High Sensitivity Amino Acid Sequence Determination. Application to Proteins Eluted from Polyacrylamide Gels†

John Bridgen

ABSTRACT: An automatic solid-phase procedure is described for determining the amino-terminal amino acid sequence of very small quantities of proteins. The sample is covalently attached to an inert support so that mechanical and physical losses during sequencing are eliminated. High sensitivity is achieved by using an initial coupling with high specific activity phenyl [^{35}S]isothiocyanate followed by a longer reaction with the unlabeled reagent. The radioactive phenylthiohydantoins are identified by autoradiography after two-dimensional thin-layer chromatography. Unlabeled phenylthiohydantoin-amino acids are added to each fraction to assist in the identification and to act as carriers, hence reducing absorptive and extractive losses of the small quantities of sample. The method may be used on proteins eluted from polyacrylamide gels containing sodium dodecyl sulfate without removal of the detergent. Sequences of up to 20 residues have been obtained on quantities of protein ranging from 2.5 to 70 pmol. Results from proteins of hitherto unknown sequence are included.

The limited availability of many proteins of biological interest has necessitated the development of new highly sensitive techniques for determining their amino acid sequence. Most of these methods (e.g., Niall et al., 1974; McKean et al., 1974; Jacobs and Niall, 1975) depend for their sensitivity on the identification of radioactive PTH[1] amino acids released from the protein after degradation in a spinning-cup sequencer. Radioactivity is introduced either by in vivo labeling techniques before sequencing (e.g., McKean et al., 1974) or by the use of a radioactive coupling agent (e.g., Jacobs and Niall, 1975) during sequencing. Unfortunately, this method of sequencing is susceptible to mechanical and chemical losses of protein from the spinning-cup and extended sequences may only be obtained on relatively large (5–15 nmol) amounts of material. Also, small quantities of urea, guanidine, or sodium dodecyl sulfate interfere with the degradation (Niall, 1974) and these, along with other nonvolatile salts, must be removed before sequencing. Weiner et al. (1972) have described a manual-sequencing method based on the dansyl–Edman procedure (Gray, 1967) that is applicable to small quantities of protein eluted directly from sodium dodecyl sulfate–polyacrylamide gels. Although potentially very useful, the technique is tedious to perform and permits only a small number of residues to be identified.

The solid-phase sequencing procedure of Laursen (1971) is an automatic method that has the advantage of complete elimination of mechanical and physical losses during sequencing and is thus very well suited for adaptation to high-sensitivity work. As modified by Laursen et al. (1972), peptide or protein amino groups are cross-linked to the amino groups of an inert support, in this case an amino derivative of porous glass (Machleidt et al., 1973; Bridgen, 1975) using the bifunctional reagent, phenylene diisothiocyanate. In this paper I would like to describe an automatic solid-phase method for sequencing picomolar quantities of protein. The method is shown to be applicable to proteins dissolved in sodium dodecyl sulfate, 6 M guanidine hydrochloride, or aqueous buffer, as well as to proteins eluted directly from sodium dodecyl sulfate gels. By using a brief initial coupling with high specific activity [^{35}S]PITC, followed by an exhaustive coupling with the unlabeled reagent, sequences of up to 20 residues have been obtained on as little as 70 pmol of protein.

Materials and Methods

Sequencing reagents, dimethylformamide, *N*-methylmorpholine (all Sequenal grade), 3-aminopropyltriethoxysilane, *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane, and PTH-amino acid standards were obtained from Pierce Chemical Co. 0.4 M *N,N*-dimethyl-*N*-allylamine buffer in pyridine–water (3:2, v/v) buffered to pH 9.5 with trifluoracetic acid was purchased ready made from Pierce. [^{35}S]PITC (200 mCi/mmol in acetonitrile) was obtained from the Radiochemical Centre, Amersham, U.K., and stored at −20 °C. Porous glass (75 and 125 Å pore diameter, 200–400 mesh) was from Electro-Nucleonics, N.J. Phenylene diisothiocyanate (recrystallized from acetone) was from Eastman. Polyamide TLC plates were obtained from Analtech (ML 1010) and from Schleicher & Schuell, Dassel, W. Germany (F 1700), and were cut into 5 × 5 cm squares before use.

