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GLUTAMATE DEHYDROGENASE IN *NITROSOMONAS EUROPAEA* AND THE EFFECT OF HYDROXYLAMINE, OXIMES AND RELATED COMPOUNDS ON ITS ACTIVITY

W. WALLACE AND D. J. D. NICHOLAS

Department of Agricultural Biochemistry, Waite Institute, University of Adelaide, Adelaide (Australia)

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SUMMARY

An NADPH-specific glutamate dehydrogenase is described in the ammonia-oxidizing chemoautotroph, *Nitrosomonas europaea*. Both the amination and deamination reactions have been characterized and their relative activities found to be similar to those established previously for the glutamate dehydrogenase enzyme (EC 1.4.1.4). Hydroxylamine and α-ketoglutarate oxime are uncompetitive inhibitors of the amination reaction, but it is shown that the inhibition by hydroxylamine is not due to the prior formation of the oxime of α-ketoglutarate in the assay mixture. The amination reaction is not inhibited by pyruvate oxime or succinamic acid, but glutamate, the product of the reaction, is an uncompetitive inhibitor. While α-ketovalerate is a competitive inhibitor of amination it could not be substituted for α-ketoglutarate in this reaction. The deamination system is not inhibited by hydroxylamine, but α-ketoglutarate oxime and α-ketoglutarate are competitive inhibitors while pyruvate oxime is an activator. The preparation of oximes of α-ketoglutarate and pyruvate is described as well as a spectrophotometric method for measuring them.

INTRODUCTION

Nitrifying bacteria oxidize inorganic nitrogen compounds, thus in *Nitrosomonas europaea* nitrite is produced from ammonia and Nitrobacter converts nitrite to nitrate. In both these chemoautotrophs, reductase pathways have now been characterized[1] whereby the products of the oxidase enzymes can be reduced to ammonia. The uptake of [^{15}N]nitrate, [^{15}N]nitrite and [^{15}N]hydroxylamine, into the cell protein of the nitrifiers, indicates that the reductase enzymes are assimilatory systems[1]. As a preliminary to a study on amino acid synthesis in the nitrifying bacteria, an investigation was undertaken on glutamate dehydrogenase in Nitrosomonas. Both the forward amination reaction, synthesis of glutamate, and the reverse deamination reaction, oxidation of glutamate, were studied.

An NADPH-specific glutamate dehydrogenase has recently been described in Nitrosomonas by HOOPER, HANSEN AND BELL[2]. The finding of HOOPER, HANSEN AND BELL that this NADPH-specific enzyme was not modified by purine nucleotides agrees with the suggestion of FRIEDEN[3] that only the NADH or NADPH nonspecific glutamate dehydrogenase enzymes are subject to allosteric control.

In corn BULEN[4] found that hydroxylamine did not substitute for ammonia in the amination reaction but was an inhibitor. KUN AND ACHMATOWICZ[5] have since reported that hydroxylamine is an uncompetitive inhibitor of the liver enzyme with NADPH as the electron donor but is noncompetitive with NADH. A more extensive study on the inhibition of glutamate dehydrogenase in Nitrosomonas, by hydroxylamine, α-ketoglutarate oxime, related oximes and other structural analogues of α-ketoglutarate, is described in this paper. Considerable attention is given to the preparation and determination of the oxime derivatives in enzyme systems. The work of KUN AND ACHMATOWICZ with the liver enzyme is also reinvestigated.

A study of inhibitory effects of hydroxylamine is especially relevant in Nitrosomonas, where it occurs as an intermediate in the oxidation of ammonia to nitrite[6] and also is formed in the reverse reductase system[1].

MATERIALS AND METHODS

Preparation of cell-free extract

Batch cultures of *Nitrosomonas europaea* were grown and harvested as described by NICHOLAS AND RAO[7]. The cells were washed 3 times with 0.05 M phosphate (pH 7.8) and finally suspended in 3 parts (by vol.) of the same buffer per 1 part packed cell wet weight. The cells were disrupted by treatment for 10 min with a MSE ultrasonic probe (20 kcycles/sec)[1] and the supernatant fraction obtained after centrifuging at 10 000 $\times$ g (20 min) used as the cell-free extract (30 mg protein/ml). For further fractionation of the cell extract by centrifugation, a Spinco Model L (40 rotor) was used. Except where indicated, all studies were undertaken with the cell-free extract.

