RUSSIAN CHEMICAL REVIEWS
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November 1979

Translated from *Uspekhi Khimii*, 48, 1921-1942 (1979)

U.D.C. 577.15.035+541.14+535.247+77.01

Artificial Light-sensitive Enzymatic Systems as Chemical Amplifiers of Weak Light Signals†

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The chemical principles of the amplification of weak light signals, based on photochemical and coupled chemical reactions, are examined. Systems where the dark amplification process is a catalytic reaction involving enzymes are discussed in greatest detail. Artificial enzymatic systems in which the level of catalytic activity can be varied under the influence of light is subjected to a critical analysis.
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I. INTRODUCTION

The reception of light signals constitutes the basis of a whole series of most important practical processes (ranging from photography to photoelectron multipliers) as well as unique natural phenomena such as vision, photosynthesis, phototaxis, etc. Usually (both in practical applications by man and in living nature) the primary received light signal is fairly weak and for its effective detection and utilisation it must be subjected to preliminary amplification in the receiving system.

Amplifiers are devices which amplify the transmitted power (in the given case the received light signal) by means of the energy of an extraneous source. Depending on the nature of this source, all amplifiers of light signals can be divided into physical (employing physical amplification principles, which are utilised, for example, in electronic devices)[1] and chemical (using chemical amplification principles, i.e. principles based on photochemical and coupled chemical reactions). In this review we shall consider only chemical amplifiers, because in the literature devoted to a general consideration of this problem unduly little attention has been given to such amplifiers.

The operation of any light-sensitive chemical system must include two stages: the absorption of light and the chemical reaction induced thereby. The amplification factor of a light signal in such a system can be naturally defined as the ratio of the number of molecules reacting

† Certain general aspects of this problem were discussed in our recent communication: K. Martinek and I. V. Berezin, Photochem. Photobiol., 29, 637 (1979).

under the influence of light (or the number of molecules of the reaction products formed) to the number of absorbed light quanta: the greater this quantity, the greater the light sensitivity of the system, i.e. the smaller the number of quanta which it can detect in principle. However, in conformity with the Stark–Einstein photochemical equivalence law, the yield of the primary photochemical reaction cannot be greater than unity. In this case the absorbed light quantum cannot give rise to more than one molecule of a chemical substance, so that the problem of the detection of a small number of light quanta is therefore transformed into the problem of the detection of a small number of molecules, which is just as difficult. Only a system where the primary intrinsic photochemical process is coupled to a subsequent "dark" process can therefore have a high amplification factor (i.e. an effective quantum yield exceeding unity). Ultimately the efficiency of the amplification will depend mainly on the nature of this secondary (dark) process. We shall examine certain examples.

Classical chemical amplifiers of light signals are silver halides suspended in gelatin gels, which are very widely used for the production of photographic images[2,3]. After absorbing a light quantum, the silver halide decomposes, giving rise to silver atoms, which play the role of catalysts of the subsequent dark development process. Another example of the chemical amplification of light signals is provided by the well known chain reactions induced by light[4]. The absorbed light quantum generates free radicals in the system, which then propagate the chemical chain growth stages (dark process).

In principle, the nature of the dark process which follows the primary photochemical reaction can be also non-chemical[2,3,5] (for example, in the "bubble" method based on the photochemical decomposition of azo-compounds with subsequent thermal expansion of nitrogen bubbles in a polymer film). However, the sensitivity (and sometimes also the resolving power) of the latter systems is low. Systems with a light-induced chain reaction, where the chain termination stages inevitably lead to a decrease of the amplification factor, suffer from the same disadvantage. On the whole, after analysing these and other examples[2,3,5], we concluded that one should regard as optimum from the standpoint of sensitivity systems with a *catalytic* dark process, which functions in accordance with the following general mechanism: catalyst molecules are generated under the influence of light, whereupon (in the dark reaction) they catalyse the chemical transformation of a substance present in the system (insensitive to light); in principle, the "turnover number" of a single catalyst molecule is unlimited (if it is possible to avoid the "poisoning" of the catalyst) and the amplification factor can therefore be as large as is required.

The principal and unfortunately evidently insurmountable disadvantage of silver halide catalytic system is that the world resources of silver are now being exhausted[5]. This makes it necessary to seek other, non-silver light-sensitive catalytic systems. There is no review in the literature dealing with the possibility of using biological catalysts for this purpose, but we believe that enzymes in fact give rise to excellent prospects in this field. Our confidence is based on the fact that, firstly, enzymes have a catalytic activity exceeding by many orders of magnitude that of the usual chemical catalysts[6] and, secondly, owing to their unique macromolecular structure, their action can be regulated in very fine detail, i.e. one can alter sharply and adequately the catalytic activity (and other processes) under the influence of even slight changes in the external medium[7]. On this basis, we put forward, justified, and applied experimentally the principle of the applification of light signals with the aid of light-sensitive enzymatic systems[8–10].

II. THE IDEA OF A CHEMICAL AMPLIFIER OF WEAK LIGHT SIGNALS USING ENZYMES

In order to elucidate the essential feature of the proposed approach, we shall consider a system containing a catalytically inactive derivative of an enzyme E_{inact} (which, however, is capable of being converted into an active catalyst E under the influence of light) and a substrate S of the given enzyme. When such a system is illuminated with light at the appropriate wavelength, the photochemical [reaction (1)] and dark [reaction (2)] reactions begin to take place in it:

$$E_{inact} \xrightarrow{light} E, \qquad (1)$$

$$E + S \rightarrow E + P. \qquad (2)$$

If theenzymatic reaction (2) obeys the Michaelis-Menten equation and the substrate concentration is sufficiently high to "saturate" the enzyme (see our previous publication[8]), the effective quantum yield Φ of the product is defined by the following expression[10]:

$$\Phi = \varphi k_{cat} t, \qquad (3)$$

where k_{cat} is the catalytic rate constant for the enzymatic reaction (2) determined from the maximum rate of reaction and equal to the turnover number of the enzyme per unit time, φ is the quantum yield of the primary reaction (1), and t is the time of the dark enzymatic reaction. For the specified value of k_{cat} and in the presence of an inexhaustible supply of the substrate, the effective quantum yield Φ in such a system can evidently be as high as one wishes, since it is determined exclusively by the time of the dark reaction.

In practice the amplification of the light signal takes place as follows: if reaction (2) results in a coloured product P, then the "response" to a single light quantum (which cannot be actually perceived by the eye) is a number of the molecules of the coloured substance sufficiently large to be readily perceived even by the naked eye.

