***N*⁶-Trimethyl-lysine Metabolism**

STRUCTURAL IDENTIFICATION OF THE METABOLITE 3-HYDROXY-*N*⁶-TRIMETHYL-LYSINE

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¹H and ¹³C nuclear-magnetic-resonance spectroscopy and functional-group analysis were used to determine the molecular structure of an isolated metabolite (II_b) of trimethyl-lysine as 3-hydroxy-*N*⁶-trimethyl-lysine, an important intermediate in the conversion of trimethyl-lysine into trimethylammoniobutyrate and carnitine [Hoppel, Cox & Novak (1980) *Biochem. J.* **188**, 509–519]. Functional-group analysis revealed the presence of a primary amine and reaction of metabolite (II_b) with periodate yielded 4-*N*-trimethylammoniobutyrate as a product, showing 2,3-substitution on the molecule and suggesting that the 3-substitution on the molecule may be an alcohol (>CH–OH), amine (>CH–NH₂) or carbonyl (>C=O) functional group. ¹H integration ratios, ¹H and ¹³C chemical-shift data and ¹H and ¹³C signal multiplicities from the sample (II_b) were used to complete the identification of metabolite (II_b) as 3-hydroxy-*N*⁶-trimethyl-lysine. For example, the proton multiplet at δ 4.2 p.p.m. and doublet at δ 4.1 p.p.m., positions representative of amine or alcohol substitution on methylene carbon atoms, integration ratios of 1:1:2:9:4 and a positive ninhydrin test suggest 3-hydroxy-*N*⁶-trimethyl-lysine as the molecular structure for metabolite (II_b). ¹³C chemical-shift data obtained from the sample (II_b) and compared with several model compounds (trimethylammoniohexanoate, trimethyl-lysine and 3-hydroxylysine) resulted in generation of the spectrum of the metabolite and allowed independent identification of metabolite (II_b) as 3-hydroxy-*N*⁶-trimethyl-lysine. The ¹H spectrum of *erythro*- and *threo*-3-hydroxylysine are presented for comparison, and the ¹H and ¹³C n.m.r. spectra of the *erythro*-isomer support this analysis.

Proton nuclear-magnetic-resonance spectroscopy has been extensively employed as a standard analytical technique in molecular-structural analysis. The relatively small range of chemical-shift values (approx. 10–15 p.p.m.), however, coupled with potentially incomplete signal resolution at conventional frequencies and lack of direct observation of exchangeable functional-group protons in a ²H₂O medium (e.g. –NH₂, –OH, –CO₂H etc.) frequently necessitates a search for supplementary information that permits complete signal assignment and thereby provides unambiguous molecular-structural analysis.

Natural-abundance ¹³C Fourier-transform n.m.r. spectroscopy and functional-group analysis rep-

resent methods for acquiring such supplementary information on molecular structure. In contrast with proton n.m.r. spectroscopy, ¹³C chemical shifts encompass a much larger range at conventional frequencies (e.g. 250 p.p.m.) and as a result may be used to identify signals resulting from carbon atoms bonded to substituents that differ only slightly in electronic nature (e.g. –NH₂, –OH). Although the ¹³C experiment suffers from a small natural abundance (1.1%) and low sensitivity compared with proton n.m.r. spectroscopy, it is nevertheless possible to record high-quality ¹³C n.m.r. spectra of dilute solutions through the combined use of Fourier-transform technique and wide-band proton decoupling.

We had previously suggested in a preliminary abstract (Hoppel *et al.*, 1976) the molecular structure of an intermediate active in the conversion of

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trimethyl-lysine into carnitine. We have subsequently confirmed through ^1H and ^{13}C n.m.r. spectroscopy and functional-group analysis the identity of this metabolite as 3-hydroxy-*N*⁶-trimethyl-lysine. Comparison of the proton n.m.r. spectrum of the isolated metabolite, 3-hydroxy-*N*⁶-trimethyl-lysine, with that of synthetic *erythro*- and *threo*-hydroxylysine suggests that this active metabolite may exist as the *erythro*-isomer.

