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A Colorimetric Micro Method for the Determination of Formyl Groups

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The characteristic purple colour formed by *N*-formyl-*N'*-2,4-dinitrophenylhydrazine in the presence of piperidine and acetone was made the basis of a new quantitative method for the determination of formyl groups. Samples containing *N*-formyl groups (up to 0·4 μmole) are hydrazinolysed at 97–98° for 1hr. and are dinitrophenylated after the removal of excess of hydrazine. Interference from 2,4-dinitrophenylhydrazine is eliminated by subjecting the dinitrophenylated samples to chromatography on an alumina column. Interference arising from the formation of *N*-acetyl-*N'*-2,4-dinitrophenylhydrazine, when determining formyl groups in samples containing acetyl, can be avoided by a paper-chromatographic separation before analysis. A standard procedure is described. The method gives satisfactory results when applied to *N*-formyl-amino acids. Gramicidin, when analysed by this method, was found to contain 0·89 mole of formyl group/mole for a molecular weight of 1880. The method indicated the absence of formyl groups from lysozyme, a protein known not to contain such groups. Generally, the analytical values obtained by the method are within 100 $\pm$ 4% of theory.

Available methods for the quantitative determination of *N*- and *O*-formyl groups depend generally on the determination of the formic acid liberated on acid or alkaline hydrolysis, by chemical or enzymic methods (Wood & Gest, 1957), or after reduction of the formic acid to formaldehyde (Grant, 1948), which is then measured by the chromotropic acid colour reaction. It should also be possible to determine *O*-formyl groups colorimetrically by the acylhydroxamate method of Lipmann & Tuttle (1945). The possibility of determining acyl groups by converting them into acylhydrazines, which are then treated with 1-fluoro-2,4-dinitrobenzene to form the highly coloured DNP-acylhydrazines, has been explored by Phillips (1963). The yields were satisfactory for many acyl groups, but it did not prove possible to measure the formyl group since the yields from compounds such as formamide and formylisoleucine turned out to be as low as 3–17%. We have established optimum conditions for the hydrazinolytic removal of the formyl group, and examined and adapted the novel colour reaction reported by Ramachandran (1967) for *N*-formyl-*N'*-DNP-hydrazine for the quantitative determination of formyl groups. The results of the study are reported in the present paper. The applicability of the procedure is demonstrated for formyl derivatives of amino acids and for gramicidin, which is a naturally occurring pentadecapeptide containing an *N*-formyl group (Sarges & Witkop, 1964, 1965).

EXPERIMENTAL

Preparation of acylhydrazines and their DNP derivatives. All acylhydrazines were prepared by treating the methyl or ethyl esters of the corresponding carboxylic acids with anhydrous hydrazine in ethanol. The isolated acylhydrazines were treated with excess of 1-fluoro-2,4-dinitrobenzene, in 66% (v/v) ethanol in the presence of excess of $NaHCO_3$, to obtain the corresponding *N'*-DNP derivatives. The derivatives so made include: *N*-formyl-*N'*-DNP-hydrazine, m.p. 273–274° (literature m.p. 272–274°); *N*-acetyl-*N'*-DNP-hydrazine, m.p. 199–200° (literature m.p. 199·5°); *N*-*n*-butyryl-*N'*-DNP-hydrazine, m.p. 165–166° (literature m.p. 168°); *N*-isobutyryl-*N'*-DNP-hydrazine, m.p. 203–205° (literature m.p. 207°); *N*-benzoyl-*N'*-DNP-hydrazine, m.p. 227–228° (literature m.p. 230°); *N*-(*N*-acetyltyrosyl)-*N'*-DNP-hydrazine, m.p. 240–241°. *N*-Formyl derivatives of amino acids (valine, aspartic acid, asparagine, methionine and glycine, and the *NO*-diderivative of serine) were prepared by the method of Sheehan & Yang (1958) and had the reported melting points.

