# Effects of $Mg^{2+}$, $Ca^{2+}$ and $Mn^{2+}$ on sheep liver cytoplasmic aldehyde dehydrogenase

F. Mark DICKINSON and Graham J. HART*
*Department of Biochemistry, University of Hull, Hull HU6 7RX, U.K.*

Sheep liver cytoplasmic aldehyde dehydrogenase is strongly inhibited by $Mg^{2+}$, $Ca^{2+}$ and $Mn^{2+}$. The inhibition is only partial, however, with 8–15% of activity remaining at high concentrations of these agents. In 50 mM-Tris/HCl, pH 7.5, the concentrations giving half-maximal effect were: $Mg^{2+}$, 6.5 $\mu$M; $Ca^{2+}$, 15.2 $\mu$M; $Mn^{2+}$, 1.5 $\mu$M. The esterase activity of the enzyme is not affected by such low metal ion concentrations, but appears to be activated by high concentrations. Fluorescence-titration and stopped-flow experiments provide evidence for interaction of $Mg^{2+}$ with NADH complexes of the enzyme. As no evidence for the presence of increased concentrations of functioning active centres was obtained in the presence of $Mg^{2+}$, it is concluded that the effects of $Mg^{2+}$ (and presumably $Ca^{2+}$ and $Mn^{2+}$ also) are brought about by trapping increased concentrations of NADH in a $Mg^{2+}$-containing complex. This complex must liberate products more slowly than any of the complexes involved in the non-inhibited mechanism.

It has been reported that the pI-5 (mitochondrial) isoenzyme of horse liver aldehyde dehydrogenase is dissociated from tetramer to dimer in the presence of $Mg^{2+}$ and that this dissociation is accompanied by a 2-fold increase in the activity of the enzyme and a doubling of the concentration of functioning active sites (Takahashi & Weiner, 1980; Takahashi *et al.*, 1980). The F1 (cytoplasmic) isoenzyme of horse liver is also activated by some bivalent metal ions at low concentration, but inhibited by others (Venteicher *et al.*, 1977). These results were of particular interest to us, because our best preparations of the sheep liver cytoplasmic enzyme contain considerably less than the expected number of active sites (Dickinson *et al.*, 1981), given a tetrameric structure of very similar or identical subunits (MacGibbon *et al.*, 1979). In the event we have found that our preparations are very strongly inhibited by low concentrations of bivalent metal ions and that other aspects of the processes are also different from those reported for the horse pI-5 isoenzyme. We now report these results.

## Experimental

### Materials

NADH (grade I) and $NAD^+$ (grade II) were from

Boehringer Corp. (London), London W.5, U.K. $NAD^+$ was in some instances further purified before use by chromatography on DEAE-cellulose by the method of Dalziel & Dickinson (1966). Other chemicals were analytical-reagent grade where available, obtained from Fisons Chemicals, Loughborough, Leicestershire, U.K., or BDH Chemicals, Poole, Dorset, U.K. Propionaldehyde was redistilled before use.

Cytoplasmic aldehyde dehydrogenase, free from the corresponding mitochondrial enzyme, was prepared as described by Dickinson *et al.* (1981) and was assayed by the method of Hart & Dickinson (1977). Protein concentrations were calculated by using $A_{1cm}^{1\%} = 11.3$ at 280 nm, determined from dry-weight measurements. Enzyme solutions are normally prepared for use by dialysis against 50 mM- or 100 mM-sodium phosphate buffer, pH 7.0, containing 0.3 mM-EDTA and 100 $\mu$M-dithiothreitol. For most experiments done here, where the effects of metal ions were studied, enzyme solutions were dialysed against 100 mM-phosphate buffer, pH 7.5, or 50 mM-Tris/HCl, pH 7.5, with no EDTA present.

### Methods

All glassware was acid-washed, and glass-distilled water was first passed through an 'Elgastat' deionizer (Elga Products Ltd., Lane End, Bucks., U.K.) before use.

* Present address: University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, U.K.

