Measurement of 5-aminolaevulinate synthase activity by gas–liquid chromatography with electron-capture detection

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A specific assay for 5-aminolaevulinate synthase activity is described, with a sensitivity comparable with that of radiochemical assays. It is based on measurement by g.l.c. with electron-capture detection of the pentafluorobenzyl ester of the ethyl acetoacetate pyrrole derivative of 5-aminolaevulinic acid, and of the corresponding compound from 6-amino-5-oxohexanoic acid used as internal standard. Enzyme activity has been measured in homogenates of rat liver, spleen, kidney and brain, and in human lymphocytes.

INTRODUCTION

5-Aminolaevulinate synthase (ALA synthase, EC 2.3.1.37), which catalyses the formation of ALA from glycine and succinyl-CoA (3-carboxypropionyl-CoA), is the first enzyme of the haem-biosynthetic pathway [1]. Its activity has been studied extensively in the erythropoietic system and in liver, where it is the rate-controlling enzyme, and also in bacteria and yeasts. The many methods available are an indication of the difficulty its assay presents. Colorimetric assays [2,3], based on the reaction of Ehrlich's *p*-dimethylaminobenzaldehyde reagent with the pyrrole formed by the reaction of ALA with a diketone, are generally simple, rapid and cheap, but are insufficiently sensitive for many applications. Radiochemical assays with labelled glycine, 2-oxoglutarate or succinate [4–7] have been used extensively. Lack of specificity due to radiochemical impurities has been a general problem, although recently this may have been overcome by the use of h.p.l.c. to separate the pyrrole derivative of ALA [5,8]. Another sensitive assay [9] is based on measurement of the fluorescence of uroporphyrin formed from ALA by coupled sequential enzyme reactions with ALA dehydratase and hydroxymethylbilane synthase, but has not been widely used.

The present work reports an assay for ALA synthase activity based on measurement of an ALA derivative by g.l.c. with electron-capture detection. A similar method described previously to measure ALA [10,11] could not be used in assays because the high concentrations of glycine used as substrate resulted in interfering peaks. Replacement of acetylacetone by ethyl acetoacetate for pyrrole formation, however, allowed the development of a specific method with a sensitivity comparable with that of radiochemical assays. The new derivatives of ALA and of 6-amino-5-oxohexanoic acid, used as internal standard, are described, and some applications of the assay are shown.

MATERIALS AND METHODS

Chemicals

Ethyl acetoacetate was from BDH Chemicals (Poole, Dorset, U.K.), and was redistilled. Succinylacetone (4,6-dioxoheptanoic acid), CoA, GTP, ATP, pyridoxal 5'-phosphate and succinate thiokinase were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). 2-Allyl-2-isopropylacetamide (AIA) was a gift from Roche Products (Welwyn Garden City, Herts., U.K.). The

sources of other chemicals and their preparation were as previously described [10] or were of the highest grades commercially available.

Protein measurement

This was done by dye-binding [12], with BSA as a standard.

Chromatography

Ion-exchange on Dowex 50 (H⁺ form) and t.l.c. were as previously described [10], but the markers used in t.l.c. were the corresponding ethyl acetoacetate derivatives.

G.l.c.

This was done with a Pye–Umicam PU 4500 chromatograph, PU 4700 automatic injector and Spectra Physics 4270 computing integrator as previously described [10,11], but the column oven was operated isothermally at 245 °C with the injection port at 260 °C and the detector at 280 °C.

M.s. and g.c.–m.s.

These were done on a Kratos MS 890 mass spectrometer in series with a Carlo Erba gas chromatograph. A 25 m fused-silica capillary column coated with SGE BP1 was used for the g.c. with on-column injection, and a temperature program from 200 to 280 °C at 8 °C/min. For m.s. the accelerating voltage was 6 kV, the ionization energy 70 eV, the filament emission 100 μ A and the source temperature 180 °C. M.s. (direct-inlet) was done also on a VG ZAB-2F mass spectrometer, with ionization energy 70 eV, filament emission 100 μ A and source temperature 180 °C.

