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A Steady-State-Kinetic Model for Formaldehyde Dehydrogenase from Human Liver

A MECHANISM INVOLVING NAD$^+$ AND THE HEMIMERCAPTAL ADDUCT OF GLUTATHIONE AND FORMALDEHYDE AS SUBSTRATES AND FREE GLUTATHIONE AS AN ALLOSTERIC ACTIVATOR OF THE ENZYME

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The steady-state kinetics of formaldehyde dehydrogenase from human liver have been explored. Non-linearities were obtained in v-versus-v/[S] plots. It was necessary and sufficient to consider two reactants of the equilibrium mixture of formaldehyde, glutathione and their hemimercaptal adduct for a complete description of the kinetics. A random sequential reaction scheme is proposed in which adduct and β-NAD$^+$ are the substrates. In addition, glutathione can bind to an allosteric regulatory site and only the glutathione-containing enzyme is considered productive. Various alternative reaction models were examined but no simple alternative was superior to the model chosen. The discrimination was largely based on results of non-linear regression analysis. Several *S*-substituted glutathione derivatives were tested as activators or inhibitors of the enzyme, but all were without effect. Thio-NAD$^+$, nicotinamide–hypoxanthine dinucleotide and 3-acetylpyridine–adenine dinucleotide could substitute for β-NAD$^+$ as the nucleotide substrate. α-NAD$^+$ and ADP-ribose were competitive inhibitors with respect to β-NAD$^+$ and non-competitive with glutathione and the adduct. When used simultaneously, the inhibitors were linear competitive versus each other, indicating a single nucleotide-binding site or, if more than one, non-co-operative binding sites.

Formaldehyde dehydrogenase is an NAD-linked dehydrogenase found in animal tissues, bacteria and yeast (Strittmatter & Ball, 1955; Rose & Racker, 1962; Uotila & Koivusalo, 1974). The enzyme catalyses the oxidation of formaldehyde only in the presence of reduced glutathione (GSH). Recently the enzyme from human liver was thoroughly purified and characterized. It was shown that the enzyme catalyses a reversible reaction in which formaldehyde, GSH and NAD$^+$ are converted into *S*-formylglutathione and NADH (Uotila & Koivusalo, 1974).

Formaldehyde and GSH react rapidly non-enzymically to form an adduct, which is probably the hemimercaptal, *S*-hydroxymethylglutathione (Wald *et al.*, 1953). Solutions of formaldehyde and GSH thus always contain three species, the adduct (A), free formaldehyde (F) and free glutathione (G), whose concentrations at equilibrium are related to each other as described by eqn. (1):

$$F + G \rightleftharpoons A; \quad \frac{[F][G]}{[A]} = K_d \qquad (1)$$

where K_d is the dissociation constant. The adduct has been postulated to be the true substrate of formaldehyde dehydrogenase (Strittmatter & Ball, 1955), but no convincing experimental evidence has been reported to verify this assumption.

The present work has been carried out to investigate by steady-state kinetic studies which of the species present in the solution of formaldehyde and GSH are true reactants in the reaction catalysed by human liver formaldehyde dehydrogenase. The effects of NAD$^+$ analogues as substrates or inhibitors of the enzyme have also been studied.

Materials and Methods

β-NAD$^+$, β-NADH, GSH, ADP, ADP-ribose and *S*-methylglutathione were obtained from Sigma, St. Louis, MO, U.S.A. α-NAD$^+$ was a product of Boehringer, Mannheim, Germany, further purified in the laboratory of Dr. Carl-Ivar Brändén, Department of Chemistry, Agricultural College, Uppsala, essentially by the method of Pfleiderer *et al.* (1965). Thio-NAD$^+$, nicotinamide–hypoxanthine dinucleotide, 3-acetylpyridine–adenine dinucleotide, nicotinamide mononucleotide and 3′:5′-cyclic AMP were

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products of P–L Biochemicals, Milwaukee, WI, U.S.A. Formaldehyde (Uotila & Koivusalo, 1974) and *S*-formylglutathione (Uotila, 1973) were prepared and standardized as previously described. The solutions of the pyridine nucleotide derivatives were standardized by measuring the absorbance of fresh solutions at 260nm. The molar absorbance values at this wavelength reported for β-NAD^+ and its derivatives (P–L Biochemicals, 1961) and α-NAD^+ (Pfleiderer *et al.*, 1965) were used in calculations. Glutathione solutions were prepared immediately before use and were standardized by the method of Ellman (1959). Formaldehyde dehydrogenase was purified from human liver by the method of Uotila & Koivusalo (1974). Some experiments were carried out with enzyme obtained by a modified method, which involved affinity chromatography on NAD^+–Sepharose (L. Uotila & M. Koivusalo, unpublished work).

Determination of initial velocity

The assay mixture for the forward reaction catalysed by formaldehyde dehydrogenase contained 100mM-sodium phosphate buffer, pH8.0, various amounts of β-NAD^+, GSH and formaldehyde, enzyme and water to 1.0ml. The reaction was started with enzyme after 5min preincubation of the other reaction components. The preincubation was done to allow equilibration to occur in the non-enzymic reaction of formaldehyde and GSH. The temperature used in the preincubation and activity measurement was 30°C. The rate of the reaction was then measured at 340nm on an Aminco DW-2 UV/VIS double-beam spectrophotometer. The highest sensitivity used was 0.01 absorbance unit for full-scale deflexion on the recorder. The progress curves were approximately linear for at least 20s at the lowest substrate concentrations and for several minutes for the highest concentrations used. The reproducibility of the determinations of initial rates was measured; the coefficient of variation was 1.7% at the highest and <10% at the lowest velocity values.

