Sequential Degradation of Peptides with an Insoluble Edman Reagent*

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ABSTRACT: An insoluble Edman reagent, analogous to phenyl isothiocyanate, has been synthesized from polystyrene beads (cross-linked with 0.25% divinylbenzene) by nitration, reduction, and reaction with carbon disulfide and ethyl chloroformate. To enable the NCS–polystyrene to swell in polar solvents, it was essential to reduce its hydrophobicity by coupling 60% of the NCS groups to glucosaminol. The resulting resin, which swells extensively both in a solvent containing 20% water, 75% pyridine, and 5% *N*-ethylmorpholine (used for addition of peptide to resin) and in a mixture of 80% trifluoroacetic acid and 20% acetic acid (used for cleavage), has been used to degrade several peptides sequentially from their amino termini, for a maximum of 10 cycles. Recoveries of degraded peptide average about 75% per stage. This relatively poor recovery is primarily responsible for the current restriction of the method to peptides of moderate size. Degradations have been followed by determining the amino acid composition of the peptide after each cycle. The results are unambiguous even after many cycles have been performed since the terminal amino acid is lost after each degradation. One complete cycle takes about 2 hr and several peptides can be degraded in tandem very easily.

The procedure for sequential degradation of peptides with phenyl isothiocyanate, originally described by Edman (1950) and extensively modified and improved by Edman and his coworkers (Edman, 1956, 1963; Ilse and Edman, 1963; Edman and Begg, 1967) and by others (Konigsberg, 1967; Gray, 1967; Schroeder, 1967), has been the major chemical technique responsible for the elucidation of a very large number of protein sequences during recent years (Dayhoff and Eck, 1968). Several years ago, when the work reported in the present communication was initiated, the procedures available for sequential degradation of peptides were laborious and too often gave equivocal or incorrect results. We decided to attempt to simplify, speed up, and improve the reliability of the Edman procedure by making the reagent insoluble. During the course of our work, procedures for rendering peptides insoluble during the course of degradation have been developed (Laursen, 1966; Waterfield *et al.*, 1968) and an automatic apparatus for degrading proteins has been developed by Edman and Begg (1967) and extended to the degradation of peptides by Niall *et al.* (1969). Our procedure, in its present state of development, seems especially well suited for rapid, unambiguous, and complete sequential degradation of peptides of moderate size by manual methods when 0.5–1 μmole of peptide is available. Improvements in the recovery of degraded peptide after each cycle would extend considerably both the sensitivity and the scope of the method.

Preparation of the Reagent

The preparation is illustrated schematically in Figure 1.

Nitropolystyrene. Polystyrene beads cross-linked with 0.25% divinylbenzene (no. L982) were obtained from Sondell Scientific Instruments, Palo Alto, Calif. We have used the fraction which passes through a 100-mesh screen when dry. Wash the beads several times with chloroform to remove residual linear polystyrene, then dry them in a hood after most of the solvent has been removed by suction filtration on a coarse sintered-glass funnel. Nitration and reduction are carried out as first described by Chen (1955). With rapid mechanical stirring, add 40 g of resin slowly, over a period of about 30 min, to about 1.5 l. of fuming nitric acid (specific gravity 1.5) precooled in an ice–water bath. Do not allow the temperature of the reaction mixture to rise above about 15°. As nitration proceeds, the resin will swell greatly. If stirring becomes too difficult, add small amounts of concentrated nitric acid. About 30 min after the last of the polystyrene has been added, remove the stirrer and allow the reaction mixture to stand overnight at about 4°, to complete the nitration. Pour the reaction mixture slowly, with vigorous mechanical stirring, into an ice–water slurry, adding more ice as required (use a 4-l. beaker). Filter the resin on a coarse sintered-glass funnel and wash it five or six times with distilled water. Divide the damp nitropolystyrene into halves; store one-half at 4° for a future preparation and use the other for the remainder of the reactions described below.

Aminopolystyrene. Wash the nitro resin on the sintered-glass funnel several times with reagent dimethylformamide. The beads should collapse initially, then swell. Be sure that there are no clumps of unswollen resin remaining and that all of the beads are transparent before proceeding with the reduction. Transfer the resin to a 2-l. erlenmeyer flask in a total volume of about 1 l. of dimethylformamide. Heat the suspension to 100° with stirring (a water bath atop a stir plate is convenient), and then add to it a solution of 200 g of

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$SnCl_2 \cdot 2H_2O$ in 400–500 ml of dimethylformamide. Allow the mixture to stir at 100° for about 6 hr. Add 200 ml of 12 M HCl and continue to stir at 100° for about 6 hr more.

