A Study of the Coenzyme-Binding Characteristics of Rabbit Muscle L-Glycerol 3-Phosphate Dehydrogenase

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1. The binding of NAD^+ and NADH to glycerol 3-phosphate dehydrogenase was studied in the pH range 6.0–9.0 at 25°C and in the temperature range 16–43°C at pH 7.0. 2. The second-order velocity constants for the combination of NADH with the enzyme in the pH range 6.0–9.0 and for the combination of NAD^+ with the enzyme at pH 6.0 were determined. 3. The velocity constant for the dissociation of the enzyme– NAD^+ complex at pH 6.0 was measured.

Detailed information about coenzyme binding is essential in any attempt to elucidate the mechanism of action of NAD^+ -linked dehydrogenases. We decided to obtain information about the detailed mechanism of the reaction brought about by glycerol 3-phosphate dehydrogenase (L-glycerol 3-phosphate oxidoreductase, EC 1.1.1.8) and we have thus required information about the stability of the enzyme–coenzyme complexes and of the rate constants describing the combination and dissociation of NAD^+ and NADH from the enzyme. This information is presented here. The results of detailed initial-rate studies together with discussion of possible mechanisms of catalysis are presented in Bentley & Dickinson (1974). A certain amount of information about coenzyme binding to glycerol 3-phosphate dehydrogenase may be found in the literature (Kim & Anderson, 1969; Reynolds, 1971) but usually for conditions of temperature, pH and reaction-medium composition slightly different from those of our kinetic studies. In these cases we have repeated the determinations under our experimental conditions. In other cases we have provided measurements of quantities not previously determined.

Experimental

Materials

Glass-distilled water was used throughout. EDTA to a final concentration of 0.3 mM was included in all buffers and dialysed enzyme preparations. Phosphate buffers, I 0.1, were used throughout. The buffer at pH 9.0 was made from 20 mM-glycine–NaOH, pH 9.0, with sufficient disodium hydrogen phosphate added to yield the required ionic strength. NADH (grade I) and NAD^+ (grade II) were purchased from Boehr-

ringer Corp. (London) Ltd., London W.5, U.K. NAD^+ was purified by chromatography on DEAE-cellulose (Dalziel, 1963).

Crystalline L-glycerol 3-phosphate dehydrogenase was prepared from rabbit muscle as described by Bentley *et al.* (1973). Enzyme concentrations were determined spectrophotometrically by using $E_{1\text{cm}}^{1\%} = 5.15$ at 280 nm and a subunit molecular weight of 38 000 (Bentley *et al.*, 1973). NADH concentrations were measured spectrophotometrically by using $\epsilon_{340} = 6.22 \times 10^3$ litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ (Horecker & Kornberg, 1948). NAD^+ solutions were assayed enzymically with yeast alcohol dehydrogenase by using the conditions described by Dalziel (1963).

Estimation of the dissociation constant of the enzyme–NADH complex

The dissociation constant ($K_{\text{E}\cdot\text{NADH}}$) for the enzyme–NADH complex ($\text{E}\cdot\text{NADH}$) was estimated from the enhancement of the NADH fluorescence which occurs as NADH binds to glycerol 3-phosphate dehydrogenase. Measurements were performed in a recording filter fluorimeter of similar design to that described by Dalziel (1962) at 25°C in sodium phosphate buffers, over the pH range 6–9 and in sodium phosphate buffer, pH 7.0, over a temperature range from 16° to 43°C. With the filters used (Corning glass numbers 5840 and 3389 and 1 M- NaNO_2 liquid filter) the fluorescence of $\text{E}\cdot\text{NADH}$ is 5.6 times greater than free NADH. The fluorescence of solutions containing known amounts of glycerol 3-phosphate dehydrogenase and NADH was measured and the concentration of $\text{E}\cdot\text{NADH}$ calculated by using eqn. (1). In the derivation of this equation it is assumed that the fluorescence is a linear function of concentration and that the fluorescence of a mixture of $\text{E}\cdot\text{NADH}$ and NADH is equal to the sum of their individual fluorescences. Under the conditions of these experiments

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with low concentrations of NADH these assumptions are justified.

$$[E \cdot NADH] = \frac{\Delta F - f_1 [NADH_i]}{f_2 - f_1} \quad (1)$$

In eqn. (1) ΔF is the fluorescence increase occurring when NADH is added to a glycerol 3-phosphate dehydrogenase solution to a final concentration of $[NADH_i]$, f_1 is the specific fluorescence of free NADH and f_2 the specific fluorescence of $E \cdot NADH$ determined under the same conditions in separate experiments.

