Biochem. J. (1972) **129**, 31–37
Printed in Great Britain

δ-Aminolaevulinate Dehydratase, the Regulatory Enzyme of the Haem-Biosynthetic Pathway in *Neurospora crassa*

By S. MUTHUKRISHNAN,* K. MALATHI and G. PADMANABAN
Department of Biochemistry, Indian Institute of Science, Bangalore-12, India

(*Received* 11 *February* 1972)

The activity of δ-aminolaevulinate dehydratase is very low in the mould *Neurospora crassa* compared with the activities detected in bacterial and animal systems. The enzyme is inducible in iron-deficient cultures by addition of iron and is repressed by protoporphyrin. The properties of the purified enzyme indicate its allosteric nature and susceptibility to feedback inhibition by coproporphyrinogen III. *Neurospora* extracts also contain a protein inhibitor of the enzyme and a small-molecule activator, which appears to be associated with the enzyme. The regulatory function of this enzyme *in vivo* is correlated with the accumulation of δ-aminolaevulinic acid in normal cultures of *N. crassa*. The decay curve of the iron-induced enzyme *in vivo* shows a biphasic pattern, with one of the components showing a half-life of 4–5 min.

δ-Aminolaevulinate dehydratase, the second enzyme of the haem-biosynthetic pathway, catalysing the formation of porphobilinogen from δ-aminolaevulinate, has been studied in a wide variety of animal, plant and microbial systems and is considered to be present in non-limiting amounts (Gibson *et al.*, 1958; Batlle *et al.*, 1967; Nandi & Waygood, 1967; Tigier *et al.*, 1968; Burnham & Lascelles, 1963; Ho & Lacelles, 1971). However, the possibility has been indicated that in *Neurospora crassa* (Muthukrishnan *et al.*, 1969) the enzyme could regulate the haem-biosynthetic pathway.

The present study concerns the purification of δ-aminolaevulinate dehydratase from *N. crassa* and properties of the enzyme *in vitro* and *in vivo*. These studies lend support to the contention that δ-aminolaevulinate dehydratase regulates haem biosynthesis in this mould. Part of this work has already been presented in preliminary form (Padmanaban & Malathi, 1972).

Experimental

Organism and growth conditions

Neurospora crassa (wild-type, Em 5297a) was used in these experiments. The organism was grown under iron-deficient or normal conditions in stationary cultures as described earlier (Padmanaban & Sarma, 1965; Muthukrishnan *et al.*, 1969). The composition of the medium was (g/100ml): glucose, 2; KH_2PO_4, 0.3; NH_4NO_3, 0.2; ammonium tartrate, 0.1; $MgSO_4,7H_2O$, 0.05; NaCl, 0.01; $CaCl_2$, 0.01. The following components were also included as salts (μg/100ml): zinc, 20; manganese, 20; copper, 8; iron, 20; molybdenum, 2; biotin was added to a final concentration of 0.5μg/100ml.

For the preparation of the iron-deficient medium, the major nutrients were extracted with 8-hydroxyquinoline in chloroform. The excess of 8-hydroxyquinoline was removed by repeated chloroform extraction and excess of chloroform was removed by aeration at 45°C. Glucose (A.R.) was treated with Dowex-50 resin (H^+ form) to free it of metal contaminants. The iron-deficient medium contained 0.2μg of iron/10ml of medium. For induction studies 40h-old mycelia were used.

Purification of δ-aminolaevulinate dehydratase

The enzyme was induced in 40h-old iron-deficient cultures by the addition of iron (1μg/ml) as ferric citrate or ferric chloride. The cultures were shaken for 30min, when maximal induction of the enzyme occurred. The mycelia (16g fresh wt.) were ground with 80ml of potassium phosphate buffer (pH8.0, 0.05M, containing 0.01M-cysteine) by using glass powder in a VirTis homogenizer. The homogenate was centrifuged at 10000*g* for 10min and the sediment was re-extracted with 60ml of the same buffer. The supernatants were pooled and represented the crude extract. This was subjected to ammonium sulphate fractionation, and the precipitate formed between 0.25 and 0.4 saturation was collected by centrifugation and suspended in 10ml of phosphate buffer (pH6.5, 0.05M). 1% Protamine sulphate soln. (0.5ml) was then added drop by drop with stirring and the precipitated nucleoproteins were removed.

