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Kinetics and mechanism of the reaction of an arenediazonium ion with hydrophilic aminocarboxylic acids in aqueous buffered solution

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ABSTRACT: The reaction of 4-nitrobenzenediazonium ion, 4NBD, with the aminocarboxylic acids (AA) glycine and serine was studied under acidic conditions by using Linear Sweep Voltammetry (LSV), which allows simultaneous monitoring of 4NBD loss and product formation. Voltammograms of the reaction mixture are complex, showing up to five reduction peaks. The reduction peaks at $E_p = -0.5$ and -1.0 V, not detected in the absence of AA, are associated to products formed in the course of the reaction. The variation of their peak current, i_p, with time shows a complex behavior; that of i_p ($E_p = -1.0$ V) follows a biphasic profile with i_p increasing with time up to a maximum after which a decrease is detected, suggestive of formation and subsequent decomposition of a transient intermediate, meanwhile i_p ($E_p = -0.5$ V) increases with time after an induction period. The peaks at $E_p = -0.1$ and -0.8 V are associated to the reduction of the diazonium group of 4NBD and, in the presence of AA ([AA] >>> [4NBD]), their peak currents decrease exponentially with time following clean first-order kinetics for more than $3t_{1/2}$. The variation of k_{obs} with [AA] at a given pH is linear with an intercept equal to zero and that of $\log(k_{obs})$ with pH at constant [AA] is also linear. Kinetic evidence is consistent with a reaction mechanism involving an irreversible, rate-limiting bimolecular step which leads to the formation of an unstable triazene, which further decomposes yielding 4-nitroaniline among other reaction products. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: arenediazonium ions; amino acids; triazene; kinetics; N-coupling

INTRODUCTION

Arenediazonium ions, ArN_2^+, virtually react with all known nucleophiles because they behave as Lewis acids in which the β-nitrogen is the center of electrophilic character.[1–4] Among others, reactions with ionic nucleophiles such as hydroxyl, alkoxide, cyanide, halides and sulphite, and sulphate ions are well known.[1,2,4] Neutral nucleophiles can also react with arenediazonium ions through the β-nitrogen and C-, N-, O-, P-, and S-coupling reactions may take place depending on the nature of the atom of the nucleophile providing the lone pair of electrons.[5–8] The C- and N-coupling reactions attracted special interest because they are the basis for azo dye and triazene formation.[4]

Mechanistic understanding of azo coupling reactions was initiated by Conant *et al.* [9] and Wistar *et al.* [10] but in the last decades a number of phenomena found many years ago in azo coupling and other substitution reactions have been elucidated with regard to their structural and mechanistic basis, including the detection of charge-transfer[11] and diazoether complexes.[12–14] C-coupling reactions are the basis for azo dye production but N-coupling reactions have attracted considerable attention from the biochemical point of view because they are employed as specific reagents and probes of protein conformation[15] and because the coupling of diazonium ions with free histidine and tyrosine has been studied as a basis for quantitative investigation of the behavior of proteins.[16] For instance, Tracey *et al.* [17] characterized azo coupling adducts of benzenediazonium ions with aromatic peptides and proteins and Patt *et al.* [18] prepared radiolabeled peptides by reacting [^{18}F] 4-fluorobenzenediazonium ion with cysteine.

We are currently determining the distribution of polar organic molecules in emulsified systems by using arenediazonium ions as chemical probes.[19,20] Knowledge of antioxidant distribution in food emulsions is important because the efficiency of antioxidants in inhibiting lipid

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peroxidation depends, among others, on their distribution within the different regions of the system.[20–22] Because a significant number of food emulsions are prepared by using proteins as emulsifiers,[23,24] we first investigated the reaction between arenediazonium ions and amino acids prior to exploring antioxidant distributions in protein-based emulsions.

A literature survey indicates that there are not many kinetic studies on the reaction of arenediazonium ions with amino acids or proteins.[1,2,4] Most of the studies have been carried out in alkaline solution and contradictory reports appear. For instance, Howard *et al.* [25] investigated the reaction of diazonium compounds with amino acids under alkaline conditions and reported that the products of the reaction are pentaz-1,4-dienes, but later investigations by Remes *et al.* [26] claimed that at pH ~ 8–10 only triazenes are formed.