*Determination of coenzyme and substrate concentrations*

$NAD^+$ and NADH were assayed enzymically with yeast alcohol dehydrogenase by the methods of Dalziel (1962, 1963) and by using $\varepsilon = 6.22 \times 10^3$ litre·mol$^{-1}$·cm$^{-1}$ for NADH at 340nm (Horecker & Kornberg, 1948). Aldehyde concentrations were checked by following the loss of NADH at 340nm by using yeast alcohol dehydrogenase and excess of NADH in 0.1 M-phosphate buffer, pH 7.0

*Activity assays and the effect of metal ions*

Assays of the dehydrogenase activity were carried out in a filter fluorimeter in 50 mM-phosphate buffer, pH 7.5, or 50 mM-Tris/HCl buffer, pH 7.5 at 25°C. The reactant concentrations were $NAD^+$ 250 μM and propionaldehyde 1 mM. For spectrophotometric assays of the esterase activity the same buffers were used at 25°C and the substrate concentration was 1.25 mM. The p-nitrophenyl acetate was prepared in acetone, and all assays thus contained 2.5% (v/v) acetone as a result of substrate addition. For the dehydrogenase activity determinations, it was established that addition of EDTA (30 μM) had no effect on observed rates when no metal ion had been previously added. The esterase activity seemed to be slightly inhibited (approx. 20%) on addition of the EDTA.

*Stopped-flow experiments*

These were performed in an apparatus modified (Hart & Dickinson, 1982) from the design of Gibson & Milnes (1964). The apparatus is not ideally suited to experiments of the type described here, because the valve-block and observation tubes are made from stainless steel. For these experiments, then, all buffers contained 1 mM-EDTA and the effect of metal ions was observed after adding excess (5 mM) metal ion.

**Results and discussion**

Fig. 1 shows the effects of $MgCl_2$, $CaCl_2$ and $MnCl_2$ on the activity of aldehyde dehydrogenase in assays containing 50 mM-Tris/HCl buffer, pH 7.5. As 5 mM-NaCl gave no more than 7% inhibition and 5 mM-KCl no more than 3% inhibition under the same conditions, it is evident that the dehydrogenase is strongly inhibited by low concentrations of bivalent metal ions. It may be noted that these effects occur within the time of mixing (approx. 5 s), and progress curves are linear for several minutes. The effects are fully and immediately reversed on addition of excess EDTA. Analysis of the data of Fig. 1 by using the plot in Fig. 2 indicates that the inhibition is hyperbolic, with the concentrations giving half-maximum effect being $Mn^{2+}$ 1.5 μM, $Mg^{2+}$ 6.5 μM, $Ca^{2+}$ 15.2 μM. The results with $Mn^{2+}$ are rather similar to those reported for the horse
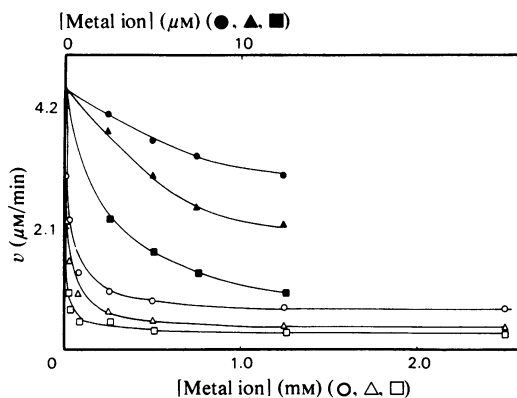


Fig. 1. *Effect of bivalent metal ions on the activity of aldehyde dehydrogenase*
Assays were performed at pH 7.5, 25°C, as described in the text. The additions were $MnCl_2$ (□, ■), $MgCl_2$ (△, ▲) and $CaCl_2$ (○, ●).
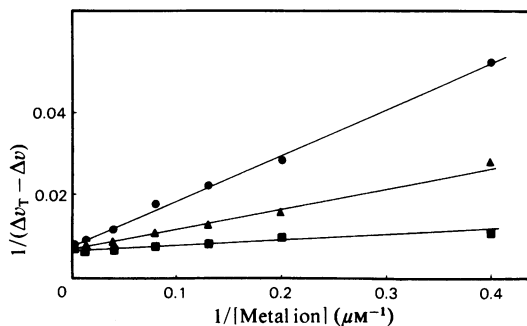


Fig. 2. *Alternative plot of some of the data from Fig. 1*
$\Delta v_T$ is the change in rate on changing from assays containing no metal ion to those containing a very high concentration of the same ion. $\Delta v$ is the change in rate on changing from a given metal ion concentration to a very high concentration of the same ion. The additions were $MnCl_2$ (■), $MgCl_2$ (▲) and $CaCl_2$ (●).

