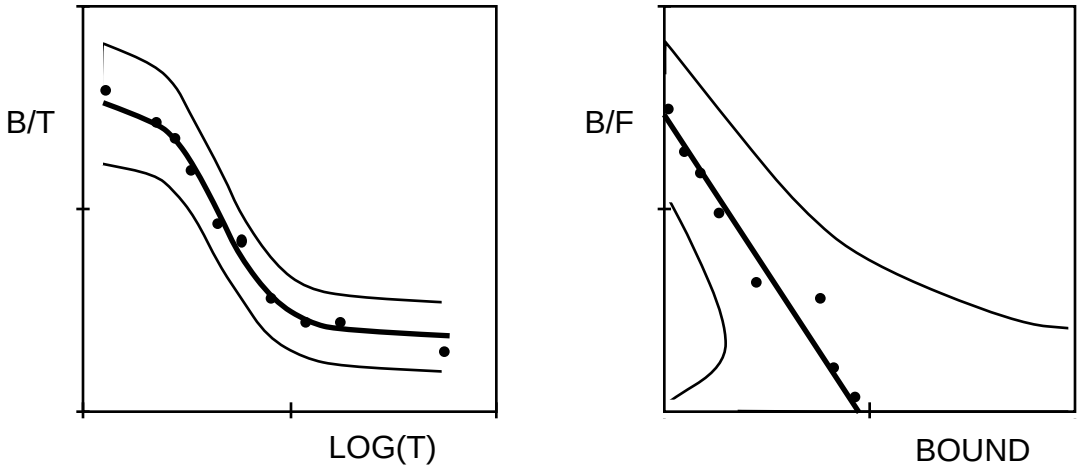
A User's Guide to

LIGAND

Data analysis and curve-fitting
for ligand binding experiments



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Dr. David Rodbard provided the original impetus, guidance, stimulation, substantial advice and friendly support for the development of this computer program and its application to numerous laboratory studies. Dr. Andre Delean collaborated in the development of the algorithms for curve fitting and function evaluation. This program is based in part on algorithms developed by Dr. Henry A. Feldman.

Dr. Richard Clayton contributed data used in some of the examples. Jeanne Grillo and Girija Chandrasekhar began the work on the Fortran version of LIGAND, which was completed by Michael Beveridge. Michael Beveridge also adapted the Fortran and Basic versions to the IBM-PC. Jeffrey Coburn completed the conversion to the IBM-PC. Michael Hinchcliff produced the Microsoft Fortran version of LIGAND for the Macintosh with a fully developed Macintosh interface, and reduced the MAC-LIGAND system to two programs: SCAPRE and Scafit. Arnie Cushing rewrote the SCAGRF package for the IBM-PC in Pascal. The IBM-PC version of SCAGRF in now a single executable. This eliminated the need for a Basic run time library, a graphics print screen driver and the installation procedure. The print screen utility for SCAGRF was rewritten to support EGA and VGA and support was developed for Postscript printers. Mr. Cushing also redid the Macintosh interface and added many new features including the ability to import curve data from the IBM-PC and the ability to create Saturation and B/Bo plots.

Dr. Vincenzo Guardabasso provided help in testing and verifying the accuracy of the Fortran version, including extensive Monte-Carlo testing. Dr. Siegfried Schwartz provided a careful and critical review of the User's Guide. Dr. Juan Calvo contributed improvements to the preprocessing programs for the IBM-PC (Scahot/Scapre).

We would also like to acknowledge the valuable suggestions and comments of numerous users at LTPB, NICHD including but not limited to Drs. R. Cruciani, D. Lichtstein, E. Rovatti, R. Lutz, G. Pesce, and others at NIH and from around the world.

PROGRAM AVAILABILITY

The Ligand program is currently available for the IBM-PC and clones, CONVEX UNIX, and Apple Macintosh computers. Please address all requests to the authors at NIH, DCRT, Bldg 12a, Room 2041, Bethesda, Md, 20892, USA. We do not generally distribute the source code for these programs. We do ask that you read, sign and return the enclosed "License Agreement". Feel free to make copies of this Agreement for your colleagues who may also wish to use the program.

Please address all inquiries, complaints, suggestions, praise, etc. in writing to us at the above address. While we can offer only a minimal level of support to users, we are very interested in fixing bugs and incorporating your suggestions into new versions.

LIGAND PROGRAMS LICENSE AGREEMENT

In requesting the LIGAND program, I understand, accept and agree to be bound by the following terms and conditions:

1. I agree that this software, known as LIGAND-PC, SCAFIT, SCAPRE, SCAHOT, and SCAGRF, is provided for my own personal, educational, and scientific research purposes only.

2. I agree not to sell, re-distribute, modify or commercialize this software, without the express written permission of the authors.

3. I will protect the source, object and executable code, whether on paper or stored in electronic, magnetic, optical or other storage medium, from unauthorized access or copying.

4. I understand that any rights of ownership, proprietary interests and/or copyrights of this software, remain with the authors of the Ligand programs. Furthermore, I understand that the license for use hereby granted to me may be revoked at any time, with reasonable notice, by the authors at their sole discretion.

5. I agree to accept all responsibility regarding the accuracy, suitability, correctness and appropriateness of use of this software.

6. I agree to acknowledge or make reference to published descriptions of this software in scientific publications and presentations of studies which make substantial use of results calculated by this software.

7. I understand that no warrantee, either expressed or implied, including but not limited to fitness for a particular purpose is made by the authors or by NIH. Neither the authors of this program nor NIH, nor its agents shall be held liable for any direct, indirect or consequential damages arising out of the use or inability to use this software, loss of data or damage to equipment.

Requestor

Circle One

IBM-PC or clone Macintosh

_____

Signature Date

_____

Full name (Print)

Mail to: Peter Munson
Bldg. 12A, Room 2041
NIH
Bethesda, MD 20892 USA

_____

Title/Institution

_____

Address

_____

Phone number

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1. INTRODUCTION

LIGAND is a multipurpose computer system for the analysis of ligand binding data, model fitting and parameter estimation. It is simple to use and does not require learning a computer language. You operate LIGAND by answering a series of simple, usually YES/NO questions about your data and the problem you are trying to solve. LIGAND is a very powerful system that will handle any multiple-ligand, multiple binding site problems. Data for which this program is applicable may arise from in vitro receptor binding studies, radio-immunoassay, competitive protein binding studies, etc.

LIGAND is essentially a non-linear model fitting program, which allows you to fit any of a large number of physical-chemical binding models to your data. It can be used to find the most appropriate model for your data as well as the best parameter estimates for that model.

The LIGAND system has many important features of interest to the laboratory investigator and statistician:

1) Exact physical-chemical model of equilibrium binding is used to describe the data. No computational approximations or simplifications are used.

2) Non-specific binding can be handled as a fitted parameter in the model, which means that one can practically omit the setup of non-specific binding tubes in the experiment.

3) More than one ligand may be handled simultaneously.

4) Correction factors for varying concentration of receptors (binding proteins) between different experiment runs may be used, or estimated from the data.

To facilitate the use of the LIGAND system, the program incorporates these features.

1) Easy data entry and editing.

2) Interactive, user-controlled curve fitting.

3) Easy model specification - you need only specify the number of ligands and number of binding sites you wish to consider.

4) Model parameters may be fixed, fitted to the data, or may have their values shared with other fitted parameters.

5) Model parameters may be re-adjusted, if necessary, during the course of the fit.

6) Graphics are readily available.

7) Statistical comparison and goodness-of-fit tests are performed automatically.

8) A session log is kept, especially useful on CRT or TV screen terminals (mainframe versions only).

This manual provides instructions to the user about how to run the LIGAND programs. It assumes that the user is running on the CONVEX computer at NIH, but most of the manual will be applicable at other centers. Most of the program features are identical on other machines. Many of notes within these sessions are valuable in other contexts. The User's Guide assumes that you are already reasonably familiar with logging on to the CONVEX. If you are not yet at ease with this computer, get a copy of the CONVEX UNIX Primer, and talk to the CONVEX staff at 496-4823.