Experimental Procedure

Trimethyl-lysine and trimethylammoniohexanoate were prepared as previously described (Cox & Hoppel, 1974). The *erythro*-3-hydroxylysine and *threo*-3-hydroxylysine samples were obtained through the courtesy of Dr. C. Stammer, University of Georgia, Athens, GA, U.S.A. For the n.m.r. studies, all solutions were prepared in $^2\text{H}_2\text{O}$ (99.8% ^2H ; Aldrich). NaOH (1M) was used to adjust pH when required; the pH values of all solutions for spectral investigation were in the range 1–3, unless otherwise indicated.

Proton n.m.r. spectra were recorded at 90MHz with a Perkin-Elmer R32 spectrometer interfaced for pulsed Fourier-transform operation with a 12K Nicolet TT-7 computer package. Acetone was used as the internal reference and ^1H chemical shifts are reported in p.p.m. from tetramethylsilane. Acetone occurs 2.20p.p.m. downfield from tetramethylsilane in the ^1H spectrum. Acetone was used as the internal reference because of its volatile nature, which allowed removal of the reference material and thus permitted use of metabolite (II_b) in biological studies after completion of the n.m.r. investigation. Suitability as a standard in aqueous solution has been established (Mieyal *et al.*, 1974).

Natural-abundance ^{13}C spectra were obtained with a Varian XL-100-15 spectrometer operating at 25.2MHz in the pulsed Fourier-transform mode with ^1H wide-band decoupling. The free-induction decays were stored in a Varian 620/L computer (16K memory) and were Fourier-transformed after a sufficient number of acquisitions had been completed, usually 50 000–100 000. Before transformation all free-induction decays were smoothed with an exponential filter to enhance signal-to-noise ratios. The instrument was internally locked on the ^2H signal from the solvent, $^2\text{H}_2\text{O}$, and chemical shifts were determined from the signal of benzene which was contained in a coaxial capillary placed inside the n.m.r. tube. ^{13}C chemical shifts are reported in p.p.m. from tetramethylsilane using the conversion δ (tetramethylsilane) – δ (benzene) = 128.7 p.p.m.

The method of Sanger (1945) was used to form dinitrophenyl derivatives of amines. Samples of trimethyl-lysine and of metabolite (II_b) were placed in 0.1g of NaHCO_3 , 2.0ml of water and 0.1ml of

fluorodinitrobenzene with 5.0ml of ethanol added. The mixture was shaken for 2h at room temperature. The sample was evaporated to dryness, 2ml of water was added and the solution was extracted three times with 5ml of diethyl ether, followed by four extractions with 5ml of ethyl acetate. The aqueous layer was evaporated to dryness and redissolved in 0.2ml of water. The compounds remained in the aqueous layer because of their quaternary amine groups.

The acetylation of metabolite (II_b) and trimethyl-lysine was done as described by Neuberger & Sanger (1943). The sample was taken up in 1ml of water and 20mg of NaHCO_3 was added, followed by 5 μl portions of acetic anhydride every 20 min for 60 min. pH was maintained at 8.0. The sample was evaporated, redissolved in water and chromatographed (Hoppel *et al.*, 1980). A portion was hydrolysed in 6M-HCl for 18h at 105°C, evaporated, redissolved in water and also chromatographed.