When substrate analogues of β-NAD^+ were studied the wavelength corresponding to the absorption maximum of the reduced form of each analogue was used for determination of reaction velocity. Calculations were based on the following molar absorbance values (cm^{-1}): β-NADH, 6220 (340nm); thio-NADH, 11300 (395nm); reduced 3-acetylpyridine–adenine dinucleotide, 9100 (365nm) and reduced nicotinamide–hypoxanthine dinucleotide, 6200 (338 nm) (P–L Biochemicals, 1961).

Regression analysis of the kinetic data

The reaction velocities at different substrate, coenzyme and effector concentrations were calculated from the initial slopes of the recorded curves and then fitted to alternative rate equations by a Gauss–Newton non-linear regression program (BMDP3R, University of California, Los Angeles, CA, U.S.A.). The details of the methods used in the comparison of alternative mathematical models fitted to the experimental data have been described (Bartfai & Mannervik, 1972; Mannervik & Bartfai, 1973). Regression of a good model on the data is expected to yield normally distributed residuals, which lack correlation (Askelöf *et al.*, 1976), low standard errors of the parameters, and a small residual sum of squares. The effect of experimental error on the regression was also considered (cf. Askelöf *et al.*, 1976), but under the conditions investigated in the present study no significant dependence of the experimental error on velocity was found. Therefore each velocity value was assigned the same weight in the regression analysis.

Data sets from separate experiments carried out under identical conditions, but on different days, were sometimes combined in the regression analysis. In this way a data set in which, e.g., the concentration of free glutathione was constant could be analysed together with an experiment in which the adduct concentration was constant. A similar combination of blocks of data has been used by Hurst *et al.* (1973).

In the present study the input contained measured velocities, total concentrations of formaldehyde, GSH, β-NAD^+ and possible effector(s). The calculation of the concentrations of free formaldehyde, free GSH and the adduct at equilibrium (eqn. 1) was performed by the computer. A value of 1.5mM was used for the dissociation constant (K_d) of the adduct of formaldehyde and GSH (Uotila & Koivusalo, 1974).

The rate equations for the different reaction schemes have been derived in coefficient form by the structural rule (King & Altman, 1956; Wong & Hanes, 1962) to evaluate which terms would be expected in the rate law and to examine the effect of saturation with a particular reactant (Wong, 1975). The equations given in this paper are simplified to the extent that only variables for a particular experiment are given explicitly, whereas the non-variable reactants are considered as coefficients. Furthermore, the coefficients are not written out and, for example, the classical eqn. (2) for a sequential mechanism (cf. Cleland, 1970):

$$v = \frac{V[A][B]}{K_m^A K_m^B + K_m^B[A] + K_m^A[B] + [A][B]} \quad (2)$$

is accordingly written:

$$v = \frac{AB}{K + A + B + AB} \quad (3)$$

where K denotes a constant term and A is the concentration of A multiplied by a coefficient etc.

Results

Effect on velocity of formaldehyde, glutathione and their adduct

Several combinations of concentrations of free glutathione, free formaldehyde and the adduct were investigated at a fixed concentration of β-NAD^+. Fig. 1 shows an Eadie–Augustinsson–Hofstee plot (Mannervik, 1975*a*) of an experiment in which the concentration of β-NAD^+ was high (1 mM, which can be regarded as saturating; cf. Table 1). The glutathione concentration was kept at several constant values, whereas the adduct concentration was varied. According to eqn. (1) the concentration of formaldehyde varies proportionally to the concentration of adduct under these conditions. The corresponding graph of the same data in which v is plotted versus $v/[G]$ was similar to Fig. 1.

Fig. 2 presents an experiment similar to that shown in Fig. 1, except that the fixed concentration of β-NAD^+ was low (5 μM). The non-linearities become more marked when β-NAD^+ is non-saturating. Both experiments show that the rate of the reaction is increased by increasing either adduct or glutathione concentration (except with the highest concentrations; see Fig. 2). Thus, if the adduct is regarded as the true substrate, glutathione is an activator of the enzyme. An experiment was also carried out in which the adduct was varied at different constant low concentrations of formaldehyde and at a fixed low β-NAD^+ concentration. The curves of the Eadie–Augustinsson–Hofstee plot were non-linear, resembling those of Fig. 1, and the rate was apparently increased by increasing either adduct or formaldehyde concentration. Glutathione was used at high concentrations in this experiment and appears to be an inhibitor under these conditions. In the corresponding experiment with saturating β-NAD^+, the result was similar, except that the curves of the plot did not differ significantly from linearity.

