Purification and Properties of Glycerol Dehydrase*

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SUMMARY

Glycerol dehydrase, a coenzyme B₁₂-dependent enzyme, from Aerobacter aerogenes, has been purified to a stable, inactive, homogeneous complex with hydroxocobalamin. The inactive complex can be converted to the active holo-enzyme by replacement of hydroxocobalamin with coenzyme B₁₂ in the presence of Mg⁺⁺ and SO₄²⁻. The molecular weight of the hydroxocobalamin apoenzyme complex is 188,000 and 1 mole of hydroxocobalamin is bound to 1 mole of the apoenzyme. There is a significant difference between the spectra of the dissociated and the protein-bound hydroxocobalamin. The greater stability of the protein hydroxocobalamin complex as compared to the apoenzyme was shown by various methods. Complete dissociation of the apoenzyme is caused by low pH, Na⁺, or EDTA, whereas the hydroxocobalamin apoenzyme complex does not dissociate under the same conditions.

EDTA markedly inhibits the activity of the enzyme whereas other chelating agents have little or no inhibitory effect. Partial reversal of the inhibitory effect of EDTA is achieved by increased concentrations of glycerol or by adding an excess of either subunit, A or B. The experimental evidence suggests that the effect of EDTA on the apoenzyme does not involve a divalent cation.

Glycerol dehydrase, a coenzyme B₁₂-requiring enzyme, isolated from Aerobacter aerogenes (strain 572, PZH, Warsaw), catalyzes the conversion of glycerol to β-hydroxypropionaldehyde (1). The enzyme dissociates into two different protein subunits, previously designated as A and B. The dissociation and association of the apoenzyme into its subunits, A and B, are strongly influenced by pH, the presence of certain monovalent cations, and glycerol (2).

The apoenzyme formed from the apoenzyme and DBCC undergoes rapid, irreversible inactivation in the presence or absence of substrate. The inactive enzyme contains hydroxocobalamin firmly bound to the apoenzyme. The inactive complex containing hydroxocobalamin can also be obtained by addition of hydroxocobalamin to solutions of the apoenzyme AB. Other cobamide derivatives can also form inactive complexes with AB (3, 4). The intact apoenzyme is required for the binding of the cobamide derivatives since no binding of cobamide derivatives by the separate subunits, A or B, was observed. Since the cobamide derivatives appear to be firmly bound to the active site, conditions for the replacement of the B₁₂ derivatives by DBCC to restore the active holoenzyme were studied. Furthermore, these complexes thus provided a means of studying the number of active centers on the enzyme.

The apoenzyme and the separate subunits, A and B, are very unstable compared to the complex formed from AB and hydroxocobalamin. Therefore, the enzyme was purified as the complex, AB-B₁₂OH.

This paper describes improved methods for the purification of the enzyme as the inactive AB-B₁₂OH complex and presents further studies on the properties of the enzyme.

EXPERIMENTAL PROCEDURE

Materials and Methods—Hydroxocobalamin was obtained from Merck, dl-tryptophan from Nutritional Biochemicals, hexadecyltrimethylammonium bromide from Eastman Kodak, and p-chloromercuribenzoate from Calbiochem. Sephadex G-25 and G-50 were obtained from Sigma, and hydroxyapatite from Bio-Rad. The crystalline DBCC was a gift from the Yamanouchi Pharmaceutical Company, Japan. Labeled DBCC and hydroxocobalamin were isolated from Propionibacterium shermanii cultures grown on a medium containing ⁶⁰CoCl₂ (5). One nanomole of radioactive cobalamin had 3100 cpm. Bacteriological reagents were obtained from Difco. Reagents for disc electrophoresis were obtained from Canaleo. Yeast alcohol dehydrogenase (1.1.1.1) and NAD were obtained from Worthington. All other reagents used were analytical grade.

Determination of Enzyme Activity—The method of Smiley and Sobolov (6), which involves conversion of the β-hydroxypropionaldehyde to acrolein, was used to determine the β-hydroxypropionaldehyde produced in the glycerol dehydrase assay. The optical density was proportional to the concentration of acrolein over the range of 0.5 to 3.0 µmoles per sample.

