[¹⁹F]Fluorine Nuclear-Magnetic-Resonance Study of the Interaction of Difluoro-oxaloacetate with Aspartate Transaminase

By PATRICIA A. BRILEY, ROBERT EISENTHAL, ROGER HARRISON
and GEOFFREY D. SMITH

Biochemistry Group, School of Biological Sciences, University of Bath, Bath BA2 7AY, U.K.

(Received 12 October 1976)

Difluoro-oxaloacetate interacts with the aldimine form of aspartate transaminase to give a complex, the dissociation constant of which has been determined spectrophotometrically and by ¹⁹F n.m.r. (nuclear magnetic resonance). The ¹⁹F n.m.r. line-width–pH and chemical-shift–pH profiles of difluoro-oxaloacetate in the presence of the aldimine form of the enzyme both show inflexion points in the pH 5 and pH 8 regions, which may arise from variations in the binding of difluoro-oxaloacetate as specific groups on the enzyme are successively protonated. Difluoro-oxaloacetate also interacts with apoenzyme to form a complex, the dissociation constant of which was determined by ¹⁹F n.m.r. The ¹⁹F n.m.r. line-width–pH and chemical-shift–pH profiles of difluoro-oxaloacetate in the presence of apoenzyme show a single inflexion point in the region of pH 8. The absence, in this case, of an inflexion in the pH 5 region indicates that the latter, present in the corresponding profiles for the aldimine form of the enzyme, results from ionization of an enzyme group associated with the pyridoxal phosphate cofactor.

Difluoro-oxaloacetate behaves as an effective analogue of oxaloacetate in being transaminated by the aminic (pyridoxamine) form of aspartate transaminase (Briley *et al.*, 1977). In view of the known binding of oxaloacetate and of a number of other dicarboxylic acids to the aldimine (pyridoxal) form of aspartate transaminase (hereinafter called ‘aldimine enzyme’) (Haarhoff, 1969), difluoro-oxaloacetate might be expected to act as an analogue of oxaloacetate in this respect also and to form an ‘abortive complex’ with the aldimine enzyme. Although such a complex was not detected by kinetic analysis of the inhibition of transamination of 2-oxoglutarate and aspartate by difluoro-oxaloacetate (Briley *et al.*, 1977), an abortive complex having a dissociation constant in the millimolar range would not be apparent under the conditions of the experiments.

Like perfluorosuccinate, which has been used to study binding of dicarboxylic acids to the enzyme (Martinez-Carrion *et al.*, 1973), difluoro-oxaloacetate gives rise to a single sharp ¹⁹F n.m.r.* signal and so constitutes a convenient probe with which to study binding to both holoenzyme and apoenzyme, particularly so in the case of the apoenzyme, which is not readily susceptible to spectrophotometric study. The present paper describes the use of the ¹⁹F n.m.r. signal of difluoro-oxaloacetate to report on the nature of the binding to both forms of aspartate transaminase.

* Abbreviation: n.m.r., nuclear magnetic resonance.

Experimental

Materials

Difluoro-oxaloacetic acid was prepared as described by Briley *et al.* (1977) and perfluorosuccinic acid was purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Unless otherwise stated, both acids were neutralized to pH 7.4 with KOH before use. Cysteinesulphinic acid was from Sigma (London) Chemical Co., London S.W.6, U.K., and all other reagents were from BDH Chemicals Ltd., Poole, Dorset, U.K.

The aldimine and aminic forms of cytoplasmic aspartate transaminase (L-aspartate–2-oxoglutarate aminotransferase, EC 2.6.1.1) were prepared from pig heart as described by Briley *et al.* (1977). The aldimine enzyme typically showed an A_{280}/A_{362} ratio of 7.25 at pH 7.4. The apoenzyme was prepared from the aldimine enzyme via the aminic form as described by Scardi *et al.* (1963), except that cysteinesulphinic acid was substituted for glutamate. This procedure consistently achieved greater than 95% resolution as determined by residual transaminase activity.