The sample sessions provide a tutorial for learning the operation of LIGAND. Running the program involves answering relatively simple questions at each step. Default answers are printed within square brackets ([]) and which you may accept by simply entering carriage-return. A very large number of models may be tested using SCAFIT, so that we cannot cover all possibilities in the examples. After reading the sessions, it should be clear how to formulate and test the particular model which applies to your data. If you wish to do Scatchard plot analysis and your experiment involves only one ligand (that is, if you may assume that hot and cold ligand have the same affinity), then you should read the section on Scatchard plot analysis, and perhaps skip the discussions of multiple ligand systems.

The program can handle an extremely wide variety of problems. However, it does make a few limiting assumptions. The analysis technique assumes that all experiments are carried out until equilibrium is achieved. Further, the program only treats the case of non-cooperative, single step molecular binding reactions. However, the technique and program could be generalized to include multi-step, possibly cooperative binding reactions without undue difficulty. (Cooperativity may be modeled in 1981 or later versions of SCAFIT) (see Munson and Rodbard, 1984).

The numerical method for analysis of the n-ligand, m-binding site problem was originally developed by Feldman (1972), and later enhanced by us to include

new parameters for non-specific binding and for "correction factors" for varying receptor concentration between experiments (Munson and Rodbard, 1980).

We feel that this method represents a substantial improvement over the more common graphical, Scatchard plot approach for estimating the affinity constants in ligand binding studies. In some senses, the technique is the "best" possible analysis technique, since it fits the raw experimental data in an untransformed coordinate system, where the errors are most likely to be normally distributed and uncorrelated with the independent variable. Many approximations sometimes used in the analysis of binding studies (e.g. assuming that Total ligand = Free ligand, or that the ratio of affinities of two different ligands (K11/K21) is approximately equal to the ratio of their ED50's, or that the tracer ligand concentration is negligible, etc.) may sometimes lead to erroneous and misleading results. Our method avoids such problems by solving the exact equations which represent the mass-action law binding equations:

$$\frac{[HR_{ij}]}{[H_i]\,[R_j]} = K_{ij}$$

These equations model the chemical reaction scheme:

$$H_i + R_j = HR_{ij}$$

where [H] is free hormone concentration, [R] is free receptor concentration and [HR], the bound hormone-receptor complex concentration, and i indexes ligands, and j indexes the different receptor classes. Of course, these symbols may be interpreted to fit any bimolecular reaction, e.g. Antibody + Antigen = Antibody-Antigen complex, etc. The program is designed to facilitate the estimation of the equilibrium constant of association of the i-th ligand for the j-th binding site, K_{ij}, and the receptor binding capacities of the j-th receptor class, R_j.

A common pitfall confronting the user is that, in general, he doesn't know, a priori, what the correct model for his data should be. Rather there is an entire series of models, all of which must be considered as possibilities. The experimentalist may rule out some of them on the basis of biological or chemical considerations. Then the program can choose, from the remaining possibilities, the model which gives the statistically "best" representation of the data. Having established the appropriate model, one can then evaluate the parameter estimates. Since the final fit can be influenced by wildly incorrect initial parameter estimates, one must always verify that the converged results are within a reasonable range, i.e., that no parameter value has become vanishingly small or that its standard error has become excessively large. Further, if in doubt, one can restart the program with new initial estimates, and hopefully verify that the converged results are the same as before.

With a correct choice of the binding models, and with initial parameter estimates to within an order of magnitude or so, the program is usually able to find the best estimates within less than 10 iterations.

2.Program operation at the DCRT CONVEX Computer Facility.

This chapter section is oriented primarily toward users of the CONVEX at NIH. Users on other systems (IBM-PC,Macintosh) should also read this chapter, taking account of the slight differences relating to system operations.

The LIGAND system consists of four separate programs and their shared data sets. The first two programs, SCAPRE and SCAHOT, are preprocessor programs designed to take data directly from the investigator's notebook, in more or less raw form, and convert them to the standardized input format required by SCAFIT, the fitting program. SCAPRE is designed around the "hot plus cold" or displacement experiment where a constant, usually small, amount of tracer ligand and varying amounts of cold, unlabeled ligand are added to each tube in the experiment. If your experiment does not fit this regime, you may need to modify the program, or do some of the simpler calculations by hand and go directly to SCAFIT. Alternately, you may use SCAHOT for the "hot only" or saturation experiment where increasing doses of a single ligand are used in both labeled and unlabeled form. Typically, a large dose of unlabeled is used for the "nonspecific" tubes.

The standard input format for SCAFIT requires that the Total and Bound ligand concentrations be converted to Moles/Liter. In addition, certain identification information is required in each data set. The preprocessor allows you to edit and update the raw data, if necessary.

The FORTRAN program, SCAFIT, is the main fitting program which actually analyzes the data and finds the best parameter estimates. As with all the programs, it is run interactively. Thus, the user answers a series of questions which directs the course of the computation. When a certain stage in the computation is reached, the program pauses, again asking the user for further direction. Thus, the user may formulate and test hypotheses during the program execution, and as a result of early computations, revise these hypotheses, formulate new ones and test their validity.

After a run of the fitting program, the user may graphically display the fit using SCAGRF. This facility allows the user to visually evaluate the goodness of fit, detect outliers, and possibly, to develop intuition about what new models and hypotheses may be suggested by the data. This program produces two types of plots: Displacement (B/T vs. log(T)) and Scatchard (B/F vs. B). Confidence limits for a point around the fitted curve may also be plotted. Scatchard plots of the data are useful only when a single ligand is used in the experiment.

Five EASY steps to running LIGAND on the CONVEX.

The following section describes the steps necessary to use the LIGAND program on the CONVEX computer at the NIH DCRT computer center. Additional information about the CONVEX is available from the computer center, Bldg 12A, Rm1017. The user should already be familiar with the mechanics of logon, of connecting the terminal with the CONVEX via telephone lines, with simple CONVEX monitor commands.

Step 1 Obtain documentation for LIGAND program and for CONVEX. Also get the program copied into your account on the computer. This can be accomplished by typing the following command.
~floyd/ligand/distribution/.install_ligand
This will install the CONVEX LIGAND system into the directory from which the command was issued.

Step 2 GET STARTED
a) Connect your terminal with the CONVEX via a telephone link (phone 492-2224). The mechanics of this vary with each terminal type, so ask someone who knows how to operate your terminal.
b) Logon to the CONVEX. This involves giving your account number and password. If you don't have an account, call the Project Control Office at 496-6146.

Step 3. PREPARE DATA
Run the data preparation program by typing "scapre" or "scahot". From now on you will be talking to the program SCAPRE (or SCAHOT), which will assist you in formatting the data from your experiment in a form which the computer will be able to process. You may avoid SCAPRE and SCAHOT and format your data directly for input to SCAFIT. See the section about preparing your data.
At this point, you may run another program by going to Step 3 or 4.

Step 4. FIT DATA
Type "scafit" to run the fitting program. For details of how to operate the program, see the sample session.

Step 5. GRAPH DATA
At a Tektronix 4105 graphics terminal emulator, type "scagrf", to run the graphics program. This program asks a series of questions and then produces a displacement plot (B/T vs. Total

ligand concentration) or a Scatchard plot (B/F vs. Bound).

3. Saturation Binding Data -- Scatchard Analysis

Saturation binding is one of two main types of binding experiments. Using this approach, increasing doses of labeled ligand or drug are added to a tissue preparation until the receptor is "saturated". The other approach is a "displacement study", which is described in section 4.

Typically, only a single ligand is used in both labeled and unlabeled forms in a saturation study. The raw data must be put into a standardized format for the fitting program. The amount of radioactivity ("Total counts") added to each tube, and the amount in the bound fraction ("Bound counts") are entered into the computer program. This information is used to calculate Total Concentration and Bound Concentration in Molar units for input to SCAFIT. Additional information (specific activity, counting time, etc) is also entered.

