Evaluation of 1-Fluoro-2-nitro-4-trimethylammonio benzene Iodide, a Protein-Solubilizing Reagent

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The new protein reagent 1-fluoro-2-nitro-4-trimethylammonio benzene iodide reacts with model amino acids to give derivatives that are very stable to hydrolysis. In a dimethyl sulphoxide–water medium it reacts rapidly (3h) with bovine insulin, and substitution occurs quantitatively at the *N*-terminal amino groups and at the ϵ -amino groups of lysine residues. Two of the four tyrosine residues react, and it is assumed that these are the exposed groups leaving the buried groups unattacked. Unlike 1-fluoro-2,4-dinitrobenzene and related reagents, it imparts hydrophilic properties to the protein derivative, thus facilitating structural and other studies on the derivative. Circular-dichroism spectra of the modified insulin suggest that no conformational changes have occurred during reaction. These spectra also reveal the presence of an extrinsic Cotton effect at 410nm.

To Sanger (1945) goes the credit for uncovering the potential of activated phenyl halides as reagents valuable for structural studies on proteins. Sanger's (1945) reagent, 1-fluoro-2,4-dinitrobenzene (N_2ph-F),* was used initially as an end-group reagent only, but subsequently it and related compounds have found applications in the study of binding sites and tertiary structure of complex proteins.

Over the last decade there has been considerable activity aimed at the selective modification of proteins, and several reagents that react with *N*-terminal amino and other reactive groups have been developed. The most successful reagent for quantitative end-group analysis 5-dimethylaminonaphthalene-1-sulphonyl chloride (dansyl chloride, Dns-Cl), introduced by Gray & Hartley (1963), is some 100 times as sensitive as N_2ph-F because it is strongly fluorescent. One serious drawback of N_2ph-F and many related reagents has been their limited solubility in water. This problem has been overcome in part by employing suitable solvent mixtures (such as water–ethanol and water–acetone), which enhance reagent solubility, during reaction with the protein. Although formation of insoluble protein derivatives makes isolation of the latter a relatively simple matter, it also has serious drawbacks if precipitation occurs before complete

reaction or when it becomes impossible to evaluate the derivative either for homogeneity or for its chemical or biological properties because of its low solubility.

Sutton *et al.* (1965) found that the perfectly hydrophilic protein gelatin was rendered increasingly hydrophobic when its amino groups reacted with an increasing number of hydrophobic benzenesulphonyl groups. Use of other hydrophobic reagents, including benzoyl chloride, phenyl isothiocyanate and ninhydrin, also conferred hydrophobicity whereas methanesulphonyl chloride did not. However, methanesulphonyl chloride undergoes very rapid hydrolysis with water, so that its value is limited under purely aqueous conditions.

Hydrophobic reagents are unsuitable for reaction with relatively hydrophobic proteins such as insulin if it is desired to achieve complete reaction of the available functional groups and to study the protein derivative in solution. Thus when benzenesulphonyl chloride reacted with insulin (D. A. Sutton & B. W. Berman, unpublished work, cited by Berman, 1968) the protein was converted into an insoluble mass that was precipitated out when only about one-half of the available glycine, phenylalanine and lysine (ϵ -amino groups) residues and all of the tyrosine residues had reacted. The use of N_2ph-F with insulin also led to its precipitation (Sanger, 1945), but in this case reaction with the *N*-terminal residues was almost complete.

The desirability of finding reagents that preserve or enhance the solubility of the corresponding protein derivatives is being increasingly recognized. Thus Cohen (1968) has emphasized the need for the introduction of a water-soluble modification of N_2ph-F .

* Abbreviations [in accordance with IUPAC–IUB Rules given in *Biochem. J.* (1972) 126, 773]: N_2ph-F , 1-fluoro-2,4-dinitrobenzene; Dns-Cl, 5-dimethylaminonaphthalene-1-sulphonyl chloride; $HO_3S-Nph-F$, 1-fluoro-2-nitro-4-sulphoxybenzene (4-fluoro-3-nitrobenzene sulphonate); $I-Me_3N^+-Nph-F$, 1-fluoro-2-nitro-4-trimethylammonio benzene iodide [*N*-(4-fluoro-3-nitrophenyl)trimethylammonium iodide].