N.m.r. spectroscopy

¹H-n.m.r. spectra were obtained on a Bruker 250 spectrometer at 250 MHz.

Microanalysis

This was done by Butterworth Laboratories (Teddington, Middx., U.K.).

Preparation of compounds

4-Ethoxycarbonyl-5-methylpyrrol-3-ylpropanoic acid [C5-EAA-pyrrole, compound (1) in Fig. 1]. This was synthesized and recrystallized essentially as previously described [13].

Abbreviations used: AIA, 2-allyl-2-isopropylacetamide; ALA and C5, 5-aminolaevulinic acid; C6, 6-amino-5-oxohexanoic acid; EAA, ethyl acetoacetate; AA, acetylacetone; ALA-pyrrole, 4-acetyl-5-methylpyrrol-3-ylpropanoic acid; C6-pyrrole, 4-acetyl-5-methylpyrrol-3-ylbutanoic acid; C5-EAA-pyrrole, 4-ethoxycarbonyl-5-methylpyrrol-3-ylpropanoic acid; C6-EAA-pyrrole, 4-ethoxycarbonyl-5-methylpyrrol-3-ylbutanoic acid; pfb, pentafluorobenzyl; g.c.–m.s., gas chromatography–mass spectrometry.

Fig. 1. Structure of pentafluorobenzyl esters of pyrrole carboxylic acids

Compounds (1)–(4) are derived from ethyl acetoacetate; compounds (5)–(8) are derived from acetylacetone [10].

4-Ethoxycarbonyl-5-methylpyrrol-3-ylbutanoic acid [C6-EAA-pyrrole, compound (2) in Fig. 1]. This was synthesized similarly from 6-amino-5-oxohexanoic acid. M.p. was 143 °C. M.s. (direct-inlet) gave peaks at m/z 239 (M^+ , 87%), 194 (M^+ –CH₃CH₂O, 18%), 193 [M^+ –(CH₃CH₂O+H), 37%], 180 (M^+ –CH₂CO₂H, 44%), 166 (M^+ –CH₃CH₂CO₂H or M^+ –CH₃CH₂CO₂, 67%), 150 [M^+ –(CH₃CH₂CO₂+CH₃+H), 41%], 138 [M^+ +1–(CH₂CH₂CO₂H+CH₃CH₂), 100%, base peak] 134 [M^+ –(CH₂CO₂H+CH₃CH₂O+H), 91%], 120 [M^+ –(CH₂CH₂CO₂H+CH₃CH₂O+H), 56%] and 93 [M^+ –(CH₂CH₂CO₂H+CH₃CH₂CO₂), 29%]. ¹H n.m.r. (reference compound tetramethylsilane) gave δ (p.p.m.) [(²H)dimethyl sulphoxide] 11.80 (1H, very broad s, CO₂H), 10.93 (1H, broad s, NH), 6.39 (1H, d, NCH), 4.11 (2H, q, CH₃CH₂), 2.56 (2H, t, CH₂CH₂CH₂CO), 2.36 (3H, s, NCCCH₃), 2.18 (2H, t, CH₂CH₂CH₂) 1.70 (2H, quint, CH₂CH₂CH₂CO) and 1.25 (3H, t, CH₃CH₂).