NCS-Polystyrene. The procedure for converting amino groups into NCS groups with CS_2, triethylamine, and ethyl chloroformate is adapted from Garmaise *et al.* (1958). Filter the resin, using a sintered-glass funnel, and wash it alternately with dimethylformamide and 6 M HCl, six times each. The beads should collapse in the HCl and swell again in dimethylformamide. If they do not swell very much, add a little 6 M HCl. Finish the washes with dimethylformamide and, this time, allow the beads to collapse partially. Add to the resin a mixture of dimethylformamide and about 40 ml of triethylamine (redistilled from ninhydrin). Take about 10 min to break up the clumps of resin as well as possible with a large spatula, to ensure complete neutralization. Filter, then wash the resin with dimethylformamide once or twice, to remove excess triethylamine and to aid in dispersing the beads. Transfer the slurry of resin to a 2-l. three-necked flask in a total volume of about 1.2 l. of dimethylformamide. Cool the flask in ice–water and, with rapid mechanical stirring, add dropwise and simultaneously 40 ml of CS_2 (purified according to Leonis and Levy (1954) and stored in a freezer) and 90 ml of redistilled triethylamine. Remove the ice, fill the flask almost to capacity with dimethylformamide, and allow the reaction mixture to warm up to room temperature over a period of about 4 hr, with continued rapid stirring. Cool the flask in ice–water again and add 85 ml of ethyl chloroformate (redistilled and stored in the freezer) dropwise with stirring. Remove the ice–water and stir the reaction mixture slowly at room temperature overnight. Filter the resin and wash it with chloroform several times, until all visible triethylammonium chloride has been removed, then two or three times with pyridine (redistilled from ninhydrin and stored at 4°).

Glucosaminol–NCS–Polystyrene. Following the final wash, allow the resin to remain on the filter with suction for about 5 min after the bulk of the solvent has passed through. Remove small portions of the damp resin for determination of capacity (see below) and, without delay, weigh all of the remainder into a 2-l. erlenmeyer flask. Add about 1.2 l. of pyridine and stir the resin with cooling for several hours while the determination of capacity is being completed, to disperse the beads as well as possible. Add all at once 75 ml of *N*-ethylmorpholine (redistilled from ninhydrin and stored in the freezer), then add a solution of glucosaminol hydrochloride in water dropwise with rapid stirring over a period of about 10 min, with enough cooling to prevent the temperature from rising above about 25°. The volume of this solution in milliliters should be about twice the weight of glucosaminol hydrochloride in grams. The number of millimoles of glucosaminol added should be 0.60 times the total number of milliequivalents of NCS, determined in the capacity experiment. After the glucosaminol has been added, continue to stir the solution for 1.5 hr at room temperature. Add dropwise, but rapidly, 60 ml of triethylamine (redistilled) and, after 15 min, filter the resin and wash it immediately three times with pyridine. Store the resin under pyridine at 4°. The capacity of the glucosaminol–NCS–polystyrene can bed etermined as described below, but remember to use about three times as much glucosaminol–NCS–resin as NCS–resin, since the capacity will be lower.

