Volume 4 Number 6 June 1977 Nucleic Acids Research

A program for least squares analysis of reassociation and hybridization data

W.R. Pearson, E.H. Davidson and R. J. Britten*

Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA

Received 21 December 1976

ABSTRACT

A computer program is described for the rapid calculation of least squares solutions for data fitted to different functions normally used in reassociation and hybridization kinetic measurements. The equations for the fraction not reacted as a function of Cot follow: First order, $\exp(-kCot)$; second order, $(1+kCot)^{-1}$; variable order, $(1+kCot)^{-n}$; approximate fraction of DNA sequence remaining single stranded, $(1+kCot)^{-.44}$; and a function describing the pairing of tracer when the rate constant for the tracer (k) is distinct from the driver rate constant (k_d): $\exp\{k[1-(1+k_dCot)^{1-n}]/[k_d(1-n)]\}$. Several components may be used for most of these functional forms. The standard deviations of the individual parameters at the solutions are calculated.

INTRODUCTION

The quantitative examination of reassociation and hybridization kinetic measurements has become increasingly important as the sophistication of the measurements has grown. In this paper we describe a general computer program which can conveniently apply the variety of functions now used to interpret kinetic measurements. We have chosen to use a non-linear least squares method so that the solutions give equal weight to all of the individual measurements and no preliminary assumptions need be made about the initial or terminal values of the reaction.

Least squares computer programs have been applied to the problem of resolving repetitive and single copy kinetic components in DNA renaturation experiments carried out on many organisms (e.g. 1,2,3). They are also used to determine rate constants in RNA excess hybridization reactions (e.g. 4,5). The use of cDNA probes to determine the complexity of RNA populations by kinetic rather than saturation measurements (e.g. 6,7) also relies heavily on the resolution of abundance classes by accurate determination of their rate constants.

The five functions listed in Table 1 are used for the examination of the following kinds of measurements. The second order equation (FINGER) describes

TABLE 1 Functional forms used by the program

Number	Name	Form	Use
1	FINGER	$f_i(1 + k_iCot)^{-1}$	second order reaction: DNA renaturation measured by hydroxyapatite chromatography
2	WHATOR	$f(1 + kCot)^{-n}$	variable form reaction: to determine values of n when the apparent order of the reaction is unknown
3	NUFORM	$f_i exp\{k_i\lfloor 1-(k_dCot)^{1-n}\rfloor/\lfloor k_d(1-n)\rfloor\}$	describes rate of tracer reaction when tracer rate constant k differs from driver rate constant k_d
4	EXCESS	$f_i exp(-k_iCot)$	first order function: for RNA excess experiments
5	WHTCMP	$f_i(1 + k_iCot)^{-0.44}$	modified second order function: S-1 nuclease assay of hybridization

accurately the form of DNA reassociation kinetics assayed by hydroxyapatite chromatography (8,9) though for fairly complex reasons (10,11). The pseudo-first- order equation (EXCESS) applies when the nucleic acid driving the reaction remains unpaired as in RNA driven reactions. The third function (WHATOR) has a form which can be varied by changing the value of the exponent, and is useful when the apparent order of the reaction is not known or there is a need to test the heterogeneity of the reacting components. When the exponent n=1, it reduces to second order form. When high quality single component hydroxyapatite measurements are analyzed, the least squares solution yields n=1.0 (11). As n becomes large this equation approaches first order and values of n as high as 10 yield a form which is indistinguishable from first order. The next functional form (WHTCMP) is applicable to DNA reassociation when the reaction is assayed by S-1 nuclease (11,12) and expresses the fraction of the length of the DNA sequence present which remains single stranded. The last equation (NUFORM) applies when the rate of reassociation of tracer with the driver and the rate of driver renaturation itself differ. The amount of driver available is assumed to follow the equation $(1+kCot)^{-n}$. A value of n=.44 is usually used and gives a good approximation to the actual capacity of the remaining single stranded regions to reassociate with tracer molecules (13).

The non-linear least squares program described in this paper is in use on a PDP-10 timesharing computer. The program provides for interactive input but

has been designed for ease of conversion to a batch processing system. A typical run of the program is presented in Appendix I, with user responses underlined. In a Batch processing environment the underlined inputs would be submitted on cards.