The oxidation of trimethyl-lysine and metabolite (II_b) was accomplished by two methods. First, samples were added to water and fresh 2% KMnO_4 was added with shaking. This mixture was heated for 1h at 67°C, cooled and chromatographed on AG-50 with the HCl-gradient elution system. The second method used the special cleavage reactions of periodic acid, which were first applied by Malaprade (1934) to vicinal glycols and by Criegee (1937) to α -amino alcohols. The latter reaction was investigated by Nicolet & Shinn (1939, 1941*a,b*) and incorporated in a scheme for quantification of serine and threonine in protein hydrolysates. Van Slyke *et al.* (1940, 1941) later confirmed the stoichiometry and specificity, but not the rapid rate of 3-hydroxy amino acid oxidation reported by Nicolet and Shinn; Van Slyke and his co-workers did discover that the reaction of the hydroxy amino acids with alkaline NaIO_4 proceeded to completion almost instantaneously, whereas the reaction with ordinary amino acids was several orders of magnitude slower. The sample was added to 0.45 ml of 0.1 mM- NaIO_4 and 1 ml of 1M- K_2CO_3 with mixing. After 30–50s, 0.45 ml of 0.1 mM- NaHSO_3 was added with mixing. The pH was adjusted with HCl and the sample chromatographed as described by Hoppel *et al.* (1980).

Results and Discussion

Metabolite (II_b) isolated from the urine of rats chronically injected with trimethyl-lysine co-chromatographed (both column and thin-layer) with the radioactive metabolite (II_b). The region of the thin-layer chromatogram that contained the radioactivity showed a positive reaction to both iodoplatinate and ninhydrin spray, demonstrating the

presence of quaternary and primary amine groups respectively. To further explore the nature of the substitution on the metabolite, samples of trimethyl-lysine and metabolite (II_b) were treated with acetic anhydride. The mobility on t.l.c. of both these compounds was increased by the acetic anhydride procedure, suggesting acylation of the amino group. The derivatives formed with acetic anhydride were purified by column chromatography, acid hydrolysed (6M-HCl at 105°C for 20h), and rechromatographed by t.l.c. The sample derived from trimethyl-lysine now chromatographed as trimethyl-lysine and not as the acetylated derivative. Metabolite (II_b) is acid-labile; after acid hydrolysis of the derivative from metabolite (II_b), the acetylated derivative was not observed, but radioactivity was present both at the origin and in the region of metabolite (II_b), and the distribution was comparable with that observed when metabolite (II_b) itself was acid-hydrolysed, suggesting some decomposition. Fluorodinitrobenzene reacts with amines; hence, derivatives were formed from trimethyl-lysine and metabolite (II_b). By the method of Sanger (1945), samples of trimethyl-lysine and metabolite (II_b) showed higher *R_F* values, suggesting that both compounds are converted into the dinitrophenylated compounds.

KMnO₄ oxidation cannot be used to analyse for the position of the substituents because both trimethyl-lysine and metabolite (II_b) are oxidized. Periodate oxidation under the controlled conditions we used resulted in the conversion of 90% of metabolite (II_b) into trimethylammoniumbutyrate, whereas only 10% of trimethyl-lysine was oxidized. This suggests that an appropriate functional group is present in the 3-position, and that the substituents on metabolite (II_b) are in the 2,3-positions. Since metabolite (II_b) can be hydrolysed in 6M-HCl at 105°C, the additional substituent in the 3-position may be an alcohol (>CH-OH) or carbonyl oxygen (>C=O).

¹H n.m.r. characterization

The ¹H n.m.r. spectrum of trimethyl-lysine is shown in Fig. 1(a). An analysis of this spectrum yielded the following assignment of signals to particular groups of protons on the trimethyl-lysine molecule, as deduced from reported chemical-shift values (Silverstein *et al.*, 1974) and multiplicities. The methyl groups on the quaternary nitrogen [-N-(CH₃)₃] gave rise to an intense sharp singlet at 3.1 p.p.m. The methylene protons (>N-CH₂-) produced an apparent triplet at 3.4 p.p.m. (Although this signal is described as triplet, additional coupling is anticipated as a result of ¹H-¹⁴N interaction involving the amine nitrogen atom.) The unsubstituted methylene protons (-CH₂-CH₂-CH₂-) appeared as broad multiplets in the range

Fig. 1. ¹H spectra of (a) trimethyl-lysine and (b) the active metabolite of trimethyl-lysine (II_b).

