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The Estimation of Purines in Nucleic Acids: a Method Applicable to Materials with Low Concentration of Nucleic Acid

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During the course of studies on the metabolism of protein in the rumen of sheep, it became desirable to obtain an estimate of the content of nucleic acids in the digesta; this material consists of a heterogeneous mixture of plant material and a wide variety of bacteria and protozoa. No success was achieved in attempting to apply the procedures based on phosphorus partition (Schmidt & Thannhauser, 1945), colorimetric tests for pentoses (Schneider, 1945) or separation of the purines as the copper complexes (Graff & Maculla, 1935; Vendrely & Sarciron, 1944). Direct spectrophotometry, as used for example by Mitchell (1950), or chromatography, as developed by Markham & Smith (1949), could not be applied owing to the low concentration of nucleic acid in the materials analysed. No procedure has yet been devised for the quantitative separation of the nucleic acids from plant material. It was therefore decided to estimate the nucleic acids in terms of the purines, adenine and guanine; this was achieved by mild acid hydrolysis followed by precipitation of the purines as the silver salts (Schmidt & Levene, 1938; Gulland, Jordan & Threlfall, 1947), the separation of the two purines by chromatography (Markham & Smith, 1949) and final estimation by spectrophotometry. The method has been applied to preparations of protozoa and bacteria, plant leaves and other feeding stuffs as well as to digesta.

PROCEDURE

Preparation of sample. Before hydrolysis it is necessary to remove chloride and purine-containing compounds other than nucleic acids. Samples containing about 1 mg. of purine-N were extracted with 80% (v/v) ethanol containing approximately 0.001 N-H₂SO₄, in the case of dry samples, or treated with absolute ethanol to a final concentration of 80% in the case of aqueous suspensions.

The mixture was filtered with gentle suction over a thin layer of Celite, in a sintered glass funnel, and the precipitate was washed twice with 80% ethanol, once with absolute ethanol and once with acetone. The major part of the acetone was removed by suction and the last traces by drying the filter in an oven at low temperature.

Hydrolysis. The residue was hydrolysed in 17 ml. of N-H₂SO₄ in the boiling water bath for 1 hr. When handling coarse, fibrous plant material, considerable care was required at this stage. To prevent charring of polysaccharides the hydrolysis was performed in glass-stoppered tubes; the dry filter cake was thoroughly wetted with a little of the acid and transferred to the hydrolysis tube; the remainder of the acid was used to complete the quantitative transfer of the sample. At first, the mixture was cautiously warmed in the bath until occluded air was removed; the glass stopper was then inserted and the hydrolysis allowed to proceed.

In order to avoid errors due to change in volume of the acid during hydrolysis, the hydrolysate was separated from the bulky fibrous residue by straining through a disk of fine stainless steel gauze fitted in the bottom of an all-glass syringe; the fluid was expressed with the aid of compressed air and the pad compressed with the glass piston; the fibrous pad was washed in a similar manner several times with water. The completeness of washing could readily be verified by titration of residual acid in an aqueous extract of the fibre pad. The filtrate and washings were collected into a 25 ml. flask, made to volume and filtered through Whatman no. 42 paper.

Separation of the silver-purines. To 20 ml. of the filtrate was added 1 ml. of 0.5M-AgNO₃ and the mixture was chilled in the refrigerator for 1-2 hr.; the inner wall of the centrifuge tube was then carefully rubbed down with a rubber-tipped glass rod to loosen any adherent precipitate. The rod was rinsed down with about 1 ml. of saturated Ag₂SO₄, in 0.01 N-H₂SO₄. After chilling again, the tubes were centrifuged at 0°, the supernatant fluid was removed and the wall of each tube carefully washed down with a small volume of the Ag₂SO₄ solution; this washing was best performed with a coarse Pasteur pipette carrying a short

length of rubber tubing (bicycle valve tubing) at the tip. The purpose of the washing is to ensure collection of the precipitate at the bottom of the tube rather than to remove contaminants. The tubes were again chilled and centrifuged; the supernatant was removed as completely as possible.

