A Colorimetric Method for the Determination of Aliphatic Amines in the Presence of Ammonia

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During studies on the formation of lower aliphatic amines by bacteria (King, 1953; Ekladius & King, 1956) a method was needed for estimating these compounds in culture filtrates. Alkaline distillation will separate them from nearly all other components save ammonia, hence the problem was to find a reagent which would discriminate between ammonia and the amines. Weber & Wilson (1918) reported that mercuric oxide would adsorb ammonia but not the amines, and Pugh & Quastel (1937) suggested this as a means of separating and estimating the amines. We attempted to devise a micro-method embodying this principle, but found it difficult to prepare mercuric oxide which would completely remove ammonia without adsorbing significant amounts of amine as well. After trying various reagents known to react with $-NH_2$ or ammonia (picryl chloride, toluene-*p*-sulphonyl chloride, Nessler's reagent) 1-chloro-2:4-dinitrobenzene (CDNB) proved suitable. This reacts with aliphatic amines to give *N*-alkylanilines (Willgerodt, 1876):

$$R.NH_2 + C_6H_3(NO_2)_2Cl = R.NH.C_6H_3(NO_2)_2 + HCl.$$

Blanksma & Schreinmachers (1933) determined the velocity constants for the reaction of CDNB with the amines, with M CDNB and 2M amine in 50% (v/v) ethanol at 25°. They found values of the order $k = 0{\cdot}055$ for *n*-butylamine, *n*-propylamine and *iso*amylamine. With ammonia the reaction was much slower (k 0·0024). CDNB should therefore be suitable for differentiating between ammonia and the amines, and we have found this to be so in practice. The chlorodinitroanilines are yellow compounds, and Duin (1953) has used them for the chromatographic separation and characterization of the amines. We have found that they are suitable for colorimetric determination. The reaction is not stoicheiometric but the colour developed is proportional to amine concentration over a wide range, and replicate assays—even when performed on different occasions—agree well.

EXPERIMENTAL

Preparation of dinitroanilines. A number of 2:4-dinitroanilines were prepared (Willgerodt, 1876) by condensing CDNB with the appropriate amine. Derivatives of several primary amines and of dimethylamine and diethylamine were prepared (Table 1). Di-*n*-propylamine and di-*n*-butylamine also react readily (see Fig. 3), but di*iso*propylamine and di*iso*butylamine and adrenaline both failed to react appreciably. Ammonia itself does not react readily, a yield of only 3% of dinitroaniline being obtained even after refluxing for 7 hr. (cf. Blanksma & Schreinmachers, 1933). The derivatives of the primary amines gave absorption spectra which are very similar to each other (Fig. 1). The two secondary amines examined gave slightly stronger absorption, but the form of the curve was similar. *N*-*n*-Butyl-2:4-dinitroaniline (Fig. 1) gave $E^{1\%}_{1\,cm.} = 725$, and $\epsilon = 17\,400$ at $\lambda_{max} = 358$ mμ. This would normally be the best wavelength for determinations but in practice it is better to use a longer wavelength. CDNB itself has an appreciable absorption at 358 mμ. and as it has to be used in large excess this makes reading difficult at the $\lambda_{max.}$. Examination of the spectra shows that the region of 450 mμ.

Table 1. *Properties of* 2:4-*dinitroanilines* [$RR'.N.C_6H_3(NO_2)_2$]

Extinction coefficients were measured at $\lambda_{max.}$.

R	R′	M.p. Found	M.p. Reference	$\lambda_{max.}$ (mμ.)	$E^{1\%}_{1\,cm.}$	ϵ
n-C_3H_7-	H	98°	97° (1)	358	827	18 700
n-C_4H_9-	H	92	90 (2)	358	725	17 400
$(CH_3)_2.CH.CH_2$-	H	80	80 (3)	358	777	18 600
$(CH_3)_2.CH.CH_2.CH_2$-	H	98	97 (2)	358	695	18 200
$C_6H_5.CH_2.CH_2$-	H	154	154 (4)	348	686	19 100
CH_3-	CH_3-	74	(5)	370	900	19 000
C_2H_5-	C_2H_5-	80	(5)	370	900	21 500

References: (1) Romberg & Jansen (1911). (2) Kam (1926). (3) Romberg (1885). (4) Jansen (1931). (5) Romberg (1881) reports m.p. 87° and 80° for the dimethyl and diethyl compounds respectively. Blanksma & Schreinmachers (1933) state that the compounds are dimorphic with m.p. 87° and 74° (dimethyl) and 80° and 69° (diethyl).

