Pathways of reducing equivalents in hepatocytes from rats

Estimation of cytosolic fluxes by means of ^3H -labelled substrates for either A- or B-specific dehydrogenases

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The metabolism of $[2\text{-}^3\text{H}]\text{lactate}$ and $[2\text{-}^3\text{H}]\text{glycerol}$ was studied in isolated hepatocytes from fed rats. In order to estimate the rate of equilibrium between the 4A and 4B hydrogen atoms of NADH, we compared the flow of ^3H from $[2\text{-}^3\text{H}]\text{lactate}$ and $[2\text{-}^3\text{H}]\text{glycerol}$, the oxidations of which are catalysed by A- and B-type dehydrogenases, respectively. Hepatocytes were incubated with lactate, glycerol and ethanol and tracer amounts of $[2\text{-}^3\text{H}]\text{lactate}$ or $[2\text{-}^3\text{H}]\text{glycerol}$ and the labelling rates of lactate, ethanol, glucose and glycerol phosphate were determined. The data were used to calculate the oxidation rate of NADH catalysed by lactate dehydrogenase, alcohol dehydrogenase, triosephosphate dehydrogenase and glycerol phosphate dehydrogenase. The rates were calculated by obtaining the best fit of a model to the experimental data by using a least-squares procedure. The results support our model and suggest that the fluxes through various dehydrogenases are sufficient to equilibrate the 4A and 4B hydrogen atoms of cytosolic NADH. The validity of the metabolic models used was evaluated by comparison of rates of NADH oxidation catalysed by cytosolic dehydrogenases as calculated by two different models.

INTRODUCTION

Reactions catalysed by NAD-linked dehydrogenases can be classified as type A or type B depending on the stereospecificity of the hydride transfer (Levy *et al.*, 1962). The A-stereospecific enzymes transfer the hydride ion to the A-side (*re*-face) of the pyridine ring of NAD^+ and the B-stereospecific enzymes to the B-side (*si*-face). $[2\text{-}^3\text{H}]\text{Lactate}$ generates $[4\text{A-}^3\text{H}]\text{NADH}$ via lactate dehydrogenase (Loewus *et al.*, 1953), whereas $[2\text{-}^3\text{H}]\text{glycerol}$ via $[2\text{-}^3\text{H}]\text{glycerol phosphate}$ and glycerol phosphate dehydrogenase generates $[4\text{B-}^3\text{H}]\text{NADH}$ (Levy & Vennesland, 1957). In systems involving branched or competing pathways with more than one labelled product, the specificity of the dehydrogenases involved can affect the isotope yields in the products. It is only when the rates of the reversible dehydrogenases are greatly in excess of the net fluxes in the system that the differences caused by the stereospecific transfer of hydride ions become negligible.

In previous studies the rate of NADH oxidation catalysed by cytosolic dehydrogenases have been estimated in hepatocytes incubated with $[2\text{-}^3\text{H}]\text{lactate}$ (Vind & Grunnet, 1984, 1987).

In the present paper we have estimated the rate of equilibration between the 4A and the 4B hydrogen atoms of NADH catalysed by reversible dehydrogenases. For this purpose hepatocytes were incubated with $[2\text{-}^3\text{H}]\text{glycerol}$ or $[2\text{-}^3\text{H}]\text{lactate}$ under identical conditions and the labelling rates of ethanol, glucose, lactate and glycerol phosphate were used to calculate the rate of NADH oxidation catalysed by cytosolic dehydrogenases.

The validity of the metabolic models used was evaluated by comparison of rates of oxidation of NADH catalysed by cytosolic dehydrogenases calculated by two different models. The rate of equilibration between the 4A and the 4B hydrogen atoms of NADH was estimated

with the two labelled substrates, which generate $[4\text{A-}^3\text{H}]\text{NADH}$ or $[4\text{B-}^3\text{H}]\text{NADH}$.

MATERIALS AND METHODS

Reagents

Enzymes and coenzymes were from Boehringer-Mannheim. NaB^3H_4 , $[2\text{-}^{14}\text{C}]\text{glycerol}$, $\text{DL-}[1\text{-}^{14}\text{C}]\text{lactic acid}$ and $\text{L-}[U\text{-}^{14}\text{C}]\text{lactic acid}$ were purchased from Amersham International, and $[2\text{-}^3\text{H}]\text{glycerol}$ was from NEN Research Products.

