SUMMARY

1. Ultracentrifugal examination of clover-leaf extracts has shown two components of high sedimentation coefficient to be present.

2. The heavier of these has been isolated and shown to be a ribonucleoprotein containing 50–55 % of ribonucleic acid. Its properties have been studied by ultracentrifuging and electrophoresis.

3. In common with similar ribonucleoproteins from pea shoots, yeast or liver cells, this component dissociates into subunits in the presence of certain buffer solutions.

4. The second component is considerably less stable, and has not yet been isolated. It is suggested that it may also be a ribonucleoprotein.

5. The possibility of contamination of plant-virus preparations with these normal constituents of leaf is discussed.

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The Estimation of Aminoacetone and δ -Aminolaevulinic Acid

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Since the discovery of δ -aminolaevulinic acid as a precursor of porphyrins (Shemin & Russel, 1953; Neuberger & Scott, 1953) two methods for the determination of this compound have been published. One of these involves the conversion of the amino ketone into a pyrrole by condensation with a second ketone such as acetylacetone or ethyl acetoacetate; the pyrrole so formed is then estimated by means of the Ehrlich reaction. Mauzerall & Granick (1956) used a modified Ehrlich reagent, which gave more stable colours and greater sensitivity than that described by Shuster (1956), and their procedure is generally the method of choice, particularly when small amounts of amino ketone are to be measured. It suffers, however, from the disadvantage that any excess of Ehrlich-reacting compounds such as porphobilinogen or indole derivatives must first be removed.

The other method for the determination of δ -aminolaevulinic acid is based on the observation (Shuster, 1956) that when the amino ketone is mixed with a solution of picric acid and excess of sodium hydroxide is added, a complex is formed

which gives a red-brown colour on subsequent acidification with hydrochloric acid. The mechanism of the reaction and the nature of the complex are not known.

During work on the production of aminoacetone by *Staphylococcus aureus* (Elliott, 1958, 1959), it was necessary to have a suitable assay method. Aminoacetone could be estimated by both procedures, but the picrate method was chosen because of its greater freedom from interference from other substances and because of its simplicity. However, the original procedure (Shuster, 1956) gave erratic results in the determination of both δ -aminolaevulinic acid and aminoacetone and the conditions of the estimation were therefore re-investigated.

The present paper describes a simple procedure for the determination of both of these metabolites; although it is less sensitive than the procedure of Mauzerall & Granick (1956), the method is valuable since it can be used in conditions where the latter is inapplicable. It has the additional advantage that, when combined with a simple resin treatment, only amino ketones, among biologically occurring

compounds, are known to react. Methods for the separation of δ -aminolaevulic acid and aminoacetone are also described.

MATERIALS

δ -Aminolaevulic acid hydrochloride was a Parke Davis and Co. product kindly given by Dr J. E. Falk of C.S.I.R.O., Canberra. Aminoacetone hydrochloride was synthesized by the method of Gabriel & Colman (1902). Aminoacetone toluene-*p*-sulphonate was prepared by adding excess of toluene-*p*-sulphonic acid in ethanol to an ethanolic solution of aminoacetone hydrochloride. The salt was crystallized by the cautious addition of ether and was twice recrystallized from the same solvents. M.p. 130.5° (uncorr.) (Found: C, 49.1; H, 6.4; N, 5.9; S, 12.9%. $C_9H_9ON, C_7H_8O_3S$ requires: C, 49.0; H, 6.2; N, 5.7; S, 13.1%).

RESULTS

Absorption spectra of coloured complexes. In the original procedure with picric acid it was recommended (Shuster, 1956) that measurements of the extinction of the coloured complex formed by δ -aminolaevulic acid (ALA) be made at 450–470 $m\mu$. However, the absorption due to picric acid at these wavelengths (Fig. 1) is such that a slight error in the amount of picric acid added can introduce a large error in the estimation and markedly affect the reproducibility. These difficulties may be avoided if measurements are made at 495 $m\mu$, where the absorption due to picric acid becomes negligible; although there is a slight loss in sensitivity (cf. spectrum of amino ketone–picric acid complex, Fig. 1), this modification avoids the necessity of removing the excess of picric acid as

Fig. 1. Absorption spectra of amino ketone picrate complexes. Upper curve: absorption spectrum given by 0.5 μ mole of ALA in the standard procedure described in the text measured against the picrate-reagent blank. Aminoacetone gives a curve identical in shape but a few per cent lower in absorption values, and is not shown. Lower curve: spectrum of the picric acid blank measured against water.

recommended by Laver, Neuburger & Udenfriend (1958).