Pentafluorobenzyl 4-ethoxycarbonyl-5-methylpyrrol-3-ylpropionate [compound (3) in Fig. 1]. This was synthesized by allowing a suspension of C5-EAA-pyrrole (112 μ mol) in 0.5 ml of acetonitrile to react with pentafluorobenzyl bromide (1 mmol) and *N*-ethyl-*NN*-diisopropylamine (716 μ mol) at 25 °C for 20 min in a stoppered vial. Excess reagents were removed by a stream of N₂, and the compound was crystallized in high yield from acetonitrile at –18 °C and recrystallized under the same conditions. On t.l.c. a single spot, R_f 0.55, giving a purple colour in the cold with Ehrlich's reagent (0.5% *p*-dimethylaminobenzaldehyde in 6M-HCl) was obtained. The m.p. was 112 °C (Found: C, 53.0; H, 4.1; F, 23.5; N, 3.6; C₁₈H₁₆F₅NO₄ requires C, 53.3; H, 4.0; F, 23.5; N, 3.5%). M.s. (direct-inlet) and g.c.–m.s. gave peaks at m/z 405 (M^+ , 97%), 360 (M^+ –CH₃CH₂O, 21%), 332 (M^+ –CH₃CH₂CO₂, 14%), 224 (M^+ –C₇H₂F₅, 12%), 208 (M^+ –C₇H₂F₅O, 11%), 181 (C₇H₂F₅, 88%), 178 [M^+ –(C₇H₂F₅+CH₃CH₂O+H), 88%], 166 [M^+ –(C₇H₂F₅+CH₂CO₂), 22%] and 134 [M^+ –(C₇H₂F₅+CO₂+CH₃CH₂O+H), 100% base peak]. ¹H n.m.r. (reference compound tetramethylsilane) gave δ (p.p.m.) [(²H)chloroform] 7.9 (1H, broad s, NH), 6.36 (1H, d, NCH), 5.17 (2H, s, OCH₂), 4.29 (2H, q, CH₃CH₂), 3.02 (2H, t, CH₂CH₂CO), 2.67 (2H, t, CH₂CH₂), 2.48 (3H, s, CCH₃) and 1.32 (3H, t, CH₃CH₂).

Pentafluorobenzyl 4-ethoxycarbonyl-5-methylpyrrol-3-ylbutanoate [compound (4) in Fig. 1]. This was synthesized in analogous fashion to the C5 derivative. The m.p. was 86 °C. M.s. gave peaks at m/z 419 (M^+ , 83%), 374 (M^+ –CH₃CH₂O, 26%), 346 (M^+ –CH₃CH₂CO₂, 10%), 238 (M^+ –C₇H₂F₅, 4%), 222 (M^+ –C₇H₂F₅O, 12%), 192 [M^+ –(C₇H₂F₅+CH₃CH₂O+H), 48%], 181 (C₇H₂F₅, 100%, base peak), 180 [M^+ –(C₇H₂F₅+CH₂CO₂), 53%], 166 [M^+ –(C₇H₂F₅+CH₂CH₂CO₂), 40%], 148 [M^+ –(C₇H₂F₅+CO₂+CH₃CH₂O+H), 40%] and 134 [M^+ –(C₇H₂F₅+CH₂CO₂+CH₃CH₂O+H), 66%]. ¹H n.m.r. (reference compound tetramethylsilane) gave δ (p.p.m.) [(²H)chloroform] 7.9 (1H, broad s, NH), 6.36 (1H, d, NCH), 5.18 (2H, s, OCH₂), 4.24 (2H, q, CH₃CH₂), 2.7 (2H, t, CH₂CH₂CH₂CO), 2.48 (3H, s, NCCCH₃), 2.36 (2H, t, CH₂CH₂CH₂), 1.9 (2H, t, CH₂CH₂CH₂) and 1.33 (3H, t, CH₃CH₂).

CH₂CO₂), 53 %], 166 [M^+ –(C₇H₂F₅+CH₂CH₂CO₂), 40 %], 148 [M^+ –(C₇H₂F₅+CO₂+CH₃CH₂O+H), 40 %] and 134 [M^+ –(C₇H₂F₅+CH₂CO₂+CH₃CH₂O+H), 66 %]. ¹H n.m.r. (reference compound tetramethylsilane) gave δ (p.p.m.) [(²H)chloroform] 7.9 (1H, broad s, NH), 6.36 (1H, d, NCH), 5.18 (2H, s, OCH₂), 4.24 (2H, q, CH₃CH₂), 2.7 (2H, t, CH₂CH₂CH₂CO), 2.48 (3H, s, NCCCH₃), 2.36 (2H, t, CH₂CH₂CH₂), 1.9 (2H, t, CH₂CH₂CH₂) and 1.33 (3H, t, CH₃CH₂).