The precipitate was cautiously transferred with N -HCl, in small fractions, into a 1 ml. graduated pipette (previously recalibrated to contain known volumes of water) held vertically in a clamp; the transfer was controlled with a screw-plunger attached by pressure tubing to the pipette. This procedure was found to be simpler than transferring the precipitate to a 1 ml. volumetric flask. The contents were brought to the required volume and, after drying the tip of the pipette, were transferred to a small glass-stoppered tube; mixing was achieved by taking the fluid into and out of the pipette several times.

The preparation was allowed to stand for at least an hour with occasional mixing. The AgCl was then centrifuged down and the supernatant solution removed to a small glass-stoppered tube.

Chromatographic analysis. The purines were separated by the technique of Markham & Smith (1949, 1951) using Whatman no. 3 paper, and n -butanol:water:formic acid (77:13:10, v/v) in the descending direction. The purine spots were identified under ultraviolet light as described by Holiday & Johnson (1949). The spots and blanks were cut out in equal areas, extracted with 0.1*N*-HCl and the densities measured at wavelengths of 250 $m\mu$. for guanine and 262 $m\mu$. for adenine. For calculation, the molecular extinctions given by Smith & Markham (1950) and Wyatt (1951) were used.

Two procedures were found to be satisfactory for controlling the errors due to the paper blanks. In the first, triplicate portions of the sample were spotted on to the paper with alternating spots of solvent (N -HCl) for the blanks; in the second, multiple volumes (e.g. 0, 4, 8, 12, 16 μ l.) were put on the paper and the final readings of density plotted on a graph to give a line the slope of which yields an estimate of the concentration corrected for blanks.

COMMENT ON PROCEDURE

Hydrolysis. Smith & Markham (1950) found that hydrolysis of ribonucleic acid with N -HCl at 100° for 1 hr. liberated the purine bases quantitatively; the pyrimidine bases remained in the form of nucleotides with traces of nucleosides. These latter compounds are not precipitated by Ag^+ in acid solution (Kerr & Seradarian, 1945). Wyatt (1951) observed that the purines are rapidly liberated from deoxyribonucleic acids. Vendrely & Sarciron (1944) and Gulland *et al.* (1947) showed that these conditions of hydrolysis did not lead to a significant loss of purines by acid hydrolysis.

Paper blanks. Markham & Smith (1951) noted the importance of the paper blank as a contributing factor to analytical error, and overcame the difficulty by increasing the quantity of nucleic acid hydrolysate put on to the paper, and by using a chromatographic solvent containing strong hydrochloric acid. This tactic could not be used in the

present work owing to the difficulty in obtaining the highly concentrated solutions required (equivalent to about 10% solution of nucleic acid). The use of replicates or of multiple amounts proved to be satisfactory alternatives.

Solubility of silver-purines. The solubility of the silver salts of adenine and guanine in presence of excess Ag^+ was measured spectrophotometrically. For convenience the solvent chosen was Ag_2SO_4 (0.025*N*- Ag^+) in 0.6*N*- H_2SO_4 . The solubility of Ag-guanine at 0° was found to be approximately 2.3×10^{-6} *M*. The solubility of Ag-adenine was too low to be estimated by this means and hence may be taken as less than 1×10^{-7} *M*. In the procedures given here, these solubilities would indicate a source of error of about 0.5–1% for guanine and less than 0.05% for adenine.

Micro-pipettes. For delivery of small volumes of fluid on to the papers, a modification of the micro-pipette described by Wigglesworth (1937) has been used. The pipettes were made from short lengths of Pyrex capillary tubing with external diameter of about 5 mm. The tube was drawn out to a fine capillary, cut off and sealed into a suitable glass tube to act as a holder. The free end of the capillary was then drawn out, an approximate calibration made and the tube was finally cut at the required length. The pipettes were calibrated for delivery by weighing repeated deliveries of water on to a stack of filter paper disks in a small weighing bottle. The pipette is automatic in action, simple to use, accurate and sturdy. A similar pipette has been described by Meinhard & Hall (1950).

