Carbon-13 Nuclear-Magnetic-Resonance Spectroscopy of Whole Cells and of Cytochrome *c* from *Neurospora crassa* Grown with [*S*-Me-¹³C]Methionine

By R. T. EAKIN and L. O. MORGAN

Department of Chemistry, University of Texas, Austin, Texas 78712, U.S.A.

and N. A. MATWIYOFF

University of California Los Alamos Scientific Laboratory, Los Alamos, New Mexico 87545, U.S.A.

(Received 9 June 1975)

Neurospora crassa cytochrome *c* biosynthetically labelled with [*S*-Me-¹³C]methionine was prepared and analysed by ¹³C nuclear-magnetic-resonance spectroscopy. The methyl group of methionine is extensively incorporated into an *N*-trimethyl carbon species similar to tetra-alkyl ammonium compounds, such as choline. The results were interpreted as an indication that the methyl carbons of the *N*-trimethyl-lysine-72 residue arise from *S*-adenosylmethionine transmethylation, and that the methyl carbons of methionine residues are sufficiently close to the haem centre to experience chemical shifts from the ring currents of the tetrapyrrole π electrons and broadening due to binding of methionine-80 with the haem, as well as interaction of the *S*-[¹³C]methyl groups with the paramagnetic iron centre. Although whole cells of the labelled *Neurospora* produced a ¹³C resonance at the expected position for the methionyl methyl group most of the methyl label was diverted into *N*-tetra-alkyl ammonium compounds. After an active state of growth these labelled *N*-methyl compounds appear, in the main, to be low-molecular-weight derivatives of choline which, if associated with membrane, are in a sufficiently fluid environment to have short rotational correlation times. During a subsequent dormant growth period these compounds become associated to some extent with relatively more immobile phases as a result of membrane binding or an increase in membrane rigidity.

Selective ¹³C enrichment at various carbon-atom positions within a protein molecule provides a good experimental system for studies of static and dynamic conformational phenomena by ¹³C n.m.r. (nuclear-magnetic-resonance) spectroscopy. Specific biosynthetic labelling with [2-¹³C]histidine for such purposes has been demonstrated with α lytic protease (Hunkapillar *et al.*, 1973*a,b*), the α subunit of tryptophan synthetase (Browne *et al.*, 1973*a,b*) and mouse haemoglobin (London *et al.*, 1975). Selective labelling of *Clostridium acidii urici* ferridoxin has also been accomplished with [1-¹³C]glycine (Packer *et al.*, 1972). Similar experiments can be conducted with ¹³C-labelled cytochrome *c* but, as in the case of ferridoxin and haemoglobin, the presence of a paramagnetic (haem) centre and the occurrence of significant conformational changes in the protein on reduction of that centre (Takano *et al.*, 1973) expands the scope of experimental possibilities.

In choosing a biosynthetic system for labelling a protein such as cytochrome *c* there are considerations for producing a sufficient quantity of material and selectively introducing the label into desired molecular positions (Scott, 1974). For n.m.r.

experiments milligram quantities of purified protein are desirable and for specific labelling a precursor is required that does not become widely incorporated into many amino acids in a protein by intermediary metabolism.

The organism of choice for these studies is the fungus, *Neurospora crassa*, which can be genetically manipulated to: (a) produce a large quantity of cytochrome *c*; and (b) require an external source of particular amino acids for growth. In addition to considerations of efficiency and specificity of label incorporation we chose to study *Neurospora* cytochrome *c* because it is one of the few cytochromes *c* in which a secondary modification of a lysine residue by *N*-trimethylation occurs. This modification is apparently associated with membrane-binding properties of the protein (Scott & Mitchell, 1969; DeLange *et al.*, 1969) and we have been interested in the use of ¹³C n.m.r. in the study of the architecture and dynamics of the constituents of membranes of intact cells (London *et al.*, 1975; Matwiyoff & Needham, 1972; Eakin *et al.*, 1972). An additional eventual goal of this study is to compare conformational states of cytochrome *c* in homogen-

eous solution with that observed in whole cell or membrane-cytochrome *c* systems.