The assay mixture contained 0.06 mM glycerol, 0.1 mM potassium phosphate (pH 8.6), 0.05 mM potassium sulfite, 1 nmole of DBCC, and apoenzyme (AB) containing 0.03 to 0.10 unit of glycerol dehydrase activity in a total volume of 1 ml. The mixture was
incubated for 30 min at 30°C. The reaction was started by the addition of DBCC and stopped with 1 drop of HCl. One unit of AB activity is defined as the amount of AB which produces 1 μmole of β-hydroxypropionaldehyde per min. The activity of the AB-B₂OH was estimated by adding to the previously described assay mixture 30 μmoles of magnesium acetate and an AB-B₂OH preparation instead of AB, containing 0.004 to 0.012 unit of activity. This mixture was incubated for 4 hours at 30°C. One unit of AB-B₂OH activity is defined as the amount of enzyme which produces 1 μmole of β-hydroxypropionaldehyde per min. The conversion of 1 unit of AB into AB-B₂OH yields 0.08 unit of the enzyme activity.

Estimation of Activity of Subunits, A and B—The activity tests on the subunits were performed in the way described for the apoenzyme except that an excess (0.2 to 0.4 unit) of B was added to 0.03 to 0.10 unit of A for the determination of A. An excess of A (0.2 to 0.4 unit) was added to 0.03 to 0.10 unit of B for the determination of B.

Determination of Cobalamin—Concentrations of DBCC and hydroxocobalamin were determined from the millimolar extinction coefficient of the dicyanate at 580 nm produced by treating hydroxocobalamin were determined from the millimolar extinction coefficient of A (0.2 to 0.4 unit) was added to 0.03 to 0.10 unit of B for the determination of B. An excess of A (0.2 to 0.4 unit) was added to 0.03 to 0.10 unit of B for the determination of B.

Determination of Cobalamin—Concentrations of DBCC and hydroxocobalamin were determined from the millimolar extinction coefficient of the dicyanate at 580 A (0.2 to 0.4 unit) was added to 0.03 to 0.10 unit of B for the determination of B. An excess of A (0.2 to 0.4 unit) was added to 0.03 to 0.10 unit of B for the determination of B.

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**AB-BtOH was eluted as two fractions by the 0.02 and 0.04 M \( K_2HPO_4 \). Rechromatography of the fraction eluted with 0.04 M \( K_2HPO_4 \) resulted in about 60% of the enzyme activity being eluted with 0.02 M \( K_2HPO_4 \) and about 40% with 0.04 M \( K_2HPO_4 \). The combined fractions eluted with 0.02 M \( K_2HPO_4 \) were used for further purification.**

**Step V—Final purification was achieved by acrylamide electrophoresis according to Davis (11).** Approximately 2 mg of a concentrated solution of AB-BtOH were applied to a column \( 9 \times 100 \) mm. The electrophoresis was performed in the Canalco analytical apparatus with 3 mA per tube for 3 hours at 4° with an electrode buffer containing 14.4 g of glycine and 3 g of Tris per liter. The stacking and separating gels contained, respectively, 5% and 10.2% acrylamide and 0.09% and 0.185% bis. Riboflavin was used as the catalyst to avoid the inactivation of the AB-BtOH by ammonium persulfate. The migration of a reddish orange AB-BtOH band could readily be observed during the electrophoresis. Two other colored bands were also resolved.

**Table I summarizes the purification of the enzyme.** The scanning diagrams presented in Fig. 1 illustrate the progressive purification of AB-BtOH at each step. Hexadecyltrimethylammonium bromide inactivated the apoenzyme, but had no detectable effect on AB-BtOH at the concentrations used. The relatively high ionic strength of 0.5 M \( K_2HPO_4 \) increased the quantity of inactive protein precipitated at 72°. KCl solutions of equal potassium concentration treated in the same manner produced considerably less precipitate. Highly viscous nonprotein impurities were removed by hydroxylapatite chromatography.

**Fig. 1. Scanning diagrams of polyacrylamide gels portraying progressive purification of AB-BtOH.** Approximately 100 \( \mu \)g of protein were fractionated with polyacrylamide gel for each step of purification checked. The 10% gel and the electrophoretic procedure were done according to Davis (11).