Biological methods

Human lymphocytes. Blood was collected with EDTA as anticoagulant, and lymphocytes were isolated [14] and resuspended in medium containing Tris/KH₂PO₄ buffer, pH 7.4 (40 mM), glycine (50 mM), sucrose (250 mM), EDTA (10 mM), MgCl₂ (20 mM), succinylacetone (0.1 mM), pyridoxal 5'-phosphate (0.4 mM), succinate (2 mM), GTP (5 mM), CoA (1 mM) and succinate thiokinase (0.3 unit) in a final volume of 0.5 ml. Cells were disrupted by irradiation at 0 °C with two bursts of 5 s at 10–12 μ A with an MSE 150 W ultrasonicator.

Rat tissues. White male Wistar rats (250–300 g) and female Lewis rats (180 g) were kept without food for 24 h but allowed water *ad libitum*, and were killed by stunning and exsanguination. The liver or other organs were quickly removed and cut into small pieces with scissors in ice-cold medium containing Tris/HCl buffer, pH 7.4 (50 mM), sucrose (250 mM), EDTA (0.5 mM) and pyridoxal 5'-phosphate (0.2 mM). After a washing with one change of the medium a 20% (w/v) suspension was made with a Teflon/glass Potter–Elvehjem homogenizer and diluted to 10% (w/v).

Treatment of rats with AIA. Rats deprived of food for 16 h were injected subcutaneously with AIA (40 mg/100 g) dissolved in sterile 0.154 M-NaCl and killed 16 h later. Tissue homogenates were prepared as described for the untreated rats.

Assay of ALA synthase activity. Portions of rat tissue homogenates were incubated at 37 °C in a final volume of 0.6 ml in unstoppered polypropylene centrifuge tubes in a shaking water bath. Up to 0.1 ml of homogenate (10%, w/v) was added, smaller volumes being adjusted to 0.1 ml with the suspension medium described above. The basic assay medium contained (final composition) Tris/phosphate buffer, pH 7.4 (40 mM), glycine (50 mM), sucrose (250 mM), EDTA (10 mM), MgCl₂ (20 mM), succinylacetone (0.1 mM), pyridoxal 5'-phosphate (0.4 mM) and succinate (2 mM). When cofactors were added, these consisted of ATP (10 mM), CoA (0.1 mM) and KF (50 mM) or of GTP (10 mM), CoA (0.1 mM) and succinate thiokinase (0.3 unit) with or without KF (50 mM). After incubation, 6-amino-5-oxohexanoic acid hydrochloride (C₆HCl, 826 pmol in 0.15 ml of water) was added as internal standard, and the reaction was terminated by adding 1 ml of 5% (w/v) trichloroacetic acid. After centrifugation at 2500 g for 15 min, the supernatants were diluted to 3 ml with ion-free water and loaded on the Dowex 50 (H⁺ form) columns for measurement of ALA as previously described [10], but with the use of ethyl acetoacetate instead of acetylacetone for the pyrrole formation step.

RESULTS AND DISCUSSION

The structures of the pentafluorobenzyl derivatives of the pyrroles obtained by Knorr-type condensation of ALA and C6 with ethyl acetoacetate were found to be analogous to the compounds obtained with acetylacetone [10] (Fig. 1). From the mass spectrum and elemental analysis a single pentafluorobenzyl

Fig. 2. G.l.c. of pentafluorobenzyl esters of ethyl acetoacetate pyrrole carboxylic acids