For spectrum, (a), integration of the signals gives an area ratio of 1:2:9:6. For spectrum (b), integration of the signals gives an area ratio of 1:1:2:9:4. Spinning side bands have been deleted from both spectra. The peaks are assigned to groups in the structure with the corresponding letter. Abbreviation: ref., acetone.

1.5–2.0 p.p.m. The proton of the 2-methylene group (HC-NH₃⁺) gave a triplet at 4.1 p.p.m. The integration of the spectrum gave areas in the ratio 1:2:9:6, consistent with spectral assignments.

The ¹H spectrum of sample (II_b) is given in Fig. 1(b). Analysis of this spectrum may, in part, be based on the observed spectrum for trimethyl-lysine. The intense singlet occurring at 3.15 p.p.m. is attributed to the -N-(CH₃)₃ group, the triplet at 3.5 p.p.m. is attributed to >N-CH₂-, and a multiplet occurred at 1.5–2.0 p.p.m., indicative of protons of unsubstituted methylene groups. However, it appeared that two signals now occurred in the range 4.1–4.2 p.p.m.; the signal at 4.1 p.p.m. appeared to

be an asymmetrical doublet with a coupling of $J \sim 3$ Hz, whereas the signal at 4.18 p.p.m. appeared to be a broad multiplet. The signal at 4.1 p.p.m. may be the proton of the 2-methylene group, which is bonded to the primary amine, whereas the multiplet at 4.18 p.p.m. might occur as a result of the 3-methylene group being bonded to substituents such as $-\text{OH}$ or $-\text{NH}_2$. The integrated areas under the signals gave ratios of 2:2:9:4 (1:1:2:9:4), suggesting that the structure of metabolite (II_b) may be:

An absolute and unambiguous assignment of substituents, however, cannot be made from these data since the chemical shifts of the signals of interest are close in value (4.18 and 4.1 p.p.m.) and the ^1H splittings are not that clearly defined. Attempts to further determine the multiplicity of each signal by use of a 270 MHz high-resolution n.m.r. spectrometer were unsuccessful; the high frequency, however, did achieve separation of the individual signals, thereby allowing integration and ^1H homonuclear decoupling. The decoupling experiment was used to establish that certain of the signals were indeed coupled and belonged to the parent molecule.

The proton n.m.r. data in conjunction with results of functional-group analysis suggest that the structure of the metabolite (II_b) may be:

Additional evidence supporting this structure and identifying the 3-substituent was obtained by using ^{13}C n.m.r.

^{13}C n.m.r. characterization

Because of the larger range of chemical-shift values available in ^{13}C n.m.r., verification of the suggested structure was achieved. The ^{13}C spectrum of metabolite (II_b) is given in Fig. 2. Assignment of the signals occurring at 70, 67, 55, 54 (triplet), 30 and 20 p.p.m. may be made by using published chemical-shift values (Strothers, 1972). The signals at 30 and 20 p.p.m. are indicative of unsubstituted methylene groups. The sharp signal at 70 p.p.m. and broad signal at 67 p.p.m. are attributed to $>\text{CH}-\text{OH}$ and $>\text{N}^+-\text{CH}_2$ respectively. The broad signal with decreased intensity at 55 p.p.m. is indicative of $\text{HC}-\text{NH}_3^+$, and the triplet at 54 p.p.m. is associated with the methyl carbon atoms on the quaternary amine group [$(\text{CH}_3)_3\text{N}^+$]. [It should be noted here

Fig. 2. ^{13}C spectrum of metabolite II_b . Tetramethylsilane was used as the reference compound. The peaks are assigned to the groups in the structure with the corresponding letter.

that in contrast with the slow passage ('continuous-wave') experiment, a series of nuclear spins subjected to a rapid repetitive sequence of pulses may not produce signals that have areas (intensities) proportional to the number of spins giving rise to the signal. The intensity of signals will be a function of T_1 , T_2 pulse width and acquisition time, where T_1 and T_2 are the longitudinal and transverse relaxation times respectively (Jones & Sternlicht, 1972). The ^{13}C signal of the carboxylic acid carbon was not observed owing to its long relaxation time and our rapid pulsing sequence (Jones & Sternlicht, 1972)).

