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AN NMR INVESTIGATION OF ISOTOPE EXCHANGE INVOLVING MULTIPLY LABELLED INTERMEDIATES

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Abstract—Information about enzyme systems can be obtained from studies of isotope exchange reactions. NMR can monitor these reactions continuously, even in intact biological samples. In this paper ^1H NMR is used to study a $^1H/^2H$ exchange process which involves multiple labelling of NADH. The NADH is an intermediate in an exchange process involving the glycolytic enzymes glyceraldehyde phosphate dehydrogenase, lactate dehydrogenase, triose phosphate isomerase and aldolase. The form of the observed exchange is predicted by an extended theory of isotope exchange.

NMR techniques have recently been applied to the study of isotope exchange reactions catalysed by intact cells.[1-3] We have demonstrated that it is possible to study the properties of specific enzymes in cells by adding a rapidly transported isotopically labelled metabolite to cell suspensions and monitoring the enzyme catalysed redistribution of label by ^1H NMR. For example, we have used isotope exchange of lactate and pyruvate to compare the properties expressed by lactate dehydrogenase in intact erythrocytes with those expressed by this enzyme *in vitro*.[4,5] We are currently investigating an exchange, catalysed by erythrocytes, involving lactate and the four glycolytic enzymes lactate dehydrogenase, triosephosphate isomerase, glyceraldehyde phosphate dehydrogenase and aldolase.[6] A schematic representation of the mechanism of the lactate $^1H/^2H$ exchange is shown in Fig. 1. In this paper, a detailed study is made of an *in vitro* model of this system. Proton NMR is used to observe the time course of label incorporation into lactate, NAD^+ and NADH.

The complex kinetics are interpreted using an extended theory of isotope exchange[7] which accounts for multiple labelling by treating each isotopically labelled species as a separate entity. Previous treatments of isotope exchange kinetics in enzyme systems have dealt with radioactive tracer experiments where the probability of multiple labelling is negligible[8-10]

EXPERIMENTAL

Enzymes and biochemicals were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Other chemicals were analytical grade.

The samples were prepared in a ^2H_2O buffer (100 mM glycine, pH* 8.1). The concentrations of reagents present in the samples were: 12 mM lactate; 8 mM NAD^+; 4 mM NADH; 1 mM fructose 1, 6-diphosphate. The samples used to observe the exchange also contained: lactate dehydrogenase (rabbit muscle), 810 μ moles min^{-1}; glyceraldehyde phosphate dehydrogenase (rabbit muscle), 16 μ moles min^{-1}; triose-phosphate isomerase (yeast), 0.2 mg; aldolase (rabbit muscle), 1.8 μ moles min^{-1}.

Spectra were obtained using a Bruker WH300 spectrometer. Each spectrum was accumulated with a 30° pulse angle, 604 scans, total acquisition time 12 min, 37°. The water resonance was suppressed with a gated pulse. A time course was followed by storing sequential 12 min blocks in the computer store.

The molefractions of NADH and the monodeuterated NADH species were calculated from the spectra using the following formulae, which are derived from the assignments of the NADH multiplet given in the Results and Discussion section.

If I_1, I_2, I_3 and I_4 are the integrals of the components of the NADH multiplet as indicated in Fig. 2, and I_n is the total intensity of the multiplet before isotopic substitution, then:

(i) the molefraction of NADH, $X_{NADH(H,H)} = 2(I_1 + I_3)/I_n$

(ii) the molefraction of [4A-^{2}H] NADH, $X_{NADH(D,H)} = 2(I_4 - I_1)/I_n$

(iii) the molefraction of [4B-^{2}H] NADH, $X_{NADH(H,D)} = 2(I_2 - I_3)/I_n$

Computer simulations. Integration of the differential equations which describe the isotope exchange was carried out using an Euler predictor-corrector method, on a Computer Technology Modular 1 computer.

THEORETICAL

Any isotope exchange reaction may be dissected into a number of partial reactions. For example, the four partial reactions which describe the transfer of H and D across LDH are:

$$NADH(H, H) \rightleftharpoons LACTATE(H) + NAD^+(H)$$
$$NADH(H, D) \rightleftharpoons LACTATE(H) + NAD^+(D)$$
$$NADH(D, H) \rightleftharpoons LACTATE(D) + NAD^+(H)$$
$$NADH(D, D) \rightleftharpoons LACTATE(D) + NAD^+(D)$$

where the convention used to indicate the site of a label on NADH is that the first symbol in the brackets indicates the labelling of the C-4A position while the second symbol indicates the labelling of the C-4B position. Note that intermediates which do not have a label site (H^+ and pyruvate in our example) are eliminated from the analysis.

