A DINITROPHENYLATION METHOD FOR THE MICRO-DETERMINATION OF AMIDE NITROGEN

J H BRADBURY

Division of Textile Industry, Wool Research Laboratories, C S I R O Geelong (Australia)

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INTRODUCTION

Amino acid analysis of cuticle-rich material from three samples of wool has shown that the cuticle is consistently poor in glutamic acid plus glutamine and aspartic acid plus asparagine[1]. It is therefore important to determine the amide-nitrogen content of both cuticle and whole fibre so that the amount of glutamic and aspartic acid present can be calculated. It seemed that this might be possible on a microscale by making use of the unwanted spot of 2,4-dinitroaniline ($DNPNH_2$), produced by reaction between 1-fluoro-2,4-dinitrobenzene (FDNB) and ammonia which is always present on the two dimensional paper chromatogram of the dinitrophenyl-amino acids. The problem of quantitative evolution of ammonia from the wool protein by hydrolysis in hydrochloric acid had been thoroughly investigated by LEACH AND PARKHILL[2]. However, LOCKHART[3] found only 50% reaction of FDNB with ammonia as compared with its quantitative reaction with a number of amines and this is paralleled by kinetic evidence on the reaction between 1-chloro-2,4-dinitrobenzene and these compounds[4].

This paper describes a satisfactory method for the analysis of amide-nitrogen of 0.5-mg samples of cuticle-rich material from wool.

EXPERIMENTAL AND RESULTS

Materials

Samples of Lincoln 36's and Corriedale 46's greasy wool were separated into base and tip and the grease removed as described previously[5]. A third sample of Lincoln 36's wool processed to top form was likewise cleaned and all samples conditioned at 65% R H and 20° The 2 *N* HCl for hydrolysis was prepared by dilution of twice-distilled 6 *N* HCl with glass distilled water both freshly distilled. Acetone (A.R) was refluxed with $KMnO_4$ and then fractionated to remove all traces of alcohols[6] A sample of FDNB (British Drug Houses Ltd , Poole, Dorset) was fractionally distilled at 0.5–1 0 mm Hg-pressure at 116° to produce a light yellow product which was stored at 2° Peroxide free diethyl ether (A R) was freed from quinones before use by extraction with 1% aq. NaOH (four times), dilute HCl (once) and distilled water (four times). Laboratory grade $DNPNH_2$ was not recrystallised since it was found to be chromatographically pure Ammonium chloride, $KHCO_3$ and glycine were A R reagents used without further purification

Preliminary experiments

The reaction between NH_3 and FDNB in aqueous solution at 40° by the method of LEVY[7], produced only 25% of $DNPNH_2$ even after 7 h heating. Reaction in a homogeneous solution of ethanol and 0.5 *M* $KHCO_3$ solution (2:1) according to

SANGER[8] gave much higher yields of $DNPNH_2$ but produced large quantities of ethyl 2,4-dinitrophenyl ether[4]. This compound, together with the $DNPNH_2$, was extracted by ether from the aqueous solution after dinitrophenylation and on a one-dimensional paper chromatogram ran just faster than $DNPNH_2$, but overlapped with it in a large range of solvents. It gave a colorless spot which showed up under U.V. light and gave a strong absorption at 345 mμ, the absorption maximum for $DNPNH_2$ in *N* HCl. To eliminate the interference of this material the reaction was repeated, ethanol being replaced by methanol, dioxan and acetone. With methanol, methyl 2,4-dinitrophenyl ether was produced which interfered in the chromatography, dioxan gave low yields but acetone appeared to be a satisfactory solvent. In order to increase the rate of reaction between NH_3 and FDNB the temperature of reaction was raised to 50° and a series of experiments carried out according to the following procedure.