To minimize kinetic complications derived from reaction of ArN_2^+ with OH^- ions,[4] we have employed slightly acidic solutions (pH = 6). 4-Nitrobenzenediazonium, 4NBD, was chosen as a model substrate because suitable rate constants can be obtained and because a substantial knowledge on their thermal decomposition in aqueous acid solution and in the absence and presence of nucleophiles is available.[27] In addition, electron-withdrawing substituents in the aromatic ring make the arenediazonium ions much more reactive against electrophilic substitution as opposed to electron-releasing substituents (e.g., methyl), and in some instances making dediazoniations to proceed through homolytic pathways *via* O-coupling formation of transient diazo ethers.[12,14,28,29]

The amino acids glycine and serine were chosen as model compounds because they are hydrophilic and thus predominantly found on the exterior surfaces of proteins, where they can probably react more easily than the hydrophobic analogues owing to the expected lower steric hindrance.

Linear Sweep Voltammetry, LSV, was employed to monitor the reaction because 4NBD is electrochemically active bearing two electroactive sites, the diazonium, $—N_2^+$, and the nitro, $—NO_2$, groups, hence voltammograms display a larger number of peaks, compared with those obtained for reduction of arenediazonium ions with no electroactive substituents. In addition to 4NBD ions, their homolytic and heterolytic dediazoniation products and derivatized azo dyes possess electroactive groups thus allowing simultaneous determination of product yields and rate constants electrochemically by monitoring either the loss or the formation of a variety of products.[12,30,31] The choice of LSV as working tool was also decided on view of the results of preliminary UV–vis experiments (not shown) carried out by monitoring the absorbance at $\lambda = 370$ nm due to product formation, revealing a complex biphasic kinetic behavior with absorbances increasing with time up to a maximum after which a slow decrease was detected.

EXPERIMENTAL

Instrumentation

Voltammograms (LSV) were obtained on an Eco Chemie Autolab, PGSTAT – 10 with VA Stand model 663 (Metrohm) fitted with a thermostated electrochemical cell and operated with GPES Autolab 4.8 software on an INFO-JC computer. A three-electrode system was employed composed of a dropping mercury electrode, a carbon electrode as auxiliary electrode, and an Ag/AgCl (3 M KCl) as reference electrode.

Materials

The amino acids, glycine and serine, and 4-nitrobenzenediazonium tetrafluoroborate were obtained from Across Organics of the highest available purity. The amino acids were used without further purification but 4NBD was recrystallized three times from CH_3CN/cold ether as described elsewhere,[32] and it was stored in the dark at low temperatures to minimize its decomposition. The UV–vis spectrum of an aqueous acid (2.0×10^{-3} M HCl) 4NBD solution shows two broad bands, the main one centered at $\lambda = 258$ nm ($\varepsilon = 16450\ M^{-1}\,cm^{-1}$) and a shoulder at $\lambda = 310$ nm. The ^1H NMR spectrum of 4NBD in CD_3CN at 25 °C is a pair of doublets of equal area centered at $\delta = 8.70$ ppm ($J = 7$ Hz) and $\delta = 8.86$ ppm ($J = 7$ Hz) in agreement with the literature data. 4NBD stock solutions were prepared by dissolving the diazonium salt in aqueous HCl to minimize diazotate formation and they were freshly prepared and used immediately or stored in an ice bath to minimize their decomposition.

Methods

Kinetic data were obtained by LSV. Under the experimental conditions employed, three reduction peaks are detected for 4NBD (see Fig. 1 and text for peak assignment). Variations in their peak current with [4NBD] are linear (correlation coefficient > 0.997) for the three reduction peaks up to [4NBD] ~ 4×10^{-5} M, but the lower detection limit and the highest linear range and sensitivity (as measured by the slope of the linear plot) were achieved for peak III, and therefore they were employed to monitor the loss of 4NBD.

