Amino Acid Sequence Analysis with Methyl Isothiocyanate. Resolution of the Methylthiohydantoins by Gas–Liquid Partition Chromatography*

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ABSTRACT: Methyl isothiocyanate (MITC) has been substituted for phenyl isothiocyanate (PITC) in the Edman degradation. The methylthiohydantoins (MTH) of the amino acids have been synthesized and with the exception of MTH-arginine have been sufficiently resolved for quantitative analysis by gas–liquid partition chromatography employing two identical columns run simultaneously in a single instrument.

Columns (10 ft × 2 mm i.d.) were used containing 5% QF1 packing in the terminal 2 ft and 5% SE-52 in the remaining 8 ft. Derivatization with *N*,*O*-bis(trimethylsilyl)acetamide (BSA) or trifluoroacetic anhydride has been used to stabilize and resolve serine, threonine, and *S*-carboxymethylcysteine, and to volatilize aspartic and glutamic acids. A complete analysis which may be performed in 1 hr is made by injecting an untreated aliquot onto one column and a BSA-treated aliquot onto the second identical column. MTHs which are not resolved or are unstable such as serine-MTH and *S*-carboxymethylcysteine-MTH, which untreated cochromatograph with alanine, are identified as trimethylsilyl derivatives by simultaneous analysis on the second column. This method has been applied to the simultaneous degradation of both chains of insulin, to a Bence–Jones protein, and in a modified form to a dipeptide, Ser-Gly. The difficulty of spurious peaks derived from reagents and solvents is noted. Preliminary experiments indicate that MITC may also be substituted for PITC for the degradation of Bence–Jones proteins in the Edman sequenator.

Edman degradation of peptides or proteins with PITC[1] (Edman, 1950, 1956) has become a routine method for determination of amino acid sequence. The N-terminal amino acid removed at each step can be identified by subtractive amino acid analysis (Konigsberg, 1967), by end-group analysis of the shortened peptide by dansylation (Gray, 1967), or by direct analysis of the reaction product, the PTH. These methods are generally satisfactory for sequences up to ten residues in small peptides but then begin to yield ambiguous results.

Sequence analysis of large peptides or proteins, whether carried out by hand or by an automatic machine (Edman and Begg, 1967), is dependent on identification of the PTHs formed at each step. Great reliance must be placed on these data since no information can usually be obtained from compositional or subtractive amino acid analysis.

Qualitative identification of PTHs by thin-layer chromatography (Edman and Sjöquist, 1959) is often complicated by background and does not provide the assurance of a quantitative result. For these reasons it is also unsatisfactory for sequence analysis of mixtures of peptides (Gray, 1968). Rapid quantitative analysis of PTHs by gas–liquid partition chromatography has been described fully for only a few amino acids though more complete methods have appeared in abstract form (Pisano *et al.*, 1962; Pisano and Bronzert, 1969; H. D. Niall, 1969, personal communication).

In the present paper, MITC has been studied as an alternative to PITC for the Edman degradation, and a rapid gas–liquid partition chromatographic system has been developed for quantitative analysis of MTHs.

Materials

Reagents and solvents were distilled and stored under nitrogen in acid-cleaned glass containers. PITC, benzene, ethyl acetate, trifluoroacetic acid, and ethylene dichloride (similar to butyl chloride) were purified according to Edman and Begg (1967). MITC (Eastman Organic Chemical) was purified by vacuum sublimation employing an oil pump. Trifluoroacetic anhydride and methanol were redistilled. *N*,*N*-Dimethylallylamine and pyridine were refluxed over phthalic anhydride for 3 hr and distilled. Pyridine was further refluxed over solid potassium hydroxide for 3 hr and distilled. Reagents and solvents were stored in two-neck Wolf bottles maintained under slight nitrogen pressure through one neck (Figure 1).

MTHs were synthesized by the method of Stepanov and Krivstov (1965) and structures were established by mass spectroscopy (Richards *et al.*, 1969). These compounds have now become available from Mann Research Laboratories, Inc. (New York, N. Y.).

Materials for gas–liquid partition chromatography were obtained from Varian Aerograph (Walnut Creek, Calif.) and BSA from Regis Chemical Co. (Chicago, Ill.).

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[1] Abbreviations used are: PTH, 3-phenyl-2-thiohydantoin of amino acid; PITC, phenyl isothiocyanate; MITC, methyl isothiocyanate; MTH, 3-methyl-2-thiohydantoin of amino acid; BSA, *N*,*O*-bis(trimethylsilyl)acetamide; TMS, trimethylsilyl; TFAc, trifluoroacetyl.

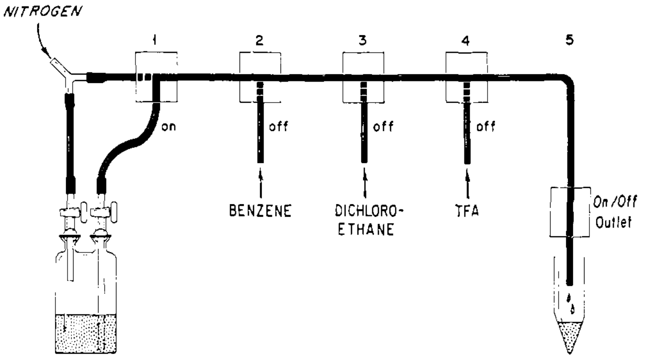

FIGURE 1: Storage and valving system of reagents for the Edman degradation. Reagents or solvents were forced by nitrogen pressure out through $^1/_{16}$-in. Teflon tubing attached by a Chromatronix (Berkeley, Calif.) G1-C fitting to the Wolf bottle (G. Finkenbiner, Waltham, Mass.). Four, three-way Chromatronix CAV 3031, valves were connected in series with a CAV 2031 two-way valve. With valves 1 and 5 in the on position reagent passed through the intervalve tubing to the sample tube. Closure of valve 1 then allowed a separate stream of nitrogen to flush reagent from the intervalve tubing into the sample tube, and to flush the sample tube with nitrogen.