Glutamate dehydrogenase assay

The reaction at 25° was followed at 340 mμ in a 1-cm cuvette with a Unicam SP700 spectrophotometer. The reaction mixture, in a total vol. of 3 ml, contained the following (μmoles): Amination reaction: phosphate buffer (pH 7.8), 240; 0.1 ml diluted cell extract (0.2 mg protein); NADPH, 0.5; $(NH_4)_2SO^4$, 40; α-ketoglutarate (pH 7.8), 10. Deamination reaction: glycine buffer (pH 9.5), 260; 0.1 ml diluted cell extract (1 mg protein); $NADP^+$, 1; potassium glutamate, 50. After a 3-min preincubation in the cuvette at 25°, the amination reaction was started by adding α-ketoglutarate, and the deamination reaction with glutamate. The reaction rates (initial 1 min) were determined as absorbance values at 340 mμ, a decrease in the amination reaction and an increase in the deamination reaction, or as μmoles NADPH oxidized or $NADP^+$ reduced. Protein precipitated with 5% trichloroacetic acid was determined by the Folin method of LOWRY *et al.*[8].

Preparation of the oximes of α-ketoglutarate and pyruvate

(A) *α-Ketoglutarate oxime.* Hydroxylamine (1 g) was dissolved in a minimum volume of water at 60°. Sodium acetate (2 g) and α-ketoglutarate (1 g) were then

dissolved in the solution to give a final vol. of 7 ml. The solution was maintained at 60° for 1 h, filtered and then cooled to room temperature. The pH was adjusted to 1 with conc. HCl (3–4 ml), and on standing in ice overnight, the α-ketoglutarate oxime crystallized. After being recrystallized twice from water, the sample was dried over P_2O_5 and the m.p. found to be 135–138° (decomposition), a crystal transition occurring between 128–130°. When α-ketoglutarate oxime was left overnight at 76° it decarboxylated to succinamic acid (m.p. 157°).

(B) *Pyruvate oxime.* When pyruvate instead of α-ketoglutarate was used in the above reaction mixture, no oxime was obtained. The following method of BACHELARD[9] was used successfully. Hydroxylamine (3.5 g) was dissolved in a minimum volume of hot ethanol and added to 58 ml sodium ethylate (2 g metallic sodium dissolved in 100 ml ethanol). The precipitated NaCl was removed, and the hydroxylamine solution added slowly at 0° to a solution of 4.4 g pyruvic acid in 30 ml ethanol. The mixture was left overnight at 0°, and the remaining ethanol removed. The crystals collected were recrystallized twice at −15° from ethyl acetate–light petroleum (boiling range 40–60°) and dried over P_2O_5. The m.p. was 178–179°. Melting points were determined on a Kofler block apparatus, and the chemical analyses were performed by Dr. K. W. ZIMMERMAN, Australian Microanalytical Service, C.S.I.R.O., Melbourne.

Reagents and cofactors

Hydroxylamine ($NH_2OH \cdot HCl$), Analar grade, was obtained from British Drug Houses, England, and a 0.1 M stock solution was prepared fresh and adjusted to pH 6.5 with 1 M NaOH on the day of use. The keto acids, α-ketoglutarate, pyruvate and α-ketovalerate and the cofactors NADH, NADPH, NAD^+ and $NADP^+$ were supplied by Calbiochem, Los Angeles, U.S.A. The sample of liver glutamate dehydrogenase (EC 1.4.1.3) was obtained from Sigma Chemical Co., St. Louis, U.S.A.

RESULTS

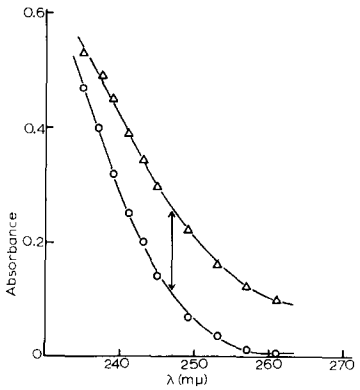
Measurement of α-ketoglutarate oxime by spectral studies

When the ultraviolet spectrum of α-ketoglutarate (3.33 mM) is compared with that of α-ketoglutarate oxime (0.16 mM) in the presence of α-ketoglutarate (3.16 mM) the latter combination results in a broadening of the absorbance peak to a longer wavelength (Fig. 1). The maximum elevation in absorbance occurs at 247 mμ. With α-ketoglutarate (3.16 mM) in the reference cell and an equivalent amount of keto acid plus oxime in the sample cell, it is possible to determine the oxime at 247 mμ. There is a linear relation between absorbance and oxime level up to 0.3 mM.