Effect on velocity of β-NAD^+ and the additional substrate(s)

At a fixed high, probably near-physiological, con-

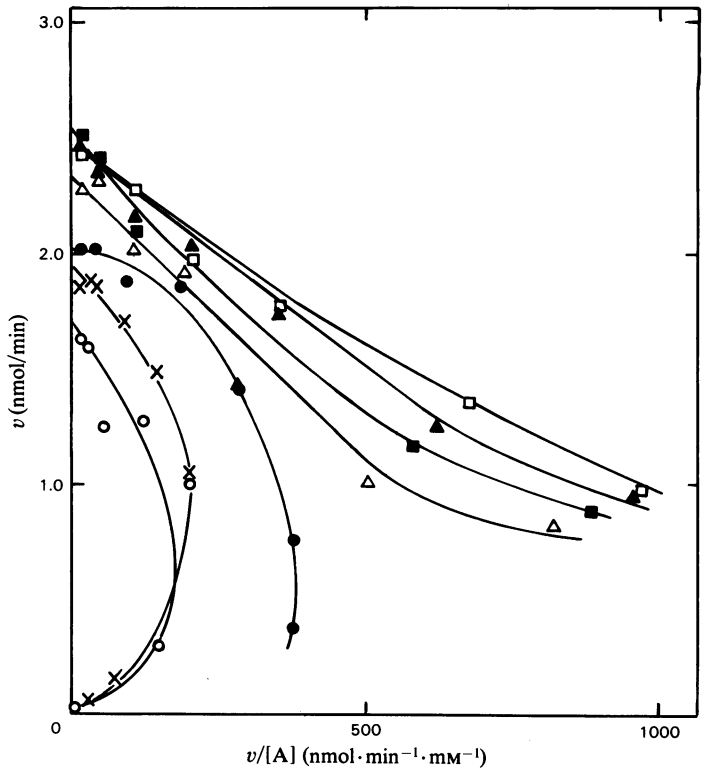


Fig. 1. *Effect on initial velocity of formaldehyde dehydrogenase of various adduct (A) concentrations (from 1 to 100 μM) at different fixed concentrations of free glutathione*

Free glutathione concentrations (μM): ○, 1; ×, 2; ●, 5; △, 10; ■, 20; ▲, 50; □, 100. The concentration of β-NAD^+ was 1.0 mM.

centration of glutathione (5mM) almost linear v versus $v/[\beta\text{-NAD}^+]$ plots were obtained for various constant concentrations of the adduct (Fig. 3*a*). The corresponding plot for variable adduct concentration at constant β-NAD^+ concentrations did not give straight lines (Fig. 3*b*). When a low fixed glutathione concentration was used, non-linearities were obtained in both plots. Fig. 4 shows the v versus $v/[\beta\text{-NAD}^+]$ diagram for 5μM-glutathione.

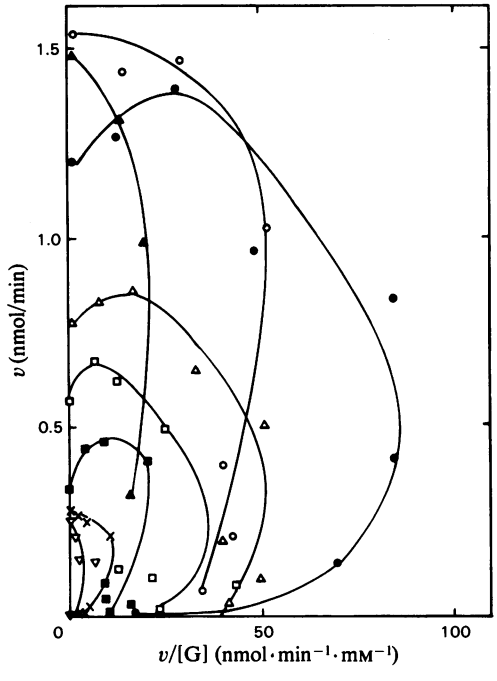


Fig. 2. *Effect on initial velocity of various glutathione (G) concentrations (from 1 to 1000μM) at different fixed concentrations of adduct*

Adduct concentrations (μM): ▽, 1; ×, 2; ■, 5; □, 10; △, 20; ●, 50; ○, 100; ▲, 1000. The concentration of β-NAD^+ was 5.0μM.

Another series of experiments was made in which the formaldehyde concentration was kept constant. Under this condition the glutathione and adduct concentrations are directly proportional (eqn. 1). At 5μM-formaldehyde apparent inhibition was obtained with excess of adduct or glutathione (Fig. 5). As the glutathione concentration is 300 times that of the adduct this effect is probably due to inhibition by excess of glutathione, as indicated previously. The corresponding v versus $v/[\beta\text{-NAD}^+]$ plot was non-linear. At 1.5mM-formaldehyde (resulting in equal concentrations of glutathione and adduct) no inhibition was detected by excess of adduct or glutathione (Fig. 6) and the v versus $v/[\beta\text{-NAD}^+]$ graphs were more linear than those of the experiment described in Fig. 5.

Effects of substrate analogues on the velocity

Several *S*-substituted glutathione derivatives (i.e. *S*-methylglutathione and corresponding higher homologues) were tested as inhibitors or activators of the enzyme but none of them showed any demonstrable effect.

Some analogues of the nucleotide substrate (β-NAD^+) were effective as alternative substrates for formaldehyde dehydrogenase (Table 1), whereas α-NAD^+ and nicotinamide mononucleotide could not serve as substrates in the enzymatic reaction. No significant deviation from linearity was obtained in Eadie–Augustinsson–Hofstee plots in a 100-fold concentration range of the active nucleotide substrates. It is clear from Table 1 that 3-acetylpyridine–adenine dinucleotide is the most efficient nucleotide substrate both on the basis of maximum velocity and on V/K_m. However, the natural substrate, β-NAD^+, has the lowest K_m value and is better than thio-NAD^+ and nicotinamide–hypoxanthine dinucleotide on the basis of the V/K_m ratio.