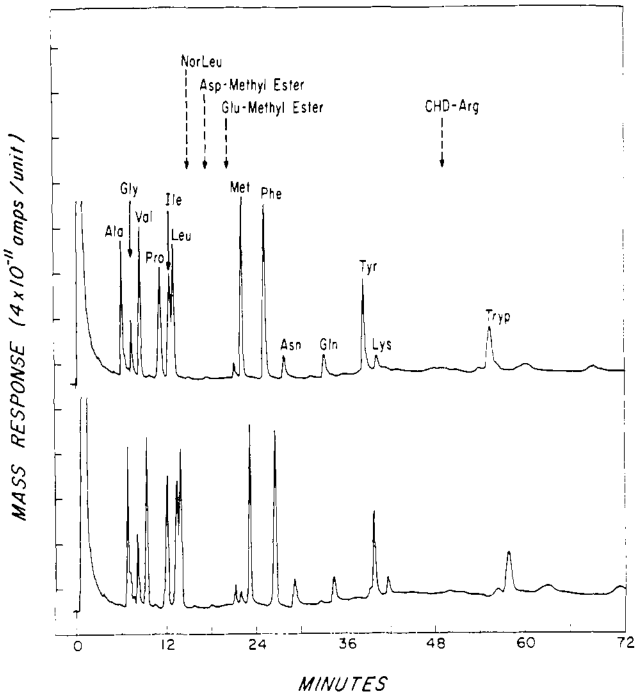

FIGURE 2: Gas–liquid partition chromatography resolution of 1 μg of each of the stable MTHs on a combined 5% SE-52–5% QF1 column. Simultaneous analyses of two identical samples are shown to indicate slight variations in elution patterns for duplicate columns. A temperature program from 187 to 275° has been used. Initial isothermal temperature was 187° for 12 min, then the temperature was increased at 6°/min to 241° where it remained for 9 min; a further increase at 4°/min to 277° over 9 min was followed by a final isothermal period.

Apparatus and Chromatography. A Varian Aerograph Model 1520 dual-column chromatograph with a Matrix temperature programmer and two-flame ionization detectors, in conjunction with a Honeywell Elektronic 16 recorder fitted with a disc integrator, was used as a dual-channel system.

Injector heaters were maintained at 250° and the detector oven at 300°. Glass columns (10 ft × 2 mm i.d.) were used with an inlet pressure of 60 psi of nitrogen at a flow rate of 25 ml/min. Glass columns were silanized before use with a 5% solution of dimethyldichlorosilane in toluene and washed with methanol to remove excess reagent. Aeroport 30 (100–120 mesh) was used as a support and was treated under vacuum with 5% dimethyldichlorosilane in toluene, exhaustively washed with methanol, and dried before use. Stationary phases were dissolved in chloroform under reflux, cooled, and added to the support. To obtain a 5% coating, 5 g of stationary phase was dissolved in 100 ml of solvent and added to 25 g of support in a 250-ml Büchner flask. The support was degassed by alternate exposure to vacuum and air. The wet support was then tipped into a sintered-glass Büchner funnel and vacuum was applied until the majority of the solvent was removed but the packing remained damp. It was then placed onto a large piece of chromatography paper and dried in an oven at 50° for 30 min. Glass columns were sealed with silanized glass wool and filled by drawing the coated support into the column under vacuum. Columns containing two different supports were filled with the support nearest the detector end first. Packed columns were initially conditioned at 290° overnight. After each day's use, 2 μl of BSA was injected and the column was left at 275° overnight. The column packing within the inlet heater (4 in.) was replaced weekly.

Gas–Liquid Partition Chromatography. MTH amino acids can be sublimed at temperatures below 250° (Richards *et al.*, 1969) and should be volatile in the gas chromatograph at similar temperatures. Indeed, the majority of the MTHs can be eluted from nonpolar columns as symmetrical single peaks within 30 min at temperatures up to 290°. However, the carboxyl and guanidino amino acid side chains have prevented elution of aspartic acid-, glutamic acid-, and arginine-MTHs even from the most nonpolar columns. In addition, MTHs with aliphatic hydroxyl and substituted sulfhydryl side chains have been unstable under all chromatographic conditions.

The resolution of mixtures of stable MTHs on many different silicone stationary phases has been examined. A combination of an SE-52 stationary phase which separates isoleucine-MTH from leucine-MTH and QF1 stationary phase which resolves ε-methylthiocarbamyllysine-MTH from tyrosine-MTH has proved the most satisfactory. These two phases have been combined into a single 10-ft column by filling the terminal 2 ft with a 5% QF1 packing and the remaining 8 ft with a 5% SE-52 packing. The separation of MTHs using this packing is shown in Figure 2. Under these conditions serine-, SCMC-, and threonine-MTHs decomposed to form compounds which cochromatograph with other MTHs (Figure 3). The peaks which cochromatograph with alanine are probably due to dehydration of serine and loss of the side chain of SCMC. A similar reaction may result in the breakdown of threonine-MTH to form the two peaks shown in Figure 3.