Eqn. (3) describes theoretically the functioning of light-sensitive enzymatic systems. However, for their experimental application, it is necessary to obtain specific derivatives of enzymes capable of changing their activity under the influence of light. Naturally, in principle one can use for this purpose light-sensitive proteins which already exist in living nature, such as, for example, the visual enzyme rhodopsin[11]. However, from the practical point of view, this procedure is quite unacceptable, because natural light-sensitive systems have an exceptionally detailed and complex organisation and are extremely unstable when isolated from their natural environment[12]. Consequently, the problem arises of synthesising artificial light-sensitive enzymatic systems, which is in fact the subject of this review.

III. GENERAL PRINCIPLES OF THE CONSTRUCTION OF ARTIFICIAL LIGHT-SENSITIVE ENZYMATIC SYSTEMS

It is logical to employ a chemo-bionic approach to the creation of artificial light-sensitive enzymatic systems[13]. After analysing photoregulatory processes *in vivo*,

Erlanger[14] concluded that the vast majority of the latter are based on the same principle: small light-sensitive molecules function as effectors (inhibitors, activators) in relation to the biologically active macromolecules, for example, enzymes (which are themselves as a rule insensitive to light). On illumination, the effector molecule is photochemically modified; this results in a change of the nature and (or) degree of its interaction with the biomacromolecule and ultimately the biological activity of the system changes. If this principle is used as the basis for the creation of artificial light-sensitive systems, then the entire vast arsenal of modern photochemistry can be used for this purpose[15,16]. In other words, one must examine suitable light-sensitive low-molecular-weight substances and select enzymes which interact with them. This is an extremely fruitful but laborious procedure, since it involves the selection of a specific light-senstitive effector–enzyme pair and there are no rules for such selection.

One can follow another approach, namely one can induce by means of light a direct change in the reactivity of either the substrate or the enzyme. The medium of the enzymatic reaction (or the microenvironment of the enzyme) can also be altered by the action of light.

The artifical enzymatic systems described in the literature, in which the level of catalytic activity can be altered under the action of light using the above principles, are subjected to a critical analysis below. Naturally, as regards applied aspects of the problem, a chemical amplifier of weak signals can be based only on systems where the light *initiates* (generates) catalytic activity. In practice a system will be regarded as being of this kind if its activity increases by 3–4 and more orders of magnitude after illumination. In other words, in practice one can use only systems where the primary photochemical process [a reaction of type (1)] can be represented in the following general form:

$$\text{System (enzyme + substrate + low-molecular-weight effector + medium) virtually without catalytic activity} \xrightarrow{\text{light}} \text{System with a high level of catalytic activity} \qquad (1a)$$

In such systems light acts either directly on the components of the enzymatic reaction (by altering the chemical or sorption properties of the enzyme itself, or the substrate, or, finally, the low-molecular-weight effector) or on the components of the medium (by altering, for example, the pH of the solution or the microenvironment of the enzyme molecule).

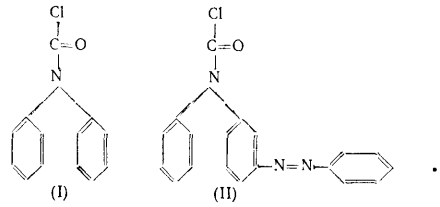
If the enzymatic activity of the system changes only within the range of 1–2 orders of magnitude under the influence of illumination, then such action of light is more correctly referred to as regulating. Despite the fact that the difference between these two types of enzymatic systems is only quantitative, we shall nevertheless consider them separately, bearing in mind the practical importance of the former. However, one must take into account the fact that in future studies only a slight modification of the method available hitherto (for example the replacement of one enzyme by another) can in principle alter sharply the quantitative characteristics of a particular system. Consequently a system which nowadays exhibits only weak regulatory effects, tomorrow (after it has been slightly modified) it can "begin to operate at full strength".

Fundamentally new possibilities for the regulation of catalytic activity have arisen from the use of immobilised enzymes (enzymes bound to a carrier)[17]. In this case sensitivity to an external stimulus can be "grafted" to the system via the carrier. This idea has already proved fruitful in the creation of sound-sensitive (mechano-sensitive) materials[18].

1. SYSTEMS WITH A LIGHT-SENSITIVE EFFECTOR

Regulation by Light of the Rates of Inactivation (or Reactivation) of Enzymes by their Interaction with Irreversible Photochromic Inhibitors

Diphenylcarbamoyl chloride (I) is a familiar irreversible inhibitor of α-chymotrypsin[19–21] and acetylcholinesterase[22]. Its photochromic azo-derivative, *N*-*p*-phenylazophenyl-*N*-phenylcarbamoyl chloride (II), can exist in the *cis*- and *trans*-configurations:



The equilibrium between these two forms can be displaced by illuminating (II) with light at a particular wavelength[13]:

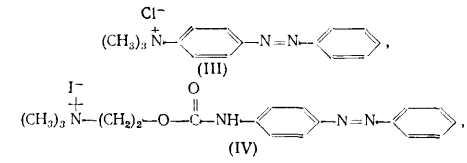
$$trans\text{-(II)} \underset{420\ nm}{\overset{320\ nm}{\rightleftharpoons}} cis\text{-(II)} . \qquad (4)$$

It was found[13] that *cis*-(II) inactivates chymotrypsin five times faster than *trans*-(II). Approximately the same differences between the rates of inactivation under the influence of the stereoisomers of (II) (as well as two other related azo-compounds) were noted also for acetylcholinesterase[23,24]. Thus, by illuminating the "enzyme + (II)" system with light having a greater or smaller wavelength, it is possible to alter the ratio of the inhibitor stereoisomers and thus regulate the rate of inactivation of the enzyme (and hence the catalytic activity of the system).

Chymotrypsin and acetylcholinesterase are inactivated under the influence of compounds of type (I) actually as a result of the carbamoylation of the active centre. The carbamoyl derivative of acetylcholinesterase can be reactivated by prolonged incubation at alkaline pH values, and for the *cis*- and *trans*-stereoisomers of inhibitor (II) slightly different rates of reactivation of the "inhibited" enzyme were found[23]. This made it possible to regulate with the aid of light also the rate of this stage of the enzyme–inhibitor interaction.

