A MATHEMATICAL MODEL FOR THE REACTION MECHANISM OF THE ENZYME ALCOHOL DEHYDROGENASE

by

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SUMMARY

1. A set of simultaneous non-linear differential equations was derived from stoichiometric equations which describe the Theorell-Chance mechanism for liver alcohol dehydrogenase.

2. Assuming that the concentrations of the intermediate complexes remained constant, an explicit solution involving three unknown parameters was obtained for the differential equations. Relaxation of the assumptions to permit the concentration of one of the intermediates to change linearly with time gave rise to a four-parameter solution. The values of the unknown parameters in the two types of solution were estimated from experimental data by the method of least squares.

3. A computer program was written to provide a numerical solution of the differential equations and the solution was used to obtain estimates of the velocity constants for the Theorell-Chance mechanism. The validity of this model for liver alcohol dehydrogenase was checked under different experimental conditions. This mechanism appears to be valid for the reaction when NAD concentrations between 80 μM and 240 μM are used.

4. The reaction of yeast alcohol dehydrogenase was also examined in relation to the Theorell-Chance mechanism. Estimates of velocity constants were obtained and numerical solutions from the computer program compared with experimental data. The results of this investigation appear to support an alternative mechanism involving the formation of a ternary complex as proposed by Theorell and Chance.

DECLARATION

I hereby declare that the work presented in this thesis has been performed by myself except where otherwise stated, and that it has not been submitted in any previous application for any degree.

M. J. ATKINSON

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INTRODUCTION

Enzymes are naturally occurring organic compounds which increase the rates of certain chemical reactions while themselves remaining unchanged. The study of enzymes is of great interest since, to quote DIXON and WEBB (1964):

> "Life depends on a complex network of chemical reactions brought about by specific enzymes, and any modification of the enzyme pattern may have far reaching consequences for the living organism."

Usually an enzyme is detected by its reactions with a substrate. The amount present is estimated from the reaction velocity.

Many workers have studied the relationships between the rate of enzymic reaction and various environmental factors, such as concentration of enzyme and of substrate, temperature and pH. These observations may be used to formulate kinetic equations which describe the reaction.

This investigation is concerned with the formulation of mathematical models of enzymic reactions, and with the testing of these models by comparing predictions based upon them with data obtained from actual experiments.

CHAPTER I

1. Early development of ideas about enzymes

PAYEN and PERSOZ (1883) were probably the first to recognise an enzymic reaction when they isolated a substance from malt extract which converted starch into sugar. The name "enzyme" was proposed by KÜHNE (1878) for such catalytic substances, which many workers were then discovering in living materials.

The mechanism of action of these enzymes was naturally of great interest and various theories were entertained. One such concept presumed that the enzyme increased the energy of the substrate molecules by some sort of action at a distance or that energy was transmitted from enzyme to substrate by inelastic collisions [MEDWEDEW (1937), HEARON and KATZMAN (1954)]. Another theory was that the enzyme and substrate combine in some way before the final product is formed. By the end of the Nineteenth Century the latter theory appears to have been in favour. For example, ARMSTRONG (1895), in a Presidential Address to the Chemical Society, included enzymic reactions among examples of the chemical changes under discussion when he asserted that

> "...complication, not simplification, precedes most, if not all, chemical change; that complex molecular systems are first formed from the interacting substances and that these, on breakdown, suffer rearrangement of the parts -

> such rearrangements taking place in consequence of elements which were previously separated being brought into one common 'sphere of activity' within which it is possible for them to interact....The function of enzymes in promoting hydrolysis (of saccharides), on this hypothesis, consists in bringing water into conjunction with the carbohydrate by combining with both."

Translation of these ideas into a precise theory of the mechanism of enzymic action which could be used to make quantitative predictions resulted from the detailed studies made by many workers about this time.

2. Formulation of reaction mechanism

HENRI (1902) suggested that the enzyme first forms a complex with its substrate and that this subsequently breaks down to give the free enzyme and the product of the reaction.

From this suggestion, MICHAELIS and MENTEN (1913), working on the enzyme invertase postulated the reaction given by the stoichiometric equations

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \qquad \ldots \text{ I.1}$$

$$ES \xrightarrow{k_2} E + P \qquad \ldots \text{ I.2}$$

where E denotes the enzyme, S the substrate, ES the enzyme-substrate complex and P the reaction product.

Equation I.1 implies that one molecule of enzyme combines reversibly with one molecule of substrate to give one molecule of enzyme-substrate complex, while equation I.2 allows for one molecule of the enzyme-substrate complex to decompose irreversibly to produce one molecule of enzyme and one molecule of reaction product.

3. Physical implications of the simple model

The reaction model described by equations I.1 and I.2 gives rise, by the Law of Mass Action, to the set of differential equations

$$\frac{d[S]}{dt} = -k_1[E][S] + k_{-1}[ES] \quad \ldots \text{I.3}$$

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES] \quad \ldots \text{I.4}$$

$$\frac{d[P]}{dt} = k_2[ES] \quad \ldots \text{I.5}$$

where [E], [S], [ES] and [P] denote the concentrations of E, S, ES and P respectively. Because the number of enzyme molecules remains constant, we have

$$[E] = [E_0] - [ES] \quad \ldots \text{I.6}$$

where $[E_0]$ denotes the initial (t=0) enzyme concentration.

Since for each substrate molecule that disappears a product molecule is produced, we also have

$$[S_0] = [S] + [ES] + [P] \quad \ldots \text{I.7}$$

where $[S_0]$ denotes the initial concentration of substrate.

The above set of equations cannot be solved explicitly* but the form of the solution can be deduced in detail and this is

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*A numerical solution was obtained by CHANCE (1943).

shown in Appendix I. From the results in Appendix I, the prediction of the behaviour of the enzyme system when the reaction is commenced by mixing substrate and enzyme may be illustrated diagrammatically as in Figure I.1.

The reaction proceeds through three distinct stages.

(a) Initially the concentration of intermediate rises rapidly, and the rate of product formation is increasing. This stage is known as the *transient stage* and persists over a very brief period (10 msecs or less) and is consequently difficult to observe.

(b) The first stage is followed by a longer period during which the amount of intermediate present is virtually constant and both the substrate and product curves are approximately linear. This is the *steady state* period.

(c) In the final stage the reaction rates decrease to zero as the *equilibrium condition* is attained.

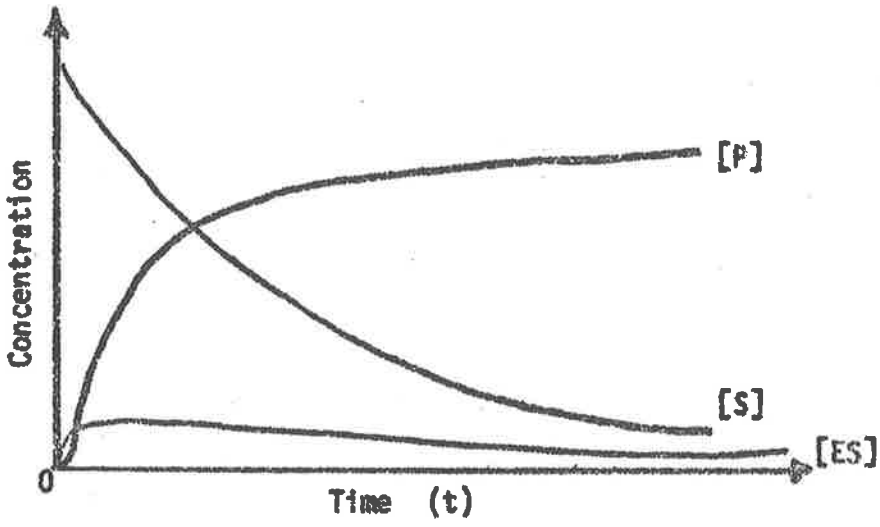

Fig. I.1 Plot of concentration of product [P], substrate [S] and intermediate [ES] against time. (Not to scale.)

4. Derivation of the Michaelis equation

MICHAELIS and MENTEN (1913) used equilibrium considerations in order to derive a relationship between initial velocity and substrate concentration. The argument presented by them is as follows.

Assuming that the reaction as described by equation I.1 has reached equilibrium, and that any effect of equation I.2 can be ignored, then by the Law of Mass Action

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] = 0 \qquad \ldots \text{I.8}$$

hence $$\frac{k_{-1}}{k_1} = \frac{[E][S]}{[ES]} \qquad \ldots \text{I.9}$$

but $$\frac{k_{-1}}{k_1} = K_s$$

the equilibrium constant for the dissociation of enzyme-substrate complex into free enzyme and substrate.

Assuming further that the substrate concentration is so much greater than that of the complex that [S] can be regarded as effectively constant, then by replacing [E] with $[E_0]$ - [ES] as in equation I.6 and rearranging I.9 we obtain

$$[ES] = \frac{[E_0][S]}{K_3 + [S]} \qquad \ldots \text{I.10}$$

In equation I.5 we have the rate of product formation given by

$$\frac{d[P]}{dt} = k_2[ES]$$

Substituting for [ES] the expression given in I.10, we get the Michaelis equation

$$\frac{d[P]}{dt} = \frac{k_2[E_0]}{1 + \frac{K_s}{[S]}} \qquad \ldots \text{ I.11}$$

Although the assumption of equilibrium may not hold for many reactions, a large number of enzymes appear to behave in this way.

5. Steady state theory

The same form of relationship as that given in equation I.11 was derived by BRIGGS and HALDANE (1925) using the assumption that the reaction was in the steady state stage (ref. section 3), that is, that

$$\frac{d[ES]}{dt} = 0$$

They also assumed, with MICHAELIS and MENTEN (1913), that [S] remained constant. Hence, from equation I.4

$$k_1[E][S] = (k_{-1} + k_2)[ES] \qquad \ldots \text{I.12}$$

Replacing [E] by $[E_0]$ - [ES] and rearranging I.4, we obtain

$$[ES] = \frac{k_1[E_0][S]}{k_1[S] + (k_{-1} + k_2)} \qquad \ldots \text{I.13}$$

The initial velocity of product formation is given by equation I.6. Substituting for [ES] from I.5, we get

$$\frac{d[P]}{dt} = \frac{k_2[E_0]}{1 + \frac{1}{[S]} \cdot \frac{(k_{-1} + k_2)}{k_1}} \qquad \ldots \text{I.14}$$

Putting $K_m = \frac{k_{-1} + k_2}{k_1}$ gives

$$\frac{d[P]}{dt} = \frac{k_2[E_0]}{1 + \frac{K_m}{[S]}} \qquad \ldots \text{I.15}$$

The above is identical with the Michaelis equation I.11 except that K_s is replaced by K_m. If k_2 is small when compared with k_{-1} it may be neglected, and K_s is equal to K_m. In this case the equations I.11 and I.15 become identical.

6. Study of transient behaviour

The first direct proof of the existence of enzyme-substrate compounds as intermediates of enzyme catalysis was provided by the experiments of CHANCE (1948), who observed the decomposition of peroxide by peroxidase during the very early stages of the reaction.

Very few enzymes are suitable for this kind of study, since the transient stages are usually of the order of msecs. This, together with the fact that some enzymes are unstable or inhibited by the reaction products, has led to most emphasis being placed on the study of the 'initial' rates of enzyme reactions. These initial rates are in most cases the steady state velocities.

7. Integrated rate equations

One approach to finding a simple equation to approximate the time course of the reaction following the Michaelis-Menten model is to integrate the steady state equation with respect to time.

Assuming [ES] is small, as before, we may put

$$[S] = [S_0] - [P] \qquad \ldots \text{I.16}$$

where $[S_0]$ is the initial concentration of substrate. Substitution for [S] in equation I.15 and rearrangement gives

$$\frac{d[P]}{dt} = \frac{k_2[E_0]}{1 + \frac{K_m}{[S_0] - [P]}} \qquad \ldots \text{I.17}$$

and integration gives

$$\int \left(1 + \frac{K_m}{[S_0] - [P]}\right) d[P] = k_2[E_0]t \qquad \ldots \text{I.18}$$

$$\text{i.e.} \quad k_2[E_0]t = [P] + K_m \ln\left(\frac{[S_0]}{[S_0] - [P]}\right) \qquad \ldots \text{I.19}$$

An equation of similar form was actually proposed by HENRI (1902). Integrated rate equations have been used by a number of workers, including STURTEVANT (1955) and JOHNSTON and DIVEN (1969). However, their use is limited, since they are really only

applicable to that part of the experiment for which the steady state assumptions hold.

In general, to obtain information from data covering the entire time course of an enzymic reaction, a more basic approach is required. One such approach for a more complicated reaction model is described in Chapter V.

CHAPTER II

1. Enzymic reactions involving two substrates and two products

Reactions of the type

$$E + A + B \rightleftharpoons E + C + D \qquad \text{....II.1}$$

where E stands for enzyme, and A, B, C and D are substrates or products depending on the direction of the reaction, are frequently encountered in biochemical processes.

The formulation of a model for such a reaction is made difficult because of the many possible combinations of intermediate complexes which can be involved in the mechanism. The problem has been considered by DALZIEL (1957) and ALBERTY (1953,1958) in particular, and the subject has been reviewed by WONG and HANES (1962).