MTHs which cannot be identified by direct chromatography may be analyzed after derivatization to form more volatile and stable compounds.

The free carboxyl group of aspartic acid- and glutamic

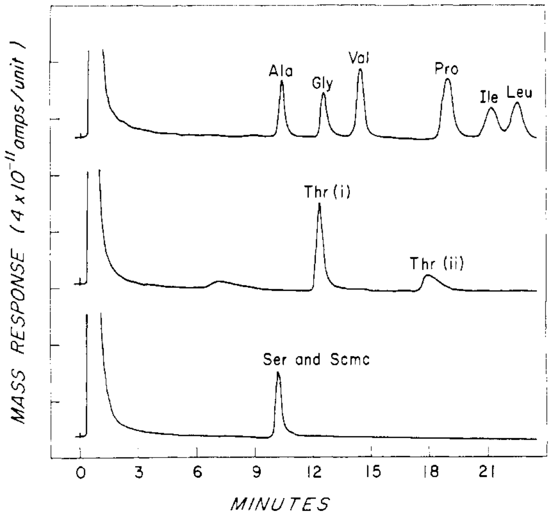

FIGURE 3: Gas–liquid partition chromatography of threonine, serine, and SCMC-MTHs. The same support is used as in Figure 2. Stable amino acids are shown for comparison. Temperature was isothermal at 175°.

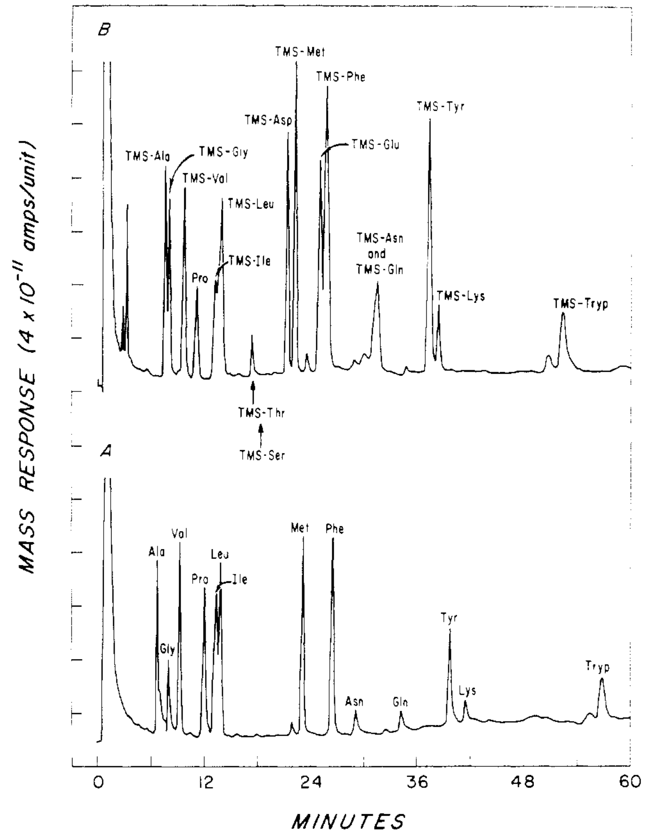

FIGURE 5: Simultaneous gas–liquid partition chromatography resolution of a standard mixture of MTHs (chromatogram a) and their trimethylsilyl derivatives (chromatogram b), 1 μg of each MTH. Conditions are identical with those in Figure 2.

acid-MTHs can be esterified with methanolic hydrogen chloride. The methyl esters are eluted between leucine-MTH and methionine-MTH in the system shown in Figure 2. However, a more rapid derivatization of the carboxyl groups can be made by using BSA to form the trimethylsilyl esters. Derivatization can be carried out using 50% BSA in ethyl acetate in a sealed capillary tube by heating at 187° for 30 sec. (Note: the sample is conveniently placed in the gas chromatograph oven which is at 187° at the start of the analysis; derivatization may also be carried out at 50° for 10 min.) If optimum conditions are not used, incomplete derivatization results in the formation of double peaks for each of the acidic MTHs, indicating mono- and disubstitution of trimethylsilyl groups. Since Harman *et al.* (1968) have shown that the nitrogen of the hydantoin ring in PTHs can be substituted with a trimethylsilyl group, a similar reaction probably occurs with the MTHs. Indeed, as expected, the retention time of all the MTHs except proline which lacks an amide nitrogen can be changed by derivatization with BSA. In addition to substitution on the hydantoin ring, BSA will react with the hydroxyl groups of serine- and threonine- (and tyrosine) MTHs forming derivatives which no longer cochromatograph with alanine- and glycine-MTHs. It is likely that the trimethylsilyl ether group prevents the dehydration of the side chains which normally results in the formation of dehydroserine- and dehydrothreonine-MTHs.