An aqueous solution containing exactly 0.020 mg of A.R. NH_4Cl was evaporated to dryness, 1.0 ml 0.5 *M* $KHCO_3$ solution added by an automatic pipette followed by 1.5 ml acetone containing FDNB. After heating at 50° in a small stoppered flask for the appropriate time, 1 ml of a 5% solution of glycine was added and the heating continued for a further 30 min in order to remove all excess FDNB[3]. Five ml 0.5 *M* $KHCO_3$ was added, the solution extracted with ether (5 × 5 ml) and the combined ether extracts divided accurately into two parts. These were evaporated to dryness, the yellow residues dissolved in the minimum of acetone and applied as spots to Whatman No. 1 paper buffered with 0.1 *M* phthalate buffer at pH 5. The chromatograms were run in one dimension using 2-methylbutan-2-ol saturated with pH 5 phthalate buffer[9] for 16 h, and the spot due to $DNPNH_2$ identified by comparison with the pure $DNPNH_2$ run on the same paper. The paper was dried, the $DNPNH_2$ spot excised and its weight, together with that of three paper blanks, determined. The yellow colour was eluted with 5 ml *N* HCl at room temperature for 30 min and measured at its absorption maximum (345 mμ) in a 1-cm matched cell of the Unicam spectrophotometer. The small blank correction, about 0.01/100 mg of paper, was subtracted from the observed optical density. A further correction, amounting to 10–30% of the total observed optical density, was made for the amount of ammonia present in the reagents used. This correction varied in amount from one series of experiments to the next and was estimated by running one control experiment with each series. From the corrected value of the optical density the % yield of $DNPNH_2$ obtained from the NH_4Cl was calculated using the experimentally obtained value

TABLE I

YIELD OF $DNPNH_2$ FROM AMMONIA BY DINITROPHENYLATION

Weight FDNB mg	*Time of heating* min	% *Yield (mean of* ≤ 2 *determinations)*
5	180	65
10	180	72
20	180	82
30	180	78
20	20	69
20	45	80
20	90	72

of the molar extinction coefficient for pure $DNPNH_2$ in N HCl of $(1.43 \pm 0.027) \cdot 10^4$.

The results in Table I show the effect of variation of the amount of FDNB and time of heating on the yield of $DNPNH_2$. It is clear that about 20 mg of FDNB is required to give about 80% yield but there is no improvement on increasing the amount to 30 mg. Similarly 45 min reaction time gives about 80% yield but there is no advantage in increasing the time to 180 min. It therefore appears that the maximum yield obtainable is about 80% and it is interesting that the 20% loss of ammonia and/or $DNPNH_2$ during reaction and chromatography agrees very closely with the losses previously found in the dinitrophenylation of the amino acids[10]. In order to estimate possible losses of $DNPNH_2$ by further substitution of the primary amino group by dinitrophenyl groups and by extraction and chromatography, micro-amounts of $DNPNH_2$ were carried through the above procedure and the % recovery of $DNPNH_2$ on the chromatogram estimated. This amounted to 91.5% showing that there is a loss of 8.5% of $DNPNH_2$ in this way. It is unlikely that the additional loss of 11.5% of material is due to incomplete reaction between ammonia and FDNB since more stringent conditions of reaction do not increase the yield. It is possible that some ammonia could react with acetone[11] but this would still not explain the very similar results obtained in the dinitrophenylation of the amino acids[10].

For practical purposes the yield is constant at 80%, giving $c = (1.14 \pm .036) \cdot 10^4$ as the mean of 10 experiments (excluding rows 1, 2 and 5 of Table I). This figure is used in all future experiments together with 20 mg FDNB and a heating period of 1 h at 50°.

Final procedure

A sample of cuticle-rich material, obtained as described previously[1] or whole fibre weighing about 0.5 mg was placed in a clean test tube, 3 ml 2 N HCl added and the tube closed with a spring-loaded ground glass stopper. The tube was heated at 100° for 2 h[2], after which time only a slight residue remained. An alternative technique of sealing the tube under vacuum and heating at 105° in the oven gave irreproducible results. The hydrochloric acid was evaporated at the water pump, 1.0 ml 0.5 M $KHCO_3$ added by an automatic pipette followed by a solution of 20 mg FDNB in 1.5 ml acetone. After heating at 50° for 1 h, 1 ml of 5% aqueous glycine was added and the heating continued for 30 min. Five ml 0.5 M $KHCO_3$ was added and the $DNPNH_2$ extracted from the aqueous solution with ether (5 × 5 ml). The ether extract was divided into two equal parts by volume, the ether evaporated at the

TABLE II

Amide nitrogen content of whole fibre and cuticle-rich material (moles/10^4 g material at 65% R.H. and 20°) with standard errors

Sample of wool	*Amide N content of*		*Mean % cuticle material removed from the fibre*
	whole fibre	*cuticle-rich material*	
Lincoln 36's, virgin wool, tips removed	7.0 ± 0.2	7.2 ± 0.5	0.69
Lincoln 36's, processed to top form	7.4 ± 0.4	7.4 ± 0.6	0.56
Corriedale 46's, virgin wool, tips removed	7.3 ± 0.2	6.1 ± 0.1	0.50

pump and the yellow residue chromatographed as described above. The spots due to $DNPNH_2$ were excised, eluted with 5 ml *N* HCl for 30 min at room temperature and the optical density measured at 345 mμ. After correction was made for the paper blank and the amount of ammonia in the reagents as described above, the optical density was used to calculate the amide-N content of the protein material in moles/10^4g material at 65% R.H. and 20°, using the value of the molar extinction coefficient of $1.14 \cdot 10^4$.

