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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PEPTIDE MAPPING AND AMINO ACID ANALYSIS IN THE SUB-NANOMOLE RANGE

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SUMMARY

By ensuring adequate gradient mixing and eliminating all major artifact peaks we have been able to obtain reproducible high-performance liquid chromatographic tryptic peptide maps on less than 50 pmol of protein. Likewise, a 10–20 fold improvement in the sensitivity of amino acid analysis has been achieved by analyzing the phenylthiocarbamyl derivatives of the free amino acids rather than the free amino acids themselves. This approach enables accurate amino acid compositions to be obtained on less than 50 ng of protein, providing that a simple correction is made for the background level of serine and glycine. We have been able to reduce the background level of these two amino acids to *ca.* 10 pmol each per sample by incinerating the hydrolysis tubes at 500°C prior to introduction of the sample and by using gas-phase as opposed to liquid-phase hydrolysis. Background corrections are not necessary when over 500 ng of protein are hydrolyzed. With this amount of protein, amino acid compositions, based on phenylthiocarbamyl amino acid analyses were, on average, found to be accurate to within ± 10%.

INTRODUCTION

Based on reports appearing in several recent publications, it appears that most reversed-phase high-performance liquid chromatographic (RP-HPLC) peptide mapping is currently being performed on digests of 1–20 nmol of protein[1–3]. We now demonstrate that even when conventional, 4-mm I.D. columns are used, it is possible to obtain comparative tryptic peptide maps on less than 50 pmol of protein routinely. Similarly, by first converting the free amino acids into their phenylthiocarbamyl (PTC) derivatives[4–6], it is possible to use RP-HPLC to obtain amino acid compositions on less than 50 ng of protein. To reach these levels of sensitivity requires that absorbance baselines be as stable and as free of artifact peaks as possible and that the "background level" of free amino acids, obtained from the acid hydrolysis of a blank tube, be reduced to the 0–10 pmol range. We give practical suggestions for meeting these requirements routinely. In addition, we present sufficient data to allow an objective comparison of the sensitivity and accuracy of amino acid analyses, obtained from ion-exchange chromatography of protein hydrolysates *versus* RP-HPLC following conversion of the resulting free amino acids into their PTC derivatives.

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EXPERIMENTAL

High-sensitivity HPLC peptide mapping

The bacteriophage T4 gene 43 protein (3.6 nmol) used for peptide mapping was precipitated by the addition of trichloroacetic acid to a final concentration of 10% (w/v). After washing the pellet with cold acetone, it was resuspended in 0.2 ml of 8 *M* urea prior to the addition of 0.6 ml of 50 m*M* ammonium hydrogen carbonate and trypsin (Cooper Biomedical, Malvern, PA, U.S.A.) at a protein:trypsin (w/w) ratio of 25. After 24 h at 37°C, suitable aliquots were diluted to 0.2 ml of 2 *M* urea prior to injection into either a 5 μm Vydac C_4 or C_{18} HPLC column (25 cm × 0.46 cm I.D., The Separations Group, Hesperia, CA, U.S.A.) or a 10 μm μBondapak C_{18} column (30 cm × 0.39 cm I.D., Waters Assoc., Milford, MA, U.S.A.) equilibrated with 0.05% trifluoroacetic acid (TFA, solvent A) at a flow-rate of 0.7 ml/min. Peptides were then eluted by linearly increasing the concentration of solvent B [80% (v/v) acetonitrile in 0.05% TFA] as follows: 0–90 min (0–37.5% B), 90–135 min (37.5%–75% B) and 135–150 min (75–100% B).