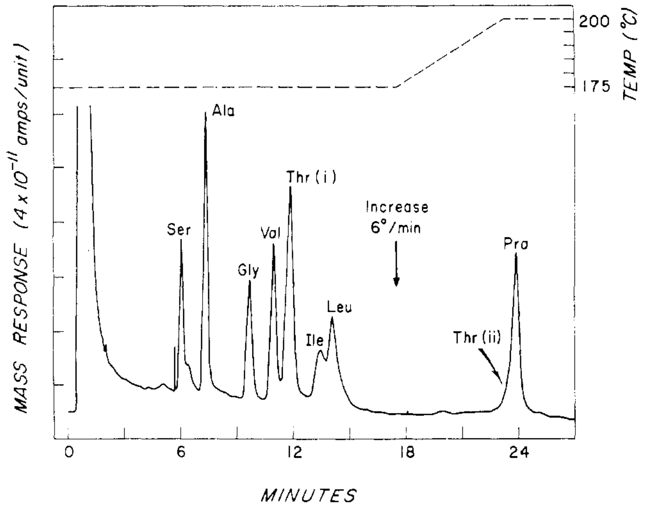

FIGURE 4: Gas–liquid partition chromatography of trifluoroacetyl derivatives of MTHs. The same support was used as in Figure 2.

Although not essential to the routine method of analysis, confirmation of the presence of serine-MTH, threonine-MTH, and SCMC-MTH may be made by derivatization with trifluoroacetic anhydride. In contrast to the TMS-MTHs, the trifluoroacetyl derivatives are more volatile than the untreated MTHs. Derivatization with trifluoroacetic anhydride was carried out by withdrawing 1 μl of the reagent into the syringe used for injection followed by 1 μl of the sample. Upon injection of this mixture, derivatization occurred in the inlet heater. Separation of a mixture of TFAc-MTH derivatives is shown in Figure 4.

Arginine-MTH can be chromatographed after blocking of the guanidino group with cyclohexanedione (Figure 2) (Toi *et al.*, 1965).

Analysis of MTH Mixtures. Derivatization with BSA is necessary to identify serine-, threonine-, SCMC-, aspartic acid,

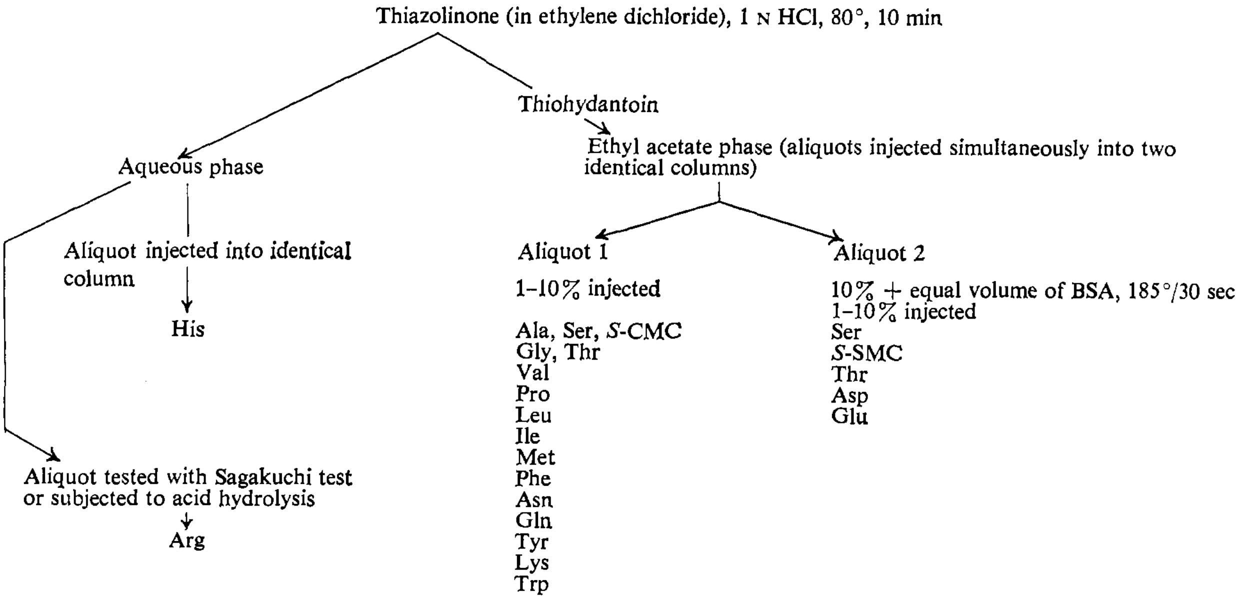

FIGURE 6: Procedure for identification of an unknown mixture of MTH by gas–liquid partition chromatography. Identity may be confirmed by amino acid analysis after hydrolysis at 150° for 24 hr, except for serine and threonine. Asparagine and glutamine MTHs are converted into aspartic and glutamic acids during hydrolysis.

and glutamic acid-MTHs. Since BSA also reacts with all the MTHs except proline, it is possible to obtain a double check on an unknown sample by measuring the change in retention time which follows derivatization. Analyses of unknown samples are carried out by injecting an untreated aliquot into one column of the gas chromatograph and a BSA-treated aliquot into the second identical column. A typical analysis of a standard mixture of MTHs is shown in Figure 5.

The retention times for identical samples injected into the two columns differ slightly but reproducibly since the amounts of packing and gas flows vary slightly between the columns. The elution of identical underivatized samples from both columns was shown in Figure 2.

