Analysis of the Acetyl Groups in Ovine Luteinizing Hormone by Gas Chromatography*

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A procedure for the analysis of acetyl groups in proteins and peptides has been developed. The procedure involves hydrolysis in strong acid, neutralization to *p*H near 3, and extraction of liberated acetic acid into an organic solvent for quantitative gas chromatography. Recovery of acetic acid from simulated hydrolysates or *N*-acetylglucosamine averaged 94%. Analysis of ovine luteinizing hormone by this procedure indicated 2.47% content of acetyl groups.

We have previously reported that ovine luteinizing hormone (LH or ICSH)[1] contains 8 glucosamine and 3 galactosamine residues per 28,000 mw as estimated after acid-hydrolysis conditions that gave a maximal yield of amino sugars (Walborg and Ward, 1963). Recently Li and Starman (1964) reported that a monomeric form of LH could be obtained at very low *p*H with an estimated molecular weight of 16,300. We have confirmed this observation (D. E. Balke and D. N. Ward, unpublished data) and a recalculation of our previous data indicates 5 (4.48) glucosamine and 2 (1.76) galactosamine residues per molecule of the monomer form. Since our titration data suggested the amino groups were not free (Ward *et al.*, 1961b) we have speculated that these amino groups were acylated, perhaps with acetyl groups. The present work was undertaken to provide a convenient method for the estimation of acetyl groups in proteins and peptides and to examine ovine LH for acetyl groups.

Acetyl groups in proteins have been determined by direct distillation from a hydrolysate, requiring impractical quantities of protein for our purposes (Johansen *et al.*, 1960), or by use of the hydrazinolysis technique (Narita, 1958). Recently, Phillips (1963) has reported a modification of the hydrazinolysis procedure with conversion of the acetyl hydrazide to the dinitrophenyl derivative for quantitation. Our approach has been to utilize gas chromatography for the estimation of acetic acid liberated after hydrolysis. This represents a convenient approach, but for technical reasons in the present form of the method it is less sensitive than the procedure of Phillips (1963).

EXPERIMENTAL PROCEDURE

Materials.—The ovine LH preparations were prepared by chromatography on carboxymethyl-cellulose as described earlier (Ward *et al.*, 1959, 1961a) followed by gel filtration on Sephadex G-75.[2] *N*-Acetyl glucosamine was a commercial preparation obtained from General Biochemicals, Inc., lot 31272. All the chemicals were reagent grade. Ethyl acetate was analyzed for traces of acetic acid before use. If acetic acid was present the bottle was discarded.

Gas Chromatography.—A Research Specialties Model 600 gas chromatograph was used. The instrument was equipped with a direct-injection, all-glass column, a ^{90}Sr ionization detector, and a Minneapolis-Honeywell recorder with a disk integrator. The carrier gas was argon and the column was 0.4 × 175 cm packed with 10% LAC 2-R-446 (a polyethylene glycol adipate obtained from Research Specialties Co.) on acid-washed Chromosorb W, 60/80 mesh (Johns Manville). This system is similar to that of Boettcher *et al.* (1960) for the gas chromatography of volatile fatty acids. The column was operated at 117° with a gas flow rate of 16.7 ml/min. The detector voltage was 1000, with the signal fed to the recorder at an attenuation of 5.

Standard solutions of acetic acid in ethyl acetate were prepared each day. Dilutions were made to obtain solutions containing from 1 to 5 μg of acetic acid per 10 μl aliquot. For reproducibility, 10 μl quantities were routinely used for sample or unknown injection into the gas chromatogram. Under our conditions acetic acid emerges after 9.0 minutes as a sharp peak with a slight trailing edge.

Hydrolysis and Extraction.—A sample estimated to yield 500–1000 μg acetic acid was weighed and transferred to a hydrolysis tube. One-half to 1 ml of 6 N HCl was added, the system was flushed with nitrogen, and the tube was sealed. The sample was then hydrolyzed for 16 hours at 110° in a silicone oil bath. The tube was positioned so that only the liquid-containing portion was submerged in the oil bath, thus allowing reflux from the upper walls.

After hydrolysis the tube was cooled in an ice bath, centrifuged to remove droplets from the walls, and opened. The sample was immediately adjusted to *p*H 2.7–3.0 with saturated NaOH (0.1 N NaOH near the end point). This step was conveniently carried out in a graduated 15-ml centrifuge tube with miniature electrodes (Leeds & Northrup No. 124138). Total volume at this point should be approximately 1.5 ml. The sample was then extracted with 1 ml of ethyl acetate using brisk agitation on a Vortex stirrer. The ethyl acetate was withdrawn with a capillary pipet and transferred to a small stoppered test tube containing approximately 100 mg of anhydrous sodium sulfate. After a lapse of a few minutes to dry the ethyl acetate, 10-μl aliquots were injected into the gas chromatograph for the analysis. Six to eight such extracts were generally required before all detectable acetic acid was removed from the aqueous sample. From the total acetic acid in the several extracts the number of acetyl groups in the original sample was estimated.