Since ^{13}C chemical shifts are not significantly affected by substitution past the 2-carbon (Horsley & Sternlicht, 1968; Horsley *et al.*, 1970), the ^{13}C spectrum of metabolite (II_b) may be generated through comparison and superimposition of ^{13}C spectra of several analogue molecules. The spectra used in this comparison were obtained from the following compounds:

If metabolite (II_b) is in fact 3-hydroxy-*N*⁶-trimethyl-lysine, then the superimposition of the ¹³C spectra of compound (a)–(c) should yield the same ¹³C spectrum as that of the metabolite (II_b), with the provision that solution pH values and ionization constants are virtually equivalent for all compounds (a)–(c). Further, ¹³C signals of carbon atoms which exist as secondary alcohols generally occur in the range 70–80 p.p.m., whereas primary amine groups usually occur in the region 50–67 p.p.m. (Strothers, 1972). Thus a signal occurring with a chemical shift of 70–80 p.p.m. would suggest a hydroxy group substitution on a methylene carbon atom.

A check of these assignments can be made by using the ¹³C spectra of compounds (a)–(c). These spectra are given in Figs. 3–5. Trimethylammoniohexanoate gave signals at 67.6, 54, 34.7, 26.2, 24.9 and 23.2 p.p.m. The signals occurring at 67.6 p.p.m. (a broad singlet) and 54 p.p.m. (a triplet) are indicative of the 6-methylene carbon atom ($\text{>N-CH}_2\text{-}$) and the methyl carbon atoms attached to the quaternary amine group [$(\text{CH}_3)_3\text{N}^+$] (¹³C¹⁴N splitting ~5 Hz) respectively (Fig. 3). The signals at 34.7, 26.2, 24.9 and 23.2 p.p.m. are the ¹³C signals of the unsubstituted methylene groups. The spectrum in Fig. 4(a) is that of trimethyl-lysine, and signals were observed at 67, 54, 34.3, 23.1 and 22.4 p.p.m. at pH ~1. However, on close inspection, the peak due to the $(\text{CH}_3)_3\text{N}^+$ group at 54 p.p.m. appears to be split into a quartet rather than into the expected triplet. The general rule for predicting splitting is $2nI + 1$, where n is the number of equivalent nuclei of spin I interacting with the nucleus being observed (Jackman & Sternhell, 1969). Thus for $(\text{CH}_3)_3\text{N}^+$, one expects to observe $2(1)(1) + 1 = 3$ lines, a triplet because $I(^{14}\text{N}) = 1$. Typical ¹³C–¹⁴N splitting values are 3–7 Hz (Quirt *et al.*, 1974). The signals occurring at 67 p.p.m. (a broad singlet) and 54 p.p.m. (a triplet and a singlet) are indicative of the

6-methylene carbon atom ($\text{>N}^+-\text{CH}_2\text{-}$), the methyl carbon atoms [$(\text{CH}_3)_3\text{N}^+$] and the 2-amino methylene carbon atom ($\text{>CH-}\text{NH}_3^+$) respectively. The signals at 34.3, 23.1 and 22.4 p.p.m. arise from the unsubstituted methylene carbon atoms.

To determine whether a signal belonging to the 2-amino carbon atom is in fact present under the signal at 55 p.p.m., a slight alteration of pH can be

Fig. 4. ¹³C spectrum of *N*⁶-trimethyl-lysine at pH ~1 (a) and pH ~3 (b)

The resulting upfield chemical shift of the 2-amino carbon atom (signal d) at pH 3 is observed. The peaks are assigned to the groups in the structure with the corresponding letter.