The algorithm used in the program was developed by Marquardt (14) for non-linear least squares problems. The implementation of the algorithm provides two additional facilities: 1) the ability to hold any number or combination of parameters constant in order to find the best solution with the remaining variable parameters; and 2) the ability to substitute any function for the NUFORM function by replacing a subroutine. Parameter fixing can provide important insight into the uniqueness of the solution parameter set. The function substitution option allows more complex functions to be fit to data as more complex phenomena - e.g. rate retardation for single strands on duplexed molecules (10) - are studied.

ANALYSIS OF HYBRIDIZATION DATA

The program strategy

This non-linear least squares program is designed to converge on a solution yielding parameters which minimize the least squares deviation of the function from a set of data. There are two main concerns: whether the final solution is biased by the input parameter estimates, and whether further iterations will improve the solution. A solution is independent of input parameter estimates and insensitive to further iterations if it has converged. Convergence is indicated by the amount of change in the RMS from one iteration to the next and by the change in the DELMX parameter. Because of the Taylor's series approximation technique used by this program, the parameter values converge very rapidly in the neighborhood of a solution. Although this neighborhood may be difficult to find for the first few iterations (the algorithm's strategy uses a "gradient" technique to find the neighborhood), once found, successive iterations will improve the parameter values by one to two significant figures with each iteration. This improvement is reflected in a 10- to 100- fold decrease in DELMX with successive iterations. This rapid decrease in DELMX may occur while the RMS changes very little. Attempts to improve the solution beyond convergence may cause the message CORRECTIVE ITERATIONS EXCEEDED to be printed.

Possible problems

This program rapidly converges to a unique solution from a wide range of parameter estimates when the data provide adequate constraints. New parameter estimates are always better (in the least squares sense) than the previous

values but the parameters may become negative during the process. Inadequate termination data may allow the FINAL parameter to become negative while the FRACTION parameter for the slowest component increases without bound. Fixing the FINAL fraction unreassociated (presumably near zero) or close scrutiny of the DELMX values to find regions of local convergence may solve the problem.

Often when the series of iterations do not immediately converge the program hesitates in a region of local convergence. When pressed to improve the RMS the program may go off in a direction (such as negative parameters) where termination is impossible. Usually DELMX starts at 1.0 to 5.0, then decreases to 0.02 to 0.1 as the fit converges (the largest parameter change is less than 2-10%). DELMX may then jump by a factor of 50 to 200 and start on another (possibly unterminated) path. The small DELMX value indicates a local low RMS region which might provide good values for the parameters but they must be carefully examined. To determine the quality of the parameter values, plot the solution.

Occasionally the program returns negative rate constants for components of the reaction. This is usually due to an attempt by the program to remove that component from the solution. Plotting the solution usually shows why this was done. Single erroneous points that do not show in the plot should be searched for and perhaps a new solution should be attempted with fewer components.

Parameter fixing

Data from other measurements may supply fixed values for parameters in a solution. For example, chemical measurements of genome size may be used to establish the rate constant of the single copy component. Slave "mini-cot" experiments (3) often provide the most reliable determinations of repetitive and single copy rate constants which may be used as fixed values to calculate the fraction of the genome associated with the different rate components in a total reassociation curve.

Fixing the single copy rate constant at a value determined from the genome size or minicot analysis may be particularly important for DNA which contains a small fraction repeated 2-20 fold. This low repetition class is virtually indistinguishable from single copy DNA but can increase the apparent single copy rate constant by a factor of two.

Parameter fixing also provides information about the variety of similar least squares solutions which describe the data equally well. A graph of RMS vs. a set of fixed values of a parameter can be informative, particularly if it turns out to be a very shallow curve near the minimum.