Observed rate constants for ArN_2^+ loss were obtained by fitting the peak current i_p–time data for at least three half-lives to the integrated first-order Eqn (1) using a non-linear least-squares method provided by a commercial computer program.

$$\ln\left(\frac{(i_p)_t - (i_p)_\infty}{(i_p)_0 - (i_p)_\infty}\right) = -k_{obs}t \qquad (1)$$

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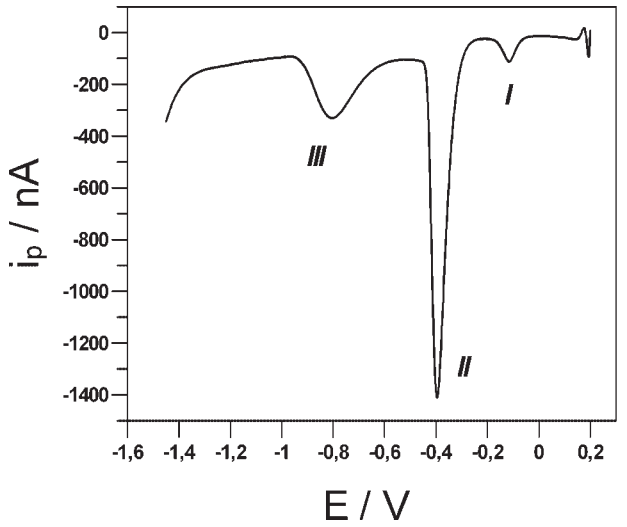
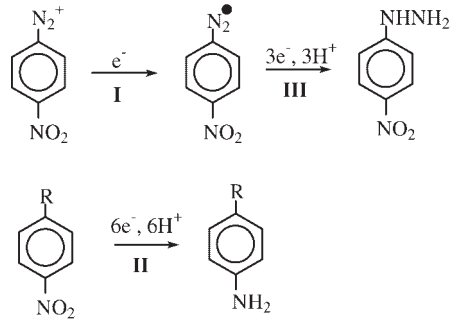


Figure 1. Linear sweep voltammogram of an aqueous buffered solution (Britton–Robinson, pH = 6.0) of 4NBD showing the three reduction peaks associated to the chemical processes indicated in Scheme 1. [4NBD] = 2.7×10^{-5} M, $T = 30\,^{\circ}$C. Step potential $\Delta E = 2$ mV, scan speed $v = 500$ mV s^{-1}

All kinetic runs were carried out at $T = 30\,^{\circ}$C in aqueous buffered (Britton–Robinson) solutions. Duplicate or triplicate experiments gave average deviations less than 7%. Stock 4NBD salt solutions were prepared by dissolving it in the appropriate acidic (HCl) mixture to minimize reaction with OH^- ions, which lead to diazotate formation.[33] Typical 4NBD solutions were of final concentrations about 1×10^{-4} M and [HCl] = 3.6×10^{-5} M and were used generally immediately or within 24 h with storage in an ice bath to minimize spontaneous decomposition.

RESULTS AND DISCUSSION

Figure 1 shows a typical voltammogram of 4NBD in Britton–Robinson buffered aqueous solutions at pH = 6.0 displaying three reduction peaks, associated to the chemical processes indicated in Scheme 1, in agreement with previous electrochemical studies.[12,31,34] Peaks I and III are connected to the one-electron reduction of the $—N_2^+$ group yielding the corresponding arenediazenyl radical, $ArN_2^{\bullet}$ (peak I) which undergoes subsequent reduction to finally yield the hydrazine $ArNHNH_2$, (peak III). Peak II is associated to the reduction of the $—NO_2$ group which yields the corresponding amine.[35]



Scheme 1. Chemical processes associated to the reduction peaks of 4NBD

Addition of glycine or serine to an aqueous acid 4NBD solution leads to more complex voltammograms, and up to five reduction peaks are detected. Figure 2, chosen as representative, shows the voltammograms obtained at different times for the reaction between 4NBD and serine, the arrows indicating the trend in the variations of the peak currents, i_p, with time. Peaks I, II, and III are associated to the reduction of the $—N_2^+$ (I and III) and $—NO_2$ groups of 4NBD, as shown in Scheme 1, and two new reduction peaks, not observed in the absence of amino acid, are detected at $E_p \sim -0.5$ V (peak IV) and $E_p \sim -1.0$ V (peak V). Under pseudo-first-order conditions ([AA] >>> [4NBD]), the i_p values of the signals associated to the reduction of the $—N_2^+$ group of 4NBD (peaks I and III at $E_p \sim -0.10$ and -0.8 V, respectively) decrease exponentially with time following a first-order kinetics for at least $3t_{1/2}$, as shown in Fig. 3 for peak III. Because of the higher sensitivity of peak III compared to that of peak I, k_{obs} values for 4NBD loss were obtained by fitting the variations in i_p ($E_p \sim -0.8$ V) with time to the integrated first-order equation (see Section 'Experimental').