Zahn & Lebkücher (1961) had previously sought to produce this very type of reagent, but the compound developed by them, 1-fluoro-2-nitro-4-sulphoxybenzene ($\text{HO}_3\text{S-Nph-F}$), though yielding soluble derivatives, suffered from other drawbacks. Subsequent work in our laboratories (D. A. Sutton, S. E. Drewes & N. D. Crozier, unpublished work, cited by Crozier, 1972) on the reaction between this reagent and insulin in aqueous medium has confirmed the findings of Zahn & Lebkücher (1961) and Carty & Hirs (1968), and shown the reagent to be insufficiently activated to be of practical value since backbone degradation occurs before reaction with functional groups is complete.

A similar trend to the above is also evident from recent developments in the field of automatic analysis of the amino acid sequences of proteins and peptides. Phenyl isothiocyanate, first introduced by Edman (1950) for determining amino acid sequences, but also valuable for effecting selective modification of proteins (Africa & Carpenter, 1970), has since been employed by Braunitzer *et al.* (1970, 1971) in modified form, as the 4-sulphonic acid derivative, to yield peptides with enhanced hydrophilic character, thereby giving improved yields of fragment peptides.

Since most non-cross-linked proteins are somewhat hydrophobic it is evident from the foregoing that any reagent conferring hydrophilic properties on the protein derivative would constitute a considerable advance on the present situation. To achieve this ideal a reagent with (i) a 'built-in' hydrophilic group and (ii) suitable substituents to ensure rapid reaction with nucleophiles was prepared and subsequently evaluated. The compound, 1-fluoro-2-nitro-4-trimethylammonio benzene iodide ($\text{I}^- \text{Me}_3\text{N}^+\text{-Nph-F}$) was tested on insulin. In dimethyl sulphoxide-water (4:1, v/v) and under conditions unlikely to cause backbone fission of the protein ($<60^\circ\text{C}$, $<\text{pH}9.5$ and $<3\text{h}$) $\text{I}^- \text{Me}_3\text{N}^+\text{-Nph-F}$ reacts quantitatively with the insulin and yields an undegraded soluble derivative. On a tentative scale depicting hydrophobicity of protein derivatives $\text{I}^- \text{Me}_3\text{N}^+\text{-Nph-F}$ occupies the position shown:

$\text{Dns-Cl} > \text{Ph-SO}_2\text{-Cl} > \text{N}_3\text{ph-F} > \text{Me-SO}_2\text{Cl}$,

$\text{HO}_3\text{S-Nph-F}$, $\text{I}^- \text{Me}_3\text{N}^+\text{-Nph-F}$

Experimental

Preparation of $\text{I}^- \text{Me}_3\text{N}^+\text{-Nph-F}$ (I)

4-Fluoro-3-nitroaniline (a gift from Olin Chemicals, Stamford, Conn., U.S.A.) was converted into $\text{I}^- \text{Me}_3\text{N}^+\text{-Nph-F}$ by the method of Bevan *et al.* (1968). This involved heating the fluoronitroaniline in methanol with methyl iodide in a sealed tube at 90°C for 3 h. The resulting crystals were washed with ether and recrystallized from hot water, m.p. 215°C

(decomp.) (Found: C, 33.1; H, 4.1; $\text{C}_9\text{H}_{12}\text{FIN}_2\text{O}_2$ requires C, 33.1; H, 3.7%).

Preparation of model derivatives of $\text{I}^- \text{Me}_3\text{N}^+\text{-Nph-F}$

In all these syntheses and for the kinetic studies described below the reaction was carried out under N_2 at $40.00 \pm 0.02^\circ\text{C}$ (water bath) and at constant pH 9.0 (Radiometer Titrimeter). Solutions were agitated gently by means of a magnetic stirrer. The solvent dimethyl sulphoxide-water (4:1, v/v) was used throughout.