Regulation of Enzyme Activity by Light with the Aid of Light-sensitive Reversible Inhibitors

The *cis–trans* stereophotoisomerisation of azo-compounds has also been used[23,25] for the reversible regulation of the catalytic activity of acetylcholinesterase. *N*-*p*-Phenylazophenyltrimethylammonium chloride (III) and *N*-*p*-phenylazophenylcarbamoylcholineiodide (IV),

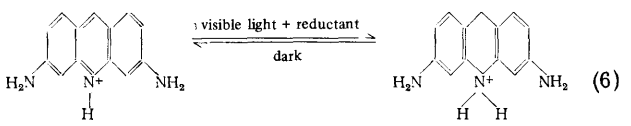


which are competitive inhibitors of this enzyme, exist in the *cis*- and *trans*-forms capable of photochemical interconversions, for example[25]:

$$trans\text{-(IV)} \underset{\text{dark}}{\overset{\text{light (348 nm)}}{\rightleftharpoons}} cis\text{-(IV)} \quad . \qquad (5)$$

The inhibition constants for the *trans*- and *cis*-isomers of (IV) are 1.6 and 3.6 μmole litre^{-1} respectively[25]; the difference is due[26] to the specific structure of the active centre of acetylcholinesterase. Using the differences between the binding capacities of the stereoisomers in relation to the active centres, it is possible (by switching light on and off) to alter reversibly the degree of inhibition of the enzyme and hence the level of the catalytic activity of the system.

We used another photochemical reaction for the reversible regulation of the catalytic activity of α-chymotrypsin. Proflavin (3,6-diaminoacridine), which is a competitive inhibitor of this enzyme[27,28], is capable of being reduced photochemically under the action of visible light in the presence of an organic reductant with formation of 3,6-diaminoacridan: the latter is reoxidised in the dark to proflavin[29]:



(6)

We have shown[30] that, in the course of its photochemical reduction, the ability of proflavin to inhibit α-chymotrypsin disappears (probably as a result of the breakdown of the planar structure of the molecule because the $C_{(9)}$-atom becomes tetrahedral). Consequently, when the "α-chymotrypsin + proflavin + reductant (ascorbic acid)" system is illuminated, the level of its catalytic activity increases (because the inhibitor is inactivated), while in the dark it decreases again.

Regulation by Light of the Potential Difference across a Biological Membrane with the Aid of Photochromic Effectors

The potential difference across certain biomembranes, whose composition includes acetylcholinesterase, diminishes (the membrane depolarises) in the presence of agents interacting specifically with the enzyme (such as acetylcholine or carbamoylcholine)[31–33]. This fact has been used[34,35] for the creation of a system for the photoregulation of the membrane potential using both irreversible[13,23,24] and reversible[23,25] photochromic inhibitors of acetylcholinesterase: under the influence of light, it is possible to alter the ratio of the *cis*- and *trans*-isomers of the inhibitor and hence (bearing in mind their different reactivities in relation to the enzyme) the degree of inhibition of acetylcholinesterase. It was found[34,35] that the membrane potential of the electric organ of the ray can be regulated in this way.

Thus, in systems with light-sensitive effectors, examined above, it is indeed possible to regulate the level of catalytic activity within fairly wide limits. However, this is clearly insufficient for the creation of chemical amplifiers of weak signals. As already mentioned [see Eqn. (1*a*)], it is in fact necessary to *initiate* catalytic activity by the action of light; this can be achieved if the photochemical reaction results in the appearance in the system of an effector (for example, an activator or a cofactor) without which the enzyme is in principle incapable of catalysis.

Initiation by Light of the Catalytic Activity of Haem-containing Enzymes

The active centre of the majority of haem-containing oxidation enzymes includes bivalent iron. For example, peroxidase is an enzyme whose globule contains, apart from the protein component, a prosthepic group (haemin), consisting of a complex of an iron ion and protoporphyrin. Neither apoperoxidase, which contains no haemin, nor haemin, nor the complex of apoperoxidase with protoporphyrin have any appreciable enzyme activity.

The principle of initiating activity by light in such systems has been developed in our laboratory[36]. One takes a mixture of apoperoxidase, protoporphyrin, and a complex iron salt [for example, potassium oxalatoferrate(III)], which does not interact with protoporphyrin; such a system should evidently have no catalytic activity. After illumination of the complex iron salt, the iron ion is liberated[37,38]:

$$\text{potassium oxalatoferrate(III)} \xrightarrow{\text{visible light}} Fe^{2+}, \qquad (7)$$

and this is followed by complex formation processes:

$$\text{protoporphyrin} + Fe^{2+} \rightarrow \text{ferroprotoporphyrin} \xrightarrow{O_2} \text{haemin}, \qquad (8)$$

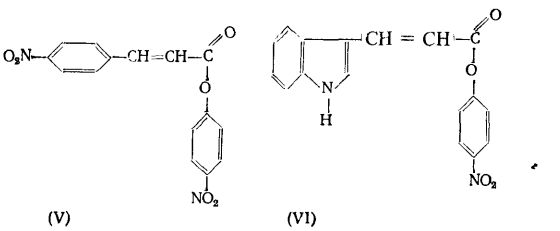
$$\text{apoenzyme} + \text{haemin} \rightarrow \text{peroxidase}. \qquad (9)$$

Thus an active enzyme can be "constructed" as a result of the primary photochemical process (7) and the subsequent dark reactions (8) and (9).

2. SYSTEMS WITH A LIGHT-SENSITIVE SUBSTRATE (OR QUASI-SUBSTRATE)

Regulation by Light of the Rates of Reaction of Enzymes with Synthetic Substrates Capable of Photoisomerisation

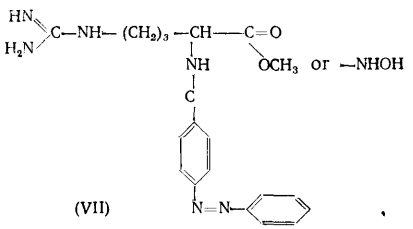
(a) The esters of β-arylacrylic acids can be regarded as structural analogues of specific α-chymotrypsin substrates[39]. The second-order rate constants for their hydrolysis by α-chymotrypsin are much higher for the *trans*-stereoisomers than for the *cis*-stereoisomers: by a factor of 5×10^3 for *p*-nitrophenyl esters of *p*-nitrocinnamic acid (V) [40] and by a factor of 10^3 for the *p*-nitrophenyl esters of indolylacrylic acid (VI) [41]:



(V) (VI)

Consequently, by illuminating a mixture of the enzyme and the *cis*-isomer of the substrate, it is possible to accelerate the enzymatic process greatly[40].

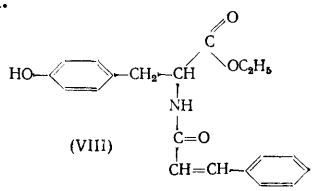
(b) Amides and esters of *N*-α-benzoyl-L-arginine — classical low-molecular-weight substrates of trypsin[42–44]. The stereoisomers of their azo-derivatives (VII).