Chemicals were of analytical grade. Serum albumin from Armour Pharmaceutical Co. was treated with charcoal to remove fatty acids (Chen, 1967). Dowex 1 was from Fluka, Sephadex G-50 from Pharmacia and Instafluor from Packard. $\text{L-}[2\text{-}^3\text{H}]\text{Lactate}$ was prepared as described by Vind & Grunnet (1984).

Preparation and incubation of hepatocytes

Hepatocytes from female Wistar rats (150–200 g), starved for 24 h, were prepared as described by Kondrup *et al.* (1980). More than 90% of the cells excluded Trypan Blue.

Packed cells (0.8–1.1 ml, approx. 0.9–1.2 g wet wt.) were incubated in a 300 ml conical flask at 37 °C in Krebs–Henseleit (1932) bicarbonate buffer containing 1% serum albumin and 7.5 mM-glucose in a total volume of 22 ml. The gas phase was O_2/CO_2 (19:1).

To the incubations were added 2.5 mM-lactate (giving a final concentration of about 3.5 mM due to the lactate content of the hepatocyte suspension), 0.25 mM-pyruvate, 8 mM-glycerol and 20 mM-ethanol, 10 min prior to addition (at time zero) of a trace amount of either $[2\text{-}^3\text{H}]\text{lactate}$ or $[2\text{-}^3\text{H}]\text{glycerol}$ to an initial specific radioactivity of approx. 500 c.p.m./nmol and 150 c.p.m./nmol, respectively. Samples (2 ml) were taken from the incubation at –10, 0, 10, 20, 30, 40, 50, 60 and

70 min and added to HClO_4 to 0.7 M. The supernatant was neutralized by KOH containing triethanolamine.

Analytical procedure

The neutralized HClO_4 supernatant was used for determinations of the metabolic concentrations and the distribution of ^3H from $[2\text{-}^3\text{H}]\text{lactate}$ or from $[2\text{-}^3\text{H}]\text{glycerol}$ in water and ethanol (Vind & Grunnet, 1984) and glucose, lactate, glycerol and glycerol phosphate. DL- $[1\text{-}^{14}\text{C}]\text{Lactate}$ was added as an internal standard in the ion-exchange chromatography (Vind & Grunnet, 1981). Lactate was eluted with 4 M-acetic acid from a Dowex 1 (acetate form) column. Glycerol phosphate was eluted with 4 M-formic acid and other anions were eluted with 2 M-potassium formate, pH 3. Glucose and glycerol were isolated as the neutral fraction and separated by Dowex 1 ion-exchange chromatography after addition of $[2\text{-}^{14}\text{C}]\text{glycerol}$ as internal standard, and phosphorylation of glycerol with ATP and glycerol kinase (Vind & Grunnet, 1984). The ^3H removed from $[2\text{-}^3\text{H}]\text{lactate}$ or $[2\text{-}^3\text{H}]\text{glycerol}$ was recovered in water, ethanol, glucose, glycerol phosphate and glycerol or lactate respectively at 90–110%. The relative yield of ^3H found in the glycerol fraction from labelled lactate was less than 2% and has been neglected in the calculation.

The distribution of ^3H in lactate after metabolism of the labelled substrates was examined by transferring the H-2 from lactate to NADH and transaminating the pyruvate produced to alanine. L- $[U\text{-}^{14}\text{C}]\text{Lactate}$ was used as internal standard. Alanine and NADH were separated by DEAE-Sephadex A-25 chromatography. Alanine was eluted with 25 mM- and NADH with 0.5 M-ammonium acetate. Less than 10% of the ^3H in lactate was found in alanine, which means that more than 90% of the ^3H was located at H-2 in lactate after metabolism of the labelled substrate.

RESULTS

Metabolic changes

Rates of glycerol, ethanol and lactate removal were linear with time over the 70 min of incubation with labelled substrate, and the same whether $[2\text{-}^3\text{H}]\text{lactate}$ or $[2\text{-}^3\text{H}]\text{glycerol}$ was added as a tracer.