**Fig. 2. Spectra of the protein-bound and dissociated cobalamin.** The optical density of a homogenous preparation of AB-BtOH in 0.1 M KCl (2.34 mg per ml; specific activity, 3.0 units per mg) was measured against a reference containing only apoenzyme protein prepared from AB-BtOH as described in the text (—). An identical sample, which had been adjusted to pH 3.0 with HCl for 3 min and neutralized by KOH, was run against the same reference (—). Inset, difference spectrum between the two samples described above.
Influence of pH on Stability of AB·BlzOH

The complex is quite stable in the pH range 5.0 to 9.5. Below pH 5.0, AB·BlzOH dissociates irreversibly, liberating BlzOH which can be readily separated from the protein by filtration through a Sephadex G-100 column. The dissociation of the BlzOH from the protein can be observed visually since the color changes from a reddish orange to a distinct pink which remains after neutralization of the acidified solution. Fig. 2 shows the spectra of AB·BlzOH and an acid-treated sample of the complex. After dissociation of BlzOH from the protein there is a shift of the peak at 361 nm to 355 nm and a disappearance of two distinct maxima at 480 and 505 nm. The cobalamin compound was not identified by chromatography, but the spectrum of the dissociated B12 is identical with a spectrum of hydroxocobalamin obtained under the same conditions.

Molecular Weight Determination and Molar Ratio in AB·BlzOH

The molecular weight of the purified AB·BlzOH is 188,000 as determined by the sucrose gradient centrifugation method (10) with alcohol dehydrogenase as a standard. The partial specific volume of the AB·BlzOH calculated from its amino acid composition is 0.755 cm³ per g (Table II) (12). The specific data and details are presented in Fig. 3 and "Materials and Methods." As indicated in Table III, the results show that 1 mole of BlzOH is bound to 1 mole of the protein, AB.

Factors Necessary for Reactivation of AB·BlzOH

The replacement of BlzOH by DBCC on AB is essential to reactivate AB·BlzOH. As the data in Table IV illustrate, sulfitre, magnesium ion, and DBCC are necessary for the reactivation. Fig. 4 depicts the absolute requirement and concentration effect of K$_2$SO$_4$. The optimal concentration of magnesium ion (Fig. 5) (30 mM) for the reactivation of AB is relatively high compared to most magnesium-requiring systems. However, it should be emphasized that the enzymatic activity of AB does not require added magnesium ion. Since many bacterial species contain an enzyme system capable of converting BlzOH to DBCC, the possibility that BizOH bound to the apoenzyme could be converted directly to DBCC on the apoenzyme and thus produce an active holoenzyme was investigated (14).

![Graph](https://example.com/graph.png)

**Fig. 3.** Centrifugation patterns for AB·BlzOH. A 100-μg portion of AB·BlzOH (3 units per mg) and 200 μg of alcohol dehydrogenase in 0.1 ml of 0.01 M K$_2$HPO$_4$ pH 7.7, were layered on a 5 to 20% sucrose gradient in 0.01 M K$_2$HPO$_4$ pH 7.7. Thirty-two fractions of equal volume with a total volume of 4.7 ml were assayed for enzyme activity after 16 hours of centrifugation at 35,000 rpm. The fraction containing the greatest activity per fraction was taken as a base line of 100%, and all other fractions were expressed as a relative percentage of this base line. The fractionation was initiated from the bottom of the tube.

**TABLE III**

Molecular ratio of cobalamin to AB in AB·BlzOH

Electrophoretically pure AB·BlzOH (3 units per mg) was used in all cases. See "Materials and Methods" for details.

**TABLE IV**

Requirements for reactivation of AB·BlzOH compared with AB activity

The conditions and quantities of reagents were the same as given under "Materials and Methods" except that 1 mole of EDTA and 4 μg of pure (specific activity 3 units per mg) AB·BlzOH or 0.1 unit of dialyzed crude AB were used.

![Table](https://example.com/table.png)
and SO₄²⁻ to check for this possibility. No glycerol dehydrogenase activity was observed under these conditions. This evidence suggests that the reactivation of AB·B₁₂OH involves an exchange reaction between DBCC and B₁₂OH.

