 287–300.

Sabat S. C., Vijayavergiya V., Tripathy B. C. and Mohanty P. (1991), Inhibitory effects of crown compound on photoelectron transport activity of beet spinach thylakoid membranes. Z. Naturforsch. **41c,** 87–92.

Tischer W. and Strotmann H. (1977), Relationship between inhibitor binding by chloroplasts and inhibition of photosynthetic electron transport. Biochim. Biophys. Acta **460,** 113–125.

Trebst A. and Draber W. (1979), Structure activity correlations of recent herbicides in photosynthetic reactions. in Advances in Pesticide Science Part 2 (H. Geissbuhler ed.) Pergamon Press, Oxford, pp. 223–234.

Van Leeuwen P. J., Nieveen M. C., van de Meent E. J., Dekker J. P. and van Gorkom H. J. (1991), Rapid and simple isolation of pure photosystem II core and reaction center particles from spinach. Photosynth. Res. **28,** 149–153.

Van Rensen J. J. S., Wong D. and Govindjee. (1978), Characterization of the inhibition of photosynthetic electron transport in pea chloroplasts by the herbicide 4, 6-dinitro-o-cresol by comparative studies with 3-(3,4-dichlorophenyl)-1, 1-dimethyl urea. Z. Naturforsch. **33c,** 413–420.

Vijayavergiya V. and Mookerjee J. (1989), Effect of Gamma-radiation on cation transport across an artificial membrane. J. Radio. Analyt. and Nucl. Chem. **134,** 39–44.