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Reactions of Orthophthalaldehyde with Nucleophiles

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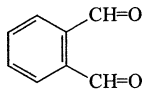
1. Introduction

Most substituted benzaldehydes (with the exception of *o*- and *p*-nitrobenzaldehydes[1,2]) show a limited reactivity to attacks by weak nucleophiles, like water.[3,4] Introduction of a second formyl group in the *para*-position results in a strong resonance interaction characterized by one of the largest values of substituent constants, $\sigma^-_{p-CHO} = 1.1$.[5] Hence, in terephthalaldehyde the presence of a second CHO group results in an increase in reactivity toward nucleophiles, reflected by hydration, which in aqueous solutions is about 15%.[6]

A similar strong mutual interaction can be expected for the two adjacent formyl groups in orthophthalaldehyde (I).



(I)

As is often the case for the effects of *ortho*-substituents, there is no single numerical value for the substituent constant for a substituent in the *ortho*-position, which would be applicable to various types of reactions. It can be assumed that resonance interaction between the two adjacent formyl groups can be comparable to those operating in terephthalaldehyde, but the reactivity of I can be affected by other factors resulting from the proximity of the two

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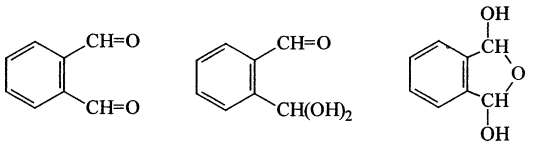
Petr Zuman was born in Prague, Czechoslovakia, where he graduated from Charles University with an RNDr degree in chemistry in 1950. In 1960 he was awarded his DrSC degree by the Czechoslovak Academy of Sciences and in 1968 his D.Sc. degree by the University of Birmingham, U.K. From 1950 to 1966 he was associated with the J. Heyrovsky Institute of Polarography in Prague, where he became Head of the Organic Division. From 1966 to 1970 he was a Senior Visiting Fellow at the University of Birmingham, England, and from 1970 to 1997 he was Professor of Chemistry at Clarkson University at Potsdam, NY, where he is currently active as Distinguished Emeritus Research Professor. His research interests involve studies of organic polarography and its applications, in particular for structure–reactivity relationships, determination of the mechanism of some organic reactions, both of fast reactions occurring in the vicinity of the electrode surface and slower reactions in the bulk of the solution, and following the interaction of xenobiotic substances with biological materials.

vicinal CHO groups, including steric and direct field effects.

The chemistry of dialdehyde I in aqueous solutions is further complicated by the consecutive formation of a cyclic hemiacetal following the addition of the first water molecule. Such ring closures result in formation of a benzofuran derivative. Thus, in aqueous solutions of dialdehyde I, three forms can be present: Unhydrated form Ia, monohydrated acyclic form Ib, and a cyclic hemiacetal form Ic.

In principle, all three forms can react with nucleophiles. The sum of the concentrations of these three forms will be further denoted as OPA.



(Ia) (Ib) (Ic)

Such a sequence of reactions also can be followed by other nucleophiles bearing two hydrogens on the heteroatom. For example, the reaction of OPA with primary amines can result in formation of isoindole derivatives.

Information available dealing with mechanisms, kinetics, and equilibria of reaction of OPA with individual nucleophiles is rather limited. This situation partly reflects the fact that the most widely used analytical application of the reaction of OPA is for the determination of amino acids, recently most frequently used in pre- and postcolumn derivatization in various variants of chromatography. In these determinations a thiol is first added to OPA before the interaction with amino acid (section 3). Existing attempts to contribute to the understanding of the solution chemistry involved in the determination of amino acids have dealt with ternary mixtures containing OPA, thiol, and amino acid. Difficulties in dealing with reactions in such complex reaction mixtures were reflected in the statement "to date (1985) the mechanisms of formation of isoindoles has not been established",[7] which is still valid. Most existing studies have made conclusions about the course of reactions involved based on identification of products or variation of concentration of a single component with time and limited evidence about the equilibria assumed to be involved.

Due to insufficient experimental evidence, this contribution cannot offer a mechanism for the reactions involved in interactions of OPA with nucleophiles. The existing information is summarized to point out areas in which further research is needed and to indicate the opportunities and limitations of individual analytical techniques used in such studies.

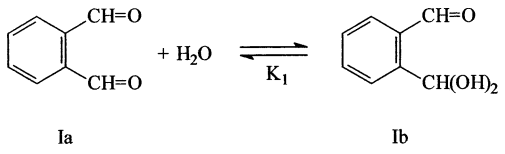
The development of analytical, recently predominantly separation, techniques in this area has been mostly based on empirical approaches. Better understanding the details of the solution chemistry involved might promise a rational development of more reliable analytical methods in the future.

This review offers a critical examination of the field of interest based predominantly on studies published between 1970 and 2002. It indicates areas where future investigations are needed.

2. Addition of a Single Nucleophile to OPA

2.1. Addition of Water and Hydroxide Ions

The covalent hydration of OPA was reported early[8] based on a phase diagram. The addition of a water molecule to the unhydrated form Ia to form an acyclic monohydrate Ib (eq 1) is shifted in favor of Ib due to the electron-withdrawing and ortho effects of the second formyl group, as mentioned above.

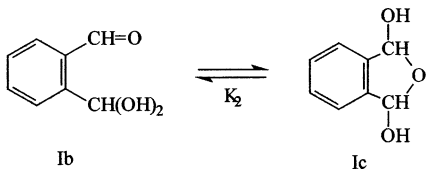


(1)

Ia Ib

Addition of a second molecule of water to Ib is less favored since the electron-withdrawing effect of the group $CH(OH)_2$ on the remaining free aldehydic group is much smaller than the effect of the second CHO group on the reactivity of the first in Ia.

Instead, an intramolecular nucleophilic attack of an oxygen of the $C(OH)_2$ group on the carbon of the unhydrated formyl group takes place (eq 2).



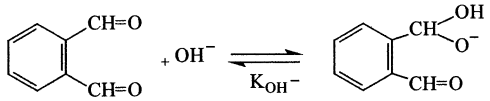
(2)

Ib Ic

In attempts to treat the equilibria between OPA and water,[9,10] an assumption has been made that at equilibrium the concentration of the acyclic monohydrate Ib is negligible. Early polarographic studies[11,12] nevertheless clearly indicated the simultaneous presence of the unhydrated (Ia), acyclic monohydrated (Ib), and cyclic forms of OPA in aqueous solutions over a wide pH range. However, only a qualitative interpretation of the recorded current−voltage curves was offered. More recent electroanalytical studies[6,13] have shown that the equilibrium (eq 1) between the unhydrated (Ia) and acyclic monohydrated (Ib) forms is established slowly ($\tau_{1/2} > 0.13$ s), whereas the equilibrium (eq 2) between the acyclic (Ib) and cyclic (Ic) hydrated forms is established faster but not extremely fast ($\tau_{1/2}$ on the order of 10^{-2} s). The equilibrium constant of reaction 1, K_1, is about 3, whereas for reaction 2 $K_2 \approx 2$.

The rates of dehydration of both the acyclic (Ib) and cyclic (Ic) hydrates are both acid and base catalyzed. This is indicated by the increases of limiting currents both at pH < 4 and pH > 6.[6,13] In the pH range between pH 4 and 6 the limiting currents are limited by the solvent-catalyzed rate of dehydration. The dependence of the measured currents in the pH range where a change of current with pH is observed on buffer kind and concentration indicates that the dehydration is both general acid and general base catalyzed.

Hydroxide ions also add to the free aldehydic form Ia in alkaline solutions, where the base-catalyzed dehydration of forms Ib and Ic is fast. This reaction follows eq 3,[13] where $K_{OH} = [Ph(CHO)CH(OH)O^-]/[Ph(CHO)_2][OH^-]$). As for the addition of water and for the same reasons, the attack of the second hydroxide ion on the adduct plays a lesser role. Conversion of K_{OH-} into a more common expression for an acid dissociation constant K_a {defined as $K_a = [Ph(CHO)CH(OH)O^-][H^+]/[Ph(CHO)CH(OH)_2]$ or $pK_a = pK_{OH-} + pK_w$} yielded (based on spectrophotometry[13]) for OPA $pK_a = 13.23$. Formation of a cyclic adduct in an intramolecular reaction of the anion $Ph(CHO)CH(OH)O^-$ cannot be excluded.



(3)

2.2. Addition of Alcohols

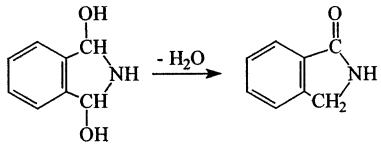
The reaction of OPA was studied electrochemically[14,15] under conditions in which methanol was both the reagent and solvent.

Similarly as in aqueous solutions, the presence of two electroactive forms—the unsolvated and acyclic solvated—was observed. As these investigations were carried out in unbuffered solutions, and as the water content of the alcohol was not reported, these studies, as with other alcohols, should be revisited as they may contribute to evaluation of the reactivity of individual forms of OPA. Acetalization of OPA can be achieved in methanol containing triethylamine as a base in the presence of $TiCl_4$ as a catalyst.[16]

2.3. Addition of Ammonia and Primary Amines

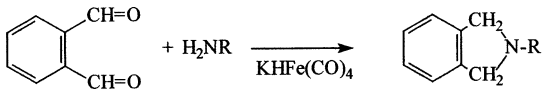
Addition of low concentrations of ammonia to solutions of OPA resulted in changes in the UV spectra that were too small to be suitable for determination of ammonia.[11,17] An electrochemical approach proved to be more promising. Reaction of OPA with NH_3 in a carbonate buffer pH 10.4 yields an electroinactive species (probably an isoindole derivative). As both the unhydrated (Ia) and acyclic hydrated (Ib) forms of OPA are reducible at the dropping mercury electrode, a decrease in limiting currents of both reduction waves of Ia and Ib enabled[17] a polarographic determination of NH_3. The reaction, which has not been tested for chemical reversibility, at concentrations used takes about 3 h to reach completion.

Under preparative conditions in cold DMSO, on the other hand, the isoindole derivative undergoes dehydration and rearrangement to phthalimidine[18] (eq 4). Primary amines under these conditions yield both phthalimidines and dimeric products.



(4)

In 99% ethanol at 0 °C primary amines react with OPA, yielding products which indicate formation of both imines and diimines as intermediates.[19] In the presence of tetracarbonylhydridoferrate as a reducing agent, 2-alkylisoindolines were selectively formed[20] (eq 5).



(5)

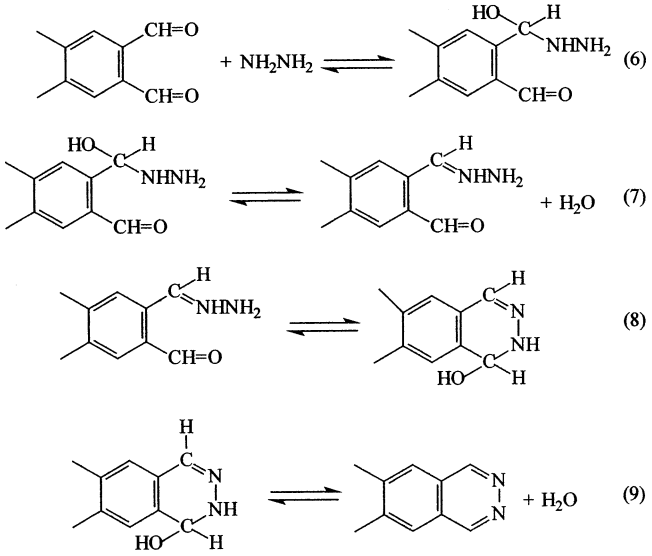
Fluorescence of an unidentified product of the reaction of OPA with methylamine was used for determination of *N*-methylcarbamate and carbamoyloxime pesticides after hydrolysis.[21]

No information is available concerning equilibria of the reaction of OPA with amines and the rate of their establishment.

2.4. Reactions with Hydrazine

Reaction of OPA with hydrazine yields a fluorescent species at pH 4−6.[22] On the basis of fluorescence of the final product, a ftalazine derivative, and comparison with reactions of analogous 1,2-dicarboxaldehydes derived from naphthalene and anthracene, the following reactions, eqs 6−9, have been proposed.[23]

In agreement with eqs 6−9, the formation of the ftalazine derivative product is first order in hydrazine and first order in dicarboxaldehyde. Ring closure in reaction 8 was assumed to be the rate-determining step. A decrease of the observed rate constant at high excess of OPA was attributed to a competing reaction. Variations in the values of the rate constant with pH were attributed to changes in protonation of the hydrazine. However, the pH ranges in which these changes were observed are not related to known



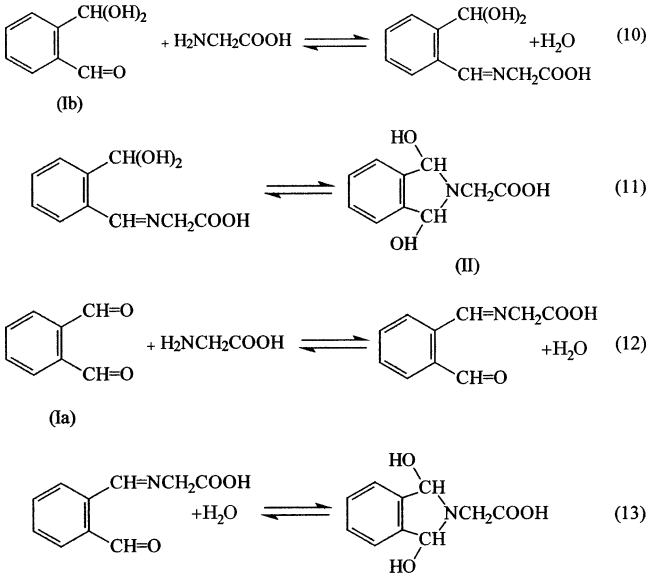
acid−base properties of hydrazine. Interpretation of the experimental results seems to be complicated by differences in the fluorescence of the protonated and unprotonated forms of the ftalazine derivative.

