Studies on the Mechanism and Kinetics of the 2-Oxoglutarate Dehydrogenase System from Pig Heart

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1. The kinetic properties of the 2-oxoglutarate dehydrogenase system were investigated. To this end, initial-velocity studies were carried out by the method of Fromm [(1967) *Biochim. Biophys. Acta* 139, 221–230]. Reciprocal plots of the results did not agree with those expected for the Hexa Uni Ping Pong mechanism previously proposed for the system. 2. The measured initial velocities were fitted to initial-rate equations corresponding to several possible mechanisms by using a computer optimization technique. Statistical analyses performed on the results of the optimization studies indicated that one mechanism was a significantly better fit to the experimental data than the other mechanisms tested. This mechanism is one in which there is a random order of binding of NAD^+ and CoA and release of succinyl-CoA, although the binding of 2-oxoglutarate and release of CO_2 is still given a Ping Pong mechanism, which precedes the binding of the other substrates. These conclusions were supported by NADH-inhibition studies. 3. The usefulness of the method of fitting initial-rate data to rate equations and the applicability of the proposed enzymic mechanism to the enzyme complex are discussed.

The 2-oxoglutarate dehydrogenase complex was first purified from pig heart muscle by Sanadi *et al.* (1952), and their method has been used with occasional slight modifications by most subsequent workers (e.g. Massey, 1960; Smith *et al.*, 1974; see, however, Hirashima, *et al.*, 1967). Sanadi (1963) was also the first to propose a catalytic mechanism for the overall reaction of the enzyme complex on the basis of his investigations of the roles and locations of the cofactors thiamine pyrophosphate, lipoic acid and FAD within the complex, and of the coenzymes, NAD^+ and CoA, which participate in the reaction (Massey, 1960; Sanadi, 1963). This mechanism is shown in Scheme 1.

Kinetic studies on the pig heart complex had, until recently, been solely concerned with the measurement of K_m (or K_i) values for one or more substrates (or products) in the presence of saturating concentrations of the other substrates (Massey, 1960; Kanzaki *et al.*, 1969; Johnson & Connelly, 1972; Smith *et al.*, 1974). The values for the kinetic constants that they reported do not agree with each other, but the conditions of assay were different in each case.

To complete our simulation studies of the citric acid cycle (McMinn & Ottaway, 1976), numerical values for the rate constants of the individual steps of the 2-oxoglutarate dehydrogenase reaction were required, and for this purpose it was necessary to check whether the mechanism proposed by Sanadi (1963) for the enzyme complex (Scheme 1) holds. This mechanism is such that the first product (CO_2)

is released before the second substrate (CoA) binds, and the second product [succinyl-CoA (3-carboxypropionyl-CoA)] is released before the third substrate (NAD^+) binds. This can be described in Cleland's (1963) terminology as a Hexa Uni Ping Pong mechanism. Establishing the mechanism is particularly important for simulation, since the Sanadi (1963) mechanism predicts that NADH is a competitive inhibitor with respect to NAD^+ . We have shown that NADH is an important factor in the control of flux through the citric acid cycle, exerted especially through malate dehydrogenase (McMinn & Ottaway, 1976; Ottaway, 1976).

We needed to simulate the 2-oxoglutarate dehydrogenase accurately enough for its importance as a cycle-regulation point to be assessed.

Hamada *et al.* (1975) attempted to establish the enzymic mechanism from studies of steady-state kinetics. They concluded that their results were compatible with the Hexa Uni Ping Pong mechanism, although at high NAD^+ concentrations there was a divergence from the expected behaviour. They reported that NADH inhibition is competitive with NAD^+ , whereas, using very similar substrate concentrations, Smith *et al.* (1974) found that this inhibition was non-competitive. Both groups showed that succinyl-CoA inhibition was competitive with CoA. The results of Smith *et al.* (1974) are not compatible with the Sanadi (1963) mechanism.

The mechanism of three-substrate enzymic reactions can be established by several steady-state

Scheme 1. *Proposed mechanism for 2-oxoglutarate dehydrogenase (Sanadi, 1963)*

Abbreviations used: TPP, thiamin pyrophosphate; Succ, succinic acid; OG, 2-oxoglutaric acid; LipS₂, lipoic acid; Succ-CoA, succinyl-CoA. Scheme 1 represents the enzyme complex which contains all three cofactors (TPP, lipoate, FAD). For the sake of clarity only those cofactors involved at each stage of the reaction are shown, although all are present within the complex (E).

kinetic methods (Cleland, 1963; Fromm, 1967; Dalziel, 1969; Fisher *et al.*, 1972). Cleland's (1963) method requires one of the substrates (S_x) to be varied within an assay, while the concentration of a second substrate (S_y) is changed between assays and the third substrate (S_z) is held constant at a saturating concentration. For the investigation to be complete the sets of assays are then carried out with S_z at non-saturating concentrations. The results are plotted in double-reciprocal form. Fromm (1967) suggested a variation of this idea, in which S_x is varied as before, but the concentrations of S_y and S_z are both changed between assays, and the ratio of S_y/S_z is kept constant. Dalziel's (1969) method also involves simultaneously varying the concentrations of all three substrates, but without fixed ratios. With all these methods, product-inhibition studies can give a fuller description of the mechanism, although initial-rate studies (in the absence of inhibitors) for a three-substrate system can be very informative (see Dalziel, 1969).