Determination of the dissociation constant of the enzyme-NAD⁺ complex

The dissociation constant ($K_{E \cdot NAD}$) of the glycerol 3-phosphate dehydrogenase - NAD⁺ complex ($E \cdot NAD^+$) was measured in experiments similar to those described above for the determination of $K_{E \cdot NADH}$, but with NAD⁺ added to the mixtures to compete with the NADH for the enzyme. Fluorescence measurements were performed in the filter fluorimeter described above at 25°C in sodium phosphate buffers, over a pH range 6-9 and in sodium phosphate buffer, pH 7.0, over a temperature range 16-43°C. Apparent dissociation constants for NADH ($K_{E \cdot NADH(app.)}$) were calculated by using eqn. (1). Values of $K_{E \cdot NADH(app.)}$ were corrected for the small fluorescence quenching effect of high concentrations of NAD⁺, which arose because of the small but significant extinction of purified NAD⁺ solutions at the exciting wavelength. Values for $K_{E \cdot NAD}$ were calculated from values of $K_{E \cdot NADH(app.)}$ by using eqn. (2):

$$K_{E \cdot NADH(app.)} = \left(1 + \frac{[NAD^+]}{K_{E \cdot NAD}}\right) K_{E \cdot NADH} \quad (2)$$

Values of $K_{E \cdot NADH(app.)}$ were measured at different NAD⁺ concentrations at each pH and temperature. Values of $K_{E \cdot NAD}$ were obtained from the slopes ($K_{E \cdot NADH}/K_{E \cdot NAD}$) and intercepts ($K_{E \cdot NADH}$) of plots of $K_{E \cdot NADH(app.)}$ versus NAD⁺ concentration.

Measurement of the velocity constants describing the formation and dissociation of the enzyme-coenzyme complexes

The rates of the reactions of glycerol 3-phosphate dehydrogenase with NAD⁺ and NADH were measured at 25°C in a thermostatically controlled stopped-flow apparatus based on the design of Gibson & Milnes (1964) which could be used to measure either fluorescence changes or extinction changes.

In fluorescence measurements the sample was excited by light of wavelengths in the range 320-400nm obtained from a Bausch and Lomb 150W xenon arc lamp used in conjunction with a Corning

filter glass number 5840. The photomultiplier was screened by a combination of a 1M-NaNO₂ liquid filter and a Corning filter glass number 3389 to remove scattered exciting radiation.

For extinction measurements at 320nm a Bausch and Lomb 50W tungsten halide lamp was used together with a Bausch and Lomb high-intensity grating monochromator. A Corning filter, glass number 9863 was placed at the exit slit to remove stray light.

For all measurements with the stopped-flow instrument changes in light intensity were followed with an E.M.I. 6256B photomultiplier (E.M.I., Hayes, Middx., U.K.) coupled to a Tektronix 546B storage oscilloscope with type 2B67 time-base and type 2A63 differential amplifier plug in units (Tektronix, Guernsey, Channel Islands, U.K.). The Bausch and Lomb optical equipment was obtained through Applied Research Laboratories, Luton, Beds., U.K. and the Corning filters were obtained through Precision Optical Instruments (Fulham) Ltd., London W.6, U.K.

Velocity constants for the reactions of enzyme and coenzymes are defined in Scheme 1 of Bentley & Dickinson (1974). The second-order velocity constants for the reaction between enzyme and NADH (k'_{+1}) were measured in sodium phosphate buffers, over a pH range 6-9 by using the stopped-flow apparatus as a filter fluorimeter. The rate of fluorescence increase was measured when 1 μ M-NADH reacted with 1 μ M-glycerol 3-phosphate dehydrogenase active sites. The concentration of $E \cdot NADH$ in solution at various time-intervals was calculated by using eqn. (1). The specific fluorescences of $E \cdot NADH$ and NADH for these calculations were determined directly in the stopped-flow apparatus.