Non-specific binding is frequently evaluated by adding excess unlabeled ("cold") ligand to a second set of tubes. In theory, this will saturate the specific receptor but not the non-specific, non-saturable binding site. The results from these "non-specific" tubes is typically subtracted from the "total binding" tubes to give "specific bound" for use in preparing a Scatchard plot.

With LIGAND, a slightly different approach is taken wherein all of the data are entered (both "specific" and "non-specific" tubes) and the program SCAFIT is required to estimate the non-specific binding as a parameter. The advantages of this approach are well-documented (Munson 1983).

Results of a saturation study are prepared for input to SCAFIT using SCAHOT. These results may later be plotted in either Scatchard or displacement curve form using SCAGRF.

3.1 Sample Session -- SCAHOT.BAS

If you are using the "hot only" saturation experiment, that is, your tracer concentration (total counts) added to each tube varies, or if you add cold ligand ("excess cold") to measure non-specific binding, then SCAHOT is the program for you. For example, estradiol binding to its receptors is commonly measured this way, except the cold ligand is often DES (diethylstibestrol) rather than cold estradiol. Strictly speaking, the cold ligand should be biochemically identical to hot, but if you can guarantee that another, perhaps cheaper, cold ligand has the same binding affinity and binding capacity, then it may be used instead.

What follows is a session using SCAHOT, which prepares the input file for SCAFIT. In order to follow the session, note that what would have appeared on your computer terminal is all in upper case. What you type to the computer is underlined. Comments which don't appear on your screen are in italicized letters.

For first time users, login to the CONVEX and run the following installation procedure:

```
ConvexOS.  Release V8.1 (helix.nih.gov)

login:  ????
password:  ????
```

The installation process copies the files and programs necessary to run LIGAND into the directory that you are currently using. So it is wise to execute the following UNIX commands to create, and move to a new LIGAND directory.

```
helix%  mkdir ligand
helix%  cd ligand
```

This is the command to install your LIGAND system:

```
helix%  /u6/floyd/ligand/distribution/.install_ligand

Installation of the LIGAND system...

Please wait while the LIGAND files are being copied
to your current directory.

LIGAND installation complete!!!

Your current directory now contains the following files:
exp1a.bas   exp2a.crv   exp4a.bas   scafit      scapre
exp1a.crv   exp3a.bas   exp4a.crv   scagrf      suz1.bas
exp2a.bas   exp3a.crv   readme      scahot      suz1.crv
```

Execution of data entry program can be done here by typing scahot.

```
helix% scahot
            ****    LIGAND SYSTEM    ****
            PROGRAM SCAHOT    --     V2.6


          THIS PROGRAM WILL PRODUCE THE INPUT FILE
                FOR THE SCAFIT PROGRAM


TO SEE LIST OF COMMANDS -- ENTER HELP

FILE NAME FOR STORING THIS DATA (NO EXTENSION) ? SUZ1
```

This is the name of the file for storing the data. You will use this name again when you run SCAFIT. It is given the extension .BAS

```
CAN'T FIND FILE suz1.dat
OPENING NEW FILE suz1.bas
```

This will create a new file.

```
1     NAME OF THIS EXPERIMENT: ?  This is the SUZ1 data file
```

This is descriptive information which helps you remember which data set is which.

2 COUNTING TIME(MIN): ? 1

This is used to convert counts to c.p.m. Use 1 if data are already in c.p.m.

3 VOLUME OF TUBE(ML.): ? .5

Reaction volume, used to calculate concentration of reagents, from mass of dose

4 SPECIFIC ACTIVITY (DPM/MOLE): ? 46.398E15

You must convert specific activities given in other units. Remember that there are 2.22E6 dpm per microcurie.

5 COUNTING EFFICIENCY (CPM/DPM): ? .43

Use 1 if data are already in dpm.

6 LIGAND #: ? 1

Every ligand in the study must be given a different number. The numbering scheme must be the same for all data sets in the study. Enter the number of the labeled ligand here.

7 BINDABILITY FRACTION (1.0 IF 100%): ? 1

This is the proportion of radioactivity attached to biologically active material.

8 MOL.WT.: ? 584.64

Molecular weight is used to convert grams to moles (see line 10)

9 BACKGROUND COUNTS [0]: ? 55

These are subtracted from total and bound counts before firsthand calculations are made.

10 DOSE UNITS OF COLD LIGAND: ? NANOMOLES

These are the units in which you are to enter. The dose (amount of cold ligand added in a tube) units. Allowable units are PICO-, FEMTO-, NANO-, MICRO-, -MOLES, -GRAMS.

VARIANCE(Y)=A0+A1 × Y+A2 × Y^{2}+A3 × Y^{A}_{4}

This is the model for the variance of the bound concentrations. It is a combination of the quadratic and exponential functions, although only one part should be used at a time. Thus either A0=A1=0 or A2=A3=A4=0. A reasonable default assumption is "constant percent error", i.e. A2=.0001, A0=A1=A3=A4=0

11 A0: ? 0
12 A1: ? 0
13 A2: ? 0.0001
14 A3: ? 0
15 A4: ? 0

COLD-AMOUNT TOTAL COUNTS COUNTS COUNTS COUNTS

The following are the actual data for the experiment, one line

per tube. Separate the columns with one or more blanks. Do not use tabs! The first column is the dose of cold (usually either zero for the "total binding" tubes or some large amount for the "non-specific" tubes). The second column is the "total counts", i.e. the total radioactivity added to this tube. The third and subsequent columns are the "bound counts", i.e. the radioactivity in the bound fraction for that tube. Any number of replicates may be used, provided they fit on a line.

```
16    :  ? 0 380666 2780 3076
17    :  ? 50 380666 655 651
```

The first two lines correspond to an "un-competed" or total binding and a "competed" or non-specific binding tube, respectively. Note that the total counts are the same for tubes 1 and 2. However, the lines may be entered in any order.

```
18    :  ? 0 287380 2878 2418
19    :  ? 50 287380 616 622
20    :  ? 0 182300 1422 153
21    :  ? 50 182300 403 340
22    :  ? 0 74406 896 865
23    :  ? 50 74406 209 250
24    :  ? 0 38073 631 517
25    :  ? 50 38073 128 172
26    :  ? 0 19249 453 344
27    :  ? 50 19249 103 127
28    :  ? 0 9563 301 210
29    :  ? 50 9563 90 86
30    :  ? 0 4871 127 140
31    :  ? 50 4871 67
32    :  ? 0 2262 81 98
33    :  ? 50 2262 57 26
34    :  ? <return>
```

A blank or null entry puts you back into the command mode.

```
COMMAND ? REV
```

The "REVIEW" command is used to display the data you have already entered.

```
1     NAME OF THIS EXPERIMENT: This is the SUZ1       experiment
2     COUNTING TIME(MIN): 1
3     VOLUME OF TUBE(ML.): .5
4     SPECIFIC ACTIVITY (DPM/MOLE): 46.398E15
5     COUNTING EFFICIENCY (CPM/DPM): .43
6     LIGAND #: 1
7     BINDABILITY FRACTION (1.0 IF 100%): 1
8     MOL.WT.: 584.64
9     BACKGROUND COUNTS [0]: 55
10    DOSE UNITS OF COLD LIGAND: NANOMOLES
11    A0: 0
12    A1: 0
```

```
13    A2: 0.0001
14    A3: 0
15    A4: 0
      COLD-AMOUNT TOTAL COUNTS  COUNTS  COUNTS   COUNTS
16    : 0 380666 2780 3076
17    : 50 380666 655 651
18    : 0 287380 2878 2418
19    : 50 287380 616 622
20    : 0 182300 1422 1530
21    : 50 182300 403 340
22    : 0 74406 896 865
23    : 50 74406 209 250
24    : 0 38073 631 517
25    : 50 38073 128 172
26    : 0 19249 453 344
27    : 50 19249 103 127
28    : 0 9563 301 210
29    : 50 9563 90 86
30    : 0 4871 127 140
31    : 50 4871 67
32    : 0 2262 81 98
33    : 50 2262 57 26

COMMAND ? REP 33
```