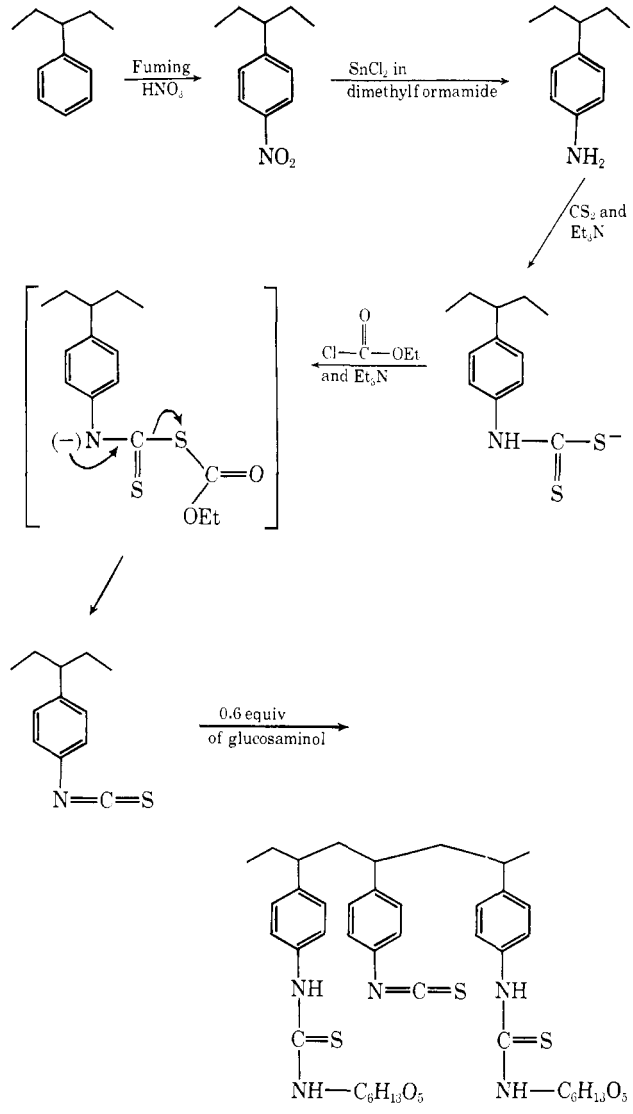

FIGURE 1: Scheme for the synthesis of glucosaminol–NCS–polystyrene.

Determination of the Capacity of NCS–Polystyrene. Place about 1 g of damp resin into each of three tared 25-ml erlenmeyer flasks and reweigh the flasks to determine the exact weight of the resin. Dry one of the samples in a vacuum desiccator over H_2SO_4 for several hours, using an oil pump, then reweigh the flask to determine the dry weight of the resin. To each of the other two flasks, add 10.0 ml of a mixture of nine volumes of pyridine and one volume of triethylamine, then 1.0 ml of 1.50 M glycine ethyl ester hydrochloride. Agitate the mixtures for several hours at room temperature along with a nonresin blank, dilute the supernatant solutions 1:500, and determine the amount of ninhydrin-positive material remaining in each. Calculate the capacity of the dry resin in milliequivalents per gram, including a correction for the volume of pyridine introduced along with the resin.

Glucosaminol Hydrochloride. Dissolve 50 g of commercially available glucosamine hydrochloride in 150 ml of water and, with cooling, adjust the pH to 10 with concentrated NaOH. Add 0.08 mole of solid $NaBH_4$ in small portions at room

TABLE I: Degradation of Thr-Val-Gln-Val-Arg.[a]

Amino Acid	Molar Ratios of Amino Acids					
	Theory	Initial Peptide	Round 1	2	3	4
Arg	1	*b*	*1.0*	*1.0*	*1.0*	*1.0*[c]
Thr	1	*b*	**0.1**	0		
Glu	1	*b*	1.0	0.9	**0.1**	0
Val	2	*b*	1.9	**1.2**	0.9	**0**
Yield, %			80	73	66	56

[a] This legend is for Tables I–IV. In each table, the number used as the basis of the calculation of molar ratios is italicized. The residue that should be removed in each round is in boldface type. [b] Not analyzed. [c] No hydrolysis.

TABLE II: Degradation of Thr-Val-Val-Gln-Arg (Tryptic Peptide 2 of Tobacco Mosaic Virus Protein).

Amino Acid	Molar Ratios of Amino Acids					
	Theory	Initial Peptide	Round 1	2	3	4
Arg	1	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*[a]
Thr	1	1.0	**0.1**	0		
Glu	1	1.0	1.0	1.0	1.1	**0**
Val[b]	2	1.8	1.5	**1.2**	**0.2**	0
Yield, %			84	75	87	60

[a] No hydrolysis. [b] In several experiments, ratios similar to those given in the table were obtained for the initial peptide upon hydrolysis for 16 hr at 110° in 6 M HCl, but only one residue of valine was present after round 1. In the experiments reported in the table, hydrolyses were carried out for 37 hr after rounds 1, 2, and 3. The sequence then becomes clear, since only valine decreases in rounds 2 and 3. It appears that the Val-Val sequence is much easier to hydrolyze in the initial peptide, where it is preceded by Thr, than after removal of the threonine in round 1.

temperature with rapid stirring. One hour after the addition of $NaBH_4$ has been completed adjust the pH to about 2 with concentrated HCl and evaporate the mixture under a vacuum until solid begins to separate. Add enough water to redissolve the solid, then precipitate the crude glucosaminol hydrochloride with nine volumes of alcohol. Recrystallize it from alcohol–water (final yield about 35 g).

