A Micromethod for the Determination of *N*- and *O*-Acetyl Groups

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In connexion with studies on the *N*-terminal groups of histones (Phillips, 1961), a method was needed for measuring about 0.1 μ mole of aliphatic acyl groups derived from a few milligrams of protein. Initial experiments were done in which acetyl-amino acids and ovalbumin were hydrolysed in sulphuric acid and an ether extract was chromatographed, by using modifications of the method of Brown & Hall (1950). The acetate spot was readily detectable by indicators but it was transient and the method could not be made quantitative. When the extract was treated with hydrazine, acetic acid hydrazide could be demonstrated (Satake & Seki, 1950), but it could not be measured and, moreover, in the hydrazinolysate of a protein, the large amounts of amino acid hydrazides hinder the detection of unaminated hydrazides. The reaction of amino acid hydrazides with 1-fluoro-2,4-dinitrobenzene has been used in a method of analysis of *C*-terminal groups of proteins (Ohno, 1953), and it was considered that the formation of aliphatic acid DNP-hydrazides ($R \cdot CO \cdot NH \cdot NH \cdot DNP$) would allow their measurement provided that they could be separated from the very large amounts of DNP-amino acid hydrazides formed concomitantly.

DNP-hydrazides have been used for the qualitative analysis of the aliphatic acid components of natural fats and oils (Inouye & Noda, 1955), though in this case the derivatives were formed by reaction of the acid chlorides with 2,4-dinitrophenylhydrazine, a procedure which could probably not be applied to proteins.

MATERIALS AND METHODS

Materials

The hydrazides of formic, acetic acid, propionic acid and *n*-butyric acid were prepared from esters as described by Narita (1958). The DNP derivatives were prepared by coupling the hydrazides with fluorodinitrobenzene in 66% (v/v) ethanol buffered at pH 3. The yellow or orange crystalline products were recrystallized from hot ethanol. They were then free of dinitrophenol and DNP-hydrazine. Propionyl-DL-phenylalanine was prepared by a general method of Du Vigneaud & Meyer (1932). The ovalbumin was a sample of the preparation O (Johansen, Marshall & Neuberger, 1960), kindly given by Dr R. D. Marshall. It contained 90% of protein and 4 acetyl groups/molecule of protein (mol.wt. 45 000), as measured by distillation methods with the amount of acetic acid at about 100 μ moles.

The chymotrypsin was the crystalline salt-free enzyme from Armour and Co. Hydrazine was the '95%+' reagent from Kodak Organic Chemicals, Rochester, N.Y., U.S.A.

Recommended method

It is convenient to work with a sample which will yield 0.5–1 μ mole of acyl groups. This will give material sufficient for five chromatograms. The sample is weighed (or pipetted and dried) into a 13 cm. test tube with a B14 neck and about 0.3 ml. of hydrazine added. For the measurement of *O*-acetyl groups, the stoppered tube is kept at 20° for 1 hr. with shaking to dissolve the sample if necessary. For total (*N*- and *O*-) acetyl groups the stopper is greased and secured with soft wire. Both the tube and stopper have glass hooks for this purpose. The sample is then heated at 100° in an oven for 17 hr. The hydrazine is removed from the samples in 10–15 min. at 0.5 mm. Hg pressure (the pump being protected by a trap containing orthophosphoric acid), and during evaporation the tube is kept in an ice–water bath and agitated intermittently.

The residue is dissolved in 1 ml. of 0.2M-citrate–HCl buffer, pH 3, the solution adjusted to pH 3 (indicator paper) and 1.6 ml. of ethanol added. Finally 0.4 ml. of a stock solution of fluorodinitrobenzene (5 m-moles, approx. 0.66 ml. in 10 ml. of ethanol) is added and the stoppered tube placed in a water bath at 38° for 4 hr. A clear yellow–orange solution is produced. The volume is reduced to not more than 1 ml. by swirling vigorously under vacuum in a warm water bath and then made up to 2 ml. with water, and the solution extracted four times with 4 ml. of diethyl ether. The ether layer is transferred each time with a Pasteur pipette to a 15 cm. B24 test tube. For cleaner separation of the phases the tubes are centrifuged for a few minutes at low speed. The combined ether extracts are evaporated to dryness and sublimed at about 0.2 mm. Hg pressure at 90° for two periods of 1.5 min. to remove dinitrophenol, DNP-hydrazine and excess of fluorodinitrobenzene. This is done in a simple apparatus (Mills, 1952), the 'cold finger' being refilled with cold water before each sublimation period. The final residue is dissolved in 0.5 ml. of redistilled ethyl methyl ketone and 0.1 ml. taken for two-dimensional descending chromatography. A 46 cm. $\times$ 46 cm. sheet of Whatman 3MM paper is used. This is soaked in 0.05M-phthalate buffer, pH 6, and allowed to dry before the sample is applied.