The amination and deamination reaction in a cell-free extract

The pH optimum of the amination reaction was found to be 7.8, while that of the deamination reaction was 9.5 (Fig. 2). At their respective pH optima, the rate of the amination reaction is 8-fold that of the deamination process. The following K_m values were determined for the cell-free extract: Amination reaction: α-ketoglutarate, 2.5 mM; $(NH_4)_2SO_4$, 7.5 mM; NADPH, 0.11 mM. Deamination reaction: glutamate, 13.2 mM; $NADP^+$, 0.32 mM. No activity was detected with NADH as the electron donor.

When the cell-free extract was centrifuged at 144 000 × *g* for 2 h, about 95%



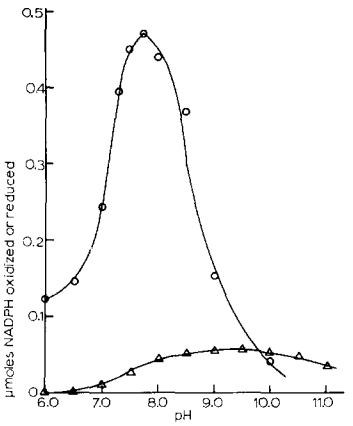


Fig. 1. Absorption spectra of α-ketoglutarate (3.33 mM) (○—○) and α-ketoglutarate oxime (0.16 mM) with α-ketoglutarate (3.16 mM) (△—△). Reference cell, water.

Fig. 2. The effect of pH on the amination (○—○) and deamination (△—△) reaction of glutamate dehydrogenase. The buffers used were: 0.1 M phosphate, pH 6.0–8.0, 0.1 M glycine. pH 8.5–11.0,

of the enzyme was found in the supernatant fraction, and from this, on centrifuging for a further 16 h, a pellet sedimented which contained all the activity.

Inhibition of the amination reaction by hydroxylamine and oxime compounds

The enzyme data were examined by Lineweaver–Burk kinetics (Fig. 3), the velocity v being the initial decrease over 1 min (amination) or increase (deamination) in absorbance at 340 mμ. With ammonia as the variable substrate, hydroxylamine was found to inhibit the amination reaction uncompetitively; a final concentration of hydroxylamine (3.33 mM) resulting in a 25% inhibition of the initial reaction rate. A similar inhibition was recorded with α-ketoglutarate oxime (0.08 mM). Thus inhibition by hydroxylamine could result from the oxime formed with α-ketoglutarate in the assay.

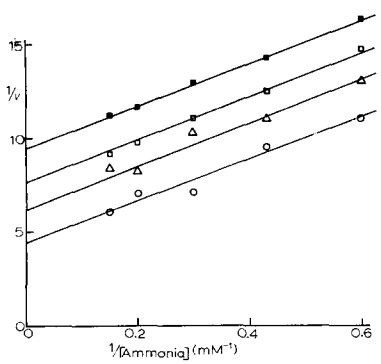
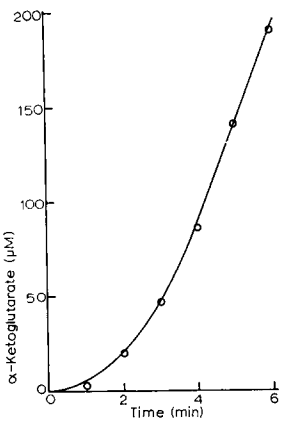


Fig. 3. Uncompetitive inhibition of the amination reaction by hydroxylamine: △—△, 1.66 mM; □—□, 3.33 mM, ■—■, 6.66 mM; ○—○, control without hydroxylamine. Hydroxylamine added at zero time.