Glucose was the major end product of glycerol metabolism. The rate of glucose production of $0.68 \mu\text{mol/min}$ per ml packed cells (Table 1) could be accounted for by glycerol disappearance minus glycerol 3-phosphate accumulation ($0.10 \mu\text{mol/min}$ per ml packed cells) plus lactate disappearance ($0.31 \mu\text{mol/min}$ per ml packed cells).

The rate of ethanol oxidation was inhibited about 30% by glycerol, from 3.09 ± 0.19 (Vind & Grunnet, 1984) to $2.20 \pm 0.04 \mu\text{mol/min}$ per ml packed cells (Table 1) and the utilization of lactate was 50% lower in the presence of glycerol (0.31 ± 0.03) (Table 1) than in its absence [$0.68 \pm 0.13 \mu\text{mol/min}$ per ml packed cells (Vind & Grunnet, 1984)].

As shown in Table 1, the rate of glycerol phosphorylation was $1.1 \mu\text{mol/min}$ per ml packed cells. The same rate of phosphorylation was found by Cronholm & Curstedt (1984), when ethanol was present. Without ethanol they found the rate of glycerol phosphorylation to be $2.0 \mu\text{mol/min}$ per g of cells. If the oxidation of ethanol is inhibited, e.g. by addition of cyanamide, glycerol phosphate is oxidized as soon as it is phosphorylated (C. Vind, unpublished work). Without inhibition of the ethanol oxidation, the rate of phosphorylation is faster than the rate of oxidation and glycerol phosphate accumulates at a rate of $0.10 \mu\text{mol/min}$ per ml packed cells (Table 1).

The inhibition of ethanol oxidation by the oxidation of glycerol phosphate and *vice versa* is in agreement with results showing that substrates of NAD^+ -dependent cytoplasmic dehydrogenases mutually inhibit the oxidation of each other. This is the case for sorbitol and glycerol (Werner & Berry, 1974) as well as xylitol and ethanol (Vind & Grunnet, 1981).

Calculation of the flux of reducing equivalents from $[2\text{-}^3\text{H}]\text{lactate}$ or $[2\text{-}^3\text{H}]\text{glycerol}$ to acetaldehyde and other cytoplasmic metabolites

Our previous approach to calculate the flux of reducing equivalents from $[2\text{-}^3\text{H}]\text{lactate}$ via $[4\text{A-}^3\text{H}]\text{-NADH}$ to pyruvate (x), acetaldehyde (y) and 1,3-bisphosphoglycerate (f) is described in detail elsewhere (Vind & Grunnet, 1984).

The metabolic model used for these calculations is designated here 'Model I'. The extension of this model to 'Model II' by the inclusion of the reactions catalysed by glycerol phosphate dehydrogenase and glycerol kinase, and with an exchange reaction between the 4A and the 4B hydrogen atoms of NADH, is shown in Fig. 1.

The model is based on the assumption that $[4\text{B-}^3\text{H}]\text{NADH}$ is produced only after $[2\text{-}^3\text{H}]\text{glycerol}$ has been phosphorylated to $[2\text{-}^3\text{H}]\text{glycerol phosphate}$. Although NAD-dependent glycerol dehydrogenase activities are found in liver (Moore, 1959), the assumption is reasonable at a glycerol concentration of 8 mM, because the K_m value of these enzymes for oxidation of glycerol to glyceraldehyde is greater than 0.5 M, and that of

Table 1. Metabolic changes

Hepatocytes were incubated for 80 min and metabolite concentrations estimated during the incubation as described in the Materials and methods section. Initial concentration of substrates were 3.5 mM-lactate, 0.25 mM-pyruvate, 8 mM-glycerol and 20 mM-ethanol. Rates were measured from 10 min after addition of unlabelled substrates (at the time labelled substrates were added). Rates are means $\pm$ S.E.M. with the number of different cell preparations in parentheses.