Lysozyme (hen egg-white) was from Sigma. Methionine tRNA synthetase (*B. stearothermophilus*) was a gift from Dr. R. Mulvey. Superoxide dismutase (*B. stearothermophilus*) was a gift from Dr. J. I. Harris. Trypanosome antigens (*Trypanosoma brucei*) were gifts from Dr. G. Cross and Dr. P. J. Beresford.

Acrylamide and *N,N'*-methylenebisacrylamide (electrophoresis grade), sodium dodecyl sulfate (specially purified grade), and guanidine hydrochloride (Aristar grade) were from BDH Chemicals Ltd., Poole, Dorset, U.K.

Proteins were oxidized according to Hirs (1967) or *S*-[^{14}C]carboxymethylated according to Kolb et al. (1974).

Polyacrylamide Gel Electrophoresis. Slab gels of average thickness 1–2 mm were prepared using the phosphate buffer system of Weber and Osborn (1969). After polymerization the polyacrylamide slab was removed from the gel plates and soaked overnight in the running buffer. The gel was then reconstituted into the supporting plates prior to electrophoresis. Protein samples (normally 5–25 μg) were boiled in loading

† From the Medical Research Council, Laboratory of Molecular Biology, Cambridge CB2 2QH, England. *Received February 3, 1976.*

[1] Abbreviations used are: PTH, phenylthiohydantoin; dansyl, 5-dimethylamino-1-naphthalenesulfonyl; PITC, phenyl isothiocyanate; TLC, thin-layer chromatography.

buffer (Weiner et al., 1972) for 3–4 min and applied to the top of the gel in a volume not exceeding 20 μl. Samples were normally run in duplicate so that one band could be used for detection and the other for sequencing. After electrophoresis at 50 V for 8 h, the gel was sliced longitudinally and the slots carrying the samples for detection were stained with Coomassie brilliant blue (0.2%) for 30 min. Slices, having the same mobility relative to the bromophenol blue marker dye as bands of interest detected on the stained reference gels, were cut from the unstained sample gels and dissected into small cubes approximately 1–2 mm square.

Protein was recovered from the gel by shaking the cubes overnight at 37 °C in 0.5 ml of the 0.4 M dimethylallylamine buffer containing 0.1% sodium dodecyl sulfate. The supernatant was then removed with a hypodermic syringe and passed through a 45-μm Millipore filter to remove small pieces of contaminating polyacrylamide. Elution yields were calculated, for radioactive proteins only, by scintillation counting of a known aliquot of the eluant.

Preparation of Activated Glass. Porous glass was incubated with a 4% (v/v) solution of 3-aminopropyltriethoxysilane or *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane (2–3 times volume of dry glass) in acetone at 45 °C for 18 h (Robinson et al., 1971). The derivatized glass was then filtered and washed alternately with large volumes of acetone and methanol. Excess solvent was removed in vacuo. The amino glass was then added in small quantities to a gently stirred solution of phenylenediisothiocyanate (5 mmol/g of glass) in dimethylformamide. Incubation at room temperature continued for 30 min after all of the amino glass had been added and the isothiocyanate glass was then removed by filtration, washed with several volumes of dimethylformamide followed by methanol, and then dried in vacuo. Activated glass was stored at −20 °C and appeared to be stable for at least 6 months.

Attachment of Proteins. Depending on the solubility of the protein, the dimethylallylamine coupling buffer (see above) was either used directly or made 6 M in guanidine hydrochloride or 1% (w/v) in sodium dodecyl sulfate before adjustment of the pH to 9.5.

The protein was dissolved in the coupling medium (ca. 0.5 ml) and isothiocyanate glass (50–100 mg) added as the dry solid. For proteins eluted from sodium dodecyl sulfate gels, the derivatized glass was added directly to the filtered supernatant obtained after elution. The mixtures were gently stirred at room temperature for 30 min and 50 μl of ethanolamine was then added to block excess isothiocyanate groups. Stirring continued for a further 20 min, the supernatant was then removed by centrifugation, and the protein–resin complex was washed with 3 × 2 ml of methanol before drying under water-pump vacuum (less than 30 cmHg).