The DNP-hydrazones of formaldehyde and benzaldehyde were prepared by treating the aldehydes with excess of DNP-hydrazine in methanol–H_2SO_4 and had m.p. 164–165° and m.p. 237° respectively. DNP-valine, 2,4-dinitroaniline, 2,4-dinitrophenol and DNP-hydrazine samples used in the work had the correct melting points. Commercial samples of 95% acetone (redistilled), 40% formaldehyde, piperidine, hydrazine (95+%) and chromatographic-grade alumina (E. Merck A.-G., Darmstadt, Germany) were used.

Colour development and calibration curve. A solution of *N*-formyl-*N'*-DNP-hydrazine in 2-chloroethanol was made so as to contain approx. 20–25 mg./100 ml. Samples containing 0·024–0·24 μmole of *N*-formyl-*N'*-DNP-hydrazine were placed in a series of test tubes; 0·3 ml. of piperidine and acetone to a total volume of 10 ml. were added to each tube. Colour development was immediate and the colour was stable for up to 15–20 hr. The presence of 40% formaldehyde (0·4 ml. added) in such tubes affected neither colour intensity nor colour stability (up to 24 hr.). The purplish-violet colour showed an absorption peak at 550 mμ, as reported by Ramachandran (1967). The calibration curves were linear. Measurements at 620 mμ, on the slope of the absorption peak, are about half as sensitive as measurements at 550 mμ. Solutions of *N*-formyl-*N'*-DNP-hydrazine in 2-chloroethanol kept in the dark for 4 months produced only 4% less colour than did fresh solutions.

RESULTS

Specificity of the reaction and sources of interference. The various DNP derivatives mentioned were tested under the conditions for colour development detailed above, and none of them even in amounts up to 10 μmoles produced the purplish-violet colour characteristic of *N*-formyl-*N'*-DNP-hydrazine. However, since some of these yellow compounds, having absorption peaks in the range 400–500 mμ, might contribute some absorption at 550 mμ, their detailed absorption spectra in the following solvents were examined: 1, piperidine–acetone (3:97, v/v); 2, piperidine–formaldehyde–acetone (3:4:93, by vol.); 3, 3% (w/v) sodium hydrogen carbonate–acetone (1:9, v/v); 4, 3% (w/v) sodium hydrogen carbonate; 5, 10–30% (w/v) sodium hydroxide–ethanol (1:9, v/v); 6, 66% (v/v) acetic acid. The results of some of these measurements are recorded in Table 1.

N-Formyl-*N'*-DNP-hydrazine has an absorption maximum at 550 mμ in solvents 1 and 3; this maximum shifts to 620 mμ in solvent 5. However, the peak at 550 mμ in solvent 1 was the most stable and exhibited ϵ_{550} 23100. *N*-Acetyl-*N'*-DNP-hydrazine showed an absorption maximum at 460–464 mμ (ϵ 17000) in solvent 1, and ϵ_{550} was 3930. In solvent 3 *N*-acetyl-*N'*-DNP-hydrazine showed ϵ values of 15700 and 3270 at 452–454 mμ ($\lambda_{max.}$) and 550 mμ respectively. DNP-hydrazine in solvent 1 showed ϵ values of 20600, 630 and 70 at 350 mμ ($\lambda_{max.}$), 550 mμ and 620 mμ respectively. In solvent 2 *N*-formyl-*N'*-DNP-hydrazine, *N*-acetyl-*N'*-DNP-hydrazine and DNP-hydrazine gave ϵ_{550} values of 23100, 100 and 0 respectively. This indicated that solvent 2 was the best for measurements of *N*-formyl-*N'*-DNP-hydrazine, since interfering absorption due to *N*-acetyl-*N'*-DNP-hydrazine and DNP-hydrazine, when present in a mixture, was at a minimum.