When the amination reaction was followed at 247 mμ with complete assay mixtures in the reference and sample cells, the formation of α-ketoglutarate oxime was determined after adding hydroxylamine to the sample cell (Fig. 4). The rate of oxime formation in the amination reaction was similar to that obtained when hydroxylamine was added to α-ketoglutarate in a phosphate buffer (pH 7.8). (The rate at pH 5.5 was over 10-fold that at 7.8). Thus α-ketoglutarate oxime is formed by a chemical reaction. In the amination reaction, hydroxylamine (3.33 mM) added at zero time resulted in a 37% inhibition of the initial reaction rate (Table I). During this period 6 μM of oxime were formed. When α-ketoglutarate oxime (20 μM) was added to the assay mixture at zero time the inhibition was only 2%. Thus the inhibition due to hydroxylamine appears to be a direct effect of this compound on the enzyme. When the hydroxylamine was included in the preincubation mixture in the presence of α-ketoglutarate and the amination reaction was started after 5 min on adding ammonia the inhibition was 65%. The amount of α-ketoglutarate oxime formed in 5 min (preincubation *plus* initial reaction) was 200 μM (Fig. 4). This level of oxime added to the amination reaction at zero time caused a 35% inhibition (Table I). The increased inhibition (28%), when hydroxylamine is included in the preincubation mixture instead of at zero time, appears to be due to the α-ketoglutarate oxime formed.



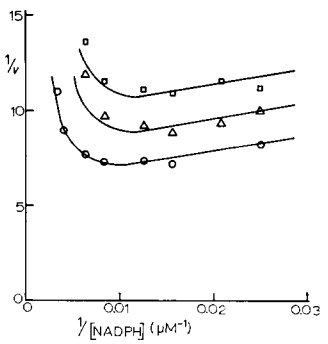


Fig. 4. Formation of α-ketoglutarate oxime by the reaction of α-ketoglutarate (3.33 mM) with hydroxylamine (3.33 mM) either in 0.1 M phosphate (a) or in the complete reaction mixture for the amination reaction (b) .Reference cell (a) α-ketoglutarate, (b) assay mixture for the amination reaction.

Fig. 5. Inhibition of the amination reaction by hydroxylamine when NADPH was the substrate variable. ○—○, control; △—△, 1.6 mM hydroxylamine; □—□, 3.33 mM hydroxylamine. Hydroxylamine added at zero time.

When NADPH was used as a substrate variable for the amination reaction (Fig. 5), high levels of the coenzyme gave substrate inhibition. With hydroxylamine in the reaction mixture, uncompetitive inhibition was again indicated, but the substrate inhibition effect was then detected at a lower level of the coenzyme substrate. Ammonia and α-ketoglutarate only promote substrate inhibition of the amination reaction at relatively high concentrations, and the uncompetitive-substrate inhibition

TABLE I

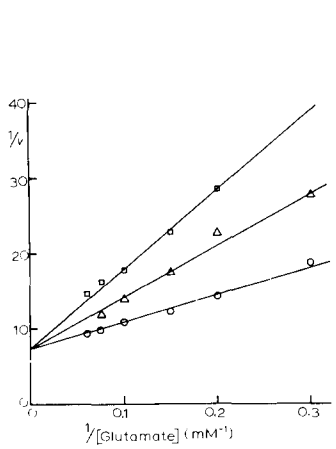
INHIBITION OF THE AMINATION REACTION BY HYDROXYLAMINE AND α-KETOGLUTARATE OXIME

Assay method as in text except that the ammonia substrate was used to start the reaction after a 5-min preincubation period.

Assay system	*Estimated level of α-ketoglutarate oxime (μM)*	*Inhibition (%)*
Hydroxylamine (3.33 mM) added at zero time	6	37.0
Hydroxylamine (3.33 mM) included in the preincubation mixture	200	65.0
α-Ketoglutarate oxime added at zero time	20	2.0
	200	35.0

effect described for NADPH was not found when either α-ketoglutarate or ammonia (Fig. 3) was the substrate variable. The phenomenon of substrate-uncompetitive inhibition for NADPH as the variable substrate was much more pronounced in assays with a purified glutamate dehydrogenase from liver. It was also found that with either NADH or NADPH as the coenzyme and α-ketoglutarate as the substrate variable, hydroxylamine (1.66 mM) was an uncompetitive inhibitor of the liver enzyme.