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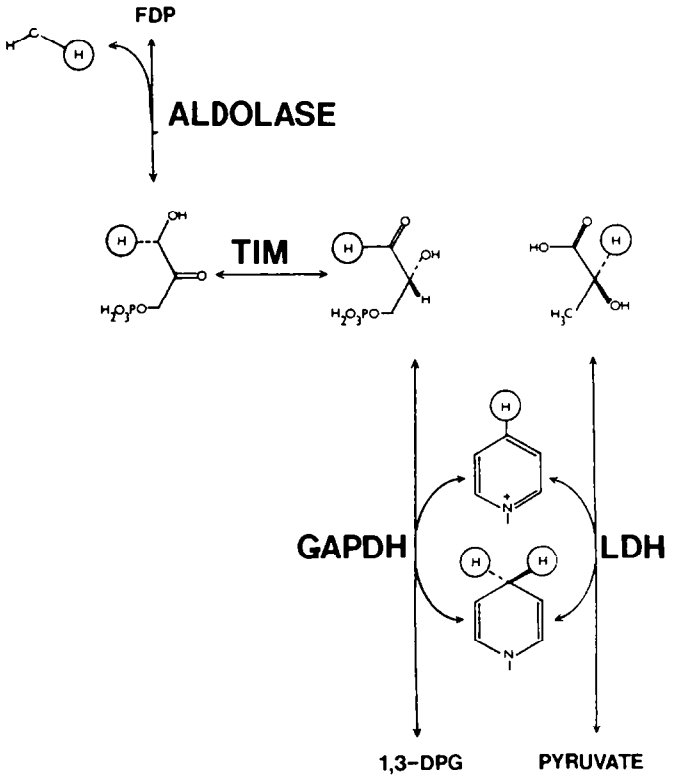

Fig. 1. An illustration of the mechanism of the lactate $^1H/^2H$ exchange system. The circled hydrogens are those exchanged. The label proceeds from the lactate, via the NAD^+/NADH coenzymes to GAP and DHAP. During the exchange NADH becomes doubly labelled because the enzymes glyceraldehyde 3-phosphate dehydrogenase and lactate dehydrogenase have opposite face specificity for NADH.

The isotope transfer fluxes of the partial reactions are of the general form

$$J = V(p(\Gamma) - p(\Delta))$$

where V is the isotope exchange equilibrium velocity of the enzyme catalysing the partial reaction and $p(\Gamma)$ and $p(\Delta)$ are the probability functions associated with label transfer between the groups of labelled species on the left (Γ) and right (Δ) hand sides of a partial reaction. The probability function for a group of labelled species is given by the product of the molefractions of each species raised to the power of their stoichiometries. For example the transfer flux equations for the lactate dehydrogenase equilibrium are:

$$J_1 = V(X_{NADH(H,H)} - X_{NAD(H)} \cdot X_{LAC(H)})$$
$$J_2 = V(X_{NADH(H,D)} - (1 - X_{NAD(H)}) \cdot X_{LAC(H)})$$
$$J_3 = V(X_{NADH(D,H)} - X_{NAD(H)} \cdot (1 - X_{LAC(H)})$$
$$J_4 = V(X_{NADH(D,D)} - (1 - X_{NAD(H)}) \cdot (1 - X_{LAC(H)})$$

where V is the enzyme equilibrium velocity.

The general form of the differential equation which describes the molefraction of a labelled species C(*), $X_{C(*)}$ is

$$[C]\dot{X}_{C(*)} = \sum_{i=1}^{i=N} c_i J_i$$

where [C] is the total concentration of the parent compound, C and N is the number of equilibria in which C is involved. The elements of the vectors c_i and J_i are the stoichiometries and transfer fluxes associated with c(*). Table 1 shows the complete set of differential equations derived for the pathway of exchange of the C-2 hydrogen of lactate given in Fig. 1. The definitions of the notation used are given in the Table caption. These non-linear differential equations can be solved either by numerical integration or by deriving approximate solutions as described in the appendix.

The assumptions required by the theory of equilibrium isotope exchange developed above are:

(1) there is a completely defined pathway for the label transfer
(2) the system is at chemical equilibrium
(3) any kinetic isotope effects should be small.

