hindered from rotation around their bonds to the tyrosyl β-carbon. Under such circumstances, the two forms will not be equivalent in their physicochemical properties, and will not be rapidly interconvertible, but this difference will not be detectable by primary structure studies. Of course, it is also possible to assume that the rotation of the phenyl rings is sterically hindered already in the native protein; the result will be the same. Work is now in progress in order to assess the reliability of this hypothesis.

Added in Proof

Dr. R. E. Dickerson has informed us that this interpretation is in the keeping with the position and environment of tyrosine-67 in his 2.8-Å resolution model of cytochrome *c*.

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Thiolysis of Dinitrophenylimidazoles and Its Use during Synthesis of Histidine Peptides*

Shmuel Shaltiel† and Mati Fridkin

ABSTRACT: The thiolytic cleavage of 2,4-dinitrophenylimidazoles was applied to the synthesis of histidine-containing peptides.

This paper describes the preparation and properties of starting materials for such syntheses and illustrates their use in the synthesis of some peptides containing histidine at either the C or the N terminus. A spectrophotometric method for following the extent of thiolysis of 2,4-dinitrophenylimidazoles was developed and used to establish optimal reaction conditions for the quantitative removal of the protecting group from histidine residues in aqueous and nonaqueous media.

Synthesis of histidine peptides can be performed without protection of the imidazole ring (Holley and Sondheimer, 1954; Hofmann *et al.*, 1957). However, the basicity of imidazole may trigger undesirable side reactions and lead to low yields of coupling and problems of purification (Schröder and Lübke, 1965). Commonly used protecting groups for the imidazole ring are the benzyl group (du Vigneaud and Behrens, 1937; Theodoropoulos and Gazopoulos, 1960) or the carbobenzoxy group (Patchornik *et al.*, 1957; Akabori *et al.*, 1958; Shaltiel and Patchornik, 1963). The benzyl group is sometimes inadequate, since its removal by catalytic hydrogenolysis is a slow process which often does not reach completion (Kopple *et al.*, 1963), while the removal by means of sodium in liquid NH_3 is a drastic procedure which may even cause rupture of peptide bonds (Guttmann, 1963). Protection

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of imidazoles with the carbobenzoxy group also has some disadvantages. *N*-Carbobenzoxyimidazoles behave as activated acyl groups and are unstable in alkaline conditions or when exposed to amines at higher temperatures.

A very promising reagent for the protection of imidazoles has been described recently by Weygand and his collaborators (1967). This protecting group (the 1-benzyloxycarbonylamino-2,2,2-trifluoroethyl group) reduces the basicity of the imidazole ring and can be removed under the conditions used for the removal of *N*-carbobenzoxy groups.

We have recently shown that DNP-imidazoles can be readily cleaved by thiols (Shaltiel, 1967) under exceptionally mild reaction conditions (neutral pH and room temperature). We wish to report here the use of this thiolytic cleavage during synthesis of histidine peptides and to describe a spectrophotometric method for following the course of thiolysis. Using this method it is possible to establish optimal conditions for unmasking the protected histidine side chain in an aqueous or nonaqueous medium.

Materials and Methods

The following compounds were prepared according to methods described in the literature: N^{α}-Z-L-histidine[1] (Patchornik *et al.*, 1957); Z-L-alanine-*N*-hydroxysuccinimide ester and Z-L-phenylalanine-*N*-hydroxysuccinimide ester (Anderson *et al.*, 1964). N^{α}-*t*-Boc-N^{im}-DNP-L-histidine (Schröder and Gibian, 1962; Hofmann *et al.*, 1965; Chillemi and Merrifield, 1969). *N*-Hydroxysuccinimide was a gift from Mr. J. Jacobson. All other chemicals were best available grades from commercial sources.

