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Purines N-Oxides. IX. Polarographic Studies[1]

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The polarographic behaviors of adenine 1-N-oxide, adenosine 1-N-oxide, 2-azadenine, 2-azaadenine 1-N-oxide, 2,6-diaminopurine and 2,6-diaminopurine 1-N-oxide were examined at various *p*H's. Only with the first two was there a discrete reduction potential attributable to the N-oxide function. Adenine 1-N-oxide and 2-azaadenine 1-N-oxide can be reduced electrolytically to adenine and 2-azaadenine, respectively.

Adenine has been shown to be polarographically reducible,[2] and it was of interest to investigate the possible differential polarographic behavior of adenine 1-N-oxide[3] and some of its derivatives. Although adenine 1-N-oxide is reduced easily to adenine by hydrogen and Raney nickel,[3] catalytic hydrogenation of 2-azaadenine 1-N-oxide[4] proceeds only with fresh Raney nickel and yields products as yet unidentified.[5] There appears to be a possible utility of electrolytic reduction with such members of the N-oxide series.

Experimental

Apparatus.—Polarograms were obtained according to the usual technique[6] with a Sargent Polarograph Model XXI. The capillary constants were t (time) = 2.73 sec., m (mass) = 1.46 mg. sec.$^{-1}$, $m^{2/3}t^{1/6}$ = 2.98 mg.$^{2/3}$ sec.$^{-1/2}$ at −1.0 v. *vs.* S.C.E. (saturated calomel electrode). The H type electrolysis cell consisted of a saturated calomel electrode separated from the purine solution by a sintered glass disk and an agar plug. Oxygen was removed from the solution by nitrogen gas which was purified by passing it through Fieser solution[7].

All *p*H measurements were made with a Beckman Model G *p*H meter. The buffer solutions[8] were prepared from 0.08 N acetic acid, 0.08 N boric acid, 0.08 M phosphoric acid and varying amounts of 0.4 N sodium hydroxide. The *p*H 1.5 buffer was 0.05 N perchloric acid. The temperature was maintained at 24 ± 1° and the concentration of the purine derivatives was 5×10^{-4} M. For adenine 1-N-oxide the height of the wave was proportional to the concentration.

Materials.—Adenine, adenine 1-N-oxide, adenosine, adenosine 1-N-oxide, 2,6-diaminopurine, 2-azaadenine and 2-azaadenine 1-N-oxide were chromatographically pure. The 2,6-diaminopurine 1-N-oxide contained a small amount of 2,6-diaminopurine, detectable by paper chromatography.

Chromatography.—Analyses were performed ascending on Whatman No. 1 paper at 25° with the (two phase) developing solvents,[9] (a) 80 g. of dibasic ammonium citrate–1600 ml. of water–80 ml. of 6 N HCl (*p*H 3.1):isoamyl alcohol, 3:2 vol./vol.[9]; (b) 95 g. of dibasic ammonium citrate–1900 ml. of water–68 ml. of 6 N sodium hydroxide (*p*H 8.7):isoamyl alcohol, 3:2 vol./vol.[9] R_f values are given in Table I. Measurements of ultraviolet absorption were performed on a Beckman DK-2 Spectrophotometer.

Electrolytic Cell.—It consisted of a 600 ml. beaker with a stationary pool of mercury (70 ml.) as the anode, and a platinum electrode screen (surface area, 100 sq. cm.) as the cathode. The system included a milliammeter, a voltmeter and a variable resistor, with current from a six volt storage battery.

TABLE I

Substance	Decomp. pt., °C.	*Rf* A	*Rf* B	Ultraviolet absorption maxima Mμ	At *p*H
Adenine 1-N-oxide	297–305	0.68	0.58	233 275	13
Adenine	360–365	.73	.43	268	13
Product of electrolysis of adenine 1-N-oxide	360–363	.72[a]	.42[a]	265	13
2-Azaadenine 1-N-oxide	>350	.56	.24	233 275 330	11
2-Azaadenine	350–355	.66	.38	220 260 295	11
Product of electrolysis of 2-azaadenine 1-N-oxide	345–358	.66[a]	.38[a]	220 260 295	11

[a] Only one spot.

Materials for Electrolytic Reduction.—(a) 200 ml. of adenine 1-N-oxide (10^{-3} M) plus 200 ml. of 0.1 N perchloric acid; (b) 200 ml. of 2-azaadenine 1-N-oxide (10^{-3} M) plus 200 ml. of 0.1 N perchloric acid.

