Journal of Chromatography, 211 (1981) 347–359
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,796

DETERMINATION OF ACETYL AND FORMYL GROUPS BY GAS–LIQUID CHROMATOGRAPHY

JASBIR CHAUHAN and ANDRE DARBRE*
Department of Biochemistry, King's College, Strand, London WC2R 2LS (Great Britain)
(Received March 3rd, 1981)

SUMMARY

A method is described for the hydrolysis of proteins, amino acids and carbohydrates for the liberation of N- and O-acetyl and formyl groups. The acetyl and formyl phenacyl esters are prepared by means of crown ether catalysis and determined by gas–liquid chromatography using the flame-ionization detector. Quantitative results are given with samples containing about 20 nmol of acetyl or formyl group. The method is also applicable to the determination of N- and O-propionyl groups.

INTRODUCTION

Narita[1] first isolated and characterized the N-acetylated peptide from the N-terminus of tobacco mosaic virus protein. Acetyl groups have since been shown to occur in proteins obtained from a variety of sources and about 80% of the soluble proteins from Ehrlich Ascites cells were reported to be N^{α}-acetylated[2]. N^{α}-Acetylamino acids have been reported in a wide range of proteins, including adenylate kinase, carbonic anhydrase, superoxide dismutase, several dehydrogenases, cytochrome *c*, parvalbumins, α-melanocyte stimulating hormone, histones and keratins[3,4]. The presence of N-formyl groups has been well established in protein chain initiation on the ribosome and N^{α}-formyl-blocked proteins have been reported[5,6].

A variety of methods for the determination of acetate were published. Acetate was determined by enzymic methods [7–18]. Acetate in proteins in the range 0.1–1.0 μmol was determined as the 1-acetyl-2-dinitrophenylhydrazine derivative[19,20]. Using 1–2 mg of protein, formyl and acetyl groups were determined as their 1-acyl-2-Dns-hydrazine derivatives, although the yield of the formyl group was only about 10% that for the acetyl group[21]. Mucopolysaccharides were hydrolyzed with methanolic HCl and the methyl acetate determined as the hydroxamate by colorimetry[22]. Acid hydrolysis was used to liberate acetic acid from protein hexosamines and the acetic acid titrated after steam distillation[23,24]. Proteins were hydrolyzed and the N-acetyl[25,26] and the N-formyl[6] amino acids were separated by high-voltage electrophoresis and the amino acid determined. The determination of N^{α}-blocking groups in proteins is possible by mass spectrometry (MS), but about 50 nmol of a short peptide carrying these groups must be isolated first[27].

0021-9673/81/0000–0000/$02.50

Gas chromatography in the free acid form was used for the determination of acetic acid in a variety of biological solutions such as plasma and urine[28–37], in biological materials[38,39] and attached to proteins[40,41] and lipopolysaccharides[42]. Protein acetyl groups were hydrolyzed by methanolic HCl and the methyl acetate formed was determined by gas–liquid chromatography (GLC)[43,44]. A method using acid fusion reaction gas chromatography for biological materials was reported[45].

We report here on a method for the direct determination of acetyl and formyl groups attached to amino acids, proteins and carbohydrates. The method is equally applicable to propyl groups, although these have not been reported in proteins. After alkaline hydrolysis of the sample the potassium salts of acetic and formic acid are converted to the phenacyl esters prepared by means of crown ether catalysis and determined by GLC. A preliminary report was published[46].

EXPERIMENTAL

Apparatus

A Pye Series 104 Model 24 gas chromatograph, fitted with dual flame-ionization detector (FID) (Pye-Unicam, Cambridge, Great Britain) was used with a Speedomax W 1 mV, 1-sec strip chart recorder (Leeds & Northrup, Birmingham, Great Britain). Integration of the peak areas was carried out with the Kent Chromalog 2 and Vidar Autolab 6300 digital integrators (Anachem, Luton, Great Britain). Nitrogen (99.9% "white spot" from BOC, Wembley, Great Britain) was used as carrier gas.

Materials

Chemicals and stationary phases were obtained as follows: phenacyl bromide, potassium acetate, N-acetylglycine, trimethylchlorosilane (BDH, Poole, Great Britain), dicyclohexyl-18-crown-6 (Aldrich, Milwawkee, WI, U.S.A.), JXR, XF-1105, XE-60, ECNSS-S and EGSS-X (Applied Science Labs., State College, PA, U.S.A.), Dexsil 300 GC and EG isophthalate (Analabs, North Haven, CT, U.S.A.), GMHS, GMS and WOL (Advita, Walton-on-Thames, Great Britain), SE-52, BDS and QF-1 (F & M Scientific, Avondale, PA, U.S.A.), Chromosorb W HP 80–100 mesh (Field Instruments, Richmond, Great Britain), EG distearate and PPSeb (Reoplex 100) (Griffin & George, Alperton, Great Britain), SE-30 (E301) (ICI, Stevenston, Great Britain), MS-200 (Hopkin & Williams, Chadwell Heath, Great Britain), Antarox CO-990 (Perkin-Elmer, Beaconsfield, Great Britain), *n*-octadecane, OV-17, OV-25, OV-61, OV-225, Celite 560, 85–100 mesh (Phase Separations, Queensferry, Great Britain), Porapak Q (Waters Assoc., Milford, MA, U.S.A.), BDA, NPGA, NPGG, NPGP, NPGS and NPGSeb were previously prepared[47], N-acetyl-DL-leucine, N-acetyl-L-tyrosine and N-acetyl-DL-phenylalanine (L. Light & Co., Colnbrook, Great Britain) and N-acetyl-L-aspartic acid, N-acetyl-L-glutamic acid, O-acetylserine, N-formyl-L-leucine, N-formyl-L-methionine, N-formyl-L-tyrosine, N-formyl-DL-valine, chicken egg albumin and egg white lysozyme (Sigma, St. Louis, MO, U.S.A.). Microcapped centrifuge tubes (0.5 ml) were obtained from Hughes & Hughes (Haroldwood, Great Britain).