Effect of reaction time. The length of time interval between the addition of NaOH and the subsequent acidification with HCl was found to influence greatly the colour yields obtained from ALA and aminoacetone. Under the conditions of the original method the colour yield from ALA is maximal if the HCl is added approx. 45 sec. after the NaOH (Fig. 2, curve 1). Increase of this time causes a marked decrease in colour yield on subsequent acidification; thus after 4 min. the intensity of colour falls to half that obtained after a 45 sec. interval.

The production of colour from aminoacetone under the same conditions is even more sensitive to the reaction time than it is with ALA. Maximum values (Fig. 3, uppermost curve) are obtained after 30 sec. in alkali, the colour decreasing to 50 % after 90 sec. This rapid fall in colour yield probably accounts for the report that aminoacetone is inactive in the picrate reaction (Shuster, 1956).

Effect of concentration of sodium hydroxide. The formation of the colour-yielding complex from ALA proceeds more slowly at lower concentrations of NaOH. The final level attained, however, is the same at concentrations of NaOH from 1.5 to 0.75N, while at 0.38N this value falls by only 20 %. It is also clear from Fig. 2 that the colour-yielding complex is more stable at lower NaOH levels, so

Fig. 2. Effect of reaction time in alkali on colour yield from ALA at different concentrations of NaOH. Colours were developed at room temperature (22°) as follows: 0.5 μ mole of ALA hydrochloride in a 2.5 ml. sample was treated with 0.25 ml. of saturated picric acid solution followed by 0.5 ml. of NaOH of appropriate normality (see below). 1.75 ml. of HCl was added at varying times and colours read at 495 $m\mu$ against a reagent blank. The HCl solution used was adjusted in each case so that the final excess over the NaOH was in all cases 0.75N. Curve 1, 1.5N-NaOH; curve 2, 0.75N-NaOH; curve 3, 0.38N-NaOH; curve 4, as 3 except that the reaction was carried out at 15° instead of 22° .

that at 0.38N the values obtained are almost constant over reaction periods from 4 to 7 min. With aminoacetone, by contrast (Fig. 3), reduction of the NaOH concentration results in lower colour yields, and this cannot be compensated by increasing the reaction time, since destruction of the colour-forming complex proceeds rapidly irrespective of the NaOH concentration.

Effect of temperature. As described above, the colour yields obtained from ALA reached a stable plateau value over a reaction period of 4–7 min. (Fig. 2, curve 3) when 0.38N-NaOH was present

Fig. 3. Effect of reaction time in alkali on the colour yield from aminoacetone at different concentrations of NaOH. Conditions as in Fig. 2 except that samples contained 1.0 μ mole of aminoacetone. ●, 1.5N-NaOH; ○, 0.75N-NaOH; ×, 0.38N-NaOH.

Fig. 4. Colour production from ALA (●) and aminoacetone (○) at 0°. 0.5 μ mole samples of the amino ketones in 2.5 ml. volume were cooled to 0° in an ice-water bath and 0.5 ml. of 5N-NaOH, previously cooled to 0°, added. At the appropriate times 1.75 ml. of 4N-HCl was added and colours were read after 10 min. at room temperature against a reagent blank.

and this seemed to offer the possibility of a suitable procedure for ALA assay. However, Fig. 2 (curves 3 and 4) shows that reduction of the temperature from 22° to 15° alters the course of the reaction so that accurate estimations are impossible without temperature control. It is also clear from Fig. 3 that estimation of aminoacetone at room temperature is impracticable. The effect of carrying out the reaction at 0° was therefore studied. Fig. 4 shows that at this temperature, in the presence of 0.75N-NaOH, stable plateau values are obtained from ALA after reaction periods of 14–16 min.; similarly, colour intensities obtained with aminoacetone are almost constant after reaction periods of 5–7 min. Under these conditions of estimation, errors due to inaccuracies in timing are negligible.