cytoplasmic enzyme, though in that case the concentration of $Mn^{2+}$ giving half-maximal effect was about 5 μM (Venteicher et al., 1977). The experiment was partially repeated but with 50 mM- and 100 mM-sodium phosphate buffer, pH 7.5, and gave similar results for the dehydrogenase activity. The concentrations of $Mg^{2+}$ required to give half-maximal effect under these conditions were 150 μM with 50 mM buffer and 300 μM with 100 mM buffer. Taking a value for the dissociation constant for $MgHPO_4$ of $1.2 \times 10^{-3}$ M (Taylor et al., 1963), the free $Mg^{2+}$ concentrations at the point of half-

maximal effect would be $4.0\,\mu M$ in 100 mM-phosphate buffer, pH 7.5, and $3.1\,\mu M$ in 50 mM-phosphate buffer, pH 7.5. These calculations are in reasonable agreement with the results in Tris/HCl buffer, pH 7.5, and indicate that it is the free $Mg^{2+}$ that interacts with the enzyme. The latter experiments could not, of course, be conducted with $MnCl_2$ and $CaCl_2$ because of immediate precipitation of the corresponding phosphates. The results in phosphate buffers explain why we have not experienced serious problems of irreproducibility in our earlier work by adventitious contamination with bivalent metal ions. It was precisely such difficulties found when using Tris buffers, together with the apparent activating effects of EDTA, which led us into this investigation. In the previous work (Hart & Dickinson, 1982), assays were performed in 50 mM- or 100 mM-phosphate buffer. Further, enzyme solutions were always prepared and stored in the presence of 0.3 mM-EDTA and $100\,\mu M$-dithiothreitol. Fortunately, then, conditions were chosen that would have minimized the effect of trace metals. However, we have on occasion experienced some problems with irreproducibility. Thus two initial-rate parameters could not be estimated accurately (Hart & Dickinson, 1982), and this was thought to be due to having to work at very low ($<1\,\mu M$) aldehyde and $NAD^+$ concentrations. In some stopped-flow work we have experienced small variations in behaviour, which we have privately attributed to differences between different preparations of enzyme. It now seems possible that this variability was to some extent due to contamination of reagents by metal ions. The stopped-flow work would probably be least affected, because in using high enzyme concentrations there could be significant carry-over of EDTA into the experiments. Indeed, many of the experiments of Hart & Dickinson (1982) have since been repeated, but with inclusion of 1.0 mM-EDTA in reagents, with no change of substance from what was then reported. However, for future work, not involving the effects of metal ions on the enzyme activity, it would seem a wise precaution to include EDTA in buffer solutions.

The esterase activity of the enzyme (MacGibbon et al., 1978) towards p-nitrophenyl acetate in assays containing 50 mM-phosphate buffer, pH 7.5, was not altered by concentrations of $Mg^{2+}$ (5 mM total) which exert the full inhibitory effect towards the dehydrogenase. The esterase activity of the horse cytoplasmic enzyme is also insensitive to $Mg^{2+}$, $Ca^{2+}$ and other bivalent metal ions (Venteicher et al., 1977). When the experiments were repeated in 50 mM-Tris/HCl buffer, pH 7.5, some activation of the esterase was noted. Thus with 5 mM-$Mg^{2+}$ 40% activation occurred, with 5 mM-$Ca^{2+}$ 30% activation, and with 0.5 mM-$Mn^{2+}$ and 5 mM-$Mn^{2+}$ the activations were 16% and 80% respectively. In the

esterase reaction hydrolysis of the acyl-enzyme intermediate is at least partially rate-limiting (MacGibbon et al., 1978). The effect of high $Mg^{2+}$ concentrations may be simply to promote acyl-enzyme hydrolysis. The $Mg^{2+}$ might act as a Lewis acid and complex with the carbonyl oxygen atom of the acyl enzyme, the attack of $OH^-$ on the acyl-enzyme being then facilitated and the rate of reaction increased.