Acetylated cholesterol and carbohydrates were a gift from Dr. N. R. Williams (Birkbeck College, University of London, London, Great Britain) and carbonic anhydrase from Dr. N. Spencer (King's College, London, Great Britain).

Hydrolysis of samples and preparation of esters

N-Acetyl compounds were hydrolysed with 1 *M* KOH in 0.5 ml polypropylene micro-capped centrifuge tubes in an autoclave at 15 p.s.i. (123°C) for 3 h. After hydrolysis, the tube contents containing 20–100 nmol of acetate or formate were transferred to glass tubes (5 × 0.5 cm I.D.) followed by 5 × 25 μl washings of distilled water. The solution was neutralised to phenolphthalein end-point with HBr and taken to dryness. Phenacyl bromide and dicyclohexyl-18-crown-6 in the proportion 10:1 (mol/mol) in benzene solution (25 μl) were added. The tube was sealed and incubated at 80°C for 30 min with occasional shaking. A five-fold molar excess of phenacyl bromide over the expected acetate was used. Toluene was not a satisfactory substitute for benzene. Aliquots of 1 μl were injected onto the GLC column.

RESULTS AND DISCUSSION

Successful methods were reported for the determination of volatile fatty acids by chromatography in their free acid form[28–42]. Acetic acid was determined after preliminary acid hydrolysis of N-acetylamino acids and N-acetylglucosamine[38] and of proteins[40,41]. Some preliminary experiments were carried out with hydrolysis of N-acetylamino acids in sealed tubes with 2 and 6 *M* HCl at 110°C for periods up to 24 h[38,41]. The acetic acid liberated was determined by GLC. Protein samples with 8–17 μmol acetate were used[40], but here with samples containing 50–100 nmol of acetate inaccurate results were obtained, despite precautions taken to prevent volatility losses. In addition, the analysis of free fatty acids with the injection of aqueous solutions leads to problems arising from the accumulation of non-volatile compounds at the injection head of the column, destruction of stationary phase and ghosting (see review ref. 48) and the known effect of water on the response of the FID[29,49]. The problems with tailing solvent[32,37,44,50] and free acid peaks[32,36,37] were shown. Some of these problems could be avoided by isolation of free acetate by steam distillation[28] or by microdistillation[15,38,39,43] and some workers extracted the acetate into a non-aqueous medium, such as *tert.*-butyl ethyl ether[11,41]. However, these methods involved additional manipulation which did not commend themselves for work at high sensitivity. The determination of small quantities of acetic acid in its acid form with the FID necessitated the use of low attenuation of the signal, *e.g.* $8 \cdot 10^{-12}$ A/mV, with the attendant problems of a noisy baseline when working near the limits of the apparatus[30].

In order to improve the sensitivity of the method for acetate and also to determine formate in addition with the FID it was considered best to work with high-molecular-weight ester derivatives. The use of crown ethers introduced by Pedersen[51] for ester formation by reaction of the potassium salt of the acid with a primary bromide[52–55] offered a method which was applicable to small samples, and phenacyl esters were selected for the determination of both acetyl and formyl groups.

It was previously shown[46] that the best results were obtained with phenacyl bromide and dicyclohexyl-18-crown-6 in 10:1 mol ratio in benzene solution at 80°C for 30 min. With phenacyl bromide:potassium acetate mol ratios 1.07 and 2.14 the yields of phenacyl acetate were 73.5 and 95.5 %, respectively. With molar excess ratios ranging from 3.2 to 32 the yields were 98.5 % ± 4.36 for $n = 32$ samples. A five-fold molar excess of phenacyl bromide over acetate was adopted. A large excess of phe-

nacyl bromide interfered with the desired derivative peaks. A similar method was reported for valproic acid in serum[56].

Solvent tailing is often a problem with GLC because of its interference with early eluting peaks. Acetonitrile was reported to be the best solvent for rapid ester formation with crown ethers[53]. However, this solvent tailed badly on our column (see ref. 56) and benzene was preferred. Other non-tailing solvents were chloroform and carbon disulphide, but the recoveries were not satisfactory using these solvents for the reaction.

In Table I the retention times for phenacyl formate, acetate and propionate relative to that of phenacyl bromide are given for 23 different stationary phases. Most of the silicone phases gave tailing peaks. The mixed silicone column[57] gave the best separation but the peaks tailed badly. Amongst the ten polyester phases examined only PPSeb and EG distearate showed any effective resolution between the formate

TABLE I

RELATIVE RETENTION DATA FOR PHENACYL ESTERS

The figures are retention times relative to phenacyl bromide taken as 1.00. The retention time in minutes for phenacyl bromide is given in brackets.

