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REDUCTION OF 3,5-DINITRO-2-PYRIDYL DERIVATIVES WITH SODIUM BOROHYDRIDE.—PART I—HYDROLYTIC CLEAVAGE OF N-TERMINAL PEPTIDE BONDS*

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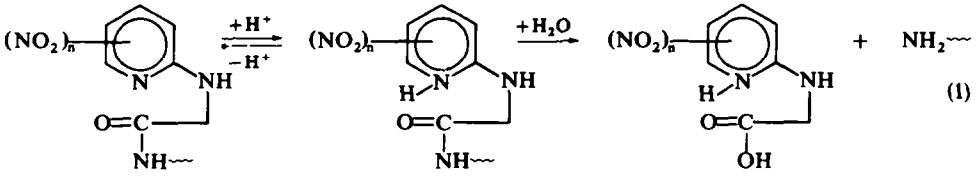
Abstract—The reaction of 2-methylamino-3,5-dinitropyridine with sodium borohydride is described. The product is a tetrahydropyridine from its mass, IR and UV spectra. This reaction applied to several dinitropyridyl-peptides leads to selective cleavage of N-terminal peptide bonds at pH values near neutrality.

SELECTIVE non-enzymatic degradative techniques are a new tool in the study of protein structure;[1-3] chemical cleavage of peptide bonds resembles in this connection enzymatic degradation in yielding information on amino acid sequences and sequence overlaps. With few exceptions enzymic reactions are performed at pH values near neutrality and at temperatures between 20° and 40°; to imitate these reactions by chemical means more drastic conditions are usually required. The criteria for a useful chemical method for the selective cleavage of peptide bonds may be summarized as follows: (a) rapid and quantitative formation of a new covalent bond between the reactive site and the reagent under mild conditions; (b) fast hydrolysis of the derivative so formed, in aqueous buffer solutions at neutral or slightly acidic pH, releasing a new terminal NH_2-group. Generally this fast hydrolysis is connected with neighboring group participation; if however this catalytic effect requires strongly acidic conditions, preferential cleavage of peptide bonds is observed as in the case of partial acid hydrolysis of non-modified peptides and proteins.

In earlier papers[4,5] it was pointed out that the acid hydrolysis of nitropyridyl-peptides involves the protonated pyridyl group; the tentative mechanism postulated for this reaction is shown in equation (1). The concentration of protonated substrate



(1)

is dependent on the acidity function H_0^6; the pH region in which the labilizing influence of the neighboring pyridyl species occurs is related to the basicity of the aza-function. Selective modification of the aromatic nucleus by reduction to dihydro- or tetrahydro-pyridine derivatives[7] should enhance the basicity of the N atom and

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displace the catalytic effect to a region of higher pH. When sodium borohydride is added to aqueous solutions of 3,5-dinitropyridyl amino acids (DNPyr-amino acids) and related derivatives, an intense color develops followed by steady fading. DNPyr-derivatives are easily prepared by reaction of the appropriate peptide with 2-chloro-3,5-dinitropyridine in aqueous solution at room temperature and at pH values ranging from 8·0 to 9·0. The rate of the reaction is higher the higher the pH; in 2% sodium bicarbonate solution the condensation proceeds at speeds conveniently rapid for preparative purposes (Fig. 1) and under these conditions the parallel

