Computer program for the kinetic equations of enzyme reactions

The case in which more than one enzyme species is present at the onset of the reaction

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This paper presents an extension of the program developed by Varón, Havsteen, García, García-Cánovas & Tudela [(1990) *Biochem. J.* 270, 825–828] for the expression of the transient-phase and steady-state kinetic equations of a general enzyme system in which the only enzyme species present at the onset of the reaction is the free enzyme. The program has been extended to situations in which more than one enzyme species may be present at the onset of the reaction. The program is given in Supplementary Publication SUP50165 (5 pages), which has been deposited at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1991) 273, 5.

INTRODUCTION

Recently, a computer program for the construction of the transient-phase (and the steady-state) equations of a general enzyme model containing n enzyme species and g ligand species was developed (Varón *et al.*, 1990). This program yields the expressions of the coefficients in the kinetic equations as functions of the rate constants and the initial concentrations of enzyme systems in which only one conformer of the free enzyme is present at the onset of the reaction. This situation is the most frequent in enzyme experiments.

However, there are enzyme reactions in which more than one enzyme form can be present at the onset of the reaction, e.g.: (a) Several conformers of the free enzyme exist (Halford, 1971; Darvey, 1977); (b) the free enzyme is premixed with one or more of the ligands of the reaction before the remaining necessary ligands are added to initiate the reaction (Luisi & Favilla, 1972; Hijazi & Laidler, 1973; Hardman & Blackwell, 1974; Darvey, 1977; Kahn & Andrawis, 1986); (c) all of the reactants of a enzyme reaction are mixed and, after the steady state has been reached, it is perturbed (e.g. by a temperature jump); Then the system relaxes to a new steady state corresponding to the new conditions (Havsteen, 1966; Ilgenfritz, 1977). The computer program previously developed by us (Varón *et al.*, 1990) is not applicable to these cases. Therefore we have extended the program to include these situations. Since the initial conditions here are less restrictive, the kinetic equations become more complex.

A detailed description of the model is given in our previous paper (Varón *et al.*, 1990). Here, only a brief outline of the solution of the differential equations under the more general initial conditions is given. Most of the details coincide with those pertaining to the more restrictive initial conditions. However, some additional symbols, given in the following section, are necessary to account for the new initial conditions.

NOTATION AND INITIAL CONDITIONS

Notation

$[X_r]_0$ ($r = 1, 2, \dots, n$). This is the initial concentration of the enzyme species X_r .

Ω . This is the set, the elements of which are the subindices in

the notation used for the enzyme species present at the onset of the reaction; i.e. the subindex r of an enzyme species X_r ($r = 1, 2, \dots, n$) belongs to the set Ω if $[X_r]_0 \neq 0$. Hence, if an enzyme system contains the enzyme species X_1 , X_2 and X_3 at the onset of the reaction, then $\Omega = \{1, 2, 3\}$.

$r \in \Omega$. This means that the index r belongs to the set Ω .

$\sum_{r \in \Omega}^{r\text{-dependent expression}}$. This is the sum extended over all the elements of the set Ω , i.e. r assumes each of the values of the elements of Ω .

$[E]_0$. This is the total concentration of the enzyme species, i.e.:

$$[E]_0 = \sum_{r \in \Omega} [X_r] \quad (1)$$

$D_{r,i}(\delta)(r \in \Omega; i = 1, 2, \dots, n)$. This is the minor of the order $(n-1)$ of $D(\delta)$ obtained by removal of the r -th row and the i -th column of $D(\delta)$. The expansion of this minor is the polynomial:

$$D_{r,i}(\delta) = \sum_{q=0}^{n-1} (a_{r,i})_q \delta^{n-q-1} \quad (2)$$

Initial conditions

We assume that if a ligand, Y_s , binds to an enzyme form, then its concentration remains approximately constant during the whole course of the reaction, either because $[Y_s]_0 \gg [E]_0$ or due to metabolic regulation of the concentration. Under these conditions, the corresponding step is of pseudo-first-order, i.e. $[Y_s] \approx [Y_s]_0$, and the set of differential equations describing the evolution of the species involved in the general model is linear.