The first of these assumptions can be checked and if additional pathways are found then they can be included in a model of the exchange (see for example Ref. 4). The second assumption is more difficult since enzyme systems in intact biological systems do not, in general, operate at equilibrium. As we have discussed elsewhere,[6] however, there are many situations in which the equilibrium assumption is valid. In the case of lactate-solvent exchange in human red cells, the assumption applies a few tens of seconds after the addition of lactate because membrane transport and induced concentration changes are rapid compared to the exchange. In addition the lactate production rate is about five times slower than the isotope-exchange

rate in the red cell.[6] The assumption about kinetic isotope effects is reasonable in many cases, especially if initial rates are considered. In the lactate-solvent exchange case the effect is small.[6]

To solve the differential equations we have used both numerical methods and approximate analytical solutions. Numerical solution of a set of differential equations is a relatively straightforward procedure in most cases[11] and this approach is completely general and flexible. A set of analytical solutions can provide a useful alternative to numerical solutions since the experimental data may then be analysed by simple graphical procedures and additional insight may be gained. The approximate analytical solutions are given in Table 2 and the procedures used to obtain them are described in the appendix.

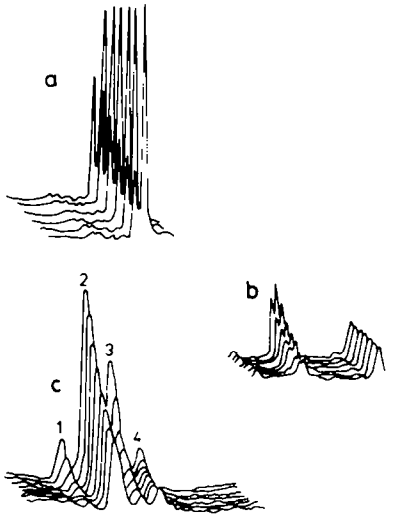

Fig. 2. Part of the exchange time course as observed in the NMR spectra of (a) the lactate methyl group, (b) and C-4 proton of the nicotinamide ring of NAD$^+$ and (c) the C-4 protons of NADH. The time axis for each stacked plot projects out of the page.

Two predictions can be made about the exchange of NADH in the lactate/solvent system from the approximate solutions listed in Table 2:

(1) Simple exponential timecourses are predicted for the molefractions X_A and X_B where

$$X_A = X_{NADH(H,H)} + X_{NADH(H,D)}$$
$$X_B = X_{NADH(H,H)} + X_{NADH(D,H)}$$

(2) The molefraction of unexchanged NADH is predicted to follow an exponential exchange curve whose rate constant is twice that associated with the exchange curves of X_A, X_B and X_{LAC}. This factor of two arises because of the opposite face stereospecificities of the enzymes glyceraldehydephosphate dehydrogenase and lactate dehydrogenase for NADH.

RESULTS AND DISCUSSION

A typical series of spectra, obtained during the course of the exchange of the C-2 hydrogen of lactate with solvent, is shown in Fig. 2. The lactate doublet

Table 1. The differential equations used to model the lactate $^1H/^2H$ exchange. The equilibrium velocities of lactate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, triose phosphate isomerase and aldolase are denoted by the symbols V_1 to V_4 respectively. The convention used to indicate the site of label on the dihydropyridine ring of NADH is that the first symbol in the brackets indicates the labelling state of the C-4A position while the second symbol indicates the labelling state of the C-4B position

Intermediate	Symbol	Variable	Differential Equations
Lactate	LAC	X_{LAC}	$[LAC]\dot{X}_{LAC} = V_1 (X_{NADH(H,H)} + X_{NADH(D,H)} - X_{LAC})$
NAD	NAD	X_{NAD}	$[NAD]\dot{X}_{NAD} = V_1(X_{NADH(H,H)} + X_{NADH(D,H)} - X_{NAD}) + V_2(X_{NADH(H,H)} + X_{NADH(H,D)} - X_{NAD})$
NADH	NADH(H,H)	$X_{NADH(H,H)}$	$[NADH]\dot{X}_{NADH(H,H)} = V_1(X_{LAC} \cdot X_{NAD} - X_{NADH(H,H)}) + V_2(X_{GAP} \cdot X_{NAD} - X_{NADH(H,H)})$
[4B-^{2}H]NADH	NADH(H,D)	$X_{NADH(H,D)}$	$[NADH]\dot{X}_{NADH(H,D)} = V_1(X_{LAC}(1-X_{NAD}) - X_{NADH(H,D)}) + V_2((1-X_{GAP})X_{NAD} - X_{NADH(H,D)})$
[4A-^{2}H]NADH	NADH(D,H)	$X_{NADH(D,H)}$	$[NADH]\dot{X}_{NADH(D,H)} = V_1((1-X_{LAC})X_{NAD} - X_{NADH(D,H)}) + V_2(X_{GAP}(1-X_{NAD}) - X_{NADH(D,H)})$
Glyceraldehyde 3-phosphate	GAP	X_{GAP}	$[GAP]\dot{X}_{GAP} = V_2(X_{NADH(H,H)} + X_{NADH(D,H)} - X_{GAP}) + V_3(X_{DHAP} - X_{GAP})$
Dihydroxyacetone Phosphate	DHAP	X_{DHAP}	$[DHAP]\dot{X}_{DHAP} = V_3(X_{GAP} - X_{DHAP}) + V_4(X_{SOL} - X_{DHAP})$
Solvent	SOL	X_{SOL}	$[SOL]\dot{X}_{SOL} = V4(X_{DHAP} - X_{SOL})$