α-NAD^+ and ADP-ribose were inhibitors of formaldehyde dehydrogenase. ADP, nicotinamide mononucleotide and 3′:5′-cyclic AMP were without inhibitory effect. Both α-NAD^+ and ADP-ribose were competitive with the substrate β-NAD^+ and the inhibition appeared to be linear with respect to inhibi-

Table 1. *Alternative nucleotide substrates for formaldehyde dehydrogenase*

The analysis was carried out with non-varied concentrations of formaldehyde, glutathione and adduct. The total concentrations of formaldehyde and glutathione were 2.0 and 1.0mM respectively. The V values correspond to about 10pmol of pure enzyme in the assay system.

Substrate	K_m (μM)	V (nmol/min)	Relative maximal velocity
β-NAD^+	4.88±0.53	3.96 ±0.09	(1.00)
Thio-NAD^+	30.1 ±2.8	17.4 ±0.6	4.39
3-Acetylpyridine–adenine dinucleotide	50.9 ±4.8	90.6 ±2.2	22.9
Nicotinamide–hypoxanthine dinucleotide	35.2 ±1.9	6.80±0.09	1.72

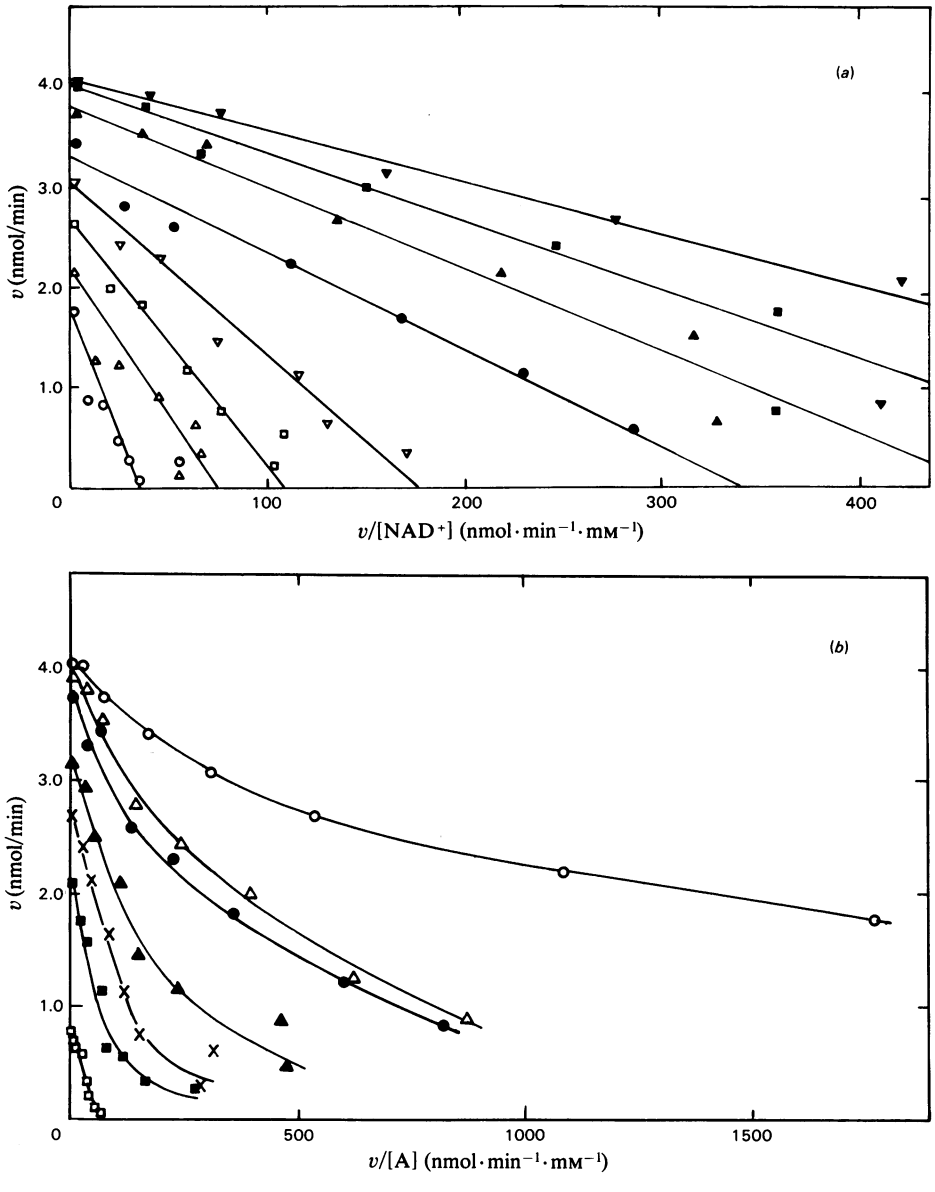


Fig. 3. *Effect on initial velocity of (a) various β-NAD⁺ concentrations at different fixed concentrations of adduct (A) and (b) various concentrations of adduct at different fixed β-NAD⁺ concentrations*

(*a*) Adduct concentrations (μM): ○, 1; △, 2; □, 5; ▽, 10; ●, 20; ▲, 50; ■, 100; ▼, 1000. (*b*) β-NAD⁺ concentrations (μM): □, 2; ■, 5; ×, 10; ▲, 20; ●, 50; △, 100; ○, 1000. The concentration of glutathione was 5.0mM.