The figures for the amide nitrogen content of whole fibre and cuticle material recorded in Table II represent the means of at least four analyses.

In order to check whether the abrasive action of the descaler had any effect on the amide-nitrogen value the sample of Lincoln 36's virgin wool was rubbed back and forth across a very sharp abrasive edge until breakage occurred. Four analyses in which an average of 5.7% of material was abraded from the fibre gave a mean value of 7.6 ± 0.4 moles/10^4g material at 65% R.H. and 20°. This is not significantly different from the whole fibre figure of 7.0 ± 0.2 hence showing that abrasion does not alter the amide-nitrogen content of the material.

DISCUSSION

From the results in Table II it is clear that the amide content of the cuticle-rich material is the same as that of the whole fibre for both samples of Lincoln 36's wool. However, this is not the case for the Corriedale 46's sample where the cuticle-rich material is significantly poorer in amide groups than the whole fibre. Differences in behaviour from one sample of wool to the next must therefore be expected. The more important use of these figures is in the calculation of the aspartic acid and glutamic acid content of the cuticle-rich material[12].

The results obtained for whole fibre analyses agree well with the value of 7.1 moles/10^4 g wool at 65% R.H. and 20° calculated from the results of LEACH AND PARKHILL[2] on Lincoln 36's wool. The mean standard error calculated over all sets of results is 4.5%. This figure is quite reasonable for such a determination which involves the use of extraction and paper chromatographic techniques and is to be compared with an overall mean standard error for amino acid determination of 6.9%[12]. The minimum amount of ammonia which can be estimated accurately by this method is about 2 μg. However if the amount of ammonia in the reagents could be reduced it should be possible to determine as little as 0.5 μg ammonia (40 μg wool) quite accurately.

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SUMMARY

A new micro-method is reported for the determination of amide nitrogen, based on the estimation of 2,4-dinitroaniline produced by the reaction between ammonia, quantitatively liberated from the protein, and 1-fluoro-2,4-dinitrobenzene. Application of the method to 0.5-mg samples of wool gives results in agreement with those obtained by other authors for the same type of wool. Analyses of cuticle-rich material obtained from these wool samples show that in one sample out of three the cuticle is significantly poorer in amide groups than the whole fibre The mean standard error taken over all the analyses is 4 5%.

RÉSUMÉ

Une nouvelle méthode microchimique est proposée pour le dosage de l'azote des amides. Elle est basée sur une détermination de la dinitro-2,4-aniline, produite par la réaction entre l'ammoniac, quantitativement libéré de la protéine et le fluoro-1-dinitro-2,4-benzène

ZUSAMMENFASSUNG

Es wird eine neue mikrochemische Methode beschrieben zur Bestimmung von Amid-Stickstoff. Das aus dem Protein in Freiheit gesetzte Ammoniak wird mit Fluoro-1-dinitro-2,4-benzol zur Reaktion gebracht und das entstandene Dinitro-2,4-anilin bestimmt

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A SIMPLE SPECIFIC TEST FOR INNER-RING *o*-QUINONES

E SAWICKI AND W. ELBERT

Air Pollution Engineering Research, Robert A Taft Sanitary Engineering Center, U S Department of Health Education and Welfare, Public Health Service, Cincinnati, Ohio (U S A)

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INTRODUCTION

In a previous paper[1] a thermochromic test for the detection of inner-ring *p*-quinones and fluorenones was described. This test gave positive results with the aromatic fraction of airborne particulates. With a modification of the procedure[2], benzofluorenes were detected in the air for the first time[3]. In another modified form the test has also been used to confirm the presence of anthracene in the air[3]. A test for terminal-ring *o*- and *p*-quinones has also been described recently[4]. Since a specific method for the detection of inner-ring *o*-quinones is not available in the literature, such a method has been developed.

The test procedure is based on the reported preparation of the bis-anils of chrysenequinone[5].