A Simple Procedure for the Estimation of Very Small Amounts of Nitrogen in Lipids

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1. About 0.1 $\mu\text{g.}$ atom of combined nitrogen, in lipids and a few other compounds, can be determined quantitatively by the gentle digestion of dry samples in 10 ml. test tubes with perchloric acid for 30 min., followed by the estimation of the resulting ammonia as the stable blue colour (λ_{max} 635 m μ) produced by the addition of phenol and nitroprusside, an alkaline phosphate buffer and alkaline hypochlorite.
2. Deionized distilled water is required, but the other reagents need no special purification if chosen and handled with care.
3. The results are linear with from 0.015 to 0.15 $\mu\text{g.}$ atom of nitrogen, or up to 1 $\mu\text{g.}$ atom if the final solutions are diluted with water after full colour development.

Some experiments on the stoichiometry of the Schiff reaction with aldehydes (Sloane-Stanley & Bowler, 1962) involved estimations of nitrogen. Direct nesslerization after perchloric acid digestion (Gottfried & Erdman, 1951; King & Wootton, 1956; Long & Staples, 1961) was convenient and rapid (also see Marinetti, Erbland & Kochen, 1957; C. Long, unpublished work, cited by McIlwain & Rodnight, 1962) but sometimes produced turbid solutions and always needed at least 0.5 $\mu\text{g.}$ atom of nitrogen for a precise determination.

When the work came to require the accurate estimation of one-tenth of this amount of nitrogen, adequate sensitivity, reliability and convenience were obtained by using a smaller volume of perchloric acid and estimating the ammonia either with ninhydrin (cf. Ballentine, 1957; Jacobs, 1960, 1962; Rosevear & Smith, 1961), or with phenol and hypochlorite (cf. Exley, 1956; Böttcher, Van Gent & Pries, 1961) in the form of the reagents developed by Lubochinsky & Zalta (1954) and Chaney & Marbach (1962); these methods and some auxiliary procedures have been demonstrated to The Biochemical Society (Sloane-Stanley & Jones, 1963). The present paper is confined to the procedure most thoroughly tested in routine use, namely the application of perchloric acid and the phenol-hypochlorite reaction to the estimation of about 0.1 $\mu\text{g.}$ atom of organically combined nitrogen.

MATERIALS AND METHODS

Apparatus. Digestion is carried out in rimless borosilicate glass test tubes, 130 mm. $\times$ 13 mm., wall thickness about 1.5 mm. (Jenaer Glaswerk Schott und Gen., Mainz, W. Germany: D.50 glass), with their bottoms re-blown to

that thickness when necessary. Bumping is prevented by 3 mm. $\times$ 3 mm. pieces of polytetrafluoroethylene rod ('PTFE rodlets'); smaller pieces are ineffective because they float (Bennett, 1947). An electrically heated digestion rack (King & Wootton, 1956) is set with its front heater low and its back heater off, so that the acid refluxes about 5 cm. up the tubes with gentle boiling.

To minimize contamination with atmospheric nitrogenous bases during digestion, the tubes' mouths may be inserted into closely-fitting holes in the side of the 'anammunium': this is a 50 mm. diam. polypropylene tube (polyethylene melts) with closed ends, supplied with a constant stream of base-free air (obtained from a water-pump, bubbled through H_2SO_4 and filtered through glass wool).

Chemicals. Aqueous solutions are prepared with deionized distilled water (initial conductivity < 1 μmho) which is stored in a borosilicate glass bottle. Other reagent chemicals are of A.R. quality, except for the NaClO , which can be any of several commercial solutions, containing about 10% (w/v) of 'available chlorine' when used. The Na_3PO_4 soln. is prepared from the calculated amounts of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and NaOH (cf. Bates, Pinching & Smith, 1950), and stored at room temp. to avoid crystallization. The phenol-nitroprusside and NaOH-NaClO reagents are prepared and stored substantially according to Chaney & Marbach (1962) (concentrated forms). Alkaline solutions are stored in polyethylene or polypropylene bottles.