Succinamic acid (1 mM) and pyruvate oxime (1.66 mM) had no effect on the amination reaction of glutamate dehydrogenase in Nitrosomonas. Glutamate, the product of the amination reaction with α-ketoglutarate as substrate, was found to inhibit the reaction uncompetitively, as described for α-ketoglutarate oxime. The reaction was inhibited 25% by a final concentration of 22.4 mM glutamate. In a study with α-ketoglutarate as the substrate variable, α-ketovalerate was found to be a



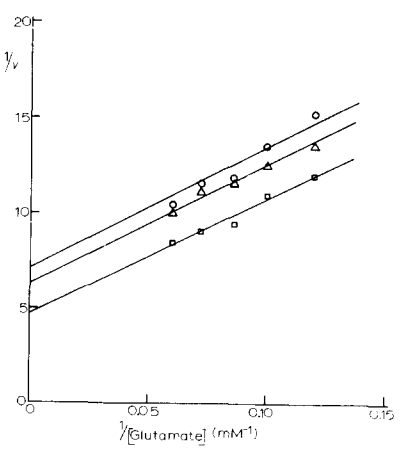


Fig. 6. Competitive inhibition of the deamination reaction by 6.6 μM α-Ketoglutarate oxime (△—△) and 16 μM α-Ketoglutarate oxime (□—□); ○—○, control without oxime.

Fig. 7. Activation of the deamination reaction by 0.33 mM pyruvate oxime (△—△) and 1.66 mM pyruvate oxime (□—□); ○—○, control without oxime.

competitive inhibitor ($K_i = 22.4$ mM). There was no activity when α-ketovaleric acid (6.66 mM) was substituted for α-ketoglutarate in the assay for the amination reaction.

Effect of hydroxylamine, oximes and related compounds on the deamination reaction

Hydroxylamine (8.33 mM) did not inhibit the deamination reaction of the enzyme, but α-ketoglutarate oxime was a very powerful competitive inhibitor (Fig. 6) ($K_i = 24\ \mu$M). At a higher concentration α-ketoglutarate was also a competitive inhibitor of the deamination step ($K_i = 1.25$ mM), whereas succinamic acid was without effect and pyruvate oxime (Fig. 7) activated the reaction.

DISCUSSION

As a result of extensive studies on glutamate dehydrogenase from yeast, HOLZER[10] has concluded that the NADPH-specific enzyme, rather than the NADH one, is the anabolic enzyme, utilizing ammonia in the cell to form glutamate. Since Nitrosomonas requires ammonia for growth, it is not surprising that it has a high activity of NADPH-specific glutamate dehydrogenase. The pH optimum of the amination reaction (7.8) in Nitrosomonas is lower than that of the deamination reaction (9.5), and the rate of the amination reaction at its pH optimum is 8-fold that of the deamination reaction. A similar distinction in pH optima has been found for the amination and deamination reactions (NADH and NADPH enzyme) in *Thiobacillus novellus*[11]. In the purified liver enzyme, OLSON AND ANFINSEN[12] found that the maximum specific activity for glutamate formation, under optimal conditions, was 950, but for glutamate oxidation it was 90. We conclude that the NADPH enzyme in Nitrosomonas is similar to that in other organisms (EC 1.4.1.4). HOOPER *et al.*[2], however, suggest that the enzyme in Nitrosomonas functions mainly in the direction of glutamate formation. They compared the activities of the two reactions at pH 7.5, since at this value optimum growth occurs, but this pH need not necessarily be that of the cytoplasm.

Hydroxylamine, an intermediate in the oxidation of ammonia by Nitrosomonas[6], is shown to be an uncompetitive inhibitor of the amination reaction. There is no evidence that hydroxylamine is interacting at the ammonia site on the enzyme. Hydroxylamine reacts readily with keto-acids chemically. Since α-ketoglutarate oxime is also an uncompetitive inhibitor (at a 40-fold lower concentration), the inhibition due to hydroxylamine may result from oxime formation.

When α-ketoglutarate oxime is prepared by a direct reaction between hydroxylamine and the keto acid, the compound formed had a m.p. of 140° (refs. 13, 14). An indirect method, *e.g.*, from triethylpropane 1,1,3-tricarboxylate by alkaline nitrosation, was shown to give the trans form of α-ketoglutarate oxime with a m.p. of 151–152° (ref. 15). Our sample of the oxime, prepared by the direct method, had a m.p. of 135–138° and is thus considered to have the cis configuration. Although KUN AND ACHMATOWICZ[5] used the direct method, their prepared sample of α-ketoglutarate oxime is reported to have a m.p. of 151–152°.