tor concentration for each of the inhibitors. Thus parallel lines were obtained in a Dixon plot of a double-inhibition experiment involving α-NAD⁺ and ADP-ribose, indicating exclusive binding of the inhibitors (cf. Yonetani & Theorell, 1964). Considering the close structural analogy between α-NAD⁺ and β-NAD⁺, the linear inhibition obtained suggests that co-operativity does not have to be taken into account in the interpretation of the non-linearities obtained with varying β-NAD⁺ (cf. Fig. 4). The results are also consistent with the assumption of a single nucleotide-binding site on the enzyme, or

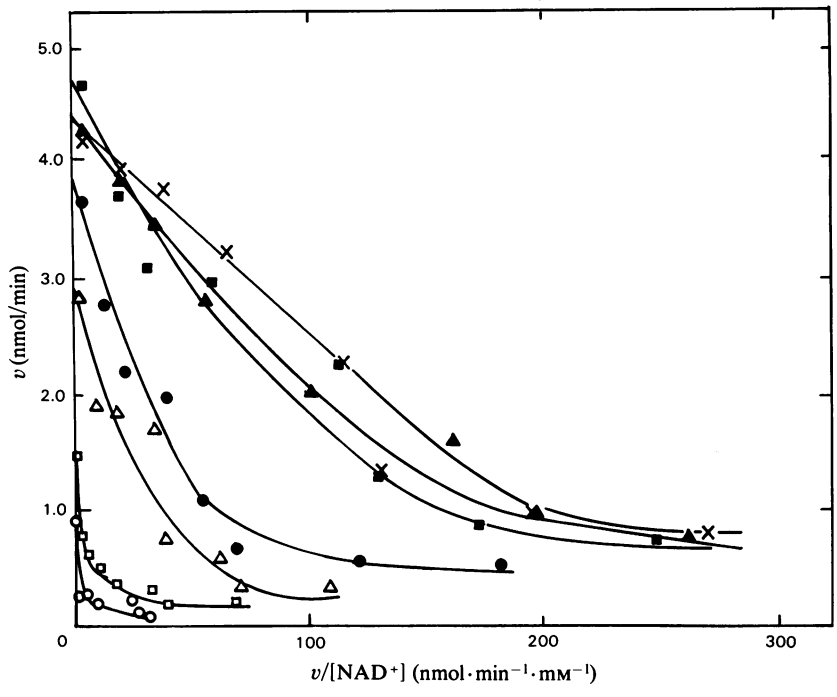


Fig. 4. *Effect on initial velocity of various β-NAD⁺ concentrations at different fixed concentrations of adduct* Adduct concentrations (μM): ○, 1; □, 2; △, 5; ●, 10; ■, 20; ×, 50; ▲, 100. The concentration of glutathione was 5 μM.

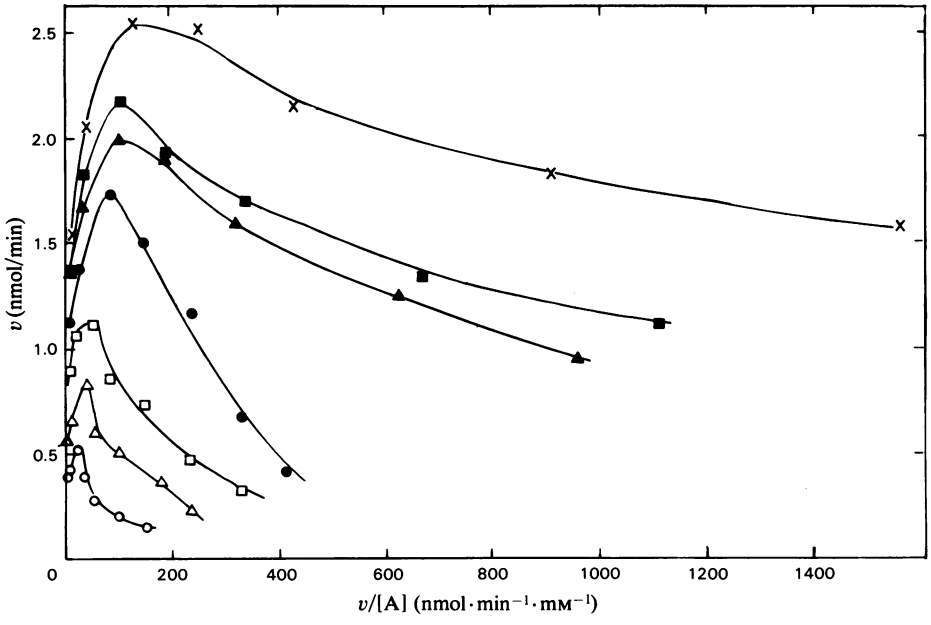


Fig. 5. *Effect on initial velocity of various concentrations of adduct (A) at different fixed concentrations of β-NAD⁺* β-NAD⁺ concentrations (μM): ○, 2; △, 5; □, 10; ●, 20; ▲, 50; ■, 100; ×, 1000. The concentration of formaldehyde was 5 μM.