The choice of [*S*-Me- ^{13}C]methionine as a substrate for these investigations was dictated by two considerations. First, there are two methionine residues within *Neurospora* cytochrome *c* whose *S*-methyl groups may be in quite different environments. Methionine-80 is directly co-ordinated to haem iron through the sulphur atom, and although methionine-98 is not, it is sufficiently close to the haem centre to provide a sensitive probe for events attendant on the oxidation-reduction process such as conformational changes. Secondly, methylation of proteins in biological systems with [*S*-Me- ^{14}C]methionine has been observed (Reporter, 1973); and partly in retrospect, the use of [^{13}C]methionine appeared a good possibility to supply a ^{13}C -labelled one-carbon pool for the synthesis of trimethylated lysine in the methionine auxotrophic mutant of *Neurospora* used.

This report describes the biosynthetic preparation and ^{13}C n.m.r. spectroscopy of whole cells of *Neurospora crassa* and *Neurospora* cytochrome *c* labelled with [*S*-Me- ^{13}C]methionine. We also consider associated aspects of methyl-group metabolism in this organism.

Materials and Methods

Strain of *Neurospora*

The strain of *Neurospora* used in this study was one that had been produced for labelling cytochrome *c* specifically with methionine or histidine. It is the product of a cross using the *poky* strain mi-1 as the protoperithecial parent and a strain containing the genetic markers histidine-4 and methionine-5 as the conidial parent. The genetic defects in the conidial parent block the formation of histidinol from histidinol phosphate in the histidine biosynthetic pathway and the formation of cystathionine from cysteine and homoserine in the methionine biosynthetic pathway. The strain selected from this cross was a triple mutant genetic recombinant containing the markers histidine-4 and methionine-5 which gave an auxotrophic requirement for these amino acids, and the mi-1 marker which causes an excessive production of cytochrome *c* (Haskins *et al.*, 1953).

Preparation of [*S*-Me- ^{13}C]methionine

Methyl-labelled methionine was synthesized by using 90 atoms of [^{13}C]methyl iodide/100 atoms of C provided by Dr. D. G. Ott of the Los Alamos Scientific Laboratory. The experimental procedure used for the synthesis was based on that described by Melville *et al.* (1947) starting with L-*S*-benzyl-homocysteine from Sigma Chemical Co., St. Louis, Mo., U.S.A. Enrichment of ^{13}C in the methyl position of the synthesized methionine was determined from proton n.m.r. by ^{13}C line splitting of the methyl

proton signal and confirmed by mass spectroscopy. We found that when natural-abundance carbon methyl iodide (1.1 atoms of ^{13}C /100 atoms of C) was used in excess to drive the reaction to completion after the addition of a stoichiometric amount of labelled methyl iodide (90 atoms of ^{13}C /100 atoms of C) the methyl group enrichment was only 70 atoms of ^{13}C /100 atoms of C. When the excess of methyl iodide was also labelled with 90 atoms of ^{13}C /100 atoms of C, the resulting methionine had 90 atoms of ^{13}C /100 atoms of C in the methyl carbon.

Culture media and growth procedures

Cultures of *Neurospora* were maintained and propagated in the vegetative state by conidial transfers to slants prepared from Vogel's minimal medium (Vogel, 1956) supplemented with 0.5 mg L-methionine/ml, 0.5 mg of L-histidine hydrochloride/ml, 20 mg of sucrose/ml and 20 mg of agar/ml. Mycelia were grown in liquid culture at 25°C in 1 litre batches in fluted 2 litre Erlenmeyer flasks on a rotary shaker, or in 10 litre batches in an aerated carboy. The liquid culture medium contained Vogel's minimal salts (Vogel, 1956) supplemented with 100 mg of L-methionine/l, 10 mg of L-histidine hydrochloride/l and 20 g of sucrose/l.