Fig. 3. ¹³C spectrum of 6-trimethylammoniohexanoate. Tetramethylsilane was used as the reference compound. The peaks are assigned to the groups in the structure with the corresponding letter.

Fig. 5. ¹³C spectrum of erythro-3-hydroxylysine. The peaks are assigned to the groups in the structure with the corresponding letter. Tetramethylsilane was used as the reference compound.

made, and this signal should shift downfield (Horsley & Sternlicht, 1968; Stammer & Webb, 1969; Horsley *et al.*, 1970). The ^{13}C spectrum of trimethyl-lysine at $\text{pH} \approx 3$ is given in Fig. 4(b); here one can observe the shifted 2-amino carbon signal, which now occurs at 56 p.p.m. This observation of a downfield shift with decreasing protonation of the amino group is in agreement with previous ^{13}C n.m.r. studies carried out on amino acids (Horsley & Sternlicht, 1968; Stammer & Webb, 1969; Horsley *et al.*, 1970). Again the signals occurring at 34, 23 and 22 p.p.m. are indicative of unsubstituted methylene carbon atoms. Comparison of the spectrum of 3-hydroxylysine with the ^{13}C spectrum of trimethyl-lysine (Fig. 5) will now complete the series. Signals occurred at 70, 60, 41, 29 and 25 p.p.m. Analysis of this spectrum yielded the following assignments: 70 p.p.m., the methylene carbon atom substituted with a hydroxy group; 60 p.p.m., the 2-amino methylene carbon atom; 41 p.p.m., the 6-amino methylene carbon atom. The remaining values, 29 and 25 p.p.m., can be ascribed to the unsubstituted methylene carbon atoms (Fig. 5).

The comparison of the ^{13}C spectra for compounds (a)–(c) yielded the following ^{13}C chemical shift values: $>\text{CH}-\text{OH}$, ~ 70 p.p.m. (sharp singlet); $>\text{N}-\text{CH}_2-$ 67 p.p.m. (broad singlet); $>\text{CH}-\text{NH}_3^+$, ~ 54 –57 p.p.m. ($\text{pH} 1$ –3) (singlet); $(\text{CH}_3)_3\text{N}^+$, 54 p.p.m. (triplet); 35–15 p.p.m., unsubstituted methylene carbon atoms. These values are in agreement with previously published data for this type of substitution (Strothers, 1972) and with the observed ^{13}C chemical shifts and assignments for sample (II_b), thus providing compelling evidence for the identity of metabolite (II_b) as 3-hydroxy- N^6 -trimethyl-lysine.

Stereochemistry of 3-hydroxy- N^6 -trimethyl-lysine

The analytical results of periodate cleavage, ninhydrin and iodoplatinate indicator tests, and altered t.l.c. R_F values from acetylation and di-nitrophenylation, as well as the ^1H spectrum and ^{13}C chemical shifts, all support the conclusion that the molecular structure of metabolite (II_b) is 3-hydroxy- N^6 -trimethyl-lysine. 3-Hydroxylysine is capable of existing as either of two diastereoisomeric pairs of enantiomers, *erythro* or *threo*, with respect to the configuration of the carbon atoms bearing the 2-amino and 3-hydroxy groups (Stammer & Webb, 1969). These isomers have distinctly different proton n.m.r. spectra, whereas the ^{13}C spectra give identical chemical-shift values. The ^1H spectra of *erythro*- and *threo*-3-hydroxylysine are given in Figs. 6(a) and 6(b). As observed, the splitting patterns of the *erythro*- and *threo*-isomers are distinctly different. The splitting pattern that appears most closely related to the ^1H spectrum of metabolite (II_b) is *erythro*-3-hydroxylysine. One question that must be