Me₃N⁺-Nph-glycine (II). $\text{I}^- \text{Me}_3\text{N}^+\text{-Nph-F}$ (1.63 g, 5 mmol) and glycine (0.3754 g, 5 mmol) were dissolved in 50 ml of solvent, the reaction vessel was lowered into the water bath (40°C) and the pH was adjusted to 9.0 with 1 M-NaOH. After 2 h fluoride analysis (von Zeppelin, 1951) indicated the extent of reaction to be 83%. The $\text{Me}_3\text{N}^+\text{-Nph-glycine}$, in the form of a yellow crystalline precipitate, was filtered off and recrystallized from water. This new betaine 2-nitro-4-trimethylammonio phenylglycine (II) had m.p. $242\text{--}244^\circ\text{C}$ (decomp.) (Found: C, 46.2; H, 6.1; N, 16.1; loss at 100°C , 7.6. $\text{C}_{11}\text{H}_{15}\text{N}_3\text{O}_4$, $1\text{H}_2\text{O}$ requires C, 46.7; H, 5.7; N, 16.1; H_2O 6.6%). This particular betaine, although insoluble in the reaction medium,

was soluble in water. It was characterized further by its n.m.r. spectrum in trifluoroacetic acid.

Me₃N-Nph-phenylalanine (III). I⁻Me₃N-NpH-F (5 mmol) and L-phenylalanine (5 mmol) were made to react as described for glycine. No precipitate was obtained on completion of the reaction (2 h). The solution was acidified (to pH 2.0) with conc. HCl. After 48 h at room temperature deep-yellow needles, m.p. 219°C (decomp.), of 2-nitro-4-trimethylammonio-phenylphenylalanine chloride (III) separated (Found: C, 56.4; H, 6.1; C₁₈H₂₂ClN₃O₄ requires C, 56.9; H, 5.8%). The n.m.r. spectrum of the compound confirmed its structure.

Me₃N-Nph-N^α-acetyltyrosine (IV). The N^α-acetyl derivative of tyrosine (m.p. 154°C) was prepared by the method of Du Vigneaud & Meyer (1932) and was treated with I⁻Me₃N-NpH-F as described above. To obtain the pure derivative it was necessary to separate it on a column of Bio-Gel P2 (100–200 mesh; Bio-Rad Laboratories, Richmond, Calif., U.S.A.) with water as eluent. The tyrosine derivative migrated near the solvent front as a distinct yellow band. Removal of solvent left a microcrystalline residue, m.p. 126°C, which proved to be extremely hygroscopic. From the n.m.r. spectrum (in ²H₂O or trifluoroacetic acid) the structure (IV) was deduced. The two sets of equivalent protons (*A₂B₂* system) from the tyrosine ring and the three protons (*ABX* system) from the Me₃N-NpH ring are clearly discernible, furthest downfield, in the aromatic-ring proton region. The three equivalent methyl groups on the quaternary nitrogen appear as a singlet at τ 6.7 and N-acetyl protons resonate at τ 8.5. The benzylic methylene group of the tyrosine ring appears at τ 7.6.

Me₃N-Nph-N^α-lysine. To prepare the N^α-acetyl derivative of lysine (m.p. 249°C) the method of Neuberger & Sanger (1943) was employed. The product was treated with I⁻Me₃N-NpH-F, but the resulting product proved difficult to purify. A fraction, which from its n.m.r. spectrum appeared reasonably pure, was used for subsequent qualitative tests.

Kinetic studies

Determination of F⁻. The ZrOCl₂ solution was prepared by the method of von Zeppelin (1951). Thus ZrOCl₂·8H₂O (50 g) was dissolved in conc. HCl (100 ml) and then made up to 1 litre with 2 M-HCl. The indicator was prepared by dissolving sodium alizarin sulphonate (0.5 g) in water (100 ml). The titration solution was standardized against NaF. It was found that 1 ml of solution $\equiv$ 10.2 mg of F⁻.

In a typical procedure the sample to be analysed was diluted to 20 ml, indicator (10 drops) was added and the solution was then carefully acidified with 1 M-HCl until the indicator just turned yellow. ZrOCl₂ solution was added from a micro-burette until the colour changed to red-brown. From the quantity of

ZrOCl₂ solution consumed the concentration of liberated F⁻ could be calculated.

All other conditions for the kinetic studies, such as temperature, pH and solvent, were identical with those described above.

Reaction of I⁻Me₃N-NpH-F with aniline and glycine. I⁻Me₃N-NpH-F (5 mmol) was dissolved in solvent (100 ml) and its pH adjusted to 9.0. To the reaction mixture at 40°C was added aniline (5 mmol). This was taken as zero time, and subsequently samples (10 ml) were withdrawn at regular intervals and analysed for F⁻ content. The same reaction was also repeated at 60°C.