(a) Approx. 80 pg of C5-EAApfb and 130 pg of C6-EAApfb in acetone were injected on to the column. The amplifier attenuation was $\times 256$. (b) Standards of ALA (50 ng) and C6 (150 ng) taken through the derivative-formation procedures but not in assay medium. A $1 \mu\text{l}$ portion from the t.l.c. eluate ($600 \mu\text{l}$) was injected. The amplifier attenuation was $\times 128$. (c) Products of an enzyme incubation with human lymphocytes (attenuation $\times 128$). The amount of ALA produced was 57 pmol/h per mg of protein. (d) Products of an assay with rat liver homogenate (attenuation $\times 128$). The amount of ALA produced was 30 pmol/h per mg of protein. The arrows mark the point of injection. The chart speed of 0.1 cm/min was increased to 0.25 cm/min between 6.5 and 13 min. 5 is C5-EAApfb and 6 is C6-EAApfb.

group was present, esterifying the propionic acid or butyric acid function. ^1H n.m.r. indicated that the ethyl group had not been removed, and that the pyrrole NH was unsubstituted.

The ALA and C6 pentafluorobenzyl derivatives did not separate in the t.l.c. system used for their partial purification, but resolved well from each other on g.l.c. (Fig. 2) and also from intervening material that had also failed to separate on t.l.c. Standards taken through the entire procedure and products of incubation from the assay of ALA synthase activity showed the same pattern. The intervening material was independent of glycine or other constituents of the assay reaction mixture, but depended on the use of ethyl acetoacetate and appeared to have several constituents. One of the compounds was likely to be acetoacetic acid pentafluorobenzyl ester on the basis of g.c.-m.s., which gave peaks at m/z 282 (M^+ , 5%), 281 ($M^+ - 1$, 33%), 198 ($\text{C}_7\text{H}_2\text{F}_5\text{O} + 1$, 11%), 181 ($\text{C}_7\text{H}_2\text{F}_5$, 100%, base peak), 84 [$M^+ - (1 + \text{C}_7\text{H}_2\text{F}_5\text{O})$, 85%], 57 (CH_3COCH_2 , 55%) and 42 (CH_2CO , 25%).

When acetylacetone was used to form Knorr pyrroles, on g.l.c. the positions of both the C5 and the C6 compounds were 'swamped' by other peaks. These compounds depended on the presence of high concentrations of glycine in the assay mixture. They may be pentafluorobenzyl derivatives of the unidentified

compounds presumed to be produced by the reaction of glycine with ALA-pyrrole [compound (5) in Fig. 1] [3] and analogously therefore with C6-pyrrole [compound (6) in Fig. 1].

The pentafluorobenzyl derivatives of C5-EAA-pyrrole and C6-EAA-pyrrole were eluted more rapidly on g.l.c. than the corresponding AA derivatives. The column could thus be operated at a lower temperature. Typical retention times for EAA derivatives at 245°C were 8.05 min and 11.8 min for the C5 and C6 compounds respectively, compared with 9.07 min and 13.44 min for the AA derivatives. On t.l.c. the EAA derivatives had higher R_F values (relative R_F 1.29). Sensitivity of detection on g.l.c. was similar, however, with a lower limit of measurement on column of 1 pg (approx. 2 fmol).

Literature values for ALA synthase activity in rat liver homogenates determined by different methods span a wide range of values, e.g. 20–120 pmol of ALA formed/h per mg of liver [3] and 39 pmol/h per mg of protein [9], which depend in part on the conditions of the assay and the preparation of the tissue. Regardless of the absolute values reported, however, activity of the enzyme has been shown to increase approx. 10-fold after treatment of the animals with an inducing agent such as AIA [15]. The activity of ALA synthase was therefore measured in normal liver and after induction with AIA as a means of

Table 1. Activity of ALA synthase in rat liver homogenates

ALA synthase activity was assayed as described in the Materials and methods section in homogenates prepared from white Wistar rats. Fresh tissue was assayed within 60 min of death. Frozen tissue refers to the same homogenates kept at -18°C for 4 weeks and thawed once only. Pretreatment with AIA is described in the Materials and methods section.

