Crystalline Pyruvate Oxidase from *Escherichia coli*

III. PHOSPHOLIPID AS AN ALLOSTERIC EFFECTOR FOR THE ENZYME*

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**SUMMARY**

Crystalline pyruvate oxidase, a soluble tetrameric flavo-protein from *Escherichia coli*, which binds both thiamine pyrophosphate (TPP) and FAD is activated 15- to 100-fold by phospholipids and long chain fatty acids. Maximal activation of the oxidase requires incubation of the enzyme for at least 6 min with the lipid activator in the presence of substrate and cofactors (pyruvate, TPP, and MgCl₂). Very little activation occurs if any of these components are omitted from the mixture. Furthermore, activation is markedly reduced if the enzyme is incubated with phospholipid before pyruvate, TPP, and MgCl₂ are added.

Phosphatides dramatically effect the kinetic parameters of the pyruvate oxidase reaction. The $K_m$ values for pyruvate and TPP are lowered 13-fold and 3- to 4-fold, respectively, in the presence of phospholipid. In addition, phosphatides bestow cooperativity to the enzyme with respect to binding of TPP in a very unusual fashion. In the absence of phospholipid, TPP binding to the enzyme follows ordinary Michaelis-Menten type saturation kinetics. In the presence of phospholipid, TPP binds cooperatively to the enzyme and shifts the $K_m$ for TPP to a lower value.

The physiological significance of pyruvate oxidase and the activation phenomenon are discussed.

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In the previous paper (1) the activation of crystalline pyruvate oxidase from *Escherichia coli* by various phospholipids and long chain fatty acids has been described. The interaction between phospholipids and the enzyme appears to involve the hydrophobic portion of the phospholipid molecule and does not appear to be specific for any particular phosphatide species.

In this paper, we examine the characteristics of the enzyme-phospholipid interaction in further detail. Kinetic parameters for the pyruvate oxidase reaction, obtained both in the absence and presence of phospholipids, are presented. Moreover, the conditions for optimal activation of the enzyme by phospholipids are described. The presence of phospholipids dramatically affects the binding of substrate and cofactors to the enzyme. Rapid kinetic studies have been undertaken to localize the rate-controlling step which is affected by the presence of lipid activator. Since most assays for pyruvate oxidase are dependent upon turnover of the enzyme, the possibility existed that the lipid activator could function either in the steps leading to reduction of the enzyme-bound FAD or alternatively in the reoxidation of the enzyme-FADH₂ complex (Equations 1 and 2). Stopped-flow experiments show that lipid activator enhances the rate of formation of reduced enzyme-bound FAD by approximately 160-fold. Thus, it is clear that the primary effect of phospholipids is associated with the reactions leading to enzyme reduction (Equation 1).

**EXPERIMENTAL PROCEDURE**

*Materials*—The materials used for the experiments described in this paper were obtained from the sources listed in the previous paper (1).

2,6-Dichloroindophenol Reductase Assay—This assay procedure is based on the utilization of 2,6-dichloroindophenol as an electron acceptor for pyruvate oxidase. The assay reaction mixture contained, in a total volume of 1 ml, 100 μmoles of potassium phosphate buffer, pH 6.0, 10 μmoles of MgCl₂, 50 μmoles of pyruvate, and enzyme. The addition of 2,6-dichloroindophenol reduced the absorbance of the assay mixture at 600 nm. The reaction was initiated by the addition of pyruvate, and absorbance changes were monitored at 600 nm.

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* The abbreviations used are: TPP, thiamine pyrophosphate; DCI, dichloroindophenol.
tassium pyruvate, 0.1 µmole of TPP, saturating amounts of micellar phospholipid (30 µg) or other activators, 0.2 µmole of DC1, and sufficient enzyme to yield a decrease in absorbance of 0.1 to 1.0 per min. The order of addition of reactants to the DC1 assay mixture is important. The phospholipid activator is normally added after all other components except DC1 which was used to initiate the reaction. The assay mixture minus DC1 is incubated for 10 min before the addition of DC1. Unless otherwise noted, the phospholipid activator for the enzyme was a water-soluble micellar preparation of E. coli phosphatidylethanolamine (1). In most assays, 30 µg of phosphatidylethanolamine were added; however, smaller amounts (5 µg) of freshly prepared micelles of phosphatidylethanolamine are sufficient to fully activate the enzyme. The reduction of DC1 is followed spectrophotometrically by measuring the loss of absorbance at 600 nm.