Degradation of Peptides

Immediately before use, pipet the resin in pyridine suspension into a small jacketed tube, thermostated at 50°, about 0.6 cm i.d. A porous Teflon disk, which is easily removed after use is very convenient for closing the bottom of the tube. Wash the resin with addition solvent (75 volumes of pyridine, 5 volumes of *N*-ethylmorpholine, and 20 volumes of glass-distilled water) and adjust the height of the bed to about 5 cm. Dissolve the dry peptide in no more than 200 μl of addition solvent and pipet as much of the solution as possible onto the column of resin. (If the peptide contains lysine, it must be carried through one degradation with soluble reagent first, to block the ϵ-NH_2 group.) Allow the sample to flow into the resin by gravity or use gentle nitrogen pressure, then wash the remaining peptide onto the resin with two 100-μl portions of addition solvent. After 1 hr, wash the resin, first two or three times with addition solvent, then thoroughly with tetrahydrofuran, using nitrogen pressure to drive the solvents through rapidly. Stir the resin–tetrahydrofuran suspension with a glass rod to ensure that no regions of addition solvent remain through channeling. The same rod can be reinserted into the damp bed of resin and used to stir it again during cleavage. After the bulk of tetrahydrofuran has been driven off, place a clean heavy-walled glass tube under the column, then add about 1.5 ml of cleavage solvent (4 volumes of trifluoracetic acid and 1 volume of acetic acid, each redistilled), stir to suspend the resin and blow the solvent into the tube with nitrogen. Resuspend the resin in about 1.5 ml more of cleavage solvent and allow the reaction to proceed for a total of 30 min at 50°. Blow the solvent into the tube, wash the resin three times with more solvent, and evaporate all to dryness, using an oil pump. Rotation during this procedure prevents bumping, and a warm-water jacket hastens evaporation. Redissolve the residue left in the tube in a known volume of addition solvent and remove a small aliquot for hydrolysis and amino acid analysis; place the remainder on a fresh column of resin for the next round of degradation. One complete cycle takes about 2 hr. It is convenient to carry out four cycles on a single peptide in a normal working day and it is easy to degrade several peptides in tandem. The resin is not used more than once: only about 1.5 ml of resin is required for a single degradation and the laboratory-scale preparation described above, which yields about 1 l., suffices for about 600 experiments.

Results and Discussion

Degradations of Peptides of Known Structure. The use of the method is illustrated in Tables I–IV. The peptides represented in Tables I and II were very kindly provided by Dr. Janis D. Young. They were synthesized to clear-up an ambiguity in the sequence of tryptic peptide 2 from tobacco mosaic virus protein, since this peptide had proved difficult to degrade with phenyl isothiocyanate in solution (Young *et al.*, 1968). As can be seen from the results of Tables I and II, the sequences of these peptides can be determined unambiguously by degradation with glucosaminol–NCS–polystyrene; tryptic peptide 2 is Thr-Val-Val-Gln-Arg. In Table III is shown the degradation of an octapeptide, derived from bovine insulin, in which the ϵ-amino group has been blocked by a phenylthiocarbamyl moiety. The peptide was prepared by blocking the free amino groups of insulin with phenyl isothiocyanate in solution, then cleaving at the single arginine residue with trypsin. The peptide was easily purified on Sephadex G-25 in 50% acetic acid. In the case of the dodecapeptide repre-

TABLE III: Degradation of Gly-Phe-Phe-Tyr-Thr-Pro-PTC-Lys[a]-Ala.

	Molar Ratios of Amino Acids								
			Round						
Amino Acid	Theory	Initial Peptide	1	2	3	4	5	6	7
Lys	0.7[b]	0.6	0.3	1.0	1.0	1.0	1.4	2.1[c]	**0.3**[c]
Thr	1	0.9	0.9	0.8	1.1	0.8	**0**		
Pro	1	1.0	1.0	1.0	0.9	0.7	0.9	**0**	
Gly	1	1.0	**0.1**	0					
Ala	1	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*[d]
Tyr	1	0.9	0.8	1.0	1.0	**0**			
Phe	2	2.1	1.9	**1.2**	**0**				
Yield, %			66	85	54	76	81	60	95

[a] Abbreviation: PTC-Lys, ϵ-phenylthiocarbamyllysine. [b] Only 0.7 residue of lysine is formed from PTC-Lys under the hydrolysis conditions used; see the text. [c] A minor contaminant, derived from the resin, cochromatographs with lysine on the amino acid analyzer and obscures the analyses for this amino acid in rounds 6 and 7. However, it is clear that lysine is penultimate since only alanine and no PTC-Lys is present *without hydrolysis* following round 7. [d] No hydrolysis.

