THE *STREPTOCOCCUS FAECALIS* OXIDASES FOR REDUCED DIPHOSPHOPYRIDINE NUCLEOTIDE

VI. INHIBITION OF THE FLAVOPROTEIN PEROXIDASE BY ITS ELECTRON DONOR

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SUMMARY

When the flavoprotein, DPNH peroxidase, is incubated with DPNH, enzyme activity slowly decays with first-order kinetics ($k = 0.066$ min^{-1}, pH 5.4). The cosubstrate, hydrogen peroxide, can prevent but cannot reverse the inhibition. The kinetics of the inactivation were investigated and shown to be consistent with the following mechanism. (a) DPNH combines with the enzyme to form a binary complex that is kinetically active and has a characteristic absorption spectrum. (b) In the absence of peroxide, the complex is slowly and irreversibly converted to an inactive form. (c) Hydrogen peroxide prevents the inhibition by combining with the binary complex to form a ternary complex that dissociates into products. The ternary complex is not inactivated except at very high DPNH concentrations; this type of inactivation is reversible.

INTRODUCTION

The enzyme DPNH peroxidase is an FAD-containing flavoprotein[1,2] that catalyzes the reaction

$$DPNH + H^+ + H_2O_2 \rightarrow DPN^+ + 2H_2O$$

Previous work from this laboratory showed that the mechanism of the reaction involves the formation of a spectrophotometrically visible complex between DPNH and flavoprotein[2,3]. Evidence that this binary complex is kinetically active has been summarized elsewhere[4,19]. Kinetic data suggest that the binary complex binds peroxide to form a rate-limiting ternary complex, which then decomposes into products[2,4].

In the present report, evidence for a rather unusual type of substrate inhibition is presented. If the oxidant (H_2O_2) in this two-substrate reaction is not present, the reductant (DPNH) causes a slow conversion of the enzyme to an inactive form. The inactivation results either from an irreversible binding of DPNH to the enzyme or

Abbreviations used: DPNH, reduced diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; and FAD, flavine adenine dinucleotide.

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from a DPNH-dependent irreversible inactivation of an unspecified nature. Peroxide can arrest or prevent, but it cannot reverse the inactivation. Although many types of substrate inhibition are known[5], the situation just summarized seems not to have been encountered previously for any two-substrate reaction.

METHODS

Assay of inhibited enzyme

The peroxidase assay has been described previously[2]. To follow the kinetics of inactivation, enzyme and DPNH were preincubated in 3-ml cuvettes in the Beckman spectrophotometer, at 24°, in the absence of peroxide. Standard assay conditions were used, except that peroxide was absent and the DPNH level was adjusted to some specified concentration (final volume, 2.9 ml). Concentration profiles (varying DPNH concentration and constant preincubation time) and time profiles (constant DPNH concentration and varying time of preincubation) were determined. At the end of the preincubation period, the active enzyme remaining was assayed by the standard technique. 0.1 ml of a DPNH–H_2O_2 solution was added to bring the DPNH and peroxide concentrations up to the assay level ($8 \cdot 10^{-5}$ *M* DPNH; $1.33 \cdot 10^{-3}$ *M* H_2O_2). On addition of peroxide, the inhibition stopped immediately and the kinetics for DPNH oxidation were zero order.

Materials

Highly purified DPNH peroxidase (minimum molecular weight approximately 120,000, based on the FAD content) was prepared as described previously[1,2]. DPN and TPN were purchased from Pabst Laboratories. DPNH was prepared from DPN with yeast-alcohol dehydrogenase[6] and TPNH from TPN with the alcohol dehydrogenase of *Leuconostoc mesenteroides*[7]. The reduced nucleotides were isolated and stored as the barium salts[6]. Just prior to use, they were converted to the potassium salts by precipitation of the barium with K_2SO_4.