(VII)

The methyl ester and hydroxamide of *N*-α-*p*-phenylazophenyl-L-arginine differ severalfold in reactivity towards trypsin[45]. Under the influence of light at different wavelengths, the *cis*- and *trans*-stereoisomers of (VII) are interconverted. Consequently, with the aid of light, one can, as it were, "switch over" the rate of enzymatic reaction from one value to another[45].

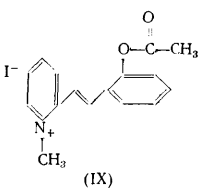
(c) When the reaction involving the photostereoisomerisation with respect to the C=C bond was used, it was possible[46] to regulate with the aid of light the rate of the chymotrypsin hydrolysis of the ethyl ester of *N*-α-cinnamoyl-L-tyrosin:



(VIII)

Compound (VIII) can be regarded as an analogue of the ethyl ester of *N*-acetyl-L-tyrosine, which is a classical specific substrate of α-chymotrypsin[39].

(d) The rates of hydrolysis of compounds of type (IX)



(IX)

catalysed by butyrylcholinesterase, differ severalfold for the *cis*-*trans*-stereoisomers[47]. Consequently, using the photostereoisomerisation of a substrate of type (IX), it is possible to regulate the rate of the enzymatic reaction. The authors[47] suggest that the rate-limiting stage in the enzymatic hydrolysis is the desorption of the product molecule (the corresponding alcohol) from the active centre. This hypothesis is extremely likely, because it is known that product molecules can be desorbed from enzyme macromolecules at a fairly low rate[48,49].

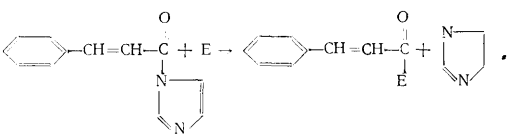
Reactions of Enzymes with Photoexcited Substrates

Molecules in photoexcited states differ sharply in their chemical properties from those in the ground state (it is therefore possible to investigate their reactions despite the extremely low steady-state concentration of the excited molecules)[59]. Consequently, in principle, one may expect that the reaction of the enzyme with the photoexcited substrate can have a different rate than that with the non-excited substrate. The foregoing may have a bearing on the surprising results obtained by Comorosan and coworkers[51–54], who showed that, when low-molecular-weight substrates of various enzymes are irradiated with low doses of ultraviolet light, long-lived excited states are formed and the rates of their reactions with enzymes can be several times higher than those observed with the non-excited substrates. This effect depends greatly on the irradiation conditions, but the mechanism of the phenomenon remains obscure.

Thus, with the aid of light-sensitive substrates it is also possible (as for light-sensitive effectors) not only to regulate the rate of the enzymatic reaction but actually to initiate the latter [this has been shown[40] in relation to compounds of type (V)]. However, the foregoing factors are insufficient for the creation of a chemical amplifier of weak signals— instead of initiating the reaction (by activating the substrate), the catalytic activity of the system must be stimulated. This can be achieved by employing "quasi-substrates", whose chemical reaction at the active centre proceeds via relatively unreactive (unstable) intermediate compounds. As a result of such "chemical modification of the active centre" by the quasi-substrate, the catalyst is, as it were, "poisoned". Using such stable and catalytically inactive "enzyme derivative" (modified by the quasi-substrate at the active centre), one can attempt to alter (increase) the enzyme reactivity under the influence of light and thereby regenerate the free active centre.

Initiation by Light of the Catalytic Activity of Enzymes with the Aid of Photochromic Quasi-substrates

(a) The possibility of initiating by light the catalytic activity of artificial enzymatic systems was demonstrated for the first time at Moscow University[8–10]. The principle of the effect is as follows. It is known[39] that acylation of the hydroxyl in the serine residues in the active centre of α-chymotrypsin (a protolytic enzyme) leads to complete inactivation of this biocatalyst. Deacylation of the resulting acylenzyme leads in its turn to the complete regeneration of the catalytic activity. Taking these factors into account, the active centre of α-chymotrypsin has been acylated[8,9] by *cis*-cinnamoylimidazole (the *trans*-stereoisomer of this reagent is a familiar titrant of α-chymotrypsin[55]):



(10)

(X)

The *cis*-cinnamoyl-α-chymotrypsin (X) formed in this process is an extremely stable acylenzyme: even at the pH optimum the deacylation rate constant is 4×10^{-4} min^{-1}, i.e. the half-deacylation time is more than 24 h. (By altering the pH away from the optimum, it is possible to increase this time still further). On illumination with ultraviolet light, the *trans*-stereoisomer is formed from the *cis*-acylenzyme, and is extremely rapidly deacylated (the half-reaction time is ~1 min). The observed difference between the reactivities of the *cis*- and *trans*-stereoisomers of the acylenzymes (by a factor of 2000) can be increased still further by introducing the nitro-group in the *para*-position of the aromatic ring in the cinnamoyl group. In this case the *trans*-acylenzyme is deacylated 2.5×10^4 times faster than the corresponding *cis*-acylenzyme[10]. The causes of the *trans*-specificity of α-chymotrypsin have been discussed[41,56]. The *cis*- and *trans*-cinnamoyl derivatives of another protolytic enzyme (trypsin) show a still greater difference in reactivity[57,58].

A chemical amplifier of weak light signals can be constructed in the following form using the systems described. We shall use, for example, *cis*-cinnamoylchymotrypsin, which is itself catalytically inactive (Fig. 1*a*) and is hardly deacylated under the experimental conditions[8,9]. If such a system is irradiated with ultraviolet light, the acylenzyme undergoes *cis-trans* photostereoisomerisation and the resulting *trans*-acylenzyme hydrolyses rapidly:

$$cis\text{-(X)} \xrightarrow{\text{light}} trans\text{-(X)} \xrightarrow{H_2O} \text{enzyme} + trans\text{-cinnamic acid.} \quad (11)$$

The kinetics of these processes can be followed on the oscillogram obtained in the flash-induced hydrolysis of *cis*-nitrocinnamoylchymotrypsin[59] (Fig. 2). Eqn. (11) shows that illumination results in the regeneration of catalytic activity (see Fig. 1*b*) and a coloured product, for example, will therefore begin to be formed in the presence of the substrate [Eqn. (2)]. The effect quantum yield Φ of the product **P** (the amplification factor for the light signal) in the system can be estimated by Eqn. (3) assuming that the quantum yield of the *cis-trans* stereoisomerisation is $\varphi = 0.3$ (at 313 nm) [56] and the catalytic constant for a specific substrate at the pH optimum for the activity of chymotrypsin is $k_{cat} \simeq 100\ s^{-1}$.[39] If the time of the dark reaction t is, for example, 1 h, then, according to Eqn. (3), $\Phi = 0.3 \times 200 \times 3600 \simeq 2 \times 10^5$, i.e. more than 10^5 product molecules are produced in the system during this period for one absorbed light quantum.