N-Formyl-*N'*-DNP-hydrazine in solvent 5 shows a blue colour absorbing maximally at 620 mμ (ϵ 22700). Under these conditions *N*-acetyl-*N'*-DNP-hydrazine and DNP-hydrazine show ϵ values of 0 and 400 respectively. However, the blue colour due to *N*-formyl-*N'*-DNP-hydrazine is not very stable, and the absorption due to *N*-acetyl-DNP-hydrazine and DNP-hydrazine shows changes with time. The interference due to DNP-hydrazine at 620 mμ in solvent 5 is nearly sixfold higher than that with measurements done at 550 mμ in solvent 2. *N*-Acetyl-*N'*-DNP-hydrazine interferes at any concentration in solvents 1 and 2, whereas in solvent 2 DNP-hydrazine does not interfere at all at a DNP-hydrazine/*N*-formyl-*N'*-DNP-hydrazine ratio 18:1 and only to the extent of 6% at a 26:1 ratio. DNP-hydrazine alters the colour of solutions containing *N*-formyl-*N'*-DNP-hydrazine to

Table 1. *Absorption characteristics of DNP-hydrazine and various substituted DNP-hydrazines*

	Solvent 1		Solvent 2		Solvent 3		Solvent 4	
Compound	λ (mμ)	$10^{-3}\epsilon$	λ (mμ)	$10^{-3}\epsilon$	λ (mμ)	$10^{-3}\epsilon$	λ (mμ)	$10^{-3}\epsilon$
N-Formyl-*N'*-DNP-hydrazine	550 (max.)	23·1	550 (max.)	23·1	550 (max.)	22·9	260 (max.)	13·4
	620	11·3	620	11·3	620	11·3	350–360 (max.)	15·9
N-Acetyl-*N'*-DNP-hydrazine	460–464 (max.)	17·0	550	0·1	452–454 (max.)	15·7	412 (max.)	11·2
	550	3·93	620	0·0	550	3·27		
					620	1·5		
DNP-hydrazine	350 (max.)	20·6	350 (max.)	16·7	260 (max.)	10·3	260–264 (max.)	11·2
	550	0·625	550	0·0	550	0·0	350–352	14·3
	620	0·07	620	0·0	620	0·0	(max.)	
N-*n*-butyryl-*N'*-DNP-hydrazine	472–474	20·0	550	0·0	470–474	19·9	264–265	22·4
	550	5·5	620	0·0	550	3·5	(max.)	
	620	3·2						

brownish-purple to brown, but the absorption at 550mμ due to *N*-formyl-*N'*-DNP-hydrazine is unaltered for DNP-hydrazine/*N*-formyl-*N'*-DNP-hydrazine ratios up to 18:1.

The *n*-butyryl, isobutyryl, benzoyl and N^{α}-acetyltyrosyl derivatives of DNP-hydrazine all show a $\lambda_{max.}$ in the range 435–490mμ (ϵ values 18000–35000) and appreciable absorption at 550mμ, which can be kept to a minimum in solvent 2. The DNP derivatives of amino acids, 2,4-dinitroaniline and 2,4-dinitrophenol showed no significant absorption at 550mμ.

To eliminate the interference due to *N*-acetyl-*N'*-DNP-hydrazine, particularly when analysis of formyl groups in compounds containing acetyl groups is contemplated, the paper-chromatographic properties of *N*-acetyl-*N'*-DNP-hydrazine, *N*-formyl-*N'*-DNP-hydrazine and DNP-hydrazine were investigated. Ascending paper chromatography on Whatman no. 1 paper with 1·5M-sodium phosphate buffer, pH 5·8, as solvent was found to be very useful for the separation of *N*-formyl-*N'*-DNP-hydrazine and DNP-hydrazine as one group from *N*-acetyl-*N'*-DNP-hydrazine, which has a high R_F value (Table 2). The recoveries are almost quantitative (Table 2), for 0·06–0·2μmole of *N*-acetyl-*N'*-DNP-hydrazine or *N*-formyl-*N'*-DNP-hydrazine, even in the presence of 24-fold contamination by DNP-hydrazine.