The method of Fisher *et al.* (1972) requires initial velocities to be measured in both directions of the reaction and is therefore only applicable to enzymic

reactions which are readily assayable in both directions.

For our kinetic studies the method of Fromm (1967) was used, for three reasons. (1) The reaction is not easily assayable in the reverse direction. (2) Ping Pong mechanisms can be readily distinguished from sequential mechanisms. (3) Full analysis by initial-rate studies alone does not require as many sets of assays as does Cleland's (1963) method.

Fromm's (1967) analysis predicts that if, the double-reciprocal plots of the results are parallel, then the mechanism is Ping Pong, but if they converge it is sequential. We found this method useful for distinguishing that the addition of 2-oxoglutarate and release of CO₂ is Ping Pong, but it was less useful in analysing the binding order of NAD⁺ and CoA, and other methods had to be applied to establish a plausible kinetic mechanism.

A preliminary report of this work has been published (McMinn & Ottaway, 1975).

Materials and Methods

CoA, NAD⁺ and 2-oxoglutarate were obtained from Boehringer (Mannheim, Germany). Cysteine hydrochloride was obtained from BDH Ltd. (Poole, Dorset, U.K.). Other chemicals used were of analytical grade. Substrate solutions were prepared fresh each day.

The 2-oxoglutarate dehydrogenase complex was prepared by the method of Sanadi (1969) from fresh pig heart muscle. Concentrations of the final enzyme solutions were between 1.3 and 3 mg/ml, with a specific activity of between 0.75 and 0.85 mol of NADH produced/min per mg of protein at 30°C. The enzyme solutions were stored at -4°C; this produced no appreciable decrease in activity. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Assays of the initial-velocity behaviour were carried out at 30°C in an SP.1800 recording spectrophotometer. Cuvettes were preincubated at 30°C for approx. 5 min before initiating the reaction by the addition of 0.01 ml of enzyme solution. CoA and NAD⁺ samples were added just before the pre-incubation stage to minimize any non-enzymic destruction. All assays were carried out in duplicate. The assay mixtures all contained (in 1 ml) 0.08 M-potassium phosphate buffer (pH 7.2) and 0.4 mM-cysteine hydrochloride (pH 7.0-7.5). The concentration ranges of substrates were: 0.025-0.5 mM-2-oxoglutarate (pH 7.2), 0.005-0.1 mM-CoA and 0.02-0.4 mM-NAD⁺ (pH 7.2).

The assay mixtures were set up as described by Fromm (1967) as outlined above. For each set of assays five concentrations of the variable substrate (S_x) and four concentrations of the 'fixed' substrates (S_y and S_z) were used, and each measurement was

made in duplicate, i.e. 40 initial velocities per set of assays, a total of 120 measurements for a complete study. (In the study reported, only 118 of the measurements were valid, as gross errors in two assays invalidated them.) The ratios S_y/S_x used for the three sets of assays were: CoA/NAD⁺, 1:8; 2-oxoglutarate/CoA, 10:1; 2-oxoglutarate/NAD⁺, 5:4. Results were plotted in double-reciprocal form.

Product-inhibition studies with NADH as the inhibitor were carried out with NAD⁺ as the variable substrate and the other two substrates, CoA and 2-oxoglutarate, at saturating and non-saturating concentrations.

The SYMAP program was developed by and is available from the Laboratory for Computer Graphics and Spatial Analysis, Harvard Center for Environmental Design Studies, Graduate School of Design, Harvard University, Cambridge, MA 02138, U.S.A. Maps were constructed with concentrations of two of the substrates as co-ordinates and the modulus of percentage differences between calculated and measured velocities as the 'height' or 'density'. The chloropleth (area) option, and not the contour plot (Ottaway & Apps, 1972), was used, because it was possible for several observations to have the same co-ordinates on a two-dimensional map. A chloropleth map represents the average value over an area (Monkhouse & Wilkinson, 1971).

Results

Qualitative analysis of the mechanism

Reciprocal plots of the initial-rate data gave a set of linear parallel plots when 2-oxoglutarate was the variable substrate S_x (Fig. 1a). However, when CoA or NAD⁺ was the variable substrate, the plots showed considerable non-linearity (Figs. 1b and 1c). These results were repeatable with different preparations of the enzyme. The only fact that can be certainly deduced from these graphs is that 2-oxoglutarate binds and is decarboxylated, and CO₂ is released from the enzyme complex, before the next substrate binds. The curvature of the other reciprocal plots makes it impossible to apply Fromm's (1967) rules to them.