TABLE 2 EFFECT OF GAUSSIAN NOISE ON PARAMETER VALUES AND ERROR ESTIMATES

Successive trials with random noise

	Trial	Number of points	Parameter values at solution					RMS
			Final	f_1	k_1	f_2	k_2	
Value	Parameters used		0.200	0.300	0.1000	0.300	10.00	
Error	to generate data		0.040[a]					
Solution	1	40	0.196	0.235	0.0536	0.328	3.69	0.0350
Error			0.017	0.037	0.0289	0.038	1.38	
Solution	2	40	0.198	0.309	0.106	0.297	13.5	0.0466
Error			0.019	0.038	0.048	0.038	6.6	
Solution	3	40	0.212	0.301	0.149	0.319	18.9	0.0392
Error			0.015	0.032	0.058	0.032	7.4	
Solution	4	40	0.208	0.274	0.0819	0.300	5.82	0.0382
Error			0.017	0.040	0.0397	0.041	2.47	
Solution	5	40	0.180	0.340	0.110	0.302	10.2	0.0379
Error			0.015	0.035	0.039	0.035	4.2	
Solution	sum of	200	0.200	0.298	0.103	0.300	9.28	0.0403
Error	trials above		0.007	0.017	0.020	0.017	1.81	

[a] Noise factor equal to FSD described in the text. For this example, F is 0.2

Parameter statistics

Parameter standard deviations and correlation coefficients calculated by the program provide information about the range of parameter values which may adequately describe the data. These numbers, along with the RMS and DELMX, indicate the significance of the parameter values obtained at the least squares solution.

The parameter standard deviation and correlation coefficient calculation is similar to standard deviation and correlation coefficient calculations in linear regression analysis. To obtain the standard deviations and correlation coefficients the data covariance matrix is inverted. The calculation assumes that the Taylor's series linear approximation is accurate in the neighborhood of the solution (indicated by a low DELMX at convergence). To examine the usefulness of the parameter standard deviations and correlation coefficients, artificial test data were generated. Some examples of solutions using the second order (FINGER) function are shown in Table 2.

The data set for each of these analyses was calculated using a Gaussian random number generator for the final unreacted quantity as follows:

$$C/Co = GAUS(F,FSD) + \sum_{i=1}^{a} f_i(1+k_i Cot)^{-1}$$

(GAUS is a computer subroutine which generates a series of values following a

Gaussian distribution with average F and standard deviation FSD.)

This method of generating variation in the "data" is a good analogue of actual fluctuations from measurement to measurement, such as the binding of DNA to hydroxyapatite. The fluctuations observed in Table 2 for the various solutions and the standard deviations shown are probably representative of what would happen in repetitions of actual measurements which showed a comparable RMS. It is clear that the standard deviations of the rate constants are much larger than those of the component quantities. When analyses of this sort are carried out with components only a factor of ten apart in rate constant (instead of the factor of 100 for the example illustrated in Table 2) the rate constant fluctations are very large. It can be seen from Table 2 that there is a reasonable quantitative relationship between the fluctuations from set to set and the calculated standard deviations.

This table exhibits one of the characteristic problems of fitting reassociation kinetic data and indicates the insight into the accuracy of individual parameter estimates provided by the standard deviation calculations. It is clear that where two kinetic components are present, even a factor of one hundred apart, very accurate data are needed to obtain estimates of rate constants with moderate accuracy.

DISCUSSION

We have described a powerful and flexible program for the least squares analysis of the kinetics of reassociation. This program has been used for the analysis of an extensive series of measurements (3,4,5,10,11,13). Least squares analysis is necessary for reproducible and clear interpretation of such measurements. We refer to these papers for examples of use and interpretation, while in this discussion we focus on problems of over-interpretation or misinterpretation of the solutions. As powerful analytical tools of this sort are developed it becomes very easy to simply accept the "output" and lose touch with its meaning.

It is important to remember that any successful least squares strategy provides parameter values which "fit the data better" in the "least squares sense". The solution does not guarantee either physical or biological reality. Components may be used to fit small peculiarities in the data and have little physical meaning. This is usually evident from a plot of the fit. Parameter values may also be the fluke of a particular set of data and have little absolute importance. The parameter standard deviations calculated by the program provide confidence limits for the parameter values given the data.

Where it is known from other measurements that a DNA fraction is homogeneous or where a single copy fraction has been purified the least squares analysis provides an excellent means for evaluating its rate constant and permits a more accurate calculation of its complexity. In general it is not known that components are homogeneous and often the values of parameters derived from the least squares solutions may be averages of a set of unresolved components. In such a case the solution makes an excellent model of the set of repetitive sequences which may be used in a variety of calculations even though the true individual components are not known. Where an important issue rests on the potential heterogeneity of a component other tools must be used. For example fractionation of double from single standed DNA could be carried out at the midpoint of its reassociation and the rate of reassociation of the two fractions carefully compared.