TABLE IV: Degradation of Thr-Thr-Gly-Gly-Gly-Asn-Pro-Val-Gly-Asp-Lys-Leu.

			Molar Ratios of Amino Acids										
			Round										
Amino Acid	Theory	Initial Peptide[a]	1[b]	2	3	4	5	6	7	8	9	10	11
Lys	1	0.9	1.0	*c*	*c*	1.0	1.2	*c*	1.0	1.5	*c*	*c*	*c, d*
Asp[e]	2	1.8	2.0	2.3	2.2	2.0	2.0	**1.2**	1.1	1.7[f]	1.0	**0.3**	0
Thr	2	1.4	**1.0**	**0.3**	0.2	0							
Pro	1	0.9	0.9	1.2	1.3	0.9	0.9	0.8	**0**				
Gly	4	3.7	3.5	3.8	**3.0**	**2.1**	**1.5**	1.6	1.2	1.7[f]	**0.5**	0.4	0
Val	1	1.0	0.9	0.9	0.9	1.0	1.1	0.8	1.1	**0.2**	0		
Leu	1	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*[d]
Yield, %			100	18[g]	66	80	65	74	90	86	43	33	98
Amount hydrolyzed, %			2.5	2.5	2.5	5.0	5.0	7.5	10	15	20	40	*d*

[a] This analysis of the initial peptide was made shortly after isolation (November, 1965) by Dr. Walter Schroeder. Our own analysis differed by less than 0.1 residue for every amino acid *except* threonine, which had decreased to 1.1 residues. [b] This round was carried out with phenyl isothiocyanate in solution; the yield at this stage is defined as 100%. Peptide (3.2 μmoles) was put onto the resin for round 2. [c] Not analyzed; lysine values are somewhat high due to an artifact from the resin. [d] No hydrolysis. [e] No attempt was made to distinguish between Asp and Asn. [f] This particular sample was contaminated by appreciable amounts of serine and alanine, which are not present in the peptide. The high values for aspartic acid and glycine probably reflect the contamination. [g] The low yield at this point probably reflects the low value for threonine in the initial peptide; see the text and footnote *a*.

sented in Table IV, a single cycle of degradation was performed with soluble phenyl isothiocyanate first, then ten cycles more were performed with glucosaminol–NCS–polystyrene. (See below for a more complete discussion of methods for degrading peptides which contain lysine.) The dodecapeptide, obtained from a chymotryptic digest of bovine catalase, was very kindly provided by Dr. Walter Schroeder. In order to test the method critically, we determined the sequence independently, then learned from Dr. Schroeder that our results and his were in agreement. The placement of amides is, however, from Dr. Schroeder's data, not ours.

The degradations represented in Tables I–IV were followed by determining the amino acid composition of the peptide after each cycle of reactions. A typical protocol for removing aliquots, designed to compensate for the steadily decreasing amount of peptide remaining, is illustrated at the bottom of Table IV. Amino acid analyses were performed as described previously (Stark, 1968), with a Spinco instrument modified

for high sensitivity, so that less than 5 mμmoles of a single amino acid could be estimated accurately. Note that the apparent recovery of lysine is consistently somewhat high in Tables III and IV. This artifact is due to some unknown material which is eluted from the resin in small amounts during degradation and which cochromatographs with lysine on the amino acid analyzer at pH 5.28, even on a 59-cm column. The artifact is not particularly troublesome unless a large fraction of the peptide is taken for analysis, as might occur near the end of a degradation.

An alternative and even more sensitive method for following the course of degradations with glucosaminol–NCS–polystyrene is the procedure described by Gray (1967), in which the end group of the peptide is determined after each cycle, using the fluorescent reagent 1-dimethylaminonaphthalene-5-sulfonyl chloride. Interpretation of such end-group determinations should be particularly clear in conjunction with degradation by the resin, since removal of the terminal residue is virtually complete each time, even after many cycles.