The kinetic studies on glycine were done by the same procedure except that the I⁻Me₃N-NpH-F was initially dissolved in pure dimethyl sulphoxide (80 ml), and to this solution was added the glycine in water (20 ml).

Preparative study

Reaction of I⁻Me₃N-NpH-F with insulin. For this study the same experimental conditions as those employed for the individual amino acids were used. Insulin (240 mg, 0.04 mmol) (bovine zinc insulin, lot no. 26918, British Insulin Manufacturers, Allen and Hanbury Ltd., London W.1, U.K.) and I⁻Me₃N-NpH-F (913 mg; 2.8 mmol; 10-fold molar excess calculated for seven nucleophilic groups in the insulin molecule) were dissolved in the solvent (24 ml), which was then warmed to 40°C and its pH raised to 9.0 with 0.1 M-NaOH. Reaction was allowed to proceed in the pH-stat for 3 h. At this stage all the requisite end groups had been substituted, but additional runs lasting 12 and 24 h were also done.

On completion of the reaction the clear yellow solution was dialysed for 48 h against water (Visking 18/32 cellophan tubing), then freeze-dried. Further purification was achieved by chromatography on a glass column (20 cm $\times$ 1.5 cm) of CM-cellulose (Whatman CM-22). Eluents were (a) 10 mM-tris+7 M-urea, and (b) 10 mM-tris+7 M-urea containing NaCl (1 M) to provide a 0–1 M salt gradient. Both solutions were adjusted with HCl to pH 4.9. All chromatography was done at room temperature and the column was equilibrated with the solution of 10 mM-tris-HCl +7 M-urea for 24 h immediately before use.

After application of the sample, dissolved in solution (a) above, the column was developed with a linear salt gradient (0–1 M-NaCl) obtained by placing 35 ml each of solution (a) and solution (b) in a Varigrad mixer. A flow rate of 25 ml/h was maintained with a Beckman Accuflow pump, and fractions were collected in a fraction collector every 12 min. Throughout the separation, eluent from the column was passed through a 1.0 cm flow-through quartz cell and monitored at 280 nm in a Beckman DB-G spectrophotometer.

Fig. 1. Rechromatography of $\text{Me}_3\text{N-Nph-insulin}$ on CM-cellulose

Experimental details are given in the text. ·····, $\text{Me}_3\text{N-Nph-insulin}$; —, unchanged insulin; ----, NaCl gradient.

After rechromatography $\text{Me}_3\text{N-Nph-insulin}$ was obtained as a single component with an elution volume of about 50ml. Under the experimental conditions the derivative is well separated from the parent insulin (Fig. 1).

$\text{Me}_3\text{N-Nph-insulin}$ is a yellow powder with solubility properties better than those of the parent insulin. It is eminently soluble in aqueous media at pH 4 and below and, in the isoelectric range of insulin (pH 4.5–7), good solubility properties are observed for the derivative.

Results

Determination of the activation energy for the reaction of $\text{I}^- \text{Me}_3\text{N-Nph-F}$ with aniline

From the equation:

it can be deduced that the progress of the reaction can be followed if the F^- concentration (x) is known at any time (t). von Zeppelin's (1951) titrimetric method is ideally suited for this purpose.

Previous work (Bunnett & Zahler, 1951; Miller, 1968) predicts that the above reaction will occur via an $\text{S}_{\text{N}}2$ mechanism. From the standard equation for calculation of the rate constant,

$$k_1 = \frac{1}{t} \cdot \frac{x}{a(a-x)} \quad (2)$$

it was possible to obtain a value for k_1 for aniline, where a is the initial concentration of $\text{Me}_3\text{N-Nph-F}$. At a temperature of 40°C and with 40mmol of each reactant/l, reagent calculation of k_1 between 8 and 240min (seven readings) gave a value of $0.192 \pm 0.015 \text{ M}^{-1} \cdot \text{min}^{-1}$ (mean $\pm$ s.d.). A straight-line plot is

obtained by plotting $1/(a-x)$ against t , thus confirming the $\text{S}_{\text{N}}2$ mechanism (Laidler, 1965).

To determine the activation energy E for the reaction an identical run was done at 60°C . Determination of k_2 at this temperature gave a value of $0.411 \text{ M}^{-1} \cdot \text{min}^{-1}$. Substitution in the Arrhenius equation then yielded a value of $E = 32.9 \text{ kJ/mol}$.