**RESULTS**

**Conditions for Maximal Activity of Enzyme**—The oxidation-reduction indicator, DC1, is an excellent electron acceptor in the pyruvate oxidase reaction. The Kₐ for DC1 determined from the Lineweaver-Burk plot shown in Fig. 1 is 9.3 x 10⁻⁵ M. This dye is useful for assaying the enzyme at pH values as low as 6; however, at lower pH values DC1 is insoluble in concentrations required for the assay (2 x 10⁻⁴ M). As evidenced from Fig. 2, the pH optimum for DC1 reductase activity is 6 or lower. This value is in agreement with the observed pH dependence of pyruvate oxidase when either ferricyanide or oxygen serve as terminal electron acceptors (2).

As indicated previously (1), maximal activity of pyruvate oxidase...
TABLE I

Requirements for activation of pyruvate oxidase

The concentration of the reactants is listed in the DC1 reductase assay under "Experimental Procedure." In this table, PE refers to the addition of 5 μg of a fresh micellar preparation of phosphatidylethanolamine. The assays were performed in the DC1 reductase assay and were modified only as designated in the table.

| Components present during first incubation | Components added with DC1 at the start of assay | DCl activity units | Maximal activation % |
|--------------------------------------------|-----------------------------------------------|-------------------|----------------------|
| Experiment 1                               |                                               |                   |                      |
| a. TPP, pyruvate, Mg++                    | None                                          | 29                | 6                    |
| b. TPP, pyruvate, Mg++                    | PE                                            | 61                | 14                   |
| c. TPP, pyruvate, Mg++, PE                | None                                          | 449               | 100                  |
| Experiment 2                               |                                               |                   |                      |
| a. None                                    | TPP, pyruvate, Mg++                           | 29                | 3                    |
| b. PE                                      | TPP, pyruvate, Mg++                           | 72                | 8                    |
| c. PE (10 min) + TPP, pyruvate, Mg++ (10 min)* | None                                          | 521               | 55                   |
| d. TPP, pyruvate, Mg++, PE                | None                                          | 940               | 100                  |
| Experiment 3                               |                                               |                   |                      |
| a. None                                    | TPP, pyruvate, Mg++                           | 32                | 4                    |
| b. Mg++, TPP, PE                           | pyruvate                                      | 55                | 7                    |
| c. Mg++, pyruvate, PE                     | TPP                                           | 287               | 31                   |
| d. TPP, pyruvate, PE                      | Mg++                                          | 215               | 28                   |
| e. Pyruvate, PE                           | TPP, Mg++                                     | 54                | 7                    |
| f. TPP, pyruvate, Mg++, PE                | None                                          | 759               | 100                  |

* In Experiment 2c, there were two 10-min incubation periods, the first period with phosphatidylethanolamine alone followed by a second 10-min incubation with TPP, pyruvate, and Mg++.  

oxidase in several assays is dependent on the presence of a lipid activator. Activation of the enzyme with phospholipid is a time-dependent process. As shown in Fig. 3, about 6 min of incubation are required for complete activation of the enzyme by phospholipid.

The conditions for maximal activation of pyruvate oxidase have been considered in detail (see Table I). All assays in Table I were performed as described under "Experimental Procedure" and modified only as designated. Experiment 1, Table I, reaffirms the observation that prior incubation of the enzyme with phospholipid is necessary for activation. However, if the enzyme is incubated with phospholipid in the absence of substrate and cofactors very little activation is noted (see Experiment 2b). Furthermore, if the flavoprotein is first incubated with phospholipid followed by a second incubation with TPP, pyruvate, and MgCl₂, maximal activation is decidedly lowered (Experiment 2c). For this reason phospholipid is routinely added after the other components of the reaction mixture to minimize any inhibitory effect. As demonstrated in Table I, Experiment 3, maximal activation of pyruvate oxidase is achieved only if the enzyme is incubated with pyruvate, TPP, MgCl₂, and phospholipid simultaneously. A lower degree of activation occurs if the activation is carried out in the presence of phospholipid, and either TPP or MgCl₂ (Experiments 3c and 3d). No activation of the oxidase is noted if only pyruvate and phospholipid are added to the incubation mixture (Experiment 3e). Furthermore, no stimulation of activity is observed by prior incubation with TPP, MgCl₂, and phospholipid (Experiment 3b).