Conditions	Cofactors	ALA synthase activity (pmol of ALA/h per mg of protein)
Fresh tissue		
Normal	None	75
AIA-treated	None	190
Frozen tissue		
Normal	None	15
AIA-treated	None	170
Normal	ATP, CoA, KF	93
AIA-treated	ATP, CoA, KF	864

validating the assay described. Activity was measured without and with added cofactors to compare endogenous with enhanced activity, and also in homogenates kept at -18°C for up to 6 weeks. The assay conditions used were otherwise based on general methods reported in the literature. Succinylacetone was used in addition to EDTA for complete inhibition of ALA dehydratase [8]. It was confirmed by adding it to standards that it did not affect pyrrole formation with ethyl acetoacetate that was used at approx. 450 times greater concentration. KF was used to inhibit ATPases [16].

ALA synthase activity was readily demonstrable in whole liver homogenates without addition of cofactors or a succinyl-CoA-generating system, but markedly greater activity was obtained with cofactor supplements. The combined use of ATP, CoA and KF enhanced activity (Table 1) more effectively than GTP, CoA and succinate thiokinase combinations (results not shown) in homogenates of liver of male Wistar rats stored for 4 weeks at -18°C and thawed once only. Pretreatment with AIA resulted in a 9.3-fold increase in ALA synthase activity assayed with added cofactors. Under these conditions of storage the normal homogenate lost 80% of its endogenous activity compared with fresh tissue, but the loss was minimal in the case of AIA-induced liver (Table 1).

Activity was proportional to the amount of liver homogenate and could be readily measured with $5\mu\text{l}$ of a 10% (w/v) homogenate (100–140 μg of protein) incubated for 1 h (Fig. 3). The reaction was linear for at least 2 h. The sensitivity of the assay is thus about 100 times greater than standard colorimetric assays requiring 500 μl of a 10% (w/v) liver homogenate [3].

Activities were determined also in homogenates of rat spleen, kidney, brain and heart from an AIA-treated rat. The low activity in brain (Table 2), even compared with the low activities in organs other than liver, is apparent, and its activity was not increased by addition of cofactors. Similar values (1.8–8.3 pmol of ALA/h per mg of protein [17] and 2.2–3.2 pmol of ALA/h per mg of protein [18]) have been reported for rat brain with the use of radiochemical methods. Values for other rat tissues with the coupled enzyme method [9] include 87 pmol of ALA/h per mg of protein for spleen, which is nearly twice the value obtained for liver by the same method, as indicated above, and 8 and 2 pmol of ALA/h per mg of protein for kidney and heart respectively. The Lewis-rat liver homogenates had higher ALA synthase activity than those of the Wistar rats, and an approx. 10-fold increase in activity after treatment with AIA was apparent even when the assay was performed in the absence of added cofactors (Table 3). Their addition resulted in a proportionately greater increase in activity in the normal as compared with the AIA-treated rats. No change in the activity of the spleen homogenates

Fig. 3. ALA production by different concentrations of liver homogenate

Portions of a 10% (w/v) liver homogenate made up where required to 50 μl with sucrose medium were added to the mixture described in the Materials and Methods section for assay of ALA synthase activity and were incubated at 37°C for 1 h. The homogenate had been stored 6 weeks at -18°C and had been thawed once previously at 4 weeks.

was found by adding cofactors or by pretreatment of the rat with AIA. The activities of multiple samples (two to four) from individual homogenates of rat tissues differed by less than 5% of the mean.

Purified human lymphocytes obtained from one normal subject had an activity of 57 pmol of ALA/h per mg of protein, which is at the lower end of activity reported for unfractionated peripheral leucocytes (50–300 pmol of ALA/h per mg of protein) [19].

No attempt has been made in this work to establish optimum conditions for assay of ALA synthase. These will vary with the individual tissue and its preparation. The different studies show, however, that the method described can provide a highly sensitive measuring system for ALA produced in such assays and avoids possible lack of specificity due to radiochemical impurities. The method may be suitable also for the measurement of ALA formation in plant tissues, provided that the enzyme systems that use the C_3 skeleton of glutamate for ALA synthesis [20] do not generate interfering compounds.

