The Determination of δ -Aminolaevulic Acid

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δ -Aminolaevulic acid (ALA) is an intermediate in the biosynthesis of porphyrins (Shemin & Russell, 1953; Neuberger & Scott, 1953) and may be involved in other pathways of glycine metabolism (Shemin & Russell, 1953). In the study of these problems it became necessary to devise methods for the identification and measurement of small amounts of ALA in biological materials.

Two different methods have been investigated. The first, based on the Knorr reaction for the synthesis of pyrroles (Knorr, 1886), depends on the condensation of ALA with a suitable carbonyl compound to form a product, presumably an α -unsubstituted pyrrole, which gives a red colour when treated with Ehrlich's *p*-dimethylamino-benzaldehyde reagent. A report of the application of such a method to the determination of ALA appeared while this work was in progress (Granick & Vanden Schrieck, 1955). The second method is somewhat similar to the Jaffé reaction for the determination of creatinine with alkaline picrate (Jaffé, 1886). This reaction can also be used for identifying ALA on paper chromatograms.

EXPERIMENTAL

Preparation of compounds

δ -Aminolaevulic acid hydrochloride, δ -succinimidolaevulic acid, methyl δ -succinimidolaevulate, δ -succinamidolaevulic acid, methyl δ -chlorolaevulate and methyl δ -aminolaevulate, prepared according to Neuberger & Scott (1954), were kindly supplied by Dr J. J. Scott. Aminoacetone and 2:5-dimethylpyrazine, prepared by the method of Gabriel & Pinkus (1893), were also furnished by Dr Scott. The preparation of 6-amino-5-oxohexanoic acid hydrochloride is described by Gibson, Neuberger & Scott (1955). β -Oxo-adipic acid was synthesized by the method of Eisner, Elvidge & Linstead (1950). δ -Diethylaminolaevulic acid was kindly supplied by Smith & Nephew Research Ltd., Ware, Herts. The preparation of the diethyl ester and one of the two monoethyl esters (ethyl α -amino- δ -carboxy- β -oxovalerate) of α -amino- β -oxoadipic acid, the *N*-benzoyloxycarbonyl derivative of methyl α -amino- δ -ethoxycarbonyl- β -oxovalerate, δ -oximinolaevulic acid,

δ -acetamidolaevulic acid and δ -amino- γ -hydroxyvaleric acid is described by Neuberger, Scott & Shuster (1956).

Porphobilinogen was isolated from human urine by the method of Cookson & Rimington (1954) after oral administration of ALA (Berlin, Gray, Neuberger & Scott, 1954).

Determination by the Knorr reaction with ethyl acetoacetate

Since ALA is an amino ketone an attempt was first made to use the method of Elson & Morgan (1933) for the determination of glucosamine. This consists in heating for 15 min. at 100° with 1% acetylacetone in 0.25M-Na₂CO₃. In the case of glucosamine the resulting product gives a red colour with Ehrlich's reagent. In preliminary tests under these conditions ALA gave a stable red colour, the intensity of which was proportional to concentration in the range of 0.5–5.0 μ mole. The maximum absorption was at 553 m μ . The recovery of added ALA from trichloroacetic acid supernatants of an extract of acetone-dried rat-liver powder was about 90%.

The usual conditions employed in the Knorr reaction are heating with 75% acetic acid or with 40% KOH (Knorr & Lange, 1902). The condensation of ALA with acetylacetone was carried out under similar conditions and was found to proceed considerably faster in 5N-NaOH than in either 0.25M-NaCO₃ or 50% acetic acid. Moreover, the colour produced with Ehrlich's reagent after heating with 5N-NaOH was greatly intensified by the addition of ethanol. In an effort to establish optimum conditions the effect of varying heating time, acetylacetone concentration and alkali concentration was determined, but, because of difficulties with high and variable blank values and the lack of proportionality at concentrations below 0.5 mM, this procedure was abandoned. Trial of several other carbonyl compounds showed ethyl acetoacetate to give far more satisfactory results, and the method finally adopted is based on condensation with this reagent.

Reagents

Ethyl acetoacetate. No special purification of the reagent-grade ester is required. A 10% aqueous solution is readily prepared by warming and this is stable for at least 2 days at room temperature.

10N-NaOH. No precautions for the exclusion of CO₂ seem necessary.