The enzyme is not activated appreciably if phospholipid is added during the course of enzyme turnover in the presence of an electron acceptor. As demonstrated in Fig. 4, the enzymic activity increased slightly over a 10 min period when the reaction was initiated by addition of a DCI-phospholipid mixture (Line A). In contrast, the activity of an enzyme preparation which had been incubated with phospholipid, substrate, and cofactors for 10 min was several-fold higher (Line B).

Alterations in Reaction Kinetics with Addition of Phospholipid—Addition of phospholipid to pyruvate oxidase has a profound effect on the kinetics of the enzymic reaction. The Kₘ values for pyruvate and the Vₘₐₓ values for the DCI reductase reaction, both in the absence and presence of phospholipid, are shown in Table II. These values were obtained from the Lineweaver-Burk plots shown in Fig. 5. The Kₘ for pyruvate is lowered...
Table II
Effect of micellar phospholipid on kinetic constants for pyruvate oxidase

Pyruvate oxidase activity was measured both in the presence and absence of micellar phospholipid using the DC1 reductase assay. The concentrations of pyruvate in the assay mixture were varied from 0.004 to 0.05 M. The concentration of pyruvate oxidase was increased 30-fold for assays containing no phospholipid. The \( K_m \) and \( V_{\text{max}} \) values were obtained from the Lineweaver-Burk plots shown in Fig. 5.

| Kinetic constant | Minus phospholipid | Plus phospholipid | Difference |
|------------------|--------------------|-------------------|------------|
| \( K_m \)        | \( 8.4 \times 10^{-3} \) M | \( 0.7 \times 10^{-2} \) M | 12.7- \( \text{fold} \) |
| \( V_{\text{max}} \) | \( 0.39 \times 10^5 \) units | \( 4.70 \times 10^4 \) units | 12.2- \( \text{fold} \) |

*The \( V_{\text{max}} \) values are expressed in DC1 reductase units per mg of protein.

Fig. 5. Reciprocal plots of the rate of pyruvate oxidation as a function of the reciprocal of the pyruvate concentration in the presence and absence of phospholipid. Assays were performed in the DC1 system in the presence (● – ●) and absence (Δ — Δ) of phospholipid at the indicated concentrations of pyruvate. The concentration of pyruvate oxidase was increased 30-fold for assays containing no phospholipid.

Fig. 6. Plot of pyruvate oxidase activity as a function of the concentration of TPP in the presence and absence of phospholipid. Pyruvate oxidase activity was measured both in the presence (● — ●) and absence (○ — ○) of micellar phospholipid with the DC1 reductase assay. The concentration of TPP was varied from 0.2 to 2.0 \( \times 10^{-2} \) M. The concentration of pyruvate oxidase was increased 20-fold for the assays containing no phospholipid.

Fig. 7. Hill plots. The Hill plot for TPP in the presence of phospholipid (△—△) and in the absence of phospholipid (○—○) is recorded. The \( V_{\text{max}} \) values used for these plots were obtained from reciprocal plots of the rate of DC1 reduction as a function of TPP concentration (absence of phospholipid) or rate of DC1 reduction as a function of the second power of TPP concentration (plus phospholipid).

The \( K_m \) for TPP in the absence of phospholipid is \( 1 \times 10^{-9} \) M. The substrate concentration giving one-half of the maximal velocity in the presence of phospholipid, estimated from a plot of initial velocity as a function of the TPP concentration, is lowered to the range 2.5 to 4.2 \( \times 10^{-7} \) M. An unusual effect for a positive allosteric effector is seen in this particular case. The increase in the \( V_{\text{max}} \) in the presence of phospholipid represents a 24-fold increase in specific activity for this particular preparation; this high increase is partially due to low activity noted for the unactivated enzyme. Most enzyme preparations have a slightly higher activity in the absence of an activator.
A. MINUS LYSOLECITHIN

B. PLUS LYSOLECITHIN

FIG. 8. Rapid kinetics of enzyme reduction. The techniques used to measure the rate of reduction of pyruvate oxidase by pyruvate, both in the absence of phospholipid (upper picture) and in the presence of lysolecithin (lower picture) are described under “Experimental Procedure.”