Liquid phase	*Liquid–solid (% w/w)*	*Column temp. (°C)*	*Phenacyl*			
			Bromide	*Formate*	*Acetate*	*Propionate*
Dexsil 300 GC	1	110	(7.38)	1.12	1.60	2.77
SE-30	3	110	(8.86)	1.16	1.71	2.84
SE-52	3	125	(5.91)	1.00	1.47	2.27
JXR	3	125	(6.30)	1.00	1.47	2.41
Mixed silicones*	1	125	(4.72)	1.54	1.79	2.92
OV-17	5	140	(10.43)	1.09	1.53	2.38
OV-25	1	125	(7.29)	1.19	1.65	2.46
OV-61	3	125	(13.40)	1.12	1.60	2.57
OV-225	1	125	(4.33)	1.43	1.67	2.41
XF-1105	2	125	(5.02)	1.24	1.63	2.57
Antarox CO-990	2	150	(10.04)	2.18	2.18	2.63
ECNSS-S	2	150	(6.89)	1.77	1.86	2.09
EGSS-X	2	150	(6.23)	1.89	1.89	2.16
BDA	2	150	(12.99)	1.53	1.53	2.00
BDS	2	150	(4.75)	1.71	1.71	2.23
NPGA	2	150	(12.01)	1.56	1.64	2.20
NPGG	2	150	(8.07)	1.51	1.51	2.07
NPGP	2	120	(7.48)	2.03	2.21	3.00
NPGSeb	2	150	(7.87)	1.25	1.40	2.15
NPGS	5	175	(9.80)	1.52	1.52	1.90
PPSeb	2	150	(15.45)	1.32	1.46	2.01
EG distearate	2	125	(16.90)	1.09	1.44	2.36
EG isophthalate	2	150	(4.92)	1.72	1.72	2.20
GMHS	2	150	(9.55)	1.20	1.58	2.33
GMS	2	125	(14.96)	1.15	1.52	2.52
WOL	2	125	(18.31)	1.17	1.55	2.52
PPSeb–GMS (3:2, w/w)	2	150	(8.37)	1.22	1.43	2.09

* XE-60–QF-1–MS-200 (46:27:27, w/w/w) on Chromosorb W HP, 80–100 mesh[57].

and the acetate. The surfactant phases (GMHS, GMS and WOL) separated phenacyl formate from phenacyl acetate, but phenacyl bromide was very close to the formate.

A liquid phase was developed which consisted of PPSeb and GMS mixed together in the proportions 3:2 (w/w) and coated (2%, w/w) either onto Celite 560 AW DMCS or onto Chromosorb W HP support material. This resolved the phenacyl esters and also avoided interference from the large peak due to the reagent phenacyl bromide which was unavoidably present in large excess, as shown in Fig. 1. During the preparation of the phenacyl esters, small amounts of phenacyl chloride were formed by interchange between phenacyl bromide and contaminating chloride ions[58]. Acetophenone also appeared as a minor component. The identity of the peaks of compounds shown in Fig. 1 were confirmed by GLC–MS. Table II presents the details of the fragmentation patterns obtained for these peaks. In all cases the mass ion was identified and the base peak at *m/e* 105 was assigned to $(C_6H_5CO)^+$. Similar artifact peaks were obtained with the GLC of pentafluorobenzyl esters prepared by crown ether catalysis[55].

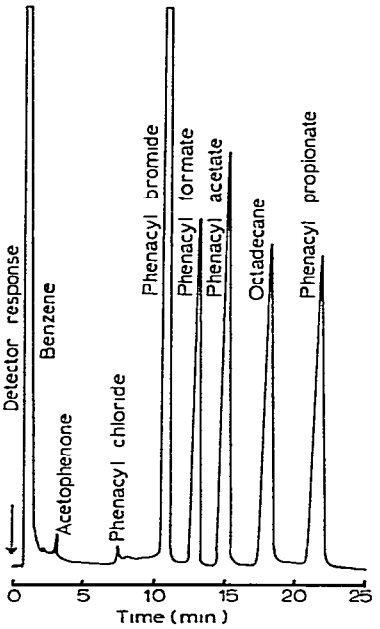

Fig. 1. GLC separation of phenacyl ester derivatives on PPSeb–GMS column. GLC conditions: Pye Series 104 Model 24. FID. Glass column (3 m × 2.5 mm I.D.) packed with 2% mixed stationary phase PPSeb–GMS (60:40, w/w) on Chromosorb W HP 80–100 mesh. Oven temperature, 150°C. Carrier gas, nitrogen, 30 ml/min, hydrogen 30 ml/min, air 450 ml/min. Sample size, 1 μl. Attenuation, $1 \cdot 10^{-9}$ A f.s.d.

The relative molar response values of phenacyl formate, acetate and propionate against octadecane determined with the FID are given in Table III. The response of phenacyl formate was lower than would be expected (by approximately 8–9%) for a linear relationship response/carbon number for the homologous series of esters.

TABLE II

MOLECULAR PEAKS AND MAJOR FRAGMENT PEAKS OF PHENACYL DERIVATIVES AND ACETOPHENONE

The compounds correspond with the peaks shown in Fig. 1.

Compound	*$[M]^+$ m/e (%)*	*Base peak (m/e)*	*Major fragment peaks, m/e (%)*			
Acetophenone	120 (68)	105	77 (71)	51 (55)	43 (48)	91 (23)
Phenacyl bromide*	198 (24) 200 (20)	105	77 (74)	91 (47)		
Phenacyl chloride*	154 (29) 156 (11)	105	77 (80)	91 (37)		
Phenacyl formate	164 (37)	105	85 (99)	29 (77)	91 (68)	119 (63)
Phenacyl acetate	178 (46)	105	77 (99)	43 (99)	91 (77)	119 (56)
Phenacyl propionate	192 (20)	105	57 (60)	77 (56)	119 (30)	91 (24)

* Two molecular ions $[M]^+$ due to natural isotopes.