The velocity constants (k'_{+1} , k_{+1}) were calculated by using eqn. (3) which applies to a second-order reaction with equal initial concentrations of both reactants where there is significant contribution from the first-order opposing reaction (Frost & Pearson, 1953):

$$\log \left[\frac{\overline{ES}(E_0^2 - \overline{ES} \cdot \overline{ES})}{(\overline{ES} - \overline{ES})E_0^2} \right] = k \frac{(E_0^2 - \overline{ES}^2)t}{\overline{ES}} \quad (3)$$

In eqn. (3) $\overline{ES}$ is the concentration of $E \cdot NAD(H)$ at equilibrium, E_0 is the initial concentration of enzyme active sites, ES is the concentration of $E \cdot NAD(H)$ at time t , and k is the second-order velocity constant to be evaluated. The concentration of $E \cdot NAD(H)$ at equilibrium ($\overline{ES}$) was calculated from the end-point of the reaction.

The first-order velocity constant describing the dissociation of the enzyme-NAD⁺ complex (k_{-1}) was measured in sodium phosphate buffer, pH 6.0, at 25°C by using the stopped-flow apparatus as a filter fluorimeter. NADH was mixed with a solution of enzyme and NAD⁺ to yield a mixture with the following initial composition: NADH, 17 μ M; NAD⁺,

100 μM ; glycerol 3-phosphate dehydrogenase, 2 μM . The increase in fluorescence was followed as the NADH reacted with free enzyme sites formed as the NAD^+ dissociated. Under the conditions of the experiment NAD^+ cannot compete effectively with NADH for vacant binding sites. As will be seen, the second-order velocity constant describing the combination of enzyme and NAD^+ at pH 6.0 is some 50 times smaller than that for the combination of enzyme and NADH, and the dissociation constant $K_{\text{E}\cdot\text{NADH}}$ is some 500 times smaller than the dissociation constant $K_{\text{E}\cdot\text{NAD}}$.

Results and Discussion

Fig. 1 shows the values of $K_{\text{E}\cdot\text{NADH}}$ measured fluorimetrically at 25°C over the pH range 6–9; NADH is bound to glycerol 3-phosphate dehydrogenase more firmly at the lower pH values. The error

Fig. 1. Variation of the dissociation constant of the enzyme-NADH complex ($K_{\text{E}\cdot\text{NADH}}$) with pH at 25°C (○)

----, Theoretical curve expected if $K_{\text{E}\cdot\text{NADH}}$ were controlled by a group with a pK of 7.2

Fig. 2. Variation of $K_{\text{E}\cdot\text{NADH}}(\text{app.})$ with the NAD^+ concentration, at 25°C at pH 7.0 (○) and pH 8.0 (●)

For details see the text.

involved in the estimation of $K_{\text{E}\cdot\text{NADH}}$ at the lower pH values was much greater than that at the higher pH values; the estimations at pH 6.0 were only reproducible to within 50%. The values found for $K_{\text{E}\cdot\text{NADH}}$ at pH 7.0 and 9.0 agree with those of Reynolds (1971), but the value of $K_{\text{E}\cdot\text{NADH}} = 0.13 \mu\text{M}$ obtained by Reynolds at pH 6.0 was rather higher than the value of $K_{\text{E}\cdot\text{NADH}} = 0.022 \mu\text{M}$ obtained in the present study. Other values of $K_{\text{E}\cdot\text{NADH}}$ agree with those of the present study, for example, values of $K_{\text{E}\cdot\text{NADH}} = 1.78 \mu\text{M}$ at pH 7.85 (Kim & Anderson, 1969) and $K_{\text{E}\cdot\text{NADH}} = 1.80 \mu\text{M}$ at pH 7.5 (van Eys *et al.*, 1959) are to be compared with respective values of 1.77 μM and 1.45 μM obtained here. The very tight binding at pH 6.0 permits accurate determination of the enzyme active-site concentration by titrating the enzyme with NADH as described by Bentley *et al.* (1973). Fig. 1 also shows the pH profile for the ionization of a compound with a pK at pH 7.2 normalized to the experimental curve at extreme alkaline pH. On the alkaline side of the pK the two curves can virtually be superimposed. However, in the region of the pK and particularly between pH 7.0 and 6.0 the change in the dissociation constant was far greater than was expected on the basis of the theoretical curve. This deviation from the normalized pH curve is outside the limits expected from the errors involved in the estimations of $K_{\text{E}\cdot\text{NADH}}$.