Table III

| Sample                        | $k_1$  | $t_1$ |
|-------------------------------|--------|-------|
| No lipid                      | 0.33   | 2.1 sec |
| No lipid                      | 0.38   | 1.8 sec |
| No lipid                      | 0.46   | 1.5 sec |
| Lysolecithin (3.4 X $10^{-4}$ M) | >35$^a$ | <20 msec$^b$ |

$^a$ A minimum value estimated from the half-life.
$^b$ This value was visually estimated from an oscilloscope signal measuring the transmittance change during the first 200-msec of the reaction.

Effect of Phospholipid on Rate of Reduction of Pyruvate Oxidase by Pyruvate—To study the rate of reduction of pyruvate oxidase in the absence of added electron acceptors, a Gibson stopped-flow apparatus was employed (3). Reduction of the enzyme-bound FAD was measured both in the presence and absence of phospholipids.

One of the difficulties encountered in these experiments was the insolubility of the phospholipid micelles in the enzyme assay reaction mixture. All cephalin type phospholipid preparations precipitated immediately when added to the standard incubation mixture. Micellar lecithin was relatively stable in the assay solution in the absence of pyruvate; however, a precipitate formed immediately upon the addition of pyruvate. These properties were particularly undesirable in stopped-flow experiments since any increase in turbidity in the mixing chamber would be reflected as an increase in absorbance. No turbidity was observed, however, in enzyme solutions upon the addition of lysolecithin. Furthermore, reaction mixtures containing lysolecithin were completely free of turbidity for at least 1 hour after pyruvate oxidase was reduced by addition of pyruvate. For this reason lysolecithin was selected as the lipid activator in the stopped-flow experiments.

Fig. 8 records the oscilloscope tracings obtained in stopped-flow experiments measuring the rate of reduction of pyruvate oxidase both in the absence and presence of phospholipid. The curve in the upper picture represents the rate of reduction of pyruvate oxidase by pyruvate in the absence of phospholipid. The 2 cps signal seen in this tracing is of unknown origin and appeared frequently, but not always, during use of the stopped-flow instrument. The rate constant for reduction of the enzyme in the absence of lipid activator in three separate experiments varied between 0.33 and 0.46 sec$^{-1}$. Table III lists the values for the first order rate constant and the half-life for the reduction of pyruvate oxidase in the absence of phospholipids.

The oscilloscope signal shown in the lower portion of Fig. 8 represents the reduction of the enzyme in the presence of lysolecithin. Pyruvate oxidase, in the presence of lysolecithin, is almost completely reduced during the flow period in the instrument. The 2 cps signal seen in this tracing is of unknown origin and appeared frequently, but not always, during use of the stopped-flow instrument. The rate constant for reduction of the enzyme in the presence of lysolecithin is 2.1. When no phosphatide is added to the reaction mixture, the slope of the Hill plot is 1 with respect to TPP.

The values below were obtained from the oscilloscope tracings shown in Fig. 8 and from the results of separate stopped-flow experiments not shown.

V$_{\text{max}}$ (6). In the case of TPP binding to pyruvate oxidase, phospholipids induce cooperativity while lowering the K$_{\text{m}}$ for TPP and raise the V$_{\text{max}}$ for the reaction by a factor of 12- to 25-fold.

Hill plots (4) for TPP, shown in Fig. 7, establish that phospholipid changes the kinetic order of the reaction with respect to TPP. The “n” value obtained if phospholipid is present in the reaction mixture is 2.1. When no phosphatide is added to the reaction mixture, the slope of the Hill plot is 1 with respect to TPP.

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This leads to a minimum value for the rate constant of the lipid-activated enzyme of 35 sec⁻¹. This rate constant is undoubtedly on the low side; however, it does clearly demonstrate that reduction of pyruvate oxidase by pyruvate is increased at least 100-fold in the presence of lyssolecithin.