Labelled substrate	Change ($\mu\text{mol/min}$ per ml of packed cells) in:				
	Lactate	Ethanol	Glucose	Glycerol	Glycerol phosphate
$[2\text{-}^3\text{H}]\text{Lactate}$	-0.31 ± 0.03	-2.27 ± 0.09	0.69 ± 0.05	-1.13 ± 0.08	0.10 ± 0.01 (9)
$[2\text{-}^3\text{H}]\text{Glycerol}$	-0.30 ± 0.02	-2.20 ± 0.04	0.66 ± 0.07	-1.09 ± 0.11	0.10 ± 0.01 (6)

Fig. 1. Model for flux of reducing equivalents from $[2\text{-}^3\text{H}]\text{lactate}$ or $[2\text{-}^3\text{H}]\text{glycerol}$

a , b and g represent the net oxidation rate of lactate, ethanol and glycerol phosphate respectively; k represents the phosphorylation rate of glycerol; $(a+x)$, $(b+y)$ and $(g+z)$ represent the rates of formation of NADH from lactate, ethanol and glycerol phosphate respectively, and x , y , z and f are the oxidation rates of NADH catalysed by lactate dehydrogenase, alcohol dehydrogenase, glycerol phosphate dehydrogenase and triosephosphate dehydrogenase, respectively. S_0 , A_0 , G_0 and F_0 represent the concentration at zero time of lactate, ethanol, glucose and glycerol phosphate, respectively. L , E , H , T , N_A and N_B represent the specific radioactivities (c.p.m./nmol) in dependence of time of lactate, ethanol, glycerol phosphate, glucose, $[4\text{-}^3\text{H}]\text{NADH}$ and $[4\text{-}^3\text{H}]\text{NADH}$. R_0 represents the specific radioactivity of glycerol, i the rate of ^3H flux between the A and B positions of NADH and α the fraction of ^3H lost to water in the aldolase reaction.

glycerol kinase 0.01 mM (Grunnet & Lundquist, 1967). The model includes only the NAD^+ -dependent cytoplasmic glycerol phosphate dehydrogenase-catalysed reaction. Carnicero *et al.* (1972) found that the detritiation of $[2\text{-}^3\text{H}]\text{glycerol phosphate}$ by the liver is primarily a cytoplasmic reaction and that less than 10% of the oxidation is a result of action of mitochondrial glycerol phosphate dehydrogenase.

The flux of reducing equivalents from $[2\text{-}^3\text{H}]\text{glycerol}$ or $[2\text{-}^3\text{H}]\text{lactate}$ was calculated from Model II (Fig. 1), expressed in the form of eqns. I–VI below. Values of the model parameters which gave the best fit to the experimental data were calculated by a least-squares fitting routine (see the Appendix).

The experimental data used in the calculations are the concentrations at zero time of lactate (S_0), ethanol (A_0), glucose (G_0), and glycerol phosphate (F_0); the rates of metabolism of lactate (a), ethanol (b), glucose (ΔG), glycerol (k) and glycerol phosphate ($k-g$); and the changes with time of the specific radioactivities of lactate (L), ethanol (E), glucose (T), glycerol phosphate (H) and glycerol (R_0) (Fig. 2). The specific radioactivity of glycerol (R_0) remained constant during the incubation.

The best fit (Fig. 2) to the model parameters allows calculation of the flux of reducing equivalents from $[2\text{-}^3\text{H}]\text{lactate}$ or from $[2\text{-}^3\text{H}]\text{glycerol}$ via NADH to pyruvate (x), acetaldehyde (y), 1,3-bisphosphoglycerate (f) and dihydroxyacetone phosphate (z) and between $[4\text{-}^3\text{H}]\text{NADH}$ and $[4\text{-}^3\text{H}]\text{NADH}$ (i).