2. Liver alcohol dehydrogenase

Crystalline alcohol dehydrogenase prepared from horse liver catalyses a reaction of the type described in equation II.1, namely the reversible transfer of hydrogen from ethanol to nicotinamide-adenine dinucleotide (NAD), producing reduced NAD (NADH) and acetaldehyde thus:

$$E + CH_3CH_2OH + NAD \rightleftharpoons E + H^+ + CH_3CHO + NADH \qquad \text{... II.2}$$

3. The Theorell-Chance mechanism

Theorell and his co-workers [THEORELL and BONNICHSEN (1951), THEORELL and CHANCE (1951), THEORELL, NYGAARD and BONNICHSEN (1955)] have formulated the following reaction mechanism for this enzyme:

$$\left.\begin{array}{l} \mathrm{NADH} + \mathrm{E} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{NADH.E} \\ \mathrm{NADH.E} + \mathrm{CH_3CHO} + \mathrm{H^+} \underset{k_6}{\overset{k_4}{\rightleftharpoons}} \mathrm{NAD.E} + \mathrm{CH_3CH_2OH} \\ \mathrm{NAD.E} \underset{k_5}{\overset{k_3}{\rightleftharpoons}} \mathrm{NAD} + \mathrm{E} \end{array}\right\} \quad \ldots \text{ II.3}$$

Because of the nature of chemical change, the following constraints are implied:

$$\left.\begin{array}{l} [\mathrm{NAD}] + [\mathrm{NAD.E}] + [\mathrm{NADH.E}] + [\mathrm{NADH}] = C_1 \\ [\mathrm{E}] + [\mathrm{NAD.E}] + [\mathrm{NADH.E}] = C_2 \\ [\mathrm{CH_3CH_2OH}] + [\mathrm{CH_3CHO}] = C_3 \\ [\mathrm{CH_3CHO}] = [\mathrm{NADH}] + [\mathrm{NADH.E}] \end{array}\right\} \quad \ldots \text{ II.4}$$

Square brackets indicate concentrations as in Chapter I; C_1, C_2 and C_3 are constants.

4. Mathematical formulation of the Theorell-Chance mechanism

Putting
- x = concentration of NADH
- w = concentration of NADH.E
- u = concentration of CH_3CHO
- z = concentration of NAD.E
- v = concentration of CH_3CH_2OH
- y = concentration of NAD
- e = concentration of enzyme

application of the Law of Mass Action to equations II.3 gives the set of differential equations

$$\left.\begin{aligned}
\frac{dx}{dt} &= -k_1 ex + k_2 w \\
\frac{dw}{dt} &= k_1 ex - k_2 w - k_4 wu + k_6 zv \\
\frac{du}{dt} &= -k_4 wu + k_6 zv \\
\frac{dz}{dt} &= k_4 wu - k_6 zv - k_3 z + k_5 ey \\
\frac{dv}{dt} &= k_4 wu - k_6 zv \\
\frac{dy}{dt} &= k_3 z - k_5 ey
\end{aligned}\right\} \quad \ldots \text{II.5}$$

Assuming that the course of the reaction is to be studied in a system where initially only NAD, ethanol and enzyme are present, the equations II.4 may be written in the form

$$\left.\begin{aligned} x + w + z + y &= y_0 \\ e + w + z &= e_0 \\ u + v &= v_0 \\ u &= x + w \end{aligned}\right\} \quad \ldots \text{II.6}$$

where y_0, e_0 and v_0 are the initial concentrations of NAD, enzyme and ethanol respectively.

For technical reasons explained in Chapter III, it is desirable to introduce the variable

$$\theta = x + w$$

and substitute θ-w for x in equations II.5 and II.6. Performing this substitution and simplifying the two sets of equations II.5 and II.6 gives rise to the reduced system

$$\left.\begin{aligned} \frac{d\theta}{dt} &= -k_4 w\theta + k_6(y_0-\theta-y)(v_0-\theta) \\ \frac{dw}{dt} &= k_1(\theta-w)(e_0-(y_0-\theta-y) - w) - k_2 w + \frac{d\theta}{dt} \\ \frac{dy}{dt} &= k_3(y_0-\theta-y) - k_5 y(e_0 - (y_0-\theta-y) - w) \end{aligned}\right\} \quad \ldots \text{II.7}$$

Details of the simplification are given in Appendix II.

5. Estimation of the velocity constants

THEORELL *et al.* (1955) have estimated the velocity constants in this mechanism by studying *initial rates of reaction* under different experimental conditions.

An alternative method of estimation would be to obtain data on the *entire course of the reaction* and fit equations II.7 to the results. Such data and methods of fitting the equations using this approach are described in detail in the following chapters.

CHAPTER III

1. Spectrophotometric methods

Information about the course of a reaction may be obtained by monitoring the concentration of one of the reactants. Spectrophotometry is one method of doing this for certain types of reactions. This procedure depends on the fact that some compounds in solution absorb light of different wavelengths and that the amount of light absorbed, that is, the optical density, is proportional to the concentration of the compound (Beer's Law) [UMBREIT, BURRIS and STAUSSER (1957)].

In the case of the reaction catalysed by liver alcohol dehydrogenase, it is possible to monitor the concentration of NADH, which has a maximum light absorption at 340 nm. At that wavelength, light is also absorbed by the second intermediate complex NADH.E, so that the optical density recorded actually indicates approximately the sum of NADH and NADH.E. It is for this reason that equations II.7 are expressed in terms of concentrations of NAD, NADH.E and NADH.E + NADH.

2. Form of data

All the data referred to in this study were obtained using a UNICAM SP 800 recording spectrophotometer. With this instrument, continuous graphs of optical density against time are produced. An example of such a record is shown in Figure III.1. Each curve represents, in our case, the production of NADH in a different reaction mixture. Because of the small scale of the chart, it is difficult to read points from the curves with sufficient accuracy. For this reason the result was also recorded on a Servoscribe unit (57718), which permitted an expansion of the scales of both the optical density and time, as shown in Figure III.2.

From the expanded chart, optical density values were read at every vertical division and the values obtained converted to concentrations of NADH using the relationship

$$\text{NADH conc. (in } \mu\text{M)} = \frac{\text{optical density reading}}{0.00623}$$

as reported by GUTFREUND (1967). The resulting tables of time *versus* concentration of NADH are given in Appendix III.

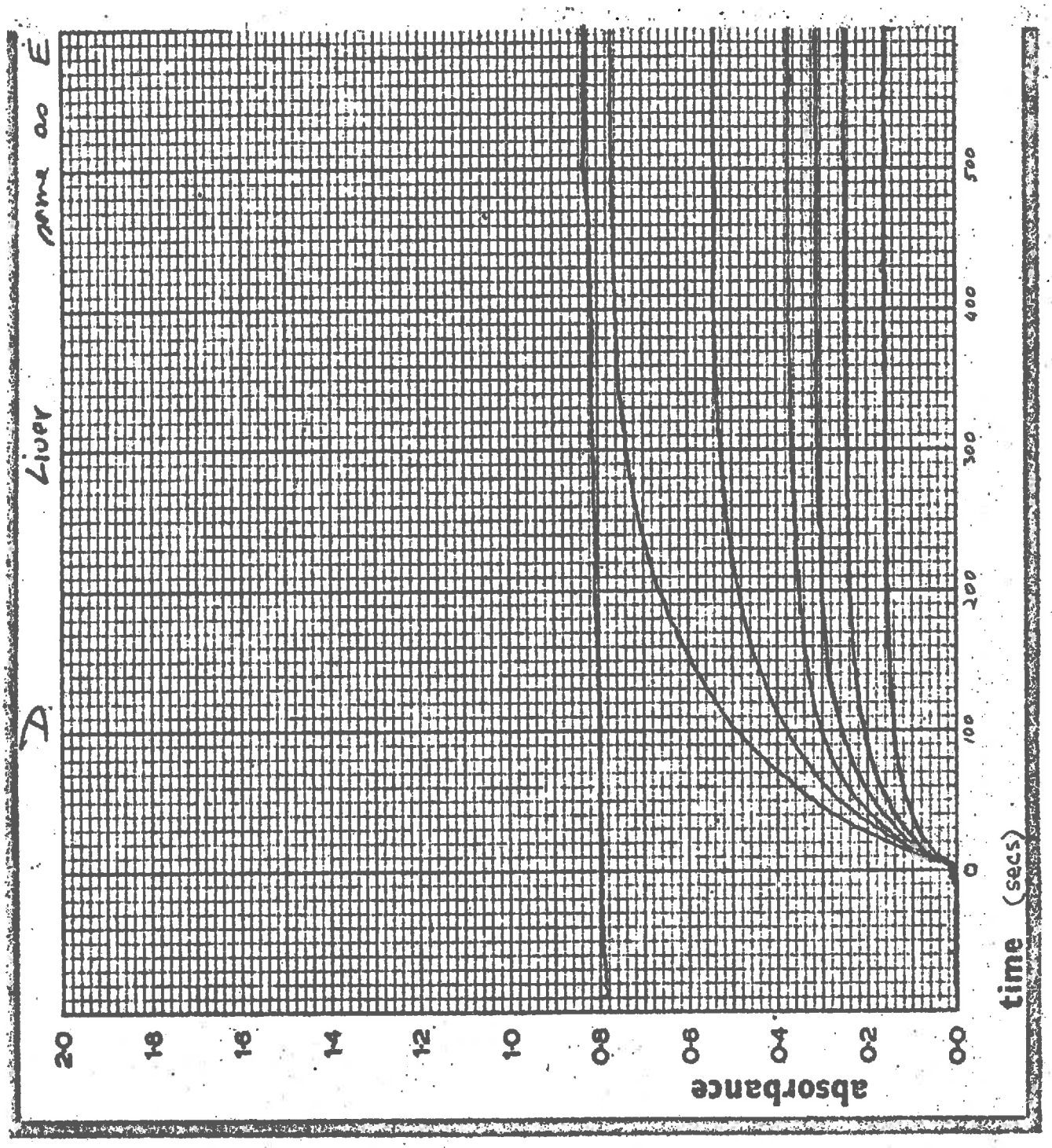

FIG. III.1 Standard form of output from SP.800 Spectrophotometer.

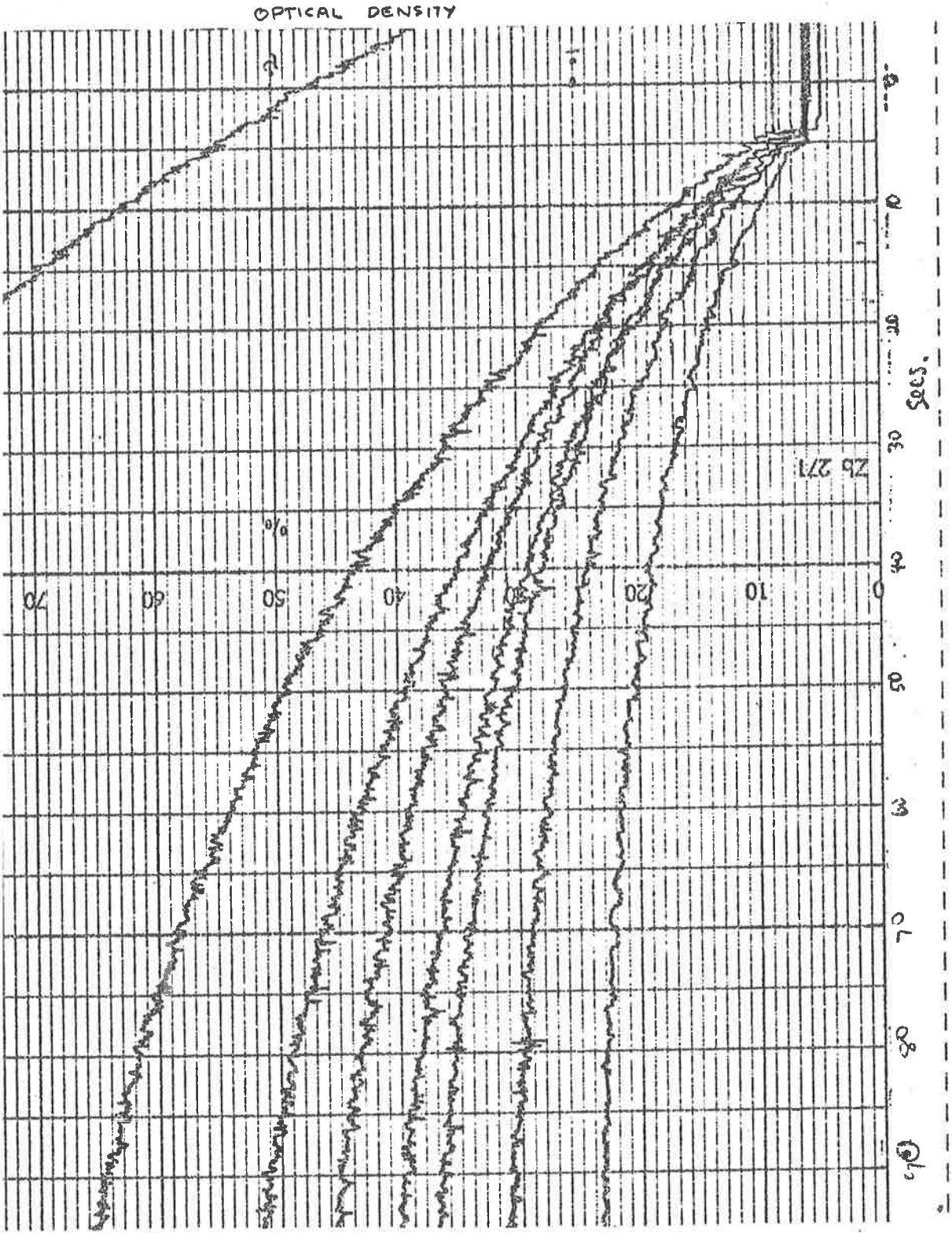

FIG III.2 Output from SP.800 Spectrophotometer recorded with expanded scales by a Servoscribe unit.

