Kinetics and Mechanism of the Nitrosation of *N*-Acetyltryptophan and of the Denitrosation of *N*-Acetyl-*N*^{1}-nitrosotryptophan

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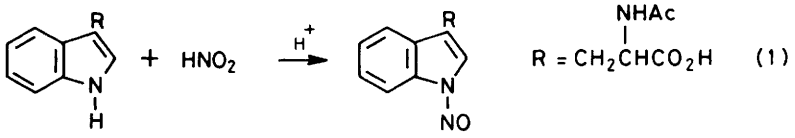
Both the formation and the denitrosation of *N*-acetyl-*N*^{1}-nitrosotryptophan have been studied kinetically in aqueous solution at 25 °C at acidities between 1 M-$HClO_4$ and pH 4. A value of 850 l mol^{-1} has been obtained for the equilibrium constant for the formation of *N*-acetyl-*N*^{1}-nitrosotryptophan. At acidities ($[H^+]$) greater than 0.1 M the rate constants for both nitrosation and denitrosation increase linearly with the concentration of acid, and the reaction rates are unaffected by the addition of nucleophiles (Br^- and SCN^-). The results are consistent with a mechanism for nitrosation where the rate-limiting step is the proton transfer from the protonated *N*-nitroso species to the medium. For denitrosation the corresponding protonation of the nitrosamine is rate-limiting. These conclusions are confirmed by the results obtained when the reactions are carried out in heavy water. However, in the pH range 1—4 the rates of both nitrosation and denitrosation are independent of the acidity of the medium and are again unaffected by the presence of nucleophiles or buffers. It is suggested that in this region nitrosation occurs at C-3. This is followed by deprotonation and an internal NO migration from C to N which is rate-limiting. This mechanism also accounts for earlier results on the denitrosation reaction at even lower acidities (pH 4—7), where acid catalysis and nucleophilic catalysis are found. Results of experiments in heavy water are compatible with this mechanism.

The *N*-nitrosation reactions of the amino groups in amino acids are by now well understood.[1,2] Reaction occurs *via* the usual reagents (NO^+, N_2O_3, nitrosyl halides, *etc.*), and it has recently been shown[2] that when the acid group is α to the amino group, one reaction pathway involves electrophilic attack at the carboxy group followed by a slow transfer of the NO group from O to N. The nitrosation of tryptophan (a biologically important species) and related compounds, however, has not received as much attention as that accorded to other amino acids.

Nitrosation reactions of tryptophan and related compounds have usually been carried out with the *N*-acetyl derivatives [equation (1)], in order to avoid the competing deamination



of the primary amino group. Such derivatives are, moreover, of greater biological interest on account of their greater similarity to peptides and proteins. It has been shown, by spectroscopic methods,[3] that the nitroso group in nitrosotryptophans is bonded to the nitrogen atom of the indole ring; this has been confirmed by studying their hydrolysis using α-chymotrypsin.[4] Not much is known however about the mechanism of nitrosation of indole derivatives. In a kinetic study, Kurosky and Hofmann[5] reported that in the pH range 2—4 the reaction was first-order with respect to both nitrous acid and substrate, but the second-order rate constant was virtually independent of the acidity of the medium. This is not compatible with the reactions of any nitrosating agents under these conditions and no explanation was offered. The reverse reaction, the denitrosation of *N*-acetyl-*N*^{1}-nitrosotryptophan, has been studied kinetically more comprehensively by one of us[6] over the acid range pH 7 to 1 M-sulphuric acid. The results suggest that two reaction pathways are involved; at the higher acidities the observed rate constant increases linearly with $[H^+]$, there is no catalysis by nucleophiles, and a primary solvent kinetic isotope effect $[k(H_2O)/k(D_2O)]$ is observed. All the results point to a mechanism involving a rate-determining proton transfer to the substrate (which in ref. 6 was taken to be at C-3), as is found in the denitrosation reactions of nitroso amides[7] and a nitroso sulphonamide.[8] However, at lower acidities another mechanism is operative; acid catalysis occurs at pH 4 (but the reaction is independent of acidity at pH 2—4), and at pH 6 the reaction is markedly subject to acceleration by nucleophilic catalysts, up to a limit attained at high catalyst concentrations. It was suggested[6] that the peculiar acidity dependence might be the result of protonation at a different site within the substrate, with pK_a about 5.5. There seems no particularly likely site for such a protonation within the nitrosamine molecule, and even though the nitroso oxygen atom was suggested, there is no independent evidence[9] that this is as basic as is necessary to explain the results. In view of this uncertainty surrounding the mechanism, particularly in the low-acid region, we have carried out a detailed kinetic study of both the formation of the nitrosotryptophan and its denitrosation.