* Present address: Department of Biophysics, University of Chicago, Ill. 60637, U.S.A.

The supernatant was adjusted to pH 7.0 and stirred with calcium phosphate gel (1 mg of gel/mg of protein). After centrifugation, the residue was discarded and the supernatant was again subjected to ammonium sulphate fractionation. The 0–0.3 saturation fraction was dissolved in 2 ml of phosphate buffer (pH 8.0, 0.05 M, containing 0.01 M-cysteine), and then loaded on to a Bio-Gel P-200 column (50 cm × 2.5 cm) equilibrated and eluted with the same buffer. Fractions (2 ml) were collected, and the fractions containing the enzyme activity were pooled and again subjected to ammonium sulphate fractionation. The 0–0.3 fraction was collected, dissolved and dialysed for 1 h against phosphate buffer containing cysteine. This preparation was tested by polyacrylamide-gel electrophoresis for homogeneity. For routine studies of the general properties of the enzyme, the preparation obtained after the second ammonium sulphate fractionation was used.

Assay of δ-aminolaevulinate dehydratase

The assay mixture, in a volume of 2 ml, contained: δ-aminolaevulinate, 2.5 μmol; potassium phosphate buffer (pH 8.0), 100 μmol; glutathione, 7.5 μmol; $ZnSO_4$, 0.2 μmol; enzyme protein. Incubation was carried out at 37°C for 2 h. No preincubation with glutathione was found necessary. The reaction was stopped with 1 ml of trichloroacetic acid–$HgCl_2$ soln. [0.1 M-$HgCl_2$ in 12.5% (w/v) trichloroacetic acid] and the porphobilinogen formed was determined with the Ehrlich reagent of Granick & Mauzerall (1958).

Determination of δ-aminolaevulinate, porphobilinogen and porphyrins

The procedures were as described by Muthukrishnan *et al.* (1968).

Determination of the half-life of δ-aminolaevulinate dehydratase

The half-life was determined in 40 h-old normal cultures or in the iron-deficient cultures, where the enzyme was induced by the addition of iron as described above. Cycloheximide (1 μg/ml) was added to normal or induced cultures to inhibit protein synthesis. The mycelia were removed at various intervals of time and δ-aminolaevulinate dehydratase activity was assayed as described above.

Protein was measured by the method of Lowry *et al.* (1951).

Results

Table 1 lists the activity of δ-aminolaevulinate dehydratase in the crude extracts of a wide variety of sources. *Saccharomyces* and *Neurospora* had the lowest activities of this enzyme.

It has already been shown (Muthukrishnan *et al.*, 1968, 1969) that δ-aminolaevulinate dehydratase is barely detectable in iron-deficient *Neurospora* but that it can be induced over the normal activities with iron. So, for purification purposes, the enzyme was induced with iron in iron-deficient cultures and the enzyme was purified by the steps described in Table 2. The final preparation gave a single protein band in polyacrylamide-gel electrophoresis. However, in some preparations an additional minor band was also observed.

The properties of the enzyme are listed in Table 3. The enzyme requires thiol groups for activity. However, preincubation with glutathione before the addition of substrate was not found necessary, unlike the animal systems (Gibson *et al.*, 1958). *p*-Hydroxymercuribenzoate inhibits the enzyme activity

Table 1. *Activities of δ-aminolaevulinate dehydratase from various sources*

δ-Aminolaevulinate dehydratase was assayed as described in the text. For the rat liver homogenate, the preparation was preincubated with glutathione before the addition of the substrate.

Source	δ-Aminolaevulinate dehydratase activity (nmol of porphobilinogen formed/h per mg of protein)
Chromatium D	295.4
Rhodopseudomonas spheroides	85.3
Rat liver	112.0
Euglena gracilis (light)	43.4
Euglena gracilis (dark)	24.3
Saccharomyces cerevisiae	1.6
Neurospora crassa (0.25–0.4-satd. ammonium sulphate fraction)	1.0
Wheat leaves (0.25–0.4-satd. ammonium sulphate fraction)	15.1

completely. The pH optimum for the enzyme reaction is 8–8.4 and the temperature optimum is at 55°C. Metal-chelating agents inhibit the enzyme activity. Dialysis of the enzyme against EDTA results in complete loss of activity but the activity could be fully regained by the addition of Zn^{2+} (10μM). Ferric iron was also found to be partly effective in restoring activity. Fig. 1 shows the velocity–substrate concentration curve as sigmoidal and the Hill plot of the data yields an n value of approx. 2.0. The K_m for δ-aminolaevulinic acid is 10^{-4}M.