Method for estimation of δ -aminolaevalic acid and aminoacetone. The procedure finally adopted for estimation of the amino ketones is as follows: the sample (0.05–0.8 μ mole of ALA or 0.05–0.5 μ mole of amino acetone) in a volume of 2.5 ml. is mixed with 0.25 ml. of a solution of picric acid saturated at room temperature and cooled to 0° in an ice-water bath. Sodium hydroxide (0.5 ml. of 5N at 0°) is then added and, after mixing, the solution is allowed to stand at 0°. After a 6 min. or a 14 min. reaction period (aminoacetone and ALA determinations respectively), 1.75 ml. of 4N-HCl is added, the solution mixed and brought to room temperature in a water bath. The absorption is read at 495 m μ after a further 10 min. period.

The absorption is proportional to the amino ketone concentration up to 0.5 μ mole for aminoacetone and to 0.8 μ mole for ALA (Fig. 5). Readings of 0.76 and 0.71 are obtained with 0.5 μ mole of ALA and aminoacetone respectively.

Errors in pipetting the reagents have negligible effects on the determination. A 20% reduction in NaOH concentration reduces colour yield by only 6% and a 20% increase has no effect. Variations of 20% in the amount of HCl added likewise have very small effects on the final colour intensity.

Colour stability and interfering substances. The colour produced with both amino ketones fades at the rate of 2%/10 min. at room temperature.

Shuster (1956) has pointed out that many carbonyl compounds such as pyruvic and α -oxoglutaric acids and acetone produce colours in the picrate reaction but that these fade at room temperature. Under the conditions described above it has been found that 50 μ moles of α -oxoglutaric acid give an absorption corresponding to 0.16 μ mole of aminoacetone and that 10 μ moles of oxoglutarate are equivalent to 0.08 μ mole of aminoacetone. In most cases, therefore, keto acids will cause little interference with amino-ketone determinations, but if present in comparatively large amounts they

may be removed by a preliminary resin treatment. In practice the following treatment has been found satisfactory. The sample is passed through a column (5 cm. $\times$ 0.8 cm.) of Dowex 50 (H form) and the column washed with 20 bed-volumes of water. The amino compounds are then eluted with 25 ml. of 2N-HCl and the eluate evaporated to dryness *in vacuo*. The residue is then dissolved in a known volume of water and a suitable sample taken for determination of the amino ketone. Recoveries of aminoacetone (Table 2) and ALA (Table 1) after such treatment are essentially quantitative.

Fig. 5. Calibration curves for ALA (●) and aminoacetone (○) by the standard method. Colours were read against a reagent blank. ALA hydrochloride and aminoacetone toluene-*p*-sulphonic acid salts respectively were used as standards.

Glucose, threonine and inorganic phosphate (0.1M) and 2.5% trichloroacetate were without effect on the colour production.

Recovery experiments. A homogenate was prepared by grinding 10 g. of rat liver in a Potter-Elvehjem apparatus with 10 ml. of 0.05M-phosphate buffer, pH 7.0. Samples (3 ml.) of this homogenate were transferred to centrifuge tubes and varied amounts of ALA or aminoacetone added together with 1.0 ml. of 20% trichloroacetic acid. The volumes were adjusted to 5.0 ml. by the addition of water and the protein removed by centrifuging. Samples (2.0 ml.) of the supernatant liquid were adjusted to pH 3.0–6.5 by the addition of 5N-NaOH and ALA and aminoacetone determined by the procedure described above. The recoveries were too high by approximately 0.07 μ mole irrespective of the amount of amino ketone present (Table 2); in view of this effect it would seem desirable to include internal standards in such determinations.

Separation of δ -aminolaevulic acid and aminoacetone

Ion-exchange resins. ALA and aminoacetone can be separated readily on the carboxylic acid resin Amberlite IRC-50 buffered at pH 4.7–5.0 with sodium acetate buffer. The column is equilibrated with the buffer, washed with water and the sample

Table 1. Recovery of δ -aminolaevulic acid from a Dowex 50 column

The ALA was contained in 20 ml. volumes of a mixture of 0.01M-phosphate buffer (pH 7.0), 1% glucose, 0.1% NaCl and 0.01M-glycine. The solution was treated on Dowex 50 columns as described in the text.