Table 2. ALA synthase activity in rat tissue homogenates

The conditions for the assays were as described in the Materials and methods section and in the legend to Table 1. The source of the homogenates was an AIA-treated rat. Values shown are for single determinations on fresh tissue without added cofactors, and for the same homogenates kept for 4 weeks at -18°C and assayed without and with the listed cofactors. Abbreviation: N.D., not determined

Tissue	Cofactors	ALA synthase activity (pmol of ALA/h per mg of protein)	
		Fresh tissue	Frozen tissue
Spleen	None	25	25
	ATP, CoA, KF	N.D.	35
Kidney	None	33	30
	ATP, CoA, KF	N.D.	70
Heart	None	11	11
	ATP, CoA, KF	N.D.	38
Brain	None	6	6
	ATP, CoA, KF	N.D.	6

Table 3. Activity of ALA synthase in fresh rat liver and spleen homogenates

The enzyme was assayed in homogenates from Lewis rats under the conditions described in the Materials and methods section and in the legends to Tables 1 and 2.

Tissue	Cofactors	ALA synthase activity (pmol of ALA/h per mg of protein)
Liver		
Normal	None	136
AIA-treated	None	1184
Normal	ATP, CoA, KF	860
AIA-treated	ATP, CoA, KF	2355
Spleen		
Normal	None	40
AIA-treated	None	44
Normal	ATP, CoA, KF	42
AIA-treated	ATP, CoA, KF	38

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REFERENCES

- Granick, S. & Sassa, S. (1971) in *Metabolic Regulation* (Vogel, H. J., ed.), pp. 77–141, Academic Press, New York and London
- Urata, G. & Granick, S. (1963) *J. Biol. Chem.* **238**, 811–820
- Lien, L.-F. & Beattie, D. S. (1982) *Enzyme* **28**, 120–132
- Strand, L. J., Swanson, A. L., Manning, J., Branch, S. & Marver, H. S. (1972) *Anal. Biochem.* **47**, 457–470
- Tikerpae, J., Samson, D. & Lim, C. K. (1981) *Clin. Chim. Acta* **113**, 65–73
- Freshney, R. I. & Paul, L. (1970) *Biochim. Biophys. Acta* **220**, 594–601
- Brooker, J. D., Srivastava, G., May, B. K. & Elliott, W. H. (1982) *Enzyme* **28**, 109–119
- Fitzsimons, E. J., May, A., Elder, G. H. & Jacobs, A. (1986) *Anal. Biochem.* **153**, 9–17
- Bishop, D. F., Devey, K. R., McBride, L. & Desnick, R. J. (1981) *Anal. Biochem.* **113**, 68–78
- Gorchein, A. (1984) *Biochem. J.* **219**, 883–889
- Gorchein, A. & Webber, R. (1987) *Clin. Sci.* **72**, 103–112
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Mauzerall, D. & Granick, S. (1956) *J. Biol. Chem.* **219**, 435–446
- Boyum, A. (1968) *Scand. J. Lab. Invest.* **21**, Suppl. **97**, 77–89
- Bonkowsky, H. L., Pomeroy, J. S. & Sinclair, P. R. (1982) *Enzyme* **28**, 130–132
- Yoda, B., Schacter, B. A. & Israels, L. G. (1975) *Anal. Biochem.* **66**, 221–233
- Percy, V. A. & Shanley, B. C. (1979) *J. Neurochem.* **33**, 1267–1274
- De Matteis, F. & Ray, D. E. (1982) *J. Neurochem.* **39**, 551–556
- Brodie, M. J., Thomson, G. G., Moore, M. R., Beattie, A. D. & Goldberg, A. (1977) *Q. J. Med.* **182**, 229–241
- Beale, S. I. & Castelfranco, P. A. (1974) *Plant Physiol.* **53**, 297–303

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