Bardsley & Childs (1975) have suggested a method of analysing non-linear double-reciprocal plots. This is essentially an analysis of v against s curves for points of inflexion and turning points, to gain information about the degree to which the substrate terms are raised in the numerator and denominator of the rate equation. We found with our data that we could come to no definite conclusions, because the points of inflexion could not be located precisely enough on v against s plots; very many more data would be needed for this method to be successful. Both more

data points for each assay and more replicates of each point measured would be required.

Quantitative analysis of the mechanism

Since no further information could be gained from the data by either Fromm's (1967) analysis or that of Bardsley & Childs (1975), it was decided to use a different approach, that of 'total fit'. The fit of the complete set of initial-velocity measurements to the rates predicted by (steady-state) initial-velocity equations corresponding to several kinetic mechanisms was examined by an optimization procedure as described by Davis & Ottaway (1972). All five mechanisms shown in Fig. 2 were fitted to the experimental data with equivalent initial estimates of the unknown kinetic constants. The best fit was obtained by minimizing the value of R_m , where R_m is defined by eqn. (1):

$$R_m = \sum_{i=1}^{118} \left(\frac{v_{\text{calc.}} - v_{\text{meas.}}}{v_{\text{calc.}} + v_{\text{meas.}}} \right)^2 \quad (1)$$

where $v_{\text{calc.}}$ is the initial velocity calculated according to the given rate equation containing the current estimates of the unknown parameters, and $v_{\text{meas.}}$ is the corresponding experimental value (Ottaway, 1973).

We found with our initial-rate data that there was a positive correlation between initial velocity and the unsigned errors. This correlation was abolished by the use of the fractional deviations as described, and we verified this, for our own results, graphically. Methods of dealing with more systematic variations have been discussed by Storer *et al.* (1975).

Experience showed that the program called SIMPLEX (see Nelder & Mead, 1965; Davis & Ottaway, 1972) was faster than more sophisticated procedures for large sets of data, and, moreover, convergence to a minimum did not depend on the initial estimates of the parameters.

The five mechanisms shown in Scheme 2 were chosen on the following basis. Mechanism 1 is the Hexa Uni Ping Pong (Sanadi, 1963) mechanism. Although the Fromm (1967) analysis predicts that this is unlikely to be the mechanism of the enzyme, it is useful as a basic model with which other mechanisms can be compared. Mechanism 2 has a sequential binding of CoA and NAD⁺. From the information that can be gained from the initial-rate data, this is as plausible a mechanism as the Hexa Uni Ping Pong, and therefore must be included. Mechanisms 4 and 5 both have random binding of CoA and NAD⁺ and both would be expected to give non-linear reciprocal plots where CoA or NAD⁺ is the variable substrate. Mechanism 3 was suggested by Hamada *et al.* (1975) as a likely explanation of their results at high NAD⁺ concentrations. Analysis of our own results by

Fig. 1. Double-reciprocal plots of the results of the initial-rate studies on the 2-oxoglutarate dehydrogenase system. Points shown on the graphs are the average of two measurements. Velocities are expressed as $\mu\text{mol of NADH produced/min per mg of protein}$. (a) 2-Oxoglutarate as the variable substrate. Concentrations of the fixed substrates were: NAD^+ , 0.333 mM, and CoA, 0.05 mM (Δ); NAD^+ , 0.133 mM, and CoA, 0.02 mM ($\bullet$); NAD^+ , 0.066 mM, and CoA, 0.01 mM ($\blacksquare$); NAD^+ , 0.033 mM, and CoA, 0.005 mM ($\blacktriangle$). Lines were fitted by computer by Cleland's (1963) method. (b) CoA as the variable substrate. Concentrations of the fixed substrates were: 2-oxoglutarate, 0.5 mM, and NAD^+ , 0.4 mM (Δ); 2-oxoglutarate, 0.1 mM, and NAD^+ , 0.08 mM ($\blacksquare$); 2-oxoglutarate, 0.05 mM, and NAD^+ , 0.04 mM ($\blacktriangle$); 2-oxoglutarate, 0.025 mM, and NAD^+ , 0.02 mM ($\bullet$). Lines were fitted by eye. (c) NAD^+ as the variable substrate. Concentrations of the fixed substrates were: 2-oxoglutarate, 0.5 mM, and CoA, 0.05 mM (Δ); 2-oxoglutarate, 0.2 mM, and CoA, 0.02 mM ($\blacksquare$); 2-oxoglutarate, 0.1 mM, and CoA, 0.01 mM ($\blacktriangle$); 2-oxoglutarate, 0.05 mM, and CoA, 0.005 mM ($\bullet$). Lines were fitted by eye.