It is evident then that the effects of metal ions on the two activities of the enzyme are very different. It has been claimed that the binding sites for aldehyde and ester are different (MacGibbon et al., 1978). The present results are consistent with the two reactions occurring at different sites on the enzyme, but they are also consistent with the view that the inhibitory effects of the bivalent metal ions are exerted on a part of the mechanism of the dehydrogenase which is not shared by the esterase. For the esterase activity one rate-limiting step occurs after acyl-enzyme formation (MacGibbon et al., 1978), and for the dehydrogenase after NADH formation, i.e. after acyl-enzyme formation (MacGibbon et al., 1977b; Hart & Dickinson, 1982). The inhibitory effects of bivalent metal ions on the dehydrogenase might be directed towards the NADH-containing complexes concerned in the mechanism. It is noted in passing that the inhibitory effects of $Mg^{2+}$ recorded in Fig. 1 are exerted over a very wide range of aldehyde concentrations, as is evident from Fig. 3. It is interesting that the activation normally seen at high propionaldehyde concentrations (MacGibbon
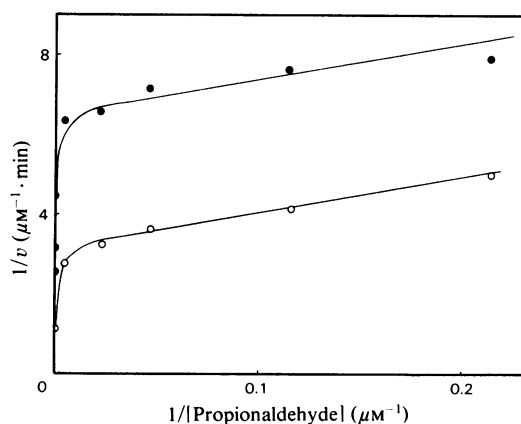


Fig. 3. *Effect of $MgCl_2$ on the kinetics of aldehyde dehydrogenase*
Assays were carried out in 50 mM-phosphate buffer, pH 7.5, at 25 °C in the absence (O) or presence (●) of $250\,\mu M$-$MgCl_2$. Purified $NAD^+$ was used (see text) and the concentration in assays was $100\,\mu M$.
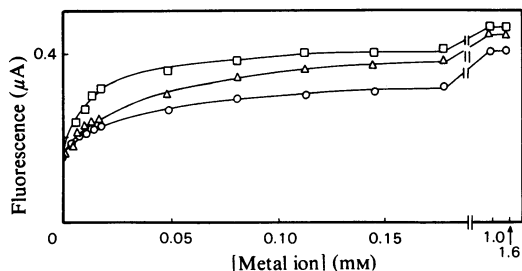
Fig. 4. *Effect of bivalent metal ions on the fluorescence of enzyme–NADH complex*

The experiment was carried out in 50 mM-Tris/HCl buffer, pH 7.5, with addition of $MnCl_2$ (□), $MgCl_2$ (△) or $CaCl_2$ (○). The mixtures initially contained 1.9 μM-enzyme and 6 μM-NADH. Measurements were performed at room temperature with a Farrand MK I spectrofluorimeter with 10 nm slits. Excitation was at 350 nm and emission at 430 nm. Under these conditions 6 μM-NADH exhibited a fluorescence of 0.04 μA.



Fig. 5. *Spectrophotometric records of the effect of $MgCl_2$ on pre-steady-state bursts with aldehyde dehydrogenase*

Enzyme (10 μM) and $NAD^+$ (250 μM) were premixed and the experiment was initiated by mixing this solution at 25°C with one containing 100 μM-propionaldehyde and 250 μM-$NAD^+$. The reaction was followed at 340 nm. All solutions contained 50 mM-Tris/HCl, pH 7.5, and 1 mM-EDTA. When $MgCl_2$ was included, it was incorporated into the propionaldehyde solution. On initiation of reaction the final $MgCl_2$ concentration was 5 mM. Experiments in which $MgCl_2$ was added to the enzyme-containing syringe gave the same result as shown above.

*et al.*, 1977*c*; Hart & Dickinson, 1982) also occurs in the presence of $Mg^{2+}$.

The effects of various concentrations of $Mg^{2+}$, $Ca^{2+}$ or $Mn^{2+}$ on the fluorescence of mixtures of enzyme and NADH at pH 7.5 are recorded in Fig. 4. The observed fluorescence increases to new plateau values, which are different for the three metal ions used, with the magnitude of the effect and the concentration of the ion giving half-maximal effect varying in the same way as in the inhibition experiments of Fig. 1. Double-reciprocal plots of the results of Fig. 4 were not linear but apparently concave downwards for $Mg^{2+}$ and $Ca^{2+}$. It is not clear what this observation means, but it should be realized that the measurements are likely to be considerably less accurate than the rate measurements of Fig. 1. It appears that the increases in fluorescence shown in Fig. 4 are principally due to the increased intrinsic fluorescence of the enzyme–NADH–$Mg^{2+}$ complex. According to MacGibbon *et al.* (1977*a*), the dissociation constant for NADH in 25 mM-phosphate buffer, pH 7.6, at 25°C is 1.2 μM. We have obtained values of this order too in fluorescence titrations. Under the initial conditions of Fig. 4 only about 25% of the NADH-binding sites will be unfilled. Since, as is documented below, the presence of $Mg^{2+}$ does not seem to increase the concentration of sites available, the 2.7–3.0-fold increase in fluorescence seen in the presence of metal ions cannot be simply due to binding to unfilled sites. Actually we find that the presence of $Mg^{2+}$ seems to have little effect on the affinity of enzyme for NADH.