Gelatin of known nitrogen content and pure amino acids were given by Dr J. E. Eastoe, and sphingosylphosphorylcholine (Kaller, 1961) by Dr H. Kaller. Lecithin and sphingomyelin were prepared in these Laboratories, and synthetic dihydrosphingosine was obtained from Yeda Research and Development Co. Ltd. (Rehovoth, Israel). Acetanilide and phenacetin were M.A.R. Standards (Hopkin and Williams Ltd., Chadwell Heath, Essex). Choline phosphate calcium chloride (phosphorylcholine), 2-aminoethyl-1-phosphoric acid (phosphorylethanolamine) and serine phosphate (phosphorylserine) were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.) and

appeared respectively 99.7%, 91.4% and 94.6% pure by P assay (Sloane-Stanley & Eldin, 1962).

Procedure. Samples, blanks and $(\text{NH}_4)_2\text{SO}_4$ standards, preferably in triplicate and each containing up to about $1\mu\text{g.}$ atom of N, are placed in digestion tubes and dried under reduced pressure. One PTFE rodlet and 0.05 ml. of HClO_4 (72%) are put into each tube, and the tubes are heated for about 45 min., then cooled to room temp. Each tube in turn is then given 0.25 ml. of 0.6 M-phenol containing mM-sodium nitroprusside, 2.5 ml. of 0.6 M- Na_2PO_4 and 0.3 ml. of 0.75 N- NaOH containing 30 mM- NaClO , with thorough mixing and minimum delay after each addition, before the next tube is treated.

The tubes are kept for at least 30 min. at room temp., after which the extinctions are measured at 635 m μ ; if any samples give $E_{635} > 1.0$, the solutions, together with appropriate standards and blanks, are suitably diluted with water and their extinctions re-determined.

Comments on apparatus. The digestion tubes are large enough for 7 ml. of solvent to be evaporated safely in each of them, and for the chromogenic reagents to be mixed by 'buzzing', but small enough to prevent the amount of HClO_4 used from boiling dry at the bottom of any tube, provided that not more than one PTFE rodlet is added to each tube and the bottom is not too thin nor the rack too hot; if the bottom does go dry during digestion, or bumping occurs, ammonia is lost. The 'anammonium' has been found to give complete protection from atmospheric contamination, but its use has rarely been found essential in this Laboratory. Bent-tipped burettes facilitate the rapid and precise admixture of successive reagents.

Details requiring attention. Very high blanks have been obtained when the reagents have been prepared with ordinary distilled water (cf. Fawcett & Scott, 1960), or with certain batches of anhydrous Na_2HPO_4 , or with concentrated volumetric solutions of NaOH (British Drug Houses Ltd., Poole, Dorset); but pellets of solid NaOH , or an approx. 47% solution (Fisons Scientific Apparatus Ltd., Loughborough, Leics.) have always been satisfactory. Alkaline reagents kept in glass bottles tend to give precipitates in the final coloured solutions.

Irregular or low extinctions have been obtained when H_2SO_4 has been added to the HClO_4 (cf. Heald, 1960), when the Na_2PO_4 soln. has been stored for more than 3 months in an old polyvinylchloride or polyethylene bottle, when the concn. of NaClO in the NaOH - NaClO reagent has been increased or decreased by 50% or more, when the nitroprusside concentration in the phenol reagent has been more than 40% high or low, when pipettes or burettes have not been pre-rinsed with the solutions to be measured out, when reagent additions have been delayed (cf. Fawcett & Scott, 1960) or mixing inadequate, when attempts have been made to simplify the procedure by using unbuffered NaOH to neutralize the HClO_4 or by combining the reagents or adding them in another order (cf. Fawcett & Scott, 1960; Searcy, Simms, Foreman & Bergquist, 1965), or when the solutions have been cooled during reagent additions (e.g. Russell, 1944) or warmed afterwards (e.g. Böttcher *et al.* 1961; Chaney & Marbach, 1962). The recommended concentrations of phenol, and consequently of NaOH , are slightly higher than those given by Chaney & Marbach (1962), because this change has led to an approx. 10% increase in the extinction given by ammonia, without any detectable rise in the blanks.

The volume of HClO_4 added to dry $(\text{NH}_4)_2\text{SO}_4$, not digested, can vary from 0.025 to 0.075 ml. (pH of final coloured solutions from 11.55 to 10.75 respectively) without affecting E_{635} , but N is lost after digestion for 90 min. with only 0.025 ml.; with 0.05 ml., 90 min. is safe and 30 min. seems adequate, but 20 min. has given low recoveries of organically combined N (cf. Marinetti *et al.* 1957). Changes of ± 0.1 ml. in the phenol or NaClO reagent volumes have little effect.