Reaction of OPA with phenylhydrazines[24] in a reaction sequence analogous to eqs 6−9 yielded a hydroxydihydroftalazine, which in acetic acid dimerized.

2.5. Reactions with Amino Acids

Color reaction of OPA with glycine at pH ≈ 10 was used early[25,26] for the detection of amino acids, but the reaction lacked sensitivity. Polarography has been used[27] at pH 10.5 to follow the decrease of the limiting currents of both the reduction of the unhydrated form (Ia) and at more negative potentials of the acyclic hydrated form (Ib) of OPA with time. The isoindole derivative formed as the final product of the reaction of OPA with amino acids is electroinactive, similar to the cyclic hemiacetal formed in the reaction with water (Ic), as stated in section 2.1.

Inspection of changes in current−voltage curves with time presented in Figure 2 of ref 27 indicates a faster conversion of the acyclic hydrated form Ib into the cyclic product (II) in eqs 10 and 11 than that of the unhydrated form Ia in eqs 12 and 13.



This observation would indicate that the nucleophilic substitution of the OH group in eq 11 is faster than the nucleophilic addition to the carbonyl group in eq 13 and that the substitution in eq 11 is faster than the establishment of equilibria between Ia and Ib. It cannot be, nevertheless, excluded that the potentials for reduction of Schiff bases formed in reactions 10 and 12 differ only slightly from the reduction potentials of the parent carbonyl compounds and that interpretation of the kinetics in Figure 2 of ref 27 is more complex.

In the presence of a small excess of the OPA reagent (1 mM) in a solution of 0.5 mM amino acid at pH 10.5 the reaction was slow. The time needed for completion of this reaction (at more than 95% conversion) varied from 2 h for glycine, to 1.8 h for tryptophan, to 1.5 h for histidine (where a different reaction is involved, see below), to 1 h for aspartic acid, and to 0.3 h for lysine.

Whereas for glycine, alanine, tryptophan, and aspartic acid the stoichiometry of their reaction with OPA was 1:1, for reactions of OPA with histidine and lysine a ratio 3:2 was reported.[27]

The indole derivatives formed in the reaction of OPA with amino acids yielded a poor fluorescence, with the exception of histidine, histamine, and some other compounds[28] discussed below. The intensity of the fluorescence of the reaction product increased considerably in the presence of borohydride and some thiols,[28] but these reactions are discussed in section 3.1.

In boiling acetic acid the isoindoles formed in reactions analogous to eqs 10−13 can undergo dehydration and conversion into N-substituted isoindol-1-ones.[29] A similar product is assumed to be formed in the reaction of OPA with cystine, which is reported[30] to react with two molecules of OPA, forming a reaction product with two pK_a values, 3.70 and 5.88.

As indicated above, histidine and histamine as well as spermidine, arginine, and seratonin yield on reaction with OPA a fluorescent species, even in the absence of borohydride or thiol[31] (reactions of OPA with cysteine and glutathione are discussed in section 2.8).

Histamine reacts with OPA in alkaline media, at pH ≈ 12.5, and yields a fluorescent species,[32] which is unstable and produces both fluorescent and nonfluorescent species.[33] To minimize effects of consecutive reactions, it was suggested[34] to prepare the primary product in the absence of air oxygen at 0 °C or even better[35] at −20 °C. Alternatively, the formation of this unstable fluorophore is followed[36] within a short interval (about 5 min after preparation of the reaction mixture) after the solution was acidified to pH ≈ 2.5 using citric and phosphoric acids. The fluorescent species, obtained under such conditions, is stable, but its structure is different[33] from that of the species yielding fluorescence at pH 12.5. The fluorescence in acidic media is quenched by halide ions.[37] The reactivity in quenching of these ions increases in the sequence $F^- < Cl^- < Br^- < I^-$. This indicates a possibility that halides react as nucleophiles in a side reaction with an intermediate in the formation of the fluorescent species.

A decrease in the fluorescence intensity was also observed[38] in the presence of as little as 1% v/v of DMSO.

Despite attempts[32,33,39] to identify the structure of the fluorescent species, the situation is most aptly described[40] by "for structures of these fluorescent end-products... the definite evidence is still lacking." From the acidified reaction mixture Rönnberg et al.[39] isolated a product which (based on MS) corresponds to reaction of one molecule of histamine with two molecules of OPA. As indicated above, the structure of this product is different from that of the species that is formed in alkaline media. It can even be questioned whether the isolated species is responsible for the fluorescence in acidified media. The authors[39] assumed formation of a tetracyclic species (presumed to be a dihydrofenantroline derivative). The formation of such species would require the following: (1) a nucleophilic attack of the C-5 carbanion of the imidazole ring on the carbon of the primarily formed Schiff base; (2) a nucleophilic addition of the imidazole NH group to the second, unhydrated CHO group of OPA; (3) a nucleophilic addition of an NH group of the piperidino ring of an intermediate to a second molecule of OPA. Considering the limiting reactivity of secondary amino groups in additions to carbonyl compounds, the above reaction sequence might be questionable.

As some of the reactions attributed[39] to the formation of the fluorescent species F_{acid} (formed in acidic media) are base catalyzed, they would occur before acidification. In this sequence of reactions the unstable fluorescent species F_{base}, formed at pH > 9, would be a precursor of the species F_{acid}. Kinetic studies[33] nevertheless demonstrated that species F_{base} is not a precursor of species F_{acid}.

The decay of species F_{base} follows two reaction paths: One is first order in OPA, and the other is independent of concentration of OPA. It was proposed[33] that the precursor of species F_{acid} is either a Schiff base derived from Ia or more likely the isoindole derivative formed by its cyclization. This was then assumed to react with a second molecule of OPA. To form the unstable fluorescent species F_{base} in alkaline solutions, it is presumed that one of the intermediates mentioned above undergoes another base-catalyzed reaction with a second molecule of OPA.

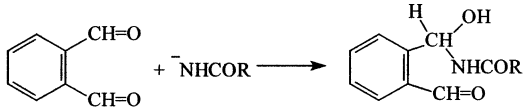
None of the proposed reaction schemes[32,33,39] is presented here in view of the uncertainties mentioned above and due to the lack of consideration of the possibilities of differences in reactivities of forms Ia, Ib, and Ic (section 2.1) toward histamine. The rates of establishment of equilibria between these three forms are acid−base catalyzed, and catalysis of their establishment may affect the kinetics of formation of the fluorescent product.

The reaction of OPA with histidine seems to follow a pattern resembling that involved in the reaction with histamine. After the initial reaction was carried out in an alkaline solution, fluorescence was measured after quenching the reaction mixture with an acid.[32,40−42] It has been proposed that the limited accuracy of the determination of histidine based on the reaction with OPA may be due to the presence of more than one active form in solutions of OPA, but this aspect has never been followed quantitatively. In some studies, where the hydration of OPA was recognized as a source of limited reproducibility, care was taken to prepare an anhydrous form of OPA, which was dissolved in xylene rather than in an alcohol. However, the reactions yielding the fluorophore were then carried out in aqueous solutions, and the role of hydration was no longer considered. In some instances, where the reaction was carried out in water−alcohol mixtures, the situation was even more complicated (cf. section 2.2).

Electroanalytical techniques offer an alternative approach to obtaining further insight into the solution chemistry involved. This was indicated by the possibility[43] of following the formation of Schiff bases based on variations of current−voltage curves with time.

2.6. Reactions with Amides, Urea, and Thiourea

In alkaline solutions (pH ≈ 13) amides with small or unbranched substituents react with OPA,[44,45] yielding isoindolines (eqs 14 and 15), whereas bulky substituents yield phthalans (eqs 16 and17).



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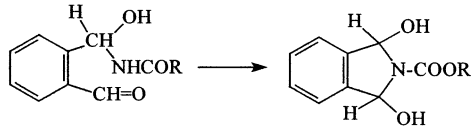

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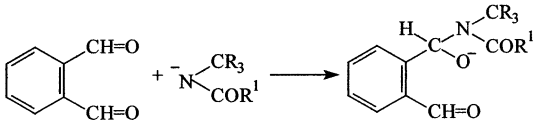

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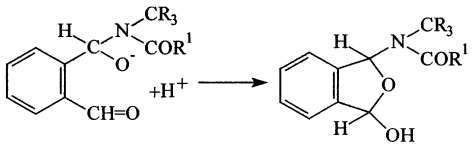


(17)

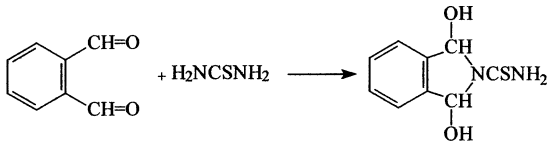
Determination of urea[46] is based on a reaction of OPA with urea, which is assumed to result in formation of either 1,3-dihydroxyindoline or 1-ureido-3-hydroxyphthalan. The product then is allowed to react with *N*-(1-naphthyl)ethylenediamine, producing an intensely colored product absorbing at 505 nm. The method has been applied to determination of urea in blood serum[47] and was automated.[46,48]

Thiourea reacts with OPA in dilute solutions of sodium hydroxide to yield an isoindoline (eq 18), which on standing forms a dimer.[49]

Under identical conditions, urea forms only the dimer.

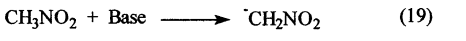
2.7. Reactions with Carbanions

An example of reactions of OPA with carbanions involves electrogeneration of a superoxide anion by



(18)

reduction of molecular oxygen in an aprotic medium.[50] This strong base reacts with nitromethane to generate a carbanion (eq 19), which can add to one of the formyl groups of OPA (eq 20).



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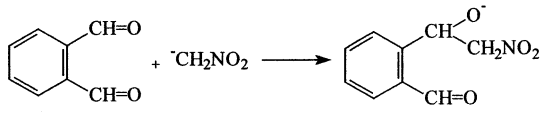
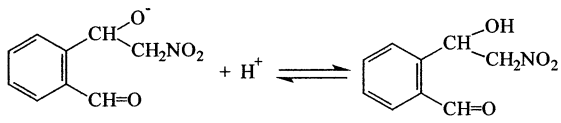


(20)

This anion can be protonated (eq 21) (possibly by a trace amount of water in the solvent, and the resulting species can react with another molecule of the base (eq 22)). Cyclization with an internal proton transfer then follows eq 23.



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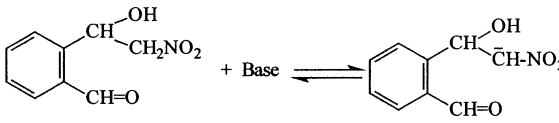

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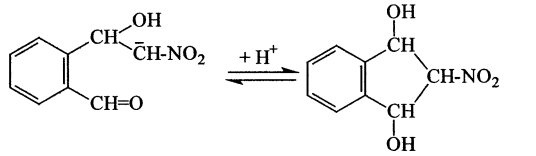
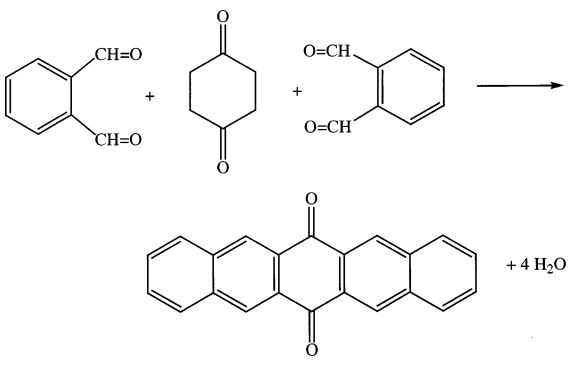


(23)

It can be questioned why a base in reaction 22 would attack the carbon rather than the hydroxy group or add to the second formyl group. Identification of the acid−base reaction involved would be needed to distinguish between these alternatives.

Another example of reactions of OPA with carbanions are reactions with cyclic 1,4-diketones in alkaline solutions,[51,52] exemplified by reaction 24.

Hence, even reactions of OPA with carbanions result in ring formation, as observed with nucleophiles bearing two hydrogens on a heteroatom.



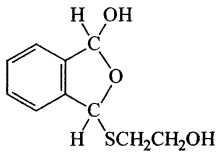
(24)

Similar additions have been reported[53,54] for reactions of OPA with indanone.[53,54] Formation of some cyclic diacetals in reactions of OPA with 2-substituted-1,3-propandiols is acid catalyzed.[55]

2.8. Reactions with Thiols and Other Sulfur Compounds

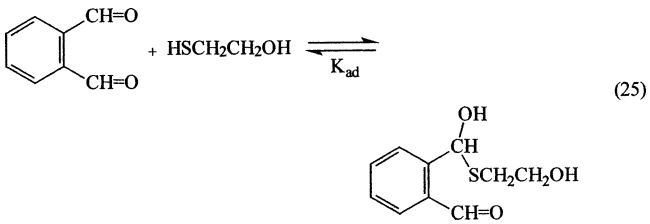
Despite the importance of the interaction of OPA with thiols, which represents the first step in procedures most frequently used for determination of amino acids, amines, ammonia, and other nucleophiles (cf. section 3), surprisingly little attention has been paid to the course and mechanism of simple reactions of OPA with thiols.