Assuming steady-state and ignoring isotope effects, which may be very close to 1 in the reversible reactions (Vind & Grunnet, 1987), we can write equations representing inflow and outflow of isotope for each metabolite in the model:

$$\frac{d(S_0 - at)L}{dt} = xN_A - (x+a)L \quad (\text{I})$$

$$\frac{d(A_0 - bt)E}{dt} = yN_A - (y+b)E \quad (\text{II})$$

$$\frac{d(G_0 + \Delta Gt)T}{dt} = \alpha fN_B \quad (\text{III})$$

$$\frac{d(F_0 + (k-g)t)H}{dt} = zN_B - (g+z)H + kR_0 \quad (\text{IV})$$

$$\frac{dQN_B}{dt} = (g+z)H + iN_A - (i+f+z)N_B \quad (\text{V})$$

$$\frac{dQN_A}{dt} = (x+a)L + (y+b)E + iN_B - (i+a+x+b+y)N_A \quad (\text{VI})$$

N_A and N_B represent the change with time of the specific radioactivities of $[4\text{-}^3\text{H}]\text{NADH}$ and $[4\text{-}^3\text{H}]\text{NADH}$, respectively. The concentration of NADH (Q) was calculated from the lactate/pyruvate ratio and the concentration of NAD^+ (Vind & Grunnet, 1984). A 10-fold change in the value of Q was without effect on the fitting and the estimation of the unknown parameters, because of its small value compared with the other measured data.

The value of α represents the relative loss of ^3H to water, in the aldolase reaction, or by cycling in the reactions catalysed by aldolase and triosephosphate isomerase, when triosephosphate is converted to glucose. If ^3H was lost only in the aldolase reaction, the value of α would be 0.5. The value of $\alpha = 0.25$ was the only value which allowed the model parameters to fit all the experimental data (Fig. 2). This value of α is in agreement with that found earlier in hepatocytes from starved rats both with and without xylitol or fructose (Vind & Grunnet, 1981, 1984).

The rate of exchange between the 4A and the 4B positions in NADH (i) had to be higher than

Fig. 2. Specific radioactivities (c.p.m./nmol) of lactate, ethanol, glucose and glycerol phosphate as a function of time

Hepatocytes were incubated for 10 min with 2.5 mM-lactate, 0.25 mM-pyruvate, 8 mM-glycerol and 20 mM-ethanol before addition of $[2\text{-}^3\text{H}]\text{lactate}$ or $[2\text{-}^3\text{H}]\text{glycerol}$. The curves are given as examples and represent the mean values for five experiments with $[2\text{-}^3\text{H}]\text{lactate}$ and five with $[2\text{-}^3\text{H}]\text{glycerol}$. The open circles represent the measured data and the lines represent the computed least-square fits to the measured data. I and II are the fits obtained by 'Model I' and 'Model II', respectively. With Model II and $i = 35$ and $\alpha = 0.25$, we find the oxidation rates of NADH catalysed by lactate dehydrogenase, alcohol dehydrogenase, triosephosphate dehydrogenase and glycerol phosphate dehydrogenase to be 1.2, 0.5, 2.1 and 0.2 or 1.5, 0.4, 2.1 and 0.3 $\mu\text{mol/min}$ per ml of packed cells with $[2\text{-}^3\text{H}]\text{lactate}$ or $[2\text{-}^3\text{H}]\text{glycerol}$ as the labelled substrate, respectively. With Model I and the experimental data with $[2\text{-}^3\text{H}]\text{lactate}$ as the labelled substrate, we find the oxidation rates of NADH catalysed by lactate dehydrogenase, alcohol dehydrogenase and triosephosphate dehydrogenase to be 1.2, 0.6 and 1.9 $\mu\text{mol/min}$ per ml of packed cells, respectively. These rates are similar to those calculated from the individual experiments (Table 2).

25 $\mu\text{mol/min}$ per ml packed cells to allow the model parameter to fit the experimental data for any of the labelled substrates ($[2\text{-}^3\text{H}]\text{lactate}$ or $[2\text{-}^3\text{H}]\text{glycerol}$).

The calculated values of x , y , f and z obtained for $[2\text{-}^3\text{H}]\text{lactate}$ with Model I and for $[2\text{-}^3\text{H}]\text{lactate}$ or $[2\text{-}^3\text{H}]\text{glycerol}$ with Model II are shown in Table 2. The calculated values for the oxidation of NADH catalysed by lactate dehydrogenase (x), alcohol dehydrogenase (y) and triosephosphate dehydrogenase (f) are the same,

whether calculated from Models I or II or from experiments with $[2\text{-}^3\text{H}]\text{lactate}$ or $[2\text{-}^3\text{H}]\text{glycerol}$. The reduction of dihydroxyacetone phosphate (z) can only be calculated from Model II and was independent of the labelled substrate used (Table 2).