Fromm's (1967) method indicated that this is highly unlikely to be the mechanism of the reaction in our conditions, but we included it for comparison. It may be that concentrations of NAD^+ at which Hamada

et al. (1975) observed this phenomenon were much higher than those we used.

It should be noted that mechanisms 2 and 4 are unlikely to be true for the oxoglutarate dehydrogenase

Scheme 2. Mechanisms fitted to the initial-rate data by SIMPLEX optimization

Abbreviations: A is [2-oxoglutarate]; B is [CoA]; C is [NAD⁺]; P is [CO₂]; Q is [succinyl-CoA]; R is [NADH].

complex, because it is known that each substrate can react with the complex if an element of the complex is in a suitable form (i.e. in the free, succinylated or reduced state), in the absence of the other two substrates. (see Sanadi, 1963). We included them in our analysis because they are kinetically feasible, i.e. we could not discount them on the basis of our initial-rate studies.

The first column in Table 1 shows the values of the minimum differences reached by SIMPLEX for each fitting. Although these results indicate that mechan-

ism 5 has a much lower residual than the others, this does not necessarily indicate a better fit to the data, since the values of the minimum differences are not directly comparable, as each equation has a different number of unknown parameters to dispose of.

The fits were examined qualitatively by the SYMAP program. The three maps produced for each mechanism give a good visual indication of the overall fit of an equation to the data. An example of two sets of maps are shown in Fig. 2. These illustrate that mechanism 5 has few areas of high (>20%) error and

Fig. 2. Plots of percentage error against substrate concentration. Errors are plotted against two of the substrates at one time, as described in the text, resulting in three 'maps', which, when the maps are: 0-10% (blank); 10-20% (•); 20-40% (×); 40-60% (*); and 60-100% (+). (a) shows the fit of mechan-

overall has a fairly even correspondence between theoretical and experimental values. Mechanism 1, however, shows wide variation in the fitting and has areas of very high (>40%) error. The fit of the other three rate equations was examined similarly and confirmed that mechanism 5 gives the best overall fit of those examined.

Statistical analysis of results

The goodness of fit of the data to the various models was examined statistically in two ways. It should be

emphasized that the statistical methods used are to some extent empirical, as the theory of non-linear least-squares inference has not yet been completely tested (R. A. Elton, personal communication). However, the need for methods of testing models of multi-enzyme systems is so great, in contrast with single-substrate enzymes for which techniques are now beginning to be agreed (Markus *et al.*, 1976), that it has been thought worth while to draw attention to the approaches outlined below, as they agree with one another in indicating that one of the models

for the fit of the initial-rate data to mechanisms 1 and 5 put together as above, give a general indication of the fit of the mechanism to the data. The error values appearing on ism 1 to the data. (b) shows the fit of mechanism 5 to the data.

tested fits the data much better than any of the others. What cannot be given at present is any precise estimate of the probability level associated with the observed difference in goodness of fit, as judged by the criteria used.

The first type of analysis we have called 'comparison of models'. It enables a basic model to be tested against a more complex one which is a 'derived version' of the first model (i.e. the equation for the second model contains all the terms in the equation

for the first model plus one or more extra terms). Thus mechanism 1 is a basic model from which each of the other four may be derived. Hence we are testing whether the extra terms in the equations corresponding to the complex models (2-5) are contributing to a significantly better fit.

For any given model x , the minimized weighted sum of squares of deviations from the estimated values is R_x (eqn. 1). If the standard deviation of $v_{\text{meas.}}$ is assumed to be proportional to the true mean value of

Table 1. Results of the 'comparison of models' test

R_m is the computed minimum differences according to eqn. (1) in the text. Mechanisms are numbered as in Scheme 2.

Mechanism	R_m	df	Mechanisms compared	$F_{calc.}$	$F(0.01)$
1	1.798	113	1 < 2	4.875	6.84
2	1.723	112	1 < 3	1.203	3.17
3	1.722	109	2 < 4	0.73	2.55
4	1.631	104	1 < 5	14.38	2.55
5	0.858	105			

v , S.D. ($v_{meas.}$) = $2\sigma v$, say, then R_x is an estimate of $\sigma^2 df(x)$, where the degrees of freedom $df(x)$ are given by: (the number of measured velocities—the number of unknown constants—1).

Suppose that model x is a 'derived version' of model y , involving extra terms, then R_y will have expected value $\sigma^2 df(y)$, and the degree of improvement from y to x resulting from fitting the extra terms will be measured by the difference $R_y - R_x$, with expected value $\sigma^2 [df(y) - df(x)]$.