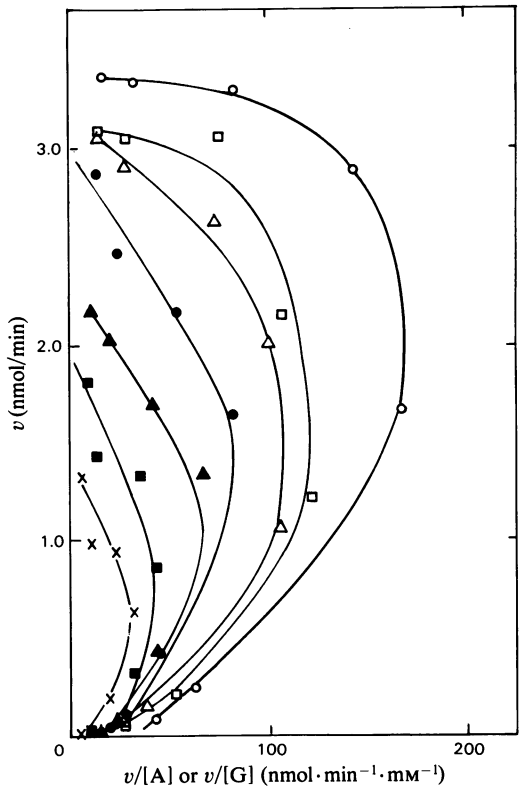


Fig. 6. *Effect on initial velocity of various concentrations of adduct (A) or glutathione (G) at different fixed concentrations of β-NAD⁺*

β-NAD⁺ concentrations (μM): ×, 2; ■, 5; ▲, 10; ●, 20; △, 50; □, 100; ○, 1000. The formaldehyde concentration was 1.5mM, a condition making the concentrations of glutathione and the adduct equal, according to eqn. (1).

independent sites if the enzyme is composed of more than one catalytically active subunit.

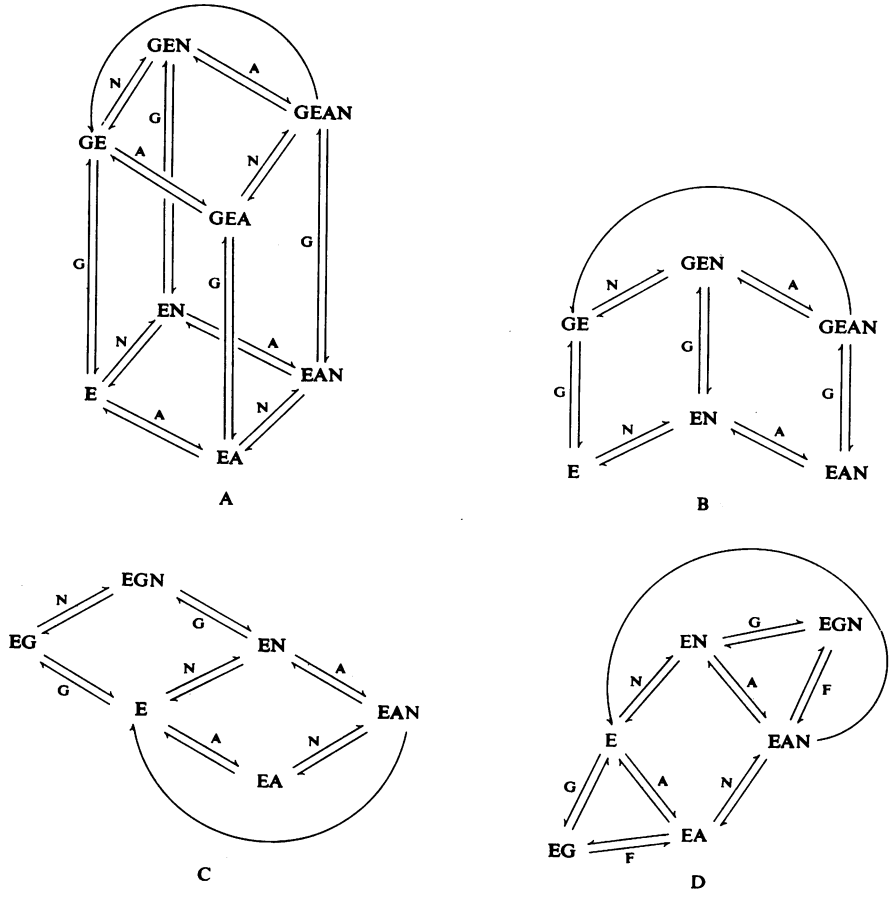
Examination of alternative reaction schemes

Our experiments clearly show that formaldehyde dehydrogenase has some kind of sequential rather than a ping-pong mechanism (cf. Cleland, 1970). Figs. 3 and 5 show kinetic patterns which seem compatible with a random sequential mechanism involving β-NAD⁺ and the adduct of formaldehyde and glutathione as the substrates for the enzyme. However, Figs. 1 and 2, which display data obtained with constant β-NAD⁺ concentration, show clearly that for a given adduct concentration v is dependent also on the concentration of glutathione and/or formaldehyde. Owing to the equilibrium relationship between formaldehyde, glutathione and their adduct (eqn. 1), it is impossible to ascribe the kinetic effects to the individual reactants separately, but two of the three species at equilibrium are necessary and sufficient to explain the kinetics.

The most likely explanation appears to be that the adduct is the true substrate and glutathione is a modifier. In this case formaldehyde does not need to have any effect on the enzyme. According to this interpretation, Figs. 1 and 2 demonstrate an activating effect of low and moderate concentrations of glutathione, whereas experiments in which the glutathione concentration is high suggest inhibition by glutathione.