Values of $K_{\text{E}\cdot\text{NAD}}$ were measured fluorimetrically by competition with NADH. The results of typical competition experiments are shown in Fig. 2 in the form of a plot of the apparent dissociation constant for NADH ($K_{\text{E}\cdot\text{NADH}}(\text{app.})$) versus the NAD^+ concentration. The values of $K_{\text{E}\cdot\text{NAD}}$ estimated from experiments of this type over a pH range 6–9 in phosphate buffer are shown in Fig. 3. The values of $K_{\text{E}\cdot\text{NADH}}(\text{app.})$ tended to increase at the higher NADH

Fig. 3. Variation of the dissociation constant of the enzyme- NAD^+ complex ($K_{\text{E}\cdot\text{NAD}}$) with pH at 25°C

At pH 7.0 and 8.0 results of duplicate experiments are shown and these give some idea of the reproducibility of these estimations.

Fig. 4. Variation of $K_{E \cdot NADH}$ (○) and $K_{E \cdot NAD}$ (●) with temperature

Experiments were performed in phosphate buffer, I 0.1, and pH 7.0 at 20°C. The enzyme was quite stable over the temperature range used. For other details see the text.

concentrations and individual measurements were only reproducible to within 30%. The value of $K_{E \cdot NAD}$ estimated at pH 9.0 is of the same order as that given by Reynolds (1971). No other estimations of $K_{E \cdot NAD}$ are available for comparison. The pH profile of $K_{E \cdot NAD}$ appears to be more complex than that of $K_{E \cdot NADH}$. However, the errors involved in the estimations were considerable and the shape of the curve may be artificially distorted.

The effects of temperature between 16° and 43°C on the values of $K_{E \cdot NADH}$ and $K_{E \cdot NAD}$ measured in phosphate buffers, pH 7.0, are shown in Fig. 4 in the form of plots of the logarithm of the dissociation constant versus the reciprocal of absolute temperature. The linear plots obtained show that, within the limits of experimental error, the enthalpies of reaction, ΔH , do not vary with temperature over the range studied. For the dissociation of the $E \cdot NAD^+$ and $E \cdot NADH$ complexes the enthalpies of reaction, from Fig. 4, are 50.8 and 35.5 kJ·mol⁻¹ respectively.

Figs. 5(a) and 5(c) show oscilloscope traces of the reaction between glycerol 3-phosphate dehydrogenase and NADH at pH 6.0 and 9.0 in phosphate buffer. The analyses of the results by using eqns. (1) and (3) are shown in Figs. 5(b) and 5(d). The values of the 'NADH on' velocity constant (k'_{+1}) estimated from such experiments over the pH range 6–9 are shown in Table 1. The values of the 'NADH-off' velocity constant (k'_{-1}) calculated from $K_{E \cdot NADH}$ and k'_{+1} are also shown. It may be noted here, that except for the estimate of k'_{+1} at pH 9.0, values of k'_{+1} and k'_{-1} are in reasonable agreement with those given by

Reynolds (1971). At pH 9.0 Reynolds (1971) obtained a value of $k'_{+1} = 4 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$. In his work estimates of k'_{-1} were obtained by displacement of bound NADH by NAD^+ and not by calculation as here. Estimation of the 'NADH-on' velocity constant was much more difficult at the higher pH values because the dissociation constant ($K_{E \cdot NADH}$) is much less favourable (Fig. 1) and the reaction rate appears to increase with pH. It is evident from Figs. 5(a) and 5(c) that much smaller signals and much higher noise values were encountered at pH 9.0. The experimental error in the values of k'_{+1} estimated at pH 8.0 and 9.0 could be as high as 100% and it is much higher than at pH 6.0 and 7.0. It seems, however, both from the present work and that of Reynolds (1971), that both k'_{+1} and k'_{-1} increase as pH is increased over the range studied. It is noteworthy that the very large and unexpected changes in $K_{E \cdot NADH}$ between pH 6.0 and 7.0 are almost entirely due to changes in k'_{-1} since k'_{+1} is essentially constant over this range.