The Waters Assoc. HPLC system used for these studies consisted of a Model 721 system controller, a WISP automated sample injector, two Model 510 pumps, and an ISCO (Lincoln, NE, U.S.A.) Model V^4 absorbance detector that was connected to a Nelson Analytical Model 4416 chromatography data station. A 1.8-ml Altex (Beckman Instruments, Palo Alto, CA, U.S.A.) dynamic mixer was placed after the two pumps, because a simple, zero dead-volume tee was found to give inadequate mixing at pressures below *ca.* 2000 p.s.i. As shown in the chromatogram in Fig. 1, this incomplete mixing resulted in less reproducible retention times as well as "wavy" baselines which would preclude the use of background subtraction. The two full-scale impurity peaks that were eluted at *ca.* 22% and 29% acetonitrile (v/v)

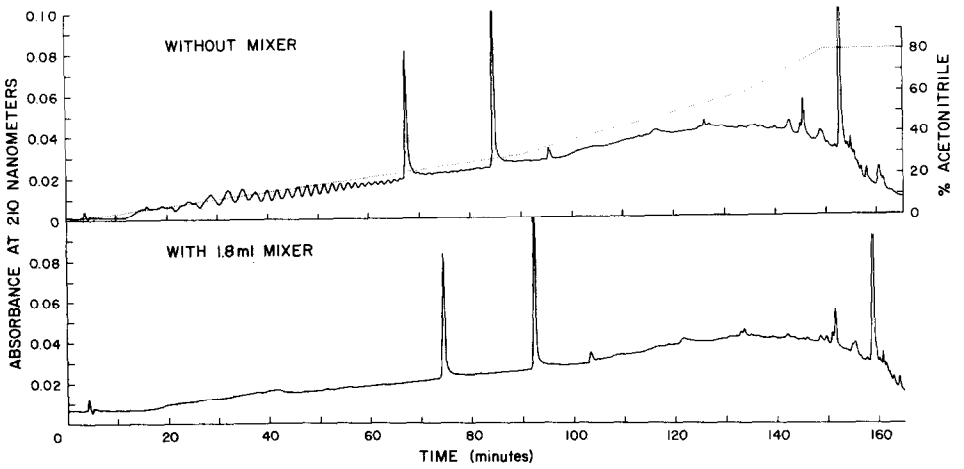

Fig. 1. Elimination of baseline instability due to incomplete buffer mixing. The μBondapak C_{18} column was eluted at 0.7 ml/min with increasing concentrations of acetonitrile, as described under Experimental. The bottom chromatogram shows the effect of installing a 1.8-ml dynamic mixer in place of the simple mixing tee after the two Model 510 pumps.

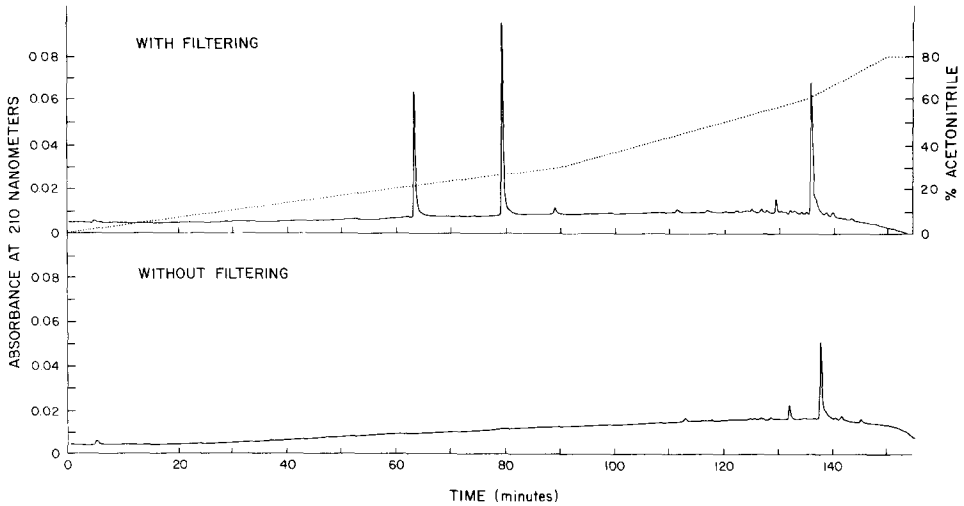

Fig. 2. Elimination of artifact peaks by elimination of filtering the mobile phases. The μBondapak C_{18} column was eluted at 0.7 ml/min with increasing concentrations of acetonitrile, as described under Experimental. In the top chromatogram both mobile phases were filtered through Ultipor Nylon 66 filters, whereas in the bottom chromatogram the solvents were not filtered prior to use.