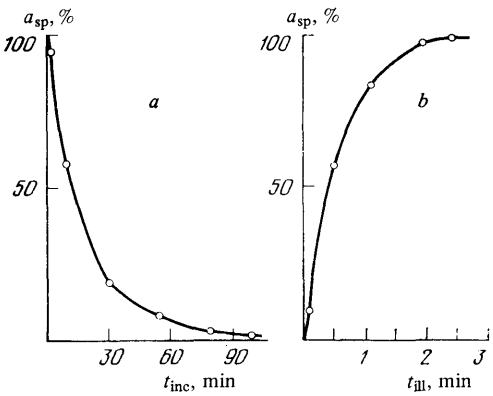


Figure 1. Variation of the specific catalytic activity (a_{sp}) of chymotrypsin incubated with *p*-nitrophenyl *cis*-4-nitrocinnamate (curve *a*; t_{inc} is the incubation time) and on subsequent illumination of the resulting acylenzyme—*cis*-4-nitrocinnamoylchymotrypsin (curve *b*; t_{ill} is the illumination time)[8,10].

Thus it is seen that the systems described are extremely sensitive light detectors (see Kazanskaya's review[60]).

(b) Another principle has been formulated in studies[61,62] where a photochemical method was proposed for the investigation of the aminoacid environment of the active centre of the enzyme. For the reaction of α-chymotrypsin with *p*-nitrophenyl diazoacetate—an analogue of *p*-nitrophenyl acetate which is a classical non-specific substrate of proteases[63]—the product is a diazoacetylenzyme—an extremely stable acylenzyme without catalytic activity.

For example, diazoacetyl-α-chymotrypsin is hardly deacylated in the course of 48 h (at pH 6.5) [44], while acetyl-α-chymotrypsin is rapidly deacylated under these conditions. The difference is associated with the diminution, as a result of conjugation with the diazo-group, of the electrophilic properties of the carbonyl carbon atom in the substrate residue. On illumination of diazoacetyl-α-chymotrypsin with ultraviolet light, nitrogen is split off[61] from the diazoacetyl residue with formation of a carbene radical. This radical hydrolyses and the resulting hydroxyacetyl-α-chymotrypsin is rapidly deacylated with regeneration of enzyme activity:

$$\text{E}-\overset{\overset{O}{\|}}{C}-CH_2-\overset{+}{N}\equiv N \xrightarrow[H_2O]{\text{u.v.light}} \text{E}-\overset{\overset{O}{\|}}{C}-CH_2-OH + N_2 \rightarrow \text{E} + HOCH_2COOH \quad . \quad (12)$$

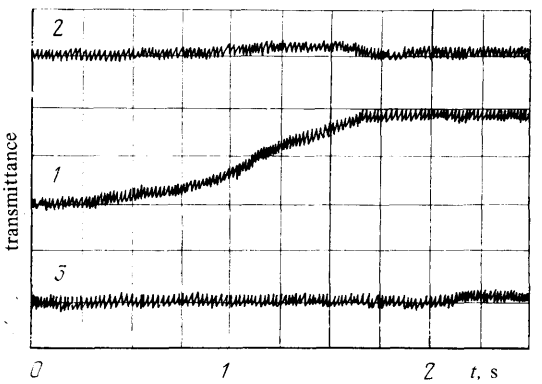


Figure 2. The oscillogram (curve *1*) of the change in the transmittance (320–360 nm) of a solution of *cis*-4-nitrocinnamoylchymotrypsin as a result of pulse irradiation (10^{-4} s) at time $t = 0$. Curves *2* and *3* correspond to 0 and 100% transmittance[59].

3. THE ACTION OF LIGHT ON THE ENZYME (INCLUDING A MODIFIED ENZYME)

Activation by Light of Catalytically Inactive Enzyme Derivatives (Modified at the Active Centre)

The principle of the approach is that the enzyme is modified initially with formation of a catalytically inactive derivative E_{inact} [see Eqn. (1)] and then the modifying agent (or its influence) is removed with the aid of light. A fairly convenient object for studies of this kind is the sulphydryl protolytic enzyme papain. The sulphydryl group of cysteine, which forms part of the active centre of the enzyme, plays the most important role in the catalytic function of papain[64]. However, the enzyme is usually in the inactive form, where the sulphydryl group of the active centre forms a mixed disulphide with an additional cysteine residue (papain–S–S-cysteine)[65,66]; for the catalytic activity to be shown by papain, the latter must be activated, for example, chemically (in this activation the S–S bond of the disulphide is cleaved).

In principle, disulphide bonds both in peptides and in proteins can be cleaved not only chemically but also photochemically[67–69]. This approach has been used[70] for the photochemical activation of papain: on illumination with ultraviolet light of a solution containing papain–S–S-cysteine, a catalytically active enzyme is formed:

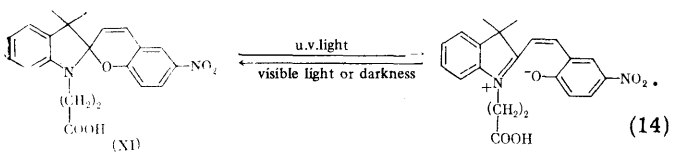
$$\underset{\text{(E-cysteine)}}{\text{E—S—S—CH}_2\text{CHCOOH}} \xrightarrow{\text{u.v.light}} \underset{\text{(E)}}{\text{E—SH}} + \underset{\text{(cysteine)}}{\text{HSCH}_2\text{CHCOOH}} \qquad (13)$$

This principle was developed further in our laboratory[71]. As a result of the oxidation of active papain catalysed by copper ions, a disulphide dimer of the enzyme, namely papain–S–S–papain, was obtained. On illumination of the dimer with ultraviolet light, the disulphide bond is cleaved with formation of two molecules of the catalytically active enzyme. The quantum yields of the photoactivation process are in both cases in the range 0.1–1.0.