This replaces the old line 33 with a new line.

```
33      : 50 2262 57 62

COMMAND ? HELP
```

```
THE LEGAL COMMANDS ARE:

INPUT       BEGIN ENTERING THE DATA AT THE NEXT AVAILABLE   LINE
HELP        TYPE THIS MESSAGE
REVIEW      REVIEWS THE DATA YOU HAVE ENTERED
FINISH      ENDS THE PROGRAM, AND STORES THE
            CALCULATED DATA IN THE FILE YOU HAVE CHOSEN
DELETE      BEGINS THE DELETION OF A LINE OF DATA
            YOU WILL BE PROMPTED FOR LINE NUMBER ON THE
            FOLLOWING LINE
INSERT      BEGINS INSERTION OF A LINE. YOU WILL BE PROMPTED
            FOR THE NEW LINE NUMBER
REPLACE     SAME AS DELETE FOLLOWED BY INSERT COMMANDS
LIST        LISTS ONE LINE OF DATA.  YOU WILL BE PROMPTED
            FOR THE LINE NUMBER IF NOT ENTERED.

ANY OTHER COMMAND WILL RESULT IN THE MESSAGE 'ILLEGAL COMMAND'


COMMAND ? FIN
```

The "FINISH" command calculates the results for input to SCAFIT and ends the program. The first two columns of the following table have concentration units (molar), not mass units.

```
DO YOU WANT A GRAPH FILE CREATED ? [Y] <return>

DOSE#       TOTAL             BOUND             B/T

1           0.381543E-07      0.288004E-09      0.0075484
2           0.100038E-03      0.157176E-06      0.0015712
3           0.288029E-07      0.259935E-09      0.0090246
4           0.100029E-03      0.196350E-06      0.0019629
5           0.182691E-07      0.142448E-09      0.0077972
6           0.100018E-03      0.173699E-06      0.0017367
7           0.745331E-08      0.827522E-10      0.0111027
8           0.100007E-03      0.234715E-06      0.0023470
9           0.381111E-08      0.520271E-10      0.0136514
10          0.100004E-03      0.249891E-06      0.0024988
11          0.192410E-08      0.344341E-10      0.0178962
12          0.100002E-03      0.312604E-06      0.0031260
13          0.953128E-09      0.200991E-10      0.0210875
14          0.100001E-03      0.347079E-06      0.0034708
15          0.482779E-09      0.786923E-11      0.0162998
16          0.100000E-03      0.249171E-06      0.0024917
17          0.221240E-09      0.345845E-11      0.0156321
18          0.100000E-03      0.203897E-06      0.0020390
STOP:  BYE
helix%
```

3.2 Output files from SCAHOT session.

The program SCAHOT creates two files. The first is the "raw" data as you input it during the session. The second (.CRV) is data to be input to SCAFIT and contains total and bound concentrations.

Listing for file SUZ1.BAS:

```
SUZ1
1.
.5
46.398E15
.43
1
1.
584.64
55.
NANOMOLES
0.
0.
0.0001
0.
0.
0.    380666.     2780. 3076.
50.   380666.     655.  651.
0.    287380.     2878. 2418.
50.   287380.     616.  622.
0.    182300.     1422. 1530.
50.   182300.     403.  340.
0.    74406.      896.  865.
50.   74406.      209.  250.
0.    38073.      631.  517.
50.   38073.      128.  172.
0.    19249.      453.  344.
50.   19249.      103.  127.
0.    9563.       301.  210.
50.   9563.       90.   86.
0.    4871.       127.  140.
50.   4871.       67.
0.    2262.       81.   98.
50.   2262.       57.   62.
```

Listing for file SUZ1.CRV:

```
"This is the SUZ1 experiment"
0 0 0.0001 0
0
1 1
0
3.81543E-8      2.88004E-10     2
1.00038E-4      1.57176E-7      2
2.88029E-8      2.59935E-10     2
1.00029E-4      1.96350E-7      2
1.82691E-8      1.42448E-10     2
1.00018E-4      1.73699E-7      2
7.45331E-9      8.27522E-11     2
1.00007E-4      2.34715E-7      2
3.81111E-9      5.20271E-11     2
1.00004E-4      2.49891E-7      2
1.92410E-9      3.44341E-11     2
1.00002E-4      3.12604E-7      2
9.53128E-10     2.00991E-11     2
1.00001E-4      3.47079E-7      2
4.82779E-10     7.86922E-12     2
0.0001          2.49171E-7      1
2.21240E-10     3.45845E-12     2
0.0001          2.03897E-7      2
```

Description of the above listing of SUZ1.CRV:

Line#	Description
1	Comments
2	Four weighing parameters a0,a1,a2,a3.
3	Fifth weighing parameter a4.
4	Varying ligand number, labeled ligand number.
5	Total hot ligand conc.[M].
6-end	Total varying ligand conc[M]; bound, labeled ligand conc[M], Replication weight.

3.3 Fitting the data -- SCAFIT.FOR

What follows is a session using the program SCAFIT. We will use the data files prepared in the previous section. The general strategy is to first attempt a simple model, in this case a "one binding site" fit, corresponding to a straight line in the Scatchard plot. After that, we look for evidence of more complexity, and try a "two-site" fit. After concluding the fits, we generate higher quality plots of the data in section 3.4.

Again, what will appear on your computer screen is in upper case, with interspersed comments in italics. User (your) responses to the computer are underlined. Default options are often presented inside square brackets. To accept the default, you need only press the "<return>" key. Otherwise, enter any valid response when the program asks a question.

```
helix% scafit


                        L I G A N D:
     Curve fitting and data analysis for ligand binding data
                 National Institutes of Health

Program: SCAFIT version 2.4MAGGI


FILE NAME FOR SESSION (NO EXTENSION) ? suz1

NUMBER OF LIGANDS (HIGHEST NUMBER USED) ? 1

TYPE THE DATA FILE NAME(S) (NO EXTENSION)
SEPARATE WITH <CR> END WITH BLANK LINE
? SUZ1
? <return>
```

The data was stored as SUZ1. Enter as many data file names as you wish to fit in this session. With multiple experiments we usually start by fitting each curve separately.

```
CURVE FILE    SUZ1.CRV
WEIGHTING PARAMETERS
0.             0.      9.9999997E-05         0.             0.
VARYING LIGAND:   1   LABELED LIGAND:   1
NUMBER OF POINTS:           18
```

If any of the above information is not as you entered it in SCAHOT there is something wrong. Go back and check your data files.

```
NUMBER OF SITES ? 1
```

The simplest model involves only a single class of receptor binding sites.

```
DO YOU WISH TO WEIGHT POINTS ACCORDING TO
NUMBER OF TUBES AT A GIVEN DOSE (Y/N) [N] ? <return>
```

This is a special option for certain statistical comparisons.It allows one to take account of different numbers of replicates at each dose level.

```
WOULD YOU LIKE THE COOPERATIVITY MODEL (Y/N) [N] ? <return>

WOULD YOU LIKE THE ADDITIONAL D PARAMETER(Y/N) [N] ? <return>

THE FOLLOWING PARAMETERS WILL BE CONSIDERED:
K11 R1  N1 C1
```

Explanations :

- *K11 - Affinity constant of first ligand for first receptor class.*
- *R1 - Binding capacity of first receptor class*
- *N1 - Fractional non-specific binding*
- *C1 - not needed here, since there is only a single curve.*

```
THE FOLLOWING PARAMETER GROUPS HAVE A SHARED VALUE:
```

There are no shared parameters in this fit.

```
DO YOU WANT THE SAME SHARED PARAMETERS AS
BEFORE (Y/N) [Y] ? <return>
```

Note the use of the default (yes) response here.

```
LIST THE CONSTANT PARAMETERS (NO COMMA)
[ C1 ] ? <return>
```