Using TLC, the formation of an adduct with OPA has been proved only for thiols carrying functional groups capable of hydrogen bonding, whereas alkyl- and arylthiols gave no apparent reaction.[56] All attempts to isolate the product of the reaction of OPA with 2-mercaptoethanol failed, but CI mass spectra indicated formation of a 1,3-dihydroisobenzofuran (III).



(III)

Variation in the absorbance of OPA at 300 nm with time in the presence of 2-mercaptoethanol followed kinetics first order in OPA and first order in thiol (eq 25).



(25)

Establishment of this equilibrium was proved by the reversibility of the ^{1}H NMR signal with variation in temperature. The reaction was investigated in a borate buffer at pH 9.0, but no information was offered about the role of pH on this equilibrium. Under these conditions, where thiolate is the predominant nucleophile, an overall equilibrium constant K_{ad} = [adduct]/[OPA]·S_{RSH} (where S_{RSH} is the analytical concentration of the thiol) was calculated (K_{ad} = 164 L mol^{-1}), corresponding to a 1:1 adduct.[57] At a large excess of the thiol, formation of another adduct was indicated, possibly a 1:2 adduct in the formation of which both formyl groups participate.

As mentioned briefly above, glutathione reacts with OPA and yields a fluorescent species.[58] The plot of the measured fluorescence intensity as a function of pH has the shape of an increasing dissociation curve with an inflection point at pH 7.7[58] or 6.5.[59] Between pH 8 and 12 the fluorescence remains practically unchanged. At pH 8 the reaction was practically complete at 20 °C within about 15 min. The activa-

tion (absorption) peak occurred at 350 nm and the fluorescence emission peak at 420 nm. When placed in diffuse light, the fluorescent species was stable for at least 30[58] or 90 min,[59] but when kept in the fluorimeter at 350 nm, the fluorescence intensity decreased with time at a rate of about 1.5%/min.[59] At large molar excess of OPA relative to glutathione, the rate of formation of the fluorescent species increased with increasing concentration of OPA, but the final fluorescence intensity was independent of concentration of OPA. The reaction involves the SH group of glutathione, as treatment of this tripeptide with *p*-chloromercuribenzoate or *N*-methylmaleinimide inhibits the development of fluorescence.[58] It has been demonstrated that one molecule of glutathione reacts with one molecule of OPA, but no attempt has been made to identify the structure of the fluorescing species.

Under the conditions used, the reaction was claimed to be practically specific for the reduced form of glutathione.[58] Small interference by cysteine can be eliminated by using shorter reaction times.[59] Carrying the reaction out at pH 7.0 decreased possible interferences by other thiols.[60] On the other hand, when the reaction of OPA with glutathione was carried out at pH 12, it was possible to determine both the reduced and oxidized forms of glutathione.[61]

At pH 8.3 in a TRIS buffer at 25 °C under nitrogen, some thiols, such as ethanethiol, 2-mercaptoethanol, and glutathione, produce strong fluorescence on reaction with OPA.[62] Fluorescence of reaction products with 3-mercaptopropionate and homocysteine is weaker, but that of products with cysteine, cysteamine, 1,2-dimercaptoethane, 2,3-dimercaptopropanol, 2-mercaptopropionate, and 2-mercaptocetate was just negligible.[62] The reaction of OPA with cysteine was too slow at 25 °C, but when the reaction mixture was kept at 50 °C for 3 h, a stable product was formed yielding fluorescence at 424 nm when excited at 364 nm.[63]

A comparable fluorescence of products of OPA with glutathione, cysteine, and homocysteine has been reported[64] in a HEPES buffer pH 7.3. The rate constants of the second-order reaction, first order in OPA and first order in thiol, have been reported at pH 7.3 as follows: $k = 25$ L mol^{-1} s^{-1} for glutathione, $k = 220$ L mol^{-1} s^{-1} for cysteine, and $k = 236$ L mol^{-1} s^{-1} for homocysteine.[64]

Glutathione can be determined after separation of glutathione-5-conjugates (produced during lipid peroxidation) using aliphatic aldehydes on a C_{13} column after reaction with OPA.[65]

Somewhat surprisingly, no anodic wave was observed in a borate buffer at pH 9.5 in solutions containing thiol and OPA (Figure 2, curve IB, ref 66), which indicates the absence of a free −SH group.

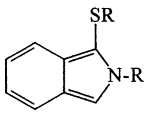
Formation of some fluorescent species has also been observed[67] in the presence of OPA with some inorganic sulfur compounds. Thus, in reaction of SO_3^{2-} the fluorescence intensity increased with increasing pH up to about pH 10 and then decreased. The highest concentration of the fluorogenic species was reached at 40 °C after about 90 min. At longer time intervals a consecutive cleavage of the fluorophore occurred. The product of the reaction of SO_3^{2-} with OPA absorbs at 360 nm. Fluorescence comparable to that in the presence of SO_3^{2-} has been reported[67] in the presence of $S_2O_4^{2-}$ and $S_2O_5^{2-}$, but negligible fluorescence has been observed for S^{2-}, SO_4^{2-}, SCN^-, $S_2O_3^{2-}$, $S_2O_6^{2-}$, $S_2O_7^{2-}$, and $S_2O_8^{2-}$.

3. Reactions in the Presence of Two Nucleophiles

As a test for detection and determination of amino acids at that time, procedures, such as the one based on reaction with ninhydrin, had limited sensitivity, Roth[28] turned his attention to conversion of amino acids into fluorescent species. On the basis of the observation[68] that *o*-diacetylbenzene can be used as a fluorogenic reagent for proteins, Roth noted[28] in a solution of this reagent in a pyrophosphate buffer at pH 8.8 a fluorescence in the presence of glycine, ornithin, histidine, and lysine. If borohydride was added to the reaction mixture, the fluorescence was observed even for alanine. He also replaced the *o*-diacetylbenzene by the more reactive orthophthalaldehyde (OPA), and instead of borohydride he included 2-mercaptoethanol (2-ME), which was assumed to act as a reducing agent. After addition of a sample to the reaction mixture containing OPA and 2-ME in a borate buffer at pH 9.0−9.5, which contained about 3% ethanol or methanol, a dramatic increase of fluorescence was observed for most amino acids. Low fluorescence has been observed (among the amino acids studied) only for cysteine, proline, lysine, and ornithin.

Perhaps the most important observation from a mechanistic point of view was that the fluorescence intensity depends on the sequence of addition of reagents. When the amino acid was added to a solution of OPA in a buffer before the thiol was added, the fluorescence was less intensive and its decrease with time faster than in the case where thiol was added to OPA first and only then the amino acid to be determined was added. This indicated "that OPA is capable of reacting with amino acid otherwise"[28] when present first, before the addition of thiol than in the preferred sequence: when OPA was mixed with thiol first and to this mixture the amino acid was added.

On the basis of NMR and MS analyses, the product of the reaction of OPA with thiol and amino acid and other amines was identified as a 1-substituted-thio-2-substituted-isoindole (IV).[69−74]



(IV)

As the species formed in the reaction of OPA with a thiol and an amino acid was difficult to isolate, products analogous reactions of OPA with 2-ME[69] or *tert*-butylthiol[56] and aliphatic amines were isolated and identified as IV. Using UV spectra it has been demonstrated that amino acids react with OPA and

2-ME forming analogous compounds with structure IV, with the exception of leucine.[56]

The reaction of OPA with primary amines, in particular amino acids, in the presence of a thiol forms the basis of numerous analytical procedures, mostly based on measurement of resulting fluorescence or absorbance.[75,76]

Literally hundreds of publications dealing predominantly with applications of determinations of amino acids and amines in the presence of OPA and thiols can be found in the literature. Still, "the mechanism of formation of the (fluorogenic) product is not completely understood" (1980, ref 57) or "to date the mechanism of the formation of the isoindole has not been established" (1985, ref 6). The situation has not improved since.

In the following discussion, existing experimental evidence will be summarized, the role of the nature and concentration of individual components of the reaction mixture will be discussed, as well as that of the composition of the reaction medium on the formation and cleavage of the fluorogen or chromogen. Proposed mechanisms will be critically evaluated.

3.1. Reagents

3.1.1. Role of the Carbonyl Compound

The fluorescence of the species formed in the reaction of a carbonyl compound and thiol with amino acids is more intense for the reaction of OPA than for the reaction of originally suggested 1,2-diacetylbenzene, as mentioned above, though use of the latter compound yielded stronger fluorescence in reactions with lysine and ornithin.[28] Hence, OPA is used as the reagent most frequently. It is commercially available in good purity, and specimens are reasonably stable.

1,2-Dicarboxaldehydes derived from naphthalene and anthracene were suggested as alternative reagents,[53,77] particularly for reactions using nucleophiles other than thiols. In particular, naphthalene-1,2-dicarboxaldehyde was claimed to offer some advantages when compared with OPA. Also, 2-acetylbenzaldehyde was stated[78] to be a useful alternative to OPA, whereas 2-benzoylbenzaldehyde offered no advantages as with this reagent the formation of the fluorescent species was slow.

3.1.2. Stability of the Mixed Reagent

It has been assumed[79] that the product of the initial reaction of OPA with thiol is stable, does not undergo cleavage even at relatively high concentrations of both components, and that on storage no fluorescent species is formed in the mixed reagent. However, it has been observed[80−87] that on standing and, in particular, at relatively high concentration of OPA and the thiol in the mixed reagent a fluorogenic species may be formed, which is manifested by additional peaks on the chromatograms.

For example, in solutions containing 10% v/v methanol with the concentration of OPA varying between 3.7 and 37 mM with the concentration of 2-ME ranging from 5 to 50 mM, on standing a fluorescent product was slowly formed and its concentration increased with time. After 48 h the fluorescence intensity of a species formed in the reagent mixture reached about 10% of the fluorescence of the product formed in a reaction containing 100 pM amino acid under the same conditions.[80]

In another example of the formation of fluorescent species in the stored reagent, OPA−3-mercaptopropionic acid mixture was kept at −4 °C for 21 days. When this aged reagent was used for determination of amino acids, the fluorescence intensity in the aged reagent had less than 5% of the value obtained using a freshly prepared reagent.[88] Generally, it is recommended to prepare mixed reagent daily fresh. The reagent solution consisting of OPA and *N*-acetyl-L-cysteine was claimed[89] to be stable for at least 1 week when stored in the refrigerator.

The rate of the decrease in reactivity of the mixed reagent over a period of days, expressed as the measured fluorescence after addition of an amino acid, also depends on the structure of the amino acid. The rate of decrease is slower for β- than α-amino acids.[90]

As the deterioration of the reagent is more marked at high concentrations of the thiol, it has been assumed that it is due to an autoxidation of the thiol,[82] but no comparison was made with solutions kept oxygen free. It has been argued that if autoxidation is the cause of lost reactivity, the process can be reversed by addition of a small amount of a thiol or bisulfite. Furthermore, the autoxidation could be minimized by eliminating catalytic effects of traces of heavy metal ions by addition of a chelating agent, such as NTA.[82] Attempts to eliminate the excess thiol from the reaction mixture by addition of iodoacetamide were unsuccessful,[86] but removal of excess OPA−*tert*-butylthiol reagent by addition of excess glycine and iodoacetamide was claimed[80] to be successful.

3.1.3. Structure of the Thiol

In addition to the most widely used 2-mercaptoethanol (2-ME)[28] and ethanethiol (ET),[91] some other thiols have been occasionally used in the reaction with OPA applied to the determination of amino acids. Among the thiols used were *n*-butanethiol,[92] *tert*-butanethiol,[56,80,93] triphenylmethanethiol,[92,93] thiophenol,[92,93] and ethanedithiol.[93] Some of the thiols bear also an OH or NH_2 group, like 3-mercapto-1-propanol,[93,94] dithiothreitol,[93,95] and *N*,*N*-dimethyl-2-mercaptoethylamine.[96] Furthermore, some carboxylic acids, their derivatives, and amino acids bearing a thiol group were used, such as methyl-2-thiolacetate,[93] cysteine,[97] and *N*-*tert*-butyloxycarbonyl-L-cysteine.[98] Among the latter group two thiols 3-mercaptopropionic acid[88,99−102] and *N*-acetyl-L-cysteine belong [79,89,91,99−105] which were recently extensively investigated. Their advantage and limitations, also compared to 2-ME and ET, were evaluated.