The variations with time of i_p of peaks IV and V are also displayed in Fig. 3, showing a much more complex pattern of behavior as that of peak III. The peak current of peak IV ($E_p \sim -0.5$ V) increases with time after an induction period but that of peak V ($E_p \sim -1.0$ V) shows a biphasic behavior, with i_p values increasing up to a

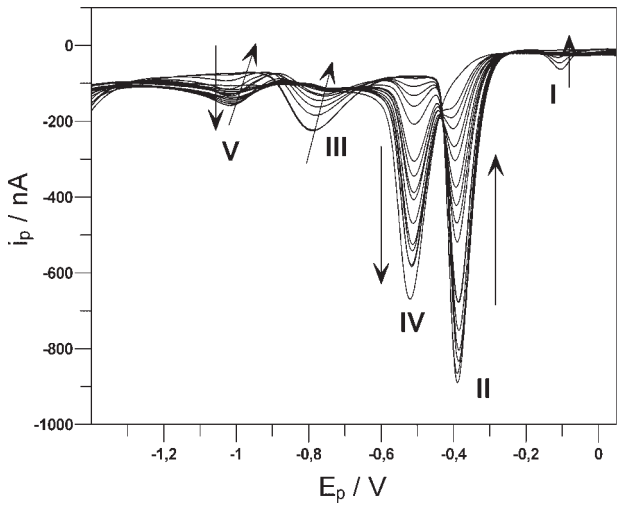


Figure 2. Overlapped linear sweep voltammograms of the reaction between 4NBD and serine in aqueous buffered (BR, pH = 6) solution obtained at different times. [4NBD] = 2.7×10^{-5} M, [Serine] = 3.8×10^{-3} M, $T = 30\,^{\circ}$C. Instrumental conditions as indicated in Fig. 1

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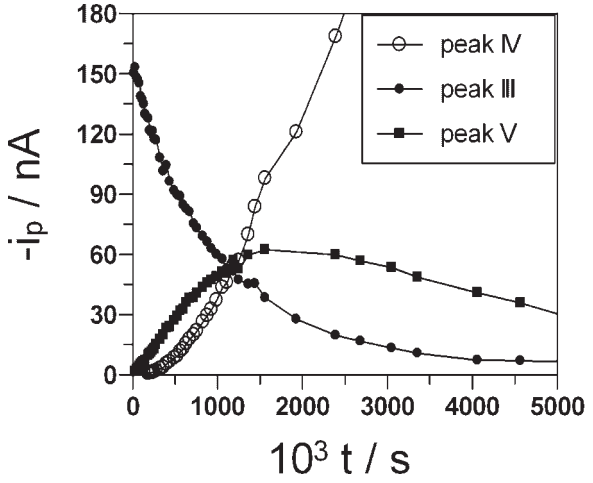

Figure 3. Variation of the peak currents of peaks III ($E_p \sim -0.8$ V), IV ($E_p \sim -0.5$ V), and V ($E_p \sim -1.0$ V) with time for the reaction between 4NBD and serine in aqueous buffered (BR, pH = 6.0) solutions. Solid lines of peaks III and V are the theoretical curves obtained by fitting the experimental data to the integrated first-order equation (see Section 'Experimental') and that derived from a consecutive reaction mechanism (Eq. 3, see Section 'Discussion'), respectively, meanwhile that of peak IV was added to help the eye. [4NBD] = 2.7×10^{-5} M, [Serine] = 3.8×10^{-3} M, $T = 30$ °C. Instrumental conditions as indicated in Fig. 1