The peptides obtained were characterized by nonaqueous titrations (Patchornik *et al.*, 1957; Patchornik and Shaltiel, 1962), as well as by thin-layer chromatography on silica gel (from Riedel-De Haen AG) using the following solvent systems: methanol, chloroform–methanol (3:1, v/v), and 1-butanol–acetic acid–water (4:1:1, v/v). R_F values are uncorrected. The peptides were revealed by charring of the plates over a flame (R. Hill, personal communication).

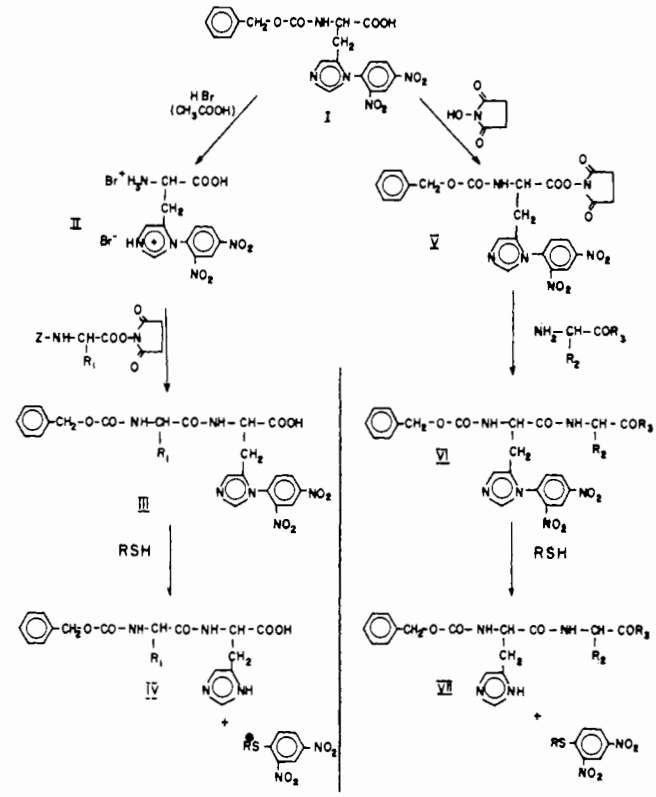
All melting points were measured on a capillary melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter and spectrophotometric measurements with a Cary Model 15 spectrophotometer.

Results

The synthesis of peptides containing histidine at the C or N terminus were usually performed according to Scheme I. In general, the activation of the carboxyl was achieved by the NHS ester method (Anderson *et al.*, 1963), except for the coupling of compound VI_b (see below) which was performed by the dicyclohexylcarbodiimide method (Sheehan and Hess, 1955).

Preparation of Starting Materials. N^{α}-Z-N^{im}-DNP-L-HISTIDINE (I). N^{α}-Z-L-Histidine (35 mmoles) was dissolved in 100 ml of water containing 80 mmoles of $NaHCO_3$. The solution was cooled to 0°, then FDNB (40 mmoles in 100 ml of

SCHEME I



dioxane) was added. The mixture was stirred for 8 hr at 4°, then concentrated *in vacuo* to about 40 ml. Upon acidification with 6 N HCl a yellow precipitate was formed which was extracted into ethyl acetate. This solution was washed with water, dried over Na_2SO_4, evaporated to dryness, and crystallized from ethyl acetate–petroleum ether: yield, 93%; mp 93–98°; R_F 0.51 (CH_3OH), 0.53 (system 2), 0.55 (system 1); $[\alpha]_D^{28}$ +104.1° (*c* 0.96, $CHCl_3$). *Anal.* Calcd for $C_{20}H_{17}N_5O_8$: C, 52.75; H, 3.76; N, 15.38; neut equiv 455. Found: C, 52.55; H, 3.80; N, 15.42; neut equiv 460 (with $NaOCH_3$ in methanol–benzene) and 472 (with $HClO_4$ in glacial acetic acid).