The chromatogram is first run with butan-2-ol saturated with 0.05M-phthalate buffer, pH 6, requiring about 50 ml. per sheet. It is important that the solvent is not allowed to reach the lower edge of the paper or crowding of the fast spots occurs. After being dried, the sheet is run in the other direction with M-phosphate buffer, pH 6, which also requires about 50 ml. per sheet. The chromatogram is dried in the dark at room temperature and placed in an atmosphere of ammonia for a few minutes, which causes the

spots of aliphatic acid DNP-hydrazides to change colour from yellow to brown. The spots of interest, as well as blank areas of similar size, are cut out and extracted by heating with 5 ml. of propan-2-ol-water (1:4, v/v) at 38° for 1 hr. To each tube 0.5 ml. of *N*-NaOH is added, which re-forms the brown colour noted with ammonia (see above), and the eluates are immediately read at 420 μ . The molecular extinction coefficients for the acetic acid and propionic acid derivatives at 420 μ were 19 500 and 20 400 respectively. Some further information on the steps in the method is given below.

(a) *Hydrazinolysis*. A temperature of 100° was chosen since the hydrazinolysis could be completed overnight (see Fig. 1), whereas at 90° at least 30 hr. was required. At temperatures above 100° the convenient technique of wiring the stoppers is insufficient to prevent the loss of hydrazine. With *O*-acyl compounds such as glucose penta-acetate, the hydrazinolysis at 20° is completed in a few minutes, and with ethyl propionate is completed on shaking for 30 min., which corresponds to the disappearance of all droplets of the ester.

(b) *Removal of hydrazine*. The hydrazine must be removed at or below room temperature since acetic acid and propionic acid hydrazides are appreciably volatile, even at 38°, though the loss is reduced if non-volatile substances such as glucose, amino acids or hydrazinolysed protein are present. In 2 min. at 85° at 0.2 mm. Hg pressure, 70% of the acetic acid hydrazide from an ovalbumin hydrazinolysate sublimed. Such sublimates also contain amino acid hydrazides ($\text{H}_2\text{N}\cdot\text{CHR}\cdot\text{CO}\cdot\text{NH}\cdot\text{NH}_2$), especially the alanine, glycine, leucine and serine derivatives, so that sublimation as a method of separation was not investigated further.

(c) *Coupling with 1-fluoro-2,4-dinitrobenzene*. Under the usual conditions of coupling amino acids and proteins at pH 8–9, acetic acid hydrazide reacts incompletely. How-

ever, the yield increases at lower pH and is complete in 3 hr., at pH 3–4, at 38°. With a mixture of amino acids, only 1% of the α -amino groups reacts with fluorodinitrobenzene at pH 4 at 38° and at pH 3 only 0.3% reacts, but proline, leucine, valine and glycine react more readily than other amino acids. Thus, after treatment with fluorodinitrobenzene at pH 3, the amino acid DNP-hydrazides retain a free basic group and are far less readily extracted by ether from aqueous acid than the unaminated DNP-hydrazides. After removal of the bulk of the hydrazine from a protein hydrazinolysate at room temperature, about 6 mg. or 200 μ moles still remain. However, hydrazine reacts only slightly with fluorodinitrobenzene at pH 3, so that in the recommended method there is a large excess of fluorodinitrobenzene at the end of the coupling period. The ability of the hydrazides to react at acid pH with fluorodinitrobenzene is presumably due to their weak basicity. Electrometric titration of acetic acid hydrazide showed a *pK* of 2.9–3.1, and *N*-acetyl-D-alanine hydrazide had a *pK* of about 2.5. As with the α -amino groups of amino acids, the uncharged form reacts far more readily with fluorodinitrobenzene. Hydrazine, with a *pK* of 8.5, would also not be expected to react very much at pH 3–4.