The apoenzyme could be re-activated with excess of pyridoxal phosphate in 50 mM-sodium acetate buffer, pH 7.4, at 22°C. Throughout experiments using apoenzyme, restorable activity was monitored and was found to be greater than 90% of original activity in experiments at pH 7.4 and greater than 70% at lower pH values. Concentrations of apoenzyme are quoted in terms of restorable activity,

Methods

Enzyme activity was assayed by determination of the rate of oxaloacetate production (A_{260}) from 2-oxoglutarate and aspartate. Protein was determined by measuring A_{280} as described by Banks *et al.* (1968). Concentrations of enzyme are quoted in terms of active sites, assuming a dimer of mol.wt. 93000 (Ovchinnikov *et al.*, 1973). Active sites were determined from A_{360} (aldimine) and A_{332} (aminic) by using absorption coefficients determined as described by Torchinsky *et al.* (1968).

N.m.r. determinations. ^{19}F n.m.r. spectra were recorded at 94.1 MHz with a JEOL PS 100 n.m.r. spectrometer in 5 mm-diameter sample tubes with co-axial capillary tubes containing either 2 M-difluoro-oxaloacetate or 2 M-perfluorosuccinate as internal lock signal. Chemical shifts were determined relative to the internal lock signal by using an electronic counter, and line widths were recorded as the peak width at half height. Where necessary, the signal-to-noise ratio was enhanced by using repeated scans with a JEOL JNM SB-3 signal-to-noise booster. Samples were maintained at $28 \pm 1^\circ\text{C}$.

For experiments at constant pH, enzyme (approx. 20 mg) was dissolved in a small volume of 20 mM-potassium pyrophosphate buffer, pH 7.4, dialysed against 2×5 litres of the same buffer and concentrated by vacuum dialysis to 0.4 ml. For determinations at varied pH values the enzyme was dialysed against 2×5 litres of double-distilled water.

Spectrophotometric determinations. These were done in a Pye-Unicam SP. 1800 instrument at $25 \pm 1^\circ\text{C}$ unless otherwise stated.

Calculation of dissociation constants and absorption coefficients by spectrophotometric titration. The interaction of an enzyme (E) with a ligand (S) to give a complex (ES)

can conveniently be followed from absorbance changes of the λ_{max} of ES provided that this differs from that of the free enzyme. If $[\text{E}_t]$ and $[\text{S}_t]$ are the total concentrations of enzyme and ligand respectively and K is the dissociation constant of the complex ES, then it can be shown that:

$$\frac{[\text{S}_t]}{\Delta A} = \frac{K + [\text{S}_t]}{(\epsilon_E - \epsilon_{\text{ES}})[\text{E}_t]}$$

where ΔA is the difference between the absorbance of the system and that of free enzyme alone, ϵ_E and ϵ_{ES} are the molar absorption coefficients of E and ES respectively at the given wavelength, and it is assumed that $[\text{S}_t] \gg [\text{E}_t]$.

$[\text{S}_t]/\Delta A$ was plotted against $[\text{S}_t]$ as described by Jenkins & Taylor (1965) to give a straight line, from which K was obtained as the negative intercept on the $[\text{S}_t]$ axis. Substitution of ϵ_E (determined directly

from aldimine enzyme alone) in the expressions for the slope and intercept gave ϵ_{ES} .

Calculation of dissociation constants and chemical shifts (Δ) by using ^{19}F n.m.r. K and Δ values for difluoro-oxaloacetate and perfluorosuccinate in the presence of aldimine enzyme and for difluoro-oxaloacetate with apoenzyme were determined by the iterative procedure described by Groves *et al.* (1967) and Smith *et al.* (1977). In this procedure, assumed values of K are varied so as to obtain the best fit of the experimental points to a straight line when δ_{obs} , (chemical-shift value) is plotted against $[\text{ES}]/[\text{S}_t]$, where $[\text{ES}]$ is the concentration of enzyme-ligand complex and $[\text{S}_t]$ the total concentration of ligand. The slope of the final line is Δ .