gives the best ratio between the absorption of CDNB itself and the derivatives of the primary amines. The absorption of the latter is still sufficient to permit adequate sensitivity ($E^{1\%}_{1\,cm.}$ = 150 at 450 mμ.). Ammonia, moreover, will not interfere at 450 mμ. If present, it will form appreciable amounts of 2:4-dinitroaniline; this has $E_{max.}$ at 335 mμ. (ϵ = 19 200) and would interfere with readings taken at 358 mμ.; but at 450 mμ. its absorption is negligible. Experiment showed that NH_3, even in large excess, does not interfere with readings taken at 450 mμ.

Reagent. 1-Chloro-2:4-dinitrobenzene (0·5%) is dissolved in 50% (v/v) aqueous propan-1-ol and stored in a dark bottle. It is stable for at least two months but should be discarded if excessive blank values are obtained.

Spectrophotometry. All measurements were made in a 1 cm. cell in either a Beckman or a Unicam instrument.

Technique. The sample for assay, containing 50–250 μg. of amine N in a volume not exceeding 10 ml., is placed in a suitable micro-steam-distillation apparatus, such as that described by Markham (1942). Propan-1-ol (2 ml.) is placed in the receiver and steam passed until approx. 5 ml. of distillate has been collected over a period of 2 min. The distillate is transferred to a 6 in. × $\frac{5}{8}$ in. test-tube, graduated at 10 ml., and 1 ml. of the CDNB reagent added. At the same time a standard is prepared containing 140 μg. of amine N in 5 ml. of 50% (v/v) propan-1-ol. The amine used for the standard should preferably be an authentic sample of the amine being assayed; otherwise, *n*-butylamine is a convenient standard. The CDNB reagent (1 ml.) is added to the standard, and also to a blank consisting of 5 ml. of 50% (v/v) propan-1-ol. Assay, standard and blank are placed in a bath containing cold water and the mouths of the tubes covered with loose glass stoppers to prevent loss of solvent. The bath is allowed to stand at room temperature for 15 min. and is then gradually raised to the boil over a period of 0·5 hr., and held at 100° for 0·5 hr. The tubes are then cooled and made up to the 10 ml. mark with 50% (v/v) propan-1-ol. Standard and unknown are read against the blank in a spectrophotometer at 450 mμ. The blank should also be read against the solvent [50% (v/v) propan-1-ol]; this reading ($\log_{10} I_0/I$) should not exceed 0·12 in a 1 cm. cell.

Time of heating. The technique described, involving standing at room temperature and slowly heating to 100°, avoids loss of the volatile amine before reaction has taken place. If the tubes are placed directly in a boiling-water bath results are erratic and tend to be low. The procedure described ensures uniform results. Similarly, propan-1-ol is chosen as solvent because of its miscibility with water combined with relatively high boiling point. During the final heating at 100°, however, the colour is fully developed after about 15 min., and there is no further change if heating is prolonged beyond the specified time.

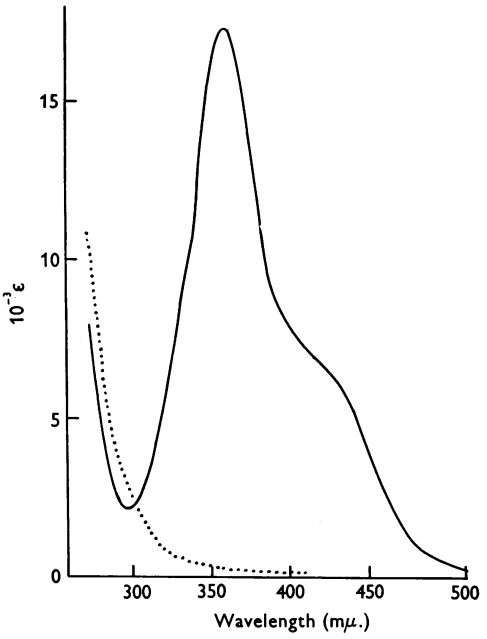

Fig. 1. Absorption spectra of 1-chloro-2:4-dinitrobenzene (···) and *N*-*n*-butyl-2:4-dinitroaniline (——) in 50% (v/v) ethanol, measured in a 1 cm. cell in a Beckman spectrophotometer.