in the blank run, shown in Fig. 2, were found to result from filtering the mobile phases through Ultipor Nylon 66 (Pall Trinity Micro Corp., Cortland, NY, U.S.A.) filters. The use of carefully deionized water, a 10-μm pump inlet filter and a 0.5-μm high-pressure, in-line filter obviated the need for filtering HPLC solvents prior to use. Maximum reproducibility with respect to retention times was obtained by first briefly degassing solvents by stirring for a few minutes under vacuum and then by keeping the two solvent reservoirs under 2 p.s.i. helium pressure in 5-l Omnifit reagent reservoirs.

High-sensitivity amino acid analysis

Samples for amino acid analysis were dried *in vacuo* in 50 × 6 mm I.D. Pyrex tubes which had been incinerated at 500°C for 16 h. Samples were then hydrolyzed with 175 μl of 5.7 *N* hydrochloric acid (Pierce, Rockford, IL, U.S.A.), containing 1% phenol, in a Pico-Tag Work Station (Waters). After 16 h at 115°C, they were transferred to a clean work station vial, dried *in vacuo* and then redried in a 2:2:1 mixture of ethanol–water–triethylamine (Pierce). Samples were then derivatized to PTC-amino acids by using ethanol–water–triethylamine–phenyl isothiocyanate (7:1:1:1) (Pierce), as described in the Pico-Tag manual. After redrying *in vacuo* for 1 h, the PTC-amino acids were dissolved in 25 μl of 5 m*M* sodium phosphate (pH 7.4) and were injected into two Nova Pak columns (15 cm × 0.39 cm I.D., Waters) connected in series and maintained at 44°C. The PTC-amino acids were eluted by increasing the concentration of solvent B [60% (v/v) acetonitrile] in solvent A [Pico-Tag eluent A: 0.15 *M* sodium acetate, 3.6 m*M* triethylamine, 6% acetonitrile (pH 6.4)], as follows: 0–8 min (0–15% B, curve 5), 8–8.5 min (15% B), 8.5–17 min (15–40% B, curve 5), 17–19 min (40–95% B, curve 6), 19–23 min (95–100% B, curve 6). The effluent was monitored at 254 nm. The flow-rate was increased from 1.0 ml/min to 1.5 ml/min

for 15 min to re-equilibrate the columns in between runs. Buffers were degassed and kept under 4 p.s.i. helium, as described above. The Waters Assoc. HPLC system used consisted of two Model 510 pumps, a refrigerated WISP sample injector, a Model 441 detector, and a Model 840 chromatography data and control system, based on a Digital Professional 350 computer equipped with Expert Chromatography Software, Revision 3.0.

RESULTS

High-sensitivity HPLC peptide mapping

Based on the chromatograms in Fig. 3, it would appear that a packing with 300-Å pore size provides better resolution than a 125-Å packing for RP-HPLC peptide mapping. At a sensitivity of $5 \cdot 10^{-6}$ absorbance units (at 210 nm)/s and a peak width of 34 s, 49 peaks were detected in the chromatogram obtained from the C_{18} μBondapak column, compared with 66 peaks for the C_{18} Vydac column. The higher degree of resolution obtained on the 300-Å column results in sharper peaks which, because of their increased height (absorbance at 210 nm), permit more sensitive peptide mapping. The total recovery of peptides seems to be similar on all three of the columns tested, *i.e.* the sum of the areas from all of the peaks in each of the chromatograms in Fig. 3 did not vary by more than ± 7%. The major differences between the C_4 and C_{18} Vydac columns occurs in the first third of the chromatogram. It is clear that relatively short peptides (fewer than about ten residues) are poorly resolved on the C_4 column. In the last two-thirds of the chromatograms, the patterns are similar, although, in general, peptides are eluted slightly later from the C_{18} than from the C_4 column. The similar patterns obtained for these two columns suggests that