The mixture analyzed in Figure 5 contains aspartic acid- and glutamic acid-MTHs which are absent from chromatogram a but appear as trimethylsilyl ester derivatives in chromatogram b. Also shown are arrows indicating the retention times of the TMS derivatives of serine- and threonine-MTHs which are completely resolved from each other. A small spurious peak running in this region may be eliminated by analysis of a reagent blank.

The retention times of MTHs and TMS-MTHs are shown in Table I.

The procedure for identification of MTHs in a sample from an Edman degradation is shown in Figure 6. Both columns a and b are run simultaneously so that all the MTHs except arginine and histidine can be analyzed in 1 hr. Analysis for histidine-MTH is best performed at the end of the dual column analysis using isothermal conditions at 275°. The resolution of a standard mixture containing histidine-MTH is shown in Figure 7. There is no satisfactory method of gas chromatographic analysis for arginine unless the guanidino group has been previously blocked, for example by treatment of the protein or peptide with cyclohexanedione (see Figure 2) (Toi *et al.*, 1965). If analyses indicate that no other MTH is present in sufficient quantity to account for the amino-terminal residue, then amino acid analysis after acid hydrolysis (at 150° for 24 hr in constant-boiling HCl) can be used to determine the presence of arginine.

Quantitation of MTH Analysis. The MTHs used in this study were prepared as crystalline compounds except lysine, methionine, histidine, and arginine. Serine-MTH was extremely unstable and it was prepared by the Edman degradation of serylglycine. Molar extinction coefficients were measured in alcoholic solutions and are shown in Table I. Comparisons between solutions of different batches of

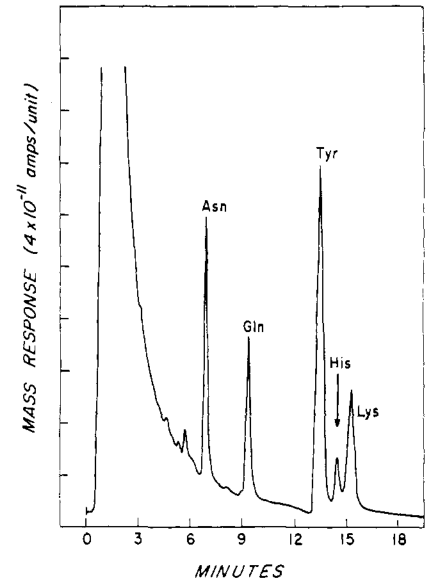

FIGURE 7: Gas–liquid partition chromatography resolution of 1 μg each of high-boiling MTHs. The column as described in Figure 2 was maintained isothermally at 275°.

TABLE I: Comparative Gas–Liquid Partition Chromatographic Data for MTHs and TMS-MTHs.[a]

		Rel Retention Time (Proline MTH = 1)		Rel Molar Peak Area (Proline MTH = 1)	
Amino Acid	ϵ_{266}	MTH	TMS-MTH	MTH	TMS-MTH
Alanine	17,200	0.56	0.69	0.61	1.50
Serine		0.63	1.33		
S-Carboxymethylcysteine		0.64	0.88		
Glycine	15,800	0.67	0.73	0.22	1.11
Threonine	15,400	0.67	1.44		
Valine	17,100	0.78	0.88	1.11	2.09
Proline	13,600	1.00	1.00	1.00	1.00
Isoleucine	17,800	1.10	*b*	1.22	*b*
Leucine	16,700	1.14	*b*	1.22	*b*
Norleucine		1.34			
Aspartic acid methyl ester		1.48			
Glutamic acid methyl ester		1.80			
Aspartic acid	18,100		1.94		2.33
Glutamic acid	20,200		2.25		3.10
Methionine	18,900	1.90	2.02	0.65	2.71
Phenylalanine	16,700	2.17	2.31	1.36	5.86
Asparagine	19,100	2.39	*b*	0.20	*b*
Glutamine	19,500	2.82	*b*	0.18	*b*
Tyrosine	17,800	3.25	3.36	0.72	4.20
Histidine	19,100	3.33		0.06	
Lysine	19,000	3.41	3.44	0.27	1.21
CHD-arginine		4.00			
Tryptophan	21,500	4.64	4.74	0.66	1.87

[a] ϵ_{266} was measured for each solution used to obtain the data for relative molar peak areas. Peak areas were measured using a disk integrator. [b] Insufficiently resolved.

TABLE II: Subtractive Amino Acid Analyses of Whole Beef Insulin and Its A Chain after Edman Degradations with MITC and PITC.[a]

		Residues Removed		
		3 Steps Whole Insulin		4 Steps Insulin A Chain
Amino Acid	Chain Position	MITC	PITC	MITC
Aspartic acid	B_3	0.52	0.59	
Glycine	A_1	0.96	0.86	0.93
Valine	B_2,A_3	1.70	1.68	0.91
Isoleucine	A_2	0.65	0.53	0.94
Phenylalanine	B_1	0.94	0.93	
Glutamic acid	A_4			0.94

[a] The manual Edman procedure described in the text was employed. Amino acid analyses were performed by the method of Spackman *et al.* (1958).

standards used to calibrate the gas chromatograph were normalized with the molar extinction coefficients obtained from pure samples.