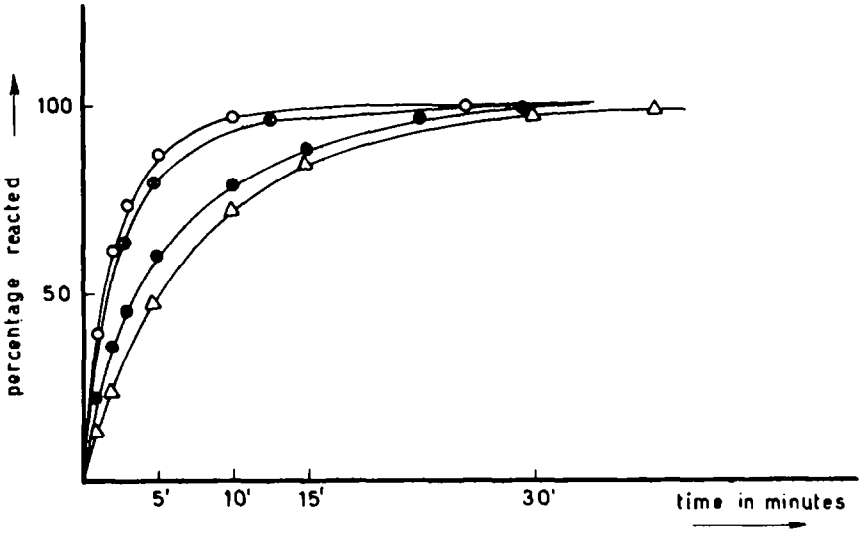
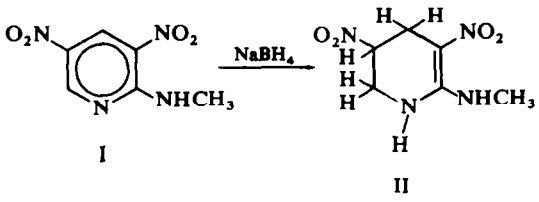


FIG. 1 Course of the reaction between 2-chloro-3,5-dinitropyridine and dl-leucine (—○—○—), dl-glycyl-leucyl-tyrosine (—⊗—⊗—), dl-alanylphenylalanine (—●—●—), dl-glycyl-serine (—△—△—). Temperature 20° and pH 8.4.

hydrolysis of the reagent may be neglected. After reduction with sodium borohydride in sodium bicarbonate solution, it was possible to establish quantitative cleavage of peptide linkage for DNPyr-Ala-Phe, DNPyr-Gly-Pro, DNPyr-Pro-Gly and DNPyr-Gly-Ser by standing the solutions at 40° and pH 5–6 for about 12 hr; the same results were obtained by warming the solutions in a boiling water-bath for about five minutes. The hydrolytic cleavage of DNPyr-Gly-Leu-Tyr quantitatively released the dipeptide Leu-Tyr, thus demonstrating that other amide bonds of a peptide chain are not attached under these conditions.

Inasmuch as heretofore aromatic nitro groups have been considered resistant to attack by sodium borohydride,[8,9] we investigated this reaction further on the model compound 2-methylamino-3,5-dinitropyridine. The isolated reduction product had very high melting point and was moderately soluble in polar organic solvents. The mass spectrum revealed a molecular ion at *m/e* 202 which may be accounted for by

the addition of four hydrogens to the substrate, to give a tetrahydro-pyridine derivative. The ultraviolet absorption in 1% sodium bicarbonate revealed two nearly symmetrical maxima in the 225– and 310 mμ regions (ε 11,800 and 13,200). These data strongly suggest conjugation of a nitro group with the remaining double bond; it is probable that the peak around 225 mμ is due to the nitro-olefin portion of the molecule[10] whereas the long wavelength peak may be associated with the auxochromic effect of the amino groups on this chromophore.[11,12] A tentative formula is shown in Eq. (2).



(2)

The IR absorption spectrum is in agreement with the amino-nitro-ölefin structure (II). There is a marked lowering of the NH-frequency which may be attributed to intermolecular hydrogen bonding (very broad NH-stretching absorption between about 3250 and 2800 cm^{-1}). The bands in the 6 μ region cannot be assigned unambiguously; some of them are undoubtedly associated with the amino-nitro-ölefin group and are probably due to C=C or C=N stretching vibrations.