KINETIC EQUATIONS

Enzyme species

The solution of the system of differential equations describing the evolution of the concentration of the enzyme species involved in our general model under the above initial conditions is:

$$[X_i] = A_{i,0} + \sum_{h=1}^u A_{i,h} \exp(\delta_h t) \quad (i = 1, 2, \dots, n) \quad (3)$$

where u is a integer which is always less than n , and $\delta_1, \delta_2, \dots, \delta_u$

are the roots of the polynomial $T(\delta) = \sum_{q=0}^u F_q \delta^{u-q}$, in which $F_0 = 1$

and the remaining coefficients, F_q ($q = 1, 2, \dots, u$), are functions

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of the rate constants and of the initial concentrations of the ligand species. The above roots are negative or complex with a negative real part (Hearon, 1963; Gálvez & Varón, 1981).

$$A_{i,0} = \frac{(-1)^{n+i+1} \sum_{r \in \Omega} (-1)^r (a_{r,i})_u [X_r]_0}{F_u} \quad (i = 1, 2, \dots, n) \quad (4)$$

$$A_{i,h} = \frac{(-1)^{i+c} \sum_{r \in \Omega} (-1)^r [X_r]_0 \sum_{q=0}^u (a_{r,i})_q \delta_h^{u-q}}{\delta_h \prod_{p=h}^{p-1} (\delta_p - \delta_h)} \quad (i = 1, 2, \dots, n; h = 1, 2, \dots, u) \quad (5)$$

where $c = n - u$. If $u = 1$, then the denominator of eqn. (4) is δ_1 , i.e. it is the only root of $T(\delta)$. Note that, in this case, $\delta_1 = -F_1$.

The coefficients $(a_{r,i})_q$ ($r \in \Omega$; $i = 1, 2, \dots, n$; $q = 0, 1, \dots, u$) in eqns. (4) and (5) are functions of the rate constants and of the initial concentrations of the ligand species.

Ligand species

The differential equation which expresses the time dependence of the concentration of any ligand species, Y_s , has the form:

$$\frac{d[Y_s]}{dt} = \sum_{(i,j)} (K_{j,i} [X_j] - K_{i,j} [X_i]) \quad (s = 1, 2, \dots, g) \quad (6)$$

where (i, j) means that the sum is extended over all pairs of values (i, j) , i and j being the subindices of the enzyme species X_i and X_j which participate in one or more of the steps of the types [2]–[6] of the model described by us previously (Varón *et al.*, 1990).

If in eqn. (6), the eqns. (3)–(5) are taken into account, then a direct integration, after some rearrangement, gives:

$$[Y_s] - [Y_s]_0 = \beta_s + \alpha_s t + \sum_{h=1}^u \gamma_{s,h} \exp \delta_h t \quad (s = 1, 2, \dots, g) \quad (7)$$

in which

$$\alpha_s = \frac{(-1)^{n+1} \sum_{(i,j)} \{K_{j,i} \sum_{r \in \Omega} (-1)^{j+r} (a_{r,i})_u [X_r]_0 - K_{i,j} \sum_{r \in \Omega} (-1)^{i+r} (a_{r,i})_u [X_r]_0\}}{F_u} \quad (8)$$

$$\beta_s = \frac{(-1)^{n+1} \sum_{(i,j)} \{K_{j,i} \sum_{r \in \Omega} (-1)^{j+r} (a_{r,j})_{u-1} [X_r]_0 - K_{i,j} \sum_{r \in \Omega} (-1)^{i+r} (a_{r+i})_{u-1} [X_r]_0\}}{F_u} - \frac{F_{u-1}}{F_u} \alpha_s \quad (9)$$

$$\gamma_{s,h} = \frac{(-1)^c \sum_{(i,j)} \{K_{j,i} \sum_{r \in \Omega} (-1)^{j+r} [X_r]_0 \sum_{q=0}^u (a_{r,i})_q \delta_h^{u-q} - K_{i,j} \sum_{r \in \Omega} (-1)^{i+r} [X_r]_0 \sum_{q=0}^u (a_{r,i})_q \delta_h^{u-q}\}}{\delta_h^2 \prod_{p=h}^{p-1} (\delta_p - \delta_h)} \quad (10)$$

If $u = 1$, then the denominator of eqn. (10) is δ_1^2 , i.e. F_1^2 .