N^{im}-DNP-L-HISTIDINE·2HBr (II).[2] A solution of HBr (5 ml) in glacial acetic acid (45%) was added to a solution of compound I in 5 ml of glacial acetic acid. The reaction was allowed to proceed for 2 hr at room temperature and the product II was precipitated with absolute ether. The precipitate was dissolved in 2-propanol, crystallized by addition of ether, and recrystallized from methanol–ethyl acetate. The resulting yellowish white crystals were very hygroscopic: yield, 85%; R_F 0.25 (CH_3OH), 0.07 (system 2), 0.48 (system 1), $[\alpha]_D^{28}$ −2.9° (*c* 1.5, *N*,*N*-dimethylformamide). *Anal.* Calcd for $C_{12}H_{11}N_5O_6 \cdot 2HBr$: Br, 33.12; neut equiv 161 (3 acidic groups). Found: Br, 32.95; neut equiv 159 (with $NaOCH_3$ in methanol–benzene).

N^{α}-Z-N^{im}-DNP-L-HISTIDINE-*N*-HYDROXYSUCCINIMIDE ESTER (V). Compound I and of *N*-hydroxysuccinimide·(2 mmoles of each) were dissolved in a mixture of 5 ml of dioxane and 5 ml of

[1] Abbreviations used are: system 1, 1-butanol–acetic acid–water (4:1:1, v/v); system 2, chloroform–methanol (3:1, v/v); *t*-Boc, *tert*-butyloxycarbonyl; Z, carbobenzoxy.

[2] N^{im}-DNP-L-histidine was previously synthesized by Siepmann and Zahn (1964) through a different method.

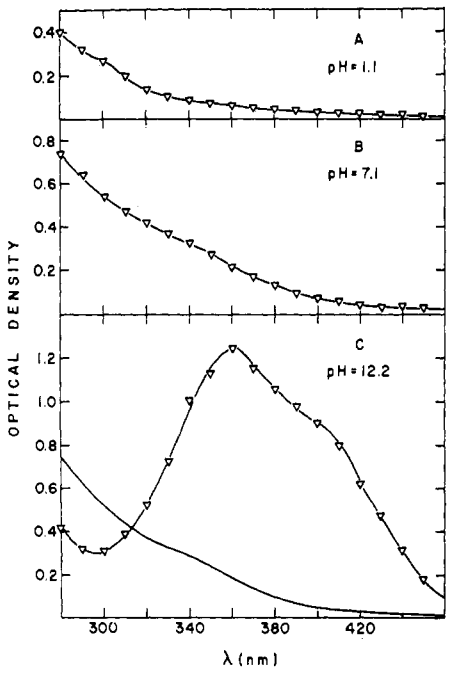


FIGURE 1: Stability of DNP-imidazoles at acidic (A), neutral (B), and alkaline (C) pH values. Solutions of N^{α}-*t*-Boc-N^{Im}-DNP-L-histidine (8.7×10^{-5} M) in various media were prepared, and their spectra were recorded immediately (——) and after standing in the dark for 20 hr at 22° (—△—).

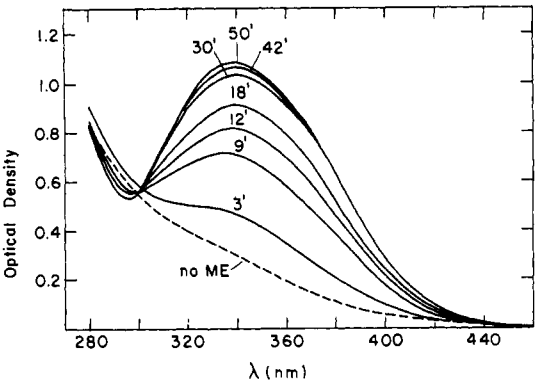


FIGURE 2: Spectrophotometric determination of the thiolytic cleavage of N^{α}-*t*-Boc-N^{Im}-DNP-L-histidine in aqueous conditions. The compound was dissolved in a phosphate buffer (0.1 M), pH 8.0 and its spectrum (---) was recorded (concentration 9.1×10^{-5} M). Thiolysis was initiated by addition of 10 μl of 9.2 M 2-mercaptoethanol to 1 ml of the sample (*ca.* 1000 moles of thiol per mole of the DNP derivative). The spectrum of the reaction mixture was recorded at the times (min) indicated in the Figure.