Always set the correction factor C1 to the constant value 1.

```
C1  = [   1.00000     ] ? <return>

DO YOU WANT AUTOMATIC INITIAL ESTIMATES (Y/N) [Y] ? <return>

AFTER ADJUSTING FOR NONSPECIFIC, THERE ARE    9 POINTS LEFT
```

The program temporarily removes the "non-specific" tubes in order to get initial estimates for K and R. All points are considered subsequently.

```
INITIAL ESTIMATE FOR K11[  5.075841E+07 ] ? <return>
INITIAL ESTIMATE FOR R1 [  3.003146E-10 ] ? <return>
INITIAL ESTIMATE FOR N1 [  2.129737E-03 ] ? <return>

THE INITIAL ESTIMATES ARE
 K11 =  5.075841E+07
```

```
 R1  =  3.003146E-10
 N1  =  2.129737E-03
 C1  =   1.00000

 ARE THESE VALUES OK (Y/N) ? y
```

Initial estimates are almost always OK for simple models such as this one.

```
 IT.         EPSILON        SUM OF SQUARES
    0             0.              9881.782

 TYPE C,R,P,D,S,X,G OR H FOR HELP ? h

***   HELP   ***

 TYPE "C" TO    CONTINUE ITERATIONS
 TYPE "R" TO RESET PARAMETERS
 TYPE "P" TO SEE PARAMETER VALUES
 TYPE "D" TO VIEW DATA AND MODEL VALUES
 TYPE "X" TO EXIT WITHOUT FINAL ESTIMATES
 TYPE "S" TO GET SUMMARY
```

```
TYPE C,R,P,D,S,G,X OR H FOR HELP ? D
```

We demonstrate the data display option before continuing the fit.

```
#     LOG(TOTAL)   B/T       B/T-PRED    DIFF       RESIDUAL PLOT
CURVE: SUZ1
1     -7.418       0.0075    0.0073      0.0003      *
2     -4.000       0.0016    0.0021      -0.0006    *|
3     -7.541       0.0090    0.0083      0.0007      *
4     -4.000       0.0020    0.0021      -0.0002     *
5     -7.738       0.0078    0.0100      -0.0022    *|
6     -4.000       0.0017    0.0021      -0.0004    *|
7     -8.128       0.0111    0.0131      -0.0020    *|
8     -4.000       0.0023    0.0021      0.0002      *
9     -8.419       0.0137    0.0147      -0.0011     *
10    -4.000       0.0025    0.0021      0.0004      |*
11    -8.716       0.0179    0.0158      0.0021      |*
12    -4.000       0.0031    0.0021      0.0010      | *
13    -9.021       0.0211    0.0164      0.0047      |*
14    -4.000       0.0035    0.0021      0.0013      | *
15    -9.316       0.0163    0.0167      -0.0004     *
16    -4.000       0.0025    0.0021      0.0004      |*
17    -9.655       0.0156    0.0169      -0.0013     *
18    -4.000       0.0020    0.0021      -0.0001     *
```

```
TYPE C,R,P,D,S,G,X OR H FOR HELP ? C
NUMBER OF ADDITIONAL ITERATIONS ? 8
```

Usually 10 iterations are sufficient for simple problems.

```
  IT.        EPSILON       SUM OF SQUARES
    1            0.             7545.734

  IT.        EPSILON       SUM OF SQUARES
    2            0.             7375.312

CONVERGED, TYPE "S" TO GET FINAL RESULTS
```

The "converged" message means the best possible parameter values have been found.

```
TYPE C,R,P,D,S,G,X OR H FOR HELP ? G
```

We now investigate the graphics option. Better quality graphs can be obtained using SCAGRF. See section 3.4.

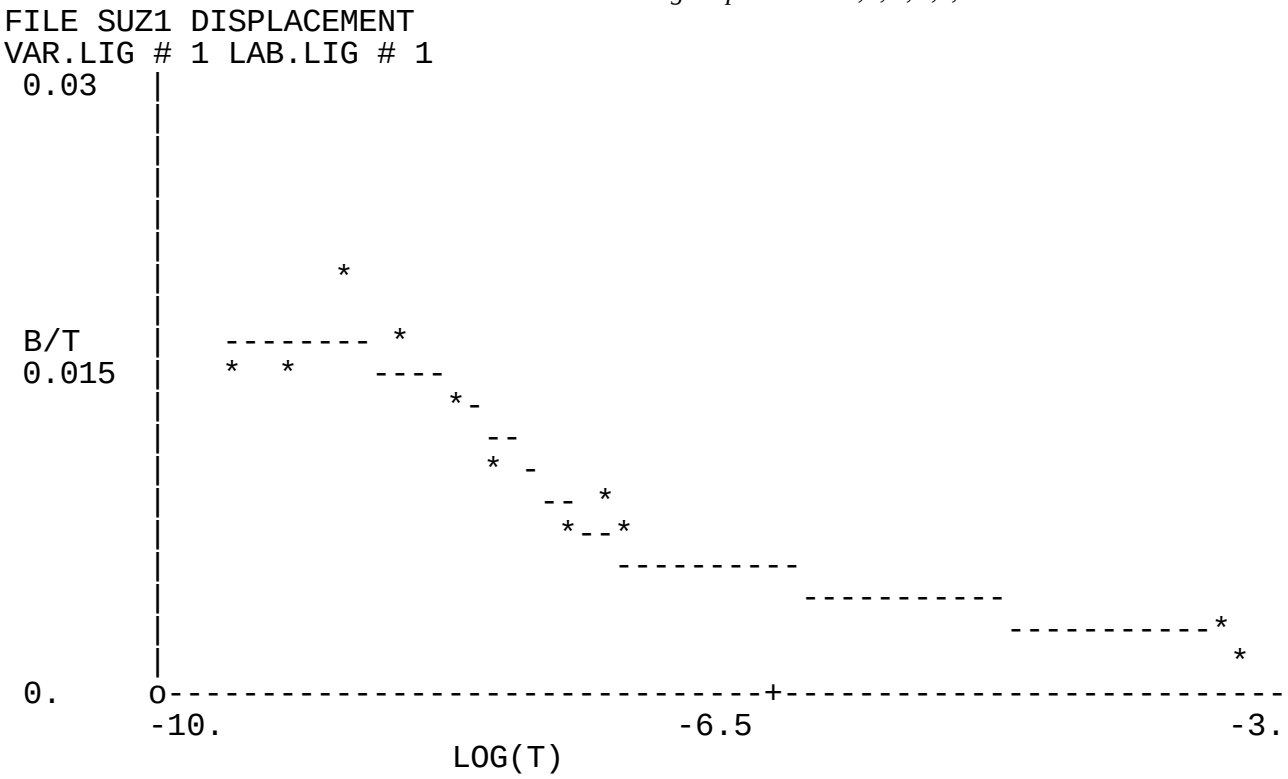
```
type  L  to change GRAPH LIMITS
type  G  to choose GRAPH TYPE [DISPLACEMENT]
```

The default graph type is DISPLACEMENT. To change the graph type to SCATCHARD, type 'G'. Also type 'G' to return to a graph type of DISPLACEMENT. Notice that 'G' is a

toggle.

```
type  C  for CONFIDENCE LIMITS (+/- 2 SIGMA) [N]
type  1  to graph the DISPLACEMENT CURVE SUZ1
type  0  to END GRAPHING SECTION

CHOOSE AN OPTION ? 1
```

Enter only one option each time you are prompted for one.
The legal options are T,L,G,C,0,1