Regulation by Light of the Catalytic Activity of Enzymes with the Aid of Photochromic Effectors Attached to the Protein Globule outside the Active Centre

When photochromic polymers are illuminated[72,73], one may expect a change in their structure. This effect has been observed experimentally[74–79]. In the above studies azo-dyes [for example compounds of type (II)–(IV)] were attached covalently or non-covalently to synthetic polymers or proteins. The *trans*-configuration of the dye is thermodynamically more stable but *trans–cis* stereoisomerisation takes place on illumination. It has been shown[74–76] that such photochemical reaction does indeed induce a considerable change in the polymer structure (revealed, for example, by a change in the viscosity of its solutions).

Enzyme macromolecules exhibit a biological activity which is extremely sensitive to the conformation of the protein globule[7]. From this point of view, it is of interest to modify the enzyme by photochromic reagents (but not at the active centre, in order to retain the catalytic activity) and to investigate the dependence of the activity of the biocatalyst on the light-regulated structure of the modifying agent. This idea has been realised experimentally[80]. A spiropyran (XI), widely used to produce photochromic polymers, served as the modifying agent for α-amylase[72,73]. On illumination with ultraviolet light, compound (XI) isomerises:



(14)

The isomerisation can be reversed by illumination with visible light or in the dark[15,81].

Namba and Suzuki[80] obtained the anhydride of compound (XI), which was then used to modify the amino-groups of α-amylase molecules. When the enzyme modified in this way is illuminated with ultraviolet light, its catalytic activity falls, while in the dark the enzyme is reactivated.

The mechanism of the regulation of systems described in this section is based on the fact that the molecular structure of the modifying agent changes under the influence of light. In principle, the mechanism of the regulation of the catalytic activity of the enzyme modified outside the active centre can also be different. The Change in the Conformational-Solvation state of the Enzyme Induced by Light. The protein molecules of enzymes contain a number of intrinsic chromophores (for example, aromatic aminoacid residues) capable of absorbing light effectively. In some cases such light absorption can activate the biocatalyst. Thus illumination of a solution of aldolase with ultraviolet light leads to a prolonged reversible increase of its enzymatic activity[82,83]. An analogous effect, but this time following the illumination of aldolase in the presence of a substrate, was observed by Volotovskii et al.[84], who proposed an interesting explanation of this effect: when light is absorbed by tryptophan residues, a radiationless dissipation of excitation energy in the form of heat can occur, in particular, in the enzyme–substrate complex, which can lead to the breakdown of the structure of water in the solvation shell of the macromolecule. This facilitates, in its turn, the allosteric conformational transition in the enzyme subunits, which leads to the formation of an aldolase conformation with an enhanced catalytic activity.

Other data[85,86] agree with this hypothesis. The difference between these results and those of the above studies[82–84] consists in the fact that the primary light acceptors were not the intrinsic chromophores of the protein but residues of diazotised *p*-aminobenzoic acid covalently attached to the sulphydryl groups of aldolase (in this case the protein absorbs visible light). Evidently such modification should not be reflected significantly in a change of the properties of the enzyme according to the mechanism of Volotovskii et al.[84] Indeed, when the modified aldolase is illuminated, its conformation changes (this can be followed from the change in the absorption spectra and the electrophoretic mobility)[86] and the kinetics of the cleavage of fructose-1,6-diphosphate catalysed by this enzyme are also altered[85]. Apparently the mechanism proposed[84] can be fairly general for different enzymes.

Another mechanism (one cannot rule out the possibility that it is to some extent similar to that already described) of the increase of the activity of enzymes under the influence of light may be proposed on the basis of Fröhlich's hypothesis[87,88], according to which biological objects, particularly proteins, can exist in long-lived excited states. In the case of enzymes the catalytic activity of these states may be different from that of the ground states; the transition from the ground to the excited state can be induced, for example, by light. An experimental study of this kind has been carried out[89], in which it has been shown that, when chymotrypsin is irradiated with a laser beam (457.9 nm), its activity increases appreciably. According to the authors[89], the picture outlined by Fröhlich actually obtains in this instance.

Photoinactivation of Enzymes

In principle, the familiar phenomenon of the photoinactivation of enzymes can be used to create light-sensitive enzymatic systems. The first study[90] devoted to this problem was published as early as 1879 and by now hundreds of such investigations have been made. We shall therefore merely refer to the most exhaustive reviews[91–97].

Inactivation under the influence of ultraviolet light is due to the photodegradation of the aminoacid residues of the protein globule (in the first place, tryptophan and cysteine residues). The photoinactivation quantum yield is in this case 0.001–0.1 and depends on the nature of the enzyme, its conformation, and the conditions in the medium. Certain flavin enzymes are inactivated under the influence of visible (blue) light. Here the cofactor (flavin

mononucleotide) plays the role of a sensitiser of the apoenzyme degradation process[98].

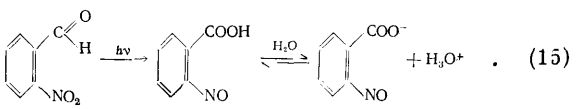
The so-called photodynamic effect, i.e. the photochemical oxidation of protein molecules sensitised by dyes, is also well known[99]. The mechanism of this effect consists in the transfer of molecular oxygen dissolved in water to the protein via the formation of an intermediate adduct of the triplet-excited dye molecule and oxygen. The photodynamic effect occurs when protein solutions are irradiated with visible light in the presence of various dyes (eosin, Methylene Blue, etc.); the enzyme photoinactivation quantum yields are usually in the range 0.001–0.1.[99]

4. THE CHANGE IN THE MEDIUM OF THE ENZYMATIC REACTION UNDER THE INFLUENCE OF LIGHT

The Light-Induced Jump in pH

Almost all enzymatic reactions are extremely sensitive to the pH of the solution: their rates decrease sharply when the pH deviates from the optimum value[6]. This factor led to the idea that the rates of enzymatic reactions can be regulated by a photoinduced change in the pH of the medium[100]. Photochemical reactions which lead to the formation of strong acids or bases have been described in the literature. We shall quote some examples of such reactions.

(a) When a solution of *o*-nitrobenzaldehyde is irradiated (436 nm), a phototautomerisation reaction takes place: *o*-nitrobenzaldehyde is converted into *o*-nitrobenzoic acid with a quantum yield $\varphi = 0.5$ (see Terenin[15], p.211):



(15)

When reaction (15) is carried out in aqueous solution, the resulting acid dissociates with formation of a hydronium ion, i.e. in this case illumination leads to a decrease of the pH of the medium.