However, the results were reproducible. The GLC of free acids [C_2 to C_7] using the FID also showed a non-linear response/carbon number relationship[30].

Fig. 2 shows the linear responses obtained when plotting peak areas (integrator counts) against increasing quantities of phenacyl formate, acetate and propionate (1.0–10 nmol) injected onto the GLC column. No breakdown of the phenacyl formate was observed within the experimental range studied.

Conditions were investigated for releasing covalently-bound acetyl groups. Hydrobromic acid was used in order to form potassium bromide for crown ether catalysis and the time course for the hydrolysis of N-acetylglycine with 6 *M* HBr at 110°C in Fig. 3 shows a maximum yield in 3 h. Previous workers used 2 *M* methanolic HCl at 100°C for 4 h to form methyl acetate directly for GLC[22,43]. Aqueous 9 *M* H_2SO_4 (ref. 11) or 3 *M* H_2SO_4 at 105°C for 2 h[41], 6 *M* HCl at 110°C for 24 h[15] and 0.2 *M* HCl at 100°C overnight[42] were used to liberate free acetic acid. This method was appplicable to N-acetyl- and N-formylamino acids. The results for acid hydrolysis are given in Table IV. (The results for alkaline hydrolysis reported for convenience in this table will be discussed later.) Except for N-acetylglycine the recoveries

TABLE III

RELATIVE MOLAR RESPONSE VALUES OF PHENACYL ESTERS DETERMINED AGAINST OCTADECANE = 1

GLC conditions as in Fig. 1. Amount injected, 1 μl.

Phenacyl ester	*Relative molar response ± S.D. (n=10)*
Formate	0.375 ± 0.0089
Acetate	0.448 ± 0.0067
Propionate	0.506 ± 0.011

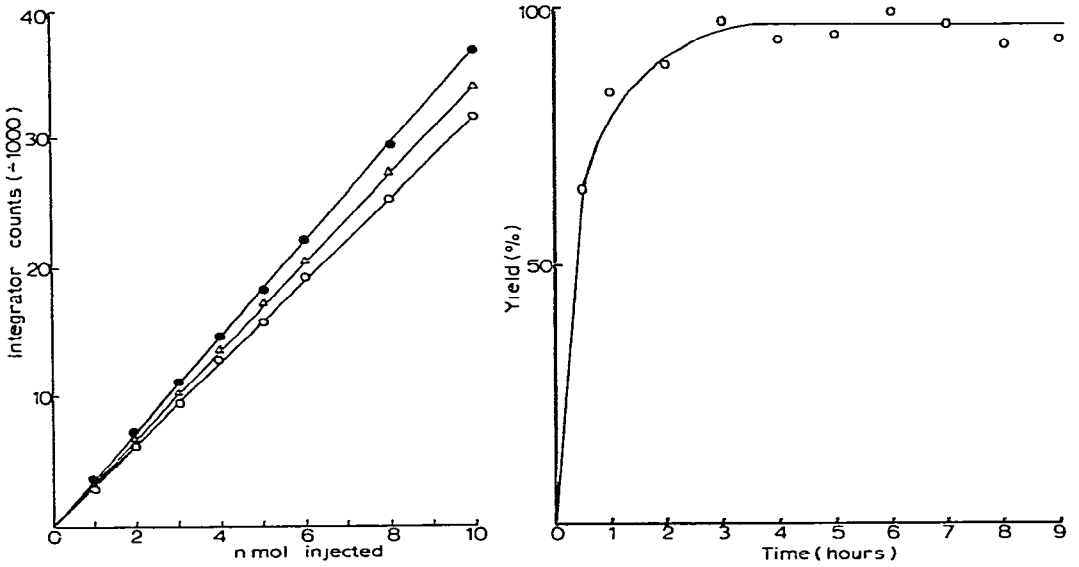

Fig. 2. Response–concentration curves for phenacyl ester derivatives. Preparation of samples: a solution was prepared containing phenacyl formate, phenacyl acetate and phenacyl propionate (10 μmol of each/ml benzene). This solution was further diluted to give the required concentration of phenacyl ester derivatives. Sample size, 1 μl. GLC conditions as in Fig. 1. ●, Propionates; △, acetate; ○, formate.

Fig. 3. Time course for hydrolysis of N-acetylglycine with 6 *M* HBr. Individual samples (683 nmol) were hydrolysed with 50 μl of 6 *M* HBr in sealed tubes at 110°C for increasing periods of time. The tubes were frozen, opened, and the contents allowed to thaw. The contents were neutralized to phenolphthalein end-point with 2 *M* KOH. The tube contents were dried under nitrogen gas at 80°C and the ester prepared as described in the Experimental section. GLC conditions as in Fig. 1.

with acid hydrolysis were below 90%. The low recoveries were attributed to losses of volatile acetic acid despite the use of sealed tubes or Reacti-vials for the hydrolysis and cooling before opening. It was claimed that N-formyl groups in peptides could be removed by refluxing with 0.5 *M* HCl in methanol for 1 h, with yields of 80–95%[59]. We did not investigate acid hydrolysis any further but studied alkaline hydrolysis.