Results

Difluoro-oxaloacetate-aldimine-enzyme complex

Spectrophotometric determination of dissociation constant and absorption coefficient. Incremental addition of difluoro-oxaloacetate (S) to a solution of the aldimine form of aspartate transaminase ($\lambda_{\text{max}} = 360\text{ nm}$) in pyrophosphate buffer at pH 7.4 gave rise to an absorbance peak with $\lambda_{\text{max}} = 435\text{ nm}$, characteristic of the difluoro-oxaloacetate-enzyme complex. A plot of $[\text{S}_t]/\Delta A_{435}$ against $[\text{S}_t]$ gave a straight line (Fig. 1) from which the dissociation constant K ($2.8 \pm 0.1\text{ mM}$) and ϵ_{435} ($7600\text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) of the difluoro-oxaloacetate-aldimine-enzyme complex were obtained as described in the Experimental section. An identical value of K was obtained

Fig. 1. Plot of $[\text{S}_t]/\Delta A_{435}$ against $[\text{S}_t]$ for addition of difluoro-oxaloacetate to aldimine enzyme

Difluoro-oxaloacetate was added incrementally to a solution of the aldimine form of aspartate transaminase ($26\text{ }\mu\text{M}$) in 20 mM-pyrophosphate buffer, pH 7.4. $[\text{S}_t]$ represents the total concentration of difluoro-oxaloacetate and ΔA_{435} the absorbance difference at 435 nm relative to that of free aldimine enzyme.

when the α -form of the aldimine enzyme (Martinez-Carrion *et al.*, 1965) was used.

¹⁹F n.m.r. determination of dissociation constant and chemical shift (Δ). Difluoro-oxaloacetate gives a single ¹⁹F n.m.r. signal which moved upfield and broadened on addition of the aldimine form of aspartate transaminase. Incremental addition of difluoro-oxaloacetate to a solution of the aldimine enzyme in pyrophosphate buffer gave a series of chemical-shift values ($\delta_{\text{obs.}}$) which were used, as described in the Experimental section, to derive values (Table 1) for the dissociation constant K and chemical shift (Δ) of the difluoro-oxaloacetate-enzyme complex. The final plot of $\delta_{\text{obs.}}$ against $[ES]/[S_i]$ is shown in Fig. 2.

pH-dependence of n.m.r. parameters. The chemical shifts and peak widths of the ¹⁹F n.m.r. signal of difluoro-oxaloacetate were measured at a range of pH values in the presence of aldimine enzyme. Plots of both parameters against pH (Fig. 3) show inflexion points at pH 5.4 and pH 8.0.

Difluoro-oxaloacetate-apoenzyme complex

Dissociation constant and chemical shift (Δ). Addition of difluoro-oxaloacetate (5 mM) to a solution of apoenzyme (0.39 mM) in 20 mM-pyrophosphate buffer, pH 7.4, gave a ¹⁹F n.m.r. signal which was shifted upfield and broadened relative to that of difluoro-oxaloacetate in buffer alone. Incremental addition of further difluoro-oxaloacetate caused the signal to shift downfield and sharpen, as with the aldimine enzyme, and chemical-shift values were used in the same way to derive values of the dissociation constant, K , and the chemical shift (Δ) of the difluoro-oxaloacetate-apoenzyme complex (Table 1).

pH-dependence of n.m.r. parameters. The pH variations of the ¹⁹F chemical shift and line width of difluoro-oxaloacetate in the presence of the apoenzyme were determined as for the aldimine form of the enzyme. Plots of both parameters versus pH (Fig. 4) showed an inflexion point at pH 8.2, but not at lower pH values.