An oscilloscope trace showing the increase in fluorescence which occurred when a solution containing glycerol 3-phosphate dehydrogenase and NAD^+ was mixed with NADH at pH 6.0 is shown in Fig. 6(a). Under these conditions of excess of NADH, the rate of this fluorescence increase was determined by the rate at which NAD^+ dissociated from the $E \cdot \text{NAD}^+$ complex. The results were analysed as for a first-order reaction (Fig. 6b) and a value of 3.9 s^{-1} was obtained for the 'NAD⁺-off' velocity constant (k_{-1}). The value of the 'NAD⁺-on' velocity constant at pH 6.0 estimated from $K_{E \cdot NAD}$ and k_{-1} was $4.2 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$.

When glycerol 3-phosphate dehydrogenase is mixed with NAD^+ a new absorption band appears in the region 300–400 nm, with a maximum absorbance at 320 nm. This effect was first drawn to our attention by Dr. J. J. Holbrook (personal communication). The magnitude of the extinction increase was unaffected by treatment of the enzyme with charcoal and increased as the enzyme concentration was increased at a fixed NAD^+ concentration. The increase in extinction could be a consequence of the reaction between enzyme and NAD^+ or caused by the reaction between NAD^+ and some other compound on the enzyme to form NADH. These two possibilities may be distinguished by the pH-dependence of the effect. The dissociation constant $K_{E \cdot NAD^+}$ is much smaller at pH 6.0 than at pH 9.0 as discussed above. The production of NADH proceeds with proton release, thus the equilibrium constant may be expected to be more favourable at high pH values than at low pH. The fact that the extinction increase, ΔE_{320} , observed at pH 6.0 was greater than that observed at pH 9.0 with the same concentrations of enzyme and NAD^+ suggests that the extinction increase is caused by the formation of an $E \cdot \text{NAD}^+$ complex. This supposition is supported by the lack of fluorescence of the reaction

Fig. 5. Reaction of NADH with glycerol 3-phosphate dehydrogenase at 25°C

a) Increase in fluorescence on mixing 1 μM -NADH and 1 μM enzyme active sites at pH 6.0. Oscilloscope sensitivity 0.5V/division with a scan speed 5ms/division. The horizontal trace represents the fully reacted mixture. (b) Analysis of results of Fig. 5(a) by using eqns. (1) and (3). (c) Increase in fluorescence on mixing 1 μM -NADH and 1 μM enzyme at pH 9.0. Oscilloscope sensitivity was 50mV/division with a scan speed of 2ms/division. (d) Analysis of results of Fig. 5(c) by using eqns. (1) and (3). Under the conditions of the experiments in Figs. 5(a) and 5(c) the signal produced by 1 μM -NADH was 0.96 V and that produced by 1 μM -E·NADH was 5.45V.

Table 1. Estimated values for velocity constants for the formation and dissociation of enzyme-coenzyme complexes
The velocity constants are those specified in the reactions:

k'_{+1} and k_{-1} were measured directly. Values of k'_{-1} were calculated from k'_{+1} and $K_{\text{E} \cdot \text{NADH}}$, and k_{+1} was calculated from k_{-1} and $K_{\text{E} \cdot \text{NAD}^+}$.

pH	$10^{-7} \times k'_{+1}$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	k'_{-1} (s^{-1})	$10^{-5} \times k_{+1}$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	k_{-1} (s^{-1})
6.0	2.5	0.5	4.2	3.9
7.0	2.6	15.4		
8.0	5.2	95		
9.0	9.4	192		

mixture in the region expected for NADH. Fig. 7 shows a plot of $1/\Delta E_{320}$ versus the reciprocal of enzyme concentration at a constant NAD⁺ concentration in phosphate buffer, pH 6.0. The intercept of the plot shows the ΔE_{320} which results from the particular NAD⁺ concentration at infinite enzyme concentration. Under these conditions all the NAD⁺ would be enzyme bound and the molar extinction coefficient of the E·NAD⁺ complex at the wavelength of maximum absorption may be calculated. The value obtained was $\epsilon_{320} = 1.42 \times 10^3$ litre·mol⁻¹·cm⁻¹ ($\epsilon_{340} = 1.3 \times 10^3$ litre·mol⁻¹·cm⁻¹).