3. Experimental method

The action of liver alcohol dehydrogenase was investigated under the following conditions: 23°C, 0.05 M phosphate buffer (pH 7.15), 0.1 M ethanol. The enzyme used was Calbiochem A-grade horse liver alcohol dehydrogenase (Lot 900323), 10 mg/ml crystalline in 0.02 M phosphate buffer.

Ten microlitres of this solution were added with a 10 µl Hamilton syringe (Scientific Glass Engineering Pty. Ltd., Melbourne, Australia) to the reaction mixture in a 3 ml cuvette to provide a concentration of the enzyme of 33 µg/ml in the final volume.

Assuming that all the protein was active enzyme, and given that its molecular weight is approximately 73,000, with two active sites per molecule [THEORELL, NYGAARD and BONNICHSEN (1955)], the maximum concentration of enzyme binding sites is calculated to be about 0.9 µM. However, because there will almost certainly be inactive protein present, the actual concentration will be less than this calculated value.

Keeping the above experimental factors constant, a set of curves was obtained showing NADH production with time for the initial levels of NAD concentration in µM: 40, 60, 80, 100, 120, 160, 240.

Using freshly prepared solutions of the reactants, a duplicate set of curves was obtained. Thus, for each initial

NAD concentration, duplicate curves were available for NADH production with time. In this way, some idea of the probable size of the experimental errors involved in the method was obtained.

4. Sources of error

The main possible sources of error, apart from variations in the performance of the recording devices of the spectrophotometer, lie in the measurement of the volumes of the reactants added to the reaction mixture, and variation in the activity of the enzyme added. The amounts of NAD and alcohol added are relatively large compared to the probable errors of measurement of these quantities; but in the case of the enzyme, the solution volume of which is measured in a syringe accurate to ± 0.1 µl, the amount added is only 10 µl, so that the variation in enzyme concentration may be significant. Another factor affecting enzyme concentration involves the instability of the enzyme itself, which may lose activity with time.

CHAPTER IV

1. Duration of transient phase

The duration of the transient phase may be approximated in the following way.

Since initially only NAD, alcohol and enzyme are present, we have, using the notation of Chapter II, section 4

$$x = w = u = z = 0 \qquad \text{when } t = 0$$

and the initial rate of build up of the intermediate z from equations II.5 is given by

$$\frac{dz}{dt} = k_5 e_0 y_0 \qquad \ldots \text{IV.1}$$

Integrating, we get

$$z = k_5 e_0 y_0 t$$

for small t. Now the build up of z causes an increase in w so that

$$\frac{dw}{dt} = k_6 z v_0 \qquad \ldots \text{IV.2}$$

and substituting for z gives

$$\frac{dw}{dt} = k_6 v_0 k_5 e_0 y_0 t \qquad \ldots \text{IV.3}$$

which gives on integration

$$w = k_6 v_0 k_5 e_0 y_0 \frac{t^2}{2} \quad \ldots \text{IV.4}$$

But initially z+w is approximately constant, so that

$$z \simeq k_5 e_0 y_0 t - k_6 v_0 k_5 e_0 y_0 \frac{t^2}{2} \quad \ldots \text{IV.5}$$

Now if the "steady state" has been reached, then $\frac{dz}{dt} = 0$ so that, differentiating z with respect to t, we get

$$k_5 e_0 y_0 - k_6 v_0 k_5 e_0 y_0 t = 0 \quad \ldots \text{IV.6}$$

$$\text{i.e.} \quad t = \frac{1}{k_6 v_0} \quad \ldots \text{IV.7}$$

provided that the time is short enough for the assumptions to hold. The value of z reached in this time is given by

$$z_s = \frac{1}{2} k_5 e_0 y_0 \cdot \frac{1}{k_6 v_0} \quad \ldots \text{IV.8}$$

The value of w will take longer to reach its steady state. Putting the steady state value into the equations for $\frac{dw}{dt}$ in equations II.5, and assuming that the value of x is still negligible, we get

$$\frac{dw}{dt} = -k_2 w - k_4 w^2 + \frac{1}{2} k_6 \cdot \frac{k_5 e_0 y_0}{k_6 v_0} (v_0 - w)$$

$$\text{i.e.} \quad \frac{dw}{dt} = \frac{1}{2} k_5 e_0 y_0 - w\left(k_2 + \frac{1}{2} k_5 e_0 \frac{y_0}{v_0}\right) - k_4 w^2 \quad \ldots \text{IV.9}$$

In the steady state, $\frac{dw}{dt} = 0$ and the resulting equation in w can be solved to give the approximate steady state value of w

$$w_s = \frac{(k_2 + k_5\frac{e_0y_0}{2v_0}) \pm \left[(k_2 + k_5\frac{e_0y_0}{2v_0})^2 + 2k_4k_5e_0y_0\right]^{\frac{1}{2}}}{-2k_4} \quad \ldots \text{ IV.10}$$

Using the equation IV.7 and inserting the value for k_6 (0.0035) estimated by THEORELL, NYGAARD and BONNICHSEN (1955), we find that the time taken for the reaction to attain the steady state is of the order of 3 msecs. Thus, the steady state will be reached before the earliest observation of product concentration can be made with the recording spectrophotometer used in this work.

2. Steady state solution

If one assumes that the steady state has been reached, then both $\frac{dw}{dt}$ and $\frac{dz}{dt}$ may be set equal to zero, and the equations II.7 can be simplified since now w and z are constants with values w_s and z_s respectively. Hence, from equations II.7 we have

$$\frac{d\theta}{dt} = -k_4 w_s \theta + k_6 z_s (v_0 - \theta) \qquad \ldots \text{IV.11}$$

The solution of the above equation (see Appendix IV) gives an equation of the form

$$\theta = a\left(1 - e^{-b(t+k)}\right) \qquad \ldots \text{IV.12}$$

3. A modification to the steady state solution

Although $\frac{dw}{dt}$ becomes very small compared with $\frac{d\theta}{dt}$ and $\frac{dy}{dt}$, the zero approximation used in section 2 above may be improved by an assumption of the form

$$\frac{dw}{dt} = gt \qquad \ldots \text{IV.14}$$

where g is a small constant. Substituting this value of the derivative in II.7 (2), we get

$$gt = k_1(\theta - w_s)(e_0 - w_s - z_s) - k_2 w_s + \frac{d\theta}{dt} \qquad \ldots \text{IV.15}$$

Solution of this equation (see Appendix IV) gives an equation of the form

$$\theta = A\left(1 - e^{-B(t+K)}\right) + Dt \qquad \ldots \text{IV.16}$$

4. Least squares estimation of the parameters

The parameters of the equations IV.12 and IV.16 were estimated by the method of least squares using the simplex technique described by NELDER and MEAD (1965). A copy of the computer program used, PROGRAM HOPE, is given in Appendix V.

4.1 Three-parameter equation

The method of estimation used requires initial estimates of the parameters. The asymptotic value of θ provides an estimate of the parameter a, and since this value may be read from the curve, a direct estimate is available. An estimate of b can be obtained using the value of a estimated as above and plotting the linear relationship of $\ln(a - \theta)$ against time (t), since taking logarithms of both sides of equation IV.12 gives

$$\ln(a - \theta) = \ln a - b(t+k)$$

and hence the slope of the line is an estimate of b. In

section 1 of this chapter it is shown that the transient phase is about 3 msecs, so that value may be used for k initially.

From these starting values, least squares estimates for a, b and k can be obtained by the iterative procedure employed in PROGRAM HOPE. This was carried out using the liver enzyme data (Rep. 2) in Appendix III. The results are shown in Table IV.1. The points on the resulting fitted curves are given in Appendix III.

4.2 Four-parameter equation

Initial values of A, B and K in equation IV.16 were based on the estimates of a, b and k above for the corresponding sets of data. D was given an initial value of 0.01.

The resulting estimates of the parameters A, B, K and D are given in Table IV.2 and the points on the corresponding fitted curves are given in Appendix III.

TABLE IV.1

Estimated values of a, b and k in the equation

$$\theta = a\left[1 - e^{-b(t+k)}\right]$$

$[S_0]$	Rep.	a	b	k	Residual Mean Square	d.f.
40 µM	1	15.577	0.0345	4.556	0.0177	17
	2	15.939	0.0327	5.394	0.0461	17
60 µM	1	21.385	0.0298	4.756	0.0979	19
	2	21.069	0.0280	5.043	0.0762	19
80 µM	1	25.596	0.0274	4.651	0.1177	23
	2	25.461	0.0250	5.854	0.1348	23
100 µM	1	28.9033	0.0231	5.038	0.1794	21
	2	29.4353	0.0235	5.079	0.1350	21
120 µM	1	33.061	0.0224	4.626	0.1167	22
	2	33.135	0.0217	5.429	0.1602	22
160 µM	1	38.628	0.0203	3.394	0.0538	18
	2	38.518	0.0208	3.747	0.1102	18
240 µM	1	49.592	0.0194	2.694	0.0730	19
	2	49.090	0.0178	4.008	0.0755	19

TABLE IV.2

Estimated values of A, B, C and D in the equation

$$\theta = A\left[1 - e^{-B(t+K)}\right] + Dt$$

$[S_0]$	Rep.	A	B	K	D	Residual Mean Square	d.f.
40 µM	1	14.391	0.0393	3.994	0.0099	0.0106	16
	2	13.925	0.0417	3.941	0.0165	0.0321	16
60 µM	1	18.381	0.0371	3.931	0.0205	0.0494	18
	2	17.965	0.0360	3.902	0.0223	0.0278	18
80 µM	1	23.222	0.0322	3.846	0.0153	0.0619	22
	2	22.076	0.0323	4.012	0.0207	0.0489	22
100 µM	1	24.208	0.0304	3.372	0.0286	0.1257	20
	2	24.717	0.0314	3.007	0.0287	0.0814	20
120 µM	1	30.182	0.0255	4.022	0.0162	0.0846	21
	2	29.124	0.0266	4.064	0.0222	0.1169	21
160 µM	1	37.165	0.0207	3.999	0.0104	0.0664	17
	2	34.150	0.0233	4.011	0.0284	0.0968	17
240 µM	1	49.824	0.0185	4.013	0.0017	1.6359	18
	2	47.116	0.0185	3.997	0.0102	0.0858	18

CHAPTER V

1. Numerical solutions

An alternative approach to the estimation of the velocity constants of the Theorell-Chance mechanism, which does not require any simplifying assumptions, is to attempt a numerical solution of the system of differential equations shown in II.7. There exists a number of well known methods for the numerical solution of simultaneous differential equations [see, for instance, McCRACKEN and DORN (1969), RICHTMEYER and MORTON (1967)].

The criterion upon which a method should be chosen for this work is primarily that of speed of computation, since it may be necessary to produce a large number of iterative solutions using different values of the velocity constants during the estimation process. For this reason Euler's method was used. The computer program used to carry out the calculations, PROGRAM PLAIN, is included in Appendix V.

2. Method of estimation of the velocity constants

Because the concentration of enzyme binding sites is difficult to determine precisely (see Chapter III, section 3.4), it was decided to regard enzyme concentration as an extra parameter to be estimated. Hence, the values of seven parameters

in equations II.7 — e_0, k_1, k_2, k_3, k_4, k_5 and k_6 — were determined.

Initial estimates of the six velocity constants were those of THEORELL, NYGAARD and BONNICHSEN (1955), and the initial estimate of enzyme concentration was taken as the maximum possible value calculated in Chapter III, section 3. Using an initial substrate concentration of 160 μM and the above initial estimates of the constants, it was found that the calculated numerical solution did not agree well with the experimental data.

It was noted that the values of the velocity constants suggested by Theorell *et al.* gave a calculated equilibrium constant

$$K_{eq} = \frac{k_2 k_5 k_6}{k_1 k_3 k_4} = 0.37 \times 10^{-11}$$

a value lower than their mean value obtained from equilibrium determination, 0.86×10^{-11}. Hence, it seemed reasonable to adjust some of the values of the velocity constants to bring the calculated equilibrium constant closer to the experimental value.