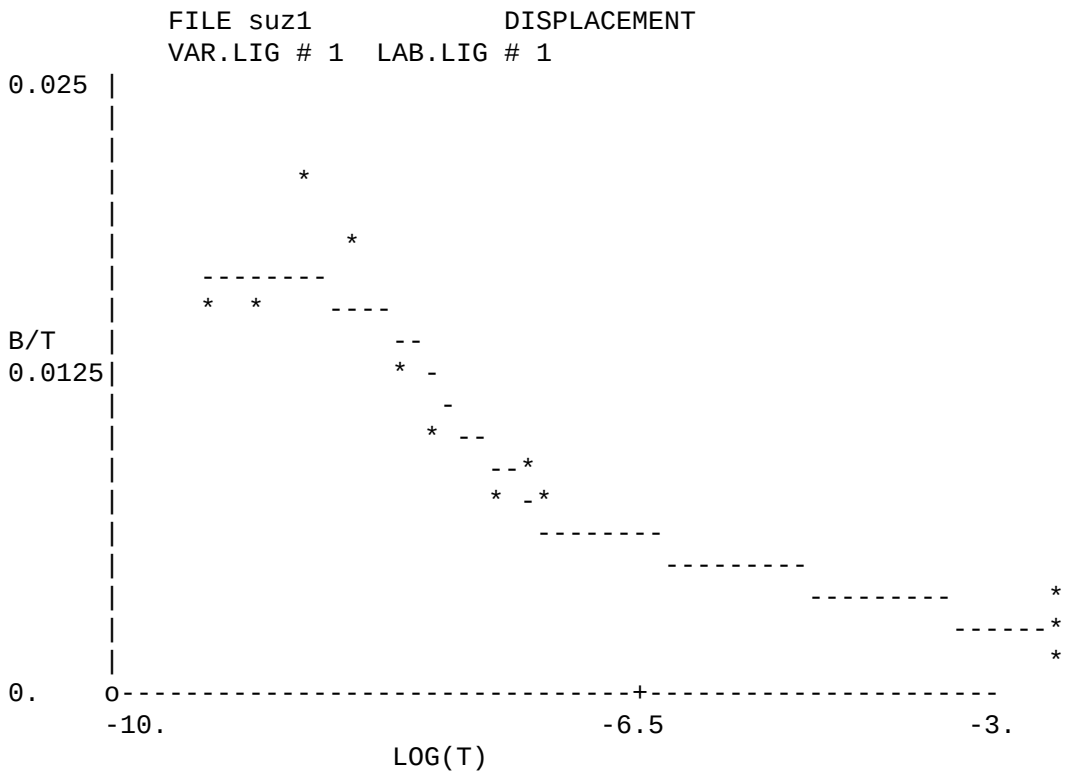



```
type  L  to change GRAPH LIMITS
type  G  to choose GRAPH TYPE [DISPLACEMENT]
type  C  for CONFIDENCE LIMITS (+/- 2 SIGMA) [N]
type  1  to graph the DISPLACEMENT CURVE SUZ1
type  0  to END GRAPHING SECTION

CHOOSE AN OPTION ? L
```

Choose 'L' to change the limits.

```
CHOOSE AN OPTION ?L
THE GRAPHING PARAMETERS ARE:
MINIMUM X, MAXIMUM X  -10.00000     -3.000000
MINIMUM Y, MAXIMUM Y        0.     3.0000001E-02
ARE THESE VALUES ACCEPTABLE (Y/N)[Y] ? N

WHAT VALUE FOR XMIN [ -10.0000    ] <return>
WHAT VALUE FOR XMAX [ -3.00000    ] <return>
```

```
WHAT VALUE FOR YMIN [ 0.000000E+00] <return>
WHAT VALUE FOR YMAX [ 0.100000    ] 0.025
```




```
type  L  to change GRAPH LIMITS
type  G  to choose GRAPH TYPE [DISPLACEMENT]
type  C  for CONFIDENCE LIMITS (+/- 2 SIGMA) [N]
type  1  to graph the DISPLACEMENT CURVE SUZ1
type  0  to END GRAPHING SECTION

CHOOSE AN OPTION ? 0
```

This option ends the graphics section and returns us to the main menu.

```
TYPE C,R,P,D,S,G,X OR H FOR HELP ? S
```

To get a summary of the fit, after the program has declared "convergence", we type "S"

```
L I G A N D: SCAFIT VERSION 2.4MAGGI

CAUTION: Results and interpretation of analysis depend upon
several important assumptions. See User's Guide for details

FINAL PARAMETER ESTIMATES, NEWEST FIT NO.   1
```

```
        VALUE          %CV       LOG-VALUE         Kd          K*R

K11     6.3189E+07     39.%       7.8006      1.5826E-08     0.0157
R1      2.4829E-10     31.%
N1      2.3679E-03      7.%
C1      1.0000E+00

K11*R1  0.0157         13.%
```

*Parameter values have units of liters/mole for K11, moles/liter for R1 and Kd, the dissociation constant. The column labeled Kd is just 1/K for the affinity constant. N1 and C1 are unitless. The %CV is percentage coefficient of variations or standard error divided by the parameter value. LOG-VALUE is base 10 logarithm of the value. K11 * R1 is a unitless measure of the amount of binding of the (in this case) first ligand to the first receptor. Compare this value to N1 in order to get a qualitative impression of the importance or significance of the specific binding. In this case, specific binding is roughly seven times larger than non-specific binding.*

```
WOULD YOU LIKE THE COVARIANCE-CORRELATION MATRIX  (Y/N) [N] ?
<return>
```

```
CURVE    SUM OF      D.F.    MEAN         RUNS     RESIDUALS
         SQUARES              SQUARE

SUZ1     7375.312    15.0    491.6874     8 O.K.   +-+------++++-+--
TOTAL    7375.312    15      491.6874              8  10
```

This table gives a summary of the fit. The columns are :

CURVE

The name of this curve. Here the only one.

SUM OF SQUARES

This is the weighted sum of squared residuals or differences between observed and model-predicted values. D.F. - degrees of freedom or number of data points less the number of parameters.

MEAN SQUARE

Sum of squares divided by degrees of freedom.

RUNS

This figure gives the number of runs of either positive or negative residuals in the curve. A good fit should give a large number of runs. The message "OK","NOK","POOR" is an expression of the quality of the fit based on the runs test.

RESIDUALS

This is a series of signs of the residuals, which allows you to quickly inspect them for apparent patterns. No pattern means a satisfactory fit. Here there are 18 residuals, with 8 positive and 10 negative.

```
      GRAPHICS FILE NOW BEING WRITTEN  FOR FIT NO.   1

              ------------------------------
```

```
#    LOG(TOTAL)  B/T        B/T-PRED   DIFF       RESIDUAL PLOT
CURVE: SUZ1
1    -7.418      0.0075     0.0069     0.0006       *
2    -4.000      0.0016     0.0024     -0.0008    * |
3    -7.541      0.0090     0.0079     0.0011       |*
4    -4.000      0.0020     0.0024     -0.0004     *|
5    -7.738      0.0078     0.0096     -0.0018     *|
6    -4.000      0.0017     0.0024     -0.0006     *|
7    -8.128      0.0111     0.0129     -0.0018     *|
8    -4.000      0.0023     0.0024     0.0000       *
9    -8.419      0.0137     0.0148     -0.0012      *
10   -4.000      0.0025     0.0024     0.0001       *
11   -8.716      0.0179     0.0161     0.0018       *
12   -4.000      0.0031     0.0024     0.0008       |*
13   -9.021      0.0211     0.0169     0.0042       |*
14   -4.000      0.0035     0.0024     0.0011       | *
15   -9.316      0.0163     0.0173     -0.0010      *
16   -4.000      0.0025     0.0024     0.0001       *
17   -9.655      0.0156     0.0175     -0.0019      *
18   -4.000      0.0020     0.0024     -0.0003     *|
```

This table displays the original data, the modeled values and a simple plot of the differences (residuals).

LOG(TOTAL)

Logarithm base 10 of the total ligand concentration in this tube.

B/T

Observed ratio of Bound to Total ligand concentration.

B/T-PRED

Predicted B/T ratio.

DIFF

Difference between observed and predicted residual.