Extraction of cytochrome *c*

Cytochrome *c* extraction was based on methods described by Hagihara *et al.* (1958) and Heller & Smith (1966). Mycelia were suspended in an equal weight of water with an equal weight of glass beads and homogenized by grinding in a porcelain mortar. The homogenate was centrifuged for 10 min at 2500g. The pellet was extracted twice more with the same amount of water and all the supernatants were pooled. The water-extracted pellet was then suspended in 100 ml of water and adjusted to pH 10 with conc. aq. NH_3 (sp.gr. 0.88). The mixture was left for 10 min, the pH adjusted to 8.5 with acetic acid and the suspension was then centrifuged for 20 min at 2500g. The resulting solution was combined with the previously pooled supernatants and diluted by a factor of three with water. This diluted solution was passed through a column (21 cm $\times$ 2.5 cm) of Amberlite CG-50 equilibrated with 0.1 M-ammonium phosphate buffer, pH 7.0. Cytochrome *c* was then eluted from the column with 0.5 M-ammonium phosphate buffer, pH 7.0. The fractions containing cytochrome *c*, determined by the pink colour, were pooled and diluted by a factor of 10 with water. The diluted cytochrome solution was then passed through a column (22 cm $\times$ 1.0 cm) of Amberlite CG-50 equilibrated with 0.1 M-ammonium phosphate buffer, pH 7.0. The column was washed with water and the cytochrome *c* eluted with 0.5 M-ammonium phosphate buffer, pH 7.0. The eluate fractions containing cytochrome *c* were freeze-dried and resuspended in 1 ml of water.

¹³C n.m.r. spectroscopy

Proton-decoupled ¹³C n.m.r. Fourier transform spectra were obtained at 20°C and 25.2 MHz with a Varian XL-100-15 spectrometer interfaced to a Data General Supernova computer, using the ²H resonance (15.4 MHz) from a ²H₂O capillary as a lock signal. Chemical-shift values were calculated relative to the methyl carbon resonance of neat tetramethylsilane measured under the same conditions. Free induction decays of 40 μs rf pulses were accumulated as 4096 data points in the time domain over a 5000 Hz region downfield from tetramethylsilane.

Results

Amino acid requirements of the mutant strain of Neurospora

The triple mutant (methionine-5, histidine-4, mi-1) was grown in 100 ml cultures in Vogel's minimal medium supplemented with 2 g of sucrose and 2 mg of L-histidine hydrochloride. Each 100 ml culture was additionally supplemented with L-methionine in various amounts, and inoculated with equal volumes of a single conidial suspension. After 7 days of growth on a rotary shaker at 25°C the mycelial pads were harvested and dried in a vacuum drier. The dry weight as a function of methionine supplement reaches a maximum value at about 50 mg of L-

Fig. 1. Growth of the triple mutant as a function of methionine supplement

A constant histidine hydrochloride supplement of 2 mg/100 ml was used and mass values were obtained after 7 days of growth.

Fig. 2. Growth of the triple mutant as a function of histidine supplement

A constant methionine supplement of 50 mg/100 ml was used and mass values were obtained after 5 days of growth.

methionine/100 ml of medium (Fig. 1) but about 80% of the maximum can be reached with only a 10 mg/100 ml of supplement.

A similar experiment with a constant supplement of 50 mg of L-methionine in each 100 ml culture, but with various amounts of L-histidine hydrochloride, showed an optimum histidine supplement at about 1 mg of L-histidine hydrochloride/100 ml of medium. Histidine supplements above this concentration inhibit growth (Fig. 2).

Growth pattern of the triple mutant

Cultures of the triple mutant strain were grown on Vogel's minimal medium at 25°C on rotary shakers. Each culture contained 100 ml of medium supplemented with 2 g of sucrose, 10 mg of L-methionine and 1 mg of L-histidine hydrochloride. Cultures were harvested at various times and the mycelial pads dried and weighed. As Fig. 3 shows, the end of the exponential phase of growth is at about 5 days.

Cytochrome c content of the triple mutant

A cell-free homogenate of the triple mutant was obtained by extensive grinding of the mycelia in 0.1 M-ammonium phosphate buffer, pH 7.0, by using glass beads as an abrasive. The homogenate was centrifuged at 500g for 10 min and the cytochrome c concentration in the supernatant was estimated by a reduced versus oxidized difference spectrum. By

using $\Delta\epsilon_{\text{nm}} (550\text{ nm} - 540\text{ nm}) = 19 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for a difference spectrum (Lambowitz *et al.*, 1972) cytochrome *c* content was determined to be 1.58 mg/g dry wt. of mycelia.