Further proof of an exchange reaction is given in Table V. The liberation of ⁶⁶Co-labeled B₁₂OH from labeled AB·B₁₂OH is 4 times greater in the presence of magnesium than the control without magnesium. The quantity of ⁶⁶Co in the labeled AB·B₁₂OH could not be increased if the labeled AB·B₁₂OH was incubated with ⁶⁶Co-labeled DBCC. The specific activity of the labeled B₁₂OH was the same as the labeled DBCC. Therefore, the results from this experiment also suggest that replacement of B₁₂OH by DBCC occurs at the same site on the apoenzyme. Based on the slopes of the curves in Fig 6, the rate of holoenzyme formation and enzymatic reaction is approximately 22 times greater for AB than AB·B₁₂OH, which suggests that the dissociation of the B₁₂OH from AB is the rate-limiting reaction in the conversion of the AB·B₁₂OH to holoenzyme.

**Table V**

**DBCC exchange in AB·B₁₂OH by use of ⁶⁶Co cobalamins**

Crude AB was incubated with ⁶⁶CoB₁₂OH to yield AB·⁶⁶CoB₁₂OH. The excess of ⁶⁶CoB₁₂OH was removed with a column of Sephadex G-50. The AB·⁶⁶CoB₁₂OH was divided into three 5-ml aliquots. K₂SO₄, 250 μmoles, was added to all three. Magnesium acetate (150 μmoles), 10 μmoles of ⁶⁶CoDBCC, or unlabeled DBCC was added as indicated below. After a 3-hour incubation period, all three lots were fractionated with a column (3 x 50 cm) of Sephadex G-50. The total radioactivity of the protein and the nonprotein fractions were then determined. The specific activity of the radioactive cobalamins used was identical.

| Experiment | Amount contained in protein | Amount liberated from protein |
|------------|----------------------------|------------------------------|
| AB·⁶⁶CoB₁₂OH + DBCC | 8700 | 1500 |
| AB·⁶⁶CoB₁₂OH + Mg²⁺ + ⁶⁶CoDBCC | 9500 | |
| AB·⁶⁶CoB₁₂OH + Mg²⁺ + DBCC | 3600 | 6100 |

A very marked difference in the thermal stability of AB and AB·B₁₂OH was observed under the specified experimental conditions.

**Fig. 7.** Thermal stability of AB and AB·B₁₂OH at different temperatures. Samples of 0.2 ml of solution containing 100 μmoles of K₂HPO₄ (pH 8.6), 0.1 unit of crude dialyzed AB, or 0.024 unit of crude AB·B₁₂OH were heated for 4 min at various temperatures and cooled, and the activity was checked by standard methods. The inserted graph, reprinted from Reference 15, shows the thermal stability of the subunits A and B.
ditions (Fig. 7). Previous work (4) showed that the thermal lability of AB is probably due to the properties of subunit A since subunit A has minimal stability at 70° whereas B is relatively stable under the same conditions (see inset in Fig. 7). Since previous work (2) had shown the effects of the sodium and the potassium ions on the dissociation and association of the apoenzyme, the effects of these 2 ions on the stability of AB-B2OH were investigated. As shown in Fig. 8, potassium ions stabilize AB-B2OH at 78° whereas the sodium ions have no stabilizing effect. An 8-fold increase in the stability of AB-B2OH is achieved if the potassium concentration is increased from 0.02 M to 0.8 M K2HPO4. No significant difference in thermal stability of AB-B2OH in the pH range 6.0 to 8.8 was observed with potassium phosphate buffers.

**Effect of —SH Inhibitors**

p-Chloromercuribenzoate, AgNO3, and HgCl2 have a greater inhibitory effect on the activity of AB than on AB-B2OH. The greatest inhibition of AB and AB-B2OH is obtained with HgCl2 (Table VI).

**Table VI**

| Inhibitor | Inhibitor concentration | Activity remaining |
|-----------|--------------------------|--------------------|
|           | AB | AB-B2OH |
| CMBa      | 1.0 | 0 | 45 |
|           | 0.1 | 0 | 51 |
|           | 0.01 | 86 | 99 |
|           | 0.01 | 130a |
| AgNO3     | 0.1 | 3 | 81 |
|           | 0.01 | 74 | 90 |
| HgCl2     | 0.01 | 0 | 30 |
|           | 0.001 | 83 | 100 |

*p-Chloromercuribenzoate.

Activity was estimated after dialysis.