Sources of error. Two major sources of error have been noted. In work with gastric contents and with plant materials special care was required (as with all coarse heterogeneous materials) in taking the initial samples for analysis. The chief manipulative errors are associated with the paper chromatography. Provided that care is taken in the initial extraction of chloride and in removing the supernatant fluids from the Ag-purine precipitates, the volume occupied by the AgCl in the final extract produces a negligible error. In the course of 130 analyses of gastric contents, it was found that the deviation between duplicate samples averaged 2.6%.

SUMMARY

A method is described for the estimation of the purines, adenine and guanine; it is based on the precipitation of the silver purines in acid solution followed by separation of the purines by paper chromatography and estimation by spectrophotometry. The method has been applied to yield an estimate of total nucleic acid in plant material and in the digesta of the ruminant stomach.

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The Oxidation of Myoglobin to Metmyoglobin by Oxygen

3. KINETIC STUDIES IN THE PRESENCE OF CARBON MONOXIDE, AND AT DIFFERENT HYDROGEN-ION CONCENTRATIONS WITH CONSIDERATIONS REGARDING THE STABILITY OF OXYMYOGLOBIN

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In Parts 1 and 2 (George & Stratmann, 1952*a, b*) it was shown that the oxidation of myoglobin to metmyoglobin by oxygen resembles very closely the corresponding oxidation of haemoglobin (Brooks, 1931, 1935). Only quantitative differences were found: in other respects the systems were identical. At constant oxygen pressure the oxidation rate is first order with respect to unoxidized myoglobin or haemoglobin; the rate constant at low oxygen pressures is proportional to the product of the concentrations of reduced myoglobin (Mb) and oxy-myoglobin (MbO₂), or of reduced haemoglobin (Hb) and oxyhaemoglobin (HbO₂); at high oxygen pressures the rate constant is proportional either to [MbO₂] or [HbO₂], or to [MbO₂]² or [HbO₂]². Because results could not be obtained with sufficient precision at high oxygen pressures to decide between these alternatives, an attempt has now been made to decide between them by making use of the inhibition of myoglobin oxidation by carbon monoxide.

Brooks (1931) found that haemoglobin oxidation in air occurs more rapidly in more acid solutions. Similar experiments with myoglobin have now been carried out at both high and low oxygen pressures, over a range of hydrogen-ion concentrations.

Finally, some of the factors which contribute to the stability of oxymyoglobin and oxyhaemoglobin with respect to oxidation are discussed.

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MATERIALS AND METHODS

The myoglobin used was prepared as described by George & Stratmann (1952*a*). In the inhibition experiments, the myoglobin solutions were heavily buffered with K₂HPO₄ and KH₂PO₄, ratio 0.159/0.841, giving a total concentration of phosphate ions of 0.6M with a pH of 5.69 at 30°. In the experiments in which pH was varied, the myoglobin was buffered with phosphate buffer mixtures prepared from NaH₂PO₄ and NaOH, giving pH values over the range 5.35–6.64. The ionic strengths of these buffer solutions were brought to 0.8, the same value as that of the 0.6M potassium phosphate buffer, by the addition of appropriate quantities of NaCl. The pH of the myoglobin solutions was determined before each kinetic experiment using a glass electrode, calibrated with a 0.05M potassium hydrogen phthalate buffer.

O₂, N₂, and CO were obtained from cylinders.

Kinetic measurements

The concentration of myoglobin was 1.68×10^{-4} M. The reaction vessel, flow apparatus, and the procedure for analysis throughout the course of an experiment were as described by George & Stratmann (1952*a, b*). The results were recorded as the percentage of unoxidized myoglobin at various times from the start of the reaction. The log. of the percentage of unoxidized myoglobin was plotted against time in hours and a first-order rate constant, k_{obs} (hr.⁻¹), calculated from the slope of the line. In the experiments in which pH was varied, series of values for k_{obs} were obtained at two partial pressures of oxygen, 4 and 760 mm. In the inhibition experiments, CO–O₂ mixtures were used with partial pressures of CO(p_{CO}) between 0 and 45 mm. and of O₂(p_{O_2}) between 760 and 715 mm. All experiments were carried out at 30°.