Fig. 2. Plot of $\delta_{\text{obs.}}$ against $[ES]/[S_i]$ for the difluoro-oxaloacetate-aldimine enzyme

Difluoro-oxaloacetate (total concentration $[S_i]$) was added incrementally (5–16 mM) to a solution of the aldimine form of aspartate transaminase (0.46 mM) in 20 mM-pyrophosphate buffer, pH 7.4, and the chemical shift ($\delta_{\text{obs.}}$) of the ¹⁹F n.m.r. signal of difluoro-oxaloacetate relative to that of difluoro-oxaloacetate in buffer alone was observed. The plot represents the best fit of the experimental points to a straight line for a series of assumed values of K (see the Experimental section). This plot was obtained for $K = 2.9$ mM (Table 1).

Perfluorosuccinate-aldimine-enzyme complex

Dissociation constant and chemical shift (Δ). Addition of perfluorosuccinate (10 mM) to a solution of the aldimine form of aspartate transaminase (0.66 mM) in 20 mM-pyrophosphate buffer, pH 7.4, gave a single ¹⁹F n.m.r. signal which was shifted downfield and broadened relative to that of perfluorosuccinate in buffer alone. Incremental addition of further perfluorosuccinate caused the signal to move upfield and sharpen. Chemical-shift values were used as described in the Experimental section to derive values for the dissociation constant and chemical shift (Δ) (Table 1) of the perfluorosuccinate-aldimine-enzyme complex.

Chemical shifts of ¹⁹F n.m.r. signals of difluoro-oxaloacetate and perfluorosuccinate in the absence of enzyme

pH-dependence. The ¹⁹F n.m.r. signal of difluoro-oxaloacetate showed a progressive downfield shift with increasing pH up to pH 5. No further shift was observed between pH 5 and 9. Comparison of the

Table 1. Dissociation constants and chemical shifts (Δ) of enzyme-fluoroacid complexes

Positive values of Δ indicate an upfield shift relative to that of free ligand in buffer alone. Negative values indicate a corresponding downfield shift. Values for Δ are $\pm$ s.e. (Bliss, 1967).

Ligand	Aldimine		Apoenzyme	
	K (mM)	Δ (Hz)	K (mM)	Δ (Hz)
Difluoro-oxaloacetate	2.9	$+137 \pm 2$	3.4	$+99 \pm 6$
Perfluorosuccinate	3.0	-93 ± 1	—	—

Fig. 3. pH variation of ^{19}F chemical shift ($\delta_{\text{obs.}}$, ●) and line width (○) of difluoro-oxaloacetate in the presence of aldimine enzyme

Difluoro-oxaloacetate was neutralized to pH 4.9 with KOH and added to a solution of extensively dialysed aldimine form of aspartate transaminase (0.75 mM) in double-distilled water, giving a final concentration of 6 mM-difluoro-oxaloacetate and final pH 4.9. The ^{19}F n.m.r. chemical shift ($\delta_{\text{obs.}}$) and line width relative to that of free difluoro-oxaloacetate in 20 mM-pyrophosphate buffer, pH 7.4, were observed as the pH of the system was increased by stepwise addition of 10 mM-KOH.

Fig. 4. pH variation of ^{19}F chemical shift ($\delta_{\text{obs.}}$, ●) and line width (○) of difluoro-oxaloacetate in the presence of apoenzyme

Difluoro-oxaloacetic acid was neutralized to pH 4.9 with KOH, and added to a solution of extensively dialysed apoenzyme (0.41 M) in double-distilled water to give a final concentration of 6 mM-difluoro-oxaloacetate and final pH 4.9. The ^{19}F n.m.r. chemical shift, $\delta_{\text{obs.}}$, and line width relative to those of difluoro-oxaloacetate in 20 mM-pyrophosphate buffer, pH 7.4, were observed as the pH of the system was increased by stepwise addition of 10 mM-KOH.

chemical-shift-pH profile (Fig. 5) with the acid-base titration curve (Fig. 6) for difluoro-oxaloacetate indicates that the increase in chemical shift corresponds to the ionization of the second carboxyl group of difluoro-oxaloacetate, which is complete at pH 5.

The ^{19}F n.m.r. signal of perfluorosuccinate shifted downfield with increasing pH up to pH 3. No further shift was observed between pH 3 and pH 9 (Fig. 5).