If, in the scheme of a reaction there is a set of parallel steps between two enzyme species X_j and X_i , and Y_s is involved in some of them, then the same considerations as those in the previous program must be made.

In the steady state ($t \rightarrow \infty$), eqns. (3) and (7) are simplified to $[X_i] = A_{i,0}$ ($i = 1, 2, \dots, n$) and $[Y_s] - [Y_s]_0 = \beta_s + \alpha_s t$ ($s = 1, 2, \dots, g$), respectively. The parameter $A_{i,0}$ ($i = 1, 2, \dots, n$) is the steady-state concentration of the enzyme species X_i , and the parameter α_s is the steady-state initial rate of the ligand species Y_s .

It can be shown (Rescigno, 1956) that:

$$(a_{r,i})_{n-1} = (-1)^{i+r} (a_{i,i})_{n-1} \quad (11)$$

Therefore, in enzyme systems in which $u = n - 1$, which usually is the case, eqns. (4) and (8) may be written in the following alternative, and simpler, form:

$$A_{i,0} = \frac{(-1)^{n+1} (a_{i,i})_{n-1} [E]_0}{F_{n-1}} \quad (i = 1, 2, \dots, n) \quad (12)$$

$$\alpha_s = \frac{(-1)^{n+1} [E]_0 \sum_{(i,j)} \{K_{j,i} (a_{j,j})_{n-1} - K_{i,j} (a_{i,i})_{n-1}\}}{F_{n-1}} \quad (s = 1, 2, \dots, g) \quad (13)$$

THE COMPUTER PROGRAM

The program presented here is an extension of the previous version (Varón *et al.*, 1990) to render it applicable to the cases mentioned in the Introduction. The new program is therefore more general than the former, and includes the previous version as a particular case, i.e. it can also be applied when the enzyme only is present in a single conformational form at the onset of the reaction.

The program which we have developed gives the expressions for the coefficients F_q ($q = 0, 1, \dots, u$) and $(a_{r,i})_q$ ($q = 0, 1, \dots, u$; $r \in \Omega$; $i = 1, 2, \dots, n$) as well as the values of u and c . The insertion into the kinetic equations of the above expressions and values together with $\delta_1, \delta_2, \dots, \delta_u$ (which are the roots of the polynomial $T(\delta)$ and the initial concentrations of the enzyme species initially present) yields the evolution of all the species in the enzyme reaction.

A summarized description of the program follows, and the novelties with respect to the previous program are explained.

The execution of this program is divided into the following steps: the input of the data, the choice of the option and the printing of the results.

Input of the data

The input in this program is analogous to the one described in the previously described program (Varón *et al.*, 1990) but the following differences must be noted: (1) the program begins by asking for a name of the enzyme system under study; (2) the arbitrary notation for the enzyme species does not require that

the free enzyme is named X_1 ; any notation is allowed; (3) the number of the enzyme species present at the onset of the reaction and the elements of the set Ω must be entered.

Choice of the option

After the input of the rate constants, the program offers the choice in the expression of the terms between those of both the transient-phase and steady-state equations and those which are involved only in the steady-state equations.

Printing of the results

When the option has been chosen, the computer begins to process the information entered and simultaneously the printer delivers the corresponding results. These results allow the construction of the kinetic equations of any of the species involved in the reaction.

(a) **The output if option (1) is chosen.** In this case, the printer

produces the expressions $F_0, (a_{r,i})_0 (r \in \Omega; i = 1, 2, \dots, n), F_1, (a_{r,i})_1 (r \in \Omega; i = 1, 2, \dots, n), \dots, F_u, (a_{r,i})_u (r \in \Omega; i = 1, 2, \dots, n)$.

(b) **The output resulting from option (2).** The printer delivers the expressions for F_u and for the coefficients $(a_{r,i})_u (r \in \Omega; i = 1, 2, \dots, n)$.