Small changes were made in turn to the value of each constant and to the enzyme concentration parameter and solutions calculated for the equations II.7. This gave an indication of the effect of the parameters on the shape of the solution curve and permitted the selection of a new set of parameters to

TABLE V.1

Comparison of values of the velocity constants for liver alcohol dehydrogenase obtained by the iterative method with those obtained by Theorell *et al.*

Constant	Theorell's method	Iterative method
k_1 ($\mu M^{-1} sec^{-1}$)	3.7	3.7
k_2 (sec^{-1})	1.6	2.1
k_3 (sec^{-1})	37.0	37.0
k_4 ($\mu M^{-1} sec^{-1}$)	0.24	0.23
k_5 ($\mu M^{-1} sec^{-1}$)	0.30	0.30
k_6 ($\mu M^{-1} sec^{-1}$)	0.0035	0.0070

The initial enzyme concentration was estimated by the iterative method to be 0.63 μM.

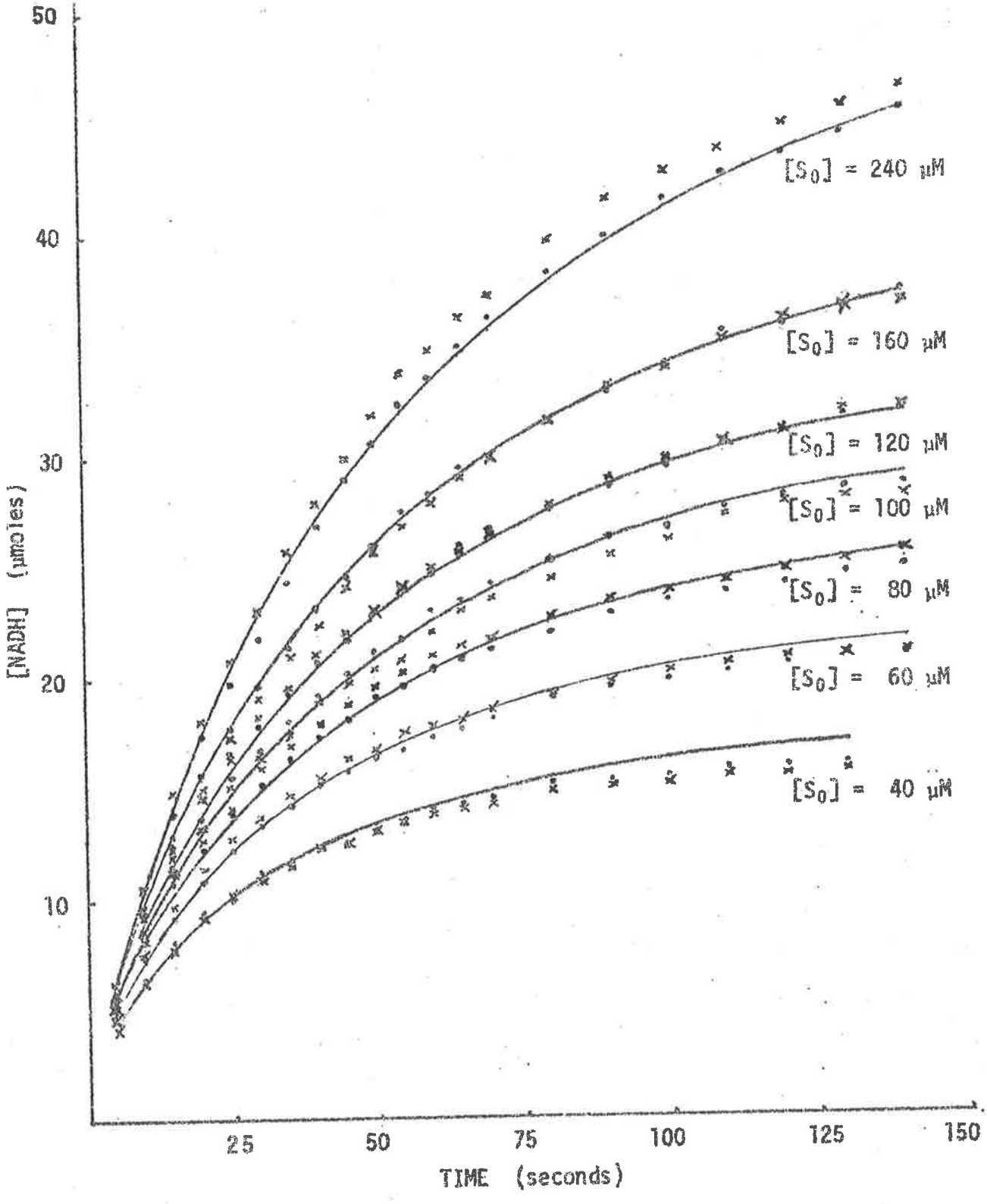

FIG. V.1 Predicted values of NADH concentration
$[E_0]$ = 0.63 μM, (rate constants as in text)

——— predicted value
× observed value, replicate I
· observed value, replicate II

CHAPTER VI

1. Mechanism of action of yeast alcohol dehydrogenase

The enzyme from yeast also catalyses the transfer of hydrogen from ethanol to NAD. NYGAARD and THEORELL (1955) showed that the Theorell-Chance mechanism for the liver enzyme did not apply to the yeast enzyme, and they postulated the alternative scheme outlined in equations VI.1.

$$\left.\begin{array}{llll} E + S_1 \rightleftharpoons ES_1 & & EP_1P_2 \rightleftharpoons EP_1 + P_2 \\ E + S_2 \rightleftharpoons ES_2 & & EP_1P_2 \rightleftharpoons EP_2 + P_1 \\ ES_1 + S_2 \rightleftharpoons ES_1S_2 & & EP_2 \rightleftharpoons E + P_2 \\ ES_2 + S_1 \rightleftharpoons ES_1S_2 & & EP_1 \rightleftharpoons E + P_1 \\ ES_1S_2 \rightleftharpoons EP_1P_2 & & \end{array}\right\} \quad \ldots \text{ VI.1}$$

where S_1 = ethanol, S_2 = NAD, P_1 = aldehyde, P_2 = NADH and E = enzyme. However, the inhibition studies with the yeast enzyme by HOCH, WILLIAMS and VALLEE (1958) gave results which were not incompatible with the Theorell-Chance mechanism. Using the method of calculation described by THEORELL, NYGAARD and BONNICHSEN (1955), Hoch and his co-workers obtained estimates of the velocity constants for the Theorell-Chance mechanism for the yeast enzyme.

2. Experimental method

Using the same methods employed in the study of liver enzyme (described in Chapter III), data were obtained on the action of yeast alcohol dehydrogenase under the following conditions: 23°C, 0.05 M phosphate buffer (pH 7.3), 0.1 M ethanol.

The yeast enzyme was obtained from the Sigma Chemical Company, St. Louis, Mo., U.S.A. (Stock no. 340-28, Lot. no. 513-9021-9). A stock solution of 1.0 mg/ml of buffer was prepared. Since the solid contained 33% w/v sucrose and 67% w/v protein, this gave an enzyme concentration of 2.2 µg protein/ml when 10.0 µl of the stock solution were added to the reaction mixture as previously described. Taking the molecular weight of the enzyme as 151,000, with four active sites per molecule [KAGI and VALLEE (1960)], the approximate concentration of active sites is calculated to be 0.05 µM.

Data were obtained for the following initial levels of NAD (in µM): 80.0, 160.0, 320.0, 640.0. The resulting tables of NADH concentration *versus* time are given in Appendix III.

3. Estimation of the velocity constants

Using the iterative method described in Chapter V, values were obtained for the velocity constants based on the data for initial substrate level 160.0 μM NAD and assuming the Theorell-Chance mechanism. The values obtained are compared in Table VI.1 with values calculated by HOCH *et al.* (1958) and with other values estimated by them from inhibition studies.

When numerical solutions of equations II.7 were calculated for other initial substrate levels, using the velocity constants based on the 160.0 μM NAD data, systematic deviations of the solutions from the experimental results were observed (see Figure VI.1). These deviations were eliminated by using a different value of enzyme concentration for each initial substrate level, as shown in Table VI.2. The calculated curves based on these enzyme concentrations appear in Figure VI.2. The calculated values used in Figures VI.1 and VI.2 are given in Appendix III.

No attempt was made to fit the more complex model set out in equations VI.1, since experimentally determined estimates of the 18 velocity constants involved were not available.

TABLE VI.1

Comparison of values of the velocity constants for yeast alcohol dehydrogenase (assuming a Theorell-Chance mechanism) obtained by three different methods.

Constant	Method of Nygaard and Theorell*	Inhibition studies*	Iterative method (in Chapter V)
k_1 ($\mu M^{-1} sec^{-1}$)	9.1	6.0	9.1
k_2 (sec^{-1})	21.0	27.0 90.0	85.0
k_3 (sec^{-1})	2100.0	4100.0 2200.0	210.0
k_4 ($\mu M^{-1} sec^{-1}$)	3.3	4.7	3.3
k_5 ($\mu M^{-1} sec^{-1}$)	0.82	0.57	0.82
k_6 ($\mu M^{-1} sec^{-1}$)	0.025	0.013	0.025

* from Hoch, Williams and Vallee (1958)

TABLE VI.2

Estimated values of enzyme concentration corresponding to initial substrate concentrations (assuming a Theorell-Chance mechanism).

Initial NAD Conc. (μM)	Estimated Enzyme Conc. (μM)
80.0	0.090
160.0	0.095
320.0	0.105
640.0	0.110

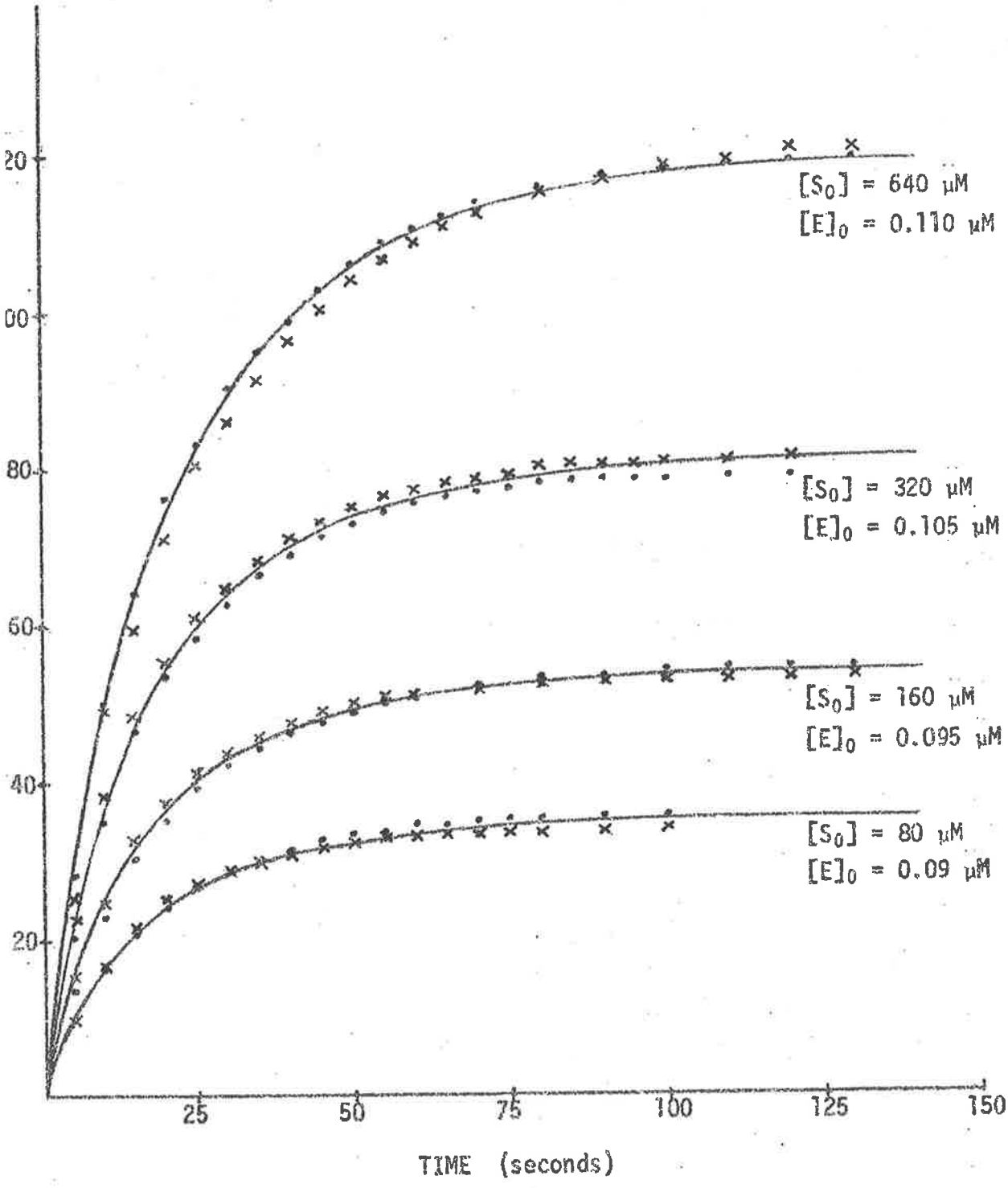

FIG. VI.2 Predicted values of NADH concentration, using different levels of initial enzyme concentration.