In the perfectly linear case, as for example in adding extra terms to a polynomial to improve the fit to a set of data, R_x and $(R_y - R_x)$ would be independent. If we assume that this is approximately true here and that the data are normally distributed, then:

$$\frac{(R_y - R_x) / [df(y) - df(x)]}{R_x / df(x)}$$

will be approximately distributed as $F[df(y) - df(x), df(x)]$ if both models are true (i.e. if the extra terms are unnecessary). Thus large F values suggest that model y is a better fit than model x . In other words, small values of F , for the appropriate numbers of degrees of freedom, can be used to support the validity of the null hypothesis (that the extra terms did not lead to any improvement in fit). The results of this test are shown in Table 1. The mechanisms compared are those shown in the third column. The large F value for mechanism 5 can be used as a guide to indicate that this is the only model of those tested which gave a better fit to the data than mechanism 1 (Hexa Uni Ping Pong mechanism).

The second statistical test is an analysis of variance. For this test R_e , that part of the deviations caused by experimental error in measuring the initial velocities, was calculated by the formula:

$$R_e = \frac{1}{2} \sum_{i=1}^{60} \left(\frac{v_{11} - v_{12}}{v_{11} + v_{12}} \right)^2 \quad (2)$$

where v_{11} and v_{12} are replicates. (Some 60 measurements were made with each enzyme preparation; see

the Materials and Methods section.) This is an unbiased estimate of $\sigma^2 df(e)$, whether or not the model is true. By using this value R_e , and the R_m values as in the previous test, the corresponding mean square (MS) values are calculated by the formulae:

$$MS_m = \frac{(R_m - R_e)}{(df_m - df_e)}$$

and

$$MS_e = \frac{R_e}{df_e}$$

The mean square values are compared by an F test, where:

$$F[(df_m - df_e), df_e] = MS_m / MS_e$$

If the value of F is significant, then the error (deviation) due to the fitting is significantly larger than that due to the measurements; hence the fit to the data is not as good as it could be. If, however, F is not significant, this implies that the error due to the fitting is no greater than the error in the measurements, and such a mechanism can be considered as an acceptable fit to the data. (The limitations on the term 'significant' discussed at the beginning of this section still hold here.)

The results of this test are shown in Table 2. Only mechanism 5 yields a non-significant value for F , which allows us to infer that this mechanism has a high probability of being an acceptable description of the experimental data, in contrast with the other mechanisms tested. However, it must be pointed out that statistical tests cannot show that any particular mechanism is a unique description of the data.

Inhibition studies with NADH

Double-reciprocal plots of the NADH-inhibition results are shown in Fig. 3. Where CoA and 2-oxoglutarate are saturating the patterns indicate a mixed-type inhibition and this also seems to be the inhibition type when both of these substrates are

Table 2. Results of the 'analysis of variance' test

Mechanisms are numbered as in Scheme 2. R_m , df_m , R_e , df_e , MS_m , MS_e and F are calculated as described in the text. The theoretical value of $F_{50,60}$ is 1.88. This is taken from *Documenta Geigy*.

Mechanism	R_m	df	R_e	df	MS_m	MS_e	F
1	1.798	113	0.4064	58	0.0253	0.00697	3.63
2	1.723	112	0.4064	58	0.0244	0.00697	3.50
3	1.722	109	0.4064	58	0.0258	0.00697	3.70
4	1.631	104	0.4064	58	0.0266	0.00697	3.82
5	0.858	105	0.4064	58	0.0096	0.00697	1.37

Fig. 3. $3s/v$ against s plots of the NADH-inhibition data where NAD^+ is the variable substrate

NAD^+ was the variable substrate. NADH concentrations were 0.0 (●), 0.01 mM (△), 0.02 mM (■) and 0.05 mM (▲). Concentrations of the fixed substrates were (a) 2-oxoglutarate, 0.05 mM, and CoA, 0.005 mM (both non-saturating), and (b) 2-oxoglutarate, 0.5 mM, and CoA, 0.05 mM (both saturating). Lines were fitted by computer by Cleland's (1963) method. The curved line in (b) was fitted by eye. Initial velocities are expressed in μmol of NADH/min per mg of protein.

Table 3. Values for the optimized constants of the rate equation for mechanism 5

The constants are numbered as in mechanism 5 in Scheme 2. Values were calculated in μmol of NADH produced/ μmol of enzyme, assuming a mol.wt. of 2.7×10^6 for the 2-oxoglutarate dehydrogenase complex.

Constant	Value
K_1	21.6 s^{-1}
K_2	$467.0 \text{ s}^{-1} \cdot \text{mm}^{-1}$
K_3	$1703.0 \text{ s}^{-1} \cdot \text{mm}^{-1}$
K_4	$0.75 \times 10^{-10} \text{ mm}$
K_5	$0.5 \times 10^{-5} \text{ mm}$
K_6	0.44
K_7	0.17
K_8	0.62 mm
K_9	0.20
K_{10}	5.18
K_{11}	17.8 mm^{-1}
K_{12}	27.1 mm^{-1}

non-saturating. We have fitted straight lines to the data, but it should be remembered that these may well be non-linear plots (see Fig. 3b). Mechanism 5 predicts that the NADH-inhibition data should yield non-linear plots; however, at the substrate concentrations used, the deviation from linearity is very slight.