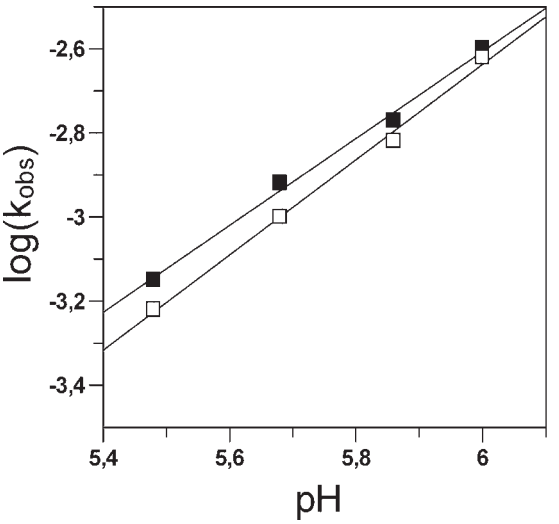

Figure 4. Variation of log(k_{obs}) with pH for the reaction of 4NBD with glycine (■) and serine (□). k_{obs} values were determined by monitoring the disappearance of peak III (see Figs 1 and 2 and text) with time. [4NBD] = 2.2×10^{-5} M, [Glycine] = 3.7×10^{-3} M, [Serine] = 8.2×10^{-3} M, $T = 30$ °C

maximum after which a decrease is observed until no reduction peak is detected. The highest peak current achieved for peak V ($E_p \sim 1.0$ V), Fig. 3, is reached when 85–90% of 4NBD has been consumed as indicated by the i_p value of peak III ($E_p \sim -0.8$ V). Similar voltammograms and variations in the peak currents with time were obtained when employing glycine instead of serine. The global kinetic pattern is suggestive, therefore, of a reaction mechanism between 4NBD and the amino acids in which an unstable transient intermediate is formed.

The effects of acidity on the reaction of 4NBD with glycine and serine were investigated by determining k_{obs} values for loss of 4NBD in buffered (BR) solutions of different pH values at a constant amino acid concentration in the pH = 5–6 range. Lower acidities were not employed because k_{obs} values were too high to be measured electrochemically and to minimize the reaction of 4NBD with OH^- ions, which yield diazotates.[3,36] At higher acidities, reactions are very slow, with k_{obs} values approaching that for the spontaneous 4NBD decomposition, whose half-life has been estimated to be $t_{1/2} \sim 215$ h at $T = 30$ °C.[37] Figure 4 shows that for both glycine and serine, the plots of log(k_{obs}) with pH are straight lines of the type $\log(k_{obs}) = \log(k_p) + b(\text{pH})$. By using a least-squares method, the calculated slopes for glycine and serine are $b = (1.03 \pm 0.05)$ and (1.13 ± 0.05), respectively, indicating an inverse dependence of k_{obs} with H^+].

Because the pK_a for the carboxylic groups of the amino acids is ~2.1, there exists the possibility of 4MBD reacting through the O-atom of the carboxylate group. Reactions between arenediazonium ions and acetate ions are known and they proceed, as most of the O-coupling reactions, through an equilibrium step; however, the equilibrium lies very much on the side of the starting ions ($K = 10^{-5}$ M^{-1})[1,2,4] and thus a hypothetical O-coupling reaction should be negligible under the present experimental conditions. The results are thus consistent with a reaction between 4NBD and the amino acids taking place through the non-protonated form of the amino group of the amino acid (with the carboxylic group completely ionized).

The effects of amino acid concentration on k_{obs} for ArN_2^+ loss were determined by increasing [AA] at a fixed pH. Figure 5 shows the variation of k_{obs} with [glycine] and [serine] at three different acidities and, in all cases, linear variations were found. By fitting the corresponding pairs of data to a linear equation, the values of the slope, intercept, and correlation coefficients given in Table 1 can