In both of the cases (a) and (b), the u and c values, as well as the time employed in the process from the moment of the completion of the data entry, are recorded. Moreover, if $u = n - 1$, the expressions of all of the coefficients $(a_{r,i})_{n-1}$ also are delivered. Before the results are written, a list of the data entered is printed.

This program has, like that previously described (Varón *et al.*, 1990), been written in the computer language BASIC. The program runs on computers with an MS-DOS operating system. The present version accepts enzyme systems with up to 120 different enzyme forms.

EXAMPLE

The present program is applicable to all cases covered by the previously described program (Varón *et al.*, 1990), i.e. the cases in which the free enzyme at the onset of the reaction is present only in one conformational form. In addition, it is applicable to the cases mentioned in the Introduction, i.e. to those in which more than one species of enzyme complex or more than one conformer of the free enzyme are present at the onset of the reaction.

In this section we apply the new program to the construction of the kinetic equations of a system to which the previously described program (Varón *et al.*, 1990) cannot be used. The system chosen has two conformers of the free enzyme which both catalyse the same reaction (Segel, 1975; Darvey, 1977; Ainslie & Neet, 1978). If we assume a Michaelis-Menten mechanism for the catalytic action of each conformer, then the simplest scheme of the reactions is:

Scheme 1.

where E_1 and E_2 are the two conformers of the free enzyme, E , present at the onset of the reaction and S and P the substrate and the product of the reaction.

Initial conditions

Before the addition of the substrate, S , to the preparation, the system only contains the conformers, E_1 and E_2 , which are interconvertible, $E_1 \rightleftharpoons E_2$. If we assume that this isolated step is in equilibrium, then the initial concentrations of E_1 and E_2 , i.e. $[E_1]_0$ and $[E_2]_0$ are:

$$[E_1]_0 = \frac{[E]_0}{1 + \frac{k_{+1}}{k_{-1}}} \quad (14)$$

$$[E_2]_0 = \frac{k_{+1}[E]_0}{k_{-1}\left(1 + \frac{k_{+1}}{k_{-1}}\right)} \quad (15)$$

$[E]_0$ being the total concentration of the enzyme forms.

The present program is applicable to Scheme 1 if we assume that the initial concentration, $[S]_0$, of the substrate is much higher than $[E]_0$ (and therefore much higher than both $[E_1]_0$ and $[E_2]_0$), i.e. $[S]_0 \gg [E]_0$.

When the reaction after the addition of the substrate starts, then the equilibrium of the isolated step $E_1 \rightleftharpoons E_2$ is displaced.

Notation of the enzyme forms

We arbitrarily choose the notation: $X_1 \equiv E_1$, $X_2 \equiv E_2$, $X_3 \equiv E_1S$ and $X_4 \equiv E_2S$.

Interaction with the computer

(a) **Input of the data.** The numeral 4 must be entered to specify the number of enzyme forms. Next, a name for the reaction is required. We choose 'Scheme 1'. Then, the rate constants are entered as the following expressions:

$$\left. \begin{array}{ll} K_{1,3} = k + 1 & K_{2,1} = k - 1 \\ K_{1,2} = k + 2[S]_0 & K_{3,1} = k - 2 + k + 4 \\ K_{2,4} = k + 3[S]_0 & K_{4,2} = k - 3 + k + 5 \\ K_{3,4} = k + 6 & K_{4,3} = k - 6 \end{array} \right\} \quad (16)$$

For the remaining rate constants $K_{i,j}$, 0 must be entered or the key 'enter' is merely pressed.

When the computer asks for the number of enzyme species present at the onset of the reaction, the numeral 2 must be entered. Then the program requests the elements of the set Ω ; since X_1 and X_2 are present at the onset of the reaction, the numerals 1, 2 must be entered successively.

(b) **Choice of the option.** Since both the transient-phase and the steady-state equations are of interest, option (1) is chosen.

Printing of the results

The results delivered by the printer are given in the Appendix. In this example a TANDOM PCA computer and an EPSON LQ-500 printer in the high-quality mode were used.