Electrolytic Reduction of Adenine 1-N-Oxide.—Adenine 1-N-oxide (200 ml., 10^{-3} M) and perchloric acid (0.1 N, 200 ml.) were mixed in the cell and subjected to a voltage of 2 v. and a current of 90–116 milliamperes for 2 hours. The temperature was maintained at 24 ± 1°. The solution was separated from the mercury and made basic with ammonia. The mixture was boiled for one hour and filtered. The filtrate was evaporated to dryness and the solid stirred with 50% potassium hydroxide and the resulting mixture was filtered. The filtrate was neutralized carefully with 35% perchloric acid and filtered. The filtrate was evaporated and the solid washed with some cold water, m.p. 360–363° dec.; R_f and ultraviolet spectrum were those of adenine (Table I).

Electrolytic Reduction of 2-Azaadenine 1-N-Oxide.—2-Azaadenine 1-N-oxide (200 ml., 10^{-3} M) and perchloric acid (0.1 N, 200 ml.) were mixed in the cell and subjected to a voltage of 2 v. and a current of 54–60 milliamperes for 3 hours and 40 minutes. The temperature was maintained at 24 ± 1°. The solution was separated from the mercury and made basic with ammonia. The mixture was boiled for one hour and filtered. The filtrate was evaporated to dryness and the solid stirred with 50% potassium hydroxide and the resulting mixture was filtered. The filtrate was neutralized carefully with 35% perchloric acid and filtered. The filtrate was evaporated and the solid washed with cold water, m.p. 345–358° dec.; R_f and ultraviolet spectrum were those of 2-azaadenine (see Table I).

Results and Discussion

The polarographic characteristics at various *p*H's of adenine 1-N-oxide, adenosine 1-N-oxide, 2,6-diaminopurine, 2,6-diaminopurine 1-N-oxide, 2-azaadenine and 2-azaadenine 1-N-oxide are shown in Table II. Figures 1 and 2 represent the tracings from which the figures enclosed in the boxes were obtained.

Reduction products of adenine 1-N-oxide and 2-azaadenine 1-N-oxide were demonstrated to be adenine and 2-azaadenine, respectively, in electrolytic reductions on a several mg. scale.

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TABLE II

Substance[a]	Half wave potential[b,c] 1.5	2.3	3.7	4.7	5.6	7.2	11
Adenine	−1.1	I.d.[d]	N.r.[e]	....	...	....	....
Adenine 1-N-oxide	−0.87	I.d.	N.r.	....	...	....	....
	−1.1						
Adenosine	−1.17	I.d.	N.r.	....	...	....	....
Adenosine 1-N-oxide	−0.84	I.d.	N.r.	....	...	....	....
	−1.14						
2-Azaadenine	−0.45	−0.54	−0.7	−0.81	−0.9	−0.99	−1.74
	−0.99	−1.11	−1.2	−1.26	I.d.	I.d.	....
2-Azaadenine 1-N-oxide	−0.54	−0.7	−0.78	−0.9	−1.0	N.r.	....
	−1.02	−1.11	−1.2	I.d.	I.d.	....	....
2,6-Diaminopurine	−1.14	I.d.	N.r.	....	...	....	....
2,6-Diaminopurine 1-N-oxide	−1.1[f]	I.d.	N.r.	....	...	....	....

[a] All concentrations were 5×10^{-4} *M*. [b] Reference electrode was the S.C.E. [c] Diffusion currents may be read from Figs. 1 and 2 for four compounds. Those for equimolar concentrations of adenosine and diaminopurine were similar to those for adenine, and the two for adenosine 1-N-oxide were similar to those for adenine 1-N-oxide. [d] I.d. is ill-defined. [e] N.r. is no reduction. [f] Very small flow of current (see text).

The polarographic characteristics observed for adenine and adenosine were those demonstrated by Heath.[2] Adenine 1-N-oxide in 0.05 *N* perchloric acid (*p*H 1.5) produced a double wave (Fig. 1), one corresponding to that for adenine[2] at −1.1 v., and one attributable to the N-oxide function at −0.87 v. At a *p*H of 2.3 the reduction was not well defined.