Sequence Determination. Amino acid sequences were determined on an Anachem SPA 1200 (Anachem Ltd., Luton, U.K.) solid-phase sequencer using the following reagents and solvents: reagent 1, [^{35}S]PITC, 2% in acetonitrile (1 mCi/ml, 10 000 cpm/nmol); reagent 2, PITC, 5% (v/v) in acetonitrile; reagent 3, sequencing buffer, pH 8.1 (Laursen, 1971); reagent 4, trifluoracetic acid; solvent 1, methanol.

The sequencing program and flow rates are shown in Table I. The specific activity of the [^{35}S]PITC was reduced to 0.5 mCi/ml if more than 1 nmol of protein was being degraded. The following modifications were made to the instrument. The standard reagent 1 bottle was replaced by a 3-ml Reacti-Vial (Pierce) connected via an 8″ length of narrow gauge (0.3-mm internal diameter) Teflon tubing to the reagent pump. The 5-ml syringe of the latter was replaced by an 0.5-ml syringe (Hamilton Gas-Tight) mounted as close as possible to the [^{35}S]PITC delivery valve. A three-way mixing block (Altex 200-22) was connected between the buffer and [^{35}S]PITC lines and the [^{35}S]PITC delivery valve was modified in a manner analogous to that described by Laursen (1971) to allow these reagents to be mixed before reaching the column. The conventional column was replaced by a smaller 10 cm × 3 mm column. Apart from these modifications, the instrument was operated in a manner essentially similar to that described by Laursen (1971) and Laursen et al. (1975).

TABLE I: Program Used for High-Sensitivity Solid-Phase Sequencing.[a]

Function	Program Time (min)	Flow Rate (ml/min)
Methanol	1–2	2.0
Buffer	3–8	0.07
[^{35}S]PITC	9	0.012
Buffer + [^{35}S]PITC	10–17	0.082
Buffer + [^{32}S]PITC	19–31	0.12
Delay	32–41	–
Buffer	42–50	0.07
Methanol	51–70	2.0
Trifluoracetic acid	72–78[b]	0.15
Methanol	82–83	2.0

[a] All program times are inclusive; thus the initial methanol delivery lasts from the start of the cycle to the end of the second minute. [b] The trifluoracetic acid delivery is timed to allow the acid to reach slightly beyond the end of the column. Cleavage then takes place for a further 3 min before the final methanol delivery to fraction collector. Column temperature was 48 °C.

Before sequencing was started, 10 μl of a standard PTH-marker mixture containing all of the conventional PTH-amino acids (100 nmol/ml in ethyl acetate) was added to each fraction collector tube.

Identification of Phenylthiohydantoins. The thiazolinone derivatives were dried at 50 °C under a stream of N_2 and 200 μl of 20% aqueous heptafluorobutyric acid added to each tube. Samples were converted to the PTH derivatives by incubation at 80 °C for 10 min and were then extracted with 2 × 0.7 ml of ethyl acetate. The organic phases were removed and dried under a stream of N_2.

After redissolving in 10 μl of ethyl acetate, the PTH amino acids were identified by two-dimensional chromatography on 5 × 5 cm polyamide layers (Summers et al., 1973) using BBOT(5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene 0.1%, w/v) as fluorescent indicator (Oroszlan et al., 1975). PTH-histidine and PTH-arginine were resolved by redeveloping the plates in the direction of solvent 1 using the solvent system, 1-propanol–formic acid (20:1) (personal communication, P. J. Lowry). Radioactive derivatives were identified by autoradiography using Kodak Autoprocess x-ray film and these were then matched to the unlabeled internal markers by visualization under uv light.