The flame ionization detector used in these studies has a theroetical linear response of 1×10^6 up to a concentration of 0.5% of an eluted compound in the gas entering the detector. Within the range tested for MTHs (0.5–4.0 μg) the response was confirmed to be entirely linear. The relative molar response factors for the MTHs and TMS-MTHs were determined and are shown in Table I.

Analysis of Amino Acid Sequence. The three-stage degradation described by Blomback *et al.* (1966) as modified by Niall and Potts (1969) was used with either MITC or PITC as coupling agent. A nitrogen atmosphere was maintained at all times (Figure 1).

COUPLING. The peptide (0.5–1.0 μmole) was dissolved in 250 μl of 0.4 M dimethylallylamine (in 50% aqueous pyridine adjusted to pH 9.5 with trifluoroacetic acid). MITC was melted by warming at 40° and 10 μl was added with a warm pipet. Samples were incubated at 50° for 30 min. The solution was then extracted twice with 2 ml of benzene. The phases were separated by centrifugation and traces of benzene remaining were evaporated in a stream of nitrogen. Samples were then lyophilized.

CYCLIZATION. Trifluoroacetic acid (50–100 μl) was added and tubes were heated at 50° for 10 min; the acid was then removed with a stream of nitrogen leaving the peptide coated onto the walls of the tube as a thin film. The peptide film was extracted with 2 ml of ethylene dichloride which was dried and thiazolinones were converted into thiohydantoins in 200 μl of 1 N HCl at 80° for 10 min (Edman and Begg, 1967). Thiohydantoins were extracted into 3 ml of ethyl acetate which was dried, and 1–10% of the sample in 1–5 μl of ethyl acetate was injected into the gas chromatograph.

A comparison between MITC and PITC as coupling reagents was made by subjecting whole beef zinc insulin to three cycles of degradation. Subtractive amino acid analysis (Table II) shows that MITC and PITC are equally effective in removing the first three residues from the A and B chains of insulin. Although removal of the second and third residues was incomplete, there was no significant difference between the degradation by MITC and PITC. Gas chromatographic analysis of steps one and two are shown in Figure 8. It should be noted that essentially equimolar yields are obtained from both chains. Similar experiments with insulin A chain show that complete removal of the first four residues can be effected by MITC indicating that the N terminus may be in part sterically hindered in whole insulin.

Complete analyses are usually carried out using derivatization with BSA to confirm the identification of each MTH. Additional components are often found in gas chromatographic analyses of BSA-treated samples; these components vary with the batch of reagents used. A dual column analysis

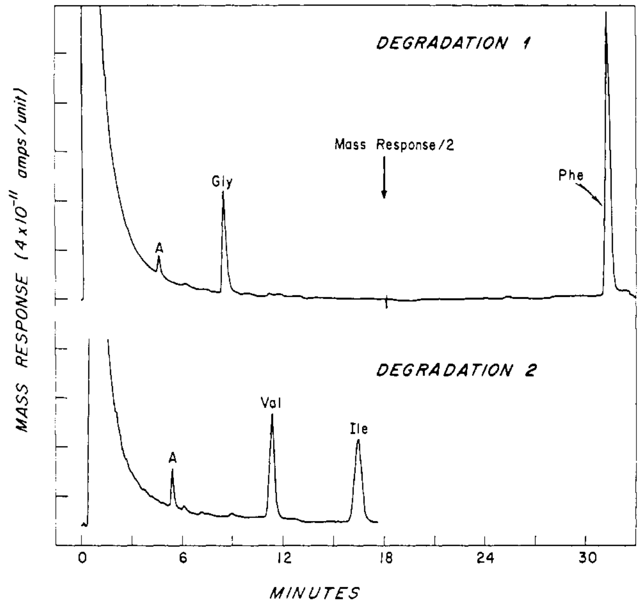

FIGURE 8: Gas–liquid partition chromatographic analysis of Edman degradations 1 and 2 of 1 μmole of whole beef insulin with MITC; 1% of each residue was analyzed by the same method as used in Figure 2. Peak A is explained in the text.

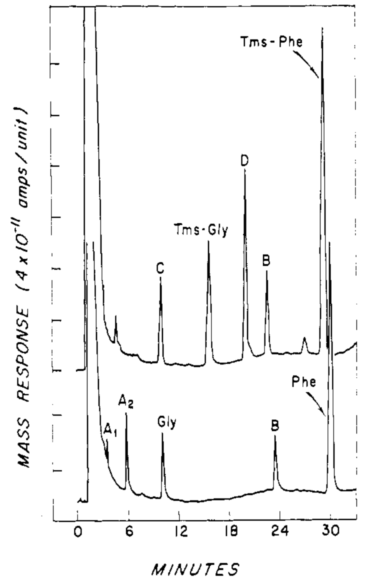

FIGURE 9: Dual-column gas–liquid partition chromatographic analysis of the first step of the Edman degradation of whole beef zinc insulin with MITC. Conditions are similar to those in Figure 4. Insulin (1 μmole) was degraded and 2% of the untreated sample injected (lower diagram) and 2% of a BSA-derivatized sample (upper diagram). The identity of the lettered peaks is discussed in the text.

of another experiment representing the first degradation of insulin is shown in Figure 9. This demonstrates that even in the presence of these additional components, an interpretation of the chromatogram can be made easily. Analysis of the underivatized sample on channel A shows peaks for glycine-MTH and phenylalanine-MTH similar to those demonstrated

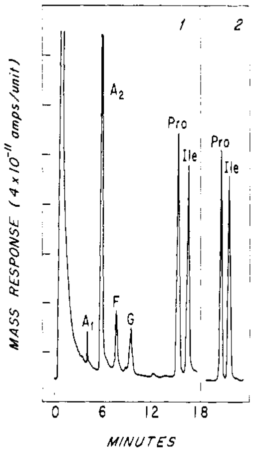

FIGURE 10: Gas–liquid partition chromatography of Edman degradation of the second step of 0.5 μmole of a Bence–Jones protein[2] with MITC. Proline-MTH (2 μg) as internal standard and 8% of the sample were injected. Analysis 2 represents a duplicate degradation; conditions are similar to those of Figure 2.