N.m.r. spectra of ethanolamine derivatives

The ^{13}C n.m.r. spectra of *N*-methyl and *NN*-dimethyl derivatives of ethanolamine were obtained from neat solutions and from aq. 0.5 M solutions at pH 1. The spectrum of a saturated aqueous solution of choline chloride was also obtained. The signals from these spectra occur at the positions listed in Table 1. The methyl signals in aqueous solution, which are the ones of concern when [*S-Me- ^{13}C*]methionine is

used as a labelled substrate, occur at 34.5 p.p.m. in the monomethyl derivative, 44.4 p.p.m. in the dimethyl derivative and at 55.2 p.p.m. (triplet) in the trimethyl derivative (choline chloride).

N.m.r. spectra of mycelia

The ^{13}C n.m.r. spectrum of a suspension of mycelia in water was obtained after growth for 5 days on [*S-Me- ^{13}C*]methionine enriched to 70 atoms of ^{13}C /100 atoms of C. This spectrum (Fig. 4a) indicates that the ^{13}C is incorporated into many biosynthetic products, predominantly into *N*⁺-trimethyl groups. The amount of methionine directly incorporated into protein or remaining as methionine in a metabolic pool is quite small compared with the amount used as a required supplement.

The most predominant feature of the spectrum is a triplet centred at 55 p.p.m. corresponding to the *N*⁺-methyl groups of choline (Table 1). There are less intense signals at 63.7 p.p.m. and 15.0 p.p.m. corresponding to a hydroxymethyl carbon and the methyl carbon of methionine respectively (Johnson & Jankowski, 1972). There are four other prominent but much less intense signals in the methyl or methylene region of the spectrum that occur at 23.2, 28.1, 34.6 and 73.4 p.p.m.

An indication of the activity of methionine and methyl-group metabolism during a relatively dormant state of growth was obtained by allowing the cells whose ^{13}C n.m.r. spectrum is shown in Fig. 4(a) to remain suspended in water at 4°C for an additional 4 days. The ^{13}C n.m.r. spectrum of the cells after this dormant period is shown in Fig. 4(b). The predominant feature in the latter spectrum is still the triplet centred at 55 p.p.m. However, it appears to be superimposed on a broadened signal in the same region. The relative integrated intensities of the signals at 23.2, 28.1 and 34.6 p.p.m. are less, whereas that of the methyl group of methionine at 15.0 p.p.m. is greater.

Fig. 3. Growth curve of the triple mutant

A methionine supplement of 10 mg/100 ml and a histidine hydrochloride supplement of 1 mg/100 ml were used.

Table 1. Chemical shifts of the carbons of methylated derivatives of ethanolamine

For details see the text.

Carbon atom	Chemical shifts from tetramethylsilane (p.p.m.)				
	<i>N</i> -Methylethanolamine		<i>NN</i> -Dimethylethanolamine		Choline chloride
	Neat liquid	Aq. solution pH 1	Neat liquid	Aq. solution pH 1	Sat. aq. solution
Methyl	36.6	34.5	46.3	44.4	55.2 (triplet)
Hydroxymethyl	60.7	58.1	60.1*	56.7	57.0
<i>N</i> -Methylene	54.8	52.1	62.5*	60.2	68.6

* Assignments made on the basis that the 62.5 p.p.m. signal was twice as broad as the 60.1 p.p.m. signal, presumably from ^{14}N quadrupolar broadening.

Fig. 4. ¹³C n.m.r. spectra of whole cells of the triple mutant grown on [*S*-Me-¹³C]methionine

(a) After 5 days of growth on nutrient medium at 25°C, 7520 pulses; (b) after 5 days of growth on nutrient medium at 25°C followed by 4 days in water at 4°C, 36016 pulses.

The background ¹³C n.m.r. spectrum of cells grown on natural abundance [*S*-Me-¹³C]methionine has been obtained and shows that all signals are within the noise level of the spectra in Fig. 4.