The feed-back inhibitory properties of the metabolites of the haem-biosynthetic pathway are given in Table 4. Haemin, protoporphyrin and protoporphyrinogen do not inhibit the enzyme significantly, but coproporphyrin III and coproporphyrinogen III are good inhibitors.

It was not possible to detect δ-aminolaevulinate dehydratase activity in crude extracts of *N. crassa* (see Table 2), and hence the possible presence of an inhibitor in crude extracts was investigated. Addition of crude extract to the purified enzyme gave inhibition of activity. The inhibitor was purified 10-fold by the following procedure. The precipitate formed between 0.5 and 0.9 saturation with ammonium sulphate was collected and dissolved in phosphate buffer (pH 7.0, 0.05M). After dialysis against the same buffer, the

Table 2. *Purification of δ-aminolaevulinate dehydratase from Neurospora crassa*

The purification steps are given in text. The units of enzyme activity are as described in Table 1.

Preparation	Protein content (mg) (A)	Specific activity (units/mg of protein) (B)	Total activity ($A \times B$)
Crude	642.0	0	0
I $(NH_4)_2SO_4$ ppt. (0.25–0.4-satd. fraction)	159.0	1.83	290.1
Protamine sulphate supernatant	135.7	2.30	312.8
Calcium phosphate gel supernatant	54.50	4.01	281.1
II $(NH_4)_2SO_4$ ppt. (0–0.3-satd. fraction)	17.60	7.88	138.3
Biogel P-200 eluate	8.00	19.38	92.8
III $(NH_4)_2SO_4$ ppt. (0–0.3-satd. fraction)	1.44	27.78	32.2

Table 3. *Properties of δ-aminolaevulinate dehydratase from Neurospora crassa*

The preparation obtained after the second ammonium sulphate fractionation was used in these studies. The absolute specific activity of the enzyme is given in parentheses.

Treatment	Specific activity of δ-aminolaevulinate dehydratase (%)
Complete system	100 (8.2)
Glutathione omitted	0
Complete system + p-hydroxymercuribenzoate (1mM)	0
Complete + EDTA (0.1mM)	0
Complete + o-phenanthroline (1mM)	3
Complete + 8-hydroxyquinoline (1mM)	27
EDTA-dialysed enzyme* (preparation A)	0
Prep. A + Zn^{2+} (10μM)	100
Prep. A + Fe^{3+} (10μM)	55
Prep. A + Fe^{2+} (10μM)	25
Prep. A + Cu^{2+} (10μM)	0

* The enzyme in 0.05M-phosphate buffer (pH 8.0) was dialysed against the same buffer containing EDTA (1mM) for 2h. This was followed by dialysis against buffer alone for 2h, with three changes of the buffer to remove EDTA.

Table 4. *Effect of the metabolites of the haem-biosynthetic pathway on the activity of δ-aminolaevulinate dehydratase from Neurospora crassa*

The preparation obtained after the second ammonium sulphate fractionation was used in these studies. Haemin and protoporphyrin were dissolved just before use in 0.05ml of 0.1M-NaOH and then made up to the required volume with phosphate buffer (pH 8.0). Coproporphyrinogen was prepared by the reduction of coproporphyrin III with sodium amalgam or sodium borohydride and neutralized before use. Additions were at 0.1mM with the exception that coproporphyrinogen III was also tested at 10μM.