(d) *Extraction of the products*. Two extractions with ethyl methyl ketone-diethyl ether (1:9, v/v) are sufficient to remove the aliphatic acid DNP-hydrazides, but the use of ether alone gave cleaner chromatograms. With protein hydrazinolysates there is a small amount of amino acid derivatives in these extracts amounting to 2–5% of the amino acids originally present, some 60% of this consisting of the glutamic acid and leucine-isoleucine derivatives. On the chromatograms these substances appear mainly in spot 8 and in the brown-yellow streak running from the origin (see Fig. 2).

Aliphatic acid hydrazides can be extracted directly from a hydrazinolysate, after removing the bulk of the hydrazine, by dissolving in *m*- or 2*M*-acetate buffer, pH 5, and extracting with ethyl methyl ketone. These extracts, however, contain over 25% of all the amino acid hydrazides and the chromatograms prepared from them after dinitrophenylation were no better than in the recommended method.

(e) *Chromatography*. An improvement in the separation of the aliphatic (formic to *n*-butyric) acid DNP-hydrazides was noted when the butan-2-ol solvent and the filter paper were buffered at pH 9–11. As it was later found that the DNP-hydrazides are unstable in alkali, this modification was abandoned.

(f) *Controls*. Runs in which only the reagents were processed generally show no spot of acetic acid DNP-hydrazide on the chromatograms but they do show a weak spot in the position of formic acid DNP-hydrazide (spot 9 in Fig. 2). The source of this compound is unknown but it is unlikely to be due to formic acid DNP-hydrazide (see Table 1 and the Results and Discussion section). Recovery experiments with acetic acid hydrazide and DNP-acetic acid hydrazide have shown that the overall loss in the method, amounting to about 30%, occurs at several stages. Some 5–10% of the product is lost in the evaporation of the excess of hydrazine, up to 5% of the DNP derivative is left behind after the four ether extractions and 10–15% further is lost in the chromatography and subsequent steps.

(g) *Stability of acetic acid dinitrophenylhydrazide*. Only about 1% of acetic acid DNP-hydrazide is hydrolysed in

Fig. 1. Hydrazinolysis of ovalbumin: the formation of acetic acid hydrazide (A), propionic acid hydrazide (B), spot 8 (probably pyrrolidonecarboxylic acid hydrazide) (C) and spot 9 (probably an artifact derived from the reagents) (D). Each reading is derived from 2 mg. of ovalbumin treated by the recommended method, except for the time of hydrazinolysis. The position of these substances on the chromatograms are shown in Fig. 2.

24 hr. at 38° at pH 3, so that it is stable under the fluorodinitrobenzene-coupling conditions. It is also stable for at least 6 hr. in ethanol-bicarbonate at 38°. In stronger alkali it is unstable. When n -NaOH is added to the spot eluates to give a final concentration of about 0.1 N -alkali, the extinction at 420 $m\mu$ falls by 0.1%/min. at room temperature. This loss is insignificant provided that there is no long delay in reading the eluates.

Fig. 2. Diagram of the paper chromatogram of the dinitrophenylated hydrazinolysis products of ovalbumin and some other substances. Solvent 1: butan-2-ol shaken with excess of 0.05 M -phthalate buffer, pH 6; upper layer used. Solvent 2: m -sodium phosphate buffer, pH 6. Spots: 1, 2,4-dinitrophenylhydrazine; 2 and 2', 2,4-dinitrophenol; 3, artifact derived from the hydrazine; 4, glutamic acid γ -DNP-hydrazide; 5, alanine DNP-hydrazide; 6, a prominent spot derived from aspartic acid; 7, phenylalanine DNP-hydrazide; 8, probably pyrrolidonecarboxylic acid DNP-hydrazide; 9, formic acid DNP-hydrazide and also the position of an artifact observed in control runs; 10, acetic acid DNP-hydrazide; 11, propionic acid DNP-hydrazide; 12, n -butyric acid DNP-hydrazide. The spots with broken edges are not seen on chromatograms prepared from ovalbumin or chymotrypsin hydrazinolysates.