Apart from speed and convenience, this completeness of the degradation is one of the principal advantages of the method. Side reactions or incomplete degradation can lead to a reduction in yield with the resin, but should not lead to contamination of the degraded peptide, because there is a selection for the desired material at two stages during each degradation. First, during the addition reaction, any compound that lacks a free amino group is not held covalently by the resin and will be washed away before the degraded peptide is released by trifluoroacetic acid. For example, if a peptide with a blocked amino group is present as a contaminant, it will be separated from peptides that do have free amino groups during the first step because it cannot add to the resin. Such a situation is sometimes encountered when a residue of glutamine is reached in degradations with soluble phenyl isothiocyanate, because glutamine cyclizes readily to pyrrolidonecarboxylic acid, which has no free amino group, under the alkaline conditions required for the addition reaction to occur. A second purification occurs during the exposure of the resin to trifluoroacetic acid, for no compound that has added to the resin will be released unless it is a peptide. For example, free amino acids would be removed from a peptide undergoing degradation. Also, if the cleavage reaction is not complete, it is obvious that the uncleaved peptide will not be released and will not contaminate the product, as it would if the degradation were being carried out with soluble reagents.

Recoveries of Peptides. The major limitation of the method is the failure to achieve complete addition of peptide to resin each time. The average recovery of peptide after each cycle varies from 69 to 78% in the degradations of Tables I–IV. Although there is room for substantial improvement in this aspect, the procedure in its present state of development is useful for degradation of peptides of about ten residues or less. The details are presented now not because we think that the recoveries cannot be improved further, but rather because we hope that other investigators will find ways to improve them during the course of their own experiments.

In an experiment to determine the rate and extent of addition of a peptide to the resin, octapeptide (Table III) was shaken in a test tube with an excess of resin under nitrogen at 37°. Hydrolysis and analysis of portions of the supernatant solution revealed that 60% of the peptide had added after 30 min, 80% after 3 hr, still 80% after 5.5 hr. Most of the small amount of peptide that fails to add to the resin after such extended reaction is incapable of adding if reapplied to a fresh portion of resin. The amino acid analysis of the material that does not add is usually somewhat low in the amino-terminal residue. An example is provided by an experiment with aspartylalanine: 5% of the peptide placed on a column was represented in a wash of the column with addition solvent after 15 min at 50°, judged by the recovery of alanine after acid hydrolysis; only 0.8 mole of aspartic acid was recovered per mole of alanine. However, there was no free peptide in this wash, judged by direct chromatography on the amino acid analyzer in comparison with a sample of the parent peptide. The implication is strong that some side reaction, the nature of which is not known at present, competes with reaction of the resin-bound NCS groups for the free amino group of the peptide. In the preparation of reagents for the sequenator, Edman and Begg (1967) stress that aldehydes must be scrupulously avoided, since they are capable of competing with isothiocyanates for the amino groups of peptides. We prepared addition solvent, scrupulously free of aldehydes according to the Tollen's test proposed by Edman and Begg (1967), but failed to achieve appreciably better recoveries of peptides than were obtained with the usual solvent, prepared as described above. Carbon dioxide, which has been shown to reduce the yield in the reaction of peptides with *N*-carboxyanhydrides of amino acids (Hirschmann *et al.*, 1967), is not responsible, since rigorous exclusion of CO_2 from the addition solvent does not improve the yields. Similarly, carbon oxysulfide, which might arise by hydrolysis of NCS groups, is not responsible, since an acid wash of the resin immediately before a degradation, which would be expected to decompose resin-bound NH—C(=O)—S⁻ groups, does not improve the recovery.

Recovery does appear to be correlated somewhat with the nature of the terminal residue; it seems somewhat worse when glycine or a polar residue such aspartic acid is terminal than when an apolar residue, such as phenylalanine, is terminal. Also, recoveries are not as good with large peptides: in attempts to degrade the oxidized phenylalanyl chain of insulin (30 residues), porcine glucagon (29 residues), or tryptic peptide 16 from oxidized ribonuclease (20 residues), average yields of only about 50% were obtained at each stage. It can be calculated that 20 g of polystyrene gives rise to approximately 55 g of glucosaminol–NCS–polystyrene. This much resin occupies about 900 ml in addition solvent; *i.e.*, about 95% of the swollen resin is liquid. It does not seem reasonable that even a large peptide should be prevented sterically from penetrating the resin beads.