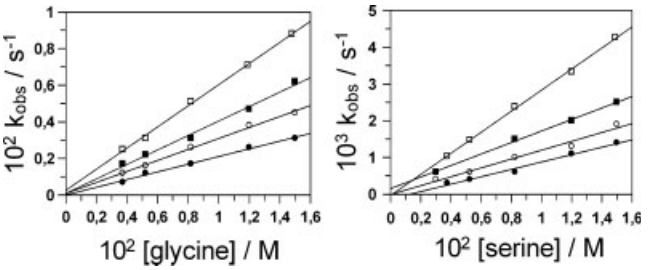

Figure 5. Variation of k_{obs} with [amino acid] at different pH values (buffer controlled). (●), pH = 5.48; (○), pH = 5.68;(■), pH = 5.86; (□), pH = 6.0. [4NBD] ~ 2.0×10^{-5} M, $T = 30$ °C. The solid lines correspond to the theoretical values calculated upon fitting the experimental data to a linear equation. See Table 1 for calculated parameters

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Table 1. Values of the intercept (*i*), slope (*s*), and correlation coefficient (cc) obtained by fitting the variation of k_{obs} with [amino acid] at different pH values to a linear equation, Fig. 5

pH	*I*	*s*	cc
Glycine			
5.48	$(5 \pm 90) \times 10^{-6}$	0.21 ± 0.01	0.9965
5.68	$(9 \pm 9) \times 10^{-5}$	0.30 ± 0.01	0.9983
5.86	$(9 \pm 20) \times 10^{-5}$	0.39 ± 0.02	0.9955
6.03	$(3 \pm 1) \times 10^{-4}$	0.58 ± 0.01	0.9990
Serine			
5.48	$(-10 \pm 7) \times 10^{-5}$	0.10 ± 0.01	0.9924
5.68	$(-8 \pm 10) \times 10^{-5}$	0.12 ± 0.01	0.9886
5.86	$(20 \pm 6) \times 10^{-5}$	0.16 ± 0.01	0.9988
6.03	$(-3 \pm 6) \times 10^{-5}$	0.29 ± 0.01	0.9993

be obtained. As shown in Table 1, the standard deviation values of the intercepts are usually larger than the own intercept value, and thus the intercepts are statistically better considered as null.

It is known that 4NBD reacts with MeOH and other alcohols under acidic conditions through the oxygen atom leading to the formation of transient diazoethers of the type Ar—N = N—O—R′.[13,14] Serine has a hydroxyl group in its structure, and so one may hypothesize that a similar reaction may take place. However, serine concentration is much lower than that needed for O-coupling reactions to compete with spontaneous decomposition reactions, and so a hypothetical reaction between 4NBD and serine through the OH group is unlikely.

In summary, results in Figs 2 and 3 are consistent with a reaction mechanism where a transient intermediate is formed, and the linear kinetic plots shown in Fig. 5 having a negligible intercept suggest that the reaction does not proceed through a reversible step.[38] Therefore, all these kinetic results are consistent with a reaction mechanism involving the irreversible formation of an unstable transient intermediate, a triazene, Scheme 2.

According to Scheme 2, the rate of disappearance of 4NBD is given by Eqn (2)

$$-\frac{\mathrm{d}[4\mathrm{NBD}]}{\mathrm{d}t} = k'_1[4\mathrm{NBD}][\mathrm{R}-\mathrm{NH_2}] \qquad (2)$$

where k'_1 is the second-order rate constant for the N-coupling reaction (first step of the proposed mechanism), and where [R—NH_2] represents the non-protonated amino group of the amino acids which otherwise, under the present experimental conditions, have the carboxylic group fully ionized. Bearing in mind the different acid–base equilibrium, the corresponding mass balances for the amino acid and that we have worked under first-order conditions, the observed rate constant for 4NBD loss is given by Eqn (3):

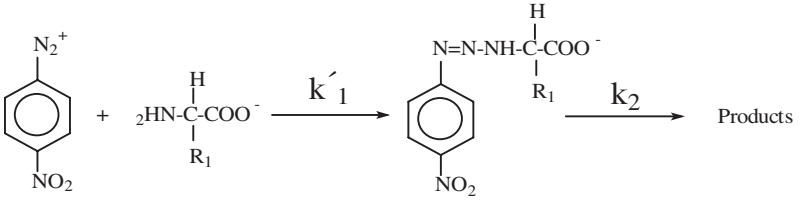
$$k_{obs} = \frac{k'_1 K_{a2}[\mathrm{AA_T}]}{K_{a2} + [\mathrm{H^+}]} \qquad (3)$$

where K_{a2} represents the second ionization constant of the amino acid (associated to the amino group), [H^+] the concentration of protons in solution, and [AA_T] the stoichiometric concentration of the amino acid.