Differences in the reactivity of individual thiols have not been for most of the thiols more extensively discussed. An example of the possible differences involved is a comparison of reaction mixtures of OPA, thiol, and *n*-propylamine in 95% ethanol.[56] Under these conditions the adduct formed in the presence of 2-ME decayed slowly at 25 °C to yield 2,3-dihydro-1*H*-isoindole.[56] Analogous reaction with ET yielded different products. As the reaction with 2-ME is not

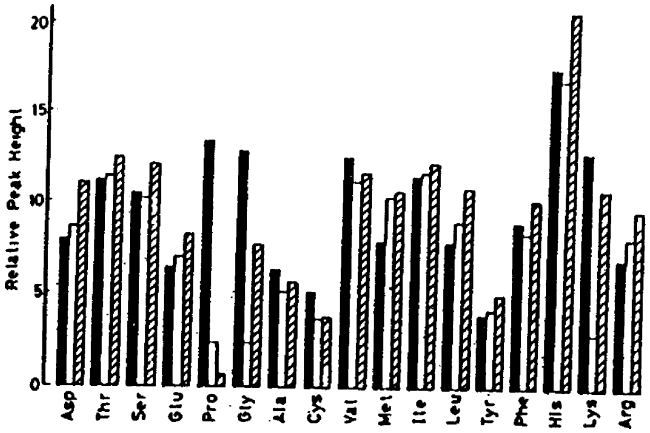


Figure 1. Comparison of relative peak heights of amino and imino acids observed with different compositions of the reagent: (full boxes) 0.5% OPA with 2-ME; (empty boxes) 0.4% OPA with 2-ME; (Shrafed boxes) 0.08% OPA with 2-ME. (Reprinted with permission from ref 103. Copyright Elsevier.)

accelerated by an increasing water content in the solvent, it was concluded that the ring closure in the presence of 2-ME as reagent involves a nucleophilic attack of the OH group of the thiol on the C−S bond.[56] Still, it cannot be excluded that the difference between the covalent addition of ethanol and water to OPA plays a role in the observed effect.

3.1.4. Structures of Amino Acids and Amines

The sequence of reactivities of individual amino acids depends strongly on the nature of the thiol used in the reaction with OPA and on concentrations of reagents used[103] (Figure 1). Besides the composition of the reaction medium, the sequence of reactivity also depends on the time interval after which the reaction between the thiol and OPA reagent and the amine was quenched or a sample taken.

Considerable differences in the reactivity of individual amino acids are manifested by the differences in values of rate constants, discussed in section 3.7. As an example,[57] the second-order rate constant (k_{obs}) for the reaction of glycine was 1.5×10^3 L mol^{-1} s^{-1}, whereas for all other 19 amino acids studied, k_{obs} varied between 0.1 and 0.6×10^3 L mol^{-1} s^{-1}.

An extensive list of α- and β-amino acids as well as of primary aliphatic amines that yield fluorescence after treatment with a mixture of OPA and 2-ME in the presence of 1% ethanol is given in ref 90. Even less common amines, like tobramycin,[112] yield a similar fluorescent product.

Using the reagent containing OPA and 2-ME it was possible to distinguish epimers, diastereoisomeric amino acids, based on differences in the rates of reactions yielding the fluorescent product.[106]

L-Leucine reacts faster than epimeric D-alloisoleucine, whereas L-threonine reacts with the product of the reaction of OPA with 2-ME somewhat slower than its D-epimer. Wider use of the reaction with OPA−thiol mixtures for separation of enantiomeric amino acids became possible after introduction of the *N*-acetyl-L-cysteine as the thiol component.[89,107−109] Diastereoisomeric derivatives of 28 amino acids were efficiently resolved on a reverse-phase column.[89]

3.2. Sequence of Addition

As mentioned in the introduction to section 3, a much stronger fluorescence can be observed when the thiol is added to OPA before the addition of the amine than when the amine or amino acid is added to OPA first. This indicates that in the first step of the reaction yielding the fluorogen the addition of at least one thiolate ion to OPA takes place. The reaction occurs in a pH range where the thiol is present, at least partly, as a thiolate. The product of the reaction between OPA and thiolate can then react further in a consecutive step with the amine or amino acid. Some authors (cf. section 3.8) considered the addition of thiol to OPA to be a side reaction in which an equilibrium is established. Several facts exclude such an interpretation. There is no reason the presence of this side reaction would result in the observed effect of the sequence of the addition of components of the reaction mixtures. Further, the equilibria between thiols and carbonyl compounds are much more shifted in favor of the product than reactions with amino compounds leading to formation of Schiff bases. Furthermore, under conditions most frequently used the initial concentration of the thiol is usually several orders of magnitude higher than that of the amino acid. Generally, S-nucleophiles are stronger than N-nucleophiles when bound on a comparable molecular frame. Thus, there is no evidence for the amine to compete with the addition of thiol to the first formyl group.

3.3. Reaction Medium

The rate of formation of the fluorescent species in the reaction of OPA with thiols and amines depends not only on the ratio of the concentrations of the three reactants, but also on the presence of other components of the reaction mixture, like buffers, on the composition of the solvent and on reaction conditions. Similar factors also affect the fluorescence intensity, measured after it reached a limiting or maximum value. Conditions for reaching the most suitable reaction rate and a sufficiently strong and time-independent (over a chosen time interval) fluorescence may differ for individual amino acids and the corresponding thiol used. Under optimal conditions in reaction mixtures, where OPA is present in excess relative to the amino acid, the fluorescence should reach a constant value within a period between 5 and 25 min. The measured fluorescence intensity increases with increasing concentration of OPA but can reach a maximum value beyond which is observed a small decrease of fluorescence with a further increase in concentration of OPA.[28] As an example, a concentration ratio of OPA to amino acid of about 18:1 was recommended.[82] On the other hand, when concentrations of OPA and the amino acid were kept constant, variation in thiol concentration was reported not to markedly affect the fluorescence intensity.[28]

Both the rate of the reaction generating it and the concentration of the fluorescent species at chosen optimal conditions depend strongly on the temperature of the reaction mixture. The effect of this adjustable variable on reactions of various amino acids depends strongly on the nature of the thiol

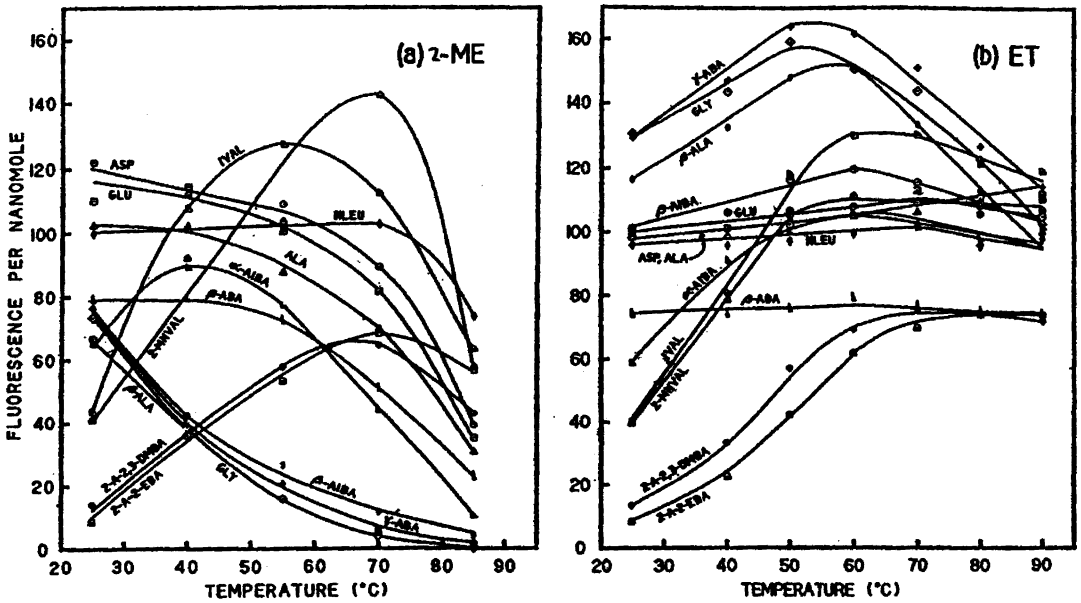


Figure 2. Effect of reaction temperature on fluorescence yield for reactions of various amino acids with OPA and (a) 2-ME and (b) ET. (Reprinted with permission from ref 110. Copyright 1979 Elsevier.)

used,[110] as shown for reactions of OPA with 2-ME and ET (Figure 2).

Another factor that can affect the reaction of OPA with thiol and the primary amino group is the composition of the solvent used. Organic solvents used in mixtures with water as a reaction medium for this reaction may not only affect the kinetics and equilibria by a variation of the dielectric constant and by changes in solvation of individual reactants but can be involved in covalent reactions with OPA. An increase in the concentration of the organic solvent can affect the hydration−dehydration equilibria involving OPA. When the organic cosolvent is an alcohol, it can act as a nucleophile and add to OPA (as mentioned in section 2.2) and establish additional equilibria. The position of these equilibria and rates of hydrolysis of hemiacetals and/or acetals formed might affect the reactivity of OPA in its reaction with thiols and amines or amino acids.

Another example of a solvent effect on the reaction of OPA with thiols and amino acids, which has been reported but not interpreted, is the role of DMSO in the solvent mixture. In solutions containing 0.33 mM OPA, 1.3 mM ET, and 0.06 mM amino acid the UV absorbance of the generated isoindole derivative is little affected by the presence of DMSO as a cosolvent. On the other hand, the fluorescence intensity under these conditions sharply increases with increasing fraction of DMSO in the reaction mixture. The increase is particularly marked in the presence of between 60% and 83% v/v DMSO in the reaction mixture with water.[95] This indicates a solvent−solute interaction in one of the excited states rather than in the ground state.

A similar type of solvent effect was observed for the reaction of OPA with ET in the presence of *n*-propylamine.[95] Comparison (Figure 3) indicates limited solvent effects for excitation spectra of the reaction product in aqueous solution of a borate buffer in 95% ethanol and in isooctane but considerable variations in emission spectra. Decreasing solvent polarity results in a marked blue shift in λ_{max} values in the emission spectra. The variation in fluorescence intensity with solvent polarity depends strongly on the thiol used[93] (Figure 4).

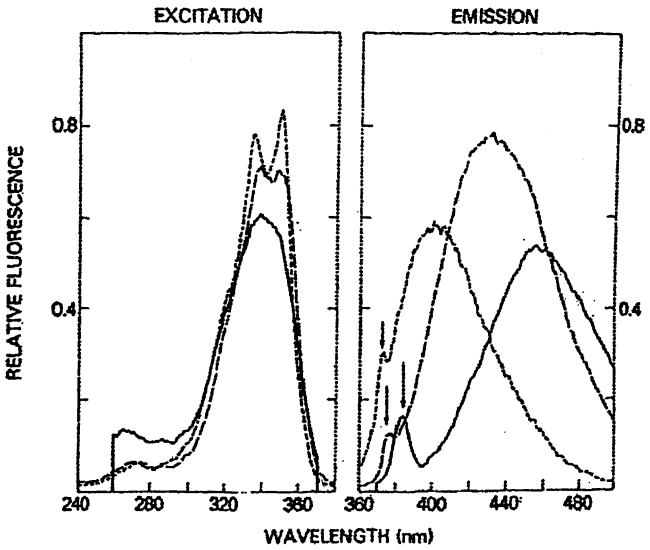


Figure 3. Excitation (left) and emission (right) spectra of an adduct formed in the reaction of OPA ethanethiol (ET) and *n*-propylamine. Concentration of the adduct: 3.3×10^{-7} M in borate buffer pH 9.3 (—), 6.7×10^{-7} in 95% ethanol (- - -), and 2.7×10^{-6} M in isooctane (· · ·). λ_{max} values for emission were 453 nm in aqueous, 435 nm in ethanolic, and 400 nm in isooctane solution. λ_{max} value for excitation was (338 ± 1) nm for all solvents. Due to differences in concentrations, only shapes and λ_{max} should be compared. (Reprinted with permission from ref 93. Copyright 1978 Elsevier.)

The fluorescence of the product of the reaction of OPA with thiol and amine or amino acid was quenched by $CHCl_3$, CH_3CCl_3, and $ClCH{=}CCl_2$.[93] The fluorescence was also suppressed in the presence of Cu^{2+} or Ni^{2+} ions. This effect can be minimized by addition of a chelating agent, such as EDTA.[98]

Last but not least, the effects of pH and the composition of the buffer on the fluorescence in

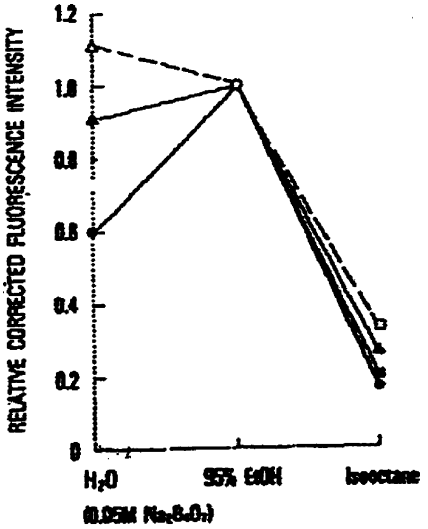


Figure 4. Effect of solvent composition on the relative fluorescence intensity of various isoindoles at their emission λ_{max}, expressed relative to the fluorescent intensity in 95% ethanol. The thiol in the reaction with OPA varied: (—) ET, (▲) 2-ME, and (■) butylthiol, all with *n*-propylamine; (△) 2-ME adduct with leucine; (□) dibutylthiol adduct. (Reprinted with permission from ref 93. Copyright 1978 Elsevier.)

mixtures of OPA with thiol and amine should be considered. The effect of pH on the observed fluorescence enables us to obtain information about the role of acid−base equilibria on individual reaction steps. In the interpretation of such effects it should be kept in mind that buffer components can also react with OPA and/or reaction intermediates.

In the majority of reactions of amino acids with the product of the reaction of OPA with thiol the maximum fluorescence intensity is obtained between pH 8 and 11, but for the reaction product with lysine, the strongest fluorescence was reported between pH 6 and 7. For histamine the highest fluorescence was observed[28] at pH 6, but also a limiting, pH-independent intensity was reached at pH > 10.5. On the other hand, when an isoindole derivative resembling the product of the reaction of OPA with thiol and amino acid was synthesized and transferred to various buffers, the fluorescence has been reported to vary little between pH 6 and 11.5. Thus, the pH independence of fluorescence described above can be attributed to pH effects on the rate, extent of the reaction, and consecutive reactions of the adduct in the reaction mixture containing OPA, thiol, and amino acid or amine.