——— predicted value
x observed value, replicate I
. observed value, replicate II

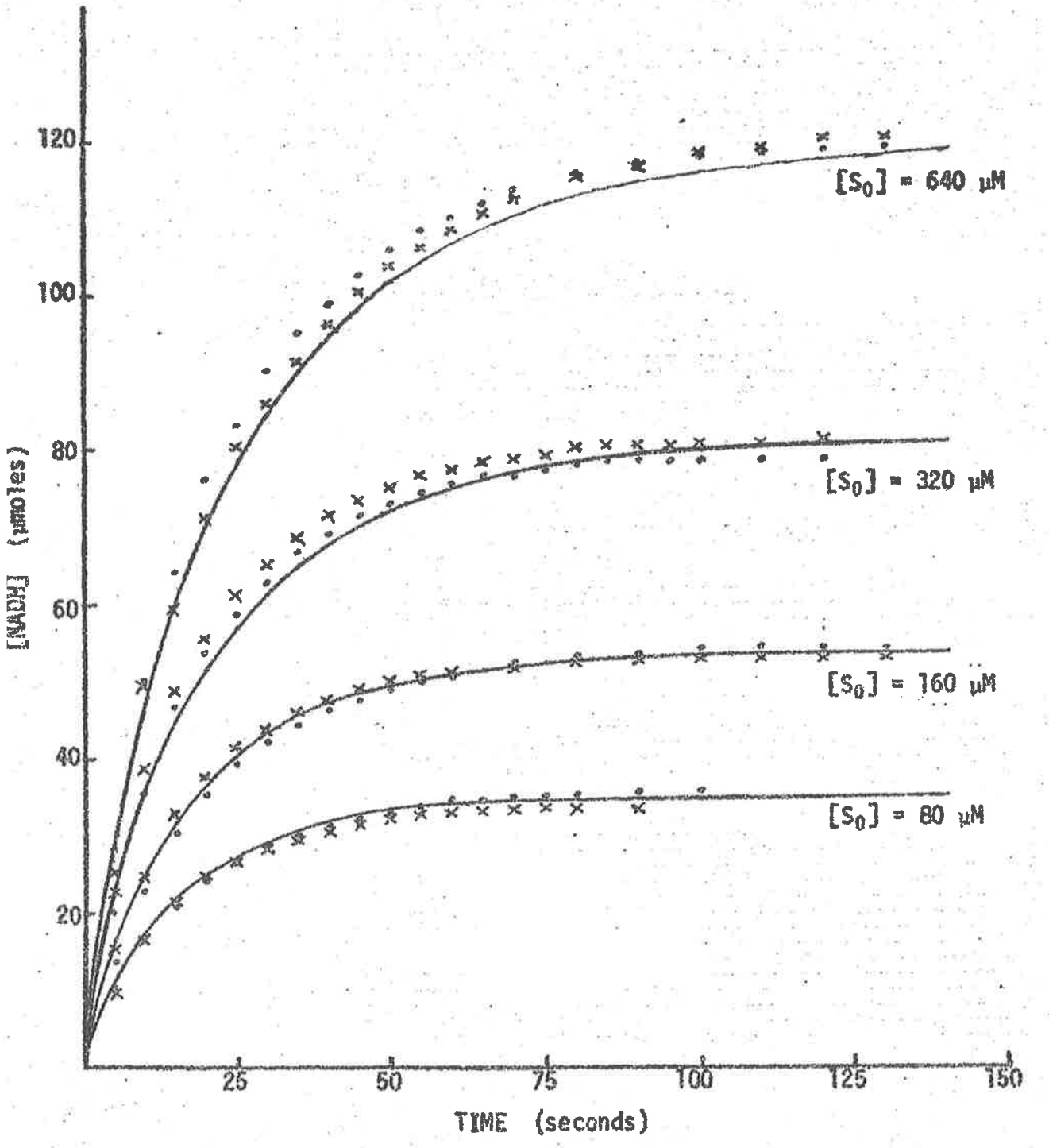

FIG. VI.1 Predicted values of NADH concentration assuming a constant level (0.095 μM) of initial enzyme concentration.

——— predicted value
x observed value, replicate I
. observed value, replicate II

CHAPTER VII

1. Fitting of exponential curves to the data

Although the steady state assumptions made in Chapter IV, sections 2 and 3 lead to explicit solutions of the differential equations II.7, and the resulting exponential equations (IV.12, IV.16) may be made to fit the experimental data very closely (see Tables IV.1, IV.2 and Appendix III), the interpretation of the parameters obtained in this way presents several difficulties.

Firstly, a choice must be made between the *three-parameter model* (Equation IV.12) and the *four-parameter model* (Equation IV.16). Normally, the introduction of an extra parameter into a regression equation which is to be fitted by the method of least squares leads to a reduction of the residual sum of squares. To determine whether the reduction is a significant one, the ratio

$$\frac{\left(\begin{array}{c}\text{Residual Sum of Squares}\\ \text{with three parameters}\end{array}\right) - \left(\begin{array}{c}\text{Residual Sum of Squares}\\ \text{with four parameters}\end{array}\right)}{\text{Residual Mean Square with four parameters}}$$

is calculated. This ratio is distributed as F^{1}_{n-4} (where n denotes the number of data points) and it may be tested for significance from known probability tables in the usual way.

It will be noted from Table VII.1 that, for all sets of data with initial substrate levels of 120 μM or less, the addition

TABLE VII.1

Test of significance of the reduction in the residual sum of squares obtained by fitting the four-parameter equation $\theta=A\left[1-e^{-B(t+K)}\right]+Dt$ to the experimental data instead of the three-parameter equation $\theta=a\left[1-e^{-b(t+k)}\right]$.

Initial substrate concentration (μM)	Replicate No.	F^1_{n-4} $(=\frac{RSS_3-RSS_4}{RMS_4})$	Significance level (%)	Degrees of freedom
40	1	44.41	0.1	16
40	2	8.39	5.0	16
60	1	19.67	0.1	18
60	2	12.82	1.0	18
80	1	21.71	0.1	22
80	2	41.38	0.1	22
100	1	9.59	1.0	20
100	2	14.83	0.1	20
120	1	9.50	1.0	21
120	2	9.15	1.0	21
160	1	< 0	n.s.	17
160	2	5.39	n.s.	17
240	1	0	n.s.	18
240	2	0	n.s.	18

of the fourth parameter significantly improves the fit of the model. However, for the higher levels (160 μM and 240 μM NAD), the addition of the fourth parameter actually increases the residual sum of squares in three cases and does not reduce it significantly in the fourth. This is accounted for by the shape of the curves at the higher substrate levels. The curves of NADH production with time are steeper, have less curvature and provide less information about the equilibrium level. In cases where a simplex method, such as that employed in PROGRAM HOPE, is used to fit a non-linear equation containing too many parameters for the information available in the data, this form of anomalous result is not uncommon [NELDER (1967)]. Hence, for the 240 μM and 160 μM results, the three-parameter model is the most detailed one that can be accepted. For the other sets of results, although the four-parameter equation provides a better fit, it may be more consistent to use the three-parameter model.

Secondly, it must be remembered that the steady state assumptions made in Chapter IV do not hold for the entire course of the reaction. Although the discrepancies between the steady state model and the Theorell-Chance mechanism will be slight in the initial stages of the reaction since the transient stage is short (Chapter IV, section 1), greater differences would be expected as the reaction approaches equilibrium. Inclusion of data outside the range of applicability of the steady state assumptions will produce invalid estimates of the parameters in

equations IV.12 and IV.16.

Finally, the major difficulty associated with this method is the interpretation of the estimated parameters in terms of the velocity constants. As can be seen in Appendix IV, the parameters in equations IV.12 and IV.16 are not only very complicated functions of the velocity constants, but also include constants of integration whose values are unknown.

2. Direct estimation of the velocity constants by numerical solution of the differential equations

This method, described in Chapter V, does not have the disadvantages outlined in the previous section, in that no simplifying assumptions are made and the velocity constants are obtained directly. Computing procedures for the solution of a set of non-linear differential equations such as II.7 are slow when compared with the method of least squares. The availability of a high-speed computer, such as the CDC 6400 used in this work, makes this approach a practical one provided the computer program is made as simple and efficient as possible. Because of the large number of iterations of the algorithm usually required, it is desirable to avoid the use of such time-consuming program operations as testing procedures.

An apparent major disadvantage of this method is that the errors of estimates of the constants cannot be statistically calculated along lines similar to the method described by NELDER and MEAD (1965) for the least squares procedure. The validity of such errors is doubtful in any event since a true estimate of error requires each data point to be independently obtained, a situation far from being realised in this experimental arrangement.

The sources of error in the method involving direct estimation of the reaction constants are twofold, the first involving the lack of fit of the calculated solution to the experimental data, which is directly regulated by the number of iterations performed, and the second associated with the errors involved in reading the observed data points. These two errors are confounded since neither is capable of direct and separate estimation. The first type of error is non-statistical, since the decision on when the fit is 'good enough' will depend upon some pre-determined stopping rule in the search for the values of the reaction constants. The second type of error, being calculated from the deviations of the observed points about the 'agreed' curve of best fit, is conditioned upon the first.

Considerations of the shape of the calculated curve, as well as the individual deviations of the data points from it, enter into the determination of when the search for the correct values of the constants is deemed complete; the method used here

has been one of plotting and visual inspection. As suggested in Chapter V, differences between pairs of replicated curves may be used to obtain the order of magnitude of the variation resulting from experimental errors. The process of adjustment of constants and recalculation of the solution provides some information as to the sensitivity of the model to changes in the constant values. This information, together with a knowledge of the experimental variation, may permit the construction of confidence intervals based upon subjective probabilities.

An alternative approach, when a number of replications are taken, consists of fitting the model to each replicate in turn to obtain sets of values of the velocity constant estimates. From such sets, a meaningful variance-covariance matrix for the velocity constants could then be calculated. Such an evaluation, although desirable, constitutes a project both large and complicated enough to be beyond the scope of this present work.

3. Mechanism of action of liver alcohol dehydrogenase

It can be seen from Figure V.1 that for initial substrate levels in the range 80 μM-240 μM NAD the agreement with the Theorell-Chance mechanism is close. Deviations shown by the 40 μM and 60 μM curves after an elapsed period of approximately

60 seconds could possibly be attributed to the instability of NADH in dilute solution.

The estimated values of k_1, k_3 and k_5 agree with those quoted by THEORELL *et al.* (1955). Comparison of the remaining velocity constants show varying deviation: k_2, 30% greater; k_4, 5% smaller and k_6, half the value given by Theorell. The estimated enzyme concentration (0.63 μM) appears a reasonable value when compared with the theoretical maximum of 0.9 μM given in Chapter III, section 3. These results appear to support the Theorell-Chance mechanism for the liver enzyme.

4. Mechanism of action of yeast alcohol dehydrogenase

Although, as is seen in Figure VI.2, it is possible to get numerical solutions to equations II.7 which will fit the data from reactions catalysed by the yeast enzyme, a number of aspects of these solutions suggest that the Theorell-Chance mechanism may not apply in this instance.

From values of the velocity constants given in Table VI.1, it can be seen that the estimated value of k_3 is one-tenth that obtained by HOCH *et al.* (1958), while the estimated values of all other constants appear to agree reasonably closely. A more serious discrepancy involves the estimated enzyme concentration

(0.095 μM for the 160 μM data), which is almost twice that calculated in Chapter VI, section 2 as the maximum theoretical value, namely 0.05 μM. Again, examination shows that the estimated enzyme concentration for each experiment depends upon the initial substrate concentration.

While the significance of the discrepancy between the two values of k_3 is not clear, the values obtained for the initial enzyme concentration appear to lend support to the mechanism proposed by NYGAARD and THEORELL (1955). Part of this mechanism is shown diagrammatically in Figure VII.1. Since all the experiments were carried out in the presence of excess ethanol (0.1 M), the formation of the ternary complex would be expected to proceed largely through pathway I. An increase in the initial concentration of NAD would lead to a greater proportion of the enzyme being involved in pathway II.

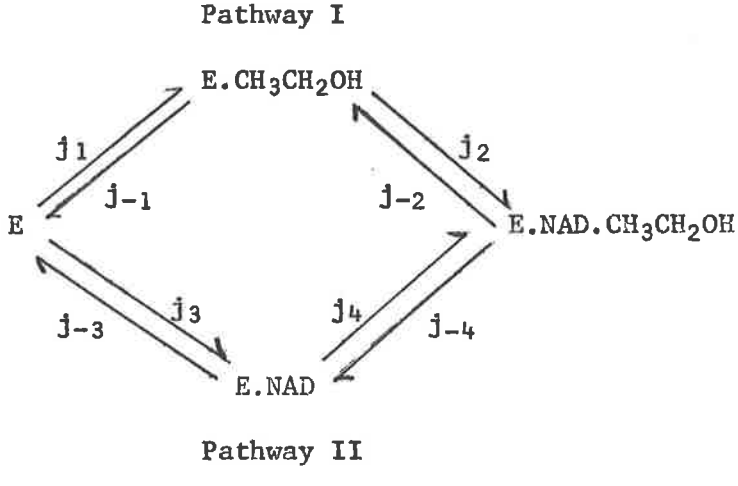

Fig. VII.1 Part of the proposed mechanism for the reaction of yeast alcohol dehydrogenase. (j_1, j_{-1} etc. are velocity constants).