EXPERIMENTAL

Inhibition of DPNH peroxidase by DPNH

The data of Fig. 1 demonstrate some of the main features of the inactivation phenomenon. Curve A shows that the inactivation obtained by preincubating DPNH and enzyme for 10 min (in the absence of peroxide) is a function of the DPNH concentration in the preincubation mixture. At very low DPNH concentrations, the percentage inactivation is proportional to the DPNH concentration, but at concentrations in excess of $1 \cdot 10^{-6}$ *M*, the inactivation becomes virtually independent of the DPNH level. The percentage inactivation at which saturation occurs depends on the period of preincubation of DPNH and enzyme—the longer the preincubation period, the greater the percentage inactivation obtained at saturation. The kinetics of decay in the region of DPNH saturation are shown in curve B. In a 30-min period, during which 87 % of the initial enzyme activity was lost, the decay of activity followed first-order kinetics. Virtually the entire course of the reaction, therefore, can be described by these simple kinetics and there seem to be no complicating side effects. The results shown in curves A and B suggest that DPNH combines with the

flavoprotein to form a complex that, in the absence of peroxide, is slowly converted with first-order kinetics ($k = 0.065$ min^{-1}) to an inactive form. The half-saturation value for DPNH determined from curve A ($0.37 \cdot 10^{-6}$ M) is in good agreement with the dissociation constant determined from a more rigorous mathematical treatment discussed in a later section.

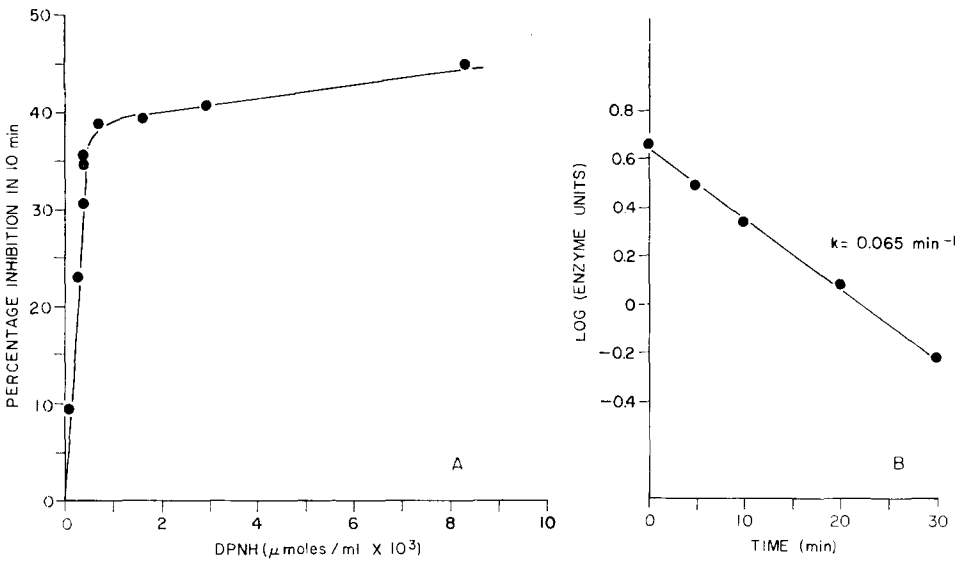

Fig. 1. Inhibition of DPNH peroxidase by DPNH. Standard conditions (METHODS section) were used to measure the activity remaining after preincubation of enzyme and DPNH. Temperature, 24°, pH 5.4. Curve A: DPNH peroxidase, equivalent to $1.5 \cdot 10^{-9}$ M bound FAD, incubated 10 min with DPNH in concentrations shown. Volume, 2.9 ml. At the end of the preincubation period, 0.1 ml of a DPNH–H_2O_2 mixture was added to complete the assay system. Curve B: DPNH peroxidase concentration as for curve A, incubated with DPNH, $8.3 \cdot 10^{-5}$ M. The peroxidase activity remaining at the times indicated was assayed after the addition of H_2O_2, $1.33 \cdot 10^{-3}$ M (final concentration).

Protective effect of H_2O_2

As mentioned in the METHODS section, the addition of peroxide to the enzyme–DPNH incubation mixture immediately stops the inactivation reaction. It is this that makes possible the accurate assay of active enzyme in the experiments described here. Hydrogen peroxide can also protect the enzyme against inactivation during prolonged exposure to DPNH. These results are shown in Fig. 2. First, it is evident that incubation of enzyme alone, or enzyme plus peroxide, causes only a small loss in activity. When the flavoprotein is exposed to DPNH, activity decays in the usual manner; however, in the presence of enough peroxide to ensure that the flavoprotein is all in the form of its ternary complex (composed of enzyme, DPNH, and peroxide) most of the decay in activity is prevented. The loss in activity is a little greater than that which takes place in the presence of peroxide alone. In this experiment, the addition of peroxide completes the assay system, and DPNH oxidation takes place at a constant rate. During the entire time course of the oxidation, however, the DPNH level was deliberately maintained above saturation (for both the inactivation and

peroxidase reactions) so that the protective effect of peroxide cannot be ascribed to a decrease in the DPNH concentration. The data are in accord with the hypothesis that peroxide exerts its protective effect by combining with the binary complex of flavoprotein and DPNH to form a ternary complex that does not undergo inactivation.