Another consideration is as important as questions of parameter meaning: solution uniqueness. If a parameter can change over a wide range without affecting the quality of the fit measured by the RMS or GOODNESS OF FIT, conclusions based on a specific parameter value are suspect. In many cases, this problem will be indicated by a high parameter standard deviation. More quantitative insight into the significance of a particular parameter value can be gained by plotting the RMS or GOODNESS OF FIT criterion as a function of the best fit using <u>different</u> fixed values for the parameter in question. For example, if the best fit of the data gives a rate constant of 0.05 for a slow component with a GOODNESS OF FIT of 0.02, other solutions should be found with the rate constant fixed at 0.15 and 0.015. If the variation in the GOODNESS OF FIT is less than 10% over this range of rate constants, no conclusions can be based on the 0.05 value which would be different for the other values. If the GOODNESS OF FIT changes by 50- to 100% with variations in the parameter value the value is probably uniquely determined by the data. While the method of fixing one parameter value and varying the others to find the best least squares solution can be used for any parameter, it is particularly important when measuring rate constant parameter values. Data with two rate constants differing by a factor of 10-30 can usually be described by a very wide range of rate constants.

In summary, once a fully convergent least squares solution has been established for a set of data we must consider four issues of interpretation: 1. There may be systematic errors in the measurement such as DNA degradation or unknown fragment size. 2. The individual set of data may be atypical, particularly if only a few measurements are available and the standard

deviations are then a poor measure of the possible error. Deviations in reassociation kinetic measurements are typically not due to random statistical variables such as sampling from a population or radioactive decay, but are more likely due to variations in assay procedures such as hydroxyapatite batch or temperature or volume of samples. This weakens the significance of the standard deviations. 3. The components may not represent true individual rate components but be averages of unresolved sets of components. 4. The least squares minimum in the region of the solution may be shallow. In that case, the set of solution parameter values may not uniquely fit the data; other parameter sets with substantially different values might provide equally valid interpretations of the data.

In many cases such problems can be shown to be of minor quantitative significance. Least squares solutions such as those generated by this program represent the best presently known approach to the interpretation of reassociation and hybridization kinetics.

The program described in this paper is available as a punched card deck from the authors. Two versions are available, one for a DECsystem-10 timesharing computer and a second for an IBM 370 Batch processor. Both programs are written in FORTRAN and can be easily modified for other systems; the two programs differ in minor system dependent features. Inquiries should be directed to William Pearson, California Institute of Technology, 101 Dahlia, Corona del Mar, CA 92625.

* Also Staff Member, Carnegie Institute of Washington

REFERENCES

1 Davidson, E. H. and Britten, R. J., (1973) Quart. Rev. Biol. 48,565-613
2 Davidson, E. H., Galau, G. A., Angerer, R. C. and Britten, R. J., (1975) Chromosoma 51,253-259
3 Britten, R. J., Graham, D. E. and Neufeld, B. R. (1974) in Methods in Enzymology (L. Grossman and K. Moldave, eds.) Vol. 29 Part E pp. 363-418
4 Galau, G. A., Britten, R. J. and Davidson, E. H., (1974) Cell 2,9-20
5 Galau, G. A., Klein, W. H., Davis, M. M., Wold, B. J. Britten, R. J. and Davidson, E. H. (1976) Cell 7,487-505
6 Bishop, J. O., Morton, J. G., Rosbash, M. and Richardson, M. (1974) Nature 250,199-204
7 Ryffel, G. U. and McCarthy, B. J. (1975) Biochemistry 14,1379-1384
8 Britten, R. J. and Kohne, D. E. (1966) Carnegie Inst. Wash. Yearbook 65,78-106
9 Wetmur, J. G. and Davidson, N. (1968) J. Mol. Biol. 31,349-370
10 Britten, R. J. and Davidson, E. H., (1976) Proc. Nat. Acad. Sci. US 73,415-419