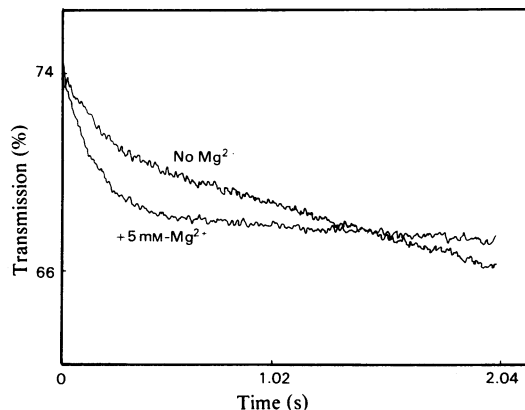The presence of excess $Mg^{2+}$ produces dramatic increases in pre-steady-state bursts seen in both spectrophotometric (Fig. 5) and fluorescence stopped-flow experiments (results not shown). The conditions (200 μM-$NAD^+$, 50 μM-propionaldehyde) chosen represent approximately maximum-rate conditions for the enzyme when no substrate activation is present (Hart & Dickinson, 1982). It may be mentioned, though, that high activating propionaldehyde concentrations (33 mM) also give much enhanced bursts in fluorescence experiments when $Mg^{2+}$ is present. Actually the increase in the burst under these conditions is apparently much greater, because the burst at 33 mM-propionaldehyde in the absence of $Mg^{2+}$ is much decreased from what is seen at 50 μM-propionaldehyde in the absence of $Mg^{2+}$ (Hart & Dickinson, 1982). The spectrophotometric record of Fig. 5 shows that the 'burst' of NADH production is increased from 0.35 mol/mol of enzyme in the absence of $Mg^{2+}$ to 0.68 mol/mol in the presence of excess $Mg^{2+}$, assuming the same absorption coefficient for bound NADH at 340 nm in both cases. The fluorescence record under the same conditions also indicates an approximate doubling of the burst in the presence of $Mg^{2+}$. The stoicheiometries here are more uncertain, however, because the intrinsic fluorescence of the NADH-

containing complexes are unknown, and in any case, as indicated above, the fluorescence of $Mg^{2+}$-containing complexes may well be different from those not containing $Mg^{2+}$.

The increased burst observed in the spectrophotometric experiment could indicate a doubling of the concentration of enzyme active centres participating in the reaction or, more likely, that an increased amount of enzyme is trapped in a terminal NADH-containing complex by the inhibitory effects of $Mg^{2+}$. Presumably the $Mg^{2+}$-containing complex would liberate products more slowly than whatever complex is rate-limiting in the absence of $Mg^{2+}$. The suggestion of an increase in the number of participating active centres seems unlikely here, if only because a strong inhibition seems incompatible with it. However, the idea requires serious consideration, because Takahashi & Weiner (1980) and Takahashi et al. (1980) have reported that the pI-5 (mitochondrial) isoenzyme of horse liver aldehyde dehydrogenase is activated by $Mg^{2+}$ and that the number of active sites doubles as the tetrameric molecule dissociates to dimers in the presence of $Mg^{2+}$. Experiment with the sheep cytoplasmic enzyme has shown that these changes do not occur with this enzyme. Thus NADH-binding experiments using the method of Hummel & Dreyer (1962) in the presence and absence of 5 mM-$Mg^{2+}$ at 25°C, pH 7.0, show that there was no increase in the number of coenzyme-binding sites. With 100 $\mu$M-NADH, which is approximately saturating, in 50 mM-phosphate + 1 mM-EDTA, and basing the estimates on the peak of the elution profile, 80 nmol of enzyme bound 76 nmol of NADH in the absence of $MgCl_2$ and 88 nmol in the presence of 10 mM-$MgCl_2$. The results are corrected for any contribution of the enzyme to the $A_{340}$ and for the fact that the enzyme–NADH–$Mg^{2+}$ complex appears to have an absorption coefficient at 340 nm some 10% less than that of free NADH (F. M. Dickinson, unpublished work). The absorbance of NADH in the enzyme–NADH complex appears to be unchanged from that of free NADH. If the calculations are based on the trough region of the elution profile, the estimates of bound nucleotide were rather higher than above (approx. 1.5 mol of NADH/mol of enzyme), but again there was no dramatic effect of $Mg^{2+}$. These results show that there are about 1.0–1.5 NADH-binding sites/molecule of enzyme. The reason for such a low result is not clear, but it is a result that we have obtained consistently using fluorescence-titration and dialysis methods with the best preparations of enzyme available to us (G. J. Hart & F. M. Dickinson, unpublished work). However, the important fact here is that the result clearly shows that $Mg^{2+}$ does not substantially alter the number of NADH-binding sites. It may be noted that the increased burst observed in Fig. 5, even in the presence of $Mg^{2+}$, is significantly less than the total number of coenzyme-binding sites measured directly. In several gel-filtration experiments on calibrated columns of Sephadex G-200 we have observed no effect of 5 mM-$Mg^{2+}$ on the elution volume of the enzyme. In all cases the enzyme ran in a position almost identical with that of the marker catalase. As the molecular weight of catalase is 230000 and that of cytoplasmic aldehyde dehydrogenase is approx. 210000 (MacGibbon et al., 1979), there is no evidence for dissociation of the enzyme into subunits in either the presence or the absence of $Mg^{2+}$. We conclude, then, that the most likely explanation for the effects of bivalent metal ions on the enzyme is that a terminal complex containing NADH and metal ion is formed which dissociates products at a much slower rate than any complex in the normal mechanism.