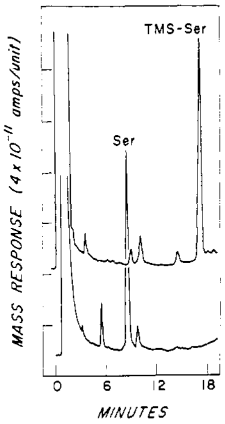

FIGURE 11: Gas–liquid partition chromatographic analysis of Edman degradation of serylglycine using the modified procedure for small peptides. Underivatized sample, lower chromatogram; BSA-treated sample, upper chromatogram. Conditions as in Figure 4.

in Figure 7. Two additional peaks were also found. Peaks A1 and A2 correspond to mono- and dimethylthioureas which are formed by hydrolysis of MITC during coupling. These compounds are often extracted after coupling with ethyl or butyl acetate (Blomback *et al.*, 1966) unless gas chromatographic analysis is used, in which case the extraction can be omitted since the peaks are eluted before any of the MTHs. The identity of peak B is not known. It does not, however, interfere with analysis of any MTH. After derivatization with BSA, the retention time of peak B was unchanged and new peaks were found for TMS-glycine-MTH and TMS-phenylalanine-MTH. In addition, three other peaks were found of which only one, peak D, which was eluted in the position of TMS-leucine-MTH, interfered with analysis of TMS-MTHs.

Edman degradation with MITC has also been used to

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[2] Bence–Jones protein 26, a gift of Corrado Baglioni.

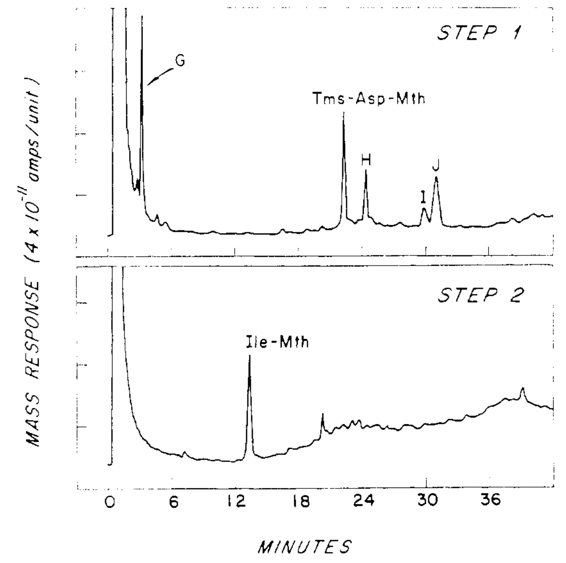

FIGURE 12: Gas–liquid partition chromatographic of residues 1 and 2 from Edman degradation of 0.5 μmole of Bence–Jones protein 26 (see Figure 10) with MITC in a sequenator; 3% of BSA-treated sample was injected in step 1 and 6% of the underivatized sample in step 2. Chromatographic conditions as in Figure 4. Peaks G, H, I, and J are background peaks which occur in BSA-treated samples only.

sequence the first 3 residues of several Bence–Jones proteins and immunoglobulin light chains. Figure 10 shows analysis of the second step of a Bence–Jones protein in which 2 μg of proline-MTH has been added as an internal standard; 8% of the sample was analyzed and a yield of 54% isoleucine-MTH was found. Superimposed in the figure is analysis of a duplicate sample showing a high degree of reproducibility of both degradation and analysis.

A modified degradation has been used for amino-terminal analysis of small peptides to avoid losses caused by extraction. After coupling, 1 ml of benzene is added to the coupling buffer and the mixture is evaporated under vacuum at room temperature. Cyclization and conversion are both carried out in one step with 1 N HCl and the MTH is extracted into ethyl acetate. One complete N-terminal analysis can then be carried out in 45 min. Figure 11 shows a gas chromatographic analysis of serylglycine degraded by this method.

Discussion

The results of Edman degradation of peptides or proteins with MITC have shown that this coupling agent can be used to replace PITC for amino-terminal sequence analysis. It was not found necessary to modify any of the reagents or solvents used in degradation by the three-stage method when MITC was substituted for PITC.