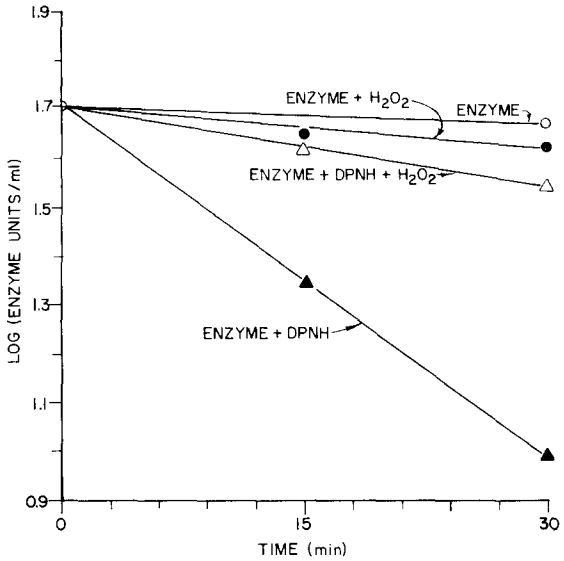

Fig. 2. Inactivation of DPNH peroxidase: protection by H_2O_2. Enzyme, equivalent to $0.89 \cdot 10^{-9}$ *M* bound FAD, was incubated with the components indicated, and at 15 and 30 min, enzyme assays were performed (standard conditions). ○, DPNH peroxidase; ●, DPNH peroxidase + H_2O_2, $1.8 \cdot 10^{-3}$ *M*; △, DPNH peroxidase + DPNH + H_2O_2 (DPNH concentration was maintained above $5 \cdot 10^{-5}$ *M* during the course of the experiment; H_2O_2, $1.8 \cdot 10^{-3}$ *M*); ▲, DPNH peroxidase + DPNH, $2.2 \cdot 10^{-5}$ *M*.

Attempts to reverse the inactivation

All attempts to reverse the inhibition brought about by DPNH have been unsuccessful. The inhibited enzyme is neither reactivated by dialysis nor by incubation with the known flavins, with hot-water extracts of *Streptococcus faecalis* cells, or with the various metal ions. Fluorometric[8] and enzymic analysis[9] show that there is no detectable difference in the FAD content of active and DPNH-inhibited enzyme.

Substrate specificity for inactivation

Neither DPN nor the primary acid modification product of DPNH is inhibitory[10]. The loss of inhibitory activity on conversion of DPNH to the modification product (presumably the trihydromonohydroxy nicotinamide analogue of DPN[11]) is evidence that the inhibitor is DPNH itself and not an incidental contaminant of the DPNH preparation. To obtain further information on this point, the following experiment was performed. DPNH was prepared from 95 % pure DPN with alcohol dehydrogenase. Two control reaction mixtures were run through the entire alcohol dehydrogenase procedure, one without alcohol dehydrogenase and one without DPN. After the DPN in the complete system had been converted to DPNH, the three reaction

mixtures were heated at 100° for 3 min, cooled to 0°, and centrifuged. When aliquots of the DPNH-containing reaction mixture were tested for their inhibitory effect they gave a saturation curve identical, within experimental error, to the one shown in Fig. 1. Aliquots from the two control reaction mixtures were not inhibitory. The results, therefore, indicate that the true inhibitor is the reduced nucleotide.

TPNH can also function as an inhibitor of the flavoprotein, however the half-maximum saturation value with this nucleotide is $3.3 \cdot 10^{-5}$ *M*, approximately 90 times as great as that found for DPNH. It had been known previously that TPNH could interact with DPNH peroxidase; TPNH is a substrate for the weak oxidase activity of the enzyme[2] but is a very poor peroxidase substrate. TPNH can also form a kinetically active, visible complex with DPNH peroxidase[4]. Comparison of the dissociation constants of the visible complexes formed between the flavoprotein and either DPNH or TPNH shows that the ratio of the constants is almost the same as the ratio of the half-maximum saturation values found for these nucleotides in the inhibition reaction.