ethyl acetate. The solution was cooled to 0°, then 2 mmoles of dicyclohexylcarbodiimide (in 5 ml of ethyl acetate) was added. The reaction mixture was allowed to stand for 1 hr at 0° then overnight at room temperature. The dicylohexylurea formed was filtered and the solution was concentrated *in vacuo* to dryness, dissolved in a minimal volume of ethyl acetate, precipitated with ether, and recrystallized from 2-propanol: yield, 92%; mp 90–93°; R_F 0.53 (CH_3OH), 0.49 (system 2) 0.52 (system 1); $[\alpha]_D^{28}$ +38.3° (*c* 1; $CHCl_3$). *Anal.* Calcd for $C_{24}H_{20}N_6O_{10}$: N, 15.29; neut equiv 552. Found: N, 15.00; neut equiv 570 (with $HClO_4$ in glacial acetic acid).

Synthesis of Protected Histidine Peptides. Z-L-ALANYL-N^{Im}-DNP-L-HISTIDINE (IIIa, R_1 = CH_3). Carbobenzyloxy-L-alanine-*N*-hydroxysuccinimide ester (2 mmoles) in 10 ml of dioxane was mixed with 10 ml of an aqueous solution containing 2 mmoles of compound II and 6 mmoles of $NaHCO_3$. After about 5 min of stirring, all the components were dissolved. The reaction mixture was allowed to stand overnight at room temperature, then acidified with 6 N HCl. The paste-like material that precipitated was extracted into ethyl acetate, washed repeatedly with 1 N HCl and water, dried over Na_2SO_4, and evaporated to dryness. The yellowish white solid was triturated with ethyl acetate and recrystallized from the same solvent: yield, 77%; mp 203–204° dec; R_F 0.55 (CH_3OH), 0.30 (system 2), 0.77 (system 1); $[\alpha]_D^{28}$ +28.3° (*c* 1, *N*,*N*-dimethylformamide). *Anal.* Calcd for $C_{23}H_{22}O_9N_6$: C, 52.47; H, 4.21; N, 15.97; neut equiv 526. Found: C, 52.20; H, 4.09; N, 15.76; neut equiv 510 (with $NaOCH_3$ in methanol–benzene) and 539 (with $HClO_4$ in glacial acetic acid).

Z-L-PHENYLALANYL-N^{Im}-DNP-L-HISTIDINE (IIIb, R_1 = $C_6H_5CH_2$). A solution of 2 mmoles of carbobenzyloxy-L-phenylalanine-*N*-hydroxysuccinimide ester in 10 ml of dioxane was mixed with an aqueous solution (10 ml) containing 2 mmoles of compound II and 6 mmoles of $NaHCO_3$. Upon stirring for 30 min all the components were dissolved and the reaction mixture was allowed to stand overnight at room temperature. The solution was concentrated *in vacuo* to a volume of about 5 ml, cooled to 4°, and acidified with 6 N HCl. A yellowish white precipitate was formed which was recrystallized from DMF–water: yield, 85%; mp 212–213° dec; R_F 0.67 (CH_3OH), 0.30 (system 2), 0.75 (system 1); $[\alpha]_D^{28}$ −10.3° (*c* 0.97, *N*,*N*-dimethylformamide). *Anal.* Calcd for $C_{29}H_{26}N_6O_9 \cdot H_2O$: C, 56.13; H, 4.55; N, 13.54; neut equiv 620. Found: C, 56.08; H, 4.45; N, 13.70; neutr equiv 600 (with $NaOCH_3$ in methanol–benzene) and 660 (with $HClO_4$ in glacial acetic acid).