The final blue colour needs at least 30 min. for full development, but is then stable for a few hours even if diluted; E_{635} decreases by about 10% overnight, but remains proportional to the quantity of N taken.

RESULTS

Blanks have given values of E_{635} varying from 0.05 to 0.15 on different occasions; the additional extinction due to 0.1 $\mu\text{equiv.}$ of ammonia has been from 0.65 to 0.75 in optically matched test tubes (cf. Sloane-Stanley, 1959) on different days, but the standard deviation has not exceeded ± 0.03 in any one analysis. Extinctions (blank values subtracted) have been proportional to quantities taken up to $1\mu\text{g.}$ atom of nitrogen.

The complete recovery of lecithin N (C. Long, unpublished work, cited by McIlwain & Rodnight, 1962) has been confirmed; acetanilide (cf. Moore & Diehl, 1962) has given 100–103% of its nitrogen and phenacetin 99–105% when nearly $1\mu\text{mole}$ is taken, but smaller amounts have given low values, perhaps due to sublimation during drying of the ethanolic solutions (lowest pressure about 10 μ Hg). Fractions of ox brain sphingomyelin, all appearing free of impurities by both chemical and chromatographic analyses (Eldin & Sloane-Stanley, 1964), have given nitrogen/phosphorus ratios varying from 1.64 to 2.11, but each fraction has given consistent values in repeated assays. Sphingosylphosphorylcholine has given N/P 2.00–2.05, and synthetic dihydrosphingosine has given nitrogen as 98.5% of theoretical. Phosphorylcholine, phosphorylethanolamine and phosphorylserine have given N/P 0.73, 1.03 and 0.98 respectively. Gelatin, and several amino acids examined singly, gave low recoveries: the best yields of ammonia were obtained from hydroxyproline (95% of theoretical) and lysine (88%), the worst from arginine (66%) and gelatin (64%).

DISCUSSION

The convenience and sensitivity of the present method are largely due to perchloric acid. For instance, the mercury-catalysed sulphuric acid digestion of various phospholipids (Sloane-Stanley, 1953) took at least 90 min. and needed considerable attention, including repeated additions of hydrogen peroxide; similar results were obtained with smaller

amounts of other lipids and choline iodide, but perchloric acid alone gave equally complete recoveries of nitrogen and very much easier and quicker digestion (C. Long & D. A. Staples, personal communication), and was therefore used subsequently (Long & Staples, 1961; C. Long, unpublished work, cited by McIlwain & Rodnight, 1962; cf. Gatt & Berman, 1963a,b).

The low recoveries given by perchloric acid with several non-lipids, whether due to over-oxidation (Kerry, 1960) or vaporization of ammonium perchlorate, can in some cases be improved by using a reflux condenser (Galanos & Kapoulas, 1966), zinc (Moore & Diehl, 1962) or hydriodic acid (Sloane-Stanley & Jones, 1963); preliminary reduction of the sample has long been known to aid Kjeldahl digestion (Bradstreet, 1965), and the quantitative conversion of lipids' nitrogen into ammonia by perchloric acid may depend on the reducing conditions provided initially by the excess of hydrocarbon which impedes sulphuric acid digestion.

Other merits of the use of perchloric acid (King, 1932) assist ultramicroanalysis, as small volumes of chromogenic reagents can be used with acid volumes large enough for digestion in ordinary test tubes. Perchloric acid digestion, moreover, does not need added mercury, which is incompatible with the phenol-hypochlorite procedure (Lubochinsky & Zalta, 1954; Exley, 1956; Böttcher *et al.* 1961).

The introduction by Lubochinsky & Zalta (1954) of nitroprusside catalysis of the phenol-hypochlorite reaction increased the yield of coloured product to at least ten times that obtained with manganese (Russell, 1944; Böttcher *et al.* 1961), and together with the recombination of the reagents in stable forms by Chaney & Marbach (1962), resulted in a procedure at least as sensitive as any using ninhydrin, although needing less manipulation and cheaper reagents which can more easily be obtained sufficiently pure (cf. Moore & Stein, 1948; Jacobs, 1960, 1962; Eastoe & Courts, 1963), besides giving stabler extinctions proportional to a wider range of quantities taken than either ninhydrin or the less-sensitive Nessler reagent.

The estimation of 0.1 $\mu\text{g. atom}$ of nitrogen inevitably, however, requires care to be taken to avoid contamination from the operator's hands (e.g. Hamilton, 1965) or the reagents (cf. Sidle, 1967).

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