A reversible inhibition caused by DPNH

A second type of inhibition caused by DPNH can be recognized. This takes place at high DPNH concentration in the presence of H_2O_2 and is reversible. Fig. 3, a plot of DPNH peroxidase activity *vs.* DPNH concentration, illustrates the effect. The plateau region of the saturation curve extends to about $5 \cdot 10^{-4}$ *M* DPNH. Higher concentrations of DPNH cause immediate inhibitions, which seem to be directly proportional to the DPNH concentration. In this region of the curve, as DPNH is used up in the peroxidase reaction, the reaction rate increases, and when the DPNH concentration falls below $5 \cdot 10^{-4}$ *M*, the velocity is almost at the experimentally

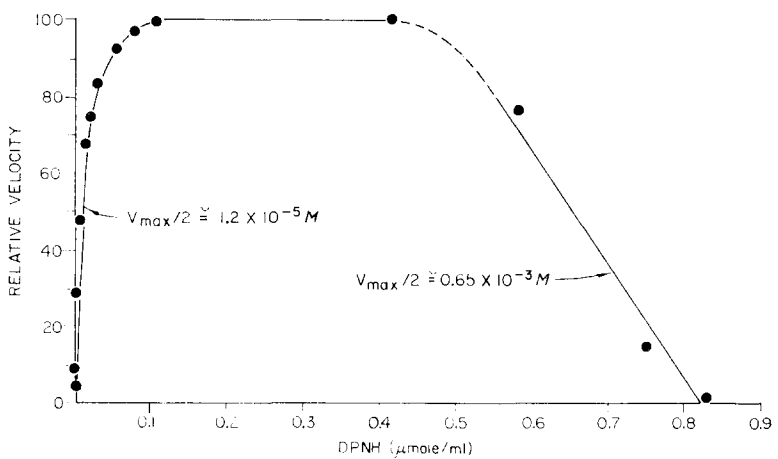

Fig. 3. DPNH peroxidase activity vs. DPNH concentration. Enzyme equivalent to $1.32 \cdot 10^{-9}$ *M* bound FAD. Standard peroxidase assay system, except for DPNH concentration, as shown.

obtainable maximum. The half-maximum saturation value for DPNH as an inhibitor in this reaction is about 55 times as great as its half-maximum saturation value as a substrate. The reversible inhibition demonstrated here may reflect the binding of an additional molecule of DPNH to the ternary complex.

Test of a proposed mechanism for the inhibition reaction

The data that have been presented thus far suggest the following mechanism for the irreversible inhibition and its prevention by peroxide:

$$\begin{array}{l} E + DPNH \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\cdot DPNH \xrightarrow{k_3} EI \\ \quad\quad - H_2O_2 \uparrow\downarrow H_2O_2 \\ \quad\quad\quad E\cdot DPNH\cdot H_2O_2 + H^+ \xrightarrow{k_6} E + DPN^+ + 2H_2O \end{array}$$

Enzyme and DPNH combine to form the binary complex E·DPNH. This is the complex that has the characteristic absorption spectrum previously dexscribed[2,3] and normally would combine with peroxide to form the ternary complex E·DPNH·H_2O_2. Decomposition of the latter complex yields DPN and $2H_2O$. If, however, peroxide is not present, the binary complex is slowly converted to the inactive form, EI, in an irreversible step. The following equation is a linear form of the integrated rate equation for the proposed mechanism (inactivation in the absence of peroxide):

$$\frac{1}{\ln [E_0|(E_0 - EI)]} = \frac{1}{k_3 t} + \frac{1}{S}\left(\frac{k_2}{k_1} \times \frac{1}{k_3 t}\right)$$

Where E_0 is total enzyme (initial enzyme activity); EI, inactive enzyme (initial enzyme activity minus activity remaining at time t); and S, DPNH concentration*.