RESULTS

Specificity of reaction. Fig. 2 shows that the three primary aliphatic amines give approximately equal colour intensities, β-phenylethylamine about 25% less. Fig. 3 gives the results with some secondary aliphatic amines. The method would probably be suitable for estimation of some secondary amines, though we have made no detailed investigations. Di*iso*propylamine and di*iso*butylamine gave no appreciable colour. Ammonia gave about 0·1%, indole 2·5%, and skatole 4% of the readings obtained with *n*-butylamine.

Linearity of response. Figs. 2 and 3 show that absorption is proportional to the amount of amine present up to at least 300 μg. of amine N. As this

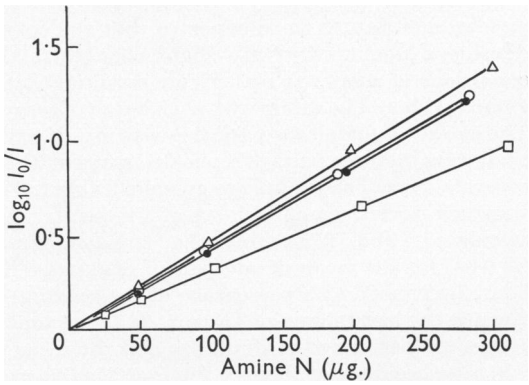

Fig. 2. Colour development with primary amines. Varying quantities of the amines were treated with CDNB in accordance with the assay procedure and the absorption was read at 450 mμ. Each point represents the mean of a pair of replicate readings. ○, *n*-Propylamine; ●, *n*-butylamine; △, *iso*amylamine; □, β-phenylethylamine.

Table 2. *Reproducibility of results of assay of* n-*butylamine*

The quantities of amine (expressed as N) in the first column were subjected to the assay procedure. Duplicates were set up and the absorptiometer readings (at 450 mμ.) are recorded in columns A1 and A2. The following day the experiment was repeated and the results are given in columns B1 and B2; a third repetition on another occasion gave the results in columns C1 and C2. The mean was calculated, and the last column gives the standard percentage error for the mean of any two readings.

Amine N (μg.)	A1	A2	B1	B2	C1	C2	Mean	S.E. (%)
24	0·115	0·110	0·114	0·092	0·097	0·100	0·105	4·1
47·5	0·220	0·206	0·210	0·203	0·210	0·200	0·212	2·1
95	0·425	0·411	0·410	0·402	0·410	0·410	0·411	2·6
190	0·830	0·825	0·800	0·810	0·810	0·825	0·815	1·8
285	1·31	1·30	1·20	1·20	1·21	1·22	1·24	5·6

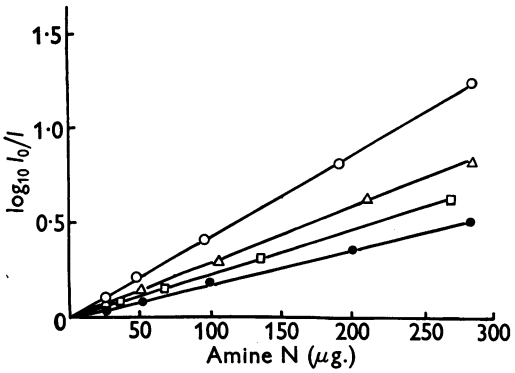

Fig. 3. Colour development with secondary amines. ○, Di-*n*-butylamine; △, di-*n*-propylamine; □, dimethylamine; ●, diethylamine.

amount gives about the maximum absorption which can be read satisfactorily in the spectrophotometer, larger amounts were not investigated.

Consistency of results. Since the reaction is not stoicheiometric it is to be expected that the colour developed might vary with slight changes in the conditions of assay. It is therefore essential that a standard should be set up with each batch of assays. The extent of fortuitous variation was investigated by setting up three pairs of replicate assays on three separate days. The results are given in Table 2. The standard deviations of each set of six results were calculated, and from this the standard error (P 0·05) for the mean of any pair of readings. This figure (expressed as a percentage of the reading) is given in the last column of Table 2. It shows that if assays are performed in duplicate and the mean is taken, an accuracy of about ± 2 % is attainable over the best part of the range.