DISCUSSION

Equilibration of the 4A and 4B hydrogen atoms of NADH

The results indicate that the flux between the 4A and 4B hydrogen atoms of NADH was higher than 25 $\mu\text{mol/min}$ per ml packed cells, i.e. of the order of equilibrium. In this case the results obtained with the two labelled substrates were in agreement (Table 2). Though each NAD^+ molecule undergoes many redox turnovers during an incubation, the equilibrium between the 4A and 4B positions in NADH will depend on the amount of scrambling by the action of both A- and B-specific NAD^+ -dependent dehydrogenases. In our model we have estimated the rate of reactions catalysed by the two A-specific dehydrogenases, lactate and alcohol dehydrogenases, and the two B-specific dehydrogenases, triosephosphate and dihydroxyacetone phosphate dehydrogenases. Beside those, the reduction of NAD^+ and the oxidation of NADH are catalysed at a high rate by the A-specific malate dehydrogenase in the cytoplasm, connected via the malate-aspartate shuttle to the mitochondria (Crow *et al.*, 1982; Cronholm, 1985). The relative yield of ^3H in water was 63% from $[2\text{-}^3\text{H}]\text{glycerol}$ and 75% from $[2\text{-}^3\text{H}]\text{lactate}$, which suggests that the activity of the malate dehydrogenase, together with the other cytoplasmic dehydrogenases, is high enough to account for the near-equilibrium of the 4A and 4B hydrogen atoms of NADH.

Evaluation of the models

The rate of reduction of dihydroxyacetone phosphate and 1,3-bisphosphoglycerate (Table 2) are of the same order of magnitude as the carbon flux in these reactions, estimated by Crawford & Blum (1983), who followed the flux of ^{14}C along the gluconeogenic and glycolytic pathways, using reducing conditions similar to those of the present work. They found the flux from dihydroxyacetone phosphate to glycerol phosphate to be 1.19 $\mu\text{mol/min}$ per g wet wt. of liver and from phosphoenolpyruvate to triosephosphate to be 0.23 $\mu\text{mol/min}$ per g wet wt. of liver.

Furthermore, the results obtained with $[2\text{-}^3\text{H}]\text{lactate}$ and calculation based on Model I were in agreement with results obtained with Model II, which includes glycerol metabolism (Table 2).

Agreement between results, obtained with a model for carbon flux, a model for ^3H and an extension of the model for ^3H , as in this case by the glycerol phosphate dehydrogenase reaction, suggests that this type of model may be useful for estimation of intracellular reaction rates.

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Table 2. Rate of reduction of pyruvate, acetaldehyde, dihydroxyacetone phosphate and 1,3-diphosphoglycerate

Hepatocytes were incubated for 10 min with 2.5 mM-lactate, 0.25 mM-pyruvate, 8 mM-glycerol and 20 mM-ethanol before addition of labelled substrate. Samples were taken every 10 min for 70 min and used to estimate the labelling pattern of lactate, ethanol, glucose and glycerol phosphate from which the oxidation rates of NADH catalysed by cytoplasmic dehydrogenases were calculated. Values are expressed as means $\pm$ S.E.M. with the number of different cell preparations in parentheses.

Labelled substrate	Calculation by Model:	Rate of reduction (μ mol/min per ml of packed cells) of:			
		Pyruvate (x)	Acetaldehyde (y)	1,3-Bisphosphoglycerate (f)	Dihydroxyacetone phosphate (z)
[2- 3 H]Lactate	I	1.18 $\pm$ 0.12	0.61 $\pm$ 0.12	1.84 $\pm$ 0.37 (5)	—
[2- 3 H]Lactate	II	1.35 $\pm$ 0.15	0.63 $\pm$ 0.12	1.96 $\pm$ 0.52 (5)	0.18 $\pm$ 0.02
[2- 3 H]Glycerol	II	1.16 $\pm$ 0.11	0.47 $\pm$ 0.09	1.95 $\pm$ 0.45 (6)	0.38 $\pm$ 0.11