The effects of the metal ions are very striking and particularly potent in buffers such as Tris/HCl. It is clear that for experiments in vitro precautions should be taken to avoid casual contamination of chemicals and glassware. Whether these effects have any physiological significance remains to be seen. However, we may note that the physiological concentration of $Mg^{2+}$ in cytosol is reported to be in the range 0.5–2.5 mM (Endo, 1975; Crompton et al., 1976). Even if only 1% of the $Mg^{2+}$ remained free, there would be marked inhibition of the aldehyde dehydrogenase.

## References

Crompton, M., Capano, M. & Carafoli, E. (1976) Biochem. J. **154**, 735–742

Dalziel, K. (1962) Biochem. J. **84**, 240–244

Dalziel, K. (1963) J. Biol. Chem. **238**, 1538–1543

Dalziel, K. & Dickinson, F. M. (1966) Biochem. Prep. **11**, 84–88

Dickinson, F. M., Hart, G. J. & Kitson, T. M. (1981) Biochem. J. **199**, 573–579

Endo, M. (1975) Proc. Jpn. Acad. **51**, 479–484

Gibson, Q. H. & Milnes, L. (1964) Biochem. J. **91**, 161–171

Hart, G. J. & Dickinson, F. M. (1977) Biochem. J. **163**, 261–267

Hart, G. J. & Dickinson, F. M. (1982) Biochem. J. **203**, 617–627

Horecker, B. L. & Kornberg, A. (1948) J. Biol. Chem. **175**, 385–390

Hummel, J. P. & Dreyer, W. J. (1962) Biochim. Biophys. Acta **63**, 530–532

MacGibbon, A. K. H., Buckley, P. D. & Blackwell, L. F. (1977a) Biochem. J. **165**, 455–462

MacGibbon, A. K. H., Blackwell, L. F. & Buckley, P. D. (1977b) Biochem. J. **167**, 469–477

MacGibbon, A. K. H., Blackwell, L. F. & Buckley, P. D. (1977c) *Eur. J. Biochem.* **77**, 93–100

MacGibbon, A. K. H., Haylock, S. J., Buckley, P. D. & Blackwell, L. F. (1978) *Biochem. J.* **171**, 533–538

MacGibbon, A. K. H., Motion, R. L., Crow, K. E., Buckley, P. D. & Blackwell, L. F. (1979) *Eur. J. Biochem.* **96**, 585–595

Takahashi, K. & Weiner, H. (1980) *J. Biol. Chem.* **255**, 8206–8209

Takahashi, K., Weiner, H. & Hu, J. H. J. (1980) *Arch. Biochem. Biophys.* **205**, 571–578

Taylor, A. W., Frazier, A. W., Gurney, E. L. & Smith, J. P. (1963) *Trans. Faraday Soc.* **59**, 1585–1589

Venteicher, R., Mope, L. & Yonetani, T. (1977) in *Alcohol and Aldehyde Metabolizing Systems* (Thurman, R. G., Williamson, J. R., Drott, H. R. & Chance, B., eds.), vol. 2, pp. 157–166, Academic Press, New York