Fig. 3. Spectrum of acetic acid 2,4-dinitrophenylhydrazide. A, 37 μM in water, pH 6; B, 35.5 μM in 0.1 N -sodium hydroxide.

(h) *Measurement of the dinitrophenylhydrazides.* There is a marked change in the spectra of acetic acid and propionic acid DNP-hydrazides in the pH range 7–11 (Fig. 3). The spot eluates are read in alkali at 420 $m\mu$ because acetic acid DNP-hydrazide in neutral solution has λ_{max} 335–340 $m\mu$ (ϵ 13 900) whereas in alkali it has λ_{max} 420 $m\mu$ (ϵ 19 500). With propionic acid DNP-hydrazide the corresponding ϵ values are 14 500 and 20 400. The spectra and spectral changes of the amino acid mono-DNP-hydrazides such as glutamic acid γ -DNP-hydrazide are very similar to those mentioned above, but they have a slightly greater extinction in the range 400–550 $m\mu$ at neutrality which gives them a brownish colour on the chromatograms. The changes in the spectra with pH are presumably due to the formation of the isonitro form.

RESULTS AND DISCUSSION

A diagram of the chromatogram produced from a hydrazinolysate of ovalbumin together with the positions of some other derivatives is given in Fig. 2. The position of acetic acid and propionic acid DNP-hydrazides on the chromatograms from ovalbumin has been confirmed by the addition of authentic samples, and their spectra in the range 300–550 $m\mu$ show the same maxima and changes with pH as the synthetic samples.

Spot 2 and the 2–2' trail are due to 2,4-dinitrophenol derived from the hydrolysis of a little fluorodinitrobenzene still present in the extract applied to the chromatogram. Spot 8 is seen on the chromatograms from many protein hydrazinolysates. It increases with time (see Fig. 1), but only part of it is extracted by ether from the coupled hydrazinolysates. It is brown, like the spots of amino acid DNP-hydrazides, but does not give a ninhydrin reaction; on acid hydrolysis it gives glutamic acid approximately equivalent to the DNP-hydrazide group extinction at the maximum at 420 $m\mu$ in alkali (assuming ϵ is 20 000 in this case). Glutamic acid α -DNP-hydrazide would give a ninhydrin reaction, so that it is very likely that spot 8 is pyrrolidonecarboxylic acid DNP-hydrazide. It appears in quantity on chromatograms run with the products from hydrazinolysed glutamic acid.

However, although amino acids can be found in hydrolysates of the ether extracts, they are present largely as spot 8 and as the DNP-amino acid DNP-hydrazides ($DNP-NH \cdot CHR \cdot CO \cdot NH \cdot NH \cdot DNP$), which are formed to a small extent even at acid pH and are responsible for the streak running from the origin of the chromatogram (see Fig. 2). The spot 8 is the only one due to amino acid mono-DNP-hydrazides seen on the chromatograms prepared from ovalbumin or chymotrypsin, and analyses of acid hydrolysates of the DNP-acetic acid and DNP-propionic acid hydrazides washed from the chromatograms have shown only traces of amino acids.

The yield of acetic acid DNP-hydrazide from ovalbumin rises to a maximum in about 15–20 hr. of hydrazinolysis and slowly declines on continued treatment (Fig. 1). This amount corresponds to about 70% of the acetyl groups known to be present in the protein (Johansen *et al.* 1960). However, the slower rate of formation and continued development of propionic acid DNP-hydrazide (Fig. 1) suggests that this substance, like spot 8, arises in another way. The propionic acid hydrazide is apparently derived from the partial degradation of cystine and cysteine, since no other amino acid occurring in ovalbumin or chymotrypsin gives rise to more than a trace of acetic acid or propionic acid hydrazides on hydrazinolysis for 17 hr. at 100° (Table 1). A carbohydrate source is also ruled out since chymotrypsin gives rise to an amount of the propionic acid derivative very similar to ovalbumin, though in this case the amount of acetic acid DNP-hydrazide is very small.

Propionyl groups can still be estimated by this method if cystine and cysteine are absent or are oxidized beforehand, since cysteic acid does not give rise to any propionic acid hydrazide on hydrazinolysis.