Compound added	Specific activity of δ-aminolaevulinate dehydratase (%)
None	100 (8.5)
Haemin	100
Protoporphyrin	100
Coproporphyrin III	10
Coproporphyrinogen III	2
Coproporphyrinogen III (10μM)	30
AMP	94
ADP	103
ATP	96
Succinate	93
α-Oxoglutarate	90

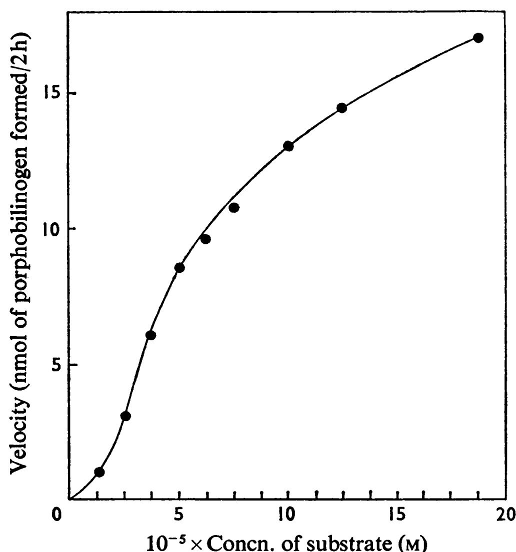

Fig. 1. *Effect of substrate concentration on the velocity of δ-aminolaevulinate dehydratase from Neurospora crassa*

The experimental details are given in the text.

preparation was stirred with calcium phosphate gel (1mg of gel/mg of protein) and centrifuged. The supernatant was used as the inhibitor fraction. The inhibitor is heat-labile and loses its activity after trypsin treatment (Table 5). These properties indicate that it is a protein. The decreased amount of porphobilinogen detected in the presence of the inhibitor could also result if the inhibitor were to be an enzyme leading to the further metabolism of porphobilinogen. This is ruled out by the finding that the decreased amount of porphobilinogen formed in the presence of the inhibitor is stoicheiometrically related to the amount of δ-aminolaevulinic acid that disappears in the reaction. Thus the protein inhibitor blocks the conversion of δ-aminolaevulinic acid into porphobilinogen and does not catalyse the further metabolism of the product.

When δ-aminolaevulinate dehydratase preparation obtained after the second ammonium sulphate fractionation was loaded on a column (20cm × 1.3cm) of DEAE-cellulose, equilibrated and eluted with potassium phosphate buffer (pH 8.0, 0.05M), the enzyme was slightly retarded on the column and was detected in fractions 16, 17 and 18 when 2ml fractions were collected. The specific activity of the enzyme in the pooled fractions increased by 2.2-fold, but the total amount of enzyme activity recovered was found to be only 25% of the units loaded on the column. No enzyme activity was detected in other fractions obtained from the DEAE-cellulose column. However, when the column was further eluted with buffer containing 1M-NaCl, and this elute was added to the enzyme fraction, the specific activity of the enzyme increased to 3.1-fold as compared with the preparation loaded on the column. The recovery of the total enzyme activity loaded on the column increased by

Table 5. *Inhibition and activation of δ-aminolaevulinate dehydratase from Neurospora crassa*

The methods for the isolation of the inhibitor (X) and activator (Y) fractions are given in the text. The enzyme obtained after the second ammonium sulphate fractionation was loaded on a column of DEAE-cellulose to isolate the activator fraction.

Treatment	Specific activity of δ-aminolaevulinate dehydratase (%)
Complete system	100 (9.2)
Complete system+X (1 mg)	15
Complete system+trypsin-treated X	100
Complete system+heat-treated X	100
DEAE-cellulose-treated enzyme	220
DEAE-cellulose-treated enzyme+Y (0.5 ml of 1 M-NaCl extract)	310
DEAE-cellulose-treated enzyme+heat-treated Y	305
DEAE-cellulose-treated enzyme+trypsin-treated Y	302
DEAE-cellulose-treated enzyme+ribonuclease-treated Y	307

Table 6. *Concentrations of haem-biosynthetic intermediates in normal, iron-deficient and iron-induced cultures of Neurospora crassa*

For the determination of δ-aminolaevulinic acid, porphobilinogen and total porphyrins 40h-old mycelia were used. Iron-induced cultures were those to which iron was added to 40h-old deficient cultures and shaken for 2h. The experimental details are given in the text.

	Concn. of intermediate (nmol/100mg dry wt.)		
Condition	δ-Aminolaevulinic acid	Porphobilinogen	Porphyrins
Normal	32.1	Nil	16.3
Iron-deficient	123.4	Nil	14.5
Iron-induced	53.1	Nil	17.3

35%. It was found that NaCl at the concentrations present (0.25 M-NaCl final concentration in the enzyme reaction mixture) in the fractions that activated the enzyme was inhibitory and thus the activation observed with the activator fractions could be an underestimate. The properties listed in Table 5 indicate the activator to be a small molecule. A survey of several of the common nucleotides, tricarboxylic acid-cycle intermediates and B-group vitamins indicates that they have no activating effect on the enzyme treated with DEAE-cellulose.