The enzyme is a dimer (Uotila & Koivusalo, 1974), but no evidence for co-operativity between the subunits has been found, and we have assumed that if more than one active site per enzyme molecule exist they act independently in the catalysis. This assumption is supported by the straight lines obtained in the double-inhibition experiment with α-NAD⁺ and ADP-ribose.

Thus four reaction schemes will be considered (Scheme 1), which might fulfil the requirements imposed by the experimental data and the above assumptions. All of them are simple from the structural point of view, involving only a minimal number of binding sites for reactants on the enzyme. In Scheme 1 (A–D) enzyme, β-NAD⁺, glutathione, formaldehyde and the adduct are denoted by E, N, G, F and A respectively; a curved arrow symbolizes the product-releasing steps. Scheme A is based on the assumption that the enzyme exists in a glutathione-free and in a glutathione-containing form, of which only the latter is catalytically active. Both forms are assumed to bind the substrates, adduct and β-NAD⁺ in random order, but only the quaternary GEAN complex will release product. Scheme B is a degenerate form of scheme A, which lacks the EA and GEA complexes. The substrates are consequently added in an ordered sequence, β-NAD⁺ being the first substrate bound. Schemes A and B both require an allosteric binding site for the essential modifier glutathione. In addition, the catalytically active site has one binding site for adduct and one for β-NAD⁺. Schemes C and D involve only the latter two binding sites. In all schemes glutathione is assumed to be capable of binding to the adduct site, but in schemes A and B only at high concentrations. (This is the proposed explanation of inhibition observed at very high glutathione concentrations.) Scheme C is a simple random mechanism extended with a branch involving enzyme forms EG and EGN, in which glutathione is bound to the adduct site. In scheme D the simple random mechanism is extended by assuming that adduct can be bound not only in the preformed state to the enzyme, but that it can also be formed in the active centre by sequential addition of glutathione and formaldehyde. A similar scheme with glutathione and methylglyoxal as the reactants has previously been



Scheme 1. *Four altenative reaction schemes*

E, Enzyme; N, β-NAD^+; G, glutathione; F, formaldehyde; A, adduct. The individual schemes A–D are discussed in the text.

considered for glyoxalase I (Mannervik *et al.*, 1973, 1974).

In this analysis, scheme C was excluded by the following reasoning. At high concentrations of β-NAD^+ (N) the dependence of velocity on adduct (A) and glutathione (G) would be

$$v = \frac{A}{K+A+G}$$

showing linear competitive inhibition of G versus A. This is in conflict with Figs. 1 and 2, which show activation by G. Furthermore, high G (corresponding to Fig. 3) would give more or less complete inhibition of the enzyme, which was not obtained. Finally, our experiments indicate an obligatory requirement of G for catalytic activity and this cannot be explained by scheme C.

The last argument is also applicable for rejection of scheme D. The latter scheme would yield the rate law

$$v = \frac{A+AF}{K+A+AF+F+G}$$

at saturation with N, but this mathematical model was inferior to other models tested by regression analysis. Likewise, saturation with A or G in scheme D would give respectively

$$v = \frac{N+NF+NF^2}{K+N+NF+NF^2+F+F^2}$$

and

$$v = \frac{NF}{K+N+F+NF}$$

Neither of these equations was acceptable by statistical criteria when compared with alternative models after regression analysis. Consequently, scheme D could also be discarded.

Scheme B could explain some of the data but was inferior to scheme A, e.g. for saturation with G (Fig. 3), a condition giving for scheme B

$$v = \frac{AN}{K+A+N+AN}$$

which predicts straight lines in both Fig. 3(*a*) and Fig. 3(*b*). Scheme A would give

$$v = \frac{AN+A^2N+AN^2}{K+A+N+AN+A^2+A^2N+AN^2+N^2}$$

which may or may not yield curvature in the Eadie–Augustinsson–Hofstee plot (cf. Fig. 3). This model was the best found to describe the conditions corresponding to Fig. 3. A comparison of the alternative models based on the results of regression analysis is made in Table 2. It is clear that reaction scheme A is the best alternative. The residuals after regression were for scheme A approximately normally distributed, as revealed by a normal probability or probit plot (cf. Askelöf *et al.*, 1976). However, the coefficients of two N^2-containing terms in the denominator of the rate equation were not well defined, as expressed by high standard deviations. This is a reflection of the lack of detectable curvature of Fig. 3(*a*) and the complexity of the rate law.

The conditions of the experiment displayed in Fig. 3, in which the free glutathione concentration is 5mM, probably are close to conditions *in vivo*. Therefore, numerical values of constants in a rate equation are of interest for descriptive purposes. The values obtained with these data and the equation

$$v = \frac{V[A][N]}{K+K_m^N[A]+K_m^A[N]+[A][N]}$$

are $V = 300 \pm 5\,\mu$mol/min per μmol of enzyme, $K = 0.00018 \pm 0.00002$ (mM)^2, $K_m^A = 0.0019 \pm 0.0002$ mM, $K_m^N = 0.0047 \pm 0.0005$ mM (where K_m^A and K_m^N are the Michaelis constants for the hemimercaptal adduct and β-NAD^+ respectively at 5mM free glutathione). However, the fit was not unbiased and velocities predicted on the basis of the above equation will be too low in the range of low adduct concentrations. The Michaelis constants are of the same order of magnitude as found previously in less-extensive experiments at 2mM-glutathione concentration (Uotila & Koivusalo, 1974).