Values of kinetic constants

The values of the complex constants for mechanism 5 found by optimization, which will be used in subsequent simulation studies, are given in Table 3. K_m values have no meaning for rate equations as complex as this, but the ' S_{50} ' value for each of the substrates was computed from the values in Table 3 by setting the other two substrates at 'saturating' concentrations. Table 4 shows our computed S_{50} values along with the values of K_m for the various substrates which have been reported by previous workers.

Table 4. Comparison of kinetic constants and S_{50} values for the pig heart 2-oxoglutarate dehydrogenase. S_{50} values are calculated as described in the text. The S_{50} value given here is the lowest concentration of CoA that will give half-maximal velocity. Mechanism 5 predicts that CoA will exhibit substrate inhibition, so there is more than one concentration of CoA that can give half-maximal velocity. OG, 2-Oxoglutaric acid.

K_m^{OG} (mM)	K_m^{CoA} (mM)	$K_m^{NAD^+}$ (mM)	Conditions	Reference
0.013	0.0001	0.0045	pH 7.4 25°C	Massey (1960)
0.22	0.025	0.05	pH 7.5 25°C	Hamada <i>et al.</i> (1975)
—	0.0027	0.021	pH 7.2 22°C	Smith <i>et al.</i> (1974)
S_{50}^{OG} (mM)	S_{50}^{CoA} (mM)	$S_{50}^{NAD^+}$ (mM)		
0.2	0.0035	0.05	pH 7.2 30°C	The present study

Discussion

The work reported here indicates that the kinetic mechanism of the 2-oxoglutarate dehydrogenase complex is not a simple sequence of substituted enzyme (Ping Pong) reactions. It appears from the statistical significance uncovered by our 'total fit' procedure that the kinetic mechanism is random, at least with respect to binding of NAD^+ and CoA, and the release of succinyl-CoA. This random binding with the consequent appearance of squared substrate terms in the denominator would account for the non-linearity of the reciprocal plots (Fig. 1). It would also account for the non-competitive inhibition of NAD^+ by NADH reported by Smith *et al.* (1974), and our own finding of a mixed inhibition by this product (Fig. 3).

It is possible that the non-linearity of the reciprocal plots could be due to homotropic allosteric effects, but we have rejected this possibility, because there is at present no evidence for allosteric behaviour of the 2-oxoglutarate complex, in contrast with the *Escherichia coli* pyruvate dehydrogenase complex (Shepherd & Hammes, 1976), for which binding of acetyl-CoA at sites other than catalytic sites has been established.

It must be borne in mind, however, that the mechanism that we have proposed for the reaction may be the simplest one which gives an acceptable fit to our data. We have assumed, in constructing the models shown in Scheme 2, that the one part of the overall reaction which does seem certain to involve an ordered substitution step, the decarboxylation of oxoglutarate, precedes the other two stages, because in the steady state neither the transfer of the succinyl residue through the lipoate succinyltransferase subunit to CoA, nor the transfer of 2H through the transferase and lipoamide dehydrogenase subunits to NAD^+ , can occur before the oxoglutarate has been decarboxylated. We have also assumed that release of NADH is the last step in the process, but this need

not be so. It is noteworthy that the NADH-inhibition plots are not simple (Fig. 3); moreover, the distribution of error, even in the best model, is worse in relation to NAD^+ than to the other two substrates (Fig. 2). These findings, together with those of Hamada *et al.* (1975), which indicate that the binding of CoA (or succinyl-CoA) retards the binding of NAD^+ (or NADH), suggest that the behaviour of the enzyme system towards NAD^+ (or NADH) is not straightforward. A more complicated sequence of intramolecular transfers, perhaps dependent on substrate concentrations, could be envisaged which could describe the sequence of events at this point in the reaction more accurately.

We do not think, however, that we could prove that more complex models give a better fit. The F test is already significant for model 5 at the 1% level of probability; other models might give an equally significant result, but there would be no criterion for choosing between them. It is theoretically possible that the 'comparison of models' test could give a positive decision, but, since any more complex model than those we have suggested would involve even more parameters in the rate equation the reduction in residual error by the optimization program would have to be very considerable indeed for statistical significance to be reached. It does not seem likely that this would happen, particularly in view of the difficulty, touched on below, of assuming that a 'global minimum' has been reached. We think that the limit of model building on the basis of steady-state measurements has been reached, and that other lines of approach are required to establish more precisely the dynamics of the reactions catalysed by the complex.