It has been shown earlier that the other enzymes of the haem-biosynthetic pathway, δ-aminolaevulinate synthetase and ferrochelatase, are also induced by iron in iron-deficient *Neurospora* cultures, although δ-aminolaevulinate dehydratase shows maximal dependence on iron nutrition (Muthukrishnan *et al.*, 1969). The results in Table 6 indicate that δ-aminolaevulinic acid, the substrate for δ-aminolaevulinate dehydratase, accumulates in normal *Neurospora* and the accumulation is accentuated under iron-deficient conditions. Under conditions of induction, there is a striking decrease in amounts of δ-aminolaevulinate, but no porphobilinogen could be detected in any of the conditions investigated. This, coupled with the observation that the amounts of porphyrin remain more or less constant under the different treatment conditions, emphasizes that δ-aminolaevulinate dehydratase is the rate-limiting step of haem biosynthesis in *N. crassa*.

It was also decided to determine the turnover rate of δ-aminolaevulinate dehydratase *in vivo*. This was done in normal as well as in induced cultures, by the addition of cycloheximide to prevent further protein synthesis and then by following the decrease in enzyme activity as a function of time. Fig. 2 indicates that, in induced cultures, the decay curve is biphasic in nature, the component turning over faster having a half-life of nearly 4–5 min. The decay curve for the enzyme from normal cultures is similar to that for the component of slow turnover of the induced cultures.

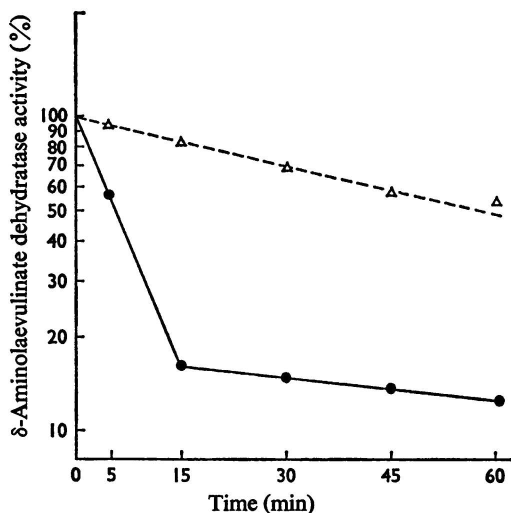

Fig. 2. *Half-life of δ-aminolaevulinate dehydratase from normal and iron-induced cultures of Neurospora crassa*

δ-Aminolaevulinate dehydratase was induced in iron-deficient cultures by the addition of iron. The flasks were shaken for 30min before the addition of cycloheximide. Other experimental details are given in the text. △, Enzyme decay in normal cultures; ●, enzyme decay in iron-induced cultures.

Discussion

δ-Aminolaevulinate dehydratase has been purified from several systems and is considered to be present in non-limiting amounts. However, Nandi *et al.* (1968) showed that the enzyme from *R. spheroides* exhibits allosteric properties, although no regulatory function has been attributed to this enzyme *in vivo.* In *Propionibacterium shermanii,* Menon & Shemin (1967) showed that δ-aminolaevulinate dehydratase activity can be related to the vitamin B_{12} content of the organism. Transfer of anaerobically grown cells to aerobic conditions results in a steep fall in vitamin B_{12} synthesis and the δ-aminolaevulinate dehydratase activity becomes almost undetectable. Ebbon & Tait (1969) showed that the increase of chlorophyll synthesis in *Euglena gracilis* on illumination is preceded by a threefold increase in δ-aminolaevulinate dehydratase activity. A report by Ho & Lascelles (1971) indicates that the activity of δ-aminolaevulinate dehydratase is lower than that of δ-aminolaevulinate synthetase in *Spirillum itersonii,* but a regulatory function for the former has been ruled out.