DISCUSSION

Comparison of the colour developed in the assay with the extinction coefficients of the 2:4-dinitroanilines shows that only about one-sixth of the amine is converted into the derivative under these conditions. The colour developed is, however, almost proportional to the concentration of CDNB even when the latter is in large excess. This suggests that the reaction proceeds to an equilibrium. Attempts were made to drive the reaction nearer completion. Increasing the concentration of CDNB was not possible, since the reagent was already almost a saturated solution; use of a larger volume would dilute the amine and the overall gain would be small. Use of more CDNB would also increase the blank. A more attractive method of driving the reaction forward was to seek some method of removing the hydrochloric acid formed from the system. This could not, however, be done by addition of alkali, which gave an intense yellow colour in the blank (presumably owing to hydrolysis of the CDNB), nor by tertiary amines such as pyridine, quinoline or triethanolamine which also gave excessive blanks. Replacement of the CDNB by fluorodinitrobenzene was also tried, but it gave a highly coloured derivative with ammonia. Even if the reaction could be made to proceed to completion, however, the gain would be solely in the sensitivity of the method; its accuracy and specificity have already reached the limits normally attained in a method of this kind.

Though the method, as described, depends on the use of a spectrophotometer, a simpler instrument will give satisfactory results. We have used the EEL photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, England) with a narrow-band blue filter (EEL no. 621). The range over which a linear response is obtained is somewhat restricted, however, and will depend on the characteristics of the instrument and filter used.

SUMMARY

A method is described for the micro-determination of lower aliphatic amines in biological material. The amine is steam-distilled at alkaline pH and allowed to react with 1-chloro-2:4-dinitrobenzene. The

yellow product is estimated spectrophotometrically. Ammonia does not interfere with the determination.

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Studies on Sulphatases

14. A PRELIMINARY ACCOUNT OF THE CHONDROSULPHATASE OF *PROTEUS VULGARIS**

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The existence in putrefactive bacteria of an enzyme which liberated sulphuric acid from chondroitin sulphate was first suspected by Neuberg & Rubin (1914). Subsequently, a concentrate of this enzyme, to which the name 'chondrosulphatase' was given, was obtained from an organism resembling *Bacterium fluorescens* 'non-liquifaciens' (syn. *Pseudomonas fluorescens* 'non-liquifaciens') (Neuberg & Hofmann, 1931*a*, *b*). Other putrefactive bacteria, *Bacterium proteus* (syn. *Proteus vulgaris*) and *Bact. pyocyaneus* (syn. *Pseudomonas aeruginosa*) were also shown to possess chondrosulphatase activity (Neuberg & Hofmann, 1931*a*, *b*). The ability of micro-organisms, including those isolated from the oral flora (Pincus, 1950), to desulphate mucopolysaccharides isolated from human enamel and dentine (Pincus, 1949; Candelli & Tronieri, 1951) has suggested a relationship between bacterial chondrosulphatase and the production of dental caries. A chondrosulphatase is present in living cultures of *Penicillium spinulosum* (Pincus, 1950) and in the digestive organs of the marine mollusc *Charonia lampas* (syn. *Triton nodiferus*) (Soda & Egami, 1938), but it is not yet clear whether the enzyme is present in mammalian tissues. Thus although no activity could be detected in a number of different rat tissues by sensitive chemical or histochemical methods (Dohlman & Friedenwald, 1955), it was possible to detect appreciable amounts of $^{35}SO_4^{2-}$ in rat urine after the administration of ^{35}S-containing chondroitin sulphate (Dr C. H. Dohlman, personal communication). There is also a certain amount of somewhat confused evidence which suggests that the enzyme elastase contains more than one component, one of which may be a chondrosulphatase type of enzyme (Pepler & Brandt, 1954; Hall & Gardiner, 1955).

In the studies mentioned above and in other investigations on chondrosulphatase activity in bacteria (Beuhler, Katzman & Doisy, 1951; Reggianini, 1950*a*, *b*; Konetzka, Pelczar & Burnett, 1954) liberation of sulphuric acid from chondroitin sulphate was always accompanied by the appearance of degradation products possessing reducing activity. Although the mucopolysaccharase (chondroitinase) system responsible for the appearance of reducing activity has in no case been completely separated from the associated chondrosulphatase, in the mollusc preparations a partial separation of the two enzymes has been achieved and there seems to be no doubt as to their separate identities (Soda & Egami, 1938).

In spite of the possible value of a chondrosulphatase as a tool in establishing the structure of chondroitin and related sulphates little is known of the properties of the enzyme. The present report presents the results of a preliminary study of the chondrosulphatase of a strain of *Proteus vulgaris*.

* Part 13: Dodgson, Spencer & Williams, (1956).