N.m.r. spectra of cytochrome c

The ¹³C n.m.r. spectrum of cytochrome *c* labelled biosynthetically with [*S*-Me-¹³C]methionine and isolated by purification on Amberlite CG-50 columns is dominated by a narrow multiplet centred at 55.1 p.p.m. with a broader, less-intense, upfield shoulder. There are also lower intensity signals at 40.3, 28.8 and 24.2 p.p.m. As monitored by ¹³C n.m.r. spectroscopy, dialysis of the cytochrome *c* solution for 48 h against 0.05 M-ammonium phosphate buffer, pH 7.0, resulted in the removal of small labelled molecules that had remained associated with the protein throughout the previous purification procedures. After dialysis the ¹³C n.m.r. spectrum of cytochrome *c* exhibits a signal at 54.3 p.p.m. (Fig. 5) which is shifted upfield to 53.6 p.p.m. on reduction with dithionite, whereas the resonances of the undialysed product at 55.1, 40.3, 28.8 and 24.2 p.p.m. are absent. A second signal in the ferricytochrome *c* can be seen at 13.8 p.p.m. which has an integral approx. one-quarter of that of the predominant signal at 54.3 p.p.m. On reduction of the ferricytochrome *c* this resonance shifts to 13.6 p.p.m.

Discussion

*Metabolic fate of [*S*-Me-¹³C]methionine*

The ¹³C n.m.r. spectra of *Neurospora* whole cells and cytochrome *c* indicate that in the triple mutant the methyl group of exogenous methionine is utilized more extensively in the one-carbon or methyl-group pool than in the direct incorporation as the methyl group of methionine residues of protein. This is not surprising in view of the ubiquitous nature of *S*-adenosylmethionine as a biological methylating agent and the genetic defect eliminating an endogenous metabolic source of methionine. A comparison of Figs. 1 and 2 shows that maximum growth of the triple mutant requires 50 times as much methionine as histidine hydrochloride on a weight basis, corresponding to a ratio of about 65 on a molar basis. The large differential requirement can be attributed to the need for exogenous methionine as a methyl group and one-carbon unit source. Fig. 4 shows that the major use of the exogenous methionine methyl group is in the formation of the *N*⁺-trimethyl group of choline derivatives. In *Neurospora*, phosphatidylcholine can be synthesized by sequential methylation of phosphatidylethanolamine (Hall & Nyc, 1952) and cytidyl diphosphocholine can be synthesized by sequential methylation of cytidyl diphosphocholine ethanolamine (Matysiak *et al.*, 1974). Small molecules such as these in the cytoplasm or those associated with

Fig. 5. ¹³C n.m.r. spectra of dialysed cytochrome *c* from the triple mutant grown on [*S*-Me-¹³C] methionine

Upper trace, [*S*-Me-¹³C]methionine used as the labelled substrate, 1000 pulses; lower trace, ferricytochrome *c*, 10 mg/ml, 80126 pulses.

membranes that are fluid enough to permit rapid tumbling will have short rotational correlation times and exhibit a narrow-line methyl resonance with triplet structure from ^{14}N splitting. If association is with more rigid and immobilized phases so that rotational correlation times are long, the methyl-group resonance would be expected to appear as a single broad line. Thus the ^{13}C n.m.r. spectra of Fig. 4 suggest that in the active phase of cell proliferation a significant concentration of choline and small derivatives accumulate in an environment that allows relatively unrestricted molecular tumbling. Subsequently, during a relatively dormant, nutrient-deficient metabolic state these accumulated molecules become associated with more immobilized phases such as rigid membrane structures.