Rate of reaction of $\text{I}^- \text{Me}_3\text{N-Nph-F}$ with glycine

Since glycine is present as an N -terminal amino acid in insulin its rate of reaction with the new reagent was established. Conditions of reaction are those reported in the Experimental section. For this reaction the rate constant for the total glycine was found to be $0.302 \text{ M}^{-1} \cdot \text{min}^{-1}$. Calculation of the half-life ($t_{1/2}$) for glycine from the equation:

$$t_{1/2} = \frac{1}{k} \cdot a$$

gave $t_{1/2} = 41.4 \text{ min}$.

Stability of $\text{I}^- \text{Me}_3\text{N-Nph-F}$ towards hydrolysis

$\text{I}^- \text{Me}_3\text{N-Nph-F}$ (100mg) in water (25ml) was heated at 40°C for 12h at different pH values. The degree of hydrolysis was established by measurement of the amount of F^- released. The procedure was repeated with dimethyl sulphoxide–water (4:1, v/v) as the solvent. Results are shown in Table 1.

Hydrolysis of $\text{Me}_3\text{N-Nph-amino acid derivatives}$

$\text{Me}_3\text{N-Nph-glycine}$ (5mg) and $\text{Me}_3\text{N-Nph-phenylalanine}$ (5mg) were hydrolysed with 6M-HCl for 24h at 110°C in evacuated sealed tubes. After this period of time no free amino acids could be detected in the reaction mixture by the amino acid analyser. The

Table 1. Rates of hydrolysis of $I^-Me_3\dot{N}$ -Nph-F in water and dimethyl sulphoxide–water (4:1, v/v)

For experimental details see the text.

Solvent	pH	Extent of hydrolysis (%)
Water	9	7.0
	10	30.0
	11	82.0
Dimethyl sulphoxide–water	9	0.0
	10	1.6
	11	4.8

unchanged $Me_3\dot{N}$ -Nph-glycine (54.7%) and $Me_3\dot{N}$ -Nph-phenylalanine (73.0%) could be eluted from the long column after 240min and 340min respectively with the pH 5.26 buffer after normal elution with the pH 3.25 and 4.26 buffers.

Hydrolysis of $Me_3\dot{N}$ -Nph-insulin

The $Me_3\dot{N}$ -Nph-insulin derivative obtained after 3, 12 and 24h was purified as described, then hydrolysed for 16h at 110°C *in vacuo*. Amino acid analyses on the hydrolysate and on the parent insulin were carried out in the normal way with a Beckman model 116 analyser. The results are presented in Table 2.

Paper chromatography of $Me_3\dot{N}$ -Nph-amino acid derivatives

These derivatives are well suited to paper-chromatographic techniques since they are readily detected by their yellow colour. In suitably modified solvent systems (Peraino & Harper, 1961) they travel as discrete spots, with very little tailing. The R_f values for four $Me_3\dot{N}$ -Nph-amino acid derivatives are listed in Table 3.

Circular-dichroism spectra

The spectra were recorded on a Jasco model J-20 spectropolarimeter. As solvent dil. HCl at pH 4.0 was used since both the parent insulin and the $Me_3\dot{N}$ -Nph-insulin (after reaction for 3h) were eminently soluble in it. The circular-dichroism spectra of these two compounds had negative Cotton effects at 275 and 210nm. At higher wavelengths the spectra of the two substances remained on the base-line except at 410nm, where the $Me_3\dot{N}$ -Nph-insulin exhibited a pronounced positive Cotton effect.

Discussion

Although $I^-Me_3\dot{N}$ -Nph-F was developed in the first instance as an end-group reagent that would yield

hydrophilic protein derivatives, it seems probable that it will be equally useful to effect selective modification of proteins, thereby allowing study of conformational and other changes in the derivative. The potential of $I^-Me_3\dot{N}$ -Nph-F is now discussed in the light of the above properties.

Khorana (1952) laid down certain conditions for a successful end-group reagent. These included specific and quantitative reaction with the protein under conditions that did not cause degradation of the latter, a bond between reagent and terminal amino acid that was resistant to hydrolysis, and finally, formation of an amino acid derivative that was amenable to quantitative determination on a small scale. The extent to which $I^-Me_3\dot{N}$ -Nph-F meets these requirements is considered below.