N^{α}-Z-N^{Im}-DNP-L-HISTIDYLGLYCINE (VIa, R_2 = H; R_3 = OH). A solution of compound V (1.5 mmoles in 5 ml of dioxane) was mixed with an aqueous solution of 1.7 mmoles of glycine and 3.4 mmoles of $NaHCO_3$. After stirring for a few minutes all the components were dissolved and the reaction mixture was allowed to stand at room temperature overnight. The dioxane was evaporated in high vacuum. Upon acidification, a solid paste precipitated which was extracted into ethyl acetate, washed with water repeatedly until neutrality was reached, dried over Na_2SO_4, and evaporated to dryness. The solid residue was crystallized from ethanol–petroleum ether and recrystallized from ethanol in yellowish white crystals: yield, 84%; mp 164–166°; R_F 0.62 (CH_3OH), 0.17 (system 2), 0.75 (system 1); $[\alpha]_D^{28}$ +4.3° (*c* 0.94, *N*,*N*-dimethylformamide). *Anal.* Calcd for $C_{22}H_{20}N_6O_9 \cdot H_2O$: C, 49.81; H, 4.18; N, 15.84; neutr equiv 530. Found: C, 49.80; H, 4.16; N, 15.83; neut equiv 516 (with $NaOCH_3$ in methanol–benzene) and 508 (with $HClO_4$ in glacial acetic acid).

N^{α}-Z-N^{Im}-DNP-L-HISTIDYL-L-PHENYLALANINAMIDE (VIb, R_2 = $C_6H_5CH_2$; R_3 = NH_2). A solution of 3 mmoles of compound I, 3 mmoles of $PheNH_2 \cdot HCl$ and 3 mmoles of triethylamine in 10 ml of chloroform was cooled to 0° and 3 mmoles of dicyclohexylcarbodiimide in 5 ml of chloroform was added. The reaction mixture was allowed to stand at 4°

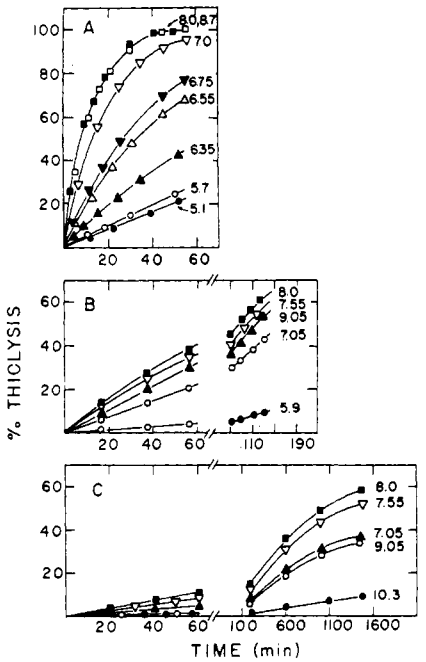


FIGURE 3: Effect of pH on the rate of thiolysis of N^{α}-*t*-Boc-N^{Im}-DNP-L-histidine. Thiolysis was initiated and performed as described in the legend to Figure 2 with either 1000 moles of 2-mercaptoethanol per mole of the DNP derivative (A), 100 moles/mole (B), and 10 moles/mole (C). The extent of thiolysis at various times was determined spectrophotometrically by the increase in absorption at 340 nm. Buffers used were: 0.1 M phosphate–citrate for the pH range 5.1–6.75, 0.1 M phosphate for the range 7.0–8.0, and 0.1 M carbonate–bicarbonate for the range 8.7–10.3.