Table 2. The approximate analytical solutions used to predict the label distribution during the lactate $^1H/^2H$ exchange. λ is the time constant which characterises the exchange and $X(\infty) = [*]_T/([*]_T + [0]_T)$; $1/V_T = 2/V_1 + 2/V_2 + 1/V_3 + 1/V_4$

Intermediate	Symbol	Variable	Approximate Solution
Lactate	LAC	X_{LAC}	$X_{LAC} = \exp(\lambda t)$
NAD	NAD	X_{NAD}	$X_{NAD} = (1-V_T(1/V_1+1/V_2)\exp(\lambda t)$
NADH	NADH(H,H)	$X_{NADH(H,H)}$	$X_{NADH(H,H)} = (1-V_T(1/V_1+1/V_2))(1-2V_T/V_1)\exp(2\lambda t)$
[4B-^{2}H]NADH	NADH(H,D)	$X_{NADH(H,D)}$	$X_{NADH(H,D)} = (1-V_T/V_1)\exp(\lambda t) - (1-V_T(1/V_1+1/V_2))(1-2V_T/V_1)\exp(2\lambda t)$
[4A-^{2}H]NADH	NADH(D,H)	$X_{NADH(D,H)}$	$X_{NADH(D,H)} = (1-V_T(2/V_1+1/V_2)\exp(\lambda t) - (1-V_T(1/V_1+1/V_2))(1-2V_T/V_1)\exp(2\lambda t)$
Glyceraldehyde 3-phosphate	GAP	X_{GAP}	$X_{GAP} = (1-V_T(2/V_1+2/V_2))\exp(\lambda t)$
Dihydroxyacetone Phosphate	DHAP	X_{DHAP}	$X_{DHAP} = (1-V_T(2/V_1+2/V_2+1/V_3))\exp(\lambda t)$
Solvent	SOL	X_{SOL}	$X_{SOL} = X(\infty)(1-\exp(\lambda t))$

resonance collapses to an apparent singlet because the deuterium coupling is relatively small and there is a small upfield isotope shift which results in the resonance of the singlet overlapping the upfield component of the doublet. The doublet spectrum of the C-4 proton of NAD^+ simply decreases as the exchange proceeds. The C-4 proton resonances of NADH appear as a quartet while the [4A-^{2}H] and [4B-^{2}H] resonances appear as singlets. Due to upfield deuterium isotope shifts these singlet resonances from the partially deuterated species coincide with the second and fourth lines (as numbered in Fig. 2) of the quartet from the unexchanged NADH species. The resonance coincident with the second line is assigned to [4B-^{2}H] NADH in agreement with Arnold and You.[12]

The molefractions of the various species were calculated from the spectra. In the case of the NADH species they were calculated from the integrals of the components of the multiplet as described in the Methods section. This involves normalizing the data to the intensity of the NADH resonance from a separate non-exchanging sample. Any error introduced by this procedure takes the form of a constant scaling factor.

The exchange of lactate and NAD^+ were found to follow exponential timecourses with rate constants of $1.5 \pm 0.2 \times 10^{-2}\,min^{-1}$. The experimental timecourses for X_A, X_B and $X_{NADH(H,H)}$ are shown in Fig. 3 and 4. It is clear that the basic forms of these are exponential, in agreement with the predictions. The rate constant associated with the exchange curves for X_A and X_B was again found to be $1.5 \pm 0.2 \times 10^{-2}\,min^{-1}$. This rate constant can be compared with the value of $3.1 \pm 0.2 \times 10^{-2}\,min^{-1}$ obtained for the rate constant of the timecourse of $X_{NAD(H,H)}$. The ratio of these rate constants is, within experimental error, in agreement with the theoretical value of 2.