MITC possesses several advantages over PITC. The reagent itself and the coupled peptide or protein are both more soluble in the semiaqueous coupling buffer. This is particularly true for peptides with a high lysine content. The major advantage of using MITC is found in the increased ease of identification of the thiohydantoin derivatives, where the chromatographic properties are more influenced by the amino acid side chain in the methyl derivative than with the bulky phenyl analog. This difference can be observed in qualitative thin-layer chromatography where a complete identification of MTHs can be made with solvent systems D and E of Edman and Sjöquist (1959) (Smith, 1969) while complete resolution of PTHs needs a pretreatment of plates with formamide and three to four systems (Edman and Sjöquist, 1959). Similar advantages were found on gas–liquid partition chromatography. For example, the MTHs of leucine and isoleucine can be 60% resolved but the analogous PTHs cannot be resolved more than 5%. Gas chromatographic resolution of PTH derivatives at present requires two columns which are operated isothermally at different temperatures (H. D. Niall, 1969, personal communication). This means that only one column can be used at a time unless two gas chromatographs are available. The system for identification of MTHs described here has considerable advantage over the PTH system since all analyses can be performed on a single column. If a dual-channel machine is available, a second column can be used to provide simultaneous analysis of a derivatized aliquot of the unknown material, thus providing identification of unstable compounds by a change in retention time. With the gas chromatograph used in this study relative retention times could be reproduced with a 1–2% accuracy, which was quite adequate for identification. Further confirmation of certain MTHs can be made by forming the trifluoroacetyl derivatives (Figure 4). Complete analysis of a residue obtained from an Edman degradation with MITC can be made in 1 hr unless histidine-MTH or arginine-MTH is present. Histidine-MTH can be identified by gas chromatography (Figure 7) but arginine-MTH must be identified by the Sakaguchi test, by amino acid analysis after hydrolysis, or by thin-layer electrophoresis (Edman and Begg, 1967). Since in practice the yield of MTH or PTH is known to drop steadily from residue to residue the sequence of amino acids is best established by relating the yields at successive steps to one another. Thus, analysis for arginine-MTH or histidine-MTH need not be performed unless the N terminus cannot be accounted for quantitatively by any other residue.

Manual Edman degradations by the method described here may be routinely carried out on samples of 0.1–0.25 μmole of peptide. The limitation of the method is not in the sensitivity of the analytical method, which could theoretically handle 0.001 μmole, but in the losses due to extraction of peptides in solvents and resolution of MTHs from background. With the recent introduction of commercially produced solvents (Beckman Instruments, Palo Alto, Calif.), a significant improvement in background and hence sensitivity is evident.

In addition to ease of separation on thin-layer chromatography and gas–liquid partition chromatography, the MTHs may be identified as amino acids after hydrolysis. Hydrolysis of MTHs in constant-boiling HCl at 150° for 24 hr gave similar yields to those obtained by Van Orden and Carpenter (1964) for PTHs. Of those not studied by Van Orden and Carpenter (1964), histidine-MTH, arginine-MTH, and ϵ-methylthiocarbamyl lysine-MTH gave yields of 40–50% of the respective amino acid.

MITC has also been substituted for PITC in the Edman automatic amino acid sequenator (Edman and Begg, 1967). In qualitative preliminary experiments with a Bence–Jones protein, satisfactory identification of the correct sequence has been made, the first two residues of which are shown in

Figure 12. The use of MITC in the sequenator will be the subject of a subsequent communication.

We have confirmed and extended Laursen's (1966) observation that MITC may be substituted for PITC in the Edman degradation. The major advantage which accrues is the ease of quantitative separation of the MTH derivatives of all the amino acids by gas–liquid partition chromatography. This gives increased confidence in the interpretation of the expected stepwise decrease of yield in the Edman degradation as well as making possible the analysis of mixed sequences.

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Perturbations of the Proton Magnetic Resonance Spectra of Conalbumin and Siderophilin as a Result of Binding Ga^{3+} or Fe^{3+}*

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ABSTRACT: Conalbumin binds specifically two Ga^{3+} per protein molecule with the ionization of four tyrosyl side chains, resulting in a shift to higher field of the aromatic region of the proton magnetic resonance spectrum. In contrast, binding of Fe^{3+} to conalbumin or siderophilin (transferrin) results in a decrease in proton magnetic resonance signal intensity in the aromatic region of the protein. This finding is consistent with the paramagnetic broadening or shift of the resonance of tyrosyl side chains specifically coordinated to high-spin Fe(III).

The hypothesis that phenolate side chains of certain tyrosyl residues serve as ligands in iron binding proteins such as conalbumin (Warner and Weber, 1953) from hen's egg white and siderophilin (transferrin) from human blood plasma (Hazen, 1962) has been substantiated by spectral (Wishnia *et al.*, 1961; Hazen, 1962; Tan and Woodworth, 1969a) and chemical modification studies (Line *et al.*, 1967; Komatsu and Feeney, 1967). As another probe of the effect of metal binding on these proteins, we have studied the interaction of conalbumin with Ga^{3+} and Fe^{3+} and of siderophilin with Fe^{3+} by proton magnetic resonance. The binding of Ga^{3+} results in shifts to high field of the resonances of aromatic protons in the bonding tyrosyl residues, whereas the binding of paramagnetic Fe^{3+} results in loss of signal intensity in the aromatic region owing to broadening or large shifts of the bonding tyrosyl signals.

Materials and Methods

Chemicals were reagent grade and were used without further purification. Deionized water was used to make all solutions and dilutions. Glassware was freed of iron by soaking in 1 mM EDTA—0.01 M sodium acetate (pH 4.5), then rinsing with deionized water. Standard iron solutions (0.020 M) were made by dissolving $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in

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