A study of the kinetics of reaction between $I^-Me_3\dot{N}$ -Nph-F and a model amine, aniline, revealed that an S_N2 reaction was involved. From this study it was possible to calculate the activation energy for reaction to be 32.9 kJ/mol (7.87 kcal/mol). The corresponding values for N_2 ph-F (Chapman & Parker, 1951) and HO_3S -Nph-F (Zahn & Lebkücher, 1961) are 24.3 kJ/mol (5.8 kcal/mol) and 48.1 kJ/mol (11.5 kcal/mol) respectively, thus showing that the activity of $I^-Me_3\dot{N}$ -Nph-F is intermediate between the two longer-established reagents. Since it was known from the work of Bunnett & Hermann (1970) that it was possible to accelerate dinitrophenylation of amino acids by means of dimethyl sulphoxide–water mixtures, this system was chosen for the present reaction. A 4:1 (v/v) mixture, containing 0.05M $I^-Me_3\dot{N}$ -Nph-F, maintained at pH 9 and 40°C, was found to be suitable. Under these conditions, for example, glycine had a half-life of 41.4 min. It was also evident from the circular-dichroism spectrum of the $Me_3\dot{N}$ -Nph-insulin derivative, in comparison with parent insulin, that the above reaction conditions were sufficiently mild not to have effected conformational changes in the insulin molecule.

Of the 51 amino acid residues in insulin only the *N*-terminal glycine, *N*-terminal phenylalanine, the ϵ -amino group of lysine-29 and phenolic hydroxyl groups of four tyrosine residues should react with $I^-Me_3\dot{N}$ -Nph-F at pH 9.0. At this pH it can be expected that all free amino groups (with the exception of the arginine side-chain amino group) and phenolic groups will be largely unprotonated and hence reactive (Tanford & Epstein, 1954). The four amino acids above (the last two with their α -amino groups blocked by acetylation) were used as model substances for reaction with the new reagent. The glycine betaine (II) was readily isolated in pure crystalline form and the phenylalanine derivative (III) was obtained pure as the crystalline chloride, but the corresponding derivatives of the two remaining amino acids proved difficult to purify. Hydrolysis of compounds (II) and (III) (with 6M-HCl in evacuated tubes at 110°C for

Table 2. *Amino acid analyses of Me₃N-Nph-insulin hydrolysates*

Experimental details are given in the text.

Amino acid	Residues in parent insulin (mol/mol)	Residues remaining (mol/mol) after reaction for:		
		3h	12h	24h
Lysine*	1.07	0.26	0.25	0.27
Histidine	1.82	1.62	1.32	0.92
Arginine (standard)	1.00	1.00	1.00	1.00
Aspartic acid	3.00	3.00	3.08	3.10
Threonine	1.06	1.03	0.95	0.99
Serine	3.02	2.87	2.74	2.76
Glutamic acid	7.10	6.90	7.12	7.10
Proline	1.01	1.00	1.05	1.03
Glycine*	4.26	3.18	3.14	3.17
Alanine (standard)	3.00	3.00	3.00	3.00
Cystine (half)	4.61	3.81	3.43	3.99
Valine	2.99	2.79	2.73	2.98
Isoleucine	0.27	0.27	0.32	0.38
Leucine	5.97	5.76	5.73	5.75
Tyrosine*	4.09	2.19	1.99	1.94
Phenylalanine*	3.02	2.03	2.01	2.01

* Denotes amino acid with potentially reactive groups.

Table 3. *R_F values for Me₃N-Nph-amino acid derivatives on Whatman no. 1 paper*

Solvent for the first direction was butan-2-ol–propan-1-ol–aq. NH₃ (sp.gr. 0.88)–acetic acid (20:3:3:6, by vol.), and solvent for second direction was 1.5M-phosphate buffer, pH 5.8 (Peraino & Harper, 1961).

Derivative	R _F	
	First direction	Second direction
Me ₃ N-Nph-glycine	0.38	0.56
Me ₃ N-Nph-phenylalanine	0.74	0.45
Me ₃ N-Nph-N-acetyl-lysine	0.73	0.58
Me ₃ N-Nph-N-acetyltyrosine	0.83	0.45

24h) yielded a high return of the unchanged derivatives (54.3 and 73.0% respectively), thus emphasizing the strength of the reagent–amino acid bond. Under comparable hydrolysis conditions N₂ph-glycine and HO₃S-Nph-glycine are recovered unchanged to the extent of 38.1 and 28.6% respectively (Zahn & Lebkücher, 1961).