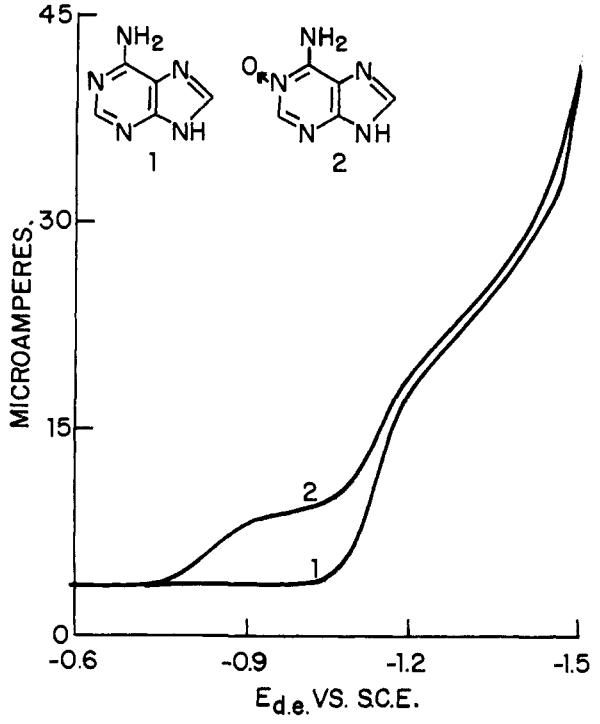

Fig. 1.—Polarograms obtained with the dropping mercury electrode of 5×10^{-4} *M* adenine (curve 1) and 5×10^{-4} *M* adenine 1-N-oxide (curve 2), at *p*H of 1.5.

The polarographic characteristics of adenosine 1-N-oxide were very similar to those of adenine 1-N-oxide (Table II).

The electrolytic reduction of adenine oxide was carried out at a *p*H of 1.5. At a voltage of 0.8 v. to 1.0 v. and 2 to 4 milliamperes, there was no significant reduction in 24 hours. At a voltage of 2 v. and a current of 90–116 milliamperes the oxide was reduced completely in 2 hours as indicated by the change in spectrum. Adenine was isolated from the solution. This extent of electrolysis results in very little loss of absorption of adenine.

2-Azaadenine and 2-azaadenine 1-N-oxide each produced very similar double waves at a *p*H of 1.5 (Table II). However, at a *p*H of 2.3, the waves for the two compounds were distinctly different (Fig. 2). In curve 2 it is obvious that no single reduction wave is attributable to the N-oxide group. However, the wave at −0.7 v. represents approximately twice the diffusion current of the first reduction wave of 2-azaadenine. Perhaps the first reduction (possibly of the 1,6-double bond) of the azaadenine ring is blocked by the presence of the N-oxide. When the N-oxide function is reduced at the higher voltage the reduction of the ring may proceed simultaneously. In more basic solutions

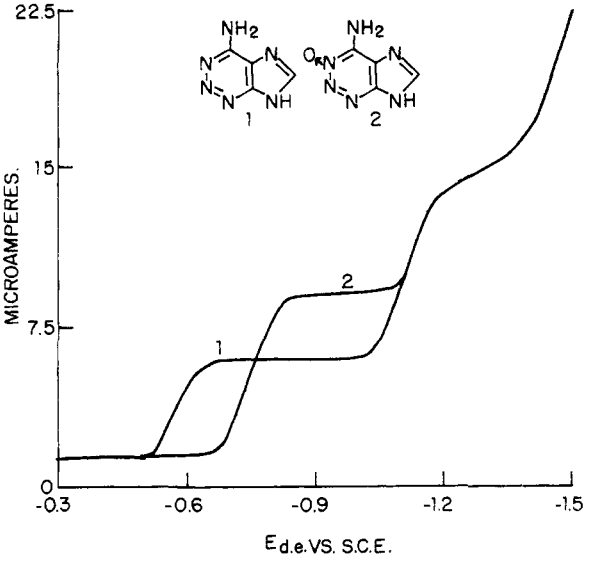

Fig. 2.—Polarograms obtained with the dropping mercury electrode, of 5×10^{-4} *M* 2-azaadenine (curve 1) and of 5×10^{-4} *M* 2-azaadenine 1-N-oxide (curve 2), at *p*H of 2.3.

the half wave potential is shifted to more negative voltage (Table II). At a *p*H of 7.2 there was no reduction of the oxide. 2-Azaadenine was, however, still being reduced at this *p*H.

Under electrolysis conditions, which were similar to those for the reduction of adenine 1-N-oxide, the oxide was reduced and the 2-azaadenine was identified by its ultraviolet spectra and paper chromatograms (Table I).