Solvent effects. The chemical shifts of the ^{19}F n.m.r. signals of solutions of both difluoro-oxaloacetate and perfluorosuccinate in organic solvent/water mixtures varied with the composition of the mixture. Increasing the content of organic solvent in 2-methylpropan-2-ol/water and in dioxan/water mixtures caused the ^{19}F n.m.r. signal of difluoro-oxaloacetate to move progressively upfield, whereas the signal of perfluorosuccinate moved downfield. Over the range studied, the chemical shifts of both fluorinated com-

pounds showed a linear dependence on the dielectric constant of both media (Fig. 7).

Discussion

Difluoro-oxaloacetate binds to the aldimine form of the enzyme to give an 'abortive complex' absorbing in the 430 nm region, and spectrophotometric titration monitoring A_{435} gave a dissociation constant for the difluoro-oxaloacetate-aldimine-enzyme complex (2.8 mM) close to that (1.8 mM) previously reported for the oxaloacetate complex (Michuda & Martinez-Carrion, 1970). Good agreement with the spectrophotometrically determined dissociation constant was obtained by titration of the enzyme with

Fig. 5. pH variation of ^{19}F chemical shift of difluoro-oxaloacetate (●) and perfluorosuccinate (○)

Solutions of the free acids (20mM) in double-distilled water were titrated in the n.m.r. tube with 200mM-KOH and the chemical-shift changes ($\delta_{\text{obs.}}$) relative to the initial values observed.

Fig. 6. Variation of pH on titration of difluoro-oxaloacetate with base

The pH of the mixture was followed as 0.2M-difluoro-oxaloacetic acid (5ml) was titrated with 0.107M-tetramethylammonium hydroxide.

difluoro-oxaloacetate, by following the ^{19}F n.m.r. chemical shift of the latter.

The ^{19}F n.m.r. signal of the difluoro-oxaloacetate-alimine-enzyme system shifts upfield and broadens as the pH of the medium is decreased, and pH profiles of both chemical shift and line width show inflexion points at pH 5.4 and 8.0 (Fig. 3). It is probable that the two inflexion points result from ionization of groups associated with the enzyme, as

Fig. 7. Variation of the ^{19}F chemical shifts of difluoro-oxaloacetate and perfluorosuccinate with the dielectric constant of the medium

2-Methylpropan-2-ol (●) and dioxan (○) were added incrementally to a solution containing pre-neutralized fluoro acid (20mM) (a, difluoro-oxaloacetate; b, perfluorosuccinate) in 20mM-pyrophosphate buffer, pH 7.4, and the ^{19}F chemical shift ($\delta_{\text{obs.}}$) was determined relative to that of the acid in buffer alone. Dielectric constants were calculated as described by von Hippel (1954).

the chemical shift of difluoro-oxaloacetate alone does not vary at pH values greater than 5 (Fig. 5). The pH profiles of both line width and chemical shift can be explained in terms of a model in which each of the two carboxyl groups of difluoro-oxaloacetate binds to a specific group on the enzyme as that group is protonated. As the pH of the medium is lowered, the groups on the enzyme will be successively protonated and the difluoro-oxaloacetate becomes progressively more tightly bound. The resulting increase in correlation time is reflected in the increased line widths of the ^{19}F n.m.r. signal. Tighter binding of the carboxyl groups of difluoro-oxaloacetate to protonated enzyme might be expected to result in shifts of the ^{19}F n.m.r. signal in the same direction as when difluoro-oxaloacetate is protonated in the absence of enzyme, i.e. upfield, which is indeed the case.