Fig. 4 shows the time courses for the alkaline hydrolysis of N-acetylalanine and N-acetylglycine at 15 p.s.i. (123°C). N-Acetylalanine required 3 h for complete hydrolysis with 1 *M* KOH. About 2 h were required for N-acetylglycine using 2 *M* KOH. The use of higher concentrations of KOH to shorten the hydrolysis time was not studied, because the excess of KBr formed on neutralization subsequently interfered with the formation of the phenacyl esters.

The results for the hydrolysis of N-acetyl- and N-formylamino acids with 1 *M* KOH are given in Table IV. The values were about 10% higher than those for acid hydrolysis and ranged from 93.1% for N-formyl leucine to 97.5% for N-acetylaspartic acid.

Some O-acetylated hexoses and cholesterol acetate were hydrolysed with 0.5 *M* KOH at 123°C for 3 h. The results in Table V show that the expected molar ratios of acetate were obtained for each compound. The N-acetyl group in N-acetylmannosamine required 1.0 *M* KOH for complete hydrolysis. O-Acetyl groups are more easily hydrolyzed[19] and weaker alkali may be used than for N-acetyl groups. It was

TABLE IV

RECOVERY OF ACETATE AND FORMATE AS PHENACYL ESTERS AFTER ACID AND ALKALINE HYDROLYSIS OF N-ACETYL- AND N-FORMYLAMINO ACIDS

Acid hydrolysis for 4 h with 6 *M* HBr at 110°C ($n=3$). Alkaline hydrolysis for 3 h with 1 *M* KOH at 15 p.s.i. (123°C) ($n=5$). GLC conditions as in Fig. 1.

Compound	*Acid hydrolysis*		*Alkaline hydrolysis*	
	Calculated (nmol)	*Recovery (%) ± S.D.*	*Calculated (nmol)*	*Recovery (%) ± S.D.*
N-Acetylalanine	150	87.3 ± 1.76	59	94.9 ± 1.69
N-Acetylaspartic acid	110	83.6 ± 3.96	41	97.5 ± 1.34
N-Acetylglutamic acid	145	85.7 ± 2.11	30	96.1 ± 3.70
N-Acetylglycine	140	93.4 ± 4.67		*
N-Acetylleucine	148	86.9 ± 1.70		*
N-Acetylmethionine	125	89.1 ± 2.55	52	96.3 ± 1.38
N-Acetylphenylalanine		*	72	96.9 ± 1.16
N-Acetyltyrosine	135	85.1 ± 3.73	43	95.1 ± 4.37
N-Formylleucine	150	84.9 ± 4.67	61	93.1 ± 1.37
N-Formylmethionine	131	88.7 ± 4.62	65	96.6 ± 1.28
N-Formylvaline	141	88.6 ± 4.76	80	95.2 ± 1.63

* Not determined.

claimed that O-acetyl groups in lipopolysaccharides could be selectively determined after hydrolysis with 0.05 *M* NaOH for 3–4 h at room temperature[42].

The method was required for application to proteins and peptides and it was necessary to look both for spurious production of formate and acetate resulting from alkali treatment and also any occluded or adsorbed from buffer solutions during the isolation of the protein. Table VI presents the results for the production of formate and acetate from some amino acids and sugars after treating them with 1 *M* KOH at 123°C for 3 h. Sugars gave rise to a lot of acetate and formate. Thus, from 1.0 μmol of galactose, 25 nmol of formate and 19 nmol of acetate were obtained. Amongst the amino acids tested, the highest yields of formate and acetate were given by cysteine. One μmol of cysteine produced about 10 nmol of formate and 20 nmol of acetate, but cystine produced less than twice as much on a mol to mol basis. Serine

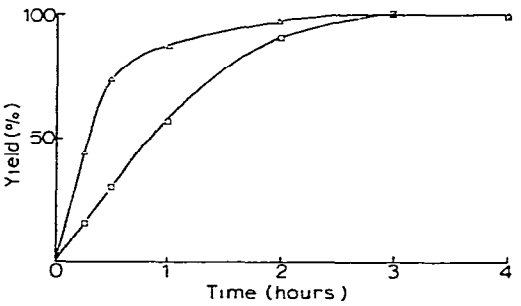

Fig. 4. Alkaline hydrolysis of N-acetylated compounds. △, 2 *M* KOH; N-acetylglycine. □, 1 *M* KOH; N-acetylalanine.

TABLE V

RECOVERY OF ACETATE FOLLOWING ALKALINE HYDROLYSIS OF ACETYLATED SUGARS AND CHOLESTEROL

Hydrolysis with 25 μl of 0.5 *M* KOH at 15 p.s.i. (123°C) for 3 h, except for N-acetylmannosamine 1.0 *M* KOH.

Compound	*Calculated (nmol)*	*Recovery (nmol ± S.D., n = 5)*	*Residues (mol/mol)*
Arabinitol pentaacetate	12.4	61 ± 2.45	5
Epi-2-inositol pentaacetate	8.97	45 ± 2.50	5
Epi-inositol hexaacetate	8.10	47 ± 1.64	6
Epi-inosamine hexaacetate	6.96	40 ± 2.16	6
N-acetylmannosamine	48.0	47 ± 2.12	1
Cholesterol acetate	35.0	32 ± 1.43	1

gave a small amount of acetate (2.5 nmol/μmol serine), whereas threonine gave 0.7 nmol acetate/μmol.