RESULTS

The gas chromatography used in this study gave a peak for acetic acid which has a slight trailing edge, so

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[1] Abbreviation used in this work: LH, luteinizing hormone (ICSH = interstitial-cell-stimulating hormone).

[2] This filtration separated an inactive impurity (5–8% of the total weight) which is present in most of the LH preparations obtained from carboxymethyl-cellulose chromatography (N. R. Ray and D. N. Ward, unpublished observations).

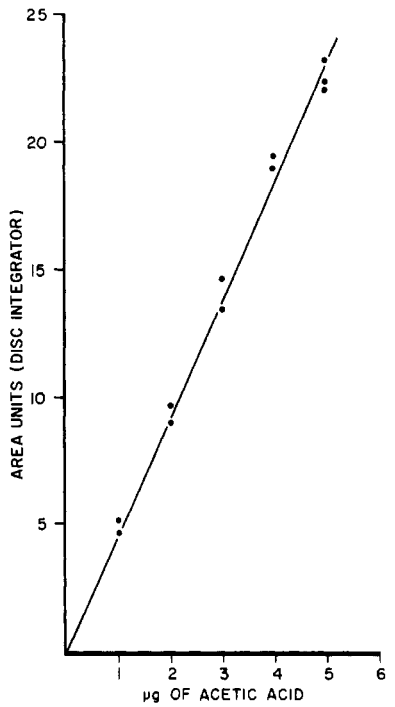

FIG. 1.—Standard curve for the gas chromatography of acetic acid in ethyl acetate. (Chromatographic conditions as indicated in text.)

it was necessary to show that the peak area could be estimated satisfactorily using the disk integrator. The disk integrator was operated so that there was always some motion for the integrator at base line. The corresponding base-line unit correction has been applied to the peak area units obtained with the disk integrator. Figure 1 shows that a linear standard curve was obtained under these conditions; reproducibility was good. For quantities of acetic acid greater than 8 μg it was necessary to use a greater attenuation. Most of the analyses reported, however, were on sample aliquots in the range of the standard curve shown in Figure 1.

To simulate hydrolysis conditions, 1 mg of acetic acid was added to 1 ml of 5.7 N HCl and the *p*H was adjusted to 2.7. The sample (1.5 ml) was then extracted eight times with 1-ml portions of ethyl acetate, the ethyl acetate was removed, and the extracts were dried over anhydrous sodium sulfate. For analysis, duplicate or triplicate 10-μl aliquots of each extract were sampled for quantitative chromatography. The last two extracts did not contain measurable acetic acid. The first six extracts accounted for 94.3% of the total acetic acid. Figure 2 shows the per cent of the total acetic acid in the individual extracts. From these figures and the relative volumes in the two phases a partition coefficient of approximately 1 can be estimated for acetic acid in this system. Two additional simulated hydrolyses as above gave recovery values of 96.8 and 99.5%.

To test the method on an acylated compound related to luteinizing hormone we next tried *N*-acetylglucosamine. The sample of *N*-acetylglucosamine was analyzed on a Spinco amino acid analyzer, Model 120B, according to conditions for the analysis of glucosamine (Walborg *et al.*, 1963) without prior hydrolysis. A second analysis, after hydrolysis under conditions giving maximal yield of amino sugars from luteinizing hormone, was also carried out; i.e., 4 hours in 2 N HCl at 100°, sealed under nitrogen. The results of these analyses are given in Table I. It can be seen that the

TABLE I

ANALYSIS OF *N*-ACETYL GLUCOSAMINE BY ION-EXCHANGE CHROMATOGRAPHY[a]

Component	After Hydrolysis (μmoles)[b]	Before Hydrolysis (μmoles)[b]	Ninhydrin-positive Material Released during Hydrolysis (μmoles)[a]
Glucosamine	0.873	0.007	0.867
Ammonia	0.168	0.022	0.146
Total			1.013

[a] Ninhydrin-positive material, before and after hydrolysis. [b] Values are expressed as the μmoles measured per theoretical μmole of *N*-acetylglucosamine in the weight of sample analyzed. Analyses as per Walborg *et al.* (1963).

sample contained only small quantities of free glucosamine and ammonia prior to hydrolysis, and no additional ninhydrin-positive material. After hydrolysis 87% of the theoretical glucosamine was recovered. The increase in ammonia would correspond to that theoretically derived from decomposition of glucosamine during hydrolysis and preparation of the sample, assuming 99% purity of the *N*-acetylglucosamine. This assumption seems reasonable in view of the analysis before hydrolysis (Table I) and the fact that only one spot was detected when the *N*-acetylglucosamine was chromatographed on paper in the system butanol–acetic acid–water (4:1:5).