(b) When complex transition metal (CO^{3+}, Cr^{3+}, etc.) ammines are irradiated with visible light, a photoaquation reaction takes place (with quantum yields of 0.01–1.0),[101,102] for example:

$$[CO(NH_3)_5Cl]^{2+} + H_2O \xrightarrow{h\nu} [Co(NH_3)_4(H_2O)Cl]^{2+} + NH_3 . \quad (16)$$

The ammonia formed as a result of reaction (16) reacts with water (i.e. with the reaction medium).

$$NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^-,$$

which leads to an increase in the alkalinity of the solution.

(c) Transition metal (Fe^{3+}, Co^{3+}) oxalates undergo a photochemical oxidation-reduction reaction with a quantum yield of approximately unity on illumination with visible light (this process constitutes the basis of the action of the oxalatoferrate (III) actinometer[37,38]), for example:

$$2Fe(C_2O_4)_3^{3-} \xrightarrow{h\nu} 2Fe(C_2O_4)_2^{2-} + C_2O_4^{2-} + 2CO_2. \quad (17)$$

The carbon dioxide formed in this process reacts with water:

$$CO_2 + H_2O \rightleftharpoons H_3O^+ + HCO_3^-,$$

which leads to an increase in the acidity of the medium.

Naturally the systems (a)–(c) enumerated above do not exhaust the photochemical reactions accompanied by a change in the pH of the medium.

Consider an enzyme and a substrate at a pH such that the rate of the enzymatic reaction is negligibly small. We then add one of the above reagents, for example, *o*-nitrobenzaldehyde [Eqn. (15)] or chloropentamminecobalt [reaction (16)]. If such a system is now illuminated, the pH of the solution changes and if the change is directed towards the pH optimum for the enzyme activity, the rate of the enzymatic reaction increases.

The above principle[100] was tested in relation to photoinitiated reactions catalysed by α-chymotrypsin [using system (a)][46] and trypsin [system (b)].[103]

Photoregulation of the Rates of Enzymatic Reactions as a Result of the Change in the Microenvironment of the Enzyme under the Influence of Light

New possibilities for the regulation of the catalytic activity by light arose when the enzyme was incorporated in a polymer film (membrane) containing a photochromic effector. Such an effector can be either introduced mechanically into the polymer carrier or can be attached covalently.

(a) Chymotrypsin and azobenzene were incorporated mechanically in an octanol/water membrane stabilised with sodium stearate[104]. Azobenzene does not interact with the enzyme, but the change in its structure as a result of the *cis–trans* photostereoisomerisation induces structural changes also in the membrane, which influences the catalytic activity of chymotrypsin (apparently as a result of the effect of the microenvironment).

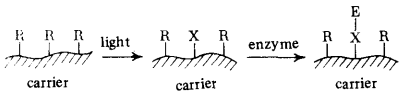
(b) An interesting idea for the photocontrol of enzymatic processes has been described in studies[105,106] where the amino-groups of collagen were modified by a photochromic compound [the anhydride of compound (XI)]. Films containing non-covalently incorporated enzymes—urease[105] or lactate dehydrogenase[106]— were then obtained from such collagen. When immobilised enzyme systems of this kind are illuminated, the structure of the modified polymer changes and hence also the microenvironment of the enzyme, which reduces the catalytic activity of the system. After the system has been kept in the dark, the photochemical reaction is reversed [in conformity with Eqn. (14)] and the activity of the enzymes rises to the initial level.

5. SYSTEMS WITH A CARRIER IN WHICH LIGHT INITIATES ENZYME IMMOBILISATION CENTRES

In virtually all the examples examined above (sub-sections 1–4) the presence of light sensitivity was indisolubly linked to a specific enzyme system. In other words, each mechanism whereby it was possible to "graft" light sensitivity artificially onto the system depends strongly either on the structure of the active centre, or on the nature of its interaction with low-molecular-weight ligands, or on the degree of its "response" both to changes in the structure of extremely remote regions of the protein globule and to changes in the reactivity of the medium, etc. Consequently the method developed for one enzyme cannot be automatically used in another system.

In order to overcome this disadvantage, we propose a fundamentally new, universal method based on the use of a carrier for the immobilisation of the enzyme. The basic

idea of this method is reflected in the following scheme:



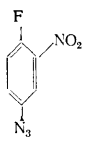
(1b)

The functional groups R of the initial carrier do not interact with the enzyme. Under the influence of light, they are transformed chemically (R — X) with formation of functional groups X capable of interacting with the enzyme either chemically or via a sorption process. Thus the enzyme is attached to the regions of the carrier surface exposed to light, while it is absent from the non-illuminated sections. The attached enzyme can be made visible by treating the system with a substrate giving rise to coloured products as a result of the enzymatic reaction.

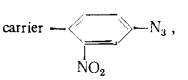
An evident advantage of this approach is that it is applicable to virtually any enzyme (because the molecules of all enzymes consist of the same aminoacid residues). Furthermore, methods for the immobilisation of enzymes on carriers have now been developed in extremely great detail[107-109]. We shall therefore indicate only photochemical methods whereby the ability to bind an enzyme can be "grafted" onto a carrier.

Photochemical Modification of the Functional Groups of the Carrier

(a) The immobilisation of aspartase with the aid of the photochemical Knowles reagent[112] [4-azido-1-fluoro-2-nitrobenzene (XII)] has been described[110,111]. This reagent was added to a carrier, namely to alkylamino-glass, in order to immobilise enzymes. As a result of the substitution of fluorine by the nucleophilic NH_2 groups of the carrier, compound (XIII) was formed:



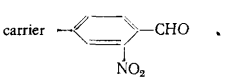
(XII)



(XIII)

which is capable of splitting off nitrogen under the influence of light with formation of an extremely reactive nitrene radical. If the illumination is carried out in the presence of an enzyme, then at the sites exposed to light the enzyme is bound covalently to the modified carrier (XIII) by interacting with the nitrene, the prospects for this approach in the photoimmobilisation of enzymes have been very fully described by Guire[111].

(b) Another method was proposed by ourselves[128]. In an optically transparent polymer carrier, containing *o*-nitrobenzaldehyde groups (XIV), the photochemical reaction (15) should proceed with formation of carboxy-groups:

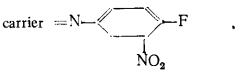


(XIV)

The remaining aldehyde groups (in the non-illuminated regions) must be blocked by an amine and the existing carboxy-groups (at the illuminated sites) must be activated, for example, by carbodiimide and the enzyme must be grafted to them (by a standard method[107-109]).