Ehrlich's reagent. A 2% solution of reagent-grade *p*-dimethylaminobenzaldehyde in 5N-HCl is stable for at least 3 weeks at room temperature.

ALA standard. An aqueous solution of recrystallized δ -aminolaevulic acid hydrochloride is stable for several weeks at room temperature.

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General procedure

1.0 ml. of 10% ethyl acetoacetate is added to the sample, containing 0.1–0.5 μ mole of ALA in a volume of 1.0 ml., followed by 0.5 ml. of 10N-NaOH. The solution is mixed and heated in a boiling-water bath for 2 min., then cooled, and 0.5 ml. of water is added, followed by 1.0 ml. of 5N-HCl to neutralize the NaOH. Then 1.0 ml. of Ehrlich's reagent is added, and the pink colour which appears on mixing is intensified by the addition of 5.0 ml. of 95% ethanol. After mixing, the tubes are allowed to stand for 10 min. at room temperature; the colour is then read against a reagent blank in a colorimeter with a green filter (500–570 $m\mu$.) or in a spectrophotometer at 550 $m\mu$. An EEL portable colorimeter (Evans Electro Selenium Ltd., Harlow, Essex) was used for most measurements. Standards of about 0.1 and 0.2 μ mole of ALA should always be estimated concurrently with every set of experimental samples.

RESULTS

Absorption spectrum of the colour

Maximum absorption under the conditions of the standard assay occurs at 550 $m\mu$., as compared with 552 $m\mu$. for the Ehrlich colour with porphobilinogen (Cookson & Rimington, 1954), and 553 $m\mu$. for the condensation product of ALA with acetylacetone. The molar extinction coefficient in terms of ALA at 550 $m\mu$. is 11 500.

Effect of varying different factors

Ethyl acetoacetate concentration. The colour produced increases with acetoacetate concentration up to a level of about 3%. Even at the highest concentrations tested there was little or no colour in the reagent blank, as contrasted with acetylacetone, which tends to give very high blank values at higher concentrations of the diketone. The precipitation of sodium ethyl acetoacetate is avoided by the use of a 10% solution of acetoacetate instead of the undiluted ester.

NaOH concentration. Maximum colour development is obtained with NaOH concentrations of about 2.5N. While the colour obtained initially with concentrations down to 0.1N is almost as intense as with 2.5N-NaOH, it is weakened rather than strengthened by addition of ethanol, so that the final intensity is much less.

Heating time. The rate at which chromogen forms during heating at 100° is illustrated in Fig. 1. In other experiments it was found that at the end of 2 min. the reaction is about 70% complete. Since volume changes resulting from evaporation during such a short period may be neglected, this period was chosen as a convenient heating time. Provided that suitable standards are run with each batch of tubes, slight variations in the time of heating need not affect the accuracy of the procedure.

Alcohol concentration. Ethanol is used to intensify the Ehrlich colour in the acetylacetone determina-

tion of glucosamine (Schloss, 1951). At a concentration of 20–50% (v/v), it has a similar effect in the acetoacetate determination of ALA, increasing the colour yield by about 50%. When the condensation is carried out under acid, neutral or weakly alkaline conditions ethanol either dilutes the colour or actually causes it to fade.

Proportionality of colour intensity to ALA concentration

Colour formation by this method is directly proportional to the amount of ALA present over the range 0.05–0.5 μ mole (Fig. 2). This allows the

Fig. 1. Effect of heating time on colour yield by the acetoacetate method. Conditions were those of the standard assay (see text), with 0.23 μ mole of ALA.

Fig. 2. Calibration curve for the ethyl acetoacetate method. Conditions were those of the standard assay.

Table 1. *Colour yields of some compounds compared with ALA*

Compound	Relative molar colour yield	
	Acetoacetate method	Picrate method
δ -Aminolaevulic acid hydrochloride	100	100
Aminoacetone hydrochloride	10	10
Diethyl α -amino- β -oxoadipate hydrochloride	6	31
α -Monoethyl ester of α -amino- β -oxoadipic acid hydrochloride	24	57
Ethyl methyl <i>N</i> -benzoyloxycarbonyl- α -amino- β -oxoadipate	0	0
Acetamidolaevulic acid	14	16
Methyl aminolaevulate hydrochloride	75	87
δ -Amino- γ -hydroxyvaleric acid lactone	0	0
δ -Oximinolaevulic acid	0	3
6-Amino-5-oxohexanoic acid hydrochloride	111	95
Methyl δ -chlorolaevulinate	0	8
δ -Diethylaminolaevulic acid hydrochloride	0	4
δ -Succinimidolaevulic acid	6	20
Methyl δ -succinimidolaevulate	6	23
δ -Succinamidolaevulic acid	138	35
Glucosamine hydrochloride	10	0
<i>N</i> -Acetylglucosamine	5	0
Acetone	0	1
Ethyl acetoacetate	0	2
α -Oxoglutaric acid	0	5
β -Oxoadipic acid	0	1
2,5-Dimethylpyrazine	0	0