It is of course possible that other factors apart from such charge-neutralization effects will contribute to the upfield shift of the ^{19}F n.m.r. signal of difluoro-oxaloacetate on binding to the enzyme. In this context it is of interest to consider the effect of alimine enzyme on the ^{19}F n.m.r. signal of perfluorosuccinate. The ^{19}F n.m.r. line-width-pH profile for perfluorosuccinate in the presence of

aldimine enzyme (Martinez-Carrion *et al.*, 1973) is very similar to that for difluoro-oxaloacetate, suggesting that binding of both dicarboxylic acids is dependent on protonation of the same two enzyme groups. For perfluorosuccinate, however, the effect of enzyme is to shift the ^{19}F n.m.r. signal downfield, the opposite direction to that for difluoro-oxaloacetate. In the absence of enzyme, perfluorosuccinate behaves like difluoro-oxaloacetate in that protonation shifts the ^{19}F n.m.r. signal upfield (Fig. 5). The net downfield shift on addition of enzyme cannot therefore be attributed to effective protonation of perfluorosuccinate on binding to the enzyme. A possible explanation is suggested by the effect on the ^{19}F n.m.r. chemical shifts of perfluorosuccinate and difluoro-oxaloacetate of increasing the content of organic solvent in the systems dioxan/water and 2-methylpropan-2-ol/water. As the dielectric constant of the medium is decreased, the ^{19}F signal of perfluorosuccinate shifts downfield and that of difluoro-oxaloacetate upfield (Fig. 7). Although a number of factors contribute to the effects of the medium on fluorine chemical shifts (Emsley & Phillips, 1971), it is noteworthy that, in both cases, the effect of transfer to a less polar environment is to shift the ^{19}F n.m.r. signal in the same direction as when the fluorinated acids bind to the enzyme. The data can be qualitatively explained if the dominant effect on the chemical shift is one associated with binding in an apolar environment on the enzyme. With difluoro-oxaloacetate, the upfield shift arising from the apolarity of the enzyme surface would be reinforced by that caused by effective protonation of its carboxyl groups as they bind to protonated groups on the enzyme. With perfluorosuccinate, however, shifts caused by the two effects would be in opposite directions, and it may be significant that Δ for the perfluorosuccinate-aldimine-enzyme complex (95 Hz downfield) is some 50% lower than that for the corresponding difluoro-oxaloacetate complex (140 Hz upfield). The suggestion of an apolar environment at the binding site of aspartate transaminase is in accord with spectrophotometric data (Metzler *et al.*, 1973). In the absence of detailed three-dimensional structural data for aspartate transaminase, discussion of factors influencing chemical-shift effects in an enzyme complex is necessarily crude, and it is quite possible that further factors such as hydrogen-bonding or ring-current effects may be important in influencing chemical shifts of the fluorine atoms of the fluorinated dicarboxylic acids in their enzyme environment.

The inflexion point at pH 5.4, which appears in the chemical-shift-pH and the line-width-pH profiles of difluoro-oxaloacetate in the presence of the aldimine form of the holoenzyme, is absent from the corresponding profiles with apoenzyme. This suggests that the low-pH inflexion point in the profiles of the

aldimine enzyme results from ionization of a group associated with cofactor. The enamine group linking pyridoxal phosphate to a lysine residue in the aldimine enzyme is known (Cheng *et al.*, 1971) to be protonated with pK 5.4 in the absence of buffer ions, and the data are consistent with binding of a carboxylate group of difluoro-oxaloacetate to the protonated form of this internal Schiff base. This grouping has been previously suggested as the low-pH carboxylate-binding site for glutamate to the aldimine enzyme (Jenkins & D'Ari, 1966).

The n.m.r.-parameter-pH profiles of difluoro-oxaloacetate in the presence of both holoenzyme and apoenzyme show an inflexion point at pH 8.0, indicating that binding at high pH is influenced by ionization of an enzyme grouping that is not associated with cofactor. It is possible that this inflexion point reflects binding of a carboxylate group of difluoro-oxaloacetate to the protonated form of a histidine residue on the enzyme, as has been suggested for other dicarboxylic acids (Martinez-Carrion *et al.*, 1973). The pK for protonation of this enzyme group is, however, rather higher than that normally associated with histidine residues, and further evidence is necessary before such an assignment can be made.