Photochemical Addition to the Carrier of Functional Groups Capable of Interacting with Enzymes

The photochemical immobilisation of enzymes using the Knowles reagent (XII) can be achieved in two ways (see the discussion of this problem by Guire[111]): either by attaching (XII) to the carrier with the aid of a nucleophilic substitution reaction involving the aromatic ring (see above) or by illuminating a mixture of (XII) and a carrier without nucleophilic groups. In the latter case the reactive nitrene radical formed at the illuminated sites is attached covalently to the carrier with formation of compound (XV):



(XV)

If an enzyme solution is then added, the amino-groups of the protein react with the fluoronitrophenyl residue (nucleophilic substitution of fluorine) in compound (XV), which leads to covalent immobilisation of the biocatalyst. In order to increase the rate of the last reaction, the Knowles reagent can be replaced by its more reactive derivative[113], a whole series of which have been synthesised by Guire[114].

Photochemical Formation of a Carrier

We used the photogel-formation process for the photoimmobilisation of enzymes[115]. For example, the classical polyacrylamide gel for the immobilisation of enzymes[116] and other polyacrylate gels can be obtained by illuminating solutions of the corresponding monomers with visible light in the presence of the dye riboflavin[117] or by illuminating with ultraviolet light[118]. Thus, when a thin layer of a solution of monomers is deposited on a plate the illuminated, a gel, retained on the plate, is formed at the illuminated sites, at the non-illuminated sites the monomer solution can be readily washed off with water. The enzyme is retained in the carrier mechanically (when it is added beforehand to the polymerisation mixture)[118] or it can be "grafted" chemically after polymerisation to the gel already formed[115].

An important feature of the methods which we proposed[115] consists in the fact that the light-initiated radical polymerisation processes are characterised (by virtue of the chain mechanism) by high quantum yields, greatly exceeding unity. This implies that one light quantum can ensure, in principle, the immobilisation of more than one enzyme molecule. This leads to additional possibilities [apart from the coupled enzymatic reaction — see Eqn. (2)] for the amplification of the light signal.

IV. THE USE OF ARTIFICIAL LIGHT-SENSITIVE ENZYMATIC SYSTEMS IN PRACTICAL APPLICATIONS AND IN FUNDAMENTAL BIOCHEMICAL RESEARCH

Analysis of literature data combined with the new approaches which we proposed shows that catalytic activity in an enzyme system can be indeed "induced" by artificial means [via mechanisms (1a) or (1b)]. In conclusion we shall note the following three factors.

(1) There is no doubt that extremely effective amplifiers of weak light signals, including new non-silver photographic processes (see the relevant reviews[60,129,130]), will be created in the immediate future on the basis of artificial

light-sensitive enzymatic systems. The success of future research will depend on the following two characteristics of the enzymatic system employed: the amplification factor and the spectral sensitivity.

(a) As already mentioned above [see the discussion of Eqn. (3)], the effective quantum yield in light-sensitive enzyme systems is extremely high. Furthermore, there exist at least two ways of increasing it further.

In the first procedure the activation of the corresponding zymogen is used as the first of the dark reactions (2) (zymogens are catalytically inactive natural enzyme precursors capable of enzymatic activation). For example, trypsinogen has no enzyme activity, but is converted into an active enzyme under the influence of trypsin[119]. This process is autocatalytic: in principle, even one trypsin molecule can activate the entire available "store" of the zymogen. Consequently, trypsinogen is extremely sensitive to even very small concentrations of trypsin. Hence the idea of creating the following light-sensitive enzymatic system naturally arises: a catalytically inactive, but capable of being activated by light, trypsin derivative + trypsinogen + trypsin substrate. When such a system is illuminated, the active enzyme is formed from the inactive trypsin derivative and activates the zymogen, while the product of the activation process hydrolyses the substrate. Evidently in this case the trypsin formed under the influence of light as it were "multiplies" due to the activation of the zymogen, which leads to a transition from a linear dependence of the effective quantum yield on the dark reaction time [Eqn. (3)] to an exponential dependence[120] and this ensures increased primary light signal amplification factors; see also Refs. 57, 58, and 103 and a review[60].

In the second procedure the photochemical formation of the carrier ensures the possibility of immobilising more than one enzyme molecule with the aid of a single light quantum[115] (see Section III.5).

(b) As regards the second important characteristic of the light-sensitive enzymatic system, namely its spectral sensitivity, the latter can be altered by varying the molecular structure of light-sensitive low-molecular-weight components, i.e. the substrate (see, for example, Varfolomeev and coworkers[10,56]) or of the effector for the enzymatic reaction or, finally, of the enzyme modifying agent.

New possibilities arise from the use of sensitised photochemical reactions, because in this case it is sufficient to vary the molecular structure (and hence the spectral sensitivity) of the sensitiser. So far it has not been possible to find a sensitiser of photochemical reactions occurring directly in the active centre of the enzyme[121], which is apparently associated with the extremely complex organisation of the active centre, i.e. with different kinds of steric hindrance. The photopolymerisation process which results in the formation of a carrier for the subsequent immobilisation of enzymes is therefore extremely promising[115]. It is significant that photopolymerisation processes do not impose unduly drastic limitations on the structure of the sensitiser molecule[122].

(2) The approaches examined in the present review to the regulation under the influence of light of the rates of enzymatic reactions should find applications also in fundamental enzymological research. Particularly wide prospects arise in photochemical approaches when the latter are used to investigate the kinetics and mechanisms of reactions occurring in frozen or crystalline states (where, in particular, diffusion-dependent obstacles[123] interfere with the introduction of the required effectors or the substrate by a sufficiently rapid procedure).

We shall indicate two examples. Thus, the flash-induced change in the reactivity of the substrate (or the effector) can be used as a basis for a method whereby the kinetics of the rapid stages of the enzymatic process can be investigated[40,124]; the use of the flash-induced jump in pH merits particular attention in this connection (see Section III, 4).

Another example involves the use of reaction (11) for the investigation of the mechanism of enzymatic catalysis. Fig. 2 shows that the photostereoisomerisation of the substrate residue at the active centre of the acylenzyme proceeds almost instantaneously (there is a rapid increase of the optical density of the solution), but the subsequent chemical stage, namely the hydrolysis of the acylenzyme (a slow increase of the optical density), proceeds with an appreciable induction period. It may be that the observed induction period is due to the conformational-solvation rearrangements in the active centres ensuring the complementarity of the catalyst to the specific substrate produced under the influence of light (as a result of photostereoisomerisation). This explanation is consistent with the hypothesis[125–127] of the conformational (solvation) changes in the active centre, induced by the substrate, which are important for catalysis.

(3) Many of the enzyme systems examined in this review constitute extremely realistic models of more complex light-sensitive systems which function *in vivo*. Analysis of the simple models can therefore assist in a deeper understanding of the principles used by nature to regulate biological activity[14].

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