**DISCUSSION**

The activation of pyruvate oxidase by phospholipids and fatty acids meets the criteria established for allosteric effectors, although the activation is unique in some respects because it represents a hybrid situation between the K and V systems as originally defined by Monod, Wyman, and Changeux (6). In the presence of phospholipids, the $K_m$ values for pyruvate and TPP in the pyruvate oxidase reaction are altered and the enzyme can be desensitized with respect to the activator as would be expected in a K type allosteric system; however, the effect of phospholipid on the $V_{max}$ of the reaction is also representative of a V type system. In addition, the effect of phospholipids on the kinetic parameters of TPP binding is very unusual. A positive allosteric effector of the K type (6) shifts an enzyme from sigmoidal kinetics in the absence of effector to normal saturation kinetics in the presence of effector. In the case of TPP and pyruvate oxidase, the opposite is true. The enzyme shows normal saturation kinetics in the absence of activator and shifts to sigmoidal kinetics in the presence of phospholipid. The large stimulation of the $V_{max}$ of the pyruvate oxidase reaction by phospholipids is unusual. In some V type systems, $V_{max}$ values in the presence and absence of effector are not nearly so drastically changed (5-7); in other V type systems, the activating effect of the allosteric effector is due to its antagonistic action with respect to allosteric inhibitors (8). In the case of pyruvate oxidase, depending on the assay system used, $V_{max}$ increases as much as 100-fold in the presence of the phospholipid effector.

The rapid kinetics experiments clearly indicate that the rate-limiting step which is altered by the presence of phospholipids occurs at or before the reduction of enzyme-bound flavin. Perhaps the simplest interpretation would be that phospholipids promote interaction between TPP and FAD prosthetic groups in the enzyme, and thus increase the overall $V_{max}$ for reduction.

The incubation period required for complete activation of pyruvate oxidase suggests that the interaction between the flavoprotein and phospholipid triggers a slow conformational change in the oxidase which leads to the activated form of the enzyme. Phospholipid alone, in the absence of cofactors and substrates, is not sufficient to activate the enzyme. Moreover, incubation of the enzyme with phospholipid prior to the addition of substrate and cofactors results in a lower degree of activation.

This observation suggests that phospholipid causes a deleterious change in the enzyme structure in the absence of substrate and cofactors.

The requirement for the combined presence of pyruvate, TPP, MgCl₂, and phospholipid for maximal activation complements the earlier observation by Hager (9) that pyruvate, TPP, and MgCl₂ are required in order to activate pyruvate oxidase by trypsin. In trypsin activation, TPP protects the enzyme from extensive proteolysis while the presence of pyruvate greatly accelerates the rate of formation of activated enzyme (9). Trypsin activation desensitizes the enzyme for activation by phospholipids.

The shift from normal saturation kinetics for TPP to sigmoidal kinetics in the presence of phospholipids deserves further comment. Since Hill plots give n values of 2 for TPP binding in the presence of phospholipids and n values of 1 in the absence of phospholipid, the obvious conclusion is that phospholipids increase interactions between TPP-binding sites, while in the absence of phospholipid, all binding sites for TPP behave independently. According to Atkinson, Hathaway, and Smith (10), the n value in the Hill equation depends both on the number of interacting binding sites and the strength of their interaction.

Where interactions are strong, the number obtained from a Hill plot is equal to the number of binding sites for the ligand involved. Equilibrium binding studies have shown that pyruvate oxidase in the absence of phospholipid binds 4 molecules of TPP per molecule of enzyme. This result is consistent with the observation that there are 4 molecules of FAD bound per enzyme molecule (2). Taken altogether, these data suggest that pyruvate oxidase is a tetramer consisting of four identical subunits, each capable of binding both a TPP and FAD molecule and on the average, in the presence of phospholipid, two of the TPP sites would have to be occupied in order to observe significant enzymic activity.

In addition to conferring cooperativity to the enzyme with respect to binding TPP, the phosphatides also lower the $K_m$ for TPP 3 to 4 fold and the $K_m$ for pyruvate is lowered 13 fold upon addition of phospholipids to the reaction mixture. In contrast to TPP, however, the saturation kinetics with respect to pyruvate is normal both in the presence and absence of phospholipids. Thus, while there undoubtedly is more than one binding site for pyruvate in the tetramer, all sites behave completely independent from each other.