The curves shown in Figs. 3 and 4 are calculated from the approximate theoretical solutions given in Table 2 (Appendix). Numerical solutions to the differential equations in Table 1 were also calculated and fits to the experimental data similar to those in Fig. 3 are obtained.

To conclude, NMR is useful for following complex isotope exchange kinetics because fractional isotopic labelling can be determined directly from the spectra. In addition the mathematical treatment of isotope

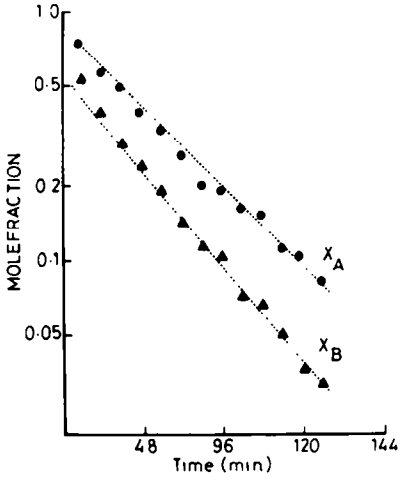

Fig. 3. The molefractions of site labelling for NADH derived from the data in Fig. 3 as described in the text. The dotted curves represent theoretical curves derived from the approximate solutions given in Table 2.

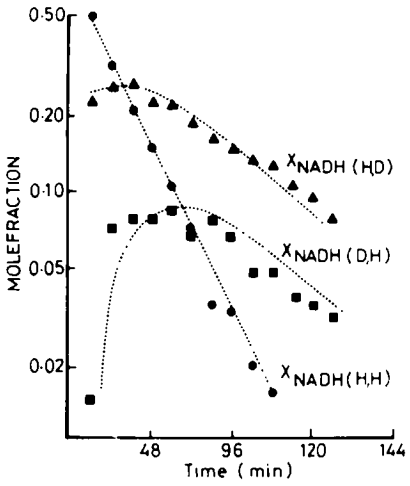

Fig. 4. The molefractions of the three ^1H labelled species derived from the data in Fig. 3. The dotted lines represent theoretical curves derived from the solutions in Table 2.

exchange discussed in the appendix represents an extension of previous theories[8-10] because it is able to describe exchange pathways in which molecules become multiply labelled. Another advantage is that it provides analytical expressions for the labelling states of all the intermediates involved in an exchange pathway. This facilitates interpretation of experimental data. The application of the treatment is not restricted to the lactate $^1H/^2H$ exchange discussed here. Multiple labelling has, for example, also been observed by ^{13}C NMR[13,14] and by ^{31}P NMR of phosphate groups with O labels.[17,18]

REFERENCES

[1]K. M. Brindle, F. F. Brown, I. D. Campbell, D. L. Foxall and R. J. Simpson, *Biochem. Soc. Trans.* **8**, 646 (1981).
[2]R. J. Simpson, K. M. Brindle, F. F. Brown, I. D. Campbell and D. L. Foxall, *Biochem. J.* **193**, 493 (1981).
[3]J. A. den Hollander, T. R. Brown K. Urgibil and R. G. Shulman, *Proc. Nat. Acad. Sci. USA* **76**, 6096 (1979).
[4]R. J. Simpson, K. M. Brindle, F. F. Brown, I. D. Campbell and D. L. Foxall, *Biochem. J.* **202**, 573 (1982).
[5]R. J. Simpson, K. M. Brindle, F. F. Brown, I. D. Campbell and D. L. Foxall, *Ibid.* **202**, 581 (1982).
[6]K. M. Brindle, F. F. Brown, I. D. Campbell, D. L. Foxall and R. J. Simpson, *Ibid.* **202**, 589 (1982).
[7]D. L. Foxall, D.Phil. Thesis, Oxford University.
[8]G. Yagil and H. D. Hoberman, *Biochemistry* **8**, 352 (1969).
[9]G. Yagil, *J. Theor. Biol.* **61**, (1976).
[10]J. Flossdorf and M. R. Kula, *Eur. J. Biochem.* **30**, 325 (1972).
[11]D. Garfinkel, C. B. Marbach and N. Z. Shapiro, *Ann. Rev. Biophys. Bioeng.* **6**, 525, (1977).
[12]L. J. Arnold and K. You, *Methods in Enzymology* **54**, 223 (1978).
[13]T. E. Walker, C. H. Han, V. H. Kollman, R. E. London and N. A. Matwiyoff, J. Biol. Chem. **257**, 1189 (1982).
[14]S. M. Cohen, S. Ogawa and R. G. Shulman, *Proc. Nat. Acad. Sci. USA* **76**, 4808 (1979).
[15]D. B. Northrop, *Isotope Effects on Enzyme Catalysed Reactions* (Edited by W. W. Cleland, M. H. O'Leary and D. B. Northrop), p. 122. University Park Press, London (1977).
[16]H. A. C. MacKay, *Nature* **142**, 997 (1938).
[17]M. Cohn and A. Hu, *Proc. Nat. Acad. Sci. USA* **75**, 200 (1978).
[18]P. Rösch, H. R. Kalbitzer and R. Goody, *Z. Naturforsch.* **36b**, 534 (1981).