11 Smith, M. J., Britten, R. J. and Davidson, E. H. (1975) Proc. Nat. Acad. Sci. US 72,4805-4809
12 Morrow, J. (1974) Ph. D. Thesis, Stanford University
13 Davidson, E. H., Hough, B. R., Klein, W. H. and Britten, R. J. (1975) Cell 4,217-238
14 Marquardt, D. W. (1963) J. Soc. Indust. Appl. Math. 11,431-441

APPENDIX I

This is a sample run of the program with user responses underlined

```
.RU NNNBAT

 1START,2ADD,3GUESS,5PLOT,6CPLOT,8LPLOT,9STOP

1                                  start by getting data
TYPE RED DATA FILENAME

RTDNA                         name of file on disk
106 PT RAT COT CURVE
TO LIST DATA TY 1

—                                  do not list data


 1START,2ADD,3GUESS,5PLOT,6CPLOT,8LPLOT,9STOP

3                                  do a fit
FUNCTION (-1HELP), ITERATIONS, RMS QUIT, DELMX QUIT, (NIT)

-1                                 check the names of the functions
OSAME,1FINGER,2WHATOR,3NUFORM,4EXCESS,5WHTCMP

FUNCTION (-1HELP), ITERATIONS, RMS QUIT, DELMX QUIT, (NIT)

1,10,.0001,.05
FINAL,FRACT(1),K(1),FRACT(2),K(2),FRACT(3),K(3)

.05,17.2,.08,.01,.65,.0005     initial parameter quesses
TYPE INDEX OF PARAMETERS TO BE FIXED.

—                                  no fixed parameters

ORIGINAL RMS=    0.0447680
GOODNESS OF FIT    0.0463237

     RMS      DELMX      PARAMETERS

 0.0304963 1.7717584
 0.393E-01 0.171E+00 0.722E+00 0.979E-01 0.985E-02 0.602E+00 0.455E-03

 0.0298000 0.2591739
 0.383E-01 0.174E+00 0.974E+00 0.100E+00 0.867E-02 0.598E+00 0.445E-03

 0.0297149 0.0705356
 0.374E-01 0.176E+00 0.105E+01 0.103E+00 0.817E-02 0.594E+00 0.438E-03

        iterations stop because RMS is
        changing by less than RMS QUIT=0.0001

        covariance matrix

I       1          2          3          4          5          6          7

 1 0.100E+01
 2-0.251E+00 0.100E+01
 3 0.175E+00-0.612E+00 0.100E+01
 4-0.699E+00 0.312E+00-0.180E+00 0.100E+01
 5 0.559E+00-0.715E+00 0.555E+00-0.825E+00 0.100E+01
 6 0.353E+00-0.526E+00 0.385E+00-0.876E+00 0.901E+00 0.100E+01
 7 0.861E+00-0.406E+00 0.289E+00-0.941E+00 0.820E+00 0.759E+00 0.100E+01

106 PT RAT COT CURVE

RMS=    0.0297149 GOODNESS OF FIT    0.0307474
    106 POINTS       99 DEG OF FREEDOM

..P(1).....P(2).....P(3).....P(4).....P(5).....P(6).....P(7)...

        parameters are printed in the same order
        they were input.  FINAL,FRACT(1),K(1),...
        the first line is the parameter values

 0.3746-01 0.176E+00 0.105E+01 0.103E+00 0.817E-02 0.594E+00 0.438E-03
 0.325E-01 0.198E-01 0.451E+00 0.695E-01 0.116E-01 0.609E-01 0.140E-03

        the second line is the parameter errors

 1START,2ADD,3GUESS,5PLOT,6CPLOT,8LPLOT,9STOP

3                                  Do anotner fit to illustrate
                                   OSAME function and a local
                                   minimum without convergence
FUNCTION (-1HELP), ITERATIONS, RMS QUIT, DELMX QUIT, (NIT)

0,10,0,,.01                        OSAME, the fit continues from
                                   where it left off