RESIDUAL PLOT

The residuals are normalized and plotted around their

ideal value of zero. A star one position to the right or left represents a +/- 1 standard deviation difference. In this case, there is no apparent pattern in the residual plot so the one-site model is probably adequate. Nevertheless, we will attempt a two-site fit next.

```
ANOTHER FIT (Y/N) [N] ? Y

NUMBER OF SITES ? 2
```

Now we will try a two-site fit, which is slightly more complex.

```
DO YOU WISH TO WEIGHT POINTS ACCORDING TO
NUMBER OF TUBES AT A GIVEN DOSE (Y/N) [N] ? <return>

WOULD YOU LIKE THE COOPERATIVITY MODEL (Y/N) [N] ?

WOULD YOU LIKE THE ADDITIONAL D PARAMETER(Y/N) [N] ?

THE FOLLOWING PARAMETERS WILL BE CONSIDERED:
 K11 K12 R1  R2  N1  C1
```

Two new parameters K12 and R2 have been introduced.

```
THE FOLLOWING PARAMETER GROUPS HAVE A SHARED VALUE:


DO YOU WANT THE SAME SHARED PARAMETERS AS
BEFORE (Y/N) [Y] ? <return>

LIST THE CONSTANT PARAMETERS (NO COMMA)
[ C1 ] ? <return>


C1  = [   1.00000     ] ? <return>

DO YOU WANT AUTOMATIC INITIAL ESTIMATES (Y/N) [Y] ? <return>

AFTER ADJUSTING FOR NONSPECIFIC, THERE ARE 9 POINTS LEFT

LOOKS LIKE A TWO SITE MODEL WONT FIT,
CANT GET INITIAL ESTIMATTES
```

The program informs us that a two-site model probably won't fit. When asked for the initial estimates, just hit the return key and accept the defaults, since we are not going to attempt a two-site fit.

```
INITIAL ESTIMATE FOR K11 [  0.000000E+00 ] ? <return>
INITIAL ESTIMATE FOR K12 [  0.000000E+00 ] ? <return>
INITIAL ESTIMATE FOR R1  [  0.000000E+00 ] ? <return>
INITIAL ESTIMATE FOR R2  [  0.000000E+00 ] ? <return>
INITIAL ESTIMATE FOR N1  [  2.129737E-03 ] ? <return>

THE INITIAL ESTIMATES ARE
```

```
K11 =  1.000000E-15
K12 =  1.000000E-15
R1  =  1.000000E-15
R2  =  1.000000E-15
N1  =  2.129737E-03
C1  =   1.00000

ARE THESE VALUES OK (Y/N) ? Y
```

Accept these as OK so we can exit the program. The program will attempt to use these values, and we'll see a few Fortran errors. Respond with "X" to the following question.

```
  IT.         EPSILON        SUM OF SQUARES
    0      0.000000E+00       2917860.750

TYPE C,R,P,D,S,G,X OR H FOR HELP ? X
```

And now we exit the program.

```
ANOTHER FIT (Y/N) [N] ? N

DO YOU WANT TO REDEFINE THE INPUT SET (Y/N) [N] ? <return>
WOULD YOU LIKE TO SEE THE GRAPH (Y/N) [N] ? <return>
SESSION LOG IN suz1.ses
GRAPHICS FILE IN suz1.ses
helix%
```

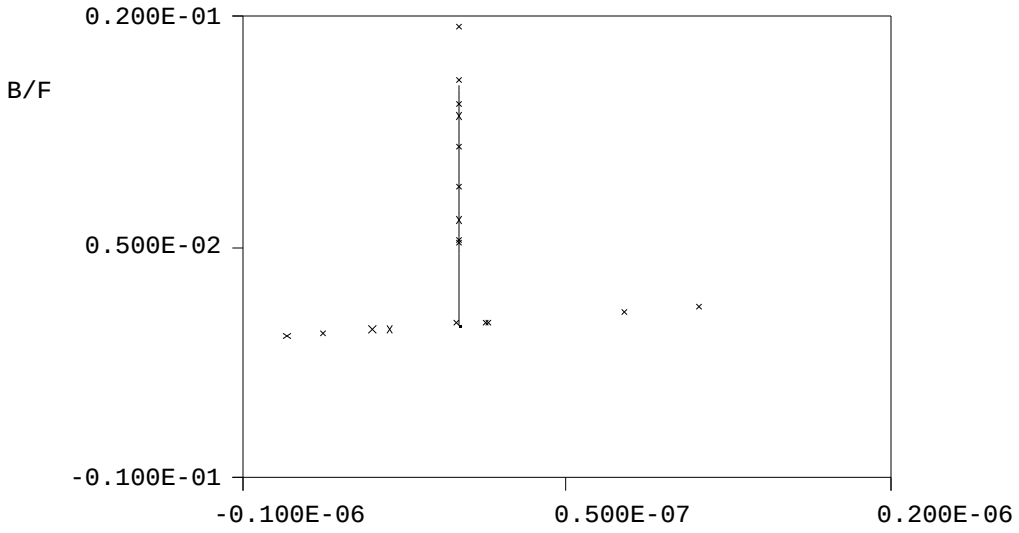
3.4 Graph the data

In this section we plot the data from the previous fit. We need the file named suz1 which contains the graphics information. Remember that this will only produce a plot on a Tektronix 4105 graphics terminal emulator.

```
helix% scagrf


          *****  LIGAND SYSTEM GRAPHICS PROGRAM  *****
      Curve fitting and data analysis for ligand binding data
                  National Institutes of Health



       CONVEX SCAGRF   v1.1     March 1991

       Graphics are designed for Tektronix 4105 display terminal


GRAPHICS FILE NAME (WITHOUT EXTENSION) ? suz1
DO YOU WANT INDIVIDUAL PLOTS?(Y/N)[N] ? Y

CURVE HEADER: "SUZ1   FIT NO.  1  N= 2.367857E-03"
VARYING LIGAND NO.  1  LABELED LIGAND NO.  1

DO YOU WANT THIS CURVE PLOTTED (Y/N)[Y] ? <return>
SCATCHARD PLOT OR DISPLACEMENT CURVE (S/D)[S]? S
NONSPECIFIC BINDING VALUE FOR CORRECTING SCATCHARD? [0.0023678570]
?  <return>

WITH CONFIDENCE LIMITS (+/- 2 SIGMA) (Y/N)[N] ? <return>
XMIN,XMAX,DELTAX      -0.100E-06      0.200E-06      0.150E-06
YMIN,YMAX,DELTAY      -0.100E-01      0.200E-01      0.150E-01
ARE THESE VALUES ACCEPTABLE (Y/N) [Y]  ? <return>
```

At this point the graphics screen is cleared and the plot is drawn on the Tektronix screen.

```
suz1.SES :  "suz1   FIT NO.  1  N= 2.367857E-03"
VAR.LIG# * LAB.LIG# 0
```

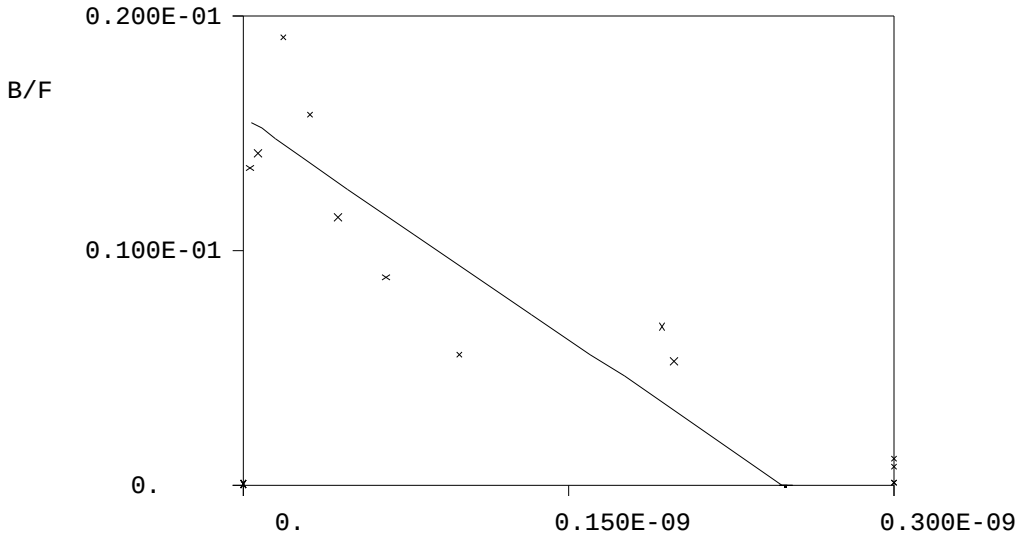



```
SCATCHARD, NONSPEC= 2.367857E-03
```