If the velocity constants j_1 and j_2 are smaller than j_3 and j_4, the turnover of enzyme in pathway II will be greater, and hence an increased use of that pathway would lead to an increased apparent enzyme concentration.

5. Value of computer techniques in assessing reaction models

The use of the numerical techniques described in Chapter V is limited to those enzymes for which reaction mechanisms have been clearly formulated and for which experimental determinations of the appropriate velocity constants have been made. In such cases it is possible to take account of data on the time course of the reaction which are normally not used when initial rate studies are undertaken. Computer techniques such as those described in this thesis can be used to obtain information about the validity of the proposed reaction models from existing experimental data.

APPENDIX I

Form of solution of equations I.3 - I.5

The equations are given by (see page 4)

$$\frac{d[S]}{dt} = -k_1[E][S] + k_{-1}[ES] \qquad \ldots \text{I.3}$$

$$\frac{d[ES]}{dt} = k_1[E][S] - (k_1 + k_2)[ES] \qquad \ldots \text{I.4}$$

$$\frac{d[P]}{dt} = k_2[ES] \qquad \ldots \text{I.5}$$

If initially $[S] = [S_0]$, $[E] = [E_0]$ and $[P] = 0$, then $[ES]$ will be zero also; and hence when $t = 0$

$$\left(\frac{d[S]}{dt}\right)_{t=0} = -k_1[E_0][S_0]$$

$$\left(\frac{d[ES]}{dt}\right)_{t=0} = k_1[E_0][S_0]$$

$$\left(\frac{d[P]}{dt}\right)_{t=0} = 0$$

The rate of disappearance of substrate will be a maximum when $t = 0$, since at later times $[ES]$ will be greater than zero; hence, $[E]$ will be less than its initial value, and $[S]$ will be decreasing as the substrate is converted into product. The difference between $k_1[E][S]$ and $k_{-1}[ES]$ will tend to diminish so

that in the limit the concentration of substrate will attain a constant level.

Initially the concentration of intermediate complex [ES] will rise rapidly but, following a pattern similar to that of the substrate, it will tend to reach a constant level. This will always happen sooner for [ES] than for [S], since

$$(k_{-1} + k_2)[ES] \quad > \quad k_{-1}[ES]$$

How much sooner will depend on the relative sizes of k_{-1} and k_2.

The concentration of product [P] is initially zero, and its initial rate of production is zero also, since it is always proportional to [ES]. This implies that the rate reaches a constant value when [ES] reaches its constant value.

The concentration of intermediate actually falls gradually after reaching its maximum because there is continual conversion of substrate into product. Thus, the term $k_1[E][S]$ in equation I.4 becomes smaller than $[ES](k_{-1} + k_2)$, and $\frac{d[ES]}{dt}$ becomes negative.

APPENDIX II

Reduction of reaction model equations

From equations II.5 (page 14) we have

$$\frac{dx}{dt} = -k_1 ex + k_2 w \qquad (1)$$

$$\frac{dw}{dt} = k_1 ex - k_2 w - k_4 wu + k_6 zv \qquad (2)$$

$$\frac{du}{dt} = -k_4 wu + k_6 zv \qquad (3)$$

$$\frac{dz}{dt} = k_4 wu - k_6 zv - k_3 z + k_5 ey \qquad (4)$$

$$\frac{dv}{dt} = k_4 wu - k_6 zv \qquad (5)$$

$$\frac{dy}{dt} = k_3 z - k_5 ey \qquad (6)$$

with boundary conditions

$$x + y + z + w = y_0 \qquad (7)$$

$$e + w + z = e_0 \qquad (8)$$

$$u + v = v_0 \qquad (9)$$

$$u = x + w \qquad (10)$$

Putting

$$x = \theta - w \tag{11}$$

equations (10) and (9) become

$$u = \theta \quad \text{and} \quad v = v_0 - \theta \tag{12}$$

and from equation (7) we get

$$z = y_0 - y - \theta \tag{13}$$

hence from (8)

$$e = e_0 - w - y_0 + y + \theta \tag{14}$$

Adding equations (1) and (2) and substituting for v and z from equations (12) and (13) respectively, gives

$$\frac{d\theta}{dt} = -k_4 w\theta + k_6(y_0 - y - \theta)(v_0 - \theta) \tag{15}$$

Substituting for x and e in equation (2) gives

$$\frac{dw}{dt} = k_1(e_0 - w - y_0 + y + \theta)(\theta - w) - k_2 w + \frac{d\theta}{dt} \tag{16}$$

Similarly, equation (6) becomes

$$\frac{dy}{dt} = k_3(y_0 - y - \theta) - k_5(e_0 - w - y_0 + y + \theta)y \tag{17}$$

From equation (12)

$$\frac{du}{dt} = \frac{d\theta}{dt}$$

from (13)

$$\frac{dz}{dt} = \frac{-dy}{dt} - \frac{d\theta}{dt}$$

and from (12) also

$$\frac{dv}{dt} = \frac{-d\theta}{dt}$$

Thus the system has been reduced to a set of three equations [(15), (16) and (17)] in the three variables θ, w and y.

APPENDIX III

Experimental results and predicted concentrations of NADH obtained by the methods described in Chapters IV and V

LIVER ENZYME

$[NAD]_0 = 40\ \mu M$

Time (secs)	Concentration of NADH (μM)				
	Experimental values		Predicted values		
			Chapter IV (Rep. 2)		Chapter V
	Rep. 1	Rep. 2	3 Parameters	4 Parameters	
5	4.18	4.18	4.60	4.42	4.09
10	6.11	6.43	6.31	6.31	6.23
15	7.85	8.04	7.76	7.85	7.81
20	9.07	9.33	9.00	9.13	9.06
25	10.16	10.03	10.04	10.17	10.10
30	10.93	11.25	10.93	11.04	10.97
35	11.58	11.64	11.69	11.76	11.71
40	12.22	12.22	12.33	12.36	12.36
45	12.54	12.54	12.88	12.86	12.92
50	13.12	13.18	13.34	13.28	13.42
55	13.57	13.51	13.73	13.64	13.87
60	13.83	14.15	14.06	13.95	14.26
65	14.08	14.28	14.35	14.21	14.61
70	14.21	14.47	14.59	14.44	14.92
80	14.79	15.05	14.96	14.83	15.45
90	14.92	15.11	15.24	15.13	15.88
100	15.18	15.43	15.43	15.39	16.23
110	15.43	15.76	15.57	15.62	16.52
120	15.47	15.76	15.68	15.83	16.76
130	15.50	15.88	15.75	16.02	16.95

LIVER ENZYME

$[NAD]_0$ = 60 μM

Time (secs)	Concentration of NADH (μM)				
	Experimental values		Predicted values		
			Chapter IV (Rep. 2)		Chapter V
	Rep. 1	Rep. 2	3 Parameters	4 Parameters	
5	4.63	4.57	5.17	5.04	4.63
10	7.59	7.40	7.25	7.30	7.23
15	9.78	9.20	9.05	9.21	9.20
20	11.58	10.93	10.63	10.82	10.78
25	12.80	12.28	11.99	12.18	12.11
30	13.83	13.50	13.18	13.34	13.24
35	14.79	14.47	14.21	14.32	14.22
40	15.43	15.11	15.11	15.17	15.07
45	16.27	15.88	15.89	15.89	15.83
50	16.85	16.53	16.56	16.51	16.49
55	17.43	16.85	17.15	17.04	17.10
60	17.88	17.43	17.67	17.51	17.63
65	18.01	17.75	18.11	17.92	18.11
70	18.65	18.14	18.50	18.28	18.55
80	19.16	18.97	19.13	18.88	19.29
90	19.61	19.42	19.60	19.37	19.91
100	20.26	19.94	19.96	19.78	20.41
110	20.58	20.13	20.23	20.13	20.83
120	20.77	20.51	20.44	20.44	21.19
130	20.90	20.90	20.59	20.73	21.48
140	21.09	20.90	20.71	20.99	21.73

LIVER ENZYME

$[NAD]_0$ = 80 μM

Time (secs)	Concentration of NADH (μM)				
	Experimental values		Predicted values		
			Chapter IV (Rep. 2)		Chapter V
	Rep. 1	Rep. 2	3 Parameters	4 Parameters	
5	5.14	5.15	6.05	5.68	4.99
10	8.49	8.36	8.33	8.24	7.96
15	11.25	10.61	10.34	10.44	10.23
20	12.86	12.54	12.12	12.33	12.09
25	14.47	14.21	13.69	13.95	13.66
30	16.08	15.43	15.07	15.34	15.00
35	17.04	16.72	16.29	16.54	16.18
40	18.01	17.43	17.37	17.58	17.22
45	18.91	18.33	18.32	18.48	18.13
50	19.61	19.16	19.16	19.26	18.95
55	20.51	19.68	19.90	19.93	19.69
60	21.09	20.58	20.55	20.53	20.35
65	21.54	20.90	21.13	21.05	20.94
70	21.99	21.35	21.64	21.50	21.48
80	22.83	22.19	22.48	22.27	22.42
90	23.47	22.83	23.14	22.88	23.20
100	23.99	23.47	23.66	23.38	23.85
110	24.24	23.86	24.06	23.80	24.39
120	24.76	24.31	24.37	24.16	24.85
130	25.08	24.63	24.61	24.47	25.23
140	25.40	24.95	24.80	24.76	25.57

LIVER ENZYME

$[NAD]_0$ = 100 μM

Time (secs)	Concentration of NADH (μM)				
	Experimental values		Predicted values		
			Chapter IV (Rep. 2)		Chapter V
	Rep. 1	Rep. 2	3 Parameters	4 Parameters	
5	4.82	5.14	6.21	5.64	5.27
10	8.68	9.00	8.79	8.57	8.51
15	11.25	11.58	11.08	11.10	11.05
20	13.18	13.63	13.11	13.29	13.14
25	15.11	15.43	14.92	15.18	14.92
30	16.53	16.91	16.53	16.81	16.46
35	17.56	18.01	17.96	18.23	17.81
40	18.84	19.16	19.24	19.46	19.00
45	19.81	20.26	20.37	20.53	20.06
50	20.64	21.22	21.37	21.47	21.01
55	20.90	21.87	22.27	22.30	21.87
60	22.19	23.09	23.06	23.02	22.64
65	23.02	23.47	23.77	23.66	23.35
70	23.47	24.24	24.40	24.23	23.98
80	24.57	25.14	25.45	25.19	25.10
90	25.40	26.24	26.29	25.97	26.03
100	26.37	26.69	26.95	26.62	26.80
110	27.01	27.33	27.57	27.16	27.47
120	27.33	27.78	27.88	27.64	28.02
130	27.78	28.30	28.21	28.07	28.50
140	27.97	28.55	28.46	28.46	28.90

LIVER ENZYME

$[NAD]_0$ = 120 μM

Time (secs)	Concentration of NADH (μM)				
	Experimental values		Predicted values		
			Chapter IV (Rep. 2)		Chapter V
	Rep. 1	Rep. 2	3 Parameters	4 Parameters	
5	5.47	5.47	6.72	6.34	5.47
10	9.32	9.65	9.44	9.30	8.96
15	11.90	12.54	11.89	11.91	11.72
20	14.79	14.79	14.07	14.20	14.02
25	16.40	16.53	16.04	16.23	15.98
30	18.20	17.88	17.80	18.01	17.69
35	19.61	19.42	19.38	19.59	19.19
40	21.09	20.90	20.80	20.98	20.53
45	22.19	21.93	22.07	22.22	21.72
50	23.15	23.15	23.21	23.31	22.79
55	24.12	24.12	24.23	24.28	23.76
60	25.08	24.82	25.15	25.15	24.64
65	25.72	25.85	25.97	25.92	25.44
70	26.50	26.56	26.71	26.61	26.17
80	27.78	27.65	27.96	27.78	27.44
90	28.94	28.62	28.97	28.73	28.51
100	29.71	29.58	29.79	29.51	29.41
110	30.55	30.55	30.44	30.16	30.18
120	30.87	30.87	30.97	30.72	30.84
130	31.51	31.25	31.39	31.19	31.39
140	31.83	31.83	31.73	31.60	31.87

LIVER ENZYME

$[NAD]_0$ = 160 μM

Time (secs)	Concentration of NADH (μM)				
	Experimental values		Predicted values		
			Chapter IV (Rep. 2)		Chapter V
	Rep. 1	Rep. 2	3 Parameters	4 Parameters	
5	5.47	5.47	6.42	6.61	5.77
10	9.45	9.97	9.59	9.80	9.64
15	12.22	12.86	12.46	12.65	12.78
20	14.92	15.43	15.04	15.21	15.42
25	17.36	17.68	17.36	17.50	17.70
30	19.04	19.55	19.45	19.55	19.70
35	21.03	21.35	21.34	21.39	21.48
40	22.51	23.15	23.04	23.05	23.06
45	24.12	24.44	24.57	24.54	24.49
50	25.40	25.72	25.95	25.88	25.78
55	26.62	27.33	27.19	27.08	26.95
60	27.97	28.10	28.32	28.18	28.02
65	28.94	29.20	29.32	29.16	28.99
70	29.90	29.90	30.23	30.06	29.88
80	31.51	31.51	31.79	31.61	31.45
90	32.99	32.93	33.06	32.89	32.78
100	33.76	33.96	34.09	33.97	33.92
110	34.73	35.05	34.92	34.88	34.89
120	35.37	35.69	35.60	35.67	35.72
130	36.33	36.33	36.15	36.35	36.43
140	36.66	36.85	36.59	36.94	37.05