It was reported that acetate in proteins could not be determined after alkaline hydrolysis because of the decompositon of certain amino acids, particularly threonine. Also, sugars yielded both formate and acetate[41]. Our results may be compared with those using acid hydrolysis. Threonine reacted with 3 *M* H_2SO_4 at 105°C for 24 h yielded 9.3 nmol acetate/μmol and with 6 *M* H_2SO_4 at 110°C for 16 h 1.0 μmol of monosaccharide gave 6.5 nmol of acetate[41].

It was claimed that propionyl groups could only be determined in the absence of cystine and cysteine using the hydrazinolysis method[19]. As reported in Table VI only threonine yielded a small amount of propionic acid and this was not sufficient to prevent propionyl group determination.

TABLE VI

PRODUCTION OF FORMATE AND ACETATE FROM SUGARS AND FREE AMINO ACIDS AFTER TREATMENT WITH ALKALI

Sugars ($n = 3$) and amino acids ($n = 4$) hydrolyzed with 50 μl of 1 *M* KOH at 15 p.s.i. (123°C) for 3 h.

Compound	*Amount hydrolyzed (μmol)*	*nmol ± S.D.*	
		Formate	*Acetate*
Galactose	1.8	45.0 ± 3.61	34.0 ± 4.72
Glucose	2.1	43.0 ± 3.99	35.0 ± 7.09
Lactose	1.85	54.0 ± 6.11	39.0 ± 6.08
Arginine	2.36	nil	nil
Aspartic acid	3.16	0.36 ± 0.08	0.66 ± 0.06
Glutamic acid	3.04	0.33 ± 0.09	0.26 ± 0.04
Cysteine	2.37	23.2 ± 3.08	45.9 ± 5.57
Cystine	1.64	26.4 ± 5.01	49.2 ± 5.22
Serine	3.64	1.22 ± 0.32	9.02 ± 0.36
Threonine*	3.06	1.89 ± 0.43	2.22 ± 0.40

* Propionic acid 1.31 nmol ± 0.23 S.D. was also found.

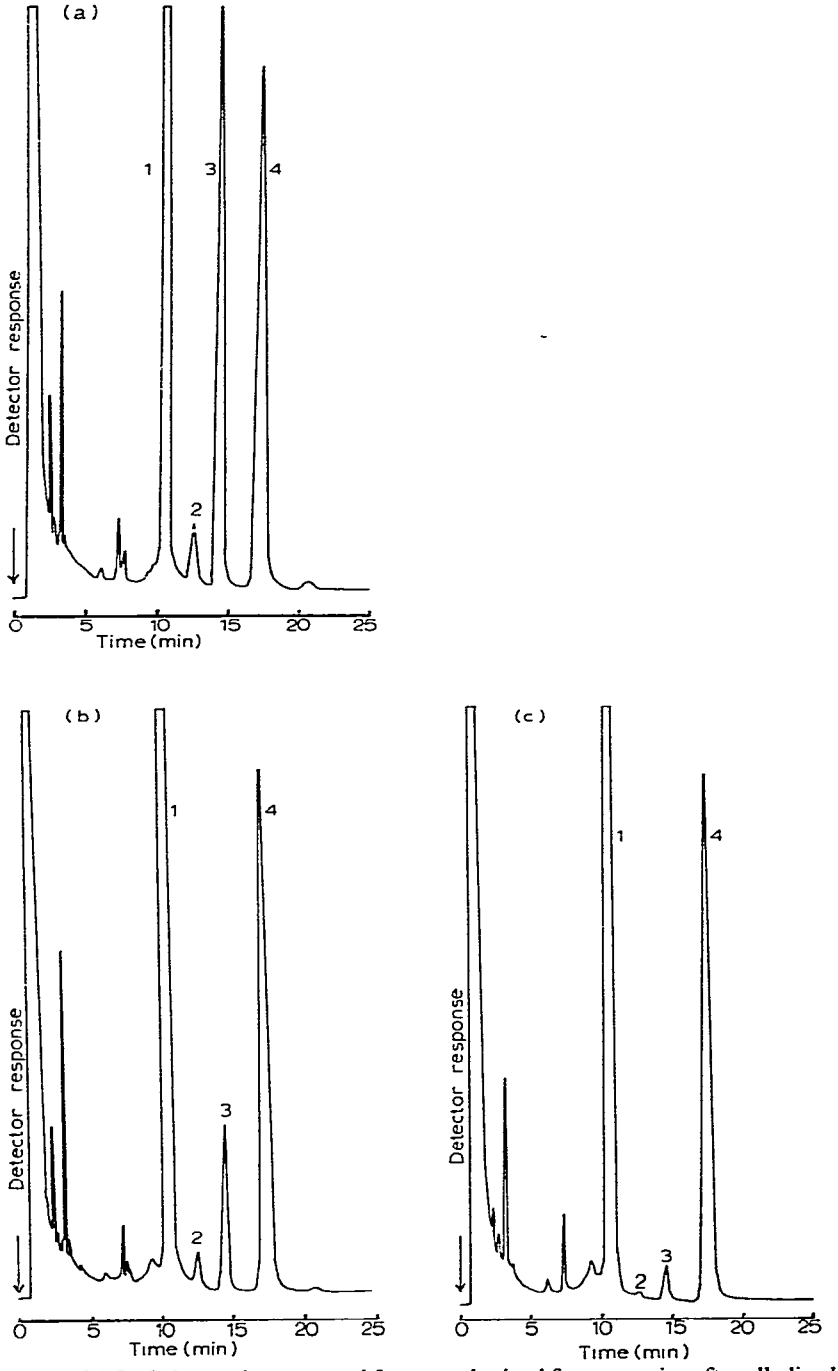

Fig. 5. GLC of phenacyl acetate and formate obtained from proteins after alkaline hydrolysis. (a) Chicken egg albumin (23 nmol), (b) carbonic anhydrase (37 nmol) and (c) egg white lysozyme (100 nmol). Each sample was dissolved finally in 25 μl of benzene and 1 μl injected. GLC conditions as in Fig. 1. Peaks: 1 = phenacyl bromide; 2 = phenacyl formate; 3 = phenacyl acetate; 4 = octadecane (internal standard).