This is obviously a bizarre plot. The problem is simply in the plotting limits MINX, MINY, etc. We next rescale the plot so that the lower left corner becomes (0,0) and MAXX is somewhat larger than the value for R1 (2.48E-10). DELTA X determines the size of the increment.

```
DO YOU WANT TO CHANGE THE AXIS PARAMETERS [N] ? y
XMIN,XMAX,DELTAX      -0.100E-06      0.200E-06      0.150E-06
YMIN,YMAX,DELTAY      -0.100E-01      0.200E-01      0.150E-01
ARE THESE VALUES ACCEPTABLE [Y] ? n

ENTER NEW VALUES FOR :
               MIN X   [    -0.100E-06] 0
               MAX X   [     0.200E-06] 3e-10
               DELTA X [     0.150E-06] 1.5e-10
               MIN Y   [    -0.100E-01] 0
               MAX Y   [     0.200E-01] 02
               DELTA Y [     0.150E-01] 01

 XMIN,XMAX,DELTAX          0.          0.300E-09      0.150E-09
 YMIN,YMAX,DELTAY          0.          0.200E-01      0.100E-01
 ARE THESE VALUES ACCEPTABLE [Y] ? y
```

```
suz1.SES :  "suz1   FIT NO.  1  N= 2.367857E-03"
VAR.LIG# * LAB.LIG# 0
```



This picture is more satisfactory.

```
DO YOU WANT TO CHANGE THE AXIS PARAMETERS [N] ? <return>
DO YOU WANT A CONGLOMERATE PICTURE ?(Y/N) n
STOP:

helix%
```

4. Displacement experiments

A common type of binding experiment is termed a "displacement study" wherein a cold ligand is used to displace or compete with a radio-labeled ("hot") ligand for the receptor binding site. A homologous displacement study uses the same ligand as label and displacer. A heterologous study uses a different ligand for the displacer. Analysis of both types of studies may be accomplished with LIGAND, but special care must be taken to correctly estimate Ki (inhibition constant) for heterologous studies. Regardless of the design of the study (saturation or displacement) a displacement curve plot may be prepared (B/T vs. log T), and is a useful coordinate system in which to present the data.

4.1 Homologous displacement experiment tracer and displacing ligand are identical

Here only a single ligand is utilized although it may come in two forms - radio-labeled and unlabeled. Therefore, fewer binding parameters need to be estimated than in the heterologous displacement study, so this is a good place to begin. Even if you are dealing with a heterologous system, you should read and understand this section first. Notice that a homologous displacement study and a saturation binding study are really quite similar. In both, graded concentrations of a single ligand are presented to the receptor site. The essential difference is in how we estimate the proportion of ligand bound to the receptor. Hence the need for the two preprocessing programs SCAHOT and SCAPRE.

4.1.1 Prepare the data

What follows is a sample session of program scapre. Your responses to the program are underlined. Annotation and comments to the session are indented in italicized letters. First, we must access the computer system.

```
ConvexOS.  Release V8.1 (helix.nih.gov)

login:  ????
password:  ????
```

Execution of data entry program can be done here by typing scahot.

```
helix% scapre
                    ***   LIGAND SYSTEM   ***
                     PROGRAM SCAPRE  --  v2.6

             THIS PROGRAM WILL PRODUCE THE INPUT FILE
                      FOR THE SCAFIT PROGRAM
               (I.E. BOUND AMOUNT VS. TOTAL AMOUNT)


                  WRITTEN BY PETER MUNSON, DEC 1978



 TO SEE LIST OF COMMANDS -- ENTER HELP

 FILE NAME FOR STORING THIS DATA (NO EXTENSION) ? exp3a
```

This is the file name. It is given the extensions .BAS and .CRV for the data files.

```
CAN'T FIND FILE exp3a.dat
OPENING NEW FILE exp3a.bas
```

This will create a new file and erase any old file with the same name. Type "N" if you are using a pre-existing file.

```
 1    NAME OF THIS EXPERIMENT:  ? HOT AND COLD ANALOG I
```

Descriptive information which helps you remember which data set is which.

```
 2    COUNTING TIME(MIN):  ? 5
```

Used to convert counts to c.p.m. Enter 1 if data are in c.p.m.

```
 3    VOLUME OF TUBE(ML.):  ?.5
```

Reaction volume, used to calculate concentration of reagents, from mass of dose.

```
 4    SPECIFIC ACTIVITY (UC/UG):  ? 1000
```

Remember, there are 2.22E6 dpm/microcurrie, so that

$$SA(uc/ug) = \frac{SA(dpm/mole)}{2.22E6 \times MW \times 1E6}$$

Also note the lack of an entry for counting efficiency (it is assumed to be 50%) and for bindability. Future versions of this program should include them.

```
5    MEAN TOTAL COUNTS:  ? 65789
```

Used to calculate mass of tracer, along with specific activity.

```
6    HOT LIGAND #:  ? 1
```

Every ligand in the study must be given a different number.The numbering scheme must be the same for all data sets in the study. Enter the number of the labeled ligand here.

```
7     COLD LIGAND #:  ? 1
```

This is the unlabeled ligand number, which has been added in varying amounts in the curve. In this case, it is the same as that of the hot ligand.

```
8     MOL.WT., MEASURED LIGAND:  ? 1130
9     MOL.WT., COLD LIGAND:  ? 1100
```

molecular weights are used to convert grams to moles

```
10   DOSE UNITS:  ? NANOGRAM
```

Enter the units in which you are later to enter the dose (amount of cold ligand) units. Allowable units are PICO-, FEMTO-, NANO-, MICRO-, -MOLES, -GRAMS.

```
11.  COUNTING EFFICIENCY :      .5

     VARIANCE(Y)=A0+A1×Y+A2×Y 2+A3×Y A4
```

This is the model for the variance of the bound concentrations. It is a combination of the quadratic and exponential functions, although only one part should be used at a time. Thus either A0=A1=0 or A2=A3=A4=0. A reasonable default assumption is "constant percent error", i.e. A2=.0001, A0=A1=A3=A4=0

```
12   A0:  ? 0
13   A1:  ? 0
14   A2:  ? 0.0001
15   A3:  ? 0
16   A4:  ? 0

DOSE  COUNTS  COUNTS   COUNTS
```

The following is the actual data, assuming a constant amount of hot, labeled "tracer" ligand has been added along with varying amount or "doses" of cold, unlabeled ligand. The first entry in each row is the dose of cold ligand. The remaining entries, separated by spaces, are the bound counts of radioactive tracer. Up to 10 replicate counts may be entered on a single line.

```
17   :  ? 0. 7997. 7891. 8081. 8265. 8262.  8213
```

```
 18   :  ? 0.05 7676. 7713
 19   :  ? 0.1 7244. 7716
 20   :  ? 0.2 7210. 6950
 21   :  ? 0.5 6451. 6268
 22   :  ? 1. 6020. 6321
 23   :  ? 3. 5477. 5317
 24   :  ? 10. 4926. 5266
 25   :  ? 30.0 5250. 4943
 26   :  ? 1000. 4931. 4724. 4479. 5072. 4385. 4694
 27   :  ? <return>
```

A blank or null entry puts the program into the command mode.

```
COMMAND ? help
THE LEGAL COMMANDS ARE:
HELP        TYPES THIS MESSAGE
REVIEW      REVIEWS THE DATA YOU HAVE ENTERED
FINISH      ENDS THE PROGRAM, AND STORES THE
                  CALCULATED DATA IN THE FILE YOU HAVE
            CREATED