The physiological significance of the pyruvate oxidase reaction in the over-all metabolism of *E. coli* remains obscure at this time. As diagrammed in Fig. 9, *E. coli* has three separate paths for production of acetate from pyruvate. The principal path for pyruvate oxidation is the dehydrogenase system since acetate-requiring mutants of *E. coli* can be readily isolated which are blocked either in the decarboxylase (11) or the transacetylase (12) component of the pyruvate dehydrogenase complex. Since these mutants contain pyruvate oxidase but still require acetate for growth, it follows that pyruvate oxidase is either not present in sufficient quantities to supply the acetate requirement for cellular growth or if present in sufficient quantities, it must be subjected to strict metabolic control and be unable to function effectively under the imposed growth conditions. Dietrich and Henning (13) favor the low level of enzyme hypothesis, whereas results in our laboratory would favor the control hypothesis.

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*Fig. 9. Scheme for metabolism of pyruvate in *E. coli.*

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1. N. Poludniak and L. P. Hager, unpublished observations.
For example, Oster and Hager (14) have shown that the specific activity of pyruvate oxidase in cell-free extracts can be 50 times greater than the activity of whole cells (studied in an acetate-requiring mutant). The observation that phospholipids and long chain fatty acids markedly activate pyruvate oxidase may offer an explanation for the apparent inactivity of the oxidase in vivo studies.

Since the early studies of Fleischer and Klouwen (15) and Fleischer et al. (16), on the effect of phospholipids on the activity of electron transport particles, several reports have indicated the requirement or stimulation of enzyme activity by phospholipid preparations (17–27). In general, phospholipid activation is usually associated with enzymes which are located in membranous organelles, such as mitochondria (16), microsomes (26), or bacterial membranes (22), and many examples of phospholipid stimulation can be found with enzymes involved in electron transport reactions (15–22, 24, 25). There appears to be a tacit assumption on the part of many investigators in this field implying that phospholipid activation merely reflects the need of the enzyme to be in a hydrophobic environment as would be found in its original membranous home. In large part, this assumption probably rests on the observations that many enzymes respond in quite a non-specific manner with respect to phospholipid activation. As is the case with E. coli pyruvate oxidase (1), a large variety of phospholipids with different bases and different fatty acid compositions are capable of activation (15). A notable exception is β-hydroxybutyric dehydrogenase which has a specific requirement for lecithin (17, 18). Although pyruvate oxidase is found among the soluble E. coli components, the oxidase must interact with the membrane-bound terminal electron transport system in order to turn over (the enzyme-substrate complex is nonautoxidizable (28)). Thus one could also argue in this case for phospholipid activation as part of the native environment for the enzyme. However, in the pyruvate oxidase case, a regulatory role with phospholipids and long chain fatty acids acting as allosteric effectors can be field formulated. As pointed out earlier, in E. coli there are three distinct paths for the conversion of pyruvate to the acetate level (Fig. 9). Two of these paths, the pyruvate dehydrogenase (29) and the phospholy catastrophic reaction (30–32), produce high energy acetyl derivatives (acetyl-CoA from the dehydrogenase system and acetyl phosphate from the cistic reaction) while pyruvate oxidase produces acetate. This leads to a deficiency of approximately 16 kcal per mole of pyruvate when pyruvate oxidase is operating since an ATP would have to be utilized in order to convert free acetate to acetyl-CoA for further metabolism and one less ATP would be available via the terminal electron transport system. Therefore, in E. coli the build-up of free fatty acids or of fatty acid-containing phosphatides would serve as a switch to turn on pyruvate oxidase, a low energy path for pyruvate utilization. In the absence of sufficient activator, the pyruvate oxidase would be inoperative. This would also explain the acetate requirement for initial growth on glucose in mutants lacking one of the pyruvate dehydrogenase components. Although pyruvate oxidase would be synthesized and present in the mutant cells, it could not function in the absence of activator. Since, the biosynthesis of activator would require acetate supplementation, no growth could occur without acetate.

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