In two separate experiments *N*-acetylglucosamine was hydrolyzed in 1 ml of 6 N HCl, adjusted to *p*H 2.8, and extracted with 1-ml portions of ethyl acetate as above. The analyses accounted for 85.7 and 96.8% of the expected acetic acid, assuming the purity of the *N*-acetylglucosamine as above. On the basis of the simulated hydrolysate and the analysis of the *N*-acetylglucosamine the average recovery of acetic acid from acetyl groups by this procedure is 94%.[3]

Luteinizing hormone was analyzed by this procedure. The values obtained from two separate analyses are listed in Table II. This represents the first direct demonstration of acetyl groups in LH. The two

TABLE II

ANALYSIS OF LUTEINIZING HORMONE ACETYL GROUPS

Weight of LH Hydrolyzed (mg)	Acetic Acid Liberated (μg)	Per cent, as Acetic Acid (%)
27.7	666	2.40
24.6	624	2.54
		2.47 (avg)

[3] Analysis of ovalbumin preparations requires over 100 mg of protein to get sufficient acetyl groups for an analysis under these conditions. The analysis is complicated by the requirement that the hydrolysis be carried out with 1–1.5 ml of HCl, which produces such a concentrated hydrolysate the extractions form emulsions and require centrifugation in closed tubes. Ovalbumin (Calbiochem., lot 33062) analyzed by this procedure gave the following values: 136 mg contained 11.3 μmoles acetic acid; 139 mg contained 12.9 μmoles acetic acid. This represents 89 and 103%, respectively, assuming 4 acetyl groups per mole as determined by Johansen *et al.* (1960). Another ovalbumin preparation (Sigma, lot A 102B-250), stated to contain three components on electrophoresis, gave 22.1 μmoles and 22.6 μmoles/140 mg (171 and 176% of theoretical, respectively).

analyses indicate 6.53 and 6.89 acetyl residues per 16,300 mw monomer of LH (Li and Starman, 1964).

Discussion

The method of analysis for acetyl groups presented here is convenient and direct. Recovery is better than the 70% reported by Phillips (1963), but sensitivity is poorer than reported by Phillips (approximately 1 μmole per determination) since our procedure requires 5–10 μmoles per determination. Further work is directed at improving the sensitivity of our procedure by using different hydrolysis conditions and different conditions of isolation of the acetic acid from the hydrolysate.

The use of ethyl acetate for extraction of acetic acid involves the risk of hydrolysis of the solvent if a *p*H below 2.0 is employed. Other experiments were done using ether for the extraction. Under these conditions the partition coefficient for acetic acid in the hydrolysate and ether is also approximately 1. However, in concentrated protein hydrolysates difficulty was encountered with the formation of emulsions and also bubble formation in the sampling with the microliter syringe for quantitative gas chromatography. Since it was also necessary to employ a solvent that would emerge ahead of acetic acid in the chromatography, ethyl acetate was finally selected.

The estimate of acetyl groups in LH indicates there are sufficient acetyl residues to acylate all the amino sugars we previously observed (Walborg and Ward, 1963) and possibly the N-terminal amino acid in addition. The assumption that the acetyl groups are associated with the amino sugars rests largely on titration data (Ward *et al.*, 1961b). We have previously reported difficulty in measuring an N-terminal amino acid by dinitrophenylation (Ward *et al.*, 1959). However, since the estimate of amino sugars represents a minimum value (Walborg and Ward, 1963), it will be necessary to obtain additional data before one can decide whether there are additional amino sugars not detected with our previous measurements which also have acylated amino groups or whether the N-terminal amino acid may also carry an acetyl group. The present data would not rule out this possibility.

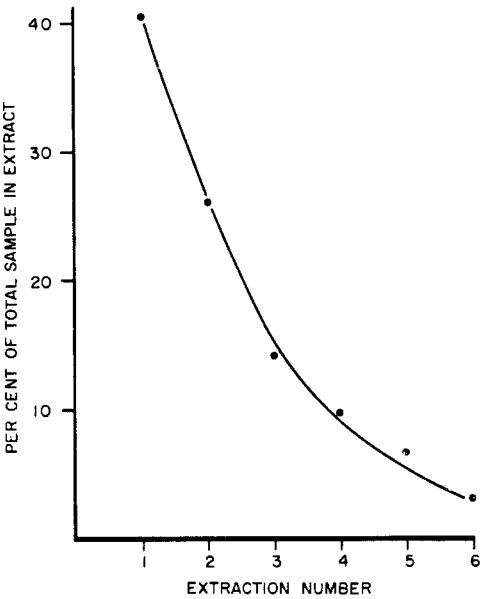

Fig. 2.—Per cent of acetic acid extracted from a 1.5-ml simulated hydrolysis at *p*H 2.7 containing 1000 μg acetic acid. Successive extractions were with 1-ml portions of ethyl acetate.

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