**Inhibition Effect of EDTA and Salicylic Acid**

Because of the possible involvement of a divalent metal ion in the protein subunit structure or enzyme activity (or both), the influence of the following chelating agents on the activity of the enzyme was investigated: 8-hydroxyquinoline, 1,10-phenanthroline, α,α′-dipyridyl, diethyldithiocarbamate, glycine, histidine, 2,4-pentaneione, 1,4-diaminobutane, inositolhexaphosphoric acid, salicylic acid, and EDTA. EDTA and salicylic acid were the only chelating agents tested which had a significant inhibitory effect. Fig. 9 illustrates the effect of increasing concentrations of EDTA on enzyme activity. This figure also shows that the inhibitory influence of EDTA could be completely reversed by Mg++ in adequate concentrations. Other divalent ions such as Ca++, Mn++, Zn++, and Co++ also reverse the EDTA inhibitory effect. Table VII contains data showing that glycerol reverses the inhibition by EDTA at the specified concentrations. The inhibition by EDTA and salicylic acid can also be overcome by adding an excess of either protein subunit as shown by the data presented in Table VIII.

**Dissociation of AB by EDTA**

EDTA at a concentration of 0.01 M and 0.1 M glycerol and K2HPO4 cause the complete dissociation of the apoenzyme, AB, into the protein subunits, A and B, on Sephadex G-100, as shown in Fig. 10, whereas a control without EDTA yields 90% of the undissociated form, AB. Removal of EDTA by dialysis prior to gel filtration also yields the undissociated form of AB. In contrast to the effect produced on AB, AB-B2OH is not dissociated on a Sephadex G-100 column equilibrated with EDTA under the same conditions.
TABLE VII
Reversal of EDTA inhibition by glycerol

Crude dialyzed AB, 0.13 unit, was used with specified concentrations of glycerol and EDTA. The apoenzyme was previously incubated with EDTA for 10 min before adding the glycerol. Other conditions were the same as the standard assay except that a 10-min incubation period was used. The remaining activity was expressed as percentage of the control containing the same amounts of glycerol without EDTA.

| Concentration of glycerol | Remaining activity |
|---------------------------|--------------------|
| mM | 10 µM EDTA | 20 µM EDTA |
| 0.5 | 13 | 7 |
| 1.0 | 25 | 13 |
| 1.5 | 27 | 15 |
| 2.0 | 37 | 28 |
| 2.5 | 41 | 34 |

TABLE VIII
Influence of A:B ratio on inhibitory effect of EDTA and salicylic acid

Subunits A and B in the ratios indicated in the table were previously incubated for 10 min prior to the addition of the inhibitor. Another 10-min period of preliminary incubation was used after addition of the inhibitor, followed by determination of enzyme activity under the standard conditions, except that a 10-min incubation period was used instead of the usual 30-min period. The enzyme activity was expressed as the percentage of activity recovered relative to an identical control except for the absence of inhibitor.

| Activity of the subunits added | A:B ratio | Activity recovered | Inhibitor concentration |
|-----------------------------|-----------|--------------------|------------------------|
| A | B | mM EDTA | salicylic acid |
| 0.19 | 0.19 | 1:1.0 | 52 | 0.25 |
| 0.19 | 0.30 | 1:2.0 | 75 | 0.39 |
| 0.19 | 0.58 | 1:3.1 | 100 | 0.25 |
| 0.17 | 0.12 | 1.5:1 | 24 | 0.5 |
| 0.26 | 0.12 | 2.1:1 | 38 | 0.5 |
| 0.38 | 0.12 | 3.1:1 | 67 | 0.5 |
| 0.44 | 0.12 | 3.7:1 | 70 | 0.5 |

Influence of High Concentrations of Potassium Phosphate on Dissociation of AB and AB-B$_3$OH

As previously described (2) potassium phosphate at pH 8.6 maintains the associated form of the apoenzyme; however, at higher concentrations, e.g. 0.3 M potassium phosphate, 40%
that a divalent cation is essential for enzyme activity. The use of a number of chelating agents and exhaustive dialysis by EDTA is that a divalent metal ion is essential for optimal enzyme stability or activity (or both). However, in this case the sharply contrasting effects of the Na$^+$ and the K$^+$ ions on the reversible dissociation of AB into subunits, A and B, are an observation that has not been previously published for other enzymes. Although a number of enzymes are known in which the Na$^+$ and the K$^+$ have an antagonistic effect on activity (19), these effects have not been related to the dissociation of the enzyme into subunits.