Kinetic equations

Since $n = 4$, $u = 3$, $c = 1$ (the values of u and c are delivered by the printer), $\Omega = \{1, 2\}$, $[X_r]_0 = [E_r]_0$ ($r = 1, 2$) and there are only two pairs of indices (i, j) , (1, 3) and (2, 4) (because P is released in both of the steps $X_3 \rightarrow X_1 + P$ and $X_4 \rightarrow X_2 + P$), we have:

$$[P] - [P]_0 = \beta_P + \alpha_P t + \sum_{h=1}^3 \gamma_{P,h} \exp(\delta_h t) \quad (17)$$

where δ_1 , δ_2 and δ_3 are the roots of the polynomial $\sum_{q=0}^3 F_q \delta^{3-q}$.

Hence:

$$\alpha_P = - \frac{\{K_{3,1}(a_{3,3})_3 + K_{4,2}(a_{4,4})_3\}[E]_0}{F_3} \quad (18)$$

(Note that because $u = n - 1$, eqn. 18 may be obtained from eqn. 13). Similarly, we obtain the expressions for β_P and $\gamma_{P,h}$ ($h = 1, 2, 3$):

$$\beta_P = - \frac{K_{3,1}\{(a_{1,3})_2[E]_0 - (a_{2,3})_2[E]_0\} + K_{4,2}\{-(a_{1,4})_2[E]_0 + (a_{2,4})_2[E]_0\}}{F_3} - \frac{F_2}{F_3} \alpha_P \quad (19)$$

$$\gamma_{P,h} = - \frac{K_{3,1}\{[E]_0 \sum_{q=0}^3 (a_{1,3})_q \delta_h^{3-q} - [E]_0 \sum_{q=0}^3 (a_{2,3})_q \delta_h^{3-q}\} + K_{4,2}\{-[E]_0 \sum_{q=0}^3 (a_{1,4})_q \delta_h^{3-q} + [E]_0 \sum_{q=0}^3 (a_{2,4})_q \delta_h^{3-q}\}}{\delta_h^2 \prod_{\substack{p=1 \\ p \neq h}}^3 (\delta_p - \delta_h)} \quad (h = 1, 2, 3) \quad (20)$$

The expressions for the coefficients $(a_{r,3})_q$ ($r = 1, 2; q = 0, 1, 2, 3$), $(a_{r,4})_{q_s}$ ($r = 1, 2; q = 0, 1, 2, 3$), $(a_{3,3})_3$, $(a_{4,4})_3$, and F_q ($q = 0, 1, 2, 3$) in eqns. (18)–(20) (the coefficients F_0 and F_1 are implicitly influencing the equations because δ_1 , δ_2 and δ_3 are the roots of the equations of the polynomial $\sum_{q=0}^3 F_q \delta^3 - q$) are given in the Appendix.

If the expressions of these coefficients given in the Appendix, as well as $K_{3,1}$ and $K_{4,1}$, are inserted into eqns. (18)–(20), then final expressions are obtained.

Note that expressions for all of the coefficients can only be obtained using the new program, since the previously described one (Varón *et al.*, 1990) only delivers the coefficients $(a_{1,i})$ ($i = 1, 2, 3, 4; q = 0, 1, 2, 3$) and F_q ($q = 0, 1, 2, 3$).

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APPENDIX

Results delivered by the printer for Scheme 1

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NAME GIVEN TO THE ENZYME SYSTEM: Scheme I

NUMBER OF ENZYME SPECIES OF THE SYSTEM: 4

NON-NULL RATE CONSTANTS OF THE SYSTEM:

K1,2 = k+1
K1,3 = k+2[S]0
K2,1 = k-1
K2,4 = k+3[S]0
K3,1 = k-2+k+4
K3,4 = k+6
K4,2 = k-3+k+5
K4,3 = k-6