It was also important to assess the stability to hydrolysis of the reagent itself under the selected reaction conditions. At pH 9.0, at a temperature of 40°C and with an aqueous reaction medium, I-Me₃N-Nph-F is 7% hydrolysed after 12h. Under otherwise identical conditions but at pH 11.0 the hydrolysis

amounts to 82%. However, in dimethyl sulphoxide–water (4:1, v/v) no hydrolysis is apparent at pH 9.0 (12h) and it amounts to only 4.8% at pH 11.0 (see Table 2).

The ability of I-Me₃N-Nph-F to react rapidly and quantitatively with model amino acids having thus been established, its value as a protein reagent was evaluated by means of its reaction with insulin. The measured rate constant obtained for glycine suggested that a reaction time of 3h at pH 9.0 and at 40°C should be adequate for complete reaction. In addition, runs lasting 12h and 24h were conducted to establish at what stage (if at all) degradation occurred. Amino acid analysis of the derivative, previously purified by dialysis and separation on CM-cellulose, showed clearly (Table 3) that after 3h all the expected glycine, phenylalanine and ε-lysine groups and one-half of the tyrosine groups had reacted. Almost identical results were obtained with the insulin derivatives obtained after reaction for 12 and 24h. The only significant difference between the three sets of results is that an increasing amount of histidine partakes in the reaction with increasing time. After 24h half of the histidine content (two residues) appears to have reacted. Chromatography of the 24h-reaction material on CM-cellulose also shows the presence of a number of unidentified by-products, but these are present in low concentration.

The eminent solubilizing properties of I-Me₃N-Nph-F are illustrated by the observation that a completely clear yellow solution of Me₃N-Nph-insulin is

obtained on termination of the reaction. Isolation of the pure derivative, free of buffer and other contaminants such as traces of residual insulin, is effected by a simple procedure. After dialysis against water the sample is freeze-dried and separated on a CM-cellulose column. By employing an eluent system with pH 4.90 the net charge difference between $\text{Me}_3\text{N}^+\text{-Nph-F}$ -insulin and insulin itself is sufficiently large to bring about a good separation on the column (Fig. 1).

If the above properties of $\text{I}^-\text{Me}_3\text{N}^+\text{-Nph-F}$ are measured against those of the ideal end-group reagent as defined by Khorana (1952), it emerges with considerable merit. The particular properties of $\text{I}^-\text{Me}_3\text{N}^+\text{-Nph-F}$, notably its protein-solubilizing attribute, extend its scope beyond that of an end-group reagent only. Although the present investigation has been concerned with a quantitative reaction with all exposed reactive groups, it should be possible to achieve a selective chemical modification of proteins. Already under the present reaction conditions $\text{I}^-\text{Me}_3\text{N}^+\text{-Nph-F}$ reacts only with the two more accessible tyrosine residues, leaving the 'buried' two (Menendez *et al.*, 1969; Menendez & Herskovits, 1969) intact. This finding provided an early indication that no conformational changes had taken place in insulin during reaction with the $\text{I}^-\text{Me}_3\text{N}^+\text{-Nph-F}$. Confirmation of this view was forthcoming from the circular-dichroism spectrum of $\text{Me}_3\text{N}^+\text{-Nph-insulin}$. The spectrum of the derivative followed closely that obtained for insulin (Ettinger & Timasheff, 1971), with negative Cotton effects at 275 and 210 nm, but, in addition, the derivative generates an extrinsic Cotton effect at 410 nm. Such effects have been observed by Fairclough & Vallee (1971) for chymotrypsinogen A modified with diazotized *p*-arsanilic acid, and this effect has been used by these authors as a means of detecting changes in local conformation that accompany activation of the modified chymotrypsinogen. Potentially $\text{I}^-\text{Me}_3\text{N}^+\text{-Nph-F}$ could be used for similar studies.

Taken together, the present work and the previous work of Sutton *et al.* (1965) demonstrate that reagents exist that enable one to increase or decrease the hydrophobicity of proteins without causing degradation of the peptide backbone.

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