Results

Gel Electrophoresis. Better resolution of the protein bands was obtained using the predialyzed sodium dodecyl sulfate gels (Weiner et al., 1972) and proteins eluted from these gels also gave better coupling yields to the porous glass and cleaner TLC plates after sequencing. The yield of eluted protein was considerably lower from gels where the protein bands had been "fixed" in methanol–acetic acid–water (5:7:88) or stained with Coomassie blue. Protein bands that had not been fixed or

TABLE II: Proteins Sequenced by the High-Sensitivity Solid-Phase Procedure.[a]

Protein	Mol Wt	Quantity	Solvent	Sequence
Lysozyme	14 300	1 μg (70 pmol)	Buffer	VFGRCE$^{L}_{I}$AAAM-RHG$^{L}_{I}$DNY
Lysozyme	14 300	10 μg (700 pmol)	Sodium dodecyl sulfate (eluted from gel)	VFGR-E$^{L}_{I}$AAAM-RHG$^{L}_{I}$DNY
Methionine-tRNA synthetase	65 000	50 μg (750 pmol)	6 M guanidine-HCl	E--TFY$^{L}_{I}$TTP$^{L}_{I}$YYP
Superoxide dismutase	20 000	50 μg (2.5 nmol)	1% Sodium dodecyl sulfate	FE$^{L}_{I}$PA$^{L}_{I}$PYPYDA$^{L}_{I}$EPH$^{L}_{I}$D
Trypanosome antigen I	65 000	10 μg (140 pmol)	Sodium dodecyl sulfate (eluted from gel)	NNHG$^{L}_{I}$-$^{L}_{I}$Q-AEA$^{L}_{I}$C
Trypanosome antigen II	65 000	50 μg (700 pmol)	6 M guanidine HCl	–EA$^{L}_{I}$EY-TWTNHCG

[a] The 1-μg sample of lysozyme and the trypanosome antigens were *S*-[^{14}C]carboxymethylated and the methionine-tRNA synthetase was performic acid oxidized before sequencing. The sequences shown all begin at residue 2, since residue 1 remains attached to the resin and is not identified. The sequences of lysozyme agree with that determined by Canfield (1963). The sequence of methionine-tRNA synthetase agrees with that reported by Mulvey and Fersht (1976). The sequence of superoxide dismutase agrees with that determined by Bridgen et al. (1975). All of the sequences were determined after attachment to 3-aminopropyl glass (Machleidt et al., 1973) except the synthetase and the second trypanosome antigen which were attached to *N*-(2-aminoethyl)-3-aminopropyl glass (Bridgen, 1975). PTH–leucine was not differentiated from PTH–isoleucine.

stained could normally be eluted with yields between 70 and 100%.

Results described here are from predialyzed gels where the protein bands have been located by staining an equivalent gel run in parallel under identical conditions.

Protein Coupling. Coupling yields were estimated by scintillation counting of a resin aliquot after attachment if the protein was radioactively labeled or, for unlabeled proteins available in sufficient quantity, by amino acid analysis of a resin aliquot after hydrolysis. Yields between 70 and 100% were found, with optimal results from porous glass of 200–400 mesh. Optimal pore diameter was 75 Å for proteins of molecular weight less than 30 000 and 125 Å for larger molecules. No consistent differences were found between the various coupling media nor was there any difference in coupling yield between fully reduced lysozyme, performic acid-oxidized lysozyme or *S*-[^{14}C]carboxymethylated lysozyme. However, carboxymethylated derivatives are preferable in that this is the easiest cysteine derivative to identify after sequencing. In no case was a PTH-amino acid detected at cycle 1 nor was any PTH-lysine detected at those cycles where this residue was known to be present, indicating the completeness of the coupling reaction. No significant difference was found between the attachment yields of native lysozyme to 3-aminopropyltriethoxysilyl glass (Wachter et al., 1973) or to *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilyl glass (Bridgen, 1975), although it has been reported (Wachter et al., 1975) that larger molecules bind more efficiently to the latter support.

Protein Sequencing. Using the program described in Table I a repetitive yield of 93% was calculated between leucine-8 and -17 of carboxymethylated lysozyme. Quantitation was achieved by cutting the relevant spots from the TLC plate and scintillation counting but since there will be variations in sample workup, the amount of sample applied to the plate and sample quenching, this figure is only an estimate.

Reversing the order of coupling in Table I, i.e., using a short pulse of unlabeled PITC (2% in acetonitrile) followed by a longer delivery of [^{35}S]PITC (5% in acetonitrile), resulted in incorporation of radioactivity into the protein corresponding to 30–40% of that expected if a single long delivery of [^{35}S]PITC had been used. This indicates that the coupling efficiency of the initial pulse of radioactive PITC (Table I) is only 60–70% and that a second coupling with unlabeled PITC is necessary for completion of the reaction.