The yield of aliphatic acid DNP-hydrazide obtained from a number of substances is given in Table 1. With acetyl- and propionyl-amino acids the uncorrected yield is, like that from ovalbumin, about 70% of the theoretical.

It has not proved possible to measure formyl groups by this method. This is presumably due to the reactivity of formic acid hydrazide (the evacuated hydrazinolysates of formyl derivatives turn

brown when air is admitted), but there may also be a loss of formic acid hydrazide by sublimation when the excess of hydrazine is evaporated, though this has not been investigated.

There remains the question of the type of linkage of the acetyl groups to the rest of the protein molecule. *N*-Acetyl groups are much more difficult to remove by hydrolysis than *O*-acetyl groups (see e.g. Uraki, Terminiello, Bier & Nord, 1957). The same appears to be true of hydrazinolysis. The maximum yield of acetyl groups from ovalbumin and from acetyl-amino acids is reached in 8–20 hr. at 100° (see Table 1 and Fig. 1), whereas with an *O*-acetyl compound such as glucose penta-acetate the acetyl groups are removed by hydrazine at room temperature in a few minutes. In 17 hr. at room temperature in hydrazine, ovalbumin gave only a slight trace of acetic acid hydrazide and this supports the view (Johansen *et al.* 1960; Marshall & Neuberger, 1961) that all the acetyl groups in this protein are in *N*-acetyl structures, 3 attached to glucosamine and 1 to the *N*-terminal amino acid.

SUMMARY

1. A method is described for the estimation of acetyl groups in proteins which involves hydrazinolysis and coupling with 1-fluoro-2,4-dinitrobenzene to form acyl dinitrophenylhydrazide. It is also applicable to acyl-amino acids and other classes of substances containing aliphatic acyl groups and to *O*-acetates and *O*-propionates.

2. It is convenient to have 0.5–1 μ mole of acetyl groups in the unknown, but the estimation can be done with 0.1 μ mole.

3. Formyl groups give very low yields, and propionyl groups can only be measured if the unknown does not contain cysteine or cystine or if these amino acids are oxidized to cysteic acid.

4. The yield of acetyl groups from ovalbumin and acyl groups from some acetyl- and propionyl-amino acids and other substances is about 70% of the theoretical.

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REFERENCES

- Brown, F. & Hall, L. P. (1950). *Nature, Lond.*, **166**, 66.
Du Vigneaud, V. & Meyer, C. E. (1932). *J. biol. Chem.* **98**, 295.

Table 1. *Yield of acyl groups (as aliphatic acid dinitrophenylhydrazides) on hydrazinolysis of various substances*

	Yield (% of theoretical)
Ovalbumin	65–70 (see Fig. 1)
Acetyl-DL-alanine	66 in 8.5 hr.; 68 in 16 hr.
Acetyl-DL-leucine	60 in 8.5 hr.; 72 in 16 hr.
<i>N</i> -Acetyl-L-tyrosine ethyl ester	72
<i>N</i> -Acetylglucosamine	70
Propionyl-DL-phenyl-alanine	83
Propionamide	82
Formyl-DL-isoleucine	3–9
Formamide	12–17
Glucose penta-acetate	66–73 in a few min. at 20° 67 in 17 hr.
Ethyl propionate	66 in 1 hr. at 20°
Cysteine	9 } as propionic acid
Cystine	16 } DNP-hydrazide
Hydrazine acetate	48; 43–52 in 17 hr. at 120° (sealed tube)

- Inouye, Y. & Noda, M. (1955). *J. agric. chem. Soc. Japan*, **19**, 214.
Johansen, P. G., Marshall, R. D. & Neuberger, A. (1960). *Biochem. J.* **77**, 239.
Marshall, R. D. & Neuberger, A. (1961). *Biochem. J.* **78**, 31P.
Mills, G. L. (1952). *Biochem. J.* **50**, 707.