LIVER ENZYME

$[NAD]_0$ = 240 μM

Time (secs)	Concentration of NADH (μM)				
	Experimental values		Predicted values		
			Chapter IV (Rep. 2)		Chapter V
	Rep. 1	Rep. 2	3 Parameters	4 Parameters	
5	6.43	6.43	7.27	7.29	6.14
10	10.61	10.61	10.83	10.88	10.54
15	14.92	13.96	14.08	14.15	14.21
20	18.01	17.36	17.06	17.13	17.38
25	20.90	19.61	19.79	19.86	20.16
30	23.15	21.99	22.28	22.34	22.63
35	25.72	24.44	24.56	24.62	24.84
40	27.97	26.69	26.65	26.69	26.83
45	29.90	28.94	28.56	28.59	28.64
50	31.83	30.55	30.31	30.32	30.29
55	33.44	32.03	31.91	31.90	31.80
60	34.73	33.44	33.37	33.35	33.19
65	36.33	34.86	34.71	34.68	34.46
70	37.30	36.08	35.93	35.89	35.64
80	39.74	38.07	38.08	38.01	37.72
90	41.61	39.87	39.87	39.79	39.51
100	42.77	41.48	41.37	41.29	41.05
110	43.73	42.44	42.63	42.55	42.38
120	44.82	43.41	43.68	43.61	43.54
130	45.66	44.37	44.56	44.51	44.54
140	46.30	45.34	45.30	45.28	45.42

YEAST ENZYME

$[NAD]_0$ = 80 μM

Time (secs)	Concentration of NADH (μM)			
	Experimental values		Predicted values*	
	Rep. 1	Rep. 2	E_0 = .095	E_0 = .09
5	10.05	9.97	11.22	10.79
10	16.72	16.72	17.63	17.07
15	20.90	21.22	21.96	21.37
20	24.44	24.44	25.07	24.49
25	26.85	26.85	27.38	26.83
30	28.62	28.54	29.12	28.62
35	30.23	29.90	30.46	30.00
40	31.35	30.87	31.49	31.08
45	32.32	31.51	32.28	31.93
50	32.96	32.15	32.91	32.60
55	33.76	32.32	33.40	33.13
60	34.41	32.96	33.78	33.55
65	34.71	33.12	34.08	33.88
70	35.05	33.44	34.32	34.15
75	35.21	33.60	34.50	34.36
80	35.37	33.76	34.65	34.53
90	35.53	33.76	34.86	34.77
100	35.69	33.76	34.99	34.93

* using parameters given in Chapter IV

YEAST ENZYME

$[NAD]_0$ = 160 μM

Time (secs)	Concentration of NADH (μM)		
	Experimental values		Predicted values*
	Rep. 1	Rep. 2	E_0 = .095
5	13.67	15.27	16.07
10	22.91	24.92	25.53
15	30.23	32.47	32.07
20	35.69	37.78	36.87
25	39.55	41.29	40.50
30	42.12	43.89	43.30
35	44.53	45.98	45.50
40	46.62	47.43	47.21
45	47.91	48.87	48.57
50	49.52	50.00	49.66
55	50.32	50.80	50.53
60	50.96	51.13	51.22
70	52.09	51.93	52.23
80	52.89	52.65	52.88
90	53.54	53.05	53.31
100	54.18	53.22	53.59
110	54.34	53.30	53.77
120	54.42	53.38	53.89
130	54.50	53.46	53.96

* using parameters given in Chapter IV

YEAST ENZYME

$[NAD]_0$ = 320 μM

Time (secs)	Concentration of NADH (μM)			
	Experimental values		Predicted values*	
	Rep. 1	Rep. 2	E_0=.095	E_0=.105
5	22.51	20.16	21.42	23.10
10	38.59	35.37	34.88	37.17
15	48.39	46.30	44.52	47.04
20	55.47	53.86	51.79	54.34
25	61.09	58.52	57.43	59.90
30	65.11	62.70	61.87	64.21
35	68.49	66.72	65.42	67.59
40	71.38	69.13	68.28	70.26
45	73.31	71.38	70.59	72.39
50	75.24	72.99	72.48	74.10
55	76.53	74.28	74.02	75.46
60	77.49	75.88	75.28	76.56
65	78.14	76.53	76.31	77.45
70	78.78	77.01	77.17	78.17
75	79.26	77.49	77.87	78.74
80	80.06	78.14	78.45	79.21
85	80.23	78.46	78.93	79.59
90	80.39	78.62	79.33	79.90
95	80.55	78.78	79.65	80.15
100	80.71	78.94	79.92	80.35
110	80.87	79.10	80.33	80.65
120	81.19	79.18	80.61	80.85

* using parameters given in Chapter IV

YEAST ENZYME

$[NAD]_0$ = 640 μM

Time (secs)	Concentration of NADH (μM)			
	Experimental values		Predicted values*	
	Rep. 1	Rep. 2	E_0=.095	E_0=.110
5	28.62	25.72	26.58	29.88
10	50.16	49.62	44.87	49.62
15	64.31	59.97	58.60	64.00
20	76.37	71.54	69.32	74.95
25	83.60	80.39	77.88	83.48
30	90.03	86.01	84.82	90.23
35	95.66	91.64	90.50	95.64
40	99.68	96.46	95.19	100.00
45	103.54	100.48	99.08	103.54
50	106.43	104.18	102.33	106.42
55	109.32	106.91	105.05	108.78
60	110.93	109.32	107.33	110.71
65	112.86	111.25	109.25	112.30
70	114.47	113.02	110.87	113.61
80	116.08	115.92	113.39	115.58
90	117.68	117.52	115.19	116.92
100	118.49	118.81	116.49	117.84
110	119.13	119.77	117.42	118.47
120	119.45	120.42	118.09	118.90
130	119.77	120.58	118.58	119.20

* using parameters given in Chapter IV

APPENDIX IV

Solutions of equations IV.11 and IV.15

1. Solution of Equation IV.11

Equation IV.11 may be written as

$$\frac{d\theta}{dt} = -\theta(k_4 w_s + k_6 z_s) + k_6 z_s v_0$$

Hence, putting $\alpha = k_4 w_s + k_6 z_s$ and $\beta = k_6 z_s v_0$, we get

$$\frac{d\theta}{\beta - \alpha\theta} = dt$$

which upon integration gives

$$-\frac{1}{\alpha}\ln(\beta - \alpha\theta) = t + K$$

where K denotes the constant of integration. Rearranging this, we get

$$\beta - \alpha\theta = e^{-\alpha(t+K)}$$

i.e. $$\theta = \frac{1}{\alpha}\left(\beta - e^{-\alpha(t+K)}\right)$$

i.e. $$\theta = a\left(1 - e^{-b(t+k)}\right)$$

where $a = \frac{\beta}{\alpha}$, $b = \alpha$ and $k = \frac{\ln\beta + \alpha K}{\alpha}$,

i.e., in terms of the velocity constants etc.,

$$a = \frac{k_6 z_s v_0}{k_4 w_s + k_6 z_s}$$

$$b = k_4 w_s + k_6 z_s$$

$$k = \frac{\ln(k_6 z_s v_0)}{k_4 w_s + k_6 z_s} + K$$

2. Solution of Equation IV.15

From equation IV.15 we have

$$gt = k_1(e_0 - w_s - z_s)\theta - k_1(e_0 - w_s - z_s)w_s - k_2 w_s + \frac{d\theta}{dt}$$

Putting

$$\alpha = k_1(e_0 - w_s - z_s)$$

and

$$\beta = \left(k_1(e_0 - w_s - z_s) + k_2\right)w_s$$

this gives

$$\frac{d\theta}{dt} = gt + \beta - \alpha\theta$$

i.e. $$\frac{d\theta}{dt} + \alpha\theta = gt + \beta$$

Multiplying by the integrating factor $e^{\alpha t}$, we get

$$e^{\alpha t}\frac{d\theta}{dt} + \alpha\theta e^{\alpha t} = \beta e^{\alpha t} + cte^{\alpha t}$$

which upon integration gives

$$e^{\alpha t}\theta = \beta\int e^{\alpha t}dt + c\int te^{\alpha t}dt$$

$$= \frac{\beta}{\alpha}e^{\alpha t} + ct\frac{e^{\alpha t}}{\alpha} - c\int e^{\alpha t}dt$$

$$= \left(\frac{\beta}{\alpha} + \frac{ct}{\alpha} - \frac{c}{\alpha^2}\right)\cdot e^{\alpha t} - Q$$

where Q denotes the constant of integration. Dividing through by $e^{\alpha t}$, we get

$$\theta = \frac{\beta}{\alpha} - \frac{c}{\alpha^2} + c\frac{t}{\alpha} - Ke^{-\alpha t}$$

i.e. $$\theta = A\left(1 - e^{-B(t+K)}\right) + Dt$$

where $A = \frac{\beta}{\alpha} - \frac{c}{\alpha^2}$, $B = \alpha$, $D = \frac{c}{\alpha}$ and $K = -\frac{1}{\alpha}\ln\frac{Q}{A} = -\frac{1}{\alpha}\ln\frac{Q\alpha^2}{\alpha\beta - c}$.

In terms of the velocity constants etc.,

$$A = -1 - \frac{k_2 w_s}{k_1(e_0 - w_s - z_s)} - \frac{c}{k_1^2(e_0 - w_s - z_s)^2}$$

$$B = k_1(e_0 - w_s - z_s)$$

$$K = \frac{-1}{k_1(e_0-w_s-z_s)} \ln\left(\frac{Qk_1^2(e_0-w_s-z_s)^2}{-k_1(e_0-w_s-z_s)[k_1(e_0-w_s-z_s)+k_2]w_s-Q}\right)$$