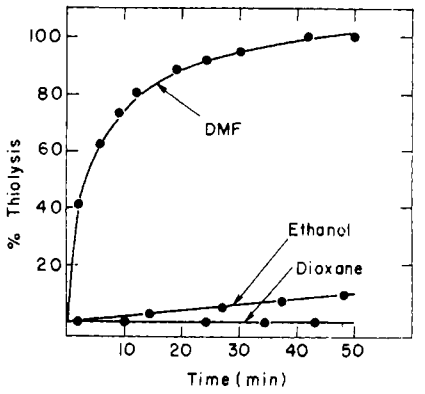


FIGURE 4: Rate of thiolysis of N^{α}-Z-N^{Im}-DNP-L-histidyl-L-phenylalaninamide with 2-mercaptoethanol in nonaqueous media. Thiolysis was initiated by addition of 2-mercaptoethanol to a solution of the peptide in the indicated solvent. The extent of thiolysis after various times was followed by the increase in absorption at 333 nm for dioxane, at 332 nm for ethanol, and 341 nm for *N*,*N*-dimethyltormamide. Final concentrations were: peptide, 9×10^{-5} M and 2-mercaptoethanol, 9×10^{-2} M (1000 moles/mole).

for 1 hr, then overnight at room temperature. The dicyclohexylurea formed was filtered and the filtrate was washed with 1 N HCl, 5% aqueous $NaHCO_3$, and water. In the course of the washings some product precipitated which was redissolved by addition of a few drops of *N*,*N*-dimethylformamide. The solution of the peptide was dried over Na_2SO_4 and evaporated to dryness and the solid residue was crystallized from ethyl acetate–ether to yield yellowish crystals; yield, 76%; mp 116–119°; R_F 0.72 (CH_3OH) 0.40 (system 2), 0.84 (system 1); $[\alpha]_D^{28} -17.4°$ (*c* 0.94, *N*,*N*-dimethylformamide). *Anal.* Calcd for $C_{29}H_{27}N_7O_8$: C, 57.90; H, 4.52; N, 16.30; neut equiv 601. Found: C, 57.71; H, 4.69; N, 16.48; neutr equiv 607 (with $HClO_4$ in glacial acetic acid).

Stability of N-DNP-imidazoles in Aqueous Media. To test the stability of *N*-DNP-imidazoles in aqueous media we incubated (for 20 hr at 22°) solutions of N^{α}-*t*-Boc-N^{Im}-DNP-L-histidine at various pH values and compared their spectra at the beginning and at the end of the incubation period. As seen in Figure 1A, B, the DNP group on the imidazole ring appears to be very stable in neutral or very acidic media. At pH 12.2 the compound decomposes within 20 hr (Figure 1C). However, at pH 10.8 (not illustrated) the extent of decomposition under the same conditions does not exceed 5%. These results were confirmed by chromatography of aliquots from the incubation mixture at the beginning and at the end of the incubation period.

Spectrophotometric Method for Following the Extent of Thiolysis of N-DNP-imidazoles. *N*-DNP-imidazoles have an end absorption above 300 nm (see Figure 1). In the case of N^{α}-*t*-Boc-N^{Im}-DNP-L-histidine the molar extinction of this absorption is 3280 at 340 nm. Upon thiolytic cleavage of the DNP-imidazole, *S*-DNP-2-mercaptoethanol is formed (ϵ_M at 340 nm 11,900 at pH 7.0). The extent of thiolysis can therefore be followed spectrophotometrically by the increase in the optical density of the solution at 340 nm. An example of the time course of the reaction is given in Figure 2.

Optimal Conditions for the Thiolytic Cleavage of N-DNP-imidazoles. In the presence of a large excess of thiol (*e.g.*, 1000 moles/mole of DNP-imidazole) the rate of thiolysis increases with increasing pH (Figure 3A). With a lower excess of thiol (100 or 10 moles per mole of the DNP-imidazole compound) the rate of thiolysis is remarkably slowed even when the pH of the reaction mixture is raised to 9 or 10 (Figure 3B,C). In fact, raising the pH above 8 brings about a reduction in the rate of thiolysis probably due to side reactions that predominate under these conditions.

The thiolytic cleavage of *N*-DNP-imidazoles is very much affected by the nature of the solvent used (Figure 4). It is therefore advisable to establish the optimal reaction conditions in each particular case. Table I gives the molar extinction and absorption maximum of *S*-DNP-2-mercaptoethanol in various solvents. These values can be used for comparing the rate of reaction in different media and choosing the most suitable solvent.