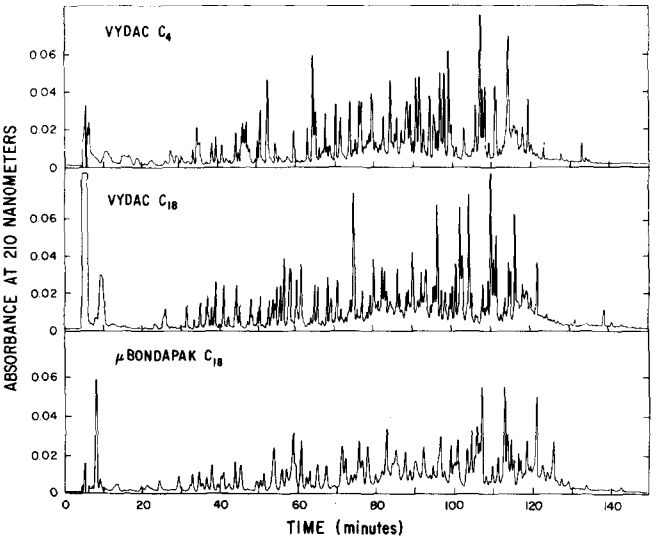

Fig. 3. Reversed-phase peptide mapping on various commercial HPLC columns. Three identical aliquots, corresponding to 490 pmol each, of a tryptic digest of the 105 000-dalton bacteriophage T4 gene 43 protein were subjected to HPLC peptide mapping on a Vydac C_4 (top), Vydac C_{18} (middle), and μBondapak C_{18} column, as described under Experimental.

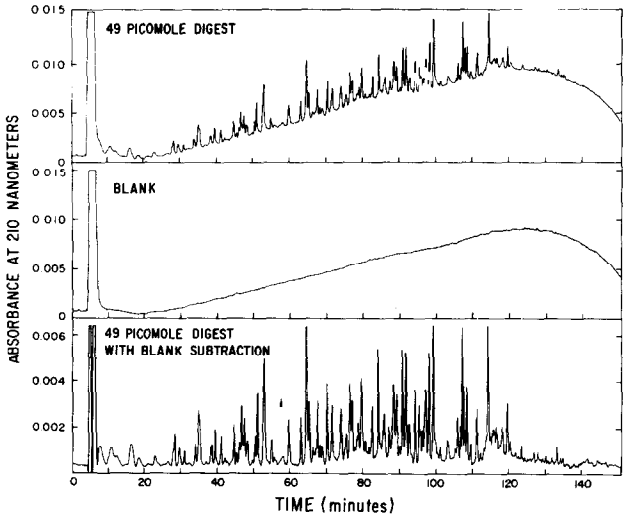

Fig. 4. High-sensitivity HPLC peptide mapping. An aliquot of a tryptic digest, similar to that described in Fig. 3 and corresponding to 49 pmol of the bacteriophage T4 gene 43 protein, was subjected to HPLC peptide mapping on a Vydac C_{18} column, as described under Experimental.

re-chromatography of peptide mixtures derived from a C_4 column under similar conditions on a C_{18} column would not be likely to result in further purification.

If care is taken to ensure stable baselines (see Experimental), it is possible (see Fig. 4) to obtain comparative peptide maps on less than 50 pmol of protein. While

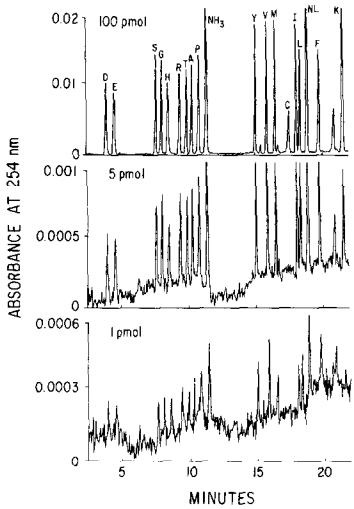

Fig. 5. Picomole quantitation of PTC-amino acids. The top chromatogram is of 100 pmol of a standard mixture of amino acids, converted into their PTC derivatives and then separated by RP-HPLC on two Nova Pak columns, as described under Experimental. The bottom two chromatograms were obtained by injecting dilutions of this mixture in 20 μl of 5 mM sodium phosphate (pH 7.4), corresponding to 5 pmol (middle) or 1 pmol (bottom) of each PTC amino acid.