A plot of the reciprocal of $\ln[E_0/(E_0 - EI)]$ *vs.* $1/S$ is a straight line, with the y intercept equal to $1/k_3 t$ and the slope equal to $k_2/k_1 \times 1/k_3 t$. Thus, the first-order rate constant, k_3, and the dissociation constant, k_2/k_1, can be obtained from this treatment. The experimental data necessary for testing the mechanism can be obtained by

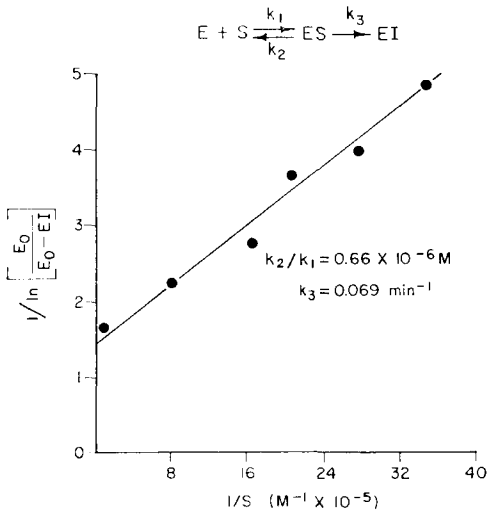

Fig. 4. Kinetics for inactivation of DPNH peroxidase. Enzyme equivalent to $1.66 \cdot 10^{-9}$ M bound FAD, incubated 10 min with DPNH in concentrations shown (sodium acetate buffer pH 5.4, 0.033 M) and then assayed in standard assay system.

* The author is indebted to Dr. W. A. ARNOLD, Biology Division, Oak Ridge National Laboratory, for deriving this rate equation. In the derivation, it was assumed that the value of k_3 (0.066 min^{-1}) is negligible when compared with the magnitude of the other rate constants.

determining the amount of active enzyme left after a given period of exposure of enzyme to a series of DPNH concentrations. Fig. 4 demonstrates that the experimental data are consistent with the proposed mechanism, at least over a 40-fold range of DPNH concentrations. The rate constant for inactivation ($k_3 = 0.069\ min^{-1}$) is in good agreement with the constant evaluated by following enzyme decay at saturating DPNH concentrations (Fig. 1). A summary of two experiments is shown in Table I. One set of data was obtained under conditions in which the enzyme shows its maximum activity (pH 5.4, acetate buffer) and the other under conditions in which peroxidase activity is low (pH 7.1, phosphate buffer). It is interesting that there is a reciprocal relation between the constants k_3 and k_6. Change of conditions from pH 7.1 (phosphate) to pH 5.4 (acetate) causes an eightfold increase in k_6 and a fivefold decrease in k_3. That is, the very conditions that cause a more rapid dissociation of the ternary complex into products also aid in maintaining the binary complex in an active form.

TABLE I

KINETIC CONSTANTS FOR DPNH PEROXIDASE

Conditions as for Fig. 4, except that for the experiment at pH 7.1, potassium phosphate (0.066 *M*) was the buffer in both the preincubation and assay phases of the experiment.

pH	k_2/k_1 *(Mole/liter)*	k_3 *(min^{-1})*	k_6 *(min^{-1})*
5.4	$0.66 \cdot 10^{-6}$	0.069	$6 \cdot 10^{3}$
7.1	$1.9 \cdot 10^{-6}$	0.34	$0.78 \cdot 10^{3}$

The ratio k_2/k_1 should be the true dissociation constant of the E·DPNH complex. As might be expected, this value is lower than the Michaelis constants obtained when either peroxide of ferricyanide serve as oxidants for DPNH oxidation[2]. Estimates for some of the individual rate constants in the mechanism just postulated have been given elsewhere[4].

Relation between the inhibited form of the enzyme and the visible complex

When substrate amounts of DPNH peroxidase are used, it can be demonstrated that the enzyme forms a spectrophotometrically visible complex with DPNH[2,3]. Three lines of evidence indicate that the visible complex is not identical with EI, the inhibited form of the enzyme. (a) Under the conditions in which the visible complex is formed (*i.e.*, with about 10^4 times the enzyme concentration used to demonstrate the inhibition reaction) inhibition either does not take place or else is reversible on dilution of the enzyme. (b) The rate constant for formation of inactive enzyme, 0.066 min^{-1} (average of 5 experiments), is equivalent to a change of 6.4 %/min (pH 5.4). At the same pH, the spectrum of the complex is fully developed as soon as DPNH is added to the flavoprotein. (c) The dissociation constants of the visible complexes formed between DPNH peroxidase and a series of DPNH analogues agree very well with the Michaelis constants derived from kinetic studies[4]. This correlation is strong evidence that the visible complex is kinetically active.