TABLE I: Spectral Properties of *S*-DNP-2-mercaptoethanol in Various Solvents.

Solvent	λ_{max}	ϵ_M
Water (pH 7.0)	340	11,900
N,*N*-Dimethylformamide	341	12,500
Ethanol	332	12,800
Ethyl acetate	332	12,800
Dioxane	333	12,300
Benzene	335	12,600

Z-L-ALANYL-L-HISTIDINE (IVa, R_1 = CH_3). To a solution of 53 mg of compound IIIa (0.1 mmole) in 0.5 ml of *N,N*-dimethylformamide we added 2.5 mmoles of 2-mercaptoethanol. The reaction was followed by subjecting aliquots of the reaction mixture at various times to thin-layer chromatography (solvent system 2). After 1 hr (22°) thiolysis was completed and the product was precipitated by addition of ether. After three recrystallizations from methanol–ether the product melted at 130° (lit. 131°, Hunt and du Vigneaud, 1938): yield, 95%; R_F 0.60 (CH_3OH), 0.05 (system 2), 0.41 (system 1); neut equiv 360. Found: 350 (with $NaOCH_3$ in methanol–benzene) and 367 (with $HClO_4$ in dioxane).

Z-L-PHENYLALANYL-L-HISTIDINE (IVb, R_1 = $C_6H_5CH_2$). To a solution of 62 mg of compound IIIb (0.1 mmole) in 0.5 ml of *N,N*-dimethylformamide we added 2.8 mmoles of mercaptoacetic acid. Thiolysis was followed by thin-layer chromatography of aliquots from the reaction mixture. The reaction was completed within 1 hr at 22° and the product was then precipitated by addition of ether and recrystallized from methanol–ether. The unprotected peptide was dissolved in 0.05 M $NaHCO_3$ (pH 8.1), precipitated by lowering the pH to 5.6 (with CH_3COOH) and recrystallized twice more from water: yield, 97%; mp 206° (lit. 198–200°, Theodoropoulos and Craig, 1955); R_F 0.60 (CH_3OH), 0.05 (system 2), 0.41 (system 1); neut equiv 426. Found: 443 (with $HClO_4$ in dioxane).

N^{α}-Z-L-HISTIDYLGLYCINE (VIIa, R_2 = H; R_3 = OH). Compound VIa (53 mg) was thiolyzed at 22° in DMF using 2.5 mmoles of 2-mercaptoethanol. After 30 min the reaction mixture was concentrated *in vacuo* and the residual oil was dissolved in methanol, precipitated with ether, washed with ether and methanol, and crystallized from ethanol–water: yield, 96%, mp 233° (lit 239°, Shaltiel and Patchornik, 1963); R_F 0.57 (CH_2OH), 0.05 (system 2), 0.25 (system 1); neutr equiv 346. Found: 337 (with $NaOCH_3$ in methanol–benzene) and 338 (with $HClO_4$ in dioxane). The product was identical in its R_F values with a marker of VIIa prepared as described previously (Shaltiel and Patchornik, 1963).

N^{α}-Z-L-HISTIDYL-L-PHENYLALANINAMIDE (VIIb, R_2 = $C_6H_5.CH_2$; R_3 = NH_2). Thiolysis of compound VIb was performed at 22° (in *N,N*-dimethylformamide using 60 mg (0.1 mmole) of the protected peptide and 2.5 mmoles of 2-mercaptoethanol. The reaction, which was followed by thin-layer chromatography (solvent system 2) was found to be completed within 30 min. The reaction mixture was concentrated *in vacuo* and the oil formed was dissolved in methanol, precipitated with ether, washed with ether, then with methanol, and recrystallized from 50% aqueous methanol: yield, 97%; mp 213–215° (lit. 214–216°, Shaltiel and Patchornik, 1963); R_F 0.77 (CH_3OH), 0.29 (system 2), 0.50 (system 1); neut equiv 435. Found: 450 (with $HClO_4$ in dioxane). The product was identical in its R_F values with a marker of VIIb prepared as described previously (Shaltiel and Patchornik, 1963).