determination of as little as 8 μ g. of ALA-HCl. Sensitivity may be increased by reducing in proportion the volumes of the various reagents and measuring the colour in a spectrophotometer at 550 $m\mu$.

Specificity and interfering substances

Other than compounds closely related to ALA, the only substances found to give rise to an appreciable colour in this determination were glucosamine and *N*-acetylglucosamine. The colour yield of both these substances is considerably less than that of ALA, but when they are known to be present it is advisable to use the picrate method described below. Table 1 gives, on a comparative molar basis, the colour yields of some compounds which have been tested. In addition it was found that pyruvic, glutamic and succinic acids, glucose and glycine gave negative results when tested at levels of 0.5–2.0 mg.

Porphobilinogen, which is formed enzymically from ALA in many tissues (Gibson *et al.* 1955), also gives a red colour with Ehrlich's reagent. Any colour due to porphobilinogen can be largely, but not completely, corrected for by running controls without added ethyl acetoacetate. Such a procedure greatly reduces interference by porphobilinogen in ALA determinations (Table 2). Extraneous interfering colours, such as caramel tints produced by heating solutions rich in carbohydrates, can also be corrected for by means of such controls.

Recovery from biological materials

The recovery of added ALA from human urine and from a haemolysate of chicken erythrocytes is essentially complete (Table 3). Similar experiments

Table 2. *Determination of ALA in the presence of porphobilinogen by the acetoacetate method*

Solutions containing known amounts of ALA and porphobilinogen were analysed by the standard assay method, and the values thus obtained were corrected for colour due to porphobilinogen by subtracting the apparent values found in control determinations without ethyl acetoacetate. All values are expressed in micromoles.

Additions		Apparent ALA		ALA (by difference)
ALA	Porpho- bilinogen	+ Aceto- acetate	– Aceto- acetate	
0.095	0	0.095	0	0.095
0.190	0	0.190	0	0.190
0	1.77	0.078	0.074	0.004
0	4.43	0.160	0.145	0.015
0.095	4.43	0.202	0.145	0.057
0.190	1.77	0.238	0.067	0.171
0.190	4.43	0.308	0.140	0.168

have shown that this method is suitable for the detection and assay of ALA in trichloroacetic acid supernatants of liver homogenates and other protein-containing extracts.

Determination by the picrate method

In attempting to isolate the pyrrole formed by the Knorr reaction of ALA with ethyl acetoacetate it was observed that alkaline solutions containing ALA gave a deep-orange colour with picric acid. The Jaffé reaction for the determination of creatinine consists in the production of such a colour with alkaline picrate (Jaffé, 1886). The addition of concentrated HCl, which bleaches the picrate colour of creatinine, intensified and deepened the picrate

Table 3. *Recovery of ALA from biological materials by the acetoacetate method*

The urine was undiluted human urine. The blood filtrate was obtained by adding an equal volume of 10% trichloroacetic acid to a 10% aqueous solution of haemolysed chicken erythrocytes. A solution of ALA was added before deproteinization.

Sample	Vol. (ml.)	Calc. (μ mole)	Found	Recovery (%)
Urine	0.2	0.190	0.194	102
Urine	0.2	0.095	0.103	108
10% trichloroacetic acid protein filtrate	1.0	0.189	0.186	98
5% trichloroacetic acid blood filtrate	0.4	0.151	0.132	88

colour of ALA. The colour with acetone, a source of interference in the Jaffé reaction, is also deepened by HCl, so that the test is not as specific as could be desired. However, the colours obtained with many carbonyl compounds, such as α -oxoglutarate, fade within 10–15 min. as contrasted with the colour from ALA, which is stable for at least several hours. Where interfering ketones are known to be present the ethyl acetoacetate test should be used instead of the picrate method.