It may be concluded that the spurious production of excess acetate and formate due to side reactions[21] when determining these groups on proteins may be overcome by the appropriate use of control samples. In addition, compensation can be made for acetate and formate contaminants of both the samples and the reagents.

The method was applied to lysozyme, carbonic anhydrase and ovalbumin which were dialysed four times against distilled water and lyophilised. The dialysis residue was then acidified with 1 *M* HBr and lyophilised four more times in order to remove free acetate and formate. However, some free acetate and formate were still detectable. Sephadex gel filtration as a method of removing bound ligands from macromolecules[60] was not satisfactory for removing formate and acetate because of the high blank values obtained from water samples passed through the column. These high values were attributed to traces of carbohydrate material from the Sephadex.

Fig. 5 shows the GLC traces obtained with carbonic anhydrase, chicken egg albumin and egg white lysozyme. All three proteins gave peaks which showed the presence of small amounts of formate. In addition lysozyme showed a peak for acetate. Control values were obtained by preparing the phenacyl esters without previous alkaline hydrolysis. The quantitative results are given in Table VII. The controls yielded values of less than 0.09 mol acetate/mol protein and if these were deducted from the values after alkaline hydrolysis, the results agreed with the expected molar ratios of 4, 1 and 0 for albumin, carbonic anhydrase and lysozyme, respectively.

TABLE VII

DETERMINATION OF ACETATE IN PROTEINS

Hydrolysis conditions: 1 *M* KOH at 123°C for 3 h.

Protein	*Acetate/protein ratio (mol/mol)*		
	Without hydrolysis (n=3)	*With hydrolysis (n=5)*	*Difference*
Albumin	0.0713 ± 0.006	3.971 ± 0.082	3.90
Carbonic anhydrase	0.0868 ± 0.004	1.128 ± 0.033	1.04
Lysozyme	0.0534 ± 0.003	0.092 ± 0.011	0.04

The determination of formate in a protein has not been determined here, although the method gives good results with N-formylamino acids. The determination of phenacyl formate presents no particular problem, but where formic acid was determined as its methyl ester by GLC there were problems with a large tailing solvent peak[50]. GLC offers a more convenient method than that involving hydrazinolysis for the determination of both formyl and acetyl groups in proteins[21]. Enzymic methods required 100–900 nmol[11], 17–250 nmol[10], a minimum of 80 nmol with an average recovery of 90%[15], 5–55 nmol[17] or 3–12 nmol[18] of acetate. It is often difficult to make a direct comparison between GLC methods because certain details are often missing, *e.g.* final volume of sample, the size of the aliquot injected or the attenuation of the recorder[34]. In general, 0.5 ml or more of biological fluid, such as plasma containing 0.05–10 μmol of acetate/ml[34] was used for GLC and no report was found where attempts had been made to scale down the amount used. Protein samples containing 8–17 μmol of acetate were analyzed by GLC[40]. The method described here allows the

determination of 20–100 nmol of covalently bound acetate (also formate and propionate). Further studies are in progress to scale down these quantities.

ACKNOWLEDGEMENTS

A.D. thanks the Science and Medical Research Councils of Great Britain and G.D. Searle Limited for financial support.

Mass spectra were obtained by courtesy of Professor V. Gold, F. R. S. and Professor A. J. B. Robertson, Chemistry Department, King's College.