When relatively large amounts of DNP-hydrazine contaminate the *N*-formyl-*N'*-DNP-hydrazine to be analysed, it is desirable that the *N*-formyl-*N'*-DNP-hydrazine be separated from the DNP-hydrazine before analysis. An alumina-column-chromatographic technique for the separation of the *N*-formyl-*N'*-DNP-hydrazine from contaminating DNP-hydrazine has been devised. A small amount of alumina was slurried with ethyl acetate and packed into a glass column (0·9cm. diam.) to a height of 19·5cm. Samples of *N*-formyl-*N'*-DNP-hydrazine and DNP-hydrazine (mixed in various ratios) were dissolved in 0·2ml. of ethyl acetate, then mixed with 0·05–0·1ml. of 40% formaldehyde, and the tube was warmed over a bunsen flame for 3–4sec. The tube was cooled and the contents were transferred quantitatively to the top of the alumina column. Ethyl acetate was passed through the column for development. The *N*-formyl-*N'*-DNP-hydrazine moves out of the top of the column as a purplish band and emerges in fractions 4–16 (1ml. each), which are coloured yellow. DNP-hydrazine remains adsorbed at the top of the column. The contents of the tubes containing *N*-formyl-*N'*-DNP-hydrazine were evaporated to dryness, or alternatively samples of the fractions in the peak region were taken, and the *N*-formyl-*N'*-DNP-hydrazine was measured after the addition of piperidine and acetone (solvent 1). Recovery of *N*-formyl-*N'*-DNP-hydrazine under these conditions amounted to 98·7%. The alumina can be regenerated, after removal from the column, by washing with butan-1-ol and drying at 110°. Under the above chromatographic conditions *N*-acetyl-*N'*-DNP-hydrazine cannot be resolved from *N*-formyl-*N'*-DNP-hydrazine.

Table 2. *R_F values for DNP derivatives in ascending paper chromatography with 1·5M-phosphate buffer, pH 5·8*

The recovery value for *N*-formyl-*N'*-DNP-hydrazine holds irrespective of whether it was being chromatographed alone or along with DNP-hydrazine. The recovery of *N*-acetyl-*N*-DNP-hydrazine is based on measurements at 470mμ.

Compound	R_F	Recovery (%)
DNP-hydrazine	0·0	—
N-Formyl-*N'*-DNP-hydrazine	0·0	98·7
N-Acetyl-*N'*-DNP-hydrazine	0·62	98·8
2,4-Dinitrophenol	0·5	—
2,4-Dinitroaniline	0·4	—

Conditions for hydrazinolytic removal of the N-formyl group. Samples of a solution of *N*-formyl-L-valine in ethanol, equivalent to 0·25–0·4μmole of the compound, were pipetted into a series of test tubes and the ethanol was removed under vacuum. The samples were heated with 0·1–0·2ml. of hydrazine or ethanolic 50% hydrazine at 97–98° for various times. The sealed tubes were cooled, then opened, and the excess of hydrazine (and ethanol) was removed by keeping the tubes in a desiccator over phosphorus pentoxide and concentrated sulphuric acid under vacuum (50–100mm. Hg) for 4–10hr. Under these conditions for removal of excess of hydrazine loss of formylhydrazine occurs only when just the pure compound alone is handled; no losses occur with formylhydrazine in hydrazine. The tubes are now removed and the contents of the tubes dinitrophenylated by the addition of 0·1–0·2ml. of 5% (w/v) 1-fluoro-2,4-dinitrobenzene in ethanol, 0·1–0·2ml. of 3% sodium hydrogen carbonate solution and 0·2–0·4ml. of ethanol so that the final concentration of ethanol was about 55–65%. The tubes were kept at room temperature (approx. 25°) for 3hr. At the end of this time the solvent in the tubes was removed under vacuum, and the tubes were then left in a desiccator over solid sodium hydroxide and connected to a pump (50–100mm. Hg) for 20–24hr. The residues left in the tubes were dissolved in 2-chloroethanol and samples (one-fifth to three-fifths of the total) chromatographed on paper, *N*-formyl-*N'*-DNP-hydrazine being eluted and determined as described under 'Recommended procedure for determination of formyl groups' below.