The mechanism of colour formation in this test is still obscure. It is of interest that a similar reaction has recently been developed for the measurement of hydantoin-3-acetic acids (Selah & Berger, 1955).

Reagents

Picric acid. A saturated aqueous solution of reagent-grade picric acid was found to be most convenient. After exposure to light for several days picric acid solutions tend to give an orange colour with strong alkali (Owen, Iggo, Scandrett & Stewart, 1954). Since any such colour is completely bleached by the addition of HCl, no protection from light is necessary, and the solution can be kept for months at room temperature.

10N-NaOH. No precautions for the exclusion of CO_2 seem necessary, although it is preferable to keep the solution clear.

General procedure

Saturated picric acid (0.1 ml.) is added to the sample containing 0.1–0.5 μ mole of ALA in a volume of 1.0 ml. Because of its strong colour the picric acid solution should be pipetted accurately. The same results can be obtained with 0.3 ml. of 0.02M picric acid. After mixing, 0.2 ml. of 10N-NaOH is added and the solution is mixed again. Then 1.0 ml. of 5N-HCl is added and, after mixing, the volume is brought to 5.0 ml. with water.

The colour develops almost immediately, and may be read in a colorimeter with a blue filter (450–500 $m\mu$.) or in a spectrophotometer (450 $m\mu$.) against a reagent blank. The absorption spectrum shows a rather broad peak in the region 440–500 $m\mu$., with a molar extinction coefficient in terms of ALA of 6300 at 450 $m\mu$.

Effects of varying different factors

Picric acid concentration. As in the Jaffé reaction, the colour developed is proportional to picric acid

concentration up to a final concentration of 0.001M (Bonsnes & Taussky, 1945). Above this level there is little change. The results obtained with more dilute solutions of picric acid (0.02M and 0.002M) were essentially similar.

Sodium hydroxide concentration. The colour increases sharply with NaOH concentration up to N-NaOH. Raising the concentration of alkali further has only a slight effect.

HCl concentration. The colour developed increases with increasing amounts of HCl up to the addition of about 3 m-moles. At higher concentrations there is a gradual decrease in colour strength. This is not due to fading, since the colour produced in concentrated acid seems to be as stable as that formed at lower HCl concentrations.

Effect of temperature. According to Hervey (1953), the colour intensity in the Jaffé reaction for creatinine varies appreciably with temperature at the time of reading. He recommended maintenance of a constant temperature during colour development and measurement. In the determination of ALA temperatures ranging from 15° to 40° were not found to affect the colour yield at time of measurement.

Specificity and interfering substances

The relative molar colour yield from a number of compounds which have been tested is given in Table 1. Glycine, glucose, and succinic acid in amounts of 10 mg. gave no appreciable colour under the conditions of the assay. Besides compounds related to ALA the only substances found to produce a stable colour were acetone and similar ketones. The picrate method cannot be used in the presence of amounts of acetone greater than 100 μ g., and this feature is its chief limitation.

The picrate method can be used to measure ALA in the presence of porphobilinogen, unless the concentration of the latter greatly exceeds that of ALA (Table 4). Any interference resulting from the pink colour of most porphobilinogen solutions can be corrected for by running controls in which the HCl is added before the NaOH.

Proportionality of colour to ALA concentration

The plot of ALA concentration against colour as measured in the EEL colorimeter with a blue filter (440–500 $m\mu$.) gives a straight-line relationship over a concentration range of 0.05–0.4 micromole (Fig. 3). Linearity in this concentration range is also obtainable in spectrophotometer readings at 450–470 $m\mu$. In all cases it is desirable to run appropriate standards with each series of determinations.

Table 4. *Determination of ALA in the presence of porphobilinogen by the picrate method*

Solutions containing known amounts of porphobilinogen and ALA were analysed by the standard procedure; the results are expressed in terms of ALA.

Additions (μ moles)		ALA recovered (μ mole)
ALA	Porphobilinogen	
0.095	0	0.095
0.190	0	0.190
0	1.77	0.006
0	4.43	0.030
0.095	4.43	0.132
0.190	1.77	0.190
0.190	4.43	0.202

Fig. 3. Calibration curve for the picrate method. Conditions were those of the standard assay. Values are corrected for colour due to picric acid by running a control without added ALA.