Experimental

N-Acetyl-DL-tryptophan (AcTry) was obtained from Sigma and used without further purification. Other reagents were supplied by Merck and dried before use. All chemical products

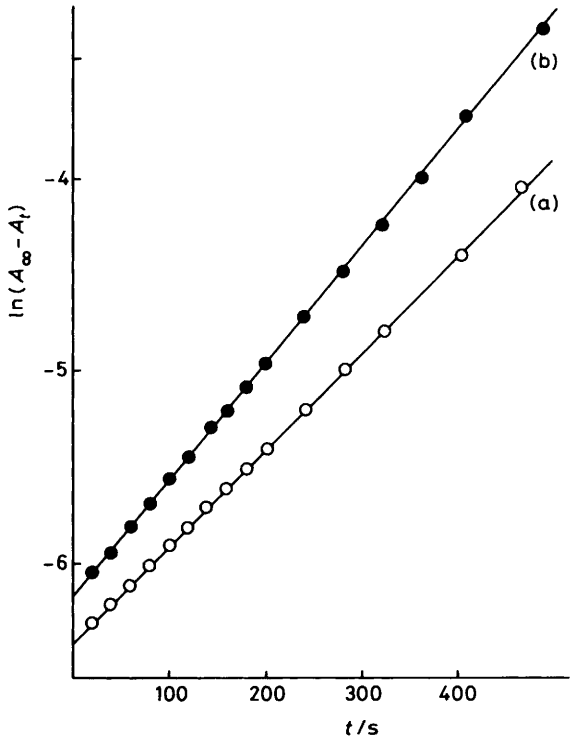


Figure 1. Typical pseudo-first-order plots of the nitrosation of AcTry at 25 °C; (a) pH 2.51, [Nit] = 1.42×10^{-4}M, [AcTry] = 7.23×10^{-3}M; (b) pH 2.70, [Nit] = 9.00×10^{-4}M, [AcTry] = 9.00×10^{-5}M

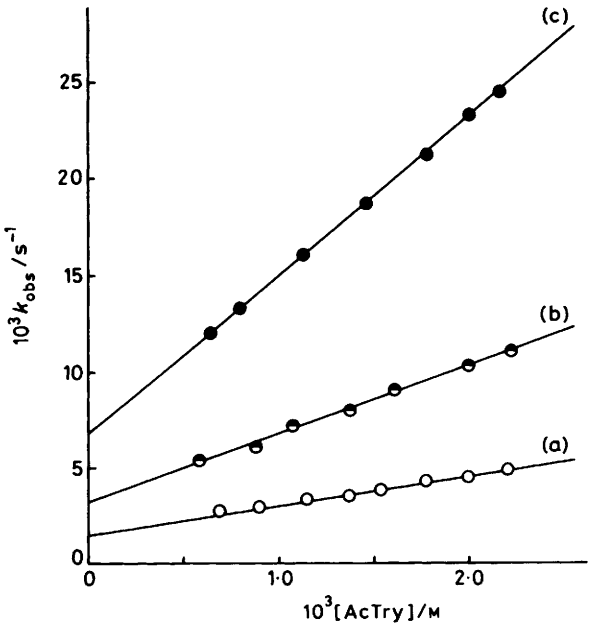


Figure 2. Influence of the concentration of AcTry upon the pseudo-first-order constants of the nitrosation of AcTry at 25 °C; (a) $[H^+]$ = 0.14M; (b) $[H^+]$ = 0.50M; (c) $[H^+]$ = 1.0M

were of the maximum commercially available purity. Heavy water containing 99.77% D_2O was supplied by the Spanish nuclear energy board.

Kinetic measurements were carried out in a Kontron Uvikon 820 spectrophotometer equipped with a thermostatically controlled cell, a printer, and a recorder. Measurements of pH were obtained with a Radiometer model 82 pH meter with a GK2401C combined glass electrode.

Table 1. Influence of acidity upon the pseudo-first-order constant of denitrosation of AcNOTry (*a*) and upon the pseudo-second-order constant of nitrosation of AcTry (*b*)

Acidity $[HClO_4]$/M or pH	$10^3a/s^{-1}$	b/l mol^{-1} s^{-1}	b'/l mol^{-1} s^{-1}†
1.0	6.76	8.03	8.03
0.50	3.63	3.11	3.11
0.29	2.67	2.16	2.16
0.20	2.03	1.50	1.50
0.14	1.76	1.40	1.40
0.094	1.90	1.00	1.01
0.047	1.82	1.20	1.23
pH 2.00	1.42	0.84	0.95
pH 2.72	1.60	0.52	0.87
pH 2.97	0.94	0.44	0.96
pH 4.01‡	0.88	0.092	1.3

† $b' = b(K_a + [H^+])/[H^+]$. ‡ Carried out in acetate buffer.