We are nevertheless gratified at the progress which we were able to make by using 'total fit'. When it became clear that the conventional double-reciprocal plots were not linear, analysis of the mechanism by

established methods became very unrewarding. The usefulness of 'total fit' was considerably enhanced by applying statistical tests, even if they rest on a somewhat insecure basis, otherwise the decision whether a particular optimization minimum represented a 'satisfactory' fit depended almost entirely on aesthetic judgment about the distribution of error on plots such as those shown in Fig. 2. Although the 'comparison of models' test is particularly valuable for preliminary decisions about the fit of models, and is very convenient for use with enzyme rate equations because they can all be regarded as 'special cases' of a basic model, that is the equation corresponding to the basic model contains all the variables that appear in the more complex equation, there are nevertheless some pitfalls in applying it. The chief of these is that no optimization minimum can ever be guaranteed to be the 'global minimum', i.e. it can never be stated unequivocally that it lies at the centre of the deepest depression on the response surface (Beveridge & Schechter, 1970; Davis & Ottaway, 1972). To guard against this difficulty, we started the optimization procedure for each model from as nearly as possible the same set of initial estimates of parameter values. It is obvious, however, that, if the equation pertaining to model *y* has more parameters than that of model *x*, some of the parameters of *y* cannot be equivalent to any of the '*x*' set. Moreover, the presence of extra parameters in equation *y* may mean that the optimum values of some of those in the '*y*' set may be different from their equivalents in set '*x*', and therefore starting from the same initial parameter estimates for both models may not in fact be giving set '*y*' an equally good opportunity to reach its minimum. There is no solution to this difficulty, and we circumvented it only by choosing several sets of initial values, letting the computer program reach a minimum for each model with each set, and comparing the lowest minima reached for each model. In doing this it was very convenient to use a very robust optimization program such as SIMPLEX, which will reach a minimum from almost any starting co-ordinates, even if it sometimes does so rather slowly, in preference to more sophisticated programs that need good initial estimates. Programs that require the parameters to be scaled internally to have about the same numerical magnitude are particularly difficult to use in the way we have suggested (Davis & Ottaway, 1972).

Although it has been known for some time that the 2-oxoglutarate dehydrogenase complex, like the pyruvate dehydrogenase complex, contains a very large number of peptide chains, and that the three functional groups are attached to three different kinds of protein (Reed, 1966; Koike *et al.*, 1971), the relation between the molecular architecture and the kinetic mechanism has not hitherto been discussed.

The reaction is still written as if only three peptide chains are involved, one for each of the partial reactions (Scheme 1; see Hamada *et al.*, 1975). One apparent difficulty is that the numbers of chains of the three types within the complex are not equal; although the values are not known so exactly as they are for pyruvate dehydrogenase, the proportions of the decarboxylase/trans-succinylase/lipoate dehydrogenase chains are probably about 6:1:6 (Tanaka *et al.*, 1972). It is, however, the average residence time of each type of chain in the substituted form that determines its reactivity with respect to the other two, and therefore, unless the structural role of the trans-succinylase as the core of the complex (Koike *et al.*, 1971) is overwhelmingly large, one may suppose that the three types of subunit are present in amounts that give them roughly equal catalytic effectiveness.

The way in which the complex is constructed suggests that each protein chain of a particular type is within range of a few, certainly more than one, molecules of the next catalyst in the sequence shown in Scheme 1. The expression 'within range' is deliberately vague, because it is not clear at present whether physical contact of the active sites is necessary for transfer to take place between the subunits; evidence suggests that, so far as the *E. coli* pyruvate dehydrogenase complex is concerned, the lipoate 'swinging arm' is out of reach of the NAD⁺-binding site (Shepherd & Hammes, 1976). We suggest that the ability of each subunit to react with more than one of another type gives the reaction within the complex its semi-random character. We may consider Scheme 3 to represent the three independent sets of subunits that are able to react with each other. Each set contains protein chains both in the substituted and in the normal states.

We have already pointed out that transfer of succinyl and 2H from a reactive site of the set 1 chains is a necessary precursor of subsequent reactions. If the complex as a whole is not working at maximum activity, it is not, however, necessary that the oxidized lipoate chain that accepts the succinyl residue is precisely the one which accepted the previous residue. The latter could remain in the reduced state for some time before being reoxidized, so long as there remains a pool of oxidized lipoate sites in set 2 capable of continuously accepting succinyl residues from a decarboxylase site. Similarly, the FAD of a chain in set 3 could conceivably remain reduced for a finite period of time, and another, within working range of the same trans-succinylase unit, could accept the next pair of oncoming hydrogens.