Regression analysis of experiments in which both adduct and glutathione concentrations varied (cf. Figs. 1 and 2) showed that all numerator terms of the rate equation must contain the glutathione concentration. When terms lacking the glutathione concentration as a factor were introduced, their coefficients became zero in the regression analysis. At high concentrations of β-NAD^+ (cf. Fig. 1) an equation

$$v = \frac{AG+A^2G+AG^2}{K+A+G+AG+A^2+G^2+A^2G+AG^2}$$

was satisfactory, but at lower β-NAD^+ concentrations higher-degree terms had to be included to describe the inhibition by excess of adduct or glutathione (cf. Fig. 2). These results are consistent with scheme A (and B) but not with schemes C and D. Furthermore, this analysis gives objective evidence for a reaction scheme involving glutathione as an essential activator.

As with glyoxalase I (Mannervik *et al.*, 1973), the discrimination between rival kinetic models as well as parameter estimation was relatively insensitive to the value of the equilibrium constant (K_d) for dissociation of adduct to formaldehyde and glutathione. In addition to $K_d = 1.5$mM (Uotila & Koivusalo, 1974) the values 0.7 and 3.0mM were used, but the results did not differ significantly (changes in the fourth digit of the residual sum of squares).

Table 2. *Comparison of alternative rate equations fitted by non-linear regression analysis to an experimental data set*

The experimental data used ($n = 56$) are those presented in Fig. 3, in which the free glutathione concentration is kept constant at a high value (5.0mM). The serial correlation coefficient is that previously used (Mannervik, 1975*b*). Reaction schemes A–D are shown in Scheme 1.

Reaction scheme	Sum of squares of residuals (arbitrary units)	No. of parameters in rate eqn.	Serial correlation coefficient
A	21.097	10	−0.074
B	68.516	4	0.397
C	Predicts zero velocity	—	—
D	68.770	4	0.397

Discussion

The model proposed for the kinetic mechanism of formaldehyde dehydrogenase (Scheme 1: A) is the best of four schemes compared explicitly in this paper. Several variants of these reaction schemes have also been examined, but they were less satisfactory in various respects. Scheme A is sufficiently elaborate to account for the complex kinetic patterns demonstrated in some of the Figures. For example, inflexions in the Eadie–Augustinsson–Hofstee plot (cf. Fig. 5) require that the rate equation is at least third degree in the concentration of the varied substrate (Bardsley & Childs, 1975). Since scheme A contains four separate steps involving the addition of A, it can, according to the structural rule, be up to fourth degree in A under the steady-state assumption (Wong & Hanes, 1962) and the curve shapes of Fig. 5 are thus explainable by this scheme.

A fundamental assumption in scheme A is that glutathione is a modifier of formaldehyde dehydrogenase. Except for very high concentrations, at which glutathione competes with adduct for the adduct-binding site in the active centre of the enzyme, glutathione is considered as an activator. A formally equivalent assumption is that formaldehyde at moderate and high concentrations is an inhibitor. However, this interpretation seems less probable because in such a case the toxic formaldehyde would prevent its own destruction. Furthermore, the concentration of free formaldehyde in the experiment depicted in Fig. 6 was only 1.5mM, whereas the highest concentration investigated was 75mM, at which concentration the enzyme was still active. Thus it appears extremely unlikely that the finding that the curves of Fig. 6 extrapolate to the origin should be interpreted as a complete inhibition of enzyme activity by a finite formaldehyde concentration rather than being interpreted as an absolute requirement of glutathione for activity. Attempts were made to find direct evidence for such a role of glutathione by studies of the reverse reaction but these failed, because the *S*-formylglutathione used was contaminated with glutathione and a glutathione-free reaction system could not be obtained. Finally, glutathione seems to be more suitable as a modifier of the enzyme, considering also its possible physiological function of detoxifying formaldehyde. The possibility that formaldehyde or glutathione acts as an irreversible inhibitor at high concentrations in the kinetic studies was also considered. However, neither 82mM-formaldehyde nor 40mM-glutathione inactivated the enzyme during a 35min incubation period at pH8 and 25°C. Higher concentrations of these reactants were never used in the kinetic experiments. The binding sites of glutathione and the adduct must be very specific for their natural ligands, because none of a variety of *S*-substituted glutathione derivatives had any demonstrable effect on the kinetics. For example, *S*-methylglutathione, which is structurally closely related to the adduct, was without influence at 10mM concentration even at low glutathione and adduct concentrations. We have also performed studies on the product inhibition of the forward and reverse reactions, of formaldehyde dehydrogenase. These experiments, which will be reported elsewhere, support the interpretation given above.

Models involving co-operative subunits in a dimeric enzyme (cf. Monod *et al.*, 1965; Koshland, 1970) have also been analysed. Such a model would have to invoke glutathione as a modifier as well as a random sequential reaction scheme to be compatible with the experimental data. Therefore further complexity would be added to the reaction scheme, and, since no evidence for co-operativity has been found for formaldehyde dehydrogenase, we prefer the simpler scheme A. Nevertheless, it is evident that the effect of glutathione in activating the enzyme in scheme A can readily be interpreted as an allosteric transition involving distinct conformational states of the enzyme without any assumptions about co-operativity in the substrate-binding properties of the two subunits. Such possibilities of co-operativity and allosteric transitions have to be explored by ligand-binding and structural studies, which require larger amounts of pure enzyme than are presently available.

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