The present studies reveal that δ-aminolaevulinate dehydratase activities of *Neurospora* and yeast are the lowest so far observed. The low activity of the enzyme in *Neurospora* reflects the rate-limiting function of this enzyme *in vivo.* This is substantiated by the fact that δ-aminolaevulinate accumulates in normal *Neurospora* and this accumulation is enhanced under iron-deficient conditions, whereas the other metabolites of the pathway are either not detectable or do not change in concentration under any of the conditions examined. This differs from bacterial systems where porphyrins accumulate under iron-deficient conditions (Lascelles, 1964). The properties of the purified δ-aminolaevulinate dehydratase from *N. crassa* indicate that it is ideally suited to fulfill its regulatory function *in vivo.* The sigmoid behaviour of the velocity–substrate concentration curve and *n* value of approx. 2.0 in the Hill plot indicate that the enzyme is possibly allosteric in nature. It is noteworthy that the *Neurospora* enzyme is feedback-inhibited by coproporphyrinogen III but not by protoporphyrin or haemin. Haemin inhibits δ-aminolaevulinate dehydratase from several other sources (Calissano *et al.*, 1966; Nandi *et al.*, 1968). In the haem-biosynthetic pathway, the enzymic steps leading to the conversion of δ-aminolaevulinic acid into coproporphyrinogen III take place in the cytoplasm, whereas δ-aminolaevulinate synthetase, coproporphyrinogen oxidase and ferrochelatase are localized in mitochondria. Thus the feedback inhibition of δ-aminolaevulinate dehydratase by coproporphyrinogen III is meaningful, with the end product controlling the activity of the first enzyme for the haem-biosynthetic steps taking place in the cytoplasm. In addition, the *Neurospora* enzyme is subject to repression control by protoporphyrin (Muthukrishnan *et al.*, 1969). The present studies also reveal the presence of a small-molecule activator and protein inhibitor for the enzyme.

Yeast has also been found to have a very low δ-aminolaevulinate dehydratase activity (Table 1). Jayaraman *et al.* (1971) showed that δ-aminolaevulinate dehydratase from *S. cerevisiae* is subject to glucose repression and is induced under conditions of glucose de-repression leading to mitochondrogenesis. δ-Aminolaevulinate, but not porphobilinogen, was detected in normal yeast cells. It appears likely that yeast and fungi form a unique group where δ-aminolaevulinate dehydratase is the primary regulatory enzyme of haem biosynthesis.

This research was supported by financial assistance from the Department of Atomic Energy, India. Requests for reprints should be addressed to G. P.

References

Batlle, A. M. del C., Ferramola, A. M. & Grinstein, M. (1967) *Biochem. J.* **104**, 244
Burnham, B. F. & Lascelles, J. (1963) *Biochem. J.* **87**, 462
Calissano, P., Bonsignore, D. & Cartasegna, C. (1966) *Biochem. J.* **101**, 550
Ebbon, J. G. & Tait, G. H. (1969) *Biochem. J.* **111**, 573

Gibson, K. D., Laver, W. G. & Neuberger, A. (1958) *Biochem. J.* **70**, 71
Granick, S. & Mauzerall, D. (1958) *J. Biol. Chem.* **232**, 1119
Ho, Y. K. & Lascelles, J. (1971) *Arch. Biochem. Biophys.* **144**, 734
Jayaraman, J., Padmanaban, G., Malathi, K. & Sarma, P. S. (1971) *Biochem. J.* **121**, 531
Lascelles, J. (1964) *Tetrapyrrole Biosynthesis and its Regulation*, p. 84, W. A. Benjamin, New York
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265
Menon, I. A. & Shemin, D. (1967) *Arch. Biochem. Biophys.* **121**, 304
Muthukrishnan, S., Padmanaban, G. & Sarma, P. S. (1968) *Biochem. Biophys. Res. Commun.* **31**, 333
Muthukrishnan, S., Padmanaban, G. & Sarma, P. S. (1969) *J. Biol. Chem.* **244**, 4241
Nandi, D. L. & Waygood, E. R. (1967) *Can. J. Biochem.* **45**, 327
Nandi, D. L., Baker-Cohen, K. F. & Shemin, D. (1968) *J. Biol. Chem.* **243**, 1224
Padmanaban, G. & Malathi, K. (1972) *Biochem. J.* **128**, 27P
Padmanaban, G. & Sarma, P. S. (1965) *Arch. Biochem. Biophys.* **111**, 147
Tigier, H. A., Batlle, A. M. del C. & Locascio, G. (1968) *Biochim. Biophys. Acta* **151**, 300