SET  $\Omega$  = {1, 2}

OPTION: BOTH THE TRANSIENT PHASE AND THE STEADY STATE EQUATIONS OF THE REACTION

DATE: 11-20-1990
=====
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RESULTS

```
F = 1
0

(ar,r 0) = -1 (r ∈  $\Omega$ )

(ar,i 0) = 0 (r ∈  $\Omega$ ; i=1,2,..., 4 ; r different of i)

F = 1

(k+1) + (k+2[S]0) + (k-1) + (k+3[S]0) + (k-2+k+4) + (k+6) + (k-3+k+5) + (k-6)
```

$$(a_{1,1}) =$$

$$-[(k-1) + (k+3[S]0) + (k-2+k+4) + (k+6) + (k-3+k+5) + (k-6)]$$

$$(a_{1,2}) =$$

$$(k+1)$$

$$(a_{1,3}) =$$

$$-(k+2[S]0)$$

$$(a_{1,4}) =$$

$$0$$

$$(a_{2,1}) =$$

$$(k-1)$$

$$(a_{2,2}) =$$

$$-[(k+1) + (k+2[S]0) + (k-2+k+4) + (k+6) + (k-3+k+5) + (k-6)]$$

$$(a_{2,3}) =$$

$$0$$

$$(a_{2,4}) =$$

$$-(k+3[S]0)$$

$$F_2 =$$

$$(k+1)(k+3[S]0) + (k+1)(k-2+k+4) + (k+1)(k+6) + (k+1)(k-3+k+5) + (k+1)(k-6) + (k+2[S]0)(k-1) + (k+2[S]0)(k+3[S]0) + (k+2[S]0)(k+6) + (k+2[S]0)(k-3+k+5) + (k+2[S]0)(k-6) + (k-1)(k-2+k+4) + (k-1)(k+6) + (k-1)(k-3+k+5) + (k-1)(k-6) + (k+3[S]0)(k-2+k+4) + (k+3[S]0)(k+6) + (k+3[S]0)(k-6) + (k-2+k+4)(k-3+k+5) + (k-2+k+4)(k-6) + (k+6)(k-3+k+5)$$

$$(a_{1,1}) =$$

$$-[(k-1)(k-2+k+4) + (k-1)(k+6) + (k-1)(k-3+k+5) + (k-1)(k-6) + (k+3[S]0)(k-2+k+4) + (k+3[S]0)(k+6) + (k+3[S]0)(k-6) + (k-2+k+4)(k-3+k+5) + (k-2+k+4)(k-6) + (k+6)(k-3+k+5)]$$

$$(a_{1,2}) =$$

$$(k+1)(k-2+k+4) + (k+1)(k+6) + (k+1)(k-3+k+5) + (k+1)(k-6)$$

$$(a_{1,3}) =$$

$$-[(k+2[S]0)(k-1) + (k+2[S]0)(k+3[S]0) + (k+2[S]0)(k-3+k+5) + (k+2[S]0)(k-6)]$$

$$(a_{1,4}) =$$

$$(k+1)(k+3[S]0) + (k+2[S]0)(k+6)$$

$$\begin{pmatrix} a \\ 2,1 \end{pmatrix}_2 =$$

$$(k-1)(k-2+k+4) + (k-1)(k+6) + (k-1)(k-3+k+5) + (k-1)(k-6)$$

$$\begin{pmatrix} a \\ 2,2 \end{pmatrix}_2 =$$

$$-[(k+1)(k-2+k+4) + (k+1)(k+6) + (k+1)(k-3+k+5) + (k+1)(k-6) + (k+2[S]0)(k+6) + (k+2[S]0)(k-3+k+5) + (k+2[S]0)(k-6) + (k-2+k+4)(k-3+k+5) + (k-2+k+4)(k-6) + (k+6)(k-3+k+5)]$$