The influence of time of heating and of the nature of the hydrazinolysis medium on the extent of release of formylhydrazine from *N*-formyl-L-valine is recorded in Table 3. A period of only 40–60min. is needed for quantitative release when hydrazine is used, whereas the use of ethanolic 50% hydrazine necessitates heating for 2–4hr. The use of sealed tubes is not essential; well-stoppered test tubes do equally well. A 1hr. period of heating is used in the experiments described below. Table 4 summarizes the results of analysis of several *N*-formyl-amino acids and *NO*-diformylserine, and Table 5 the results on the analysis of two formyl derivatives from measurements of *N* - formyl - *N'* - DNP - hydrazine at 620mμ as well as at 550mμ.

Recommended procedure for determination of formyl groups. Samples containing 0·2–0·4 μmole of formyl groups are heated with 0·1–0·2ml. of hydrazine in stoppered test tubes for 1hr. at 98°. The tubes are removed and cooled, and the hydrazine is removed by keeping the tubes in a desiccator over phosphorus pentoxide and concentrated sulphuric acid under reduced pressure (50–100mm. Hg) for not less than 10hr. The tubes are removed from the desiccator and the contents dinitrophenylated by adding excess of 1-fluoro-2,4-dinitrobenzene (0·1–0·2ml. of a 5% solution in ethanol) and excess of sodium hydrogen carbonate (0·1–0·2ml. of a 3% solution), the final ethanol concentration being adjusted to about 66%. The tubes are kept at room temperature for 3hr. The solvent and excess of reagent contained in the tubes are then removed by keeping the tubes in a desiccator connected to a vacuum pump (50–100mm. Hg) for 20–24hr. The residues left in the tubes are dissolved by the addition of 1ml. or less of 2-chloroethanol. Suitable samples (one-tenth to three-fifths of the total) are streaked on Whatman no. 1 paper for ascending paper chromatography as described above with 1·5M-sodium phosphate buffer, pH 5·8, as solvent. After a 3hr. run the sheets are removed and dried. The colour streaks left at the origin are cut out, made into small pieces and placed in test tubes to which are added 0·3ml. of piperidine, 0·4ml. of 40% formaldehyde and 4·3ml. of acetone. The tubes are stoppered and heated for 30min. at 50°, after which the tubes are cooled and opened. To each tube 5ml. of acetone is added and the contents are mixed. The *N*-formyl-*N'*-DNP-hydrazine present in the tubes is measured by transferring the coloured solutions into test-tube cuvettes (19mm. diam.), determining the extinction at 550mμ with a Coleman Junior spectrophotometer (model 6A) and reference to the calibration curve for authentic *N*-formyl-*N'*-DNP-hydrazine. Measurements can be done at 620mμ, although with lowered sen-

Table 3. *Influence of the time of heating with hydrazine on the extent of release of formylhydrazine from* N-*formyl*-L-*valine*

The first four rows relate to samples hydrazinolysed with 0·2ml. of 50% hydrazine in ethanol in sealed tubes, and the following five rows are those obtained by using 0·1ml. of hydrazine in stoppered tubes.

Amount of *N*-formyl-L-valine taken (μmole)	Time of heating at 97–98° (min.)	Amount recovered (μmole)	Amount recovered (% of theory)
0·372	40	0·24	64·5
0·372	60	0·29	77·95
0·372	120	0·36	96·77
0·246	240	0·247	100·3
0·2564	20	0·129	50·3
0·2564	40	0·234	91·2
0·2564	60	0·257	100·23
0·2564	90	0·257	100·23
0·2564	120	0·257	100·23

Table 4. *Recovery of the formyl group, as* N-*formyl*-N'-*DNP-hydrazine, in the analysis of various* N- *and* O-*formyl-amino acids*

The results for *N*-formyl-L-asparagine were obtained by using 0·2ml. of 40–50% hydrazine in ethanol for 4hr. at 97–98°. Values in rows 2, 3 and 4 were obtained by recovery of *N*-formyl-*N'*-DNP-hydrazine on chromatography on alumina. Values in rows 4 and 6 were derived from five and four separate runs respectively and the results are given as means ± S.E.M.; all other results are derived from duplicates.