The hydroxymethyl region resonance at 63.7 p.p.m. (Fig. 4) occurs at a position expected for the β carbon of serine. This hydroxymethyl carbon and the methionine methyl carbon are related metabolically through tetrahydrofolate derivatives, but the primary biosynthetic pathway is in the direction serine $\rightarrow$ methionine. Also the spectrum of cytochrome *c* shown in Fig. 5 shows no resonance at 63.7 p.p.m. even though the protein contains three serine residues. Thus the hydroxymethyl resonance may come from some metabolite other than serine. The minor peaks in the methyl region of the whole-cell spectra at 23.2, 28.1 and 34.6 p.p.m. perhaps represent precursors whose methyl groups would be expected to eventually become part of the phosphatidylcholine moiety of membrane. We have observed that formation of a sulphonium species of methionine by carboxymethylation shifts the *S*-methyl carbon resonance downfield to 24.4 p.p.m. (R. T. Eakin, unpublished work). Thus the resonance at 23.2 p.p.m. in the whole-cell spectrum is in the region expected for methionine sulphonium ion and could represent *S*-adenosylmethionine. The resonance at 34.6 p.p.m. is very close to the value 34.5 p.p.m. obtained for the methyl carbon of protonated *N*-methylethanolamine in aqueous solution (Table 1). However, the whole-cell spectrum does not show any significant resonance in the region around 44.4 p.p.m. corresponding to the methyl groups of protonated *NN*-dimethylethanolamine in aqueous solution (Table 1). The resonance at 73.4 p.p.m., which is present at about equal intensity in the two spectra of Fig. 4, is not in a region corresponding to any common metabolic product of methyl-group metabolism and is so far downfield that it almost certainly does not represent a methyl group, but is characteristic of $=\text{CHOH}$.

Incorporation of the methyl group of methionine into cytochrome c

There is a significant amount of low-molecular-weight material containing carbon atoms derived

biosynthetically from the methyl carbon of methionine which remains associated with cytochrome *c* throughout its normal purification procedure. The major associated small molecule appears to be a choline derivative, probably from the disrupted inner mitochondrial membrane to which cytochrome *c* binds *in vivo*. Also the less abundant dialysable components probably originate from inner mitochondrial membrane and remain associated with cytochrome *c* after disruption of membrane structure, but they apparently do not include dimethylethanolamine since the signals do not occur at the position for this compound as listed in Table 1.

Notable features of the n.m.r. spectra of ferricytochrome *c* in Fig. 5 are that the signal expected for a methionyl methyl carbon appears at 13.8 p.p.m. rather than 15.0 p.p.m., and a prominent peak is seen in the methyl region of *N*-tetra-alkyl ammonium compounds. The only tetra-alkyl ammonium group in *Neurospora* cytochrome *c* is in the ϵ -*N*-trimethyllysine-72 residue and presumably the dominant ^{13}C n.m.r. signal arises from those three methyl carbons. Thus it is apparent that the biosynthetic origin of the methyl groups formed in the secondary modification is the methyl group of methionine.

The resonance at 13.8 p.p.m. in the ^{13}C n.m.r. spectrum of ferricytochrome *c* (Fig. 5) is shifted to 13.6 p.p.m. in the reduced form and probably represents the methyl carbon of the methionine-98 residue. From crystallography it has been found that in horse cytochrome *c* the leucine-98 residue is quite close to the haem group (Dickerson, 1972). Evolutionary conservation of structure in cytochrome *c* would keep the position 98 close to the haem group and the resonance of the methyl carbon of methionine-98 in *Neurospora* cytochrome *c* could be subject to chemical shift from ring-current effects. In fact, the observed 1.2 p.p.m. shift from the expected position at 15.0 p.p.m. is of the same magnitude as that observed in similar circumstances for methyl protons in haemoglobin (Lindstrom *et al.*, 1972). The 0.2 p.p.m. difference in the chemical shift between the oxidized and reduced forms of cytochrome *c* is the same as that observed for the methylene carbon of the *S*-[2- ^{13}C]carboxymethyl derivative of methionine-80 in horse cytochrome *c* where the residue is no longer coordinated to the haem, but still in vicinity of the iron (Eakin *et al.*, 1975).

As the sulphur atom of methionine-80 is directly co-ordinated to the haem iron, internal motion is severely restricted and the electronic environment of the [^{13}C]methyl group is altered significantly. Failure to observe a shifted signal in the ferricytochrome *c* spectrum is probably attributable to severe line broadening. In ferricytochrome *c* the expected contact interaction with the paramagnetic iron would probably prevent observation of the [^{13}C]methyl signal in any case.