2,6-Diaminopurine at *p*H of 1.5 gave, as does adenine,[2] a well-defined reduction wave at −1.14 v. Its oxide showed a very small flow of current at this voltage, possibly attributable to the 2,6-diaminopurine present, but it was not otherwise reduced.

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Synthesis of Lysine-vasopressin by the Nitrophenyl Ester Method[1]

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The protected nonapeptide S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N$^{\epsilon}$-tosyl-L-lysylglycinamide was prepared from ethyl glycinate by stepwise lengthening of the chain according to the nitrophenyl ester method. All the protected peptide intermediates were crystalline and the over-all yield was 50%. After removal of the protecting groups from the nonapeptide derivative and oxidation to the cyclic disulfide, lysinevasopressin was isolated and purified.

Lysine-vasopressin has already been synthesized in this Laboratory.[2,3] Recently, however, a simple and straightforward method was suggested for the preparation of long peptide chains.[4] This method, employing the *p*-nitrophenyl ester of the appropriately protected amino acid, was used for the synthesis of oxytocin[4] and has now been applied to the synthesis of lysine-vasopressin.

A new active ester, *p*-nitrophenyl N$^{\alpha}$-carbobenzoxy-N$^{\epsilon}$-tosyl-L-lysinate (I), was prepared for this purpose. This ester was made to react with ethyl glycinate and the resulting protected dipeptide VIII was subjected to hydrogenolysis to remove the carbobenzoxy group. The free base, ethyl N$^{\epsilon}$-tosyl-L-lysylglycinate, was then brought into reaction with *p*-nitrophenyl carbobenzoxy-L-prolinate (II) to obtain the protected tripeptide IX. The protected tetrapeptide ester, ethyl S-benzyl-N-carbobenzoxy-L-cysteinyl-L-prolyl-N$^{\epsilon}$-tosyl-L-lysylglycinate (X), was prepared in a similar manner and transformed into the corresponding amide XI. The chain was then lengthened stepwise by successive reactions with the nitrophenyl esters of protected L-asparagine, L-glutamine, L-phenylalanine, O-benzyl-L-tyrosine and S-benzyl-L-cysteine. From the tetrapeptide through the octapeptide stage, HBr in acetic acid was used for the removal of the carbobenzoxy group. At the stage of the removal of the carbobenzoxy group from the protected octapeptide XV, the O-benzyl group of the tyrosine residue is also split off by HBr as found and previously reported in the synthesis of oxytocin by Bodanszky and du Vigneaud.[4] In the case of the protected nonapeptide, S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N$^{\epsilon}$-tosyl-L-lysylglycinamide (XVI), all the protecting groups, including the N$^{\epsilon}$-tosyl group of the lysine residue, were removed in one operation by treatment with sodium in liquid ammonia. This reduction step was followed by aeration to form the cyclic disulfide, lysine-vasopressin. The synthesis is outlined in Chart I.

The hormone thus obtained was easily purified by ion-exchange chromatography. Electrophoresis or countercurrent distribution could be used for purification in place of the chromatography if so desired. However, we found the latter procedure to be more convenient and highly satisfactory. The efficiency of the method of synthesis presented is illustrated by the fact that yields in the eight peptide bond-forming steps were all over 80% and usually over 90%, and the over-all yield for these reactions plus the amidation of the tetrapeptide ester was approximately 50%. All the protected intermediates are crystalline. The synthesis was carried out on a scale of several grams through the preparation of the protected nonapeptide XVI, and the conversion of the latter compound to lysine-vasopressin was also carried out on a scale of more than one gram.

Experimental[5]

p-Nitrophenyl N$^{\alpha}$-Carbobenzoxy-N$^{\epsilon}$-tosyl-L-lysinate (I).—N$^{\alpha}$-Carbobenzoxy-N$^{\epsilon}$-tosyl-L-lysine (152 g.) was dissolved in ethyl acetate (500 ml.) and *p*-nitrophenol (58.5 g.) was added. The solution was stirred and cooled to 0°. Dicyclohexylcarbodiimide (72 g.) was added. The reaction mixture was stirred for 0.5 hr. at 0° and for 1.5 hr. at room temperature. Glacial acetic acid (10 drops) was added and the N,N'-dicyclohexylurea was filtered off and washed 3 times with ethyl acetate. The solvent was evaporated *in vacuo* to a yellow oil (168 g.) which solidified after 10 minutes. The material was dissolved in hot ethanol (500 ml.) and crystallized as needles from the cooled solu-

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