Results from five different proteins coupled in four different solvents are shown in Table II. The autoradiogram resulting from sequence analysis of 50 μg (750 pmol) of an *S*-[^{14}C]carboxymethylated trypanosome cell-surface antigen is shown in Figure 1. Residue 1 remains attached to the resin and is not observed. Similarly, no identification has been made at position 7 and subsequent experiments (J. Bridgen and G. Cross, unpublished results) have shown this to be a lysine residue that also remains attached to the resin. PTH–asparagine at position 11 is accompanied by a characteristic spot of PTH–aspartic acid running slightly slower in solvent 2. PTH–histidine at position 12 is shown before development of the plate in solvent 3 (see Methods) to resolve this derivative from PTH–arginine.

Figure 2 shows residues 2–20 of native lysozyme (10 μg) after elution from a sodium dodecyl sulfate–polyacrylamide gel (acrylamide concentration, 15%). No identification was made at positions 5 and 12. Residue 12 is known to be lysine (Canfield, 1963) and residue 5 is cystine. PTH–cystine and PTH–cysteine are both known to be unstable (Niall, 1974) and for this reason carboxymethylated proteins are preferable for this technique. Using these solvent systems (Summers et al., 1973) PTH–leucine is not differentiated from PTH–isoleucine.

The autoradiographs obtained from the other proteins shown in Table II were broadly similar except that weakly radioactive streaks were occasionally seen along each origin line. These did not interfere with the identification. Variations in the R_f values of the PTH derivatives between different batches of TLC plates made the use of an unlabeled PTH-marker mixture essential for reliable identification. More reproducible results were obtained when solvent 1 (Summers et al., 1973) was made up fresh daily. In addition to PTH–leucine and PTH–isoleucine, incomplete resolution was occasionally observed between PTH–lysine and PTH–tryptophan and between PTH–asparagine and PTH–serine. This caused no real problem, since PTH–lysine is not encountered with this sequencing method and PTH–asparagine is invariably accompanied by a characteristic deamidation spot of PTH–aspartic acid. The use of 20% aqueous heptafluorobutyric acid for the conversion rather than the more polar M HCl (Edman and Begg, 1967) allows the PTH derivatives of histidine and arginine to be extracted directly into the ethyl acetate phase (P. J. Lowry, personal communication). They can then be identified by TLC along

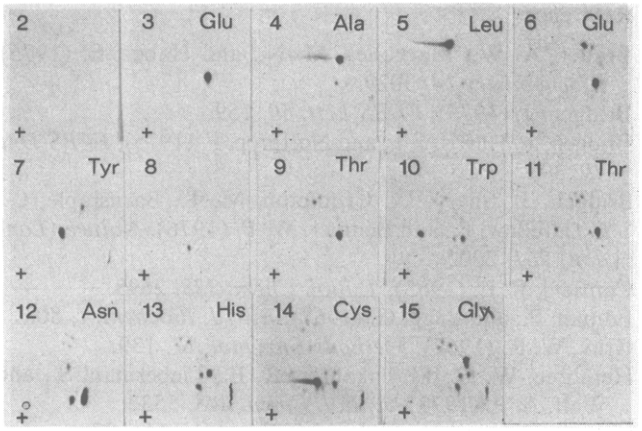

FIGURE 1: Autoradiograph of TLC plates resulting from sequence analysis of an *S*-[^{14}C]carboxymethylated trypanosome antigen (50 μg, 700 pmol attached to *N*-(2-aminoethyl)-3-aminopropyl glass). Half of the total PTH sample from each cycle was applied to the plate and development was for 7 min in solvent 1, followed by 15 min in solvent 2, and, where necessary, 15 min in solvent 3. Exposure of the autoradiograph was 18 h. The sequence was determined as: -Glu-Ala-$\frac{\text{Leu}}{\text{Ile}}$-Glu-Tyr---Thr-Trp-Thr-Asn-His-Cys-Gly, representing residues 2–15 of the protein.