In addition to their role in affecting the acid−base reactions preceding the rate-determining step or other forms of acid and/or base catalysis, buffer components can also interact chemically with starting materials or reaction intermediates. The possibility of complex formation should be, in particular, kept in mind when borate buffers are used. Boric acid and/or borates readily form complexes with 1,2-diols and can form complexes with the acyclic hydrated form Ib of OPA. Similarly, they can interact with some intermediates of the formation of the adduct. Whereas for some reactions of OPA with 2-ME and amino acids it was reported[81] that the buffer composition has no effect on measured fluorescence, higher fluorescence of the adduct formed in borate buffer pH 8 than in a phosphate buffer of the same pH was observed.[28] Following the effects of the buffer composition and concentration may enable distinguishing between effects of complex formation and general acid−base catalysis.

Other useful information, particularly about charges of reactant and/or intermediates, could be obtained by investigating the effects of ionic strength and ionic charges on rates and equilibria, but no information about such effects is available.

3.4. Kinetics of the Formation of the Primary Product

The reactions between OPA, thiol, and amino acid or amine are usually carried out under such conditions when, first, the equilibrium of the reaction between OPA and thiol is established and only then is the amino compound added. The concentrations of both OPA and thiol are usually higher (often by 1 order of magnitude or more) than that of the amine. Under such conditions the reaction resulting in formation of the adduct proceeds rapidly and the highest concentration of the fluorescent adduct is reached within minutes or their fractions. Therefore, studies of the initial stage of this reaction were carried out using stop-flow technique.[6,57,106,112] To follow the reaction, more recently[111,113−115] special spectrophotometric techniques enabling rapid measurement of absorbance instead of following the fluorescence have been introduced.

At relatively low concentrations of OPA, thiol, and amino acid in a ratio of 1:24:1, the reaction was a second-order reaction, first order in OPA and first order in amino acid.[57] When corrected for protonation of the amino group, the second-order rate constant for glycine was found to be 1.5×10^3 L mol^{-1} s^{-1}. The values for other amino acids varied between 1.5 and 4.5×10^2 L mol^{-1} s^{-1}. When the reaction was carried out in the presence of an excess of OPA and 2-ME (as used in practical analysis) and the measured pseudo-first-order rate constants were converted into second-order rate constants, these values were found to be about 30% lower than the constants obtained under second-order conditions. This was attributed to the role of the thiol, which at high concentration may shift the equilibrium by increasing the role of the reverse reaction.

First-order kinetics has also been observed in solutions containing 1−2.5 mM OPA, the same concentration of thiol, and about 0.1 mM amino acid.[79] The values of k_{obs} for reaction in the presence of 2-ME were 9.3×10^2 L mol^{-1} s^{-1} for glycine and $0.2-1.0 \times 10^2$ L mol^{-1} s^{-1} for all other amino acids, whereas in the presence of *N*-acetyl-L-cysteine the value of k_{obs} for glycine was 2.7×10^2 L mol^{-1} s^{-1} and for all other amino acid varied between 0.3 and 1.0×10^2 L mol^{-1} s^{-1}. First-order kinetics in the presence of an excess of OPA and thiol was observed[106] also for reactions of diastereoisomeric amino acids.

First-order rate constants, obtained for a given amino acid in the presence of an excess of OPA and

thiol, depend on pH. The plot of the dependence of $k_{obs} = f(pH)$ is bell shaped, with the maximum value of the rate constant for the reaction of alanine[57] between pH 10.3 and 10.7 and for the reaction of valine[111] at pH about 10. The portion of this curve which increases with increasing pH is attributed to deprotonation of the zwitterionic form of the amino acid. The decreasing part of the pH dependence at pH > 10 can be attributed to a dissociation of the acyclic hydrated form Ib of OPA or to addition of OH^- ions to one of the formyl groups (cf. section 2.1, ref 13). Similar dependence of the initial rate on pH was observed[112] for the reaction of tobramycin.

The dependence of the first-order rate constant for the reaction of amino acids in the presence of an excess of OPA and thiol on the concentration of the thiol is not simple. The observed rate constant was reported to either decrease[6] or reach a limiting value[57,106,112] at a sufficiently high concentration of thiol. The plot of the value of the rate constant k_{obs} as a function of log [2-ME] has the shape of a dissociation curve with an inflection point corresponding to an equilibrium constant K = [adduct]/[OPA][thiol] = 1.0. This behavior can be interpreted as corresponding to a reaction where the rate-determining step is preceded by an equilibrium between OPA and the thiol.

When the product of the reaction of OPA, thiol, and amino acid was formed in situ at pH ≈ 10 and transferred to buffers of varying pH, the dependence of the measured absorbance on pH had the shape of a dissociation curve with an inflection point at pH 9.0 ± 0.2. This behavior was attributed to dissociation of a proton from the nitrogen of the isoindole ring with pK_a ≈ 9.0. When a similar experiment was carried out with an alkylester of an amino acid, the observed dependence of absorbance on pH corresponds to a dissociation with pK_a = 6.1 ± 0.1. The difference between the two pK_a values resembles that between pK_a values of the dissociations of the protonated forms of the amino acid and its ester.[111]

3.5. Consecutive Reactions of the Isoindole Derivative—Stability of the Product of the Analytical Reaction

Considerable attention has been paid to the stability of the isoindole derivative formed in the reaction between OPA and a thiol in the presence of the amino acid or amine to be determined.[78,81,88,91,93−95,101,102,111,116−120] The (in principle limited) stability of the analytical response is due to a consecutive reaction of the primary product—the isoindole derivative. There are two main reasons for interest in the stability of the fluorescent or absorbing product: For direct analytical exploitation of this reaction, it is of importance to know the optimal time interval in which, after mixing, fluorescence or absorbance measurement should be carried out. The stability of the isoindole derivative is also of considerable importance when analysis of a mixture of amino acids is carried out using one of the separation techniques (usually a variant of chromatography) or by flow-injection analysis. The stability of the fluorescent species is of particular interest when the derivatization of the amino acid or the amine is carried out as a precolumn rather than a postcolumn operation.

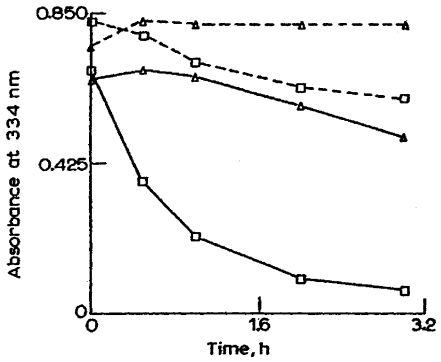


Figure 5. Dependence of absorbance of isoindole derivatives on time. Adducts were prepared from 6.7 × 10^{-4} M glycine or alanine was added. Zero time reading was within 3 min after manual mixing. Solid lines: (△) product of OPA, ET, and alanine; (□) product of OPA, ET, and glycine. Dashed lines: (△) product of OPA, 2-ME, and alanine; (□) product of OPA, 2-ME, and glycine. (Reprinted with permission from ref 95. Copyright 1979 Elsevier.)

The stability of the fluorescent species depends on numerous factors,[119] such as composition and age of the OPA−thiol reagent, pH, buffer and solvent composition, temperature, both ratios and absolute concentrations of the all three components (OPA, thiol, and the amino compound) in the reaction mixture, and furthermore on the structure of both the thiol and the amino acid or amine involved[95] (Figure 5). Consequently, it is difficult to draw general conclusions. The situation is complicated by the fact that in many instances the stability is either discussed qualitatively verbally or using graphs depicting changes in fluorescence with time without making an attempt to interpret rigorously the reactions involved. Hence, some qualitative observations will be summarized before discussing the more rigorous treatments of the kinetics of the decay of the fluorescent species.

The most widely used thiol in the reaction with OPA and the amino acid or amine is still the originally proposed 2-mercaptoethanol (2-ME), even when it has been proven that the use of ethanethiol (ET) in the reaction with OPA results in a better stability of the fluorescent product.[93,121] Excess of OPA−2-ME reagent significantly affects the stability of the adduct.[94,97,107] An even more favorable stability of the fluorescent species has been more recently reported for the use of the reagent composed of OPA with 3-mercaptopropionic acid or *N*-acetyl-L-cysteine for reactions with amines[97] and amino acids.[83,96] The stability of the fluorescent isoindole derivative has been found to be dependent also on the structure of the amine to be determined.[94,97,107] Increased concentration of an alcohol in the reaction mixture has been reported[122] to increase the stability of the adduct.

Considerable differences in the stability of the fluorescent species were observed when different methods of derivatization were used. Different information about the stability of the fluorescent species was obtained depending on whether the isocratic phase used consisted of a mixture of methanol and

acetic acid (52:48) or of acetonitrile and water (40:60).[122] This opens the question of whether chromatography is the best method for investigation of the stability of the rather reactive fluorescent species. Such species may undergo additional chemical reactions during passage through the column, particularly when the mobile phase contains alcohols, which may react with other components of the reaction mixture. To prevent this and other consecutive reactions during passage through the column, extraction with ethyl acetate has been recommended prior to injection on the column,[120,122] which is, nevertheless, another possible source of error. To minimize the decomposition of the fluorescent compound during passage through the column, use of a mobile phase was recommended, which consists of 0.1 M *N*,*N*-dimethylcyclohexylamine and 0.2 M phosphoric acid in a 70−90% methanol gradient.[122]

The fluorescence of the isoindole derivative was reported[95] to be enhanced in the presence of surfactants, like SDS or Brij, with the largest effect at concentrations below CMC (critical micelle concentration).

In some instances[61] extensive investigations have been carried out under conditions so different from those used in the majority of other studies that a comparison is impossible. Whereas the reaction is usually carried out in the presence of an excess of both OPA and thiol, in this study[61] the thiol was present in excess, but the ratio of OPA to amino acid varied only from 3:1 to 1:5.

Nevertheless, there is practically a unanimous agreement that the observed decrease of fluorescence with time is due to consecutive chemical reactions of the isoindole derivative.

Under the simplest conditions, cleavage of the isoindole derivative follows first-order kinetics in the fluorescent species but also first order in thiol and first order in OPA. Still, in certain concentration ranges of OPA and thiol used, the variation of the change in concentration of the fluorescent species with time indicates operation of a more complex reaction scheme. Deviations from first-order kinetics are sometimes observed in the presence of a large excess of thiol. When the role of concentration of OPA is followed, deviations from simple kinetics were observed both at high concentrations of OPA and when the concentrations of OPA and amino acid are comparable.

In solutions containing a large excess of thiol in the presence of 2 mM OPA and the amino acid at concentrations 1 or 2 orders of magnitude lower than that of OPA, the concentration of the adduct, followed by absorbance at 340 nm,[111] for most amino acids showed limited variation with time. The exception was the behavior of glycine and histidine, where a considerable degradation of the adduct with time was observed.

For a reaction mixture containing an (undefined) excess of 2-ME, about 2 mM OPA, and 0.1 mM amino acid, a good fit of the changes in absorbance at 340 nm with time was found for eq 26

$$\text{OPA} + \text{thiol} + \text{RNH}_2 \xrightarrow{k_1} \text{C} \xrightarrow{k_2} \text{D} \xrightarrow{k_3} \text{P} \quad (26)$$

where C is the absorbing isoindole derivative, D another absorbing intermediate, and P a product. For glycine the following set of rate constants were found: $k_1 = 930$ L mol^{-1} s^{-1}, $k_2 = 43 \times 10^{-5}$ s^{-1}, $k_3 = 43 \times 10^{-3}$ s^{-1}. For the reaction of histamine, $k_1 = 22$ L mol^{-1} s^{-1}, $k_2 = 8 \times 10^{-5}$ s^{-1}, and $k_3 = 22 \times 10^{-3}$ s^{-1}.

It is impossible to exclude that any or all rate constants correspond to establishment of equilibria or that D is formed in a competitive rather than consecutive reaction. The authors[111] assume that C is 1-alkylthio-2-substituted isoindole, D is 1-hydroxy-2-substituted iso indole, and P is an N-substituted 2,3-dihydro-1*H*-isoindol-1-one, which does not absorb at 340 nm.

Possibly of a similar origin are deviations from first-order kinetics observed in the presence of an excess of thiol when concentrations of OPA and the amino compound are comparable (between 1:15 to 15:1). Plots of the fluorescence intensity as a function of time show an induction period. The length of this period increased with decreasing concentration of OPA. The scale of Figure 3 in ref 91 does not allow one to distinguish if a short induction period is not present even at a higher concentration of OPA. Simultaneous variations in concentrations of both OPA and the thiol do not allow identifying the role of individual components.

The authors[91] attributed the presence of the induction period to a radical reaction, initiated by autoxidation, but offered no experimental evidence for formation of radicals during the induction period. However, induction periods may also result in the presence of antecedent or competitive reactions which may be ionic and may be either irreversible or lead to establishment of equilibria.

The kinetics of the cleavage of the isoindole derivative was studied[91,94,116] in more detail under conditions when the decrease of the concentration of the fluorescent species with time followed practically first order kinetics (i.e., when the above-mentioned induction period was negligibly short). Under such conditions the cleavage reaction is first order in OPA. Furthermore, as long as the initial concentration of thiol is comparable to the initial concentration of OPA, the reaction follows kinetics that is first order in thiol. To summarize, under conditions when the induction period can be neglected, the reaction follows simple kinetics laws: degradation of the isoindole derivative is an overall third-order reaction—first order in the adduct, first order, in OPA, and first order in thiol. Nevertheless, at a sufficiently high excess of thiol the reaction rate oppositely *decreases* with increasing initial concentration of the thiol used.