Note Added in Proof (Received 7 October 1975)

Since submission of this manuscript, a spectrum similar to that presented in Fig. 5 has been obtained with a higher cytochrome concentration and a larger number of pulses. A second methionine methyl resonance, presumably from methionine-80, was found at 15.8 p.p.m. in the diamagnetic ferrous protein, but not in the ferric form. Other spectral features remained the same as those reported.

We thank Dr. R. P. Wagner for the use of facilities in his laboratory for growing *Neurospora*. The technical assistance of Dr. J. D. Metter in the preparation of [$S\text{-}Me\text{-}^{13}\text{C}$]methionine is greatly appreciated. This work was performed under the auspices of the United States Energy Research and Development Administration, and was supported in part by the Robert A. Welch Foundation, Houston, Texas, under Grant No. F-041.

References

- Browne, D. T., Kenyon, G. D., Packer, E. L. & Wilson, D. M. (1973a) *Biochem. Biophys. Res. Commun.* **50**, 42–47
- Browne, D. T., Kenyon, G. L., Packer, E. L., Sternlicht, H. & Wilson, D. M. (1973b) *J. Am. Chem. Soc.* **95**, 1316–1323
- DeLange, R. N., Glazer, A. N. & Smith, E. L. (1969) *J. Biol. Chem.* **244**, 1385–1388
- Dickerson, R. E. (1972) *Sci. Am.* **226**, 58–72
- Eakin, R. T., Morgan, L. O., Gregg, C. T. & Matwiyoff, N. A. (1972) *FEBS Lett.* **28**, 259–264
- Eakin, R. T., Morgan, L. O. & Matwiyoff, N. A. (1975) *Biochemistry in the press*
- Hagihara, B., Morikawa, I., Tagawa, K. & Okunuki, K. (1958) *Biochem. Prep.* **6**, 1–7
- Hall, M. O. & Nyc, J. F. (1952) *Biochim. Biophys. Acta* **56**, 370–732
- Haskins, F. A., Tissieres, A., Mitchel, H. K. & Mitchell, M. B. (1953) *J. Biol. Chem.* **200**, 819–826
- Heller, J. & Smith, E. L. (1966) *J. Biol. Chem.* **241**, 3165–3180
- Hunkapillar, M. W., Smallcombe, S. H., Whitaker, D. R. & Richards, J. H. (1973a) *J. Biol. Chem.* **248**, 8306–8308
- Hunkapillar, M. W., Smallcombe, S. H., Whitaker, D. R. & Richards, J. H. (1973b) *Biochemistry* **12**, 4732–4743
- Johnson, L. F. & Jankowski, W. C. (1972) *Carbon-13 NMR Spectra*, John Wiley and Sons, New York
- Lambowitz, A. M., Slagman, C. W. & Slayman, C. L. (1972) *J. Biol. Chem.* **247**, 1536–1545
- Lindstrom, T. R., Noren, I. B. E., Charache, S., Lehmann, H. & Ho, C. (1972) *Biochemistry* **11**, 1677–1681
- London, R. E., Gregg, C. T. & Matwiyoff, N. A. (1975) *Science* **188**, 266–268
- Matwiyoff, N. A. & Needham, T. E. (1972) *Biochem. Biophys. Res. Commun.* **49**, 1158–1164
- Matysiak, Z., Radomska-Pyrek, A. & Chojnacki, T. (1974) *Mol. Cell. Biochem.* **3**, 143–151
- Melville, D. B., Rachele, J. R. & Keller, E. B. (1947) *J. Biol. Chem.* **169**, 419–426
- Packer, E. L., Sternlicht, H. & Rabinowitz, J. C. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3278–3282
- Reporter, M. (1973) *Arch. Biochem. Biophys.* **158**, 577–585
- Scott, A. I. (1974) *Science* **184**, 760–764
- Scott, W. A. & Mitchell, H. K. (1969) *Biochemistry* **8**, 4282–4289
- Takano, T., Kallai, O. B., Swanson, R. & Dickerson, R. D. (1973) *J. Biol. Chem.* **248**, 5234–5255
- Vogel, H. J. (1956) *Microbiol. Genet. Bull.* **13**, 42–43