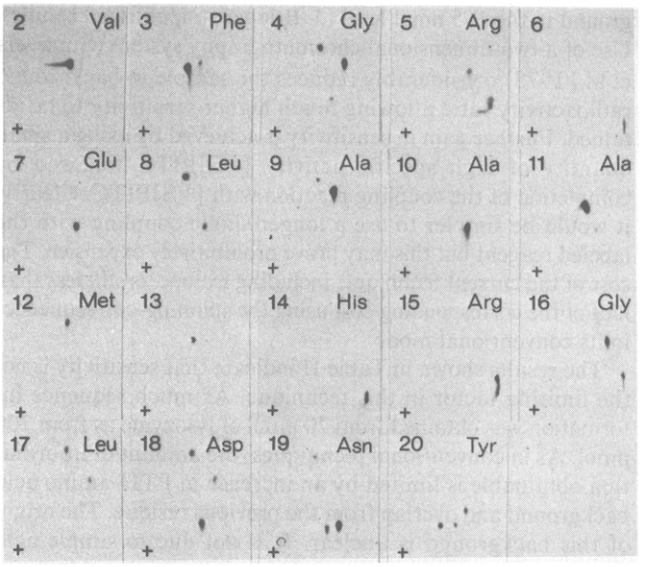

FIGURE 2: Autoradiograph of TLC plates resulting from sequence analysis of native lysozyme (10 μg, 700 pmol attached to 3-aminopropyl glass) after elution from a sodium dodecyl sulfate–polyacrylamide gel. Development conditions were as described in the legend to Figure 1. The sequence was determined as: Val-Phe-Gly-Arg---Glu-$\frac{\text{Leu}}{\text{Ile}}$-Ala-Ala-Ala-Met---Arg-His-Glu-$\frac{\text{Leu}}{\text{Ile}}$-Asp-Asn-Tyr, representing residues 2–20 of the protein (Canfield, 1963).

with the other PTH amino acids.

Discussion

Many methods have previously been described for determining the amino acid sequence of small quantities of protein. These fall broadly into three classes. The manual dansyl–Edman method of Weiner et al. (1972) has the advantage of requiring no special instrumentation or isotopic labeling. It is also the only method that, until now, has been used to derive sequences from proteins eluted from sodium dodecyl sulfate gels. However, because the dansyl technique requires an aliquot of sample to be removed at each cycle of degradation, only a small number of residues may normally be identified. Furthermore, the sensitivity of the dansyl method of identification is limited in practice to ca. 0.25 nmol (Weiner et al., 1972) so that at least 1 nmol of protein will generally be required.

The technique of in vivo labeling (e.g., McKean et al., 1974), whereby uniformly labeled proteins are isolated from cells cultured in the presence of radioactive amino acids, is theoretically capable of very high sensitivity, since only very small amounts of protein need be degraded in the presence of virtually any carrier protein. There are several drawbacks to this approach. One is that the method is clearly only applicable to the relatively small number of proteins where in vivo labeling is feasible. Another is the problem of obtaining uniformly labeled material, since the simple addition of radioactive amino acids to the culture medium does not produce uniformly labeled proteins. The third drawback is likely to be the high cost of this approach. McKean et al. (1974) have pointed out that tritiation (Hembree et al., 1973) of the protein may be a generally useful method for incorporating radioactivity but this remains to be demonstrated.

Of the automated approaches to high sensitivity sequencing perhaps the most useful is that of Jacobs and Niall (1975) where the protein is labeled first with [^{35}S]PITC and coupling is then completed with unlabeled reagent in a manner analogous to that described here. These workers used a spinning-cup sequencer for the degradation and obtained reasonably long (15–20 residue) sequences on 5–15 nmol of protein. Attempts to use less protein than this (Silver and Hood, 1975) significantly reduced the number of steps obtainable, probably as a result of chemical or mechanical losses of the small quantity of protein from the cup. Jacobs and Niall (1975) suggest that the use of carriers (e.g., Niall et al., 1974; Silver and Hood, 1974) will circumvent this problem but this does not appear to be the case (Silver and Hood, 1975). Furthermore, significant quantities of radioactivity will be incorporated into the carrier or its breakdown products rather than into the protein. Indeed, Brauer et al. (1975) have recently shown that results as good as those obtained using carriers may be obtained simply by modifying the sequencer program.