Thus, on the average, at moderate rates of catalysis, the sequence of events beyond the release of CO₂ would appear to be random. As the complex is forced nearer its saturating throughput, however, the mechanism would become more strictly sequen-

Scheme 3. Representation of the 2-oxoglutarate dehydrogenase complex showing possible relationship between structure and random-binding kinetics

This scheme shows some of the species of the three subunits of oxoglutarate dehydrogenase which could co-exist within one molecule of the complex during the catalytic process. Note that for E1 (the dehydrogenase) there is an obligatory reaction sequence for every subunit, whereas for the other two subunits (E2 and E3) this is not necessarily so. For further explanation, see the Discussion section. For explanation of abbreviations, see legend of Scheme 1.

tial, because there would no longer be a pool of unused prosthetic groups within sets 2 and 3. Finally, each succinyl group would have to 'queue up' on the decarboxylase subunit until a lipoate residue became available, and this could only happen when the residue that had been reduced immediately beforehand had been reoxidized by a subunit in set 3. The fact that the mechanism is also Ping Pong in type depends on the fact that the substituted chains are not able to revert to their normal state until the acyl residue has been transferred to another acceptor. This restriction does not hold for E3, and the kinetics with respect to NAD^+ may be slightly different from that for the other two components of the complex.

A proposal of this type explains why the previous

workers on the enzyme, who used saturating concentrations of the fixed substrates, reported the mechanism to be Hexa Uni Ping Pong in character (Massey, 1960; Reed, 1966; Hamada *et al.*, 1975). It was not until the rate of reaction began to be measured at non-saturating concentrations of all three substrates that the possibility of random transfer between components within the complex could become manifest.

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References

- Bardsley, W. G. & Childs, R. E. (1975) *Biochem. J.* **149**, 313–328
- Beveridge, C. S. G. & Schechter, R. S. (1970) *Optimization: Theory and Practice*, McGraw-Hill, New York
- Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 104–137
- Dalziel, K. (1969) *Biochem. J.* **114**, 547–556
- Davis, R. H. & Ottaway, J. H. (1972) *Math. Biosci.* **13**, 265–282
- Fisher, J. R., Priest, D. C. & Barton, J. S. (1972) *J. Theor. Biol.* **37**, 335–352
- Fromm, H. J. (1967) *Biochim. Biophys. Acta* **139**, 221–230
- Hamada, M., Koike, K., Nakaula, Y., Hiraoka, T., Koike, M. & Hashimoto, T. (1975) *J. Biochem. (Tokyo)* **77**, 1047–1056
- Hirashima, M., Hayakawa, T. & Koike, M. (1967) *J. Biol. Chem.* **242**, 902–907
- Johnson, W. A. & Connelly, J. L. (1972) *Biochemistry* **11**, 2416–2421
- Kanzaki, T., Hayakawa, T., Hamada, M., Fukuyoshi, Y. & Koike, M. (1969) *J. Biol. Chem.* **244**, 1183–1187
- Koike, K., Tanaka, N., Hamada, M., Otsuka, K., Suematsu, T. & Koike, M. (1971) *J. Biochem. (Tokyo)* **69**, 1143–1147
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Markus, M., Hess, B., Ottaway, J. H. & Cornish-Bowden, A. (1976) *FEBS Lett.* **63**, 225–230
- Massey, V. (1960) *Biochim. Biophys. Acta* **38**, 447–460
- McMinn, C. L. & Ottaway, J. H. (1975) *Biochem. Soc. Trans.* **3**, 1071–1074
- McMinn, C. L. & Ottaway, J. H. (1976) *J. Theor. Biol.* **56**, 57–73
- Monkhouse, F. J. & Wilkinson, H. R. (1971) *Maps and Diagrams*, 3rd edn., pp. 43–45, Methuen, London
- Nelder, J. A. & Mead, J. A. (1965) *Comput. J.* **7**, 308–313
- Ottaway, J. H. (1973) *Biochem. J.* **134**, 729–736
- Ottaway, J. H. (1976) *Biochem. Soc. Trans.* **4**, 371–376
- Ottaway, J. H. & Apps, D. K. (1972) *Biochem. J.* **130**, 861–870
- Reed, L. J. (1966) *Compr. Biochem.* **14**, 99–126
- Sanadi, D. R. (1963) *Enzymes 2nd Ed.* **7**, 307–344
- Sanadi, D. R. (1969) *Methods Enzymol.* **13**, 52–55
- Sanadi, D. R., Littlefield, J. W. & Bock, R. M. (1952) *J. Biol. Chem.* **197**, 851–862
- Shepherd, C. B. & Hammes, C. G. (1976) *Biochemistry* **15**, 311–317
- Smith, C. M., Bryla, J. & Williamson, J. R. (1974) *J. Biol. Chem.* **249**, 1497–1505
- Storer, A. C., Darlison, M. G. & Cornish-Bowden, A. (1975) *Biochem. J.* **151**, 361–367
- Tanaka, N., Koike, K., Otsuka, K.-I., Hamada, M., Ogasahara, K. & Koike, M. (1972) *J. Biol. Chem.* **249**, 191–198