Since the total of unadded peptide and peptide released by trifluoroacetic acid was typically very close to 100% in many experiments, peptide that has added to glucosaminol–NCS–polystyrene is released completely during cleavage. Some precautions are taken to exclude oxygen, but oxidative desulfuration (Ilse and Edman, 1963) is probably not a serious problem with the resin method, since loss of sulfur from the resin-bound phenylthiocarbamyl peptide would be expected to reduce the yield in the cleavage step. Probably small amounts of oxygen are effectively removed from solution

by the large excess of resin-bound NHC(=S)–glucosaminol groups.

Degradation of Peptides That Contain Lysine. Unless a single lysine residue is amino terminal, reaction of an ε-amino group with an NCS group of glucosaminol–NCS–polystyrene will cause a peptide to bind covalently to the resin in a manner not susceptible to cleavage by trifluoroacetic acid. In the degradation of Table IV, the ε-amino group of lysine was blocked by carrying out one round of reactions with soluble phenyl isothiocyanate, essentially as described by Konigsberg and Hill (1962) and summarized by Konigsberg (1967), including the precaution of flushing the reaction vessel and reagents with nitrogen, to avoid oxidative desulfuration (Ilse and Edman, 1963). We use triethylamine–water–pyridine as the solvent for this addition reaction, to achieve a pH high enough to assure extensive reaction of the ε-amino groups. Following cleavage, the entire reaction mixture is dissolved in the addition solvent for resin degradation and, after an aliquot has been removed for acid hydrolysis, applied directly to a column of resin. The recovery after the first cycle with the resin may reflect several different difficulties: impurity of the initial peptide, inefficient removal of the terminal residue in the cycle with soluble phenyl isothiocyanate, or poor recovery in the cycle with the resin. In the case of the dodecapeptide of Table IV, the low yield in the first cycle with resin (round 2) is probably a result of the same thing which causes threonine to be low in an acid hydrolysate of the original peptide. That is, a substantial fraction of the original preparation may have an altered amino-terminal residue, which does not react with soluble phenyl isothiocyanate. In general, recoveries are usually somewhat low following soluble rounds, but not strikingly so.

ε-Phenylthiocarbamyllysine, prepared from alanyllysine, chromatographs about 9 ml beyond phenylalanine on the 10-cm column of the amino acid analyzer at pH 5.28; on the same column at pH 4.25, the peak emerges at 63 ml. Lysine was recovered in 71% yield when ε-phenylthiocarbamyllysine was hydrolyzed for 20 hr at 110° in 6 M HCl. The ε-phenylthiocarbamyl group is apparently stable throughout degradations with glucosaminol–NCS–polystyrene, since the recoveries are as high for peptides which contain it as they are for peptides which do not, and since no appreciable amount of ε-phenyl thiocarbamyl peptide is irreversibly bound to the resin following cleavage with trifluoroacetic acid.

Digestion with carboxypeptidase B is an alternative procedure for preparing tryptic peptides for resin degradation, since such peptides are known to have their lysine residues at the carboxyl termini. No purification of the peptide should be necessary after the enzymatic digestion, since any free lysine produced, as well as any unreacted peptide, will be bound irreversibly to the resin through ε-amino groups.

Determination of Amides. We have not attempted to distinguish between glutamic or aspartic acid and their amides. However, several methods for determining amides in sequence are compatible with degradation of peptides by glucosaminol–NCS–polystyrene, for example, electrophoresis of dansyl peptides (Gray, 1967), hydrolysis with aminopeptidase M (Pfleiderer and Celliers, 1963; see also Hofmann *et al.*, 1966) followed by direct amino acid analysis for amides in the system described by Benson *et al.* (1967), or modification of the free carboxyl groups of the peptide with an amino acid ester and water-soluble carbodiimide (Hoare and Koshland, 1967). In the last case, loss of one residue of the modifying amino acid ester would be expected to accompany loss of each residue of aspartic or glutamic originally present as the free acid.