Discussion

The major advantage of the DNP group for the protection of imidazoles during peptide synthesis lies in the fact that it combines a variety of useful features. First, the protected starting materials for such syntheses can be easily prepared as crystalline, well-defined compounds. Also, the resulting protected peptides are stable enough to withstand the usual manipulations needed for their purification, such as washing under acidic and basic conditions for the removal of excess starting materials.

An important property of the DNP group is its electron-withdrawing capacity. Thus, dinitrophenylation of the imidazole imino nitrogen reduces considerably the basicity of the imidazole ring and prevents its participation in undesirable side reactions.

The thiolytic removal of DNP groups from imidazoles is a quantitative process occurring under very mild conditions which are not apt to cause removal of other protecting groups commonly used in peptide synthesis (*e.g.*, carbobenzoxy, *tert*-butyloxycarbonyl, or *p*-toluenesulfonyl groups on amines, methyl, ethyl, or benzyl esters of carboxyls, *O*-benzyl groups on serines, *S*-benzyl groups on cysteines, etc.). Therefore, the DNP group may be useful for differential protection of functional groups during peptide synthesis.

The DNP group on imidazoles can be removed by thiolysis under nonaqueous as well as aqueous conditions. Therefore it can be used not only in classical peptide synthesis but also in solid-phase synthesis (Merrifield, 1963; Gutte and Merrifield, 1969; Fridkin *et al.*, 1966, 1968). In fact, Chillemi and Merrifield (1969) have recently made use of our method during the solid-phase synthesis of a tricosipeptide from the β chain of human hemoglobin. Another potential use of this protecting group is for aqueous synthesis of peptides using *N*-carboxyanhydrides of amino acids (Denkewalter *et al.*, 1966). It should be mentioned in this connection that we have recently prepared the *N*-carboxyanhydride of N^{im}-DNP-L-histidine (M. Fridkin and S. Shaltiel, 1970, in preparation).

Even among the few peptides that were synthesized in the course of this work, remarkable differences were observed in the conditions required for optimal rate of thiolysis. From this point of view, the availability of a simple and rapid method for following the course of thiolysis is of great advantage. Thus it was possible to show that while the removal of N^{im}-DNP group from Z-L-phenylalanyl-N^{im}-DNP-L-histidine with 2-mercaptoethanol was very slow at room temperature, it could be accelerated remarkably by replacing 2-mercaptoethanol with mercaptoacetic acid. Similarly it was found that the nature of the solvent strongly affects the rate of the-thiolysis. In the case of N^{α}-Z-N^{im}-DNP-L-histidyl-L-phenylalaninamide, for example, thiolysis with 2-mercaptoethanol was rapid in DMF but very slow in ethanol and practically did not occur in dioxane (see Figure 4). The spectral properties of *S*-DNP-2-mercaptoethanol (λ_{max} and ϵ_M) in various organic solvents were determined, so that it is possible to follow the course of the reaction in a variety of media and choose the appropriate solvent and conditions in each case.

The thiolytic cleavage of DNP imidazoles in aqueous medium may involve a nucleophilic attack by the RS^- ion on the carbon through which the DNP group is attached to the imidazole imino nitrogen. If this is the case, increasing the pH of the medium should have accelerated the rate of thiolysis. In the course of this study it was found that at high pH values the rate of thiolysis may be actually slowed (see, for example, Figure 3). This is due, probably, to air oxidation of the thiol which is known to be accelerated by increasing the pH. It is therefore recommended to avoid the high pH and to perform thiolysis at pH 7–8 with a high excess of thiol (say 1000 moles of thiol per mole of protected

peptide). This ensures rapid unmasking of the peptide and minimizes undesirable side reactions.

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