δ -Aminolaevulic acid (μ mole)	
Added	Recovered
0.5	0.54
0.3	0.28
0.2	0.2
0.1	0.095

Table 2. Recovery experiments from rat-liver homogenates

Experimental details are described in the text. Figures are corrected for the blank values in homogenate alone.

Amino ketone	μ moles		
	Added	Recovered	Error
δ -Aminolaevulic acid	1.0	1.075	+0.07
δ -Aminolaevulic acid	0.5	0.575	+0.07
δ -Aminolaevulic acid	0.25	0.29	+0.04
Aminoacetone	1.0	1.07	+0.07
Aminoacetone	0.5	0.58	+0.08
Aminoacetone	0.25	0.32	+0.07
Aminoacetone (Dowex 50-treated)	1.0	1.11	+0.11
Aminoacetone (Dowex 50-treated)	0.5	0.58	+0.08
Aminoacetone (Dowex 50-treated)	0.25	0.32	+0.07

at pH 5.0 run slowly through, when aminoacetone is retained and ALA runs out. After the column has been washed with water the aminoacetone is eluted with HCl. For subsequent chromatography the eluate is evaporated to dryness *in vacuo*, the aminoacetone hydrochloride extracted with absolute ethanol and the bulky precipitate of NaCl filtered off. The solution is then sufficiently salt-free for most purposes.

Paper chromatography. Chromatographic separation of ALA and aminoacetone can be achieved by using the following solvent system: ethanol-m-acetic acid-pyridine-water (95:10:3:3, by vol.) in which ALA and aminoacetone have R_f values of 0.15 and 0.3 respectively. In five acidic solvents tested, including butanol-acetic acid, ALA and aminoacetone had identical R_f values. In view of this, and particularly since alkaline solvents cannot be used, the two compounds could easily be confused. Both give the same colour reactions with ninhydrin (yellow, turning purple at room temperature). ALA and aminoacetone can be separated by paper electrophoresis at pH 4.0–6.5 and the two can also be distinguished chromatographically by first

converting them into pyrroles (Mauzerall & Granick, 1956).

SUMMARY

Conditions for the determination of δ -aminolaevulinic acid and aminoacetone by the picrate reaction have been investigated and a simple procedure has been developed for the estimation of 0.05–0.8 μ mole of δ -aminolaevulinic acid or 0.05–0.5 μ mole of aminoacetone. Methods for separating the two compounds are also described.

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The Chromatography of L-Myosin on Diethylaminoethylcellulose

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The methods employed by most workers for the purification of L-myosin from extracts of whole muscle usually involve two main principles. These are the selective precipitation at ionic strength about 0.3 of any actomyosin which may be present and the removal of albumin and other protein impurities by repeated precipitation by dilution with water to an ionic strength at which the myosin itself is insoluble (Edsall, 1930; Bailey, 1942; Szent-Györgyi, 1945; Portzehl, Schramm & Weber, 1950; Mommaerts & Parrish, 1951; Perry, 1955). Salting-out procedures have also been employed, although when ammonium sulphate is used (Tsao, 1953) appreciable loss in adenosine triphosphatase activity occurs.

Even in the best preparations of L-myosin from rabbit skeletal muscle, identifiable impurities, such as 5'-adenylic deaminase (Summerson & Meister, 1944) and nucleic acid (Bailey, 1946; Lajtha, 1951), are known to be present. In addition, on electrophoretic and ultracentrifugal analyses of such pre-

parations it is often possible to detect small amounts of substances which have not been definitely characterized (Mommaerts & Parrish, 1951; Tsao, 1953; Raeber, Schapira & Dreyfus, 1955).

In view of the ability of myosin to interact with a number of proteins and of the limitations inherent in the standard methods of establishing protein homogeneity when applied to a substance with a molecular asymmetry comparable with that of myosin, it is very difficult to decide by the usual criteria just how homogeneous the usual preparations of L-myosin are. Certainly on electrophoretic evidence alone some workers consider the standard L-myosin preparations to contain 10–15% of impurities (Mommaerts & Parrish, 1951; Tsao, 1953). This is perhaps not surprising in view of the relative lack of specificity of the methods employed in the isolation of myosin, for it is a somewhat labile protein and the more selective methods of preparation are not readily applied. The use of synthetic ion-exchange resins has been limited to the chromatography of the more robust proteins, and until now there has been no report of the

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