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APPENDIX

Estimation of parameters

The set of equations analysed is given in (I)–(VI). First we describe how to solve (I)–(VI) for a particular choice of parameters $k = (x, y, z, i, \dots, f)$. Then we vary k to get the best possible fit of the model to experimentally recorded data on L , E , T and H . The integration of (I)–(VI) may be accomplished by standard (stiff) differential-equation-solving routines such as that described by Gear. It was found, however, that a faster method was available for this system. Consider (I) written out:

$$S_0 \frac{dL}{dt} - aL - at \frac{dL}{dt} = xN_A - (x+a)L \quad (\text{I}')$$

that is:

$$\frac{dL}{dt} = \frac{x}{S_0 - at} N_A - \frac{x}{S_0 - at} L$$

with similar expressions for the other equations (II)–(VI). If $S_0 \gg at$, etc., coefficients of the form $x/(S_0 - at)$ become virtually independent of t , and (I)–(VI) reduces to a coupled set of first-order reactions. Such a system may be written in matrix form as follows.

Let vector $c = (L, E, T, H, N_B, N_A)$, then we have:

$$\frac{dc}{dt} = Ac + d \quad (\text{I})$$

where:

$$d = (0, 0, 0, \frac{kR_0}{F_0}, 0, 0)$$

This system is readily solved by standard methods (see below). However, $S_0 - at$ (etc.) is not constant, but varies only slightly as t increases. A sufficiently accurate solution to (I)–(VI) may then be found by subdividing the time interval of interest into, say, 50 sub-intervals, within which $S_0 - at$ is treated as a constant $S_0 - at_i$. In the i th time interval, we thus solve a set of first order differential equations with constant coefficients:

$$\frac{dc}{dt} = A(k, t_i)c + d(t_i) \quad (\text{2})$$

The standard method of solving (2) is to diagonalize A , using Hessenberg reduction and the QR algorithm (Press *et al.*, 1986), i.e. finding matrix Q and eigenvalues λ_j so that:

$$Q A Q^{-1} = L$$

where L is diagonal with $L_{jj} = \lambda_j$. In the i th time step, we thus have:

$$\frac{d(Q_i c)}{dt} = (Q_i A_i Q_i^{-1})(Q_i c) + Q_i d_i \quad (\text{3})$$

or:

$$\frac{dy}{dt} = Ly + d' \quad (4)$$

As c is known on the left side of the i th t interval, so is $y (= Q_i c)$, and (4) may be integrated analytically, as L is diagonal.

The components of y satisfy:

$$\frac{dy_j}{dt} = \lambda_j y_j + d_j' \quad (5)$$

and y_j at the right side of the i th time interval is obtained directly as:

$$y_j(t = t_i + \Delta) = [y_j(t_i) + \frac{d_j'}{\lambda_j}]e^{\lambda_j \Delta} - \frac{d_j'}{\lambda_j} \quad (6)$$

As:

$$Q_i c(t_i + \Delta) = y(t_i + \Delta)$$

we can solve for c (by Gaussian elimination) at the right of the i th time interval. The steps (3)–(6) are repeated, etc., until the final value of t is reached. Hereby $c(t)$ is

found. This solution of $c(t)$ is dependent of the particular choice of parameters k . However, we may vary k and get new solutions $c(t)$ with the above method. k may be varied automatically, if its components are considered unknowns to be optimized in a standard optimizer, such as a quasi-Newton method (Press *et al.*, 1986). The function $H(k)$ we wish to minimize is the usual sum of squared deviations: four (in general q) components of $c(t)$ are measured at different times (say l times), in general ql measured values. Thus:

$$H(k) = \sum_{j=1}^q \sum_{i=1}^l [c_{ji}(\text{measured}) - c_{ji}(k)]^2 \quad (8)$$

where $c_{ji}(k)$ is a computed value from (I)–(VI). Thus, the best possible values of the parameters (k) are found by minimizing $H(k)$, with $c(k)$ being calculated from the procedure outlined in (3)–(6). This method was implemented using Hessenberg, QR, Gauss and quasi-Newton algorithms from the Numerical Algorithms Group library (available from: NAG, Mayfield House, 256 Banbury Road, Oxford OX2 7DE, U.K.).

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