Evidence is presented in this paper that the hydroxylamine inhibition of the amination reaction is not due to the prior formation of α-ketoglutarate oxime in the assay mixture. The suggestion that trace amounts of oxime on the enzyme surface

cause the inhibition is not supported by the fact that hydroxylamine did not inhibit the deamination reaction, although α-ketoglutarate oxime is a strong competitive inhibitor, and α-ketoglutarate is a reaction product.

Substrate inhibition of the amination reaction by NADPH is enhanced by increasing amounts of hydroxylamine. HOOPER *et al.*[2] found a similar interaction between the NADPH enzyme and NADH. This indicates that hydroxylamine, like NADH, influences the binding of NADPH on the enzyme.

A range of structural analogues of α-ketoglutarate were used to examine the nature of the binding at the dicarboxylic keto acid site and the inhibition by α-ketoglutarate oxime. Inhibition is not due to the oxime group since pyruvate oxime had no effect on the amination of α-ketoglutarate. Succinamic acid in which the α-carboxyl group is absent did not inhibit the amination reaction, while α-ketovalerate, in which the other carboxyl group is changed, was a competitive inhibitor. It thus appears that the α-carboxyl group is essential for the binding reaction at the keto acid site, while the other carboxyl group makes the reaction with α-ketoglutarate or its oxime irreversible.

The competitive inhibition of the deamination reaction in Nitrosomonas by α-ketoglutarate oxime or the activation by pyruvate oxime agrees with the results reported for liver enzyme[5]. We have reinvestigated the effect of hydroxylamine on the amination reaction in the liver enzyme with NADH or NADPH, but contrary to the results of KUN AND ACHMATOWICZ[5], find that it is also uncompetitive.

The amination of α-ketovalerate to give norvaline has been reported for the glutamate dehydrogenase in higher plants[16] while the liver enzyme has been shown to oxidize norvaline[17]. The cell extract of Nitrosomonas did not utilize α-ketovalerate.

In the investigations of glutamate dehydrogenase in Nitrosomonas the maximum activity recorded in a cell-free extract for the amination reaction was 576 nmoles/min per mg protein. A much lower level of the enzyme has been found in Nitrobacter, 13 nmoles/min per mg protein. For comparison the level of the NADPH enzyme in a cell-free extract of the heterotroph *E. coli* (grown on Hersheys nutrient broth medium)[18] was found to be 105 nmoles/min per mg protein. A further investigation will be necessary to establish whether there are any additional or alternative systems in Nitrobacter for synthesizing cellular amino acids.

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REFERENCES

1 W. WALLACE AND D. J. D. NICHOLAS, *Biochem. J.*, 109 (1968) 763.
2 A. B. HOOPER, J. HANSEN AND R. BELL, *J. Biol. Chem.*, 242 (1967) 288.
3 C. FRIEDEN, *J. Biol. Chem.*, 240 (1965) 2028.
4 W. A. BULEN, *Arch. Biochem. Biophys.*, 62 (1965) 173.
5 E. KUN AND B. ACHMATOWICZ, *J. Biol. Chem.*, 240 (1965) 2619.
6 H. LEES, *Biochemistry of Autotrophic Bacteria*, Butterworths, London, 1955, p. 44.
7 D. J. D. NICHOLAS AND P. S. RAO, *Biochim. Biophys. Acta*, 82 (1964) 394.

8 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
9 H. S. BACHELARD, *Anal. Biochem.*, 12 (1965) 8.
10 H. HOLZER, *Biochem. J.*, 92 (1966) 37P.
11 H. B. LE JOHN AND BARBARA-ELLEN MCCREA, *J. Bacteriol.*, 95 (1968) 87.
12 J. A. OLSON AND C. B. ANFINSEN, *J. Biol. Chem.*, 197 (1952) 67.
13 E. E. BLAISE AND H. GAULT, *Bull. Soc. Chim. France*, 9 (1911) 451.
14 J. G. WOOD, M. R. HONE, M. E. MATTNER AND C. P. SYMONS, *Australian J. Sci. Res., Ser. B*, 1 (1948) 38.
15 A. AHMAD AND I. D. SPENSER, *Can. J. Chem.*, 39 (1961) 1340.
16 L. FOWDEN, in J. BONNER AND J. E. VARNER, *Plant Biochemistry*, Academic Press, New York, 1965, p. 361.
17 J. STRUCK, JR. AND I. W. SIZER, *Arch. Biochem. Biophys.*, 86 (1960) 260.
18 M. CHASE AND A. H. DOERMAN, *Genetics*, 43 (1958) 332.