Compound	Total formyl groups present (μmole)	Amount of formyl groups found (μmole)	Amount of formyl groups found (% of theory)
N-Formyl-L-asparagine	0·2625	0·2633	100·3
	0·2625	0·26	99·1
N-Formyl-L-aspartic acid	0·31	0·321	103·2
	0·2344	0·2364 ± 0·005	100·9
N-Formyl-L-valine	0·3846	0·375	97·6
NO-Diformyl-DL-serine	0·4	0·401 ± 0·006	100·3

Table 5. *Comparison of recoveries of* N-*formyl*-N′-*DNP-hydrazine based on colour measurements at* 550mμ *and* 620mμ

All analyses were done in triplicate, and the results are given as means ± S.E.M.

Compound	Amount taken (μmole)	Amount found (550mμ measurement) (μmole)	(% of theory)	Amount found (620mμ measurement) (μmole)	(% of theory)
N-Formylglycine	0·2718	0·273 ± 0·05	100·3	0·273 ± 0·004	100·3
N-Formyl-L-methionine	0·208	0·220 ± 0·08	105·8	0·206 ± 0·008	99·0

sitivity; such values are equally reliable. When the solutions for colour measurement are deep-brown it can be inferred that DNP-hydrazine is present in a ratio higher than 8:1 relative to *N*-formyl-*N*′-DNP-hydrazine; in such cases the measurement of *N*-formyl-*N*′-DNP-hydrazine present should be made after submitting the dinitrophenylated sample to alumina-column chromatography as described above. Although even a DNP-hydrazine/*N*-formyl-*N*′-DNP-hydrazine ratio as high as 18:1 does not vitiate measurements of *N*-formyl-*N*′-DNP-hydrazine, analysis of *N*-formyl-*N*′-DNP-hydrazine after separation on alumina guards against error arising from contamination by larger amounts of DNP-hydrazine.

Application of the method to gramicidin and to lysozyme. Two different sample amounts of gramicidin (0·34mg. and 0·51mg., as corrected for weight loss at 110° in duplicate) were submitted to the procedure described in the preceding section. The 0·34mg. sample duplicates yielded respectively 0·156 and 0·164μmole of *N*-formyl-*N*′-DNP-hydrazine, corresponding to 0·863 and 0·906 mole of formyl groups/mole of gramicidin of molecular weight 1880. The 0·51mg. sample duplicates yielded respectively 0·885 and 0·893 mole of formyl groups/mole of gramicidin. The mean of all values represents a content of 0·886 ± 0·013 mole of formyl groups/mole of gramicidin.

The structure of egg-white lysozyme is established and it is known not to contain any formyl groups. Two samples of lysozyme chloride corresponding to 0·58 and 1·04μmoles respectively were hydrazinolysed and dinitrophenylated according to the procedure given above. Samples (0·05ml.) of the dinitrophenylated sample dissolved in 1ml. of 2-chloroethanol were chromatographed and the *N*-formyl-*N*′-DNP-hydrazine at the origin, after paper chromatography, was measured by colorimetry. Extinction values of 0·33 and 0·21 were obtained respectively for the samples, corresponding to 0·052 and 0·029μmole of lysozyme. There was, however, no typical purple colour in the solutions subjected to colour measurement. Therefore a 0·2ml. portion from the dinitrophenylated sample (corresponding to 0·21μmole of lysozyme) was analysed for *N*-formyl-*N*′-DNP-hydrazine after chromatography on alumina. No measurable amount of *N*-formyl-*N*′-DNP-hydrazine was detected in fractions 1–16, corresponding to a total elution volume of 16ml. The negligible extinctions at 550mμ found on colorimetric analysis of these fractions added up to a total corresponding to 0·004μmole of *N*-formyl-*N*′-DNP-hydrazine, whereas the sample used for chromatography corresponded to 0·21μmole of lysozyme. It is therefore inferred that there are no formyl groups in lysozyme.