Equation (3) predicts that, for a given [H^+], the variation of k_{obs} with [AA] should be a straight line with a zero intercept and a slope equal to $k'_1 K_{a2}/(K_{a2}+[\mathrm{H^+}])$ as found experimentally, see Fig. 5. The average values for the second-order rate constant k'_1 for the reaction between 4NBD and glycine and serine, calculated by fitting the experimental data in Fig. 5 to a linear equation, are $k'_1 = 2390 \pm 16$ and $376 \pm 7\ \mathrm{M^{-1}\,s^{-1}}$, respectively. The intrinsic reactivity of glycine is about five times higher than that of serine, which is related to the presence of the hydroxy group. Remes *et al.* [26] studied the reaction between 4-chlorobenzenediazonium ion and a number of amino acids, finding no significant correlation between the second-order rate constants and the pK_a values, and assumed that such differences were related to the presence of heteroatoms in the molecule. This important point was not further investigated and probably needs extra attention.

Alternatively, Eqn (3) predicts that for those cases where $[\mathrm{H^+}] >>> K_{a2}$, k_{obs} should decrease upon increasing [H^+], that is, in a logarithmic scale the variation of $\log(k_{obs})$ with pH should be a straight line with a slope of unity, as it was found experimentally, Fig. 4.

Qualitative auxiliary experiments (not shown) reveal that the life times for the formation of the transient intermediate depend on amino acid concentration but not that for its decomposition. In addition, a careful examination of the voltammograms obtained at different times reveals that the necessary time to reach the maximum peak current for the intermediate is very similar to that for the decomposition of 4NBD, suggesting therefore that the formation and decomposition processes



Scheme 2. Proposed reaction mechanism for the reaction between 4NBD and amino acids leading to the formation of an unstable triazene intermediate. The spontaneous thermal decomposition of 4NBD is not shown for simplicity and because it is negligible under the present experimental conditions

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of the intermediate are independent processes having substantially different half lives. Furthermore, the fact that only the formation process depends on amino acid concentration suggests that the second process is not due to a subsequent reaction of the formed triazene with the amino acid to give a pentaz-1,4-diene (Ar—N═N—N—N—N═N—R′).[2,4]

To further confirm this hypothesis, we employed a non-linear least-squares computation procedure to fit the (i_p, t) data to Eqn (4), which can be derived[38] from a reaction scheme consisting of a sequence of a bimolecular reaction and a first-order reaction of the type

$$A + B \xrightarrow{k'_1} I \xrightarrow{k_2} P$$

Where A and B represent the two reactants and $k'_1 = k_1/[B]$ is the second-order rate constant for the formation of the triazene (k_1 represents the pseudo-first-order rate constant of the process) and k_2 is the rate constant for the unimolecular decomposition of the intermediate I. Because peak currents are proportional to substrate concentration, Eqn (4) can be derived bearing in mind that we worked under pseudo-first-order conditions (e.g., [4NBD] <<< [amino acid]). The solid lines in Figs 3 and 6 were obtained by fitting the corresponding experimental data to Eqn (4) by means of a non-linear least-square method provided by a commercial computer program.