Sodium borohydride is a very mild reducing agent; amide bonds or disulfide bridges react not at all under the conditions necessary to modify the dinitropyridyl ring.[13–15] The reduction of DNPyr-derivatives represents a new reaction which by its very nature leads to the formation of a tetrahydropyridine which promotes the subsequent cleavage of amide bonds. The intramolecular catalytic effect of this intermediate with $pK_a \simeq 4{\cdot}1$ occurs at pH values near neutrality. Thus, the presence of two nitro-groups in the aromatic nucleus makes possible both the rapid coupling reaction and the fast reduction. Furthermore cleavage of the peptide bonds takes place exclusively at the linkage adjacent to the reduced dinitropyridyl group, other bonds remaining untouched; the hydrolytic conditions are comparable to those of the well known enzymatic hydrolysis.

EXPERIMENTAL

All chemicals used were of analytical reagent grade; Fluka peptides were employed without further purification. 2-Chloro-3,5-dinitropyridine (m.p. 64°) and DNPyr-peptides were synthetized according to the procedures previously described.[16,17] DNPyr-peptides were all tested for purity by elemental analysis and by chromatography on paper in at least two different solvent systems. IR spectra were determined (KBr disc) with a Perkin–Elmer model 21 recording spectrophotometer, UV spectra with a Beckman DB recording spectrophotometer and mass spectra with an A.E.I. MS 9 high resolution mass spectrometer using direct sample insertion.

Preparation of 2-methylamino-3,5-dinitropyridine (I). To an aqueous soln of 10% methylamine (22 mmoles), 1·02 g 2-chloro-3,5-dinitropyridine (5 mmoles) dissolved in EtOH (10 ml) was added. The mixture was stirred vigorously at room temp for about 30 min; during this period a voluminous yellow solid precipitated. EtOH and excess methylamine were distilled off *in vacuo*; the ppt was filtered off and washed with several portions cold water. The crude product was recrystallized from EtOH yielding 0·95 g (96%), m.p. 148°. (Found: C, 36·20; H, 3·10; N, 28·20. $C_6H_6N_4O_4$ requires: C, 36·36; H, 3·03; N, 28·28%).

Reduction of compound I *with sodium borohydride.* To a suspension of I (0·100 g; 0·5 mmoles) in abs EtOH (10 ml), 0·080 g (2·1 mmoles) solid $NaBH_4$ was added under vigorous stirring. A red color developed

and the DNPyr-derivative dissolved completely during several min. Slowly the color of the soln faded and meanwhile a flocculent white ppt formed. After about 30 min the pH of the soln was adjusted to 5–6 with a few drops glacial AcOH to decompose the excess $NaBH_4$; after centrifugating the product was washed extensively with abs EtOH and finally with ether. Recrystallization from 95% EtOH yielded 0·094 g (94%) pale yellow plates, m.p. 245–246°. (Found: C, 35·45; H, 5·45; N, 27·45. $C_6H_{10}N_4O_4$ (tetrahydropyridine) requires: C, 35·29; H, 5·88; N, 27·45%).

pK_a *Determination.* The reduced compound II (2 to $5{\cdot}10^{-3}$M) in 15% dimethylformamide–water (v/v), after addition of 5 µl conc HCl, was titrated at 25 ± 1° under N_2 with 0·22M KOH, utilizing a Radiometer type TTT 1a pH-meter equipped with automatic microburet and recorder. The resulting apparent pK value found was +4·1.

Cleavage of DNPyr-peptides. The procedure employed to determine the extent of cleavage of peptide bond is as follows: to a soln of 0·1 mmoles DNPyr-peptide in 2% $NaHCO_3$ aq (10 ml) in an ice-water bath, 7·6 mg (0·2 mmoles) solid $NaBH_4$ was added under vigorous stirring. After about 5 min, the soln was adjusted to pH 5–6 with a few drops glacial AcOH and the mixture was allowed to stand at 40° overnight. Peptide bond cleavage was estimated using a Beckman-Spinco model 120C amino acid analyzer. Yield of cleavage was calculated by reference to portions of the hydrolysis mixture subjected to complete hydrolysis by heating for 5 hr at 100°.

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