REFERENCES

1 K. Narita, *Biochim. Biophys. Acta*, 28 (1958) 184.
2 J. L. Brown and W. K. Roberts, *J. Biol. Chem.*, 251 (1976) 1009.
3 H. Bloemendal, *Science*, 197 (1977) 127.
4 L. R. Croft, *Handbook of Protein Sequence Analysis*, Wiley, Chichester, New York, Brisbane, Toronto, 2nd ed., 1980.
5 R. Sarges and B. Witkop, *J. Amer. Chem. Soc.*, 87 (1965) 2011.
6 G. Kreil and G. Kreil-Kiss, *Biochem, Biophys. Res. Commun.*, 27 (1967) 275.
7 R. W. von Korff, *J. Biol. Chem.*, 210 (1954) 539.
8 I. A. Rose, *Methods Enzymol.*, 1 (1955) 591.
9 M. Soodak, *Methods Enzymol.*, 3 (1957) 266.
10 E. Lundquist, U. Fugmann and H. Rasmussen, *Biochem. J.*, 10 (1961) 393.
11 L. D. Stegink, *Anal. Biochem.*, 20 (1967) 502.
12 L. D. Stegink and C. S. Vestling, *J. Biol. Chem.*, 241 (1966) 4923.
13 H. U. Bergmeyer and H. Moellering, *Biochem. Z.*, 344 (1966) 167.
14 F. J. Ballard, O. H. Filsell and I.G. Jarrette, *Biochem. J.*, 126 (1972) 193.
15 S. C. Kuo and E. S. Younathan, *Anal. Biochem.*, 55 (1973) 1.
16 M. Schulman and H. G. Wood, *Anal. Biochem.*, 39 (1971) 505.
17 M. Schulman and H. G. Wood, *Methods Enzymol.*, 35 (1975) 298.
18 R. W. Guynn and R. L. Veech, *Methods Enzymol.*, 35 (1975) 302.
19 D. M. P. Phillips, *Biochem. J.*, 86 (1963) 397.
20 D. M. P. Phillips, *Biochem J.*, 107 (1968) 135.
21 G. Schmer and G. Kreil, *Anal. Biochem.*, 29 (1969) 186.
22 J. Ludowieg and A. Dorfman, *Biochim. Biophys. Acta*, 38 (1960) 212.
23 P. G. Johansen, R. D. Marshall and A. Neuberger, *Biochem. J.*, 77 (1960) 239.
24 J. Z. Augustiniak and W. G. Martin, *Can. J. Biochem.*, 43 (1965) 291.
25 A. Yoshida, *Anal. Biochem.*, 49 (1972) 320.
26 H. Jörnvall, H. Ohlsson and L. Philipson, *Biochem. Biophys. Res. Commun.*, 56 (1974) 304.
27 A. D. Auffret, D. H. Williams and D. R. Thatcher, *FEBS Lett.*, 1978 (90) 324.
28 T. L. Perry, S. Hansen, S. Diamond, B. Bullis, C. Mok and S. B. Melançon, *Clin. Chim. Acta*, 29 (1970) 369.
29 M. Wadke and J. M. Lowenstein, *Methods Enzymol.*, 35 (1975) 307.
30 G. Desch and B. Descomps, *Clin. Chim. Acta*, 76 (1977) 193.
31 M. F. Laker and M. A. Mansell, *Ann. Clin. Biochem.*, 15 (1978) 228.
32 L. G. Nielsen, K. O. Ash and E. Thor, *Clin. Chem.*, 24 (1978) 348.
33 C. D. Tollinger, H. J. Vreman and M. W. Weiner, *Anal. Chem.*, 50 (1978) 1138.
34 M. Kveim and J. E. Bredesen, *Clin. Chim. Acta*, 92 (1979) 27.
35 J. P. Ryan, *Anal. Biochem.*, 108 (1980) 374.
36 R. E. Hillman, *Clin. Chem.*, 24 (1978) 800.
37 S. B. Dees and C. W. Moss, *J. Chromatogr.*, 171 (1979) 466.
38 G. R. Shepherd and B. J. Noland, *Anal. Biochem.*, 26 (1968) 325.
39 H. J. Vremen, J. A. Dowling, R. A. Raubach and M. W. Weiner, *Anal. Chem.*, 50 (1978) 1138.
40 D. N. Ward and J. A. Coffey, *Biochemistry*, 3 (1964) 1575.
41 D. N. Ward, J. A. Coffey, D. B. Ray and W. M. Lamkin, *Anal. Biochem.*, 14 (1966) 243.

42 I. Fromme and H. Beilharz, *Anal. Biochem.*, 84 (1978) 347.
43 W. A. Schroeder, J. T. Cua, G. Matsuda and W. D. Fenninger, *Biochim. Biophys. Acta*, 63 (1962) 532.
44 H. J. Hoenders and H. Bloemendal, *Biochim. Biophys. Acta*, 147 (1967) 183.
45 R. J. Williams and S. Siggia, *Anal. Chem.*, 49 (1977) 2337.
46 A. Darbre and J. Chauhan, in A. Previero and M.-A. Coletti-Previero (Editors), *Solid Phase Methods in Protein Sequence Analysis, INSERM Symposium No. 5*, Elsevier/North Holland Biomedical Press, Amsterdam, 1977, p. 21.
47 A. Darbre and K. Blau, *J. Chromatogr.*, 17 (1965) 31.
48 G. C. Cochrane, *J. Chromatogr. Sci.*, 13 (1975) 440.
49 R. B. H. Wills, *J. Chromatogr. Sci.*, 10 (1972) 582.
50 K. S. Bricknell and S. M. Finegold, *J. Chromatogr.*, 151 (1978) 374.
51 C. J. Pedersen, *J. Amer. Chem. Soc.*, 89 (1967) 7017.
52 H. D. Durst, *Tetrahedron Lett.*, (1974) 2421.
53 C. L. Liotta, H. P. Harris, M. McDermott, T. Gonzales and K. Smith, *Tetrahedron Lett.*, (1974) 2417.
54 H. D. Durst, M. Milano, E. J. Kikta, Jr., S. A. Connelly and E. Grushka, *Anal. Chem.*, 47 (1975) 1797.
55 B. Davis, *Anal. Chem.*, 49 (1977) 832.
56 S. C. Chan, *Clin. Chem.*, 26 (1980) 1528.
57 A. Darbre and A. Islam, *Biochem J.*, 106 (1968) 923.
58 H. H. Pokras and H. I. Bernstein, *J. Amer. Chem. Soc.*, 65 (1943) 2096.
59 J. C. Sheehan and D. H. Yang, *J. Amer. Chem. Soc.*, 80 (1958) 1154.
60 H. B. F. Dixon, *Biochem. J.*, 159 (1976) 161.