APPENDIX

A method for obtaining approximate analytical solutions of differential equations describing isotope exchange will be described. The first step is to remove the non-linearity in differential equations of the kind shown in Table 1. This can be brought about by a change of variables. In the lactate/solvent exchange example the non-linearity associated with the NADH species can be removed by transforming the equations into ones which describe the molefractions of site labelling for the A and B faces of the NADH molecule. If X_A and X_B are the molefractions of site labelling for the A and B faces then we have:

$$X_A = X_{NADH(H,H)} + X_{NADH(H,D)}$$
$$X_B = X_{NADH(H,H)} + X_{NADH(D,H)}.$$

These relationships can be used to linearise the differential equations in Table 1. The resulting equations may be represented in matrix notation as;

$$\dot{\mathbf{X}} = [Q]\mathbf{X}$$

where $\mathbf{X}$ is a vector whose elements are the molefractions of site labelling, and [Q] are the coefficients of the linear differential equations. The first element of $\mathbf{X}$ corresponds, by convention, to the intermediate which is the major source of label, while the last element corresponds to the intermediate which is the major sink for labelled atoms. {Note that the type of transformation used to remove non-linearity in this example is quite general[7] and can be applied to the equations arising from other systems in which multiple labelling is involved.}

An approximation to the solution of these differential equations, is:

$$\mathbf{X} = a_1\mathbf{u}\exp(\lambda t) + \mathbf{a}_0$$

where λ is the largest non-zero eigenvalue of [Q], $\mathbf{u}$ is the eigenvector of [Q] corresponding to λ and a_1 and the elements of a_0 are aribtrary constants.

The parameters a_1, $\mathbf{a}_0$, $\mathbf{u}$ and λ can be evaluated in terms of the equilibrium velocities of the enzymes involved in the exchange. This operation may be carried out as follows:

(1) The λ is expressed in terms of the overall equilibrium velocity (V_T) of an exchange by combining the approximate solution with the equation proposed by Mackay[16] to give:

$$\lambda = -V_T(1/[*]_T + 1/[0]_T)$$

where $[*]_T$ is the total concentration of labelled atoms and $[*]_T + [0]_T$ is the total concentration of label sites.

(2) The elements of $\mathbf{a}_0$ represent the distribution of label among the label sites at isotopic equilibrium.

(3) The values of the first element of $\mathbf{u}$ are defined to be unity. The remaining elements can then be determined from the simultaneous equations $[Q]\mathbf{u} = \lambda\mathbf{u}$ if the diagonal elements q_n other than those associated with the source or sink for label are such that $q_n - \lambda \simeq q_n$.

(4) The a_1 is evaluated from the expression which describes the molefraction of site labelling for the major source of label ($X_1(t)$) and is given by:

$$a_1 = X_1(0) - ([*]_T/[*]_T + [0]_T$$

(5) The relationship between the overall equilibrium velocity and the equilibrium velocities if the individual enzymes can be determined from the expression for the eigenvector element (u_x) corresponding to the intermediate

which is the major sink for labelled atoms. It can be shown that:

$$u_s = X_s(0) - ([*]_T/[*]_T + [0]_T)$$

where $X_s(0)$ is the molefraction of site labelling in the sink intermediate at the beginning of the exchange.

The non-linear equations describing the molefractions of the various species can now be linearized by substitution of their non-linear terms with the approximate expressions for the molefractions of site labelling given above. The resulting inhomogeneous linear differential equations can be solved by standard analytical methods. In our example of lactate-solvent exchange the solutions which describe the distribution of label amongst the NADH species are given in Table 2.