Table 5. *Recovery of ALA from biological materials by the picrate method*

The samples were prepared as for Table 3

Sample	Vol. (ml.)	Calc. (μ mole)	Found (μ mole)	Recovered (%)
Urine	0.5	0.238	0.227	96
Urine	0.1	0.095	0.100	105
Urine	0.3	0.285	0.260	92
Urine	0.2	0.190	0.190	100
5% trichloroacetic acid blood filtrate	0.1	0.095	0.087	92

Application to paper chromatography

Although ALA gives a typical purple colour with ninhydrin in the assay of Moore & Stein (1948), it shows up in paper chromatograms developed with ninhydrin as a yellow spot (Berlin, Neuberger & Scott, 1956). ALA can also be detected on paper chromatograms by its pale-white fluorescence under ultraviolet light. As a further check, or in cases where these two methods are not readily applicable, ALA spots can be identified by the picrate method. The method consists of spraying with saturated picric acid, then with 5N-NaOH, and then with 5N-HCl, whereupon ALA and related compounds appear as reddish-orange spots which gradually fade to a light-brown colour. It is advisable to ensure adequate ventilation during spraying.

Recovery from biological materials

Because it can readily be applied to biological fluids the picrate test has been used extensively in this Laboratory in the study of ALA metabolism (Neuberger, Scott & Gray, 1954). Provided that appropriate blanks are used (a suitable procedure is to add the HCl before the NaOH), a reasonable amount of background colour should not interfere, and this test has been used to measure ALA in some darkly coloured urines from porphyric patients. The recovery of added ALA from human urine and from trichloroacetic acid filtrates of a blood haemolysate is shown in Table 5. As in the Jaffé reaction (Owen *et al.* 1954), it is advisable to neutralize acid filtrates before applying the picrate test.

DISCUSSION

The methods here described have proved adequate for studying the metabolism of ALA, and its excretion in certain cases of porphyria (Neuberger, Scott & Gray, unpublished observations). In identifying ALA it is desirable to confirm the results of these tests by paper chromatography and by means of the specific enzyme ALA dehydrase which condenses ALA to porphobilinogen (Gibson *et al.* 1955). In the absence of interfering substances the enzymic test can detect amounts of ALA as low as 0.01 μ mole (about 1 μ g.), and this estimation can be made semiquantitative by using a dilution which falls in the straight-line portion of the enzyme substrate saturation curve, i.e. between 10^{-5} and 10^{-4} M.

These methods also establish ALA and related compounds as possible interfering substances in the Elson & Morgan (1933) assay for glucosamine and the Jaffé (1886) reaction for creatinine.

SUMMARY

1. Two colorimetric methods are described for the determination of δ -aminolaevalic acid. Both are sensitive to 0.05 μ mole.

2. One method is based on condensation with ethyl acetoacetate in 2N-NaOH to form a product which gives a red colour with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde).

3. The other method is a modification of the Jaffé picrate reaction and depends on the reaction of an alkaline picrate complex of ALA with HCl to give an orange-red colour.

4. The effects of varying different factors in the determination, specificity and application to biological materials are described for both methods.

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The Penetration of Ornithine and Citrulline into Liver Slices

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The observations of Gornall & Hunter (1943) show that, at concentrations below 100 mg./100 ml., ornithine is uniformly a more effective catalyst of urea synthesis from ammonia in rat-liver slices than is citrulline. This difference in effectiveness appears to be at variance with what is to be expected of the cycle proposed by Krebs & Henseleit (1932), in which ornithine is transformed through citrulline into arginine, which, in turn, is hydrolysed to yield urea and more ornithine.

The increase in the steady-state rate of urea formation would be expected to be the same following equimolar additions of either ornithine or citrulline to systems catalysing this cycle, and during the approach to steady-state conditions the rate of increase in velocity of urea formation would be expected to be greater when citrulline is added than

when ornithine is added. Therefore, the mean rate of urea formation over any period following the addition of a given amount of citrulline would be expected to be not less than the rate over the same period following the addition of an equimolar amount of ornithine.

It has been suggested by Krebs (1952) that the discrepancy between observation and this expectation is due to differences in accessibility of liver cells to the two amino acids. This suggestion appeared to receive some support from the observation of Christensen, Riggs, Fischer & Palatine (1952) that α -diaminobutyric acid, the next lower homologue of ornithine, is powerfully concentrated by mouse liver.

The present investigation was undertaken to determine directly whether the greater effectiveness of ornithine could be accounted for in terms of differences of ease of penetration of ornithine and citrulline into liver cells.

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