- Narita, K. (1958). *Biochim. biophys. Acta*, **28**, 184.
Ohno, K. (1953). *J. Biochem., Tokyo*, **40**, 621.
Phillips, D. M. P. (1961). *Biochem. J.* **80**, 40P.
Satake, K. & Seki, T. (1950). *J. Jap. Chem.* **4**, 557; cited from *Chem. Abstr.* (1951) **45**, 4604f.
Uraki, Z., Terminiello, L., Bier, M. & Nord, F. F. (1957). *Arch. Biochem. Biophys.* **69**, 645.

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The Effect of the Administration of Carbon Tetrachloride on the Formation of Plasma Lipoproteins in the Rat

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The subcutaneous injection of ethionine in the rat results in the development of a fatty liver, which is characterized by a specific rise in the concentration of triglyceride in the liver, and in a reduction in the concentration of all the neutral lipids of the plasma (Harris & Robinson, 1961). Soon after the ethionine has been given there is a marked fall in the rate of formation in the liver of the protein moiety of the plasma lipoproteins. Lipids are normally carried from the liver in combination with these proteins and it has been suggested, therefore, that this reduction in the rate of their formation will result in a failure to remove lipid from the liver at a normal rate and may hence be the cause of the subsequent changes in the concentrations of lipid in the liver and plasma (Robinson & Harris, 1961). A comparable inhibition of the formation of plasma-lipoprotein protein precedes the development of a fatty liver in the rat after the administration of the antibiotic, puromycin (Robinson & Seakins, 1962).

The fatty liver caused by the administration of carbon tetrachloride in rats is believed to be associated with a block in the passage of triglyceride from the liver to the plasma (Recknagel, Lombardi & Schotz, 1960; Recknagel & Lombardi, 1961; Heimberg & Weinstein, 1962). The possibility that inhibition of the formation of plasma lipoprotein occurs has been investigated in the present study. A preliminary account has been published (Robinson & Seakins, 1961).

METHODS

Female albino rats (Wistar strain) weighing 180–200 g. were used. They were starved for 16 hr. before being given, by stomach tube under light ether anaesthesia, either 1 ml. of a 1:1 (v/v) mixture of carbon tetrachloride and olive oil (test groups) or 0.5 ml. of olive oil (control groups).

Incorporation of DL-[1-¹⁴C]leucine into the proteins of rat liver and plasma

Incorporation in vivo. Rats were injected by the tail vein with 20 μ C of DL-[1-¹⁴C]leucine (7.2 μ C/m-mole) 1 hr. after they had been given either carbon tetrachloride and olive oil or olive oil alone. Then, 1.5 hr. after the injection, each animal was bled from a cannula in the abdominal aorta into 0.1 vol. of 3.8% (w/v) sodium citrate. The plasma was separated by centrifuging and stored at 0° until all the rats had been killed. The liver of each animal was removed, rinsed in 0.9% NaCl, dried on filter paper and weighed. A sample (about 0.5 g.) of each liver was homogenized immediately with 5 ml. of 10% (w/v) trichloroacetic acid and the homogenate was stored at 0°. When all the rats had been killed the liver homogenates were transferred to centrifuge tubes and the protein precipitates were aggregated in a boiling-water bath for 30 min. After being stored overnight at 0°, each precipitate was collected by centrifuging and repeatedly washed at 4° with 5% (w/v) trichloroacetic acid until the washings contained no detectable radioactivity. It was then extracted with three successive portions of hot acetone-ethanol (1:1, v/v) and once with diethyl ether.

The samples of citrated plasma were separated into protein fractions of d less than 1.063 (low-density lipoproteins), d 1.063–1.21 (high-density lipoproteins) and d greater than 1.21 (residue proteins) by high-speed centrifuging in the Spinco (model L) ultracentrifuge. After dialysis at 4° of the fractions against 0.9% NaCl adjusted to pH 7.4, they were transferred to centrifuge tubes, and ethanol and 20% (w/v) trichloroacetic acid were added to each, or to a suitable sample of the residue proteins, to give final concentrations of 70% (v/v) and 5% (w/v) respectively. The resulting protein suspensions were aggregated in a boiling-water bath for 30 min., stored overnight at 0° and centrifuged. The protein precipitates were washed with 70% (v/v) ethanol containing 5% (w/v) of trichloroacetic acid at 4° until the washings contained no detectable radioactivity, and were then extracted with three successive portions of hot acetone-ethanol (1:1, v/v) and one portion of diethyl ether.