ORIGINAL RMS=    0.0297149
GOODNESS OF FIT    0.0307474

     RMS      DELMX      PARAMETERS

 0.0296630 0.0514820
 0.366E-01 0.177E+00 0.106E+01 0.107E+00 0.777E-02 0.591E+00 0.431E-03

 0.0296151 0.0407157
 0.358E-01 0.177E+00 0.106E+01 0.110E+00 0.746E-02 0.589E+00 0.425E-03

 0.0295696 0.0350217                Note the change in DELMX after
 0.349E-01 0.177E+00 0.106E+01 0.114E+00 0.721E-02 0.586E+00 0.419E-03

 0.0295148 0.6947887                this point. A local minimum was found,
 0.264E-01 0.179E+00 0.103E+01 0.143E+00 0.425E-02 0.563E+00 0.367E-03

 0.0290103 0.4464171                going on caused a shift to a path
 0.182E-01 0.175E+00 0.112E+01 0.166E+00 0.531E-02 0.553E+00 0.331E-03

 0.0288781 1.4516340                leading to a negative parameter
 0.744E-02 0.177E+00 0.106E+01 0.189E+00 0.382E-02 0.538E+00 0.295E-03

 0.0286363 6.6211100
-0.132E-02 0.175E+00 0.112E+01 0.208E+00 0.402E-02 0.530E+00 0.268E-03

 0.0285198 0.8757134
-0.106E-01 0.176E+00 0.109E+01 0.225E+00 0.351E-02 0.521E+00 0.244E-03

 0.0284246 0.4295531
-0.187E-01 0.176E+00 0.110E+01 0.240E+00 0.337E-02 0.515E+00 0.225E-03

 0.0283610 0.2825794                now the fit will not converge
-0.260E-01 0.176E+00 0.110E+01 0.252E+00 0.317E-02 0.510E+00 0.209E-03

I       1          2          3          4          5          6          7

 1 0.100E+01
 2-0.177E+00 0.100E+01
 3 0.132E+00-0.380E+00 0.100E+01
 4-0.722E+00 0.345E+00-0.251E+00 0.100E+01
 5 0.535E+00-0.607E+00 0.490E+00-0.885E+00 0.100E+01
 6 0.967E-01-0.455E+00 0.358E+00-0.747E+00 0.828E+00 0.100E+01
 7 0.900E+00-0.305E+00 0.233E+00-0.937E+00 0.774E+00 0.498E+00 0.100E+01

106 PT RAT COT CURVE

RMS=    0.0283610 GOODNESS OF FIT    0.0293465
    106 POINTS       99 DEG OF FREEDOM

..P(1).....P(2).....P(3).....P(4).....P(5).....P(6).....P(7)...

-0.260E-01 0.176E+00 0.110E+01 0.252E+00 0.317E-02 0.510E+00 0.209E-03
 0.565E-01 0.135E-01 0.383E+00 0.829E-01 0.176E-02 0.635E-01 0.101E-03
```

```
 1START,2ADD,3GUESS,5PLOT,6CPLOT,8LPLOT,9STOP

3                                  a fit to show parameter fixing
FUNCTION (-1HELP), ITERATIONS, RMS QUIT, DELMX QUIT, (NIT)

0,15,0, 0,
FINAL,FRACT(1),K(1),FRACT(2),K(2),FRACT(3),K(3)

.05 ,17 2. ,08 ,01 ,65 ,00035      k(3) is set to rate calculated from
                                   genome size
TYPE INDEX OF PARAMETERS TO BE FIXED.

7                                  hold parameter 7 constant. note that
                                   with this constraint the fit converges
ORIGINAL RMS=    0.0608586
GOODNESS OF FIT    0.0626578

     RMS      DELMX      PARAMETERS

 0.0438545 7.8186761
 0.997E-02 0.176E+00 0.496E+00 0.134E+00 0.113E-02 0.589E+00 0.350E-03

 0.0340088 0.6862643
 0.318E-01 0.186E+00 0.772E+00 0.108E+00 0.315E-02 0.583E+00 0.350E-03

 0.0293703 0.5452864
 0.235E-01 0.170E+00 0.111E+01 0.149E+00 0.692E-02 0.570E+00 0.350E-03

 0.0292464 0.6662786
 0.228E-01 0.178E+00 0.105E+01 0.158E+00 0.415E-02 0.553E+00 0.350E-03