Under the simplest conditions the kinetics of the degradation indicates that the isoindole adduct formed in the fast initial step can undergo consecutive reactions either with another molecule of OPA and another thiolate anion or with an adduct of OPA and the thiol. These reactions can be either irreversible or equilibria and occur either as a set of consecutive reactions, as in eq 26, or in competitive side reactions. The observed decrease of k_{obs} with a large excess of

Table 1. Dependence of Rate Constants of the Decay of the Fluorescent Isoindole Derivative on Reaction Mixture Composition

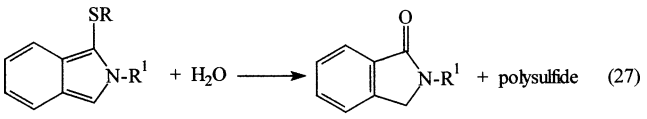
solvent	buffer	pH	*T*, °C	[OPA], mM	[RSH], mM	RSH	[RNH_2] mM	RNH_2	$k_{obs} \times 10^4$ s^{-1}	ref
25% MeOH	Carb	9.6	25	33.3	33.3	CH_3SH	0.17	$H_2N(CH_2)_3COO^-$	13	116
						CH_3CH_2SH			10	
						$(CH_3)_2CHSH$			4.5	
						$(CH_3)_3CSH$			2.8	
3% EtOH	Bor	9.7	–	1.5	1.2	$HOCH_2CH_2SH$	0.1	histamine	80[a]	111
								$H_2NCH_2COO^-$	43[b]	
25% MeOH	Carb	9.6	25	33.3	33.3	$HOCH_2CH_2SH$	0.17	H_2NCH_3	52	116
								$H_2NCH_2CH_3$	20	
								$H_2NCH(CH_3)_2$	5.5	
								$H_2NC(CH_3)_3$	slow	
								$H_2N(CH_2)_2CH_3$	9.5	
								$H_2NCH_2CH(CH_3)_2$	3.8	
								$H_2NCH_2C(CH_3)_3$	0.55	
								$H_2N(CH_2)_3CH_3$	5.2	
								$H_2N(CH_2)_2CH(CH_3)_2$	2.8	
25 MeOH	Carb	9.6	25	33.3	33.3	ET	0.17	$H_2N\text{-}CH_2COO^-$	9	116
								$H_2NCH(CH_3)COO^-$	0.23	
								$H_2NC(CH_3)_2COO^-$	slow	
								$H_2N(CH_2)_2COO^-$	5.7	
								$H_2N(CH_2)_2OH$	9.8	
								$H_2NCH_2CH{=}CH_2$	7.3	
								$H_2N(CH_2)_2OCH_3$	5.5	
								$H_2N(CH_2)_2C_6H_5$	1.8	
0.5% MeCN	Bor	8.95	40.5	0.15	1.45	2-ME		$H_2N(CH_2)_2CH_3$	6.3	94
								$H_2N(CH_2)_3COO^-$	5.5	
								$H_2N(CH_2)_2COO^-$	4.5	
								$H_2NCH_2COO^-$	3.7	
								$H_2NCH_2CH(CH_3)COO^-$	2.0	
								$H_2NCH(CH_3)COO^-$	1.2	

[a] Consecutive reaction: $k_3 = 40 \times 10^{-4}$ s^{-1}; [b] Consecutive reaction: $k_3 = 220 \times 10^{-4}$ s^{-1}.

thiol can be attributed to formation of an inactive adduct in a side reaction of OPA with the thiol.[6]

The deviations from simple kinetics at high concentrations of thiol were attributed[93] to formation of an adduct (bis-thiohemiacetal or thioacetal) in a reaction of OPA with two molecules of the thiol in a side reaction to the formation of the isoindole derivative. However, adducts of two thiolates to one molecule of OPA are known to be fluorescent also, even with a lesser intensity than the 1:1 adduct.[93] Moreover, the adduct of one OPA with two thiolates also undergoes a slow degradation into a nonfluorescent product.[93]

In this context, identification of the final product of the degradation of the isoindole derivative (P in eq 26) is of importance. Both at high (0.3 M) initial concentrations of OPA and 2-ME[93] and at lower concentrations of these reagents, resembling those used in analytical procedures,[94] the final reaction product was identified as N-substituted-phthalimidine formed by hydrolysis (eq 27).



(27)

This simplified reaction scheme does not explain the observed effects of concentrations of OPA and thiol on the reaction rate of the cleavage. An attempt to support eq 27 by the effects of acid catalysis[93] is questionable, as data in Table 3 ref 93, at pH 5.3 were obtained in unbuffered solutions.

The reported values of rate constants k_{obs} obtained under conditions of first-order kinetics are summarized in Tables 1−3. They enable comparison of some structural effects on the rate of degradation of the isoindole derivative.

Both values of rate constants k_{obs} for the degradation of the isoindole derivative obtained at a given concentration of OPA and values of k'_{obs} obtained by extrapolation to [OPA] → 0 indicate that the stability of the isoindole derivative increases with the structure of the thiol RSH in the sequence

$$R = CH_3 < CH_2CH_2OH < CH_2CH_3 < CH(CH_3)_2 < C(CH_3)_3$$

As the branching on the α-carbon has a predominant effect, it seems that a steric effect controls the reactivity of the thiol. The lower stability observed for 2-ME was interpreted as being due to a possible intramolecular nucleophilic attack.

More sterically demanding branched substituents also seem to have a predominant effect on the stability of isoindoles derived from primary alkylamines ($R-NH_2$). The stability of the adduct increases in the following sequence, indicating higher stability of adducts formed from thiols (RSH) branched on the α-carbon

$$R = CH_3 < CH_3CH_2 < CH_3(CH_2)_2 < (CH_3)_2CH < CH_3(CH_2)_3 < (CH_3)_2CHCH_2 < (CH_3)_2CH(CH_2)_2 < (CH_3)_3CCH_2 < (CH_3)_3C$$

When the role of the substituent X in the amino compound H_2NCH_2X is compared, the increase in stability follows the sequence

Table 2. Dependence of Rate Constants of the Decay of the Fluorescent Isoindole Derivative on [OPA]

solvent	buffer	pH	*T*, °C	[OPA], mM	[RSH], mM	RSH	[RNH_2]	RNH_2	$k'^{a}_{OPA} \times 10$, s^{-1}	$k'^{b}_{O} \times 10^4$, s^{-1}	ref
25% MeOH	Carb	9.6	25	varied	0 at $t = 0$	CH_3SH	0 at $t = 0$	CH_3NH_2	5.7	0.17	116
						$HO(CH_2)_2SH$			3.0	0.33	
						CH_3CH_2SH			2.2	0.33	
						$(CH_3)_2CHSH$			0.93	0.17	
						$(CH_3)_3CSH$			0.17	0.05	

solvent	buffer	pH	*T*, °C	[OPA], mM	[RSH], mM	RSH	[RNH_2]	RNH_2	$k^{c}_{obs} \times 10^4$	ref
0.5% MeOH	Bor	8.95	40. 5	0.001	1.43	$HO(CH_2)_2SH$			2.1	94
				0.038					2.7	
				0.075					3.2	
				0.15					4.6	
				0.299					7.0	
				0.448					9.6	

solvent	buffer	pH	*T*, °C	[OPA], mM	[RSH], mM	RSH	[RNH_2]	RNH_2	$k'^{a}_{OPA} \times 10$	$k'^{b}_{0} \times 10^4$	ref
0.5% MeOH	Bor	8.95	40. 5	varied	1.43	$HO(CH_2)_2SH$			17.5	2.0	

[a] k'_{OPA} =measured rate constant dependent on [OPA]. [b] k'_0 =rate constant independent of [OPA]. [c] k_{obs} =overall observed rate constant.

Table 3. Decay of Isolated Adducts (Ref 93)

solvent[a]	buffer	pH	*T*, °C	[adduct], mM	adduct RSH	components RNH_2	*k*, s^{-1}
?% EtOH	Bor	9.2	24.3	0.33-0.67	CH_3CH_2SH	$CH_3(CH_2)_2NH_2$	5.5×10^{-6}
					$HO(CH_2)_2SH$	$CH_3(CH_2)_2NH_2$	4.1×10^{-5}
					$HO(CH_2)_2SH$	$^{-}OOCH[CH_2CH(CH_3)_2]NH_2$	5×10^{-6}
	0.1 M H_3BO_3	(5.3)			CH_3CH_2SH	$CH_3(CH_2)_2NH_2$	2.3×10^{-5}
					$HO(CH_2)_2SH$	$CH_3(CH_2)_2NH_2$	6.5×10^{-4}
	0.1 M NaH_2PO_4	(5.3)			CH_3CH_2SH	$CH_3(CH_2)_2NH_2$	1.8×10^{-5}
					$HO(CH_2)_2SH$	$CH_3(CH_2)_2NH_2$	1.15×10^{-3}

[a] Reaction of the isoindole derivative in 95% EtOH and isooctane are slow.

$$X = H \ll CH_3 < CH_2OH < COO^- < CH{=}CH_2 < CH_2COO^- < CH_2OCH_3 < CH_2C_6H_5$$

Thus, the reactivity of the isoindole derivative in the consecutive reaction depends on both polar and steric effects of the substituent on nitrogen. The polar effect of the carboxylate group is larger when it is present on the β- than on the α-carbon.

For the sequence of the stability of the isoindole derivative formed from various amino acids (RNH_2), two sets of data are available

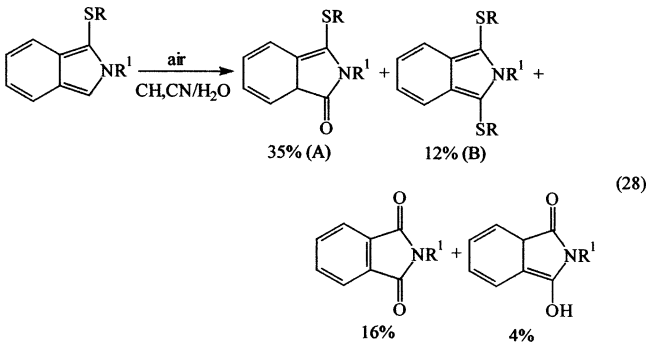
$$\text{ref 116: } R = {}^{-}OOCCH_2 < {}^{-}OOC(CH_2)_2 < {}^{-}OOCCH(CH_3) < {}^{-}OOCC(CH_3)_2$$

$$\text{ref 94: } R = {}^{-}OOC(CH_2)_3 < {}^{-}OOC(CH_2)_2 < {}^{-}OOCCH_2 < {}^{-}OOCC(CH_3)CH_2 < {}^{-}OOCCH(CH_3)_2$$

No explanation can be offered for the differences in these two sequences. For these amino acids both polar and steric effects seem to play a role in the stability of the resulting isoindole derivative.

The rates of cleavage of isolated isoindoles (Table 3), probably followed in the absence of excess concentrations of OPA and thiol, are several orders of magnitude slower than the rates of the cleavage of adducts prepared in situ.

To evaluate the effects of air oxygen and light on the degradation of isoindole derivatives, OPA was reacted with *tert*-butylmercaptan and *n*-propylamine. The isolated isoindole derivative was dissolved in a mixture of 50% v/v of acetonitrile and water and exposed to air. After 24 h complete conversion of the adduct was observed and the following reaction products were identified[117] (eq 28).



(28)

The decrease in the concentration of the parent isoindole was in the presence of air oxygen much faster than in its absence in the same medium. The kinetics of the decrease of parent isoindole as well as of the increase in concentration of the amidine main product (A) showed an induction period. It was assumed that the starting material reacts with molecular oxygen to produce a superoxide radical, which then initiates a chain reaction. Product B in eq 28 was assumed to be formed by a radical reaction involving a thiyl radical.[117] As mentioned before, no evidence was presented for the presence of radicals. Degradation paths of the adducts formed in the

presence of 2-ME and ET may be different.[117] It is questionable if reactions in unbuffered solutions containing acetonitrile resemble those in commonly used buffered aqueous solutions.

From an analytical point of view, perhaps the most important observation deals with the stability of the product of reaction of OPA with thiol and amino acids and other primary amines, generated in precolumn derivatization. Using labeling with ^{14}C it has been demonstrated[118] that the adducts formed, when ME was used as thiol, were not stable during HPLC on a reverse-phase C^{18} column. Investigation of ^{14}C-labeled degradation products indicated dependence of the stability of the isoindole derivative on the structure of the amino acid. The half-lives of adducts varied from glutamate (16 min) to arginine (40 min) and ornithin (54 min).

Stabilities of isoindole derivatives of arginine and ornithin also depend on the solvents and gradients used.[118] In particular, the content of the often used methanol, able to form hemiacetals and acetals with OPA (section 2.2), may play a role.