background subtraction has been used in this figure to enhance the appearance of the chromatogram, this procedure by itself does not significantly enhance the sensitivity of the technique. Results similar to those shown by the bottom chromatogram in Fig. 4 should be obtainable by balancing the absorbance at 210 nm of the two HPLC buffers with sodium azide.

High-sensitivity amino acid analysis

As shown by the chromatograms in Fig. 5, the PTC derivatives of all seventeen common amino acids can be separated with near-baseline resolution in less than 22 min. If baseline resolution is not essential, the analysis time can be reduced to as little as 12 min[4]. Even though 3.9-mm I.D. columns were used in Fig. 5, it is still possible to quantitate as little as 1 pmol of each PTC amino acid. Higher sensitivities could undoubtedly be obtained on 1- or 2-mm I.D. columns. In order to compare the accuracy of PTC and ion-exchange analysis, several standard proteins were hydrolyzed and then analyzed by these two different procedures. The results (Table I) indicate that when reasonably large amounts of protein are available (more than 1.5 μg), ion-exchange analysis provides a more accurate composition. Hence, for three different proteins, the average deviation from the expected amino acid composition was consistently below ±5% for ion-exchange analysis, compared with a range of ±6.1% to ±21% for PTC-amino acid analysis. When the amount of protein taken for hydrolysis is decreased to 400 ng, which is at the limit of sensitivity of the Beck-

TABLE I

AMINO ACID ANALYSIS OF STANDARD PROTEINS BY ION-EXCHANGE CHROMATOGRAPHY AND RP-HPLC OF PTC DERIVATIVES

Results presented are in each case the average of three independent hydrolyses.

	Insulin B chain			*Cytochrome c*			*Bovine serum albumin*		
	Seq.	*IE*	*RP*	*Seq.*	*IE*	*RP*	*Seq.*	*IE*	*RP*
Asp	1	1.1	1.0	8	8.2	7.9	54	54.6	50.4
Glu	3	3.2	3.0	12	12.3	12.2	79	79.3	78.1
Ser	1	1.1	1.2	0	0.3	0.4	28	26.3	27.2
Gly	3	3.1	3.3	12	12.4	13.9	16	17.8	18.1
His	2	2.0	1.7	3	3.1	3.0	17	18.3	16.6
Arg	1	1.0	1.3	2	2.1	2.9	23	23.3	25.1
Thr	1	1.0	1.0	10	9.5	9.6	34	32.3	33.7
Ala	2	2.0	1.9	6	6.2	6.6	46	45.8	46.8
Pro	1	1.2	1.0	4	3.8	4.7	28	29.4	29.2
Tyr	2	1.9	1.7	4	3.8	3.8	19	19.0	18.8
Val	3	2.9	2.6	3	3.0	3.2	36	34.3	34.5
Met	0	0	0.1	2	1.7	1.2	4	4.3	2.4
Ile	0	0	0.1	6	5.6	6.2	14	12.7	14.4
Leu	4	3.9	3.6	6	6.1	6.5	61	60.4	62.3
Phe	3	2.9	2.5	4	3.9	4.3	27	26.3	27.2
Lys	1	1.0	2.5	19	19.4	16.4	59	61.1	61.3
Average deviation (%)		4.6	21		4.3	12.0		4.2	6.1
Amount (μg)		1.7	1.5		5.4	4.7		3.2	2.8

TABLE II

HIGH-SENSITIVITY AMINO ACID ANALYSIS OF BSA

Results presented are in each case the average of three independent hydrolyses.