Properties of the Resin. Polystyrene was chosen as the supporting matrix for NCS groups because of its resistance to chemical degradation and because it is readily available in stable bead form with a low degree of cross-linking, for maximum swelling. Some experiments were attempted with NCS-Sephadex, prepared according to Axén and Porath (1966), but much of this extremely hydrophilic reagent became soluble in trifluoroacetic acid. The rate of addition of peptide to NCS-Sephadex was much slower than with glucosaminol–NCS–polystyrene and the capacity of the Sephadex was four to five times lower, even after allowing for coupling of 60% of the groups originally present in NCS–polystyrene to glucosaminol. The degree of cross-linking chosen for glucosaminol–NCS–polystyrene, 0.25%, is the lowest degree compatible with physical stability: several experiments were attempted with 0.1% cross-linked polystyrene, but most of the beads disintegrated during synthesis and the rather mushy final resin was extremely difficult to work with in small columns. Some successful experiments were carried out with 0.25% cross-linked beads initially 25 μ in diameter, but these seemed to have no particular advantages over the larger beads recommended above (minus 100 mesh; *i.e.*, smaller than 150 μ) and were much more difficult to use.

Analytical values for nitrogen were, within experimental error, equal to theory for several different preparations of nitro- and aminopolystyrene. The washing procedure that follows reduction is very effective in removing tin from the resin: one preparation of glucosaminol–NCS–polystyrene contained only 0.21% ash after combustion; most of the ash was probably NaCl, since NaOH was used to neutralize the amino resin in this particular preparation. If all of the ash were SnO_2, the resin would have contained a maximum of 0.17% tin.

The capacity of a typical preparation of NCS–polystyrene was 5.4 mequiv/g (theory is 6.2). Since it is not unlikely that a small amount of triethylammonium chloride remains trapped in the resin following the chloroform washes, the actual capacity is probably somewhat higher. Note that residual salt does not affect the calculation of total capacity, necessary for accurate an estimation of the amount of glucosaminol to be added. Glycine ethyl ester was chosen for use in the determination of capacity because it is a small molecule and uncharged at alkaline pH. Use of an amino acid rather than an ester would have given a thiourea–resin with a high density of negative charge, which would have repelled additional molecules of amino acid, also negatively charged in the presence of triethylamine, resulting in a low estimate of capacity. Glucosaminol was chosen over glucosamine because of the observation (Edman and Begg, 1967) that aldehydes are deleterious since they can react with the terminal amino group of the peptide. However, no great difference was apparent when resins prepared with glucosamine were compared with resins prepared with glucosaminol.

The manner in which glucosaminol is added is the most crucial step in the preparation of the final resin. If glucosaminol hydrochloride in water is added to a stirred suspension of resin in a mixture of pyridine and triethylamine, reaction of glucosaminol with resin is complete in less than

15 min at room temperature, but the product, although similar in appearance to resin prepared by slow addition of glucosaminol in the presence of *N*-ethylmorpholine, is very different in its properties. For example, glucosaminol (triethylamine) resin will not react with an appreciable amount of peptide in a solvent containing 20% water, whereas glucosaminol (*N*-ethylmorpholine) resin reacts with peptides readily under the same conditions. The glucosaminol (triethylamine) resin does react with some peptides when less water is present. The most probable explanation of the difference is that when glucosaminol is present as the free base (as in the presence of excess triethylamine), its rate of reaction with resin-bound NCS groups must be fast relative to its rate of diffusion into the resin beads. The result is a bead which has predominantly NHC(=S)–glucosaminol groups near the surface, and predominantly NCS groups near the center. In polar solvents containing 20% water, the peptide cannot penetrate the hydrophobic interior of the bead, where the NCS groups are to be found, although it probably does penetrate the more hydrophilic exterior. When *N*-ethylmorpholine replaces triethylamine, the glucosaminol is present predominantly as the hydrochloride, slowing the reaction with NCS groups relative to diffusion and permitting a much more even distribution of NCS groups throughout the bead, so that a greater proportion of them are near the hydrophilic exterior. In the preparation described above, only about 60% of the glucosaminol has reacted with NCS groups after 1.5 hr at room temperature in the presence of *N*-ethylmorpholine. Triethylamine is added at this point to drive the addition to completion, because prolonged exposure of NCS groups to alkaline conditions in the presence of water results in hydrolysis (Drobnica and Augustin, 1965) and loss of capacity. It is probable that the properties of glucosaminol–NCS–polystyrene could be improved further by careful control of the mode of addition of glucosaminol.

In two preparations of glucosaminol–NCS–polystyrene, addition of 0.55 or 0.65 equiv of glucosaminol per NCS group resulted in resins having a final capacity of 1.5 or 0.9 mequiv per g, respectively. These resin differs little in their physical properties (they both remained swollen during addition and during cleavage) or in the recoveries of peptides obtained. Hence, addition of 0.60 equiv of glucosaminol, an intermediate amount, is recommended.

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