Although a model in which binding of carboxylate groupings of difluoro-oxaloacetate to the enzyme is directly influenced by protonation of specific enzyme groups is attractive, it should be borne in mind that ionization of enzyme groupings could affect ligand binding in a less direct manner, such as via conformational changes in the enzyme, and such effects cannot be ruled out. The similarity of the n.m.r.-parameter-pH profiles in the difluoro-oxaloacetate-aldimine-enzyme system with those of perfluorosuccinate (Martinez-Carrion *et al.*, 1973) indicates that the two fluorinated dicarboxylic acids bind in the same manner to the enzyme. Moreover, as difluoro-oxaloacetate and perfluorosuccinate have been shown to mimic oxaloacetate (Briley *et al.*, 1977) and succinate (Martinez-Carrion *et al.*, 1973) respectively in their interactions with aspartate transaminase, it is likely that all four dicarboxylic acids, and possibly all dicarboxylic acids known to form abortive complexes, bind similarly to the aldimine enzyme.

We thank the Enzyme Chemistry and Technology Committee of the Science Research Council for a grant (to R. E. and R. H.) and for a research studentship (to G. D. S.).

References

- Banks, B. E. C., Doonan, S., Lawrence, A. J. & Vernon, C. A. (1968) *Eur. J. Biochem.* **5**, 528-539

- Bliss, C. I. (1967) *Statistics in Biology*, vol. 1, pp. 438–439, McGraw–Hill, New York
- Briley, P. A., Eisenthal, R., Harrison, R. & Smith, G. D. (1977) *Biochem. J.* **161**, 383–387
- Cheng, S., Michuda-Kozak, C. & Martinez-Carrion, M. (1971) *J. Biol. Chem.* **246**, 3623–3630
- Emsley, J. W. & Phillips, L. (1971) *Prog. Nucl. Magn. Reson. Spectrosc.* **7**, 3–36
- Groves, P. D., Huck, P. J. & Homer, J. (1967) *Chem. Ind.* 915–917
- Haarhoff, K. N. (1969) *J. Theor. Biol.* **22**, 117–150
- Jenkins, W. T. & D'Ari, L. (1966) *J. Biol. Chem.* **241**, 5667–5674
- Jenkins, W. T. & Taylor, R. T. (1965) *J. Biol. Chem.* **240**, 2907–2913
- Martinez-Carrion, M., Riva, F., Turano, C. & Fasella, P. (1965) *Biochem. Biophys. Res. Commun.* **20**, 206–211
- Martinez-Carrion, M., Cheng, S. & Relimpo, A. M. (1973) *J. Biol. Chem.* **248**, 2153–2160
- Metzler, D. E., Harris, C. M., Johnson, R. J., Siano, D. B. & Thomson, J. A. (1973) *Biochemistry* **12**, 5377–5392
- Michuda, C. M. & Martinez-Carrion, M. (1970) *J. Biol. Chem.* **245**, 262–269
- Ovchinnikov, Yu. A., Egorov, C. A., Aldanova, V. A., Feigina, M. Yu., Lipkin, V. M., Abdulaev, N. G., Grishin, E. G., Kiselev, A. P., Modyanov, N. N., Braunstein, A. E., Polyanovsky, O. L. & Nosikov, V. V. (1973) *FEBS Lett.* **29**, 31–34
- Scardi, V., Scotto, P., Jaccarino, M. & Scarano, E. (1963) *Biochem. J.* **88**, 172–175
- Smith, G. D., Eisenthal, R. & Harrison, R. (1977) *Anal. Biochem.* in the press
- Torchinsky, Yu. M., Malakhova, E. A., Livanova, N. B. & Pikelgas, V. Yu. (1968) in *Pyridoxal Catalysis: Enzymes and Model Systems* (Snell, E. E., Braunstein, A. E., Severin, E. S. & Torchinsky, Yu. M., eds.), pp. 269–290, Interscience, London
- von Hippel, A. R. (1954) *Dielectrics and Waves*, p. 231, John Wiley and Sons, New York