	6.0 pmol BSA			*RP analysis of 0.6 pmol BSA*				
	IE	*RP*	*Seq.*	*Uncorrected (residues)*	*Corrected (residues)*	*Uncorrected (pmol)*	*Background* (pmol)*	
							Average	*Range*
Asp	57.1	54.0	54	50.8	53.9	26.6	0.9	0–2.7
Glu	82.5	78.8	79	76.9	81.4	40.4	1.6	0–4.8
Ser	43.3	28.4	28	42.0	27.7	22.0	8.8	0–7.3
Gly	40.8	18.2	16	43.6	26.6	22.9	10.2	2.2–22.6
His	18.1	15.2	17	11.6	12.8	6.1	–	–
Arg	19.9	24.9	23	24.8	25.8	13.0	0.7	0–2.1
Thr	33.8	33.7	34	30.4	33.4	15.9	–	–
Ala	49.1	47.6	46	47.1	49.9	24.7	0.9	0–2.9
Pro	21.4	28.4	28	27.4	30.2	14.4	–	–
Tyr	15.3	18.7	19	17.3	19.1	19.1	–	–
Val	29.4	35.5	36	36.4	38.4	19.1	0.8	0–2.5
Met	5.0	1.9	4	10.3**	ND	5.4	5.8**	5.1–6.1
Ile	13.5	14.4	14	16.1	16.4	8.4	0.6	0–1.9
Leu	62.2	66.3	61	61.7	68.0	32.4	–	–
Phe	22.0	27.2	27	25.5	28.1	13.4	–	–
Lys	51.7	54.6	59	43.3	42.8	22.7	2.3	1.6–2.7
Average deviation (%)	21	7.5		31	12.9			
Amount (ng)	400	380		41				

* The background was determined by hydrolyzing an aliquot of the buffer (1 m*M* sodium hydrogen carbonate) equal to that used for dissolving BSA prior to lyophilization and hydrolysis.

** This peak results from a small impurity peak that is eluted with PTC-methionine.

man 121 M analyzer used in this study, then the error involved in the ion-exchange analysis increases five-fold to *ca.* ±21% (Table II). In contrast, decreasing the amount of protein over this range had no significant effect on the accuracy of the PTC amino acid analysis, so that even when only 380 ng of protein were hydrolyzed, the average percent deviation for the PTC-amino acid analysis was still only ± 7.5% (Table II). However, decreasing the amount of protein analyzed to only 41 ng does significantly decrease the accuracy of the resulting composition. Most of the 31% average deviation shown in Table II for the 41-ng sample of bovine serum albumin (BSA) results from a background level of free amino acids that can be corrected for by hydrolyzing and analyzing an empty tube. As shown in Table II, this simple expedient decreases the average error of this analysis to only ± 12.0%. In this regard, it should be noted that in a previous study[4] in which a PTC-amino acid analysis was performed on 10 ng of insulin B chain, the actual amount of protein taken for hydrolysis was 100 ng[4]. By injecting only 10% of the sample, problems due to background and to artifact peaks, resulting from the PTC conversion would, of course, be minimized. Even so, the average error in this earlier analysis, which was not