3.6. Proposed Reaction Schemes

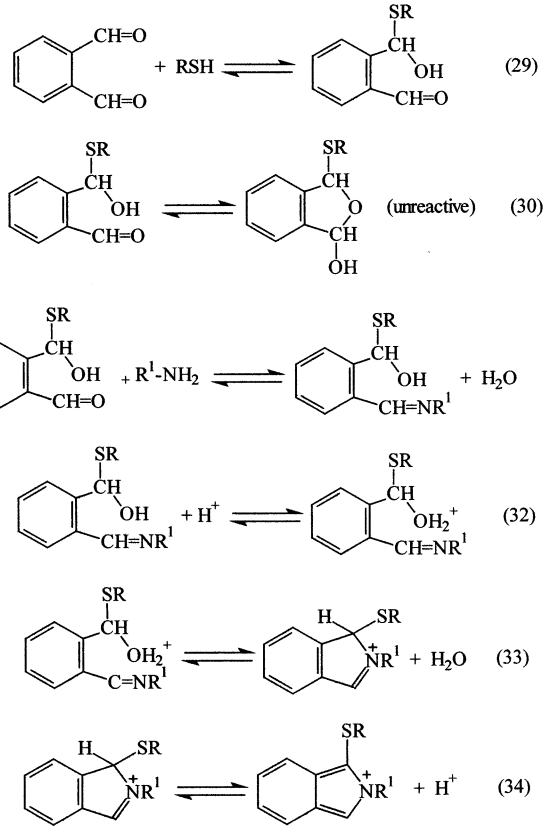
The interpretation of reaction steps taking place in reaction mixtures containing OPA, a thiol, and an amino acid or amine is predominantly based on identification of products rather than on kinetic evidence. It is thus perhaps better to discuss reaction schemes rather than mechanisms. The quotations in the introduction to section 3 indicating that mechanisms of formation and decay of the fluorescent and absorbing adduct in the reaction of OPA with thiols and amino acids "is not completely understood" is an understatement.

Before individual proposed reaction schemes are discussed, it is possible to state that none of them satisfactorily explains the significance of the sequence in which the reagents are added to the reaction mixture, nor the role of pH on the intensity of the fluorescence and on measured rate constants, nor the role of the importance of relative concentrations of individual components of the reaction mixture, neither on the formation of the isoindole derivative nor on its decay. It has not been decided which of the proposed reaction steps involve equilibria (about position of which very limited information is available) and which are practically irreversible.

In this section comments will be made on some proposed reaction schemes, and in section 3.6.7 some reasons will be given for the proposed limited understanding of the chemical processes involved in these reactions.

3.6.1. Scheme Proposed by Simmons and Johnson[56]

In eqs 29−34, based on product identification, the role of initial reaction between OPA and the thiol was recognized, but this process was considered a side reaction yielding, in eq 30, a product which did not participate in the formation of the fluorescent species. The scheme does not reflect the role of the concentration of the thiol.

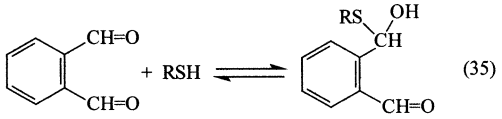


If either eq 33 or 34 were a rate-determining step, the rate of the formation of the isoindole derivative would be acid catalyzed, which has not been observed. Moreover, even kinetically controlled protonation of the hydroxy group in eq 32 seems to have low probability, taking into account that pK_a values of imines are about 6. Ring closure in eq 33 would compete with hydrolysis of the very reactive imino group. As the formation of the fluorescent species is known to be a rather fast reaction, breaking the C−H bond in eq 34 does not seem to be probable.

3.6.2. Scheme Proposed by Wong, Sternson, and Schowen[6]

In eqs 35−40, which were deduced based on the dependence of the measured rate constant of formation of the isoindole on concentration of the thiol, the initial addition of the thiol is also supposed to yield a nonfluorescent side product in eq 36.

As the equilibrium (eq 35) is shifted much more in favor of the product than equilibrium (eq 37) and since the thiol is always present at much higher concentration than the amine, the concentration of the imine formed in eq 36 would be negligible. The preferred addition of the thiol to the imino rather than to the carbonyl group in eq 38 would be logical if a higher concentration of the amine would be present. The proposed sequence is contrary to the observation that the yield of the fluorescent species is smaller when the amine or amino acid is added to the reaction mixture prior to the addition of the thiol. As in the previous scheme, the necessary cleavage of the C−H bond in reaction 40 is suspected to be too slow, even when the delocalization in the isoindole formed might be a driving force.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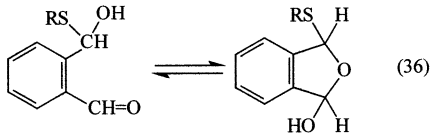
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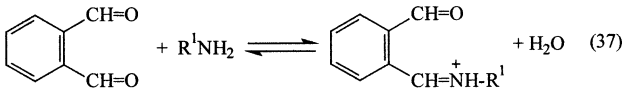
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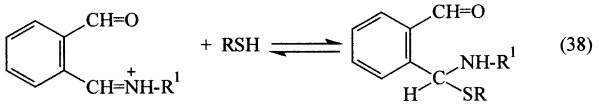
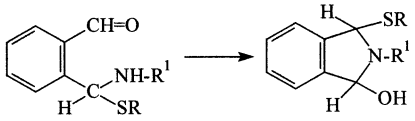
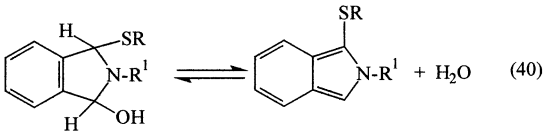
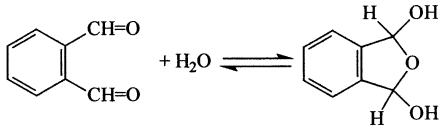

(38)

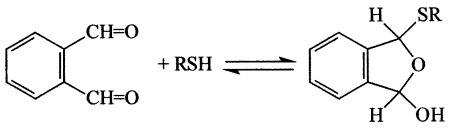

(39)

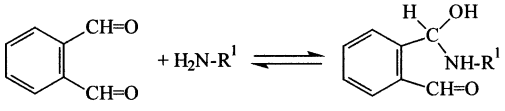

(40)

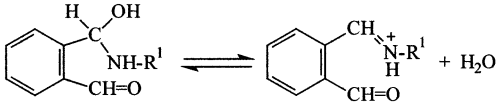
3.6.3. Scheme Proposed by Sternson, Stobaugh, and Repta[78]

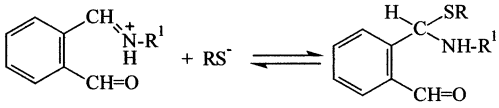
Equations 41−45 assume formation of two unreactive side products in reactions 41 and 42. Both of these reactions are at least two-step processes, where intermediates (not shown) may play an important role.

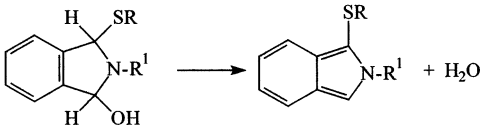

(41)


(42)


(43)


(44)

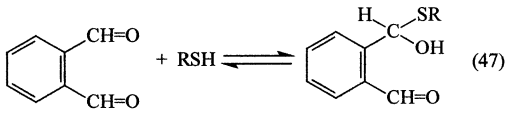

(45)

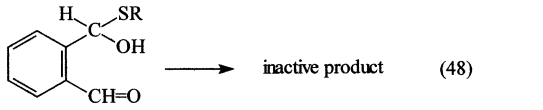

(46)

It is questionable why the addition of the amine in eq 43 should be preferred to the addition of the thiol in eqs 42 and 45, which is a stronger nucleophile and present in large excess. If the product in eq 42 is unreactive, why should thiols be added to OPA first? The cleavage of the C−H bond in eq 46 remains questionable.

3.6.4. Scheme Proposed by Gladilovich, Kartsova, and Zakharova[115]

Equations 47−53 make an attempt to include the processes involved in the degradation of the fluorescent adduct.


(47)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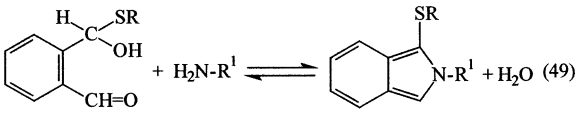
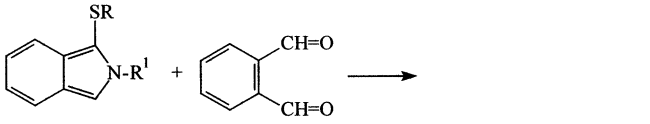
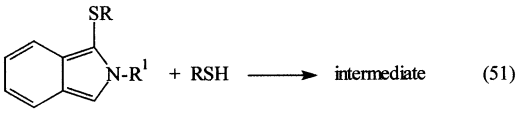
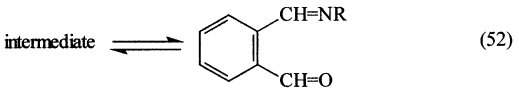
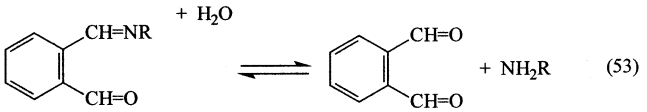

(49)


(50)


(51)


(52)

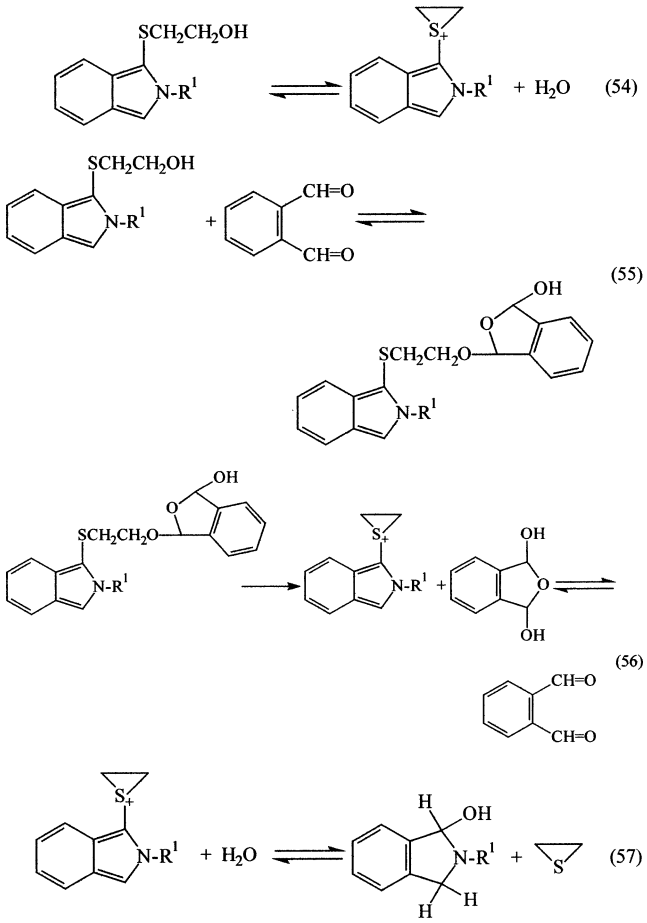

(53)

This scheme indicates the role of the sequence of addition of the two nucleophiles more successfully than previous schemes as well as the role of both OPA and the thiol on the stability of the adduct. However, without proposed structures of the inactive product, the secondary product and intermediate and considering that eqs 48−53 are or may be multistep processes, it is currently not possible to discuss this scheme in more detail.

3.6.5. Scheme of the Degradation of the Derivative of 2-Mercaptoethanol[94]

For the degradation of the product of the reaction of OPA with 2-ME in the presence of an amine, the following scheme was proposed.[94]

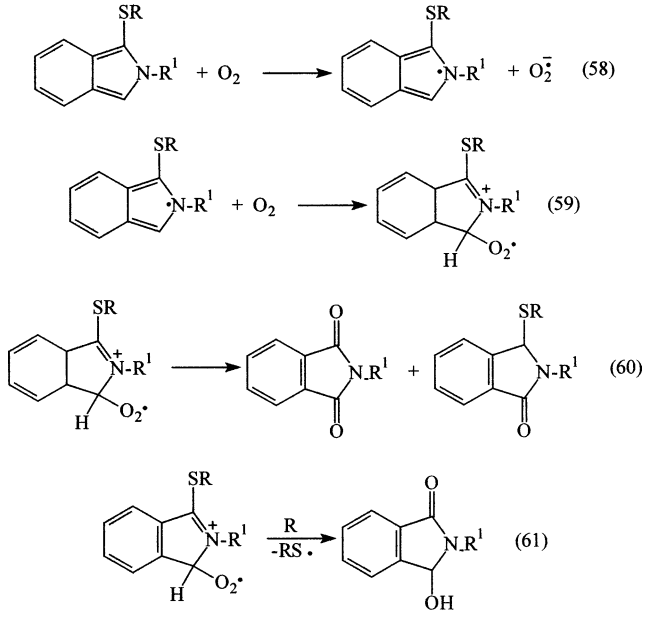
This scheme indicates the first-order kinetics in OPA but does not explain the dependence of the cleavage rate on the concentration of the thiol nor



the origin of the observed induction period. It also does not explain the similarity of the cleavage of products with 2-ME and ET reported in some contributions.

3.6.6. Scheme for Autoxidation of the Isoindole[117]

To account for products identified when 1-(*tert*-butylthio)-2-*n*-propyl)isoindole was exposed to air in 50% v/v acetonitrile−water solution (cf. section 3), the following scheme (eqs 58−61) was proposed.[117]



Absence of proof of formation of radicals and of the superoxide radical as well as of comparison with the behavior of the isoindole derivative in buffered aqueous solutions prevents a judgment of the importance of this reaction path.