The usual interpretation of the inhibition of enzyme activity by EDTA is that a divalent metal ion is essential for optimal enzyme stability or activity (or both). However, in this case the use of a number of chelating agents and exhaustive dialysis after EDTA treatment has thus far yielded no evidence indicating that a divalent cation is essential for enzyme activity. The requirement for Mg$^{2+}$ is limited to the exchange reaction involved in the conversion of the inactive AB-B$_2$OH to the active holoenzyme. Further evidence for a nonmetal effect of EDTA is the reversible dissociation of the AB into subunits by EDTA, presented in this paper. The reversal of the EDTA inhibition by the addition of either subunit in excess suggests a reversible association of inhibitor with subunits. Previous work (2) showed that glycerol increases the affinity of the subunits for each other, thus shifting the equilibrium toward the associated form, which may explain the reversal of the EDTA effect by glycerol.

Several observations have been published on the prevention of association of proteins by EDTA which were not related to its chelating properties. Shabad and Lauffer (20) found that EDTA retarded polymerization of tobacco mosaic virus protein. Yue, Nolkmann, and Kuby (21) found that EDTA completely inhibited the TPN-induced association of the apoprotein of glucose 6-phosphate dehydrogenase, but had no inhibitory effect on enzymatic activity.

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**TABLE IX**

Summary of differences in properties of AB-B$_2$OH and AB

| Factors                        | AB-B$_2$OH | AB       |
|-------------------------------|------------|----------|
| Stoichiometry                 | Not sensitive | Sensitive |
| Thermal stability at 70°C     | Quite stable | Very unstable |
| Reversible inhibition by EDTA | Complete inhibition reversible with excess of Mg$^{2+}$ | Complete inhibition reversible with divalent ions |
| pH-dependent stability        | Stable     | Complete irreversible inactivation |
| Dissociation into subunits A and B | Stable at pH 6.0-8.8 | Unstable in pH <8.0 and >9.0 |
| 1. pH < 6.0                   | Undissociated | Dissociates |
| 2. Na$^+$ 25°C                | Undissociated | Dissociates |
| 3. Salt concentration, >0.3 M | Undissociated | Partially dissociates |
| 4. EDTA                       | Stable     | Dissociates |
| Lyophilization stability      |            | Stable   |
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CORRECTIONS

In the paper by M. K. Sahib and C. R. Krishna Murti (Vol. 244, No. 17, Issue of September 10, 1969, page 4730), on page 4733, line 22 in the right-hand column, the sentence beginning “Histidine pyruvate aminotransferase ...” should be replaced with the following:

“In contrast to histidine ammonia lyase, histidine pyruvate aminotransferase did not alter either with the age of the rats or the protein content of the diets ingested by them, as reported earlier by Rao, Deodhar, and Hariharan (22a).”

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In the paper by Philip T. Cohen and Nathan 0. Kaplan (Vol. 245, No. 11, Issue of June 10, 1970, page 2825), the first author’s given name is spelled incorrectly. It should be “Philip” not “Phillip.” On page 2835, right-hand column, the word “not” should be inserted in line 8 of the second paragraph of the “Discussion” so that the sentence reads:

“Two lines of evidence led the earlier workers to believe the enzyme was not a flavoprotein.”

In the paper by Zenon Schneider, Earl G. Larsen, Gail Jacobson, B. Connor Johnson, and J. Pawelkiewicz (Vol. 245, No. 13, Issue of July 10, 1970, page 3388), the definition of DBCC given in Footnote 1 on page 3388 is incorrect. The correct definition is:

“DBCC, 5,6-dimethylbenzimidazolylcobamide 5’-deoxyadenosyl coenzyme.”

In the paper by C. Thomas Huber and Earl Frieden (Vol. 245, No. 15, Issue of August 10, 1970, page 3979), Equation 1 in the right-hand column on page 3981 is incorrect and should be replaced by the following:

$$\frac{[E]}{V_{\text{max}}} = \frac{1}{240} \frac{1}{k_{\text{HI}}} + \frac{(K_N[I] + K_NK_o[I])/(K_XV + k_{XV})}{K_XV}$$

(1)