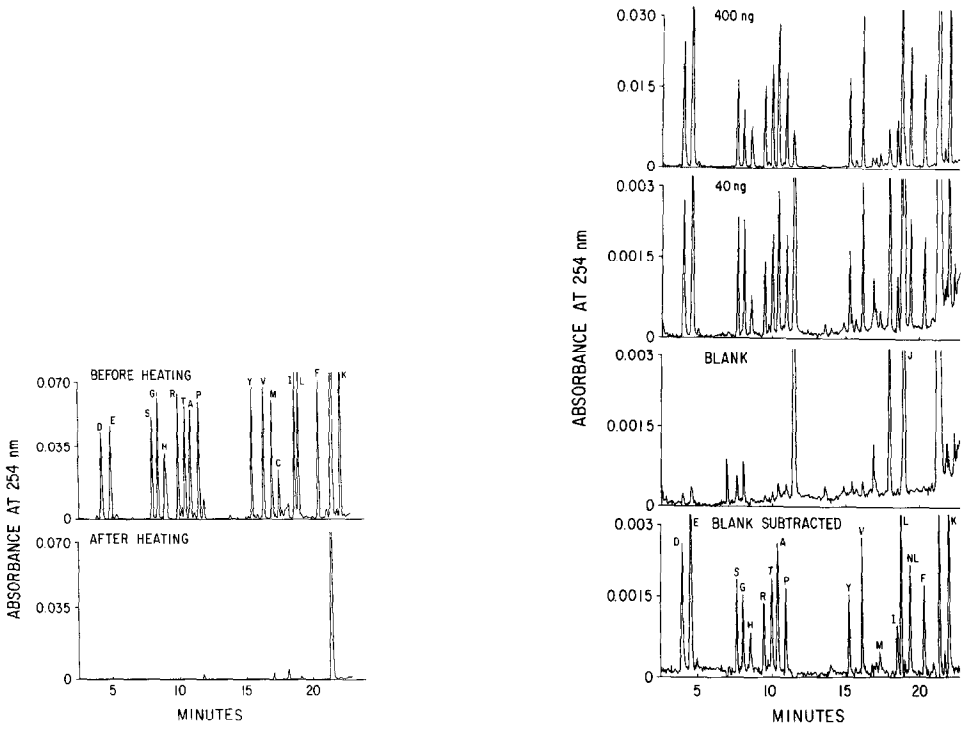

Fig. 6. Incineration at 500°C provides an effective means of cleaning hydrolysis tubes prior to use. Aliquots of an amino acid calibration standard, corresponding to 500 pmol of each individual amino acid, were dried *in vacuo* in two previously incinerated 50 × 6 mm I.D. Pyrex tubes. One sample (top chromatogram) was kept at room temperature while the other (bottom chromatogram) was kept at 500°C for 16 h. Both samples were then allowed to react with phenyl isothiocyanate and chromatographed on Nova Pak columns, as described under Experimental.

Fig. 7. PTC amino acid analysis of 600 fmol of bovine serum albumin. The top two chromatograms were obtained by hydrolyzing and then converting into PTC derivatives either 400 ng or 40 ng of bovine serum albumin, dissolved in 20 μl or 2 μl, respectively, of 20 m*M* sodium hydrogen carbonate. The chromatogram labelled "blank" was obtained by hydrolyzing and converting a comparable volume of buffer. This demonstrates the level of background contamination as well as the presence of a large artifact peak, labelled J, eluted at *ca.* 18 min and interfering with the quantitation of PTC-leucine. This "blank" chromatogram was stored by the Model 840 chromatography data and control system and then subtracted from the 40-ng chromatogram to give the chromatogram on the bottom, which corresponds to hydrolyzing 600 fmol of bovine serum albumin. In each instance, 83% of the 400-ng or 40-ng hydrolyzed sample was actually injected into the two HPLC columns.

corrected for background, was still ±23%[4]. In Table II, 83% of each sample taken for hydrolysis was actually injected into the HPLC system.

The most troublesome aspect of the "background" problem is its variability. Hence, when three identical aliquots of 20 m*M* sodium hydrogen carbonate were dried and then hydrolyzed, the amount of glycine found varied from 2.2 to 22.6 pmol, thus making it difficult to correct accurately for this error. Similar ranges and variability have been found when twenty other previously incinerated tubes were hydrolyzed and then analyzed. Although the source of this contamination has not