DISCUSSION

A detailed investigation of several *N*-acyl-*N*′-DNP-hydrazines has shown that none except *N*-formyl-*N*′-DNP-hydrazine gives the characteristic purple colour in piperidine–acetone with an absorption peak at 550mμ (ϵ 23100). The micro method for formyl groups described in this paper takes care of problems that may be encountered in the analysis of materials containing only formyl groups. But the technique should be applicable to materials containing both formyl and acetyl groups. The interference due to DNP-hydrazine could be avoided by using solvent 2 (piperidine–formaldehyde–acetone) when the DNP-hydrazine/*N*-formyl-*N*′-DNP-hydrazine ratio is not greater than 18:1; when DNP-hydrazine is present in larger amounts the use of the chromatographic procedure on alumina for the separation of *N*-formyl-*N*′-DNP-hydrazine eliminates all interference. The interference due to *N*-acetyl-*N*′-DNP-hydrazine can be avoided by using the paper-chromatographic procedure for the separation of *N*-formyl-*N*′-DNP-hydrazine from *N*-acetyl-*N*′-DNP-hydrazine.

A study of the hydrazinolytic removal of the *N*-formyl group of amino acid derivatives has shown that quantitative release of the formyl group (Table 3) is achieved in slightly over 2hr. by using ethanolic 50% hydrazine at 97–98°, and in about 1hr. by using 95% hydrazine at 97–98°, as reported

by Ramachandran (1967). Heating in hydrazine at 97–98° for 1hr. has been used as a routine for the removal of the formyl group. Longer times of heating certainly could be used, but extensive hydrazinolysis of peptide bonds in naturally occurring proteins and peptides would occur (Akabori, Ohno & Narita, 1952) leading to the formation of increasing amounts of aminoacyl-hydrazines.

The recommended procedure has been applied to samples (up to 0·4 μmole) of *N*-formyl derivatives of asparagine, aspartic acid, valine, serine, methionine, and glycine (Tables 4 and 5). Analytical values obtained are generally within 97·6–103·2% of theory, with a precision of better than 4%. The analytical result for *NO*-diformylserine (Table 4) indicates that both *N*- and *O*-formyl groups are quantitatively recovered. This is as expected, since *O*-ester bonds are very susceptible to attack by bases, hydroxylamine etc. We have noticed that the *O*-formyl group is removed by hydrazine, as formylhydrazine, even at 25° in less than 1hr. (S. Usha Lakshmi & L. K. Ramachandran, unpublished work). The analytical method used for analysis of *N*-formyl groups is therefore applicable to *O*-formyl group analyses.

The application of the present method to gramicidin indicates the presence of 0·89 mole of formyl groups/mole of the antibiotic. The gramicidins are known to contain 1 mole of formyl groups/mole (Sarges & Witkop, 1964, 1965; Ramachandran, 1967). Egg-white lysozyme (*N*-acetylmuramide glycanohydrolase) was found not to contain any formyl groups as judged by the method. This is in agreement with the structural data available on lysozyme (Canfield, 1963). These observations on gramicidin and lysozyme suggest that the method would be reliable for use on proteins, peptides and related materials.

The basic colour reaction for *N*-formyl-*N'*-DNP-hydrazine (ϵ 23100) used for formyl group analysis is of higher sensitivity than the chromotropic acid colour reaction for formaldehyde (ϵ_{570} 17000). Further, in estimating formyl groups as was done on gramicidin (Sarges & Witkop, 1964) by hydrolysis to formate and reduction of the latter to formaldehyde only one-quarter of the formyl group present is actually measured as formaldehyde. This is because the reduction of formate to formaldehyde with magnesium–hydrochloric acid proceeds only with a 29% yield (Grant, 1948). The only other micro method that approaches the present one in sensitivity is based on the spectrophotometric measurement of the increment in E_{360} (ϵ 22000) when formyltetrahydrofolate is formed in the tetrahydrofolate formylase-catalysed reaction (Goldthwait & Greenberg, 1955; Rabinowitz & Price, 1957) between formate and tetrahydrofolate. Thus the sensitivity of the method for formyl groups based on measurement of *N*-formyl-*N'*-DNP-hydrazine colorimetrically is comparable with, or better than, alternative methods that are available.

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