$$D = \frac{Q}{k_1(e_0-w_s-z_s)}$$

APPENDIX V

Computer Programs

```
      PROGRAM PLAIN (INPUT,OUTPUT)
      DIMENSION P(6)
      READ 101,SZERO,EZERO,CZERO
  101 FORMAT(6F12.0,F8.0)
      READ 101,(P(I),I=1,6)
      PRINT 107,SZERO,EZERO,CZERO
  107 FORMAT(1H1,10X*PROGRAM PLAIN*/10X*INITIAL NAD CONCENTRATION=*
     $E11.4/10X*INITIAL ENZYME CONCENTRATION=*E11.4/10X*INITIAL ALCOHOL
     *CONCENTRATION= *E11.4)
      PRINT 102,(P(I),I=1,6)
  102 FORMAT(10X* K1 =*E11.4,* K2 =*E11.4,* K3 =*E11.4/10X* K4 =*E11.4,
     ** K5 =*E11.4,* K6 =*E11.4)
      READ 101,TIME1,TH,W,Z,Y,ENZ,TLIM
      READ 110,NS,DELTA,NP
  110 FORMAT(I8,F8.0,I4)
C      DELTA IS STEP SIZE
C      NS IS NUMBER OF STEPS BETWEEN EACH PRINT OUT
      PRINT 112,NS,DELTA
  112 FORMAT(10X*NUMBER OF STEPS=*I8,* SIZE=*E11.4)
      PRINT 113
  113 FORMAT(10X*TIME*14X*TH*14X*W*14X*Z*14X*Y*14X*ENZ*)
      PRINT 114, TIME1,TH,W,Z,Y,ENZ
      DO 11 K=1,NP
      DO 1 J=1,NS
      TIME1=TIME1+DELTA
      DTH=-P(3)*W*TH+P(4)*Z*(CZERO-TH)
      DW=ENZ*P(1)*(TH-W)-W*P(2)+DTH
      DY=P(5)*Z-P(6)*Y*ENZ
      TH=DELTA*DTH+TH
      W=DELTA*DW+W
      Y=DELTA*DY+Y
      Z=SZERO-TH-Y
      ENZ=EZERO-W-Z
    1 CONTINUE
      PRINT 114, TIME1,TH,W,Z,Y,ENZ
  114 FORMAT(5XF15.6,F15.6,F15.8,F15.10,F15.6,F15.8,2E11.4)
   11 CONTINUE
      END
```

```
      PROGRAM HOPE (INPUT,OUTPUT)
      COMMON/DAT/TH(100),T(100),Y(100),NT
      DIMENSION X(10),STEP(10)
      DIMENSION TITLE(8)
  901 READ 101,NT
      IF(NT.LE.0) STOP
      READ 120,TITLE
  120 FORMAT(8A10)
      DO 1 J=1,NT
      READ 102,T(J),TH(J)
    1 CONTINUE
  102 FORMAT(2F6.0)
      READ 101,N
  101 FORMAT(4I4)
      READ 103,(X(I),STEP(I),I=1,N)
  103 FORMAT(2F10.0)
      X(1)=TH(NT)
      READ 101,MAX,IPRINT,NLOOP,IQUAD
      READ 104,A,B,C,STOPC,SIMP
  104 FORMAT(5F10.0)
      PRINT 121,TITLE
  121 FORMAT(1H19X8A10)
      PRINT 105,NT,(X(I),STEP(I),I=1,N)
  105 FORMAT(1X *PROGRAM HOPE */10X*NO OF DATA POINTS=*I4/10X*STARTING V
     $ALUE  STEP SIZE*/(10X2E11.4))
      CALL MINIM(X,STEP,N,F,MAX,IPRINT,A,B,C,STOPC,NLOOP,IQUAD,SIMP,VAR)
      PRINT 106,(T(J),TH(J),Y(J),J=1,NT)
  106 FORMAT(15X*T*6X*THETA*5X*FITVAL*/(10XF6.0,2E11.4))
      GO TO 901
      END
```

```
      SUBROUTINE GIVEF(X,SS)
      COMMON/DAT/TH(100),T(100),Y(100),NT
      DIMENSION X(1)
      SS=0.0
      A=X(1)
      AS=X(2)
      C=X(3)
      IF(AS.LT.0.OR.A.LT.0) GO TO 1
      IF(AS.GT.1000) GO TO 1
      DO 2 J=1,NT
      Y(J)=A*(1.0-EXP(-AS*(T(J)+C)))
      D=TH(J)-Y(J)
      SS=SS+D*D
    2 CONTINUE
      RETURN
    1 SS=1000000.0
      RETURN
      END
```

```
      SUBROUTINE MINIM(F,STEP,NOP,FUNC,MAX,IPRINT,A,B,C,STOPCRI,NLOOP,
     $IQUAD,SIMP,VAR)
C     NELDER AND MEAD, THE COMPUTER JOURNAL,JANUARY,1965.
      DIMENSION F(1),STEP(1)
      DIMENSION G(21,20),H(21),PBAR(20),PSTAR(20),PSTST(20)
      DIMENSION AVAL(20),BMAT(210),PMIN(20),VC(210),VAR(1)
      EQUIVALENCE(PMIN,PSTAR),(AVAL,PBAR)
C     IF PROGRESS REPORTS DESIRED,PRINT HEADING FOR THEM
      IF(IPRINT)30,30,10
   10 PRINT 20,IPRINT
   20 FORMAT(22H PROGRESS REPORT EVERY,I4,21H FUNCTION EVALUATIONS//24H
     $EVAL. N/.  FUNC. VALUE ,10X,10HPARAMETERS)
C     APPROX IS USED TO TEST CLOSENESS TO ZERO
   30 APPROX=1.0E-30
C     IF NO VALUES OF A,B,C ARE GIVEN,I.E. A IS SET =0.0,THEN THE
C     PROGRAM SETS A=1.0, B=0.5, C=2.0
      IF(ABS(A).LT.APPROX) 40,50
   40 A=1.0 $ B=0.5 $C=2.0
C     NAP IS THE NUMBER OF PARAMETERS TO BE VARIED,I.E. WITH STEP NOT 0
   50 NAP=0
      LOOP=IFLAG=0
      DO 70 I=1,NOP
      IF(ABS(STEP(I)).GT.APPROX) 60,70
   60 NAP=NAP+1
   70 CONTINUE
C     IF NAP =0, EVALUATE FUNCTION AT STARTING POINT AND RETURN
      IF(NAP) 90,80
   80 CALL GIVEF(F,FUNC)
      RETURN
C     SET UP INITIAL SIMPLEX
   90 DO 100 I=1,NOP
  100 G(1,I)=F(I)
      IROW=2
      DO 130 I=1,NOP
      IF(ABS(STEP(I)).LT.APPROX) 130,110
  110 DO 120 J=1,NOP
  120 G(IROW,J)=F(J)
      G(IROW,I)=G(IROW,I)+STEP(I)
      IROW=IROW+1
  130 CONTINUE
      NP1=NAP+1
      NEVAL=0
      DO 170 I=1,NP1
      DO 140 J=1,NOP
  140 F(J)=G(I,J)
      CALL GIVEF(F,H(I))
      NEVAL=NEVAL+1
C     ALL POINTS OF INITIAL SIMPLEX ARE OUTPUT IF IPRINT.GT.0
      IF(IPRINT) 170,170,150
```

```
  150 PRINT 160,NEVAL,H(I),(F(J),J=1,NOP)
  160 FORMAT(/3X,I4,4X,E13.6,8(1X,E13.6)/24X,8(1X,E13.6)/24X,4(1XE13.6))
  170 CONTINUE
C     NOW FOLLOWS THE BASIC LOOP,I.E. GIVEN A SIMPLEX,TO DETERMINE THE
C     NEW SIMPLEX AND TEST FOR CONVERGENCE AS REQUIRED (FOLLOWING THE
C     L
C     FLOW CHART GIVEN IN NELDER AND MEAD)
C      TO STATEMENT 13, DETERMINE MAX AND MIN POINTS OFCURRENT SIMPLEX
  180 LOOP=LOOP+1
      IMAX=IMIN=1
      HMAX=HMIN=H(1)
      DO 220 I=2,NP1
      IF(H(I).GT.H(IMAX)) 190,200
  190 IMAX=I
      HMAX=H(I)
  200 IF(H(I).LT.H(IMIN)) 210,220
  210 IMIN=I
      HMIN=H(I)
  220 CONTINUE
C     TO STATEMENT 18, FIND CENTROID OF ALL VERTICES,EXCLUDING MAXIMUM
      DO 230 I=1,NOP
  230 PBAR(I)=0.0
      DO 260 I=1,NP1
      IF(I.EQ.IMAX) 260,240
  240 DO 250 J=1,NOP
  250 PBAR(J)=PBAR(J)+G(I,J)/NAP
  260 CONTINUE
C     REFLECT MAXIMUN THROUGH PBAR TO PSTAR,EVALUATE FUNC AT PSTAR(HSTAR
      DO 270 I=1,NOP
  270 PSTAR(I)=A*(PBAR(I)-G(IMAX,I))+PBAR(I)
      CALL GIVEF(PSTAR,HSTAR)
C     NEXT 5 STATEMENTS PROVIDE PROGRESS REPORT IF REQUIRED
C     THIS PROCEDURE OCCURS FREQUENTLY IN THE PROGRAM
      NEVAL=NEVAL+1
      IF(IPRINT) 300,300,280
  280 J=NEVAL/IPRINT
      K=NEVAL-J*IPRINT
      IF(K) 300,290
  290 PRINT 160,NEVAL,HSTAR,(PSTAR(J),J=1,NOP)
  300 IF(HSTAR.LT.HMIN) 310,380
C     IF HSTAR LT HMIN REFLECT PBAR THROUGH PSTAR TO GIVE PSTST,AND
C     EVALUATE FUNCTION THERS (GIVING HSTST)
  310 DO 320 I=1,NOP
  320 PSTST(I)=C*(PSTAR(I)-PBAR(I))+PSTAR(I)
      CALL GIVEF(PSTST,HSTST)
      NEVAL=NEVAL+1
      IF(IPRINT) 350,350,330
  330 J=NEVAL/IPRINT
      K=NEVAL-J*IPRINT
```

```
      IF(K) 350,340
  340 PRINT 160,NEVAL,HSTST,(PSTST(J),J=1,NOP)
  350 IF(HSTST.LT.HMIN) 360,560
C     IF HSTST.LT.HMIN REPLACE MAXIMUM PT OF CURRENT SIMPLEX BY PSTST
C      AND HMAX BY HSTAR (QUERY) THEN TES (STATEMENT 26 ONWARDS)
  360 DO 370 I=1,NOP
  370 G(IMAX,I)=PSTST(I)
      H(IMAX)=HSTST
      GO TO 580
C     IF HSTAR NOT LT HMIN,TEST IF HSTAR GT FUNCTION VALUE AT ALL VERTIC
C         ES OTHER THA THE MAXIMUM ONE
  380 DO 400 I=1,NP1
      IF(I.EQ.IMAX) 400,390
  390 IF(HSTAR.LT.H(I)) 560,400
  400 CONTINUE
C     IF IT LESS THAN AT LEAST ONE OF THESE VERTICES, REPLACE MAX PT OF
C     CURRENT SIMPLEX BY PSTAR AND HMAX BY HSTAR THE TEST ($ 26 ONWARDS)
      IF(HSTAR.GT. HMAX) 430,410
  410 DO 420 I=1,NOP
  420 G(IMAX,I)=PSTAR(I)
      HMAX=H(IMAX)=HSTAR
  430 DO 440 I=1,NOP
  440 PSTST(I)=B*G(IMAX,I)+(1.0-B)*PBAR(I)
      CALL GIVEF(PSTST,HSTST)
      NEVAL=NEVAL+1
      IF(IPRINT) 470,470,450
  450 J=NEVAL/IPRINT
      K=NEVAL-J*IPRINT
      IF(K)470,460
  460 PRINT 160,NEVAL,HSTST,(PSTST(J),J=1,NOP)
  470 IF(HSTST.GT.HMAX) 500,480
  480 DO 490 I=1,NOP
  490 G(IMAX,I)=PSTST(I)
      H(IMAX)=HSTST
      GO TO 580
  500 DO 510 I=1,NP1
      DO 510 J=1,NOP
  510 G(I,J)=(G(I,J)+G(IMIN,J))/2.0
      DO 550 I=1,NP1
      DO 520 J=1,NOP
  520 F(J)=G(I,J)
      CALL GIVEF(F,H(I))
      NEVAL=NEVAL+1
      IF(IPRINT) 550,550,530
  530 J=NEVAL/IPRINT
      K=NEVAL-J*IPRINT
      IF(K) 550,540
  540 PRINT 160,NEVAL,H(I),(F(J),J=1,NOP)
  550 CONTINUE
```

```
    GO TO 580
560 DO 570 I=1,NOP
570 G(IMAX,I)=PSTAR(I)
    H(IMAX)=HSTAR
580 IF(LOOP.EQ.NLOOP) 590,180
590 HSTD=HMEAN=0.0
    DO 600 I=1,NP1
    HSTD=HSTD+H(I)*H(I)
600 HMEAN=HMEAN+H(I)
    HMEAN=HMEAN/NP1
    HSTD=(HSTD-NP1*HMEAN*HMEAN)/NP1
    IF(HSTD.LE.APPROX) 601,602
601 PRINT 603,HSTD
603 FORMAT(10X*HSTD=*E13.6)
    HSTD=0.0
602 HSTD=SQRT(HSTD)
    DO 620 I=1,NOP
    F(I)=0.0
    DO 610 J=1,NP1
610 F(I)=F(I)+G(J,I)
    F(I)=F(I)/NP1
620 CONTINUE
    CALL GIVEF(F,FUNC)
    NEVAL=NEVAL+1
    IF(NEVAL.GT.MAX) 630,700
630 IF(IPRINT) 690,640,640
640 PRINT 650,MAX
650 FORMAT(40H NUMBER OF FUNCTION EVALUATIONS EXCEEDS ,I4)
    PRINT 660,HSTD
660 FORMAT(51H STANDARD ERROR OF FUNCTION VALUES OF LAST SIMPLEX ,E13.
   $6)
    PRINT 670,(F(I),I=1,NOP)
670 FORMAT(28H  CENTROID OF LAST SIMPLEX  ,7(1X,E13.6)/27X,7(1X,E13.6)
   $/27X,7(1X,E13.6))
    PRINT 680, FUNC
680 FORMAT(31H  FUNCTION VALUE AT CENTROID   ,E13.6)
690 NLOOP=0
    RETURN
700 IF(HSTD.LT.STOPCRI) 720,710
710 IFLAG=0
    LOOP=0
    GO TO 180
720 IF(IPRINT) 750,730,730
730 PRINT 740
740 FORMAT(2H */33H  INITIAL EVIDENCE OF CONVERGENCE)
    PRINT 670, (F(I),I=1,NOP)
    PRINT 680, FUNC
750 IF(IFLAG) 770,760
760 IFLAG=1
```

```
    SAVEMEA=HMEAN
    LOOP=0
    GO TO 180
770 TEST=SAVEMEA-HMEAN
    IF(TEST.LT.STOPCRI) 790,780
780 IFLAG=0
    LOOP=0
    GO TO 180
790 IF(IPRINT) 850,800,800
800 PRINT 810,NEVAL
810 FORMAT(5(/),36H PROCESS CONVERGES ON MINIMUM AFTER ,I4,21H FUNCTIO
   $N EVALUATIONS///)
    PRINT 820,(F(I),I=1,NOP)
820 FORMAT(14H MINIMUM AT    ,8(1X,E13.6)/14X,8(1X,E13.6)/14X,4(1X,E13.
   $6))
    PRINT 830,FUNC
830 FORMAT(//26H MINIMUM FUNCTION VALUE    ,E13.6)
    PRINT 840
840 FORMAT(///16H END  OF  SEARCH/1X,15(1H*))
850 CONTINUE
    RETURN
    END
```

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