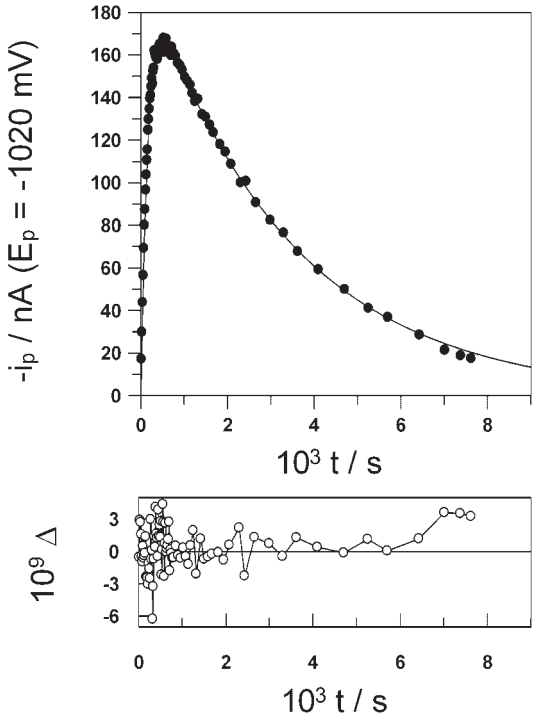

Figure 6. Variation of i_p of the reduction peak detected at $E_p = -1020$ mV in the course of the reaction between 4NBD and glycine in buffered (BR, pH = 6) aqueous solution. The solid line is the theoretical curve obtained by fitting the experimental data to Eqn (4). [4NBD] = 2.2×10^{-5} M, [glycine] = 1.2×10^{-2} M, $T = 30$ °C. In the lower graphic, the difference Δ between the calculated values according to Eqn (4) and the experimental values is plotted *versus* time

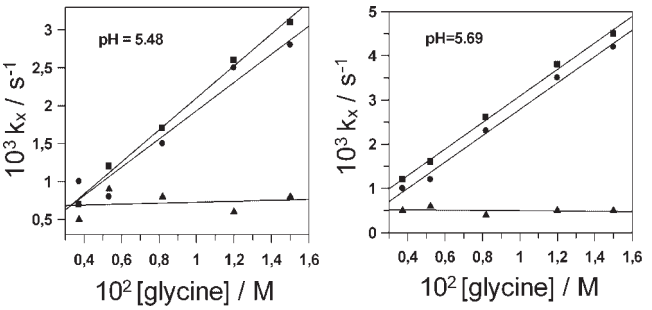

Figure 7. Variation of k_{obs} (■) for loss of 4NBD (obtained by monitoring the disappearance of the reduction peak III) and of k_1 (●) and k_2 (▲) obtained by fitting the variation of the peak current at $E_p \sim -0.1$ V to Eqn (4)

$$i_p = \frac{Dk_1[4NBD_0]}{k_2 - k_1}[e^{-k_1 t} - e^{-k_2 t}] \quad (4)$$

where D is a constant that includes parameters relating the peak current of a voltammetric peak to the concentration of the analyte. In this way, values for k_1 and k_2 can be obtained at different amino acid concentrations and at different pH s. Figure 7 shows the variations found in k_1 and k_2 with [glycine]. To associate each of the rate constants to the correct process[38] we also plotted the k_{obs} values obtained for the loss of 4NBD by monitoring the disappearance of the reduction peak III (extracted from Fig. 5). It is apparent that the k_{obs} values obtained by monitoring 4NBD (reduction peak III) are identical to the k_1 values obtained by fitting the biphasic (or biexponential) variation of the peak current (reduction peak V) with time to Eqn (4), thus providing further evidence for the proposed mechanism.

The decomposition of arylalkyltriazenes has been extensively studied revealing that the decomposition products are typically arylamines and alcohols.[39,40] For instance, Isaacs *et al.* [41] investigated the acid decomposition reaction of 3-alkyl-1-aryltriazenes with carboxylic acids, concluding that the reaction takes place through a mechanism involving a rate-determining proton transfer and departure of an alkyl cation to finally yield an arylamine and the corresponding alkylbenzoate. Thus, the reduction peak observed at $E_p = \sim -0.5$ V, whose peak current variation with time does not follow a simple kinetic equation but follows a sigmoidal variation with an induction period, Fig. 2, can be associated to the formation of 4-nitroaniline as final reaction product.

In conclusion, we have been able to show that the reaction between 4-nitrobenezenediazonium ions and hydrophilic amino acids takes place in slightly acidic or basic solutions with the anionic form of the amino acid as indicated by the inverse dependence of k_{obs} with [H^+]. The N-coupling reaction is irreversible and leads to the formation of an unstable 3-alkyl-1-aryltriazene which, under the present working conditions, further decom-

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poses (probably acid catalyzed) to yield 4-nitroaniline and other products.

Further research with hydrophobic amino acids is in due course and will be part of a future report.

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