 0.0290227 0.2421386
 0.301E-01 0.175E+00 0.112E+01 0.171E+00 0.468E-02 0.536E+00 0.350E-03

 0.0290199 0.1418406
 0.334E-01 0.177E+00 0.107E+01 0.176E+00 0.410E-02 0.525E+00 0.350E-03

 0.0290172 0.0786546
 0.320E-01 0.176E+00 0.111E+01 0.174E+00 0.445E-02 0.530E+00 0.350E-03

 0.0290166 0.0502960
 0.330E-01 0.177E+00 0.109E+01 0.176E+00 0.424E-02 0.526E+00 0.350E-03

 0.0290163 0.0301044
 0.325E-01 0.176E+00 0.110E+01 0.175E+00 0.437E-02 0.529E+00 0.350E-03

 0.0290163 0.0187524
 0.328E-01 0.177E+00 0.109E+01 0.175E+00 0.429E-02 0.527E+00 0.350E-03

 0.0290162 0.0113841
 0.326E-01 0.176E+00 0.110E+01 0.175E+00 0.434E-02 0.528E+00 0.350E-03

 0.0290162 0.0070176
 0.327E-01 0.176E+00 0.109E+01 0.175E+00 0.431E-02 0.528E+00 0.350E-03

 0.0290162 0.0042845
 0.327E-01 0.176E+00 0.110E+01 0.175E+00 0.433E-02 0.528E+00 0.350E-03

 0.0290162 0.0026309
 0.327E-01 0.176E+00 0.109E+01 0.175E+00 0.431E-02 0.528E+00 0.350E-03

 0.0290162 0.0016100
 0.327E-01 0.176E+00 0.110E+01 0.175E+00 0.432E-02 0.528E+00 0.350E-03

I      1          2          3          4          5          6
 1 0.100E+01
 2 0.216E+00 0.100E+01
 3-0.163E+00-0.413E+00 0.100E+01
 4 0.773E+00 0.131E+00-0.468E-01 0.100E+01
 5-0.547E+00-0.654E+00 0.529E+00-0.664E+00 0.100E+01
 6-0.900E+00-0.379E+00 0.291E+00-0.918E+00 0.807E+00 0.100E+01

106 PT RAT COT CURVE
```



```
RMS=     0.0290162 GOODNESS OF FIT     0.0298740
    106 POINTS       100 DEG OF FREEDOM

..P(1).....P(2).....P(3).....P(4).....P(5).....P(6).....P(7)...

 0.327E-01 0.176E+00 0.110E+01 0.175E+00 0.432E-02 0.528E+00 0.350E-03
 0.192E-01 0.142E-01 0.396E+00 0.300E-01 0.223E-02 0.509E-01 0.000E+00
           the covariance matrix does not include
       parameter 7 and the parameter 7 error is 0.000 because
       it is fixed


 1START,2ADD,3GUESS,5PLOT,6CPLOT,8LPLOT,9STOP

3                                  error estimates without iteration
FUNCTION (-1HELP), ITERATIONS, RMS QUIT, DELMX QUIT, (NIT)

1,0                                0 iterations gives error estimates
FINAL,FRACT(1),K(1),FRACT(2),K(2),FRACT(3),K(3)

.0327 ,176 1.10 ,175 .00432 ,528 .00035
TYPE INDEX OF PARAMETERS TO BE FIXED.

—                                  check the error without the parameter fixed


I      1          2          3          4          5          6          7
 1 0.100E+01
 2-0.201E+00 0.100E+01
 3 0.145E+00-0.460E+00 0.100E+01
 4-0.735E+00 0.361E+00-0.254E+00 0.100E+01
 5 0.579E+00-0.627E+00 0.466E+00-0.900E+00 0.100E+01
 6 0.465E+00-0.484E+00 0.368E+00-0.933E+00 0.937E+00 0.100E+01
 7 0.877E+00-0.341E+00 0.252E+00-0.959E+00 0.828E+00 0.816E+00 0.100E+01

106 PT RAT COT CURVE

RMS=     0.0290170 GOODNESS OF FIT     0.0300253
    106 POINTS       99 DEG OF FREEDOM

..P(1).....P(2).....P(3).....P(4).....P(5).....P(6).....P(7)...

 0.327E-01 0.176E+00 0.110E+01 0.175E+00 0.432E-02 0.528E+00 0.350E-03
 0.401E-01 0.152E-01 0.414E+00 0.106E+00 0.399E-02 0.883E-01 0.160E-03

 1START,2ADD,3GUESS,5PLOT,6CPLOT,8LPLOT,9STOP

9
STOP
```