yet been identified, our preliminary data suggests that it may be the result of an airborne contamination of the hydrolysis tubes occurring during either sample transfer or lyophilization of the sample prior to hydrolysis. This background does not seem to be due to contaminated hydrolysis tubes, because, as we have shown, incineration for 16 h at 500°C effectively and easily cleans the hydrolysis tubes prior to use. As shown in Fig. 6, this procedure easily eliminates at least up to 500 pmol of each of any "contaminating" free amino acids. Levels of free amino acids detected by performing PTC conversion in previously incinerated tubes are consistently below the 1-pmol level (data not shown). It is only when PTC conversions are performed after hydrochloric acid hydrolysis of supposedly clean, incinerated tubes that the background rises to the levels shown in the last two columns in Table II. If the background level of free amino acids observed following hydrochloric acid hydrolysis of "blank" tubes is the result of airborne contamination, which might be expected to be quite variable, then it should be possible to eliminate it by taking greater precautions in performing the required sample transfers and drying steps in a dust-free environment. Two other expected sources of contamination, the hydrochloric acid and sample buffers, have so far not posed a serious problem. By performing hydrolyses with hydrogen chloride vapor rather than with liquid hydrochloric acid, problems arising from contamination of hydrochloric acid have been eliminated. Similarly, relatively simple precautions, such as the use of ultrapure water, decreased the background obtained from hydrolysis of reasonable volumes (0.1 ml) of buffers to negligible values (data not shown). Buffer contamination could obviously become a problem if samples were so dilute as to require that large volumes of buffer be dried and hydrolyzed. However, even in this instance the background should be constant and thus, except in extreme instances, easily correctable by hydrolyzing equal aliquots of the buffer.

Another problem that becomes more evident at high sensitivity is interference by artifact peaks eluted with the PTC-amino acids. As shown in Fig. 7, an artifact peak, visible as only a slight shoulder on the leucine peak when 400 ng of protein are hydrolyzed, poses a serious problem when only 40 ng are hydrolyzed. This problem can be solved by subtraction of a blank hydrolysis tube as in Fig. 7, or perhaps also by either lengthening the analysis time for better separation of interfering peaks or by decreasing the concentration of phenyl isothiocyanate used in the conversion reaction.

DISCUSSION

While it is already possible to routinely obtain HPLC peptide maps on less than 50 pmol of a tryptic digest of a protein, the use of 1- to 2-mm I.D. columns should lower this range to 2–10 pmol, thus permitting a peptide map to be obtained on 100–500 ng of a 50 000-dalton protein. The ability to purify picomole amounts of peptides and the ease with which even a single picomole of an individual PTC-amino acid can be quantitated provide a strong impetus for further work on improving the sensitivity of amino acid analysis. However, this goal can be reached only when the background level of free amino acids, resulting from the hydrolysis of a supposedly empty tube, can be reduced to a level that more closely approximates the level of detection of the individual PTC amino acids. While decreasing the diameters of the reversed-phase columns used for PTC-amino acid analysis from 3.9

mm to 1 mm should permit quantitation of 100 fmol of an individual PTC-amino acid, this effort would surely be wasted if the background level of nine free amino acids were still in the 0.8–10.2 pmol range (Table II).

In comparing the relative merits of ion-exchange analysis of the free amino acids with reversed-phase analysis of their PTC derivatives several factors must be considered. If large amounts of protein (more than 1 μg) are available, ion-exchange amino acid analysis appears to be slightly more accurate. In this range it can provide amino acid analyses that are accurate to within $\pm 5\%$ compared with $\pm 10\%$ for PTC-amino acid analysis. Ion-exchange analysis is also more convenient in terms of sample preparation, since it does not require manual conversion of the free amino acids into their PTC derivatives. On the other hand, we have found that an automated RP-HPLC system, dedicated to PTC analysis, is considerably less expensive to set up and easier to maintain than an ion-exchange amino acid analyzer. In addition, PTC-amino acid analysis certainly has advantages in terms of speed, sensitivity and —at least below *ca.* 500 ng of protein— it appears to provide more accurate amino acid analyses. If corrections are made for background levels of serine and glycine, PTC-amino acid analysis can provide an amino acid composition on 40 ng of protein that is accurate to within $\pm 13\%$. If suitable procedures can be found for better control of this background, PTC-amino acid analysis has the potential of providing accurate analyses on less than 5 ng of protein. On balance, PTC-amino acid analysis seems to offer an excellent alternative to ion-exchange analysis, being both fast enough to keep pace with the large number of peptides that can now be routinely isolated by RP-HPLC, and matching, in terms of sensitivity, the capability of gas-phase protein sequencing.

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