3.6.7. Conclusions Regarding Reaction Scheme

Similar today as it was 20 years ago,[6,57] the mechanism of the fluorogenic reaction, so widely used in determination of amino acids, is still not understood. None of the proposed schemes agree fully with our knowledge of organic reactivity or with all available experimental evidence. A number of reported observations and experimental results remain unexplained. More detailed, quantitative, information about the hydration−dehydration and acid−base equilibria and about the role of hydroxide ions as nucleophiles is not available. Such equilibria may not involve just OPA but also some of the intermediates. Information is missing about the rates of establishment of such equilibria. It seems that elucidation of the processes taking place in complex mixtures containing OPA and two nucleophiles simultaneously in buffered solution presents problems too difficult to solve at this stage. Separate investigations of rates and equilibria in reaction mixtures containing only pairs, like OPA and thiols and OPA and amines, followed by an investigation of the role of a gradual addition of the second nucleophile seems more promising. At each stage it should be proved if the investigated reaction involves an equilibrium or not. Additional information may be obtained by following the equilibria and reaction rates simultaneously by spectrophotometric and electrochemical techniques in addition to fluorimetry. If HPLC and other separation techniques are used for following these rates and equilibria, it should be tested whether the concentrations of reactive species involved do not change during the separation process. It is questionable whether given the present state of knowledge existing analytical procedures can be validated.[123] Only when the mechanisms of the underlying processes are understood, can reliable analytical methods, including anticipation of a possible role of other components present and the effect of the matrix, be developed.

3.7. Techniques Used

Detectors based on measurement of fluorescence intensity of the primary product of the reaction between OPA, thiol, and amino compounds have been widely used in chromatographic and flow-injection analysis of mixtures of amino acids and other primary amines (cf. for examples refs 64, 76, 106, 113, 124, and 125; due to the large number of publications dealing with applications of such techniques, these references should be considered only as examples). Both precolumn[125,126] and postcolumn derivatization were used. Various types of columns were used in chromatographic methods as well as a wide variety of eluents (some including organic cosolvents or surfactants,[126] in particular in micellar LC[104]) in both isocratic and gradient regimes. Automated commercial amino acid analyzers use mostly the principle of continuous flow.[69] Stop-flow mixing was used in a kinetic fluorimetric analytical method.[106]

The use of chemiluminescence has also been proposed for detection.[127,128] In some instances measurements of absorbance in the UV region or of electrical current[129−133] may offer some advantages. Even when these techniques may be somewhat less sensitive than fluorimetric ones, they do not suffer from the possibility of quenching by other components of the reaction mixture. Spectrophotometric procedures are based on measurement of the absorbance of the isoindole derivative at 340 nm, whereas electrochemical detectors utilize the electrooxidation of the isoindole derivative. As the half-wave potentials, obtained by hydrodynamic voltammetry, of products of all amino acids studied varied between +0.2 and +0.5 V, limiting current measured at +0.7 V was reported to be a linear function of concentrations for all amino acids studied. Contrary to the above statement in a comparison of electrochemical detectors with those based on measurement of UV absorption or fluorescence,[134] it was claimed that electrochemical detection is 1 or 2 orders of magnitude more sensitive than detection based on optical methods. Because of its high sensitivity, electrochemical detection requires the use of high-purity chemicals.[135] Chemical derivatization, electrophoretic separation, and amperometric detection can all be carried out on a glass microchip.[136]

Using a phosphate−borate buffer at pH 10 with added β-cyclodextrin, it was possible to carry out analysis of a mixture of some amino acids using capillary electrophoresis, where derivatization and separation were carried out simultaneously in a capillary filled with a run buffer containing a reagent consisting of OPA and *N*-acetylcysteine.[137,138] Reaction with the same reagent was also utilized in a determination of glycine using flow-injection analysis.[139] This technique was also used in investigation of equilibria between an isoindole derivative (formed in a reaction of OPA with 2-ME or ET with lysine and serine) and cyclodextrins.[140]

3.8. Other Uses of the Reagent Formed in Reaction of OPA with Thiols

When a reagent formed in a reaction of OPA with *N*-acetylcysteine reacted with cystine, two adducts were formed, depending on the preparation of the reaction mixture.[105] In addition to the usual type of adduct resulting in a reaction of one molecule of OPA with one molecule of thiol and one molecule of cystine, another adduct was formed consisting of two molecules of OPA, two molecules of *N*-acetylcysteines, and one molecule of cystine. In the latter the central S−S bond of cystine remained intact, with two isoindole rings formed utilizing the two amino groups of cystine. When the reagent was prepared from OPA and 2-ME, cystine is reduced and the resulting cysteine forms a compound with a single isoindole ring with the reagent.

The reagent prepared from OPA and *N*-acetylcysteine can also be used for determination of ammonia.[141] Using thioglycolate as the thiol in reaction with OPA, the reaction with ammonia yielded a fluorescent species at an excitation wavelength of 415 nm and emission at 485 nm. The fluorescence was used for determination of NH_3 using a flow-injection method.[142] The use of a reagent prepared from OPA an 2-ME an a pH gradient, flow-injection analysis enabled determination of NH_3 and NH_2NH_2 in a mixture.[143]

The reagent prepared from OPA and 2-ME can also be used for a spectrophotometric determination of ethylenediamine.[144] At pH 5−6 the product absorbed at 470−480 nm, whereas at pH > 8 was observed an absorption at 430 nm. This absorption band was attributed to a product bearing two isoindole rings, formed in the reaction of two OPA molecules with one molecule of ethylenediamine. Reaction with OPA can be also utilized for determination of selenocysteine and selenomethionine.[145] Finally, secondary amines and amino acids, like proline or hydroxyproline, can be determined when first oxidized with hypochlorite.[146]

3.9. Reagents Prepared by Reaction of Orthophthalaldehyde with Nucleophiles Other than Thiols

In some recent analytical procedures, thiols were replaced in the reaction with OPA by other nucleophiles. In particular, taurine but also primary amines, bisulfite, and cyanide ions were used for this purpose.

The product of the reaction of OPA with taurine at pH 10 was used for determination of biogenic thiols.[147] After reduction with sodium bisulfite at pH 6−7, it was possible to determine disulfides, like cystine or the oxidized form of glutathione, when the reaction with a mixture of OPA and taurine was carried out at pH 9.4−10.5.[148]

To determine sulfide ions they were first converted into methylmercaptan using reaction with methyl *p*-toluenesulfonate. The resulting methyl thiolate was then converted into a fluorescent isoindole derivative by reaction with OPA and taurine at pH 8.[149]

Similarly, cyanide ions can be determined by measuring fluorescence of the 1-cyanoisoindole derivative, formed at pH 9 in a solution containing OPA and taurine.[150,151] This reaction was also utilized in a flow-injection method for determination of CN^- ions in cassava. Limarase was used in a bioreactor to liberate cyanide ions bound in cassava.[151]

The thiol can be replaced in the reaction with OPA by glycine. In the presence of cyanide ions the reagent, consisting of OPA and glycine, yielded a 1-cyanoisoindole derivative in a borate buffer at pH 8.2 containing 10% ethanol. The fluorescence was directly proportional to the concentration of CN^- ions[152] and used for flow-injection analysis. This procedure enabled detection of CN^- ions at the 10^{-8} M level. When OPA was replaced by 2,3-naphthalenedicarboxaldehyde, the detection limit was even 1 order of magnitude lower (1.2×10^{-9} M CN^-).

To detect cyanide ions using a postcolumn derivatization, to the effluent containing cyanide ions was added a 2 mM solution of OPA containing 0.5 mM EDTA and 2 mM solution of an amino acid. Among the amino acids compared, the strongest fluorescence was obtained when glycine was used. When taurine was used instead of glycine, the sensitivity was twice as high as with glycine. The resulting fluorescence

was pH independent between pH 8 and 9. With a further increase in pH, the fluorescence decreases. The plot of the fluorescence intensity as a function of pH has the shape of a decreasing dissociation curve with an inflection point (pK_a) at about 9.9.[153]

A product of the reaction of OPA with 2-aminoethanol (colamine) at pH 8.4 was used for determination of thiols in a precolumn derivatization.[154]

A product of a reaction of OPA with ammonia at pH 9.8 in a solution containing 20% methanol enabled determination of aldehyde bisulfites.[155] The fluorescence of the hydroxyalkansulfonic acid product formed was more intense than that of the analogous product with bisulfite.[156] Finally, to determine taurine, OPA was first reacted with a large excess of urea in a phosphate buffer at pH 6.8. The adduct with taurine manifested an absorption band at 560 nm that can be used for determination of taurine.[157] The absorbance of the product increased with increasing pH, and the plot of $A = f$ (pH) had a shape of an increasing dissociation curve with an inflection point (pK_a) at about pH 6. The absorbance reached a maximum value between pH 6.6 and 7.2; with further increase in pH, the absorbance slowly decreased up to pH about 9.

A reagent prepared in a reaction of OPA with sulfite was used for determination of ammonia using a flow-injection system.[158] The sensitivity of the fluorescence of the isoindole derivative in the reaction with OPA and sulfite was considerably higher when 2-ME was used as the first nucleophile.[159] A similar increase in sensitivity was observed in the determination of amino acids, when in the reaction with OPA the thiol 2-ME was replaced not only by SO_3^{2-} but also by CN^-.[160] In the latter reaction, a derivative of 1-cyanoisoindole was formed. Such compounds were isolated[161,162] under synthetic conditions in a reaction of OPA with CN^- ions and primary amines. Finally, the compound formed in the reaction of 2,3-naphthalenedicarboxyaldehyde with cyanide ions is considered to be an outstanding reagent for the determination of amino acids. The sensitivity of the determination of glycine increased, for example, by 1.5 orders of magnitude when 2-ME in the initial reaction with OPA was replaced by CN^- ions.[163] The dependence of the rate of formation of the 1-cyanoisoindole derivative of alanine on pH is bell-shaped with a maximum at pH 9.5. The value of pH at which the highest rate of formation of the fluorescent species is observed depends on the pK_a value of the dissociation of the protonated amino group of the amino acid.

When an excess of the dicarboxyaldehyde is present, the rate constant obtained at pH 9.5 increases with increasing concentration of cyanide ions. The relationship between k_{obs} and [CN^-] is not linear and at sufficiently high concentration of cyanide reaches a limiting value. This might indicate a role of the establishment of an equilibrium involving cyanide ions. In the presence of an excess of cyanide ions and varied concentration of the dicarboxaldehyde, the value of k_{obs} was a linear function of concentration of the dicarboxaldehyde. Thus, the reaction is first order in the aldehyde. In the absence of more detailed information, for example, concerning the role of the sequence of addition of components to the reaction mixture, it is possible to speculate that in the first step CN^- ions are added to the aldehyde and the resulting cyanohydrine reacts with the amino acid in the rate-determining step. The role of a possible addition to cyanide ions to a Schiff base (formed in the reaction of OPA with an amino acid) cannot be excluded. The isoindole derivatives formed in the reactions of 2,3-naphthalenedicarboxaldehyde with cyanide ions and amino acids are much more stable than the analogous adduct formed in the reaction of OPA with 2-ME and amino acids.[163]

3.10. Some Biochemical Applications

Apart from the use of OPA as a reagent in the determination of amino acids, other primary amines, and thiols as well as of some other nucleophiles (section 3.8), the interaction of OPA with −SH and $-NH_2$ groups has proved useful in identification of catalytic sites of various enzymes, for example, beef glutamate dehydrogenase,[164] succinic semialdehyde dehydrogenase,[165] yeast hexakinase,[166] 6-phosphogluconate dehydrogenase,[167] erythrocyte transglutaminase,[168] xylose reductase,[169] and xylanase.[170,171]

OPA can also be used in chemical activity labeling, which results in formation of a covalent link between selective groups M on the ligand and groups N on the macromolecule.[172] This approach was used in labeling the receptor for glucocorticoid hormones bearing at C-21 a thiol or an amino group. The labeling is particularly effective if two such groups (2 NH_2, 2 SH or 1 NH_2, 1 one SH group) are present in close vicinity in the target molecule. OPA was also proposed[172] as a reagent for specific cross-linking in proteins.

More recently, affinity labels derived from opioid ligands bearing OPA residues have been used to aid in characterization of opioid receptors. Thus, β-naltrexamine modified by OPA has been proven[173] to bind irreversibly and with high affinity to some opioid receptors. When compared with conventional affinity labels, the use of OPA-modified affinity labels offer the advantage that they are able to covalently cross-link $-NH_2$ and −SH groups in two neighboring amino acid groupings, namely, Lys 211 and Cys 216. The fluorescent species formed is a product of a highly selective process.

Similarly, natrindole modified by OPA binds covalently to a δ opioid receptor and changes the activity from an antagonist to a potent agonist.[174] As in the previous case, the binding is assumed to occur to the amino group in Lys 214 and the thiol group in Cys 216. The binding also results in conformational changes. The use of affinity labels for identification of opioid receptor recognition sites has also been reviewed.[175]

In all these applications the fluorescence intensity of the reaction product was followed, assumed to be an isoindole derivative. No attempts have been made to identify the individual steps in this analytical reaction. A better understanding of the nature of chemical processes involved might offer information useful in finding optimal conditions for the reaction, yielding the fluorescent species.

4. Conclusions

Even when the reaction products of OPA with thiols were successfully used in numerous determinations of amino acids and other primary amines, the reliability of such analyses could be improved if the mechanisms of the chemical reactions involved in the formation of the adduct were better understood. Only when reaction paths are understood can more reliable analytical procedures be developed. Perhaps the use of nucleophiles other than thiols may offer a simpler solution. It is definitely an area open for future research.

5. Acknowledgment

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