Evidence on the first point was obtained by incubation of DPNH peroxidase ($7 \cdot 10^{-5}\ M$) with an excess of DPNH under conditions that would have caused 70 % inhibition of dilute enzyme solutions. At the end of the incubation period, the enzyme

was diluted in peroxide-containing buffer (H_2O_2 was present to prevent the DPNH-dependent inactivation that takes place at low enzyme concentrations) and assayed for peroxidase activity. There was no detectable loss in activity. It seems, therefore, that, at high enzyme concentrations, the peroxidase is able to protect itself against the inactivation. It is possible that dilution of the enzyme causes a slight conformation change that makes the enzyme susceptible to substrate inhibition, whereas at high protein concentration, the enzyme conformation might prevent such inhibition. The data of Table I suggest that the binary complex may be stabilized in an appropriate environment.

DISCUSSION

The inhibition that has been described does not seem to be identical with any reported previously, although there are superficial similarities to other systems. For instance, the active catalase·H_2O_2 complex (complex I) can be slowly, but reversibly, transformed into the inactive complex II under conditions in which the peroxide concentration is maintained at a low, steady-state level for extended periods of time[12]. Certain copper-containing oxidases are progressively inactivated when they function as catalysts[13]. The kinetics of this "reaction inactivation" have been intensively investigated[13,14]. Among the flavoproteins, the DPNH oxidase of *Clostridium perfringens* undergoes a type of inhibition more nearly like the one described in the present paper[15]. The oxidase is slowly inhibited by DPNH in the absence of the oxidant (O_2). Inhibition, however, also takes place when the enzyme is "turning over". Under conditions in which the oxidase catalyzes DPNH oxidation, oxidase activity decays with first-order kinetics. Unlike DPNH peroxidase, the oxidase of *C. perfringens* is very sensitive to peroxide; the rate constant for inactivation of the oxidase increases as the peroxide level is raised.

Thus there are several examples of substrate-induced slow decay of enzyme activity not attributable to product formation. The interesting feature of the DPNH peroxidase system is that the decay is almost completely prevented by the cosubstrate, peroxide. This circumstance makes possible a complete separation of the kinetics of inactivation from those of substrate turnover. As previously discussed, the results of kinetic studies are consistent with a simple mechanism—namely, that DPNH combines with the enzyme to form a binary complex that is initially kinetically active but in the absence of peroxide is slowly converted to an inactive form. Peroxide prevents the inhibition by maintaining the enzyme in the form of its ternary complex. The cause of the inhibition is unknown. Since the inactivation is not reversed by dialysis, it may be that DPNH becomes very tightly bound to the enzyme and can no longer function as a substrate, however, there are obviously alternative interpretations. For instance, the binding of small molecules to protein or of substrate to enzyme can apparently cause conformation changes in the protein[16–18]. The manner in which a change in conformation may enhance enzyme activity has been discussed[16]. For certain two-substrate reactions, it is conceivable that a conformation change induced in the enzyme by one substrate could make the enzyme liable to denaturation unless the cosubstrate were present. It would be interesting to know whether any other two-substrate reactions show the behavior described for the DPNH-peroxidase system.

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THE EXTENT OF ENERGY MIGRATION AND CHLOROPHYLL *A* ORIENTATION IN CHLORELLA

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SUMMARY

1. A method is described by which $\bar{n}$, the average number of energy transfers between different chlorophyll *a* molecules during the mean lifetime of the excited state, can be measured *in vivo*. The effect produced by added quencher molecules on the fluorescence excitation spectrum and yield enables $\bar{n}$ to be calculated without assumptions regarding the mutual orientation of the pigment molecules.

2. The calculated mean value of $\bar{n}$ was 275. Although subject to considerable errors, this value is comparable with $\bar{n}_r$ measured in Chlorella and in concentrated chlorophyll solutions by fluorescence depolarization. Because $\bar{n}_r$ is calculated on a basis of random molecular orientation, the chlorophyll *a* molecules *in vivo* cannot possess a high degree of preferred orientation.

3. The time interval occupied by each transfer process is calculated, together with the trapping efficiencies to be expected *in vivo*.