Fig. 8(a) shows an oscilloscope trace of the increase in extinction at 320nm which occurred when glycerol 3-phosphate dehydrogenase and NAD⁺ were mixed in the stopped-flow apparatus at 25°C in phosphate buffer, pH 6.0. The results are analysed in Fig. 8(b) by using the extinction coefficient obtained above and eqn. (3). A value of $k_{+1} = 5.6 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$

Fig. 6. Determination of the 'NAD⁺-off' velocity constant at 25°C and pH 6.0

(a) Increase in fluorescence when a solution containing glycerol 3-phosphate dehydrogenase and NAD⁺ were mixed with NADH. The concentrations of reactants at the time of mixing were: NAD⁺, 100 μM; NADH, 17 μM; enzyme active sites, 2 μM. The oscilloscope sensitivity was 0.1 V/division with a scan speed of 50 ms/division. The signal produced by 1 μM-NADH was 0.086 V and that produced by 1 μM-E·NADH was 0.45 V. (b) Analysis of the results in Fig. 6(a) by using a first-order plot. $[E \cdot NAD^+]_0$ is the initial concentration of the enzyme-NAD⁺ complex and $[E \cdot NAD^+]_t$ the concentration at time t .

was obtained for the velocity constant of the reaction. This value agreed fairly well with the calculated value for k_{+1} (Table 1) and this indicates that the reaction causing the increase in extinction at 320 nm is fast enough to be involved in catalysis, and that the intermediate formed may be of catalytic significance. The value of the dissociation constant $K_{E \cdot NAD}$ estimated from the end-point of the reaction (Fig. 8a) was 9.9 μM which agrees very well with that measured fluorimetrically; the value estimated from the ratio of the directly measured values of k_{-1} and k_{+1} was 7 μM.

The studies described here have provided values for certain velocity constants and dissociation constants for the reactions of glycerol 3-phosphate dehydrogenase with NAD⁺ and NADH. These data provide

Fig. 7. Spectrophotometric titration of NAD⁺ with glycerol 3-phosphate dehydrogenase

Initially the extinction change at 320 nm was observed on mixing 193 μM enzyme active sites and 93 μM-NAD⁺ at pH 6.0 and 25°C in 1 cm-light-path cells. The extinction changes after dilution of the mixture with portions of a solution of 93 μM-NAD⁺ were then noted. A tandem cell arrangement was used for this experiment.

Fig. 8. Reaction of NAD⁺ with glycerol 3-phosphate dehydrogenase at 25°C and pH 6.0

(a) Change in transmittance at 320 nm after the mixing of 50 μM-NAD⁺ with 50 μM enzyme active sites. The experiment was performed with a 2 cm observation tube and a scan speed of 5 ms/division. The horizontal trace represents the transmittance of fully reacted mixture. (b) Analysis of the results in Fig. 8(a) by using eqn. (3).

a valuable starting point in attempting to interpret the results of initial-rate studies (Bentley & Dickinson, 1974) with a view to describing the overall mechanism of catalysis.

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References

- Bentley, P. & Dickinson, F. M. (1974) *Biochem. J.* **143**, 19–27
- Bentley, P., Dickinson, F. M. & Jones, I. G. (1973) *Biochem. J.* **135**, 853–859
- Dalziel, K. (1962) *Biochem. J.* **84**, 244–254
- Dalziel, K. (1963) *J. Biol. Chem.* **238**, 1538–1543
- Frost, A. A. & Pearson, R. G. (1953) *Kinetics and Mechanisms*, p. 174, John Wiley and Sons, New York
- Gibson, Q. H. & Milnes, L. (1964) *Biochem. J.* **91**, 161–171
- Horecker, B. L. & Kornberg, A. (1948) *J. Biol. Chem.* **175**, 385–390
- Kim, S. J. & Anderson, B. M. (1969) *J. Biol. Chem.* **244**, 1547–1551
- Reynolds, S. J. (1971) Ph.D. Thesis, University of Bristol
- van Eys, J., Nuenke, B. J. & Patterson, M. K. (1959) *J. Biol. Chem.* **234**, 2308–2313