The solid-phase sequencing method of Laursen (1971) completely eliminates mechanical and physical losses during sequencing. Thus, if a radioactive coupling reagent is used, extended solvent washes of the derivatized protein after coupling may be used to remove virtually all of the radioactive by-products. In addition, after coupling to the resin, any contaminating salt or other nonprotein material may easily be removed before sequencing commences. This allows the attachment reaction to take place in a very large number of solvent systems and renders the method useful for proteins eluted directly from polyacrylamide gels. The versatility and high resolution of polyacrylamide gel electrophoresis make it by far the most powerful means of separating protein chains and the present technique allows the determination of extended amino terminal sequences on amounts of protein only slightly greater than is required for detection.

Although originally designed for sequencing small peptides attached to derivatized polystyrene resins (Laursen, 1971), the solid-phase procedure has recently been extended to larger peptides and proteins (Machleidt et al., 1973; Bridgen, 1975). These can be attached and degraded much more efficiently on derivatized porous glass supports presumably due to their large surface area and pore diameter, allowing easy access of proteins into the interior of the resin. Using one-dimensional TLC in conjunction with [^{35}S]PITC (Bridgen, 1975), the sensitivity of this method has been limited by a high radioactive back-

ground to the 1–5 nmol level (J. Bridgen, unpublished results). Use of a two-dimensional chromatography system (Summers et al., 1973) considerably reduces the sample to background radioactivity ratio allowing much higher sensitivity to be attained. Further gain in sensitivity is achieved by using a small quantity of high specific activity [^{35}S]PITC followed by completion of the coupling reaction with [^{32}S]PITC. Clearly, it would be simpler to use a longer single coupling with the labeled reagent but this may prove prohibitively expensive. The cost of the current technique, including isotope, is still less than half of the corresponding cost using the spinning-cup sequencer in its conventional mode.

The results shown in Table II indicate that sensitivity is not the limiting factor in this technique. As much sequence information was obtained from 70 pmol of lysozyme as from 700 pmol. As in conventional techniques, the amount of information obtainable is limited by an increase in PTH-amino acid background and overlap from the previous residue. The origin of this background is unclear. It is not due to simple acid cleavage of peptide bonds, since this would result in preferential cleavage of labile bonds, e.g., aspartic acid–proline (Piskiewicz et al., 1970) and specific contaminating sequences would then appear rather than a general background. Furthermore, preincubation of proteins in either anhydrous or aqueous trifluoracetic acid (Konigsberg, 1967; Weiner et al., 1972) does not cause any increase in the background level. Control runs performed in an identical manner but with no protein attached to the glass showed virtually no background, indicating the latter to have arisen from the protein rather than from amino acid contamination. There was no obvious difference in overlap between proteins coupled in sodium dodecyl sulfate and those in guanidine or buffer solutions. However, it does appear to be true that proteins purified by conventional techniques give marginally better results than those eluted from sodium dodecyl sulfate gels.

One drawback of the present method is that for proteins or peptides not possessing lysine residues, an alternative and possibly less efficient attachment procedure would have to be used (e.g., Previero et al., 1973; Horn and Laursen, 1973). It is, however, possible to use the diisothiocyanate attachment procedure for coupling via aminoethylated (Raftery and Cole, 1963) cysteine residues (Laursen et al., 1975) and this may provide an alternative strategy. The thiazolinone and PTH derivatives of serine are both unstable and may be difficult to identify in small quantities. However, recent results using this technique on human histocompatibility antigens (Bridgen et al., 1976) have led to good positive identifications of PTH–serine. The *N*-terminal amino acid and internal lysine residues are not identified and appear as gaps in the sequence. Since *N*-terminal microsequencing is most likely to be used for comparative or idenification purposes, this should not prove a serious problem. The *N*-terminal residue may, of course, be identified by dansylation (Gray, 1967) but this could take up to ten times more material than is required to establish the remainder of the sequence.

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