Fig. 6. ^1H spectrum of *erythro*-3-hydroxylysine (a) and *threo*-3-hydroxylysine (b)

Abbreviation used: ref., reference compound (acetone). The peaks are assigned to the groups in the structure with the corresponding letter.

answered, however, is the potential change in the proton n.m.r. spectrum that might be caused by conversion of the 6-amino group in a quaternary amine. Should the quaternary amine be capable of interacting with the carboxylic acid group to form an internal ion pair, then perhaps a small alteration in the ^1H coupling (and/or chemical shift) might occur, producing a slightly different ^1H spectrum. Ion-pair formation on quaternization of the 6-amino group of the *erythro*- and *threo*-3-hydroxylysine isomers would not be expected to produce changes in the coupling constant, chemical shift or relative geometry sufficiently to render the *erythro*- and *threo*-3-hydroxylysine ^1H spectrum equivalent. Hence, we conclude from a spectral comparison that the metabolite 3-hydroxytrimethyl-lysine (II_b) appears to exist as the *erythro*-isomer. An analysis of the molecular conformation of these samples in solution

might also provide additional information on the mechanism of hydroxylation of trimethyl-lysine in the 3-position.

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References

- Cox, R. A. & Hoppel, C. L. (1974) *Biochim. Biophys. Acta* **362**, 403–413
- Criegee, R. (1937) *Angew. Chem. Int. Ed. Engl.* **50**, 153–155
- Hoppel, C. L., Novak, R. F. & Cox, R. A. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 1478
- Hoppel, C. L., Cox, R. A. & Novak, R. F. (1980) *Biochem. J.* **188**, 509–519
- Horsley, W. J. & Sternlicht, H. (1968) *J. Am. Chem. Soc.* **90**, 3738–3748
- Horsley, W. J., Sternlicht, H. & Cohen, J. S. (1970) *J. Am. Chem. Soc.* **92**, 680–685
- Jackman, L. M. & Sternhell, S. (1969) *Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry*, 2nd edn., p. 125, Pergamon Press, London
- Jones, D. E. & Sternlicht, H. (1972) *J. Magn. Reson.* **6**, 167–182
- Malaprade, L. (1934) *Bull. Soc. Chim. Biol.* **1**, 833–836
- Mieyal, J. J., Webster, L. T., Jr. & Siddiqui, U. (1974) *J. Biol. Chem.* **249**, 2633–2640
- Neuberger, A. & Sanger, F. (1943) *Biochem. J.* **37**, 515–518
- Nicolet, B. H. & Shinn, L. A. (1939) *J. Am. Chem. Soc.* **61**, 1615
- Nicolet, B. H. & Shinn, L. A. (1941a) *J. Biol. Chem.* **138**, 91–96
- Nicolet, B. H. & Shinn, L. A. (1941b) *J. Biol. Chem.* **139**, 687–692
- Quirt, A. R., Lyerla, J. R., Peat, I. R., Cohen, J. S., Reynolds, W. F. & Freeman, M. H. (1974) *J. Am. Chem. Soc.* **96**, 570–574
- Sanger, F. (1945) *Biochem. J.* **39**, 507–515
- Silverstein, R. M., Bassler, G. C. & Morrill, T. C. (1974) *Spectroscopic Identification of Organic Compounds*, p. 159, pp. 211–227, John Wiley, New York
- Stammer, C. H. & Webb, R. B. (1969) *J. Org. Chem.* **34**, 2306–2311
- Strothers, J. B. (1972) *Carbon-13 NMR Spectroscopy*, p. 375, Academic Press, New York
- Van Slyke, D. D., Hiller, A. & MacFadyen, D. A. (1940) *J. Biol. Chem.* **133**, 287–288
- Van Slyke, D. D., Hiller, A., MacFadyen, D. A., Hastings, A. R. & Klemperer, F. W. (1941) *J. Biol. Chem.* **141**, 681–705