$$\begin{pmatrix} a \\ 2,3 \end{pmatrix}_2 =$$

$$(k+2[S]0)(k-1) + (k+3[S]0)(k-6)$$

$$\begin{pmatrix} a \\ 2,4 \end{pmatrix}_2 =$$

$$-[(k+1)(k+3[S]0) + (k+2[S]0)(k+3[S]0) + (k+3[S]0)(k-2+k+4) + (k+3[S]0)(k+6)]$$

$$F_3 =$$

$$(k+1)(k+3[S]0)(k-2+k+4) + (k+1)(k+3[S]0)(k+6) + (k+1)(k+3[S]0)(k-6) + (k+1)(k-2+k+4)(k-3+k+5) + (k+1)(k-2+k+4)(k-6) + (k+1)(k+6)(k-3+k+5) + (k+2[S]0)(k-1)(k+6) + (k+2[S]0)(k-1)(k-3+k+5) + (k+2[S]0)(k-1)(k-6) + (k+2[S]0)(k+3[S]0)(k+6) + (k+2[S]0)(k+3[S]0)(k-6) + (k+2[S]0)(k+6)(k-3+k+5) + (k-1)(k-2+k+4)(k-3+k+5) + (k-1)(k-2+k+4)(k-6) + (k-1)(k+6)(k-3+k+5) + (k+3[S]0)(k-2+k+4)(k-6)$$

$$\begin{pmatrix} a \\ 1,1 \end{pmatrix}_3 =$$

$$-[(k-1)(k-2+k+4)(k-3+k+5) + (k-1)(k-2+k+4)(k-6) + (k-1)(k+6)(k-3+k+5) + (k+3[S]0)(k-2+k+4)(k-6)]$$

$$\begin{pmatrix} a \\ 1,2 \end{pmatrix}_3 =$$

$$(k+1)(k-2+k+4)(k-3+k+5) + (k+1)(k-2+k+4)(k-6) + (k+1)(k+6)(k-3+k+5) + (k+2[S]0)(k+6)(k-3+k+5)$$

$$\begin{pmatrix} a \\ 1,3 \end{pmatrix}_3 =$$

$$-[(k+1)(k+3[S]0)(k-6) + (k+2[S]0)(k-1)(k-3+k+5) + (k+2[S]0)(k-1)(k-6) + (k+2[S]0)(k+3[S]0)(k-6)]$$

$$\begin{pmatrix} a \\ 1,4 \end{pmatrix}_3 =$$

$$(k+1)(k+3[S]0)(k-2+k+4) + (k+1)(k+3[S]0)(k+6) + (k+2[S]0)(k-1)(k+6) + (k+2[S]0)(k+3[S]0)(k+6)$$

$$\begin{pmatrix} a \\ 2,1 \end{pmatrix}_3 =$$

$$(k-1)(k-2+k+4)(k-3+k+5) + (k-1)(k-2+k+4)(k-6) + (k-1)(k+6)(k-3+k+5) + (k+3[S]0)(k-2+k+4)(k-6)$$

$$\begin{pmatrix} a \\ 2,2 \end{pmatrix}_3 =$$

$$-[(k+1)(k-2+k+4)(k-3+k+5) + (k+1)(k-2+k+4)(k-6) + (k+1)(k+6)(k-3+k+5) + (k+2[S]0)(k+6)(k-3+k+5)]$$

$$(a_{2,3}) =$$

$$(k+1)(k+3[S]0)(k-6) + (k+2[S]0)(k-1)(k-3+k+5) + (k+2[S]0)(k-1)(k-6) + (k+2[S]0)(k+3[S]0)(k-6)$$

$$(a_{2,4}) =$$

$$-[(k+1)(k+3[S]0)(k-2+k+4) + (k+1)(k+3[S]0)(k+6) + (k+2[S]0)(k-1)(k+6) + (k+2[S]0)(k+3[S]0)(k+6)]$$

$$(a_{3,3}) =$$

$$-[(k+1)(k+3[S]0)(k-6) + (k+2[S]0)(k-1)(k-3+k+5) + (k+2[S]0)(k-1)(k-6) + (k+2[S]0)(k+3[S]0)(k-6)]$$

$$(a_{4,4}) =$$

$$-[(k+1)(k+3[S]0)(k-2+k+4) + (k+1)(k+3[S]0)(k+6) + (k+2[S]0)(k-1)(k+6) + (k+2[S]0)(k+3[S]0)(k+6)]$$

Number of non-null roots of the polynomial D(δ): $u = 3$

Number of null roots of the polynomial D(δ): $c = 1$

Time employed (in minutes) for the processing: 1.8153