Reaction rates were measured by monitoring the increase in 330 nm absorbance due to the formation of *N*-acetyl-N^1-nitrosotryptophan (AcNOTry) until the reaction was at least 90% complete, and analysing the results by the integration method. All experiments were carried out at constant temperature of 25 °C using a 10- to 100-fold excess of either AcTry or Nit (representing the total 'nitrous acid,' *i.e.* $HNO_2 + NO_2^-$). The value of the absorbance at time $t = \infty$ (A_∞) was optimized by the method of Davies, Swann, and Campey.[10] All graphs of $\ln(A_\infty - A_t)$ against t were perfectly linear (A_t is the absorbance at time t). All experimental reaction rates were reproducible to within 3%.

Results and Discussion

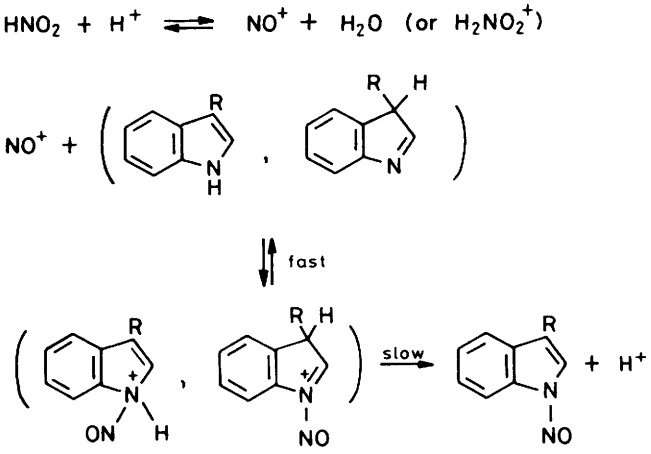
In a series of nitrosation experiments over a range of acidities, the concentration of AcTry was varied over a wide range (5×10^{-4} to 2.5×10^{-3}M), and was always in large excess over nitrous acid (2×10^{-5}M). All the kinetic results followed the first-order rate law. An example is given in Figure 1(a). At each acidity the plots of the experimental pseudo-first-order constants against [AcTry] gave good straight lines with a positive slope and a clear non-zero intercept at [AcTry] = 0, as shown in Figure 2. The final value of the absorbance (A_∞) also increased with increasing concentration of AcTry. Both these characteristics are indicative of a reversible process. The pattern is very similar to that obtained in the reversible nitrosation of alcohols to give alkyl nitrites.[11] The intercepts at [AcTry] = 0 in Figure 2 represent the pseudo-first-order rate constants for denitrosation (a), and the slopes of the plots give the second-order rate constants for the nitrosation reaction (b) as summarized in equations (2) and (3). In a similar series of experi-

$$\text{Rate (denitrosation)} = a[\text{AcNOTry}] \quad (2)$$

$$\text{Rate (nitrosation)} = b[\text{AcTry}][HNO_2] \quad (3)$$

ments with HNO_2 in large excess over AcTry [see Figure 1(b)] the pseudo-first-order rate constants again gave straight lines with non-zero intercepts when plotted against $[HNO_2]$. Values of the parameters a and b [equations (2) and (3)] obtained by this procedure agreed with the earlier values, so that it may be concluded that equations (2) and (3) are independent of which reagent is in excess.

Table 1 shows values of a and b for experiments at different acidities. The value of b is much more affected by acidity than is that of a; this is accounted for by the ionization of nitrous acid which at pH >2 significantly reduces the concentration of



Scheme 1.

Table 2. Influence of the concentration of nucleophiles upon the pseudo-first-order constant k_{obs} for the equilibrium AcTry + HNO_2 ⇌ AcNOTry at $[H^+]$ = 1.0M, [Nit] = 2.00×10^{-5}M, [AcTry] = 8.14×10^{-4}M

Nucleophile	[Nucleophile]/M	$10^2 k_{obs}/s^{-1}$
	0	1.33
SCN^-	1.48×10^{-3}	1.30
SCN^-	7.39×10^{-3}	1.20
Br^-	0.17	1.31

Table 3. Influence of the concentration of Br^- upon the pseudo-first-order constant for the equilibrium AcTry + HNO_2 ⇌ AcNOTry at pH 2.48, [AcTry] = 1.00×10^{-4}M, [Nit] = 5.77×10^{-3}M

$[Br^-]$/M	$10^3 k_{obs}/s^{-1}$
0	5.27
0.50	5.36
1.0	5.26

Table 4. Influence of the concentration of monochloroacetate buffer upon the pseudo-first-order constant k_{obs} for the equilibrium AcTry + HNO_2 ⇌ AcNOTry at pH 2.68, [AcTry] = 8.70×10^{-3}M, [Nit] = 1.55×10^{-4}M

[Buffer]/M	$10^3 k_{obs}/s^{-1}$
0.086	6.05
0.143	6.50
0.200	6.23
0.257	6.24
0.314	6.22

molecular nitrous acid, the source of the nitrosating agent. The resulting extreme slowness of the nitrosation reaction made it very difficult to obtain rate data at pH values greater than 4. If we define $b' = b(K_a + [H^+])/[H^+]$ [where K_a is the dissociation constant of nitrous acid, pK_a 2.9 (ref. 12)] then b'/a is the equilibrium constant K_1 for the formation of AcNOTry [equation (4)]. The mean value for K_1 is 850 l mol^{-1} for a series of experiments carried out at different acidities.

$$HNO_2 + AcTry \rightleftharpoons AcNOTry + H_2O \qquad (4)$$

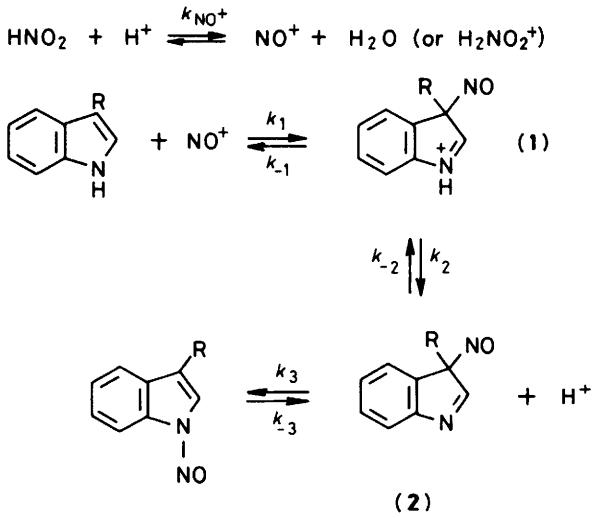
The data in Table 1 exhibit the same tendencies as were observed previously[6] when studying the denitrosation of AcNOTry. At high acidities ($[H^+]$ >0.1M) both nitrosation and denitrosation take place at rates which increase linearly with acid concentration; this represents one reaction pathway. At lower acidities both reaction rates become practically independent of pH, the slight change observed bearing little relation to the extent to which $[H^+]$ is varied. However, although no experiments were carried out in the present work at pH >4, the earlier data of Williams *et al.*[6] show that at the higher pH values the rate of denitrosation falls significantly as the pH rises. The rate constant observed in the pH-independent region is then a limiting value for the second reaction path.

The kinetic behaviour of the denitrosation reaction at high acidities was explained by one of us[6] in terms of a rate-controlling step involving a proton transfer to the substrate. Our data for nitrosation, as well as for the denitrosation reaction, support this hypothesis. It had been assumed earlier[6] that this protonation occurred at C-3 (in common with the behaviour of many indole systems), but the simpler, kinetically equivalent mechanism involving protonation of the indole nitrogen atom cannot be ruled out. This mechanism, which is now proposed, is set out in Scheme 1. In either case the slow proton transfer is supported by the absence of catalysis by SCN^- or Br^- (Table 2), and by the results of experiments carried out in D_2O. Measurements made in heavy water with 92% D_2O ($[D^+]$ = 1.0M) together with the earlier results in water, gave a value of 1.10 for $a(H_2O)/a(D_2O)$ which is typical of slow protonation reactions involving H_3O^+.[13] Moreover $b(H_2O)/b(D_2O)$ was 1.04; however, b includes K_{NO^+}, the equilibrium constant for NO^+ (or $H_2NO_2^+$) formation, and since in D_2O this constant is 2.2- (ref. 13) to 2.7- (ref. 14) times as large as in H_2O, a value of 2.3—2.8 is obtained for the isotope effect for the slow step of the nitrosation reaction, which indicates a primary kinetic isotope effect, providing further support for the mechanism outlined in Scheme 1.

The mechanism of the reaction at lower acidities is more difficult to infer. The suggestion[6] that the first step of the denitrosation reaction in this range is a fast protonation of the nitroso compound which is practically complete at pH 4 does not seem to be compatible with the acidities of the compounds involved. Clear nucleophile catalysis is however observed at pH 6, where acid catalysis is also a feature. Experiments were carried out to discover whether nucleophilic catalysis also occurs at pH values where the reaction rate is independent of the pH. Table 3 shows that at these acidities the reaction rate is unaffected by the presence of bromide ion at concentrations up to 1.0M. This implies that the rate-controlling step at pH 2—4 is different from that which obtains at pH 6. The possibility that the slow step is susceptible to general acid–base catalysis was ruled out by experiments carried out at pH 2.67 where the rate constants for both nitrosation and denitrosation are independent of the concentration of monochloroacetate buffer (Table 4). Nor does the acidity of the carboxy group in tryptophan [pK_a 2.38 (ref. 15)] allow it to be responsible for the difference in behaviour between pH 2—4 and pH 6.

The experimental observations may however be explained in terms of a mechanism based on the known behaviour of other indoles. It is known that even 3-substituted indoles can be protonated at C-3,[16] and that in the nitrosation of indoles without C-3 substituents, electrophilic substitution occurs at this position.[17] These facts suggest that the greatest electron density in the molecule occurs at C-3, and that in tryptophan (and indoles in general) we have a molecule with two nucleophilic centres, C-3 and N. It therefore seems likely that the two reaction pathways detected in the present work correspond to attack at the two different centres, although the final product in both cases is the more stable *N*-nitroso compound.

The mechanism involving attack at C-3 is shown in Scheme 2, and requires a nitroso group rearrangement from C-3 to N in step k_3. Nitroso group rearrangements of this type are known (*e.g.* the Fisher–Hepp rearrangement of *N*-nitrosoanilines)



Scheme 2.

although mechanistic details of the transfer are not known. It may well be that step k_3 occurs in more than one stage, possibly involving an intermediate with NO^+ bound to the aromatic electron cloud as a sort of π-complex. If the intermediates (**1**) and (**2**) are assumed to be in a steady state, then expressions (5) and (6) can be deduced for the rates of nitrosation and denitrosation respectively.

$$\text{Rate (nitrosation)} = \frac{k_1k_2k_3K_{NO}\cdot[\text{AcTry}][HNO_2][H^+]}{k_{-1}k_{-2}[H^+] + k_{-1}k_3 + k_2k_3} \quad (5)$$

$$\text{Rate (denitrosation)} = \frac{k_{-1}k_{-2}k_{-3}[\text{AcNOTry}][H^+]}{k_{-1}k_{-2}[H^+] + k_{-1}k_3 + k_2k_3} \quad (6)$$

At low $[H^+]$ both nitrosation and denitrosation should be acid-catalysed, but as $[H^+]$ increases the dependence upon $[H^+]$ disappears as the internal nitroso group migration becomes rate-determining for both reactions. The limiting value for k_{-3} is 2.8×10^{-3} s^{-1}. This mechanism also satisfactorily explains the influence of added nucleophiles X^- on the reaction rate. At low acidities when attack by the nitrosating agent is rate-limiting (for nitrosation) it is to be expected that catalysis by X^- occurs by reaction *via* equilibrium concentrations of NOX in the normal way, as noted experimentally for the denitrosation reaction at pH 6.[6] However, at the higher acidities where the rate-limiting step for both forward and reverse reactions is the internal NO group migration no X^- catalysis is predicted; this again is borne out experimentally by our present results at pH 2.46.

In order to determine whether the proposed mechanism also explains the kinetic behaviour of the reaction in D_2O, experiments were carried out at 0.01M-D^+. The results gave $a(H_2O)/a(D_2O) = 1.04$, *i.e.* no significant isotope effect, as expected of the internal rearrangement in the slow step. The value of 1.3 found for $b'(H_2O)/b'(D_2O)$ for the nitrosation reaction is also consistent with a rate-determining internal rearrangement, since this ratio also includes the isotope effect on $[NO^+]$ (an inverse effect, *see earlier*), the k_1/k_{-1} step (negligible), and the k_2/k_{-2} step (where the value may be taken as *ca.* 3, as found for other equilibria of similar acidity).[18] These considerations predict that $k_3(H_2O)/k_3(D_2O)$ is *ca.* 1, as expected from the mechanism outlined in Scheme 2.

Finally it is worth pointing out that the two reaction pathways discussed in this paper (for nitrosation attack at C-3 or indole N) are probably involved in the nitrosation and denitrosation of all 3-substituted indoles, and may well be the cause of 'anomalous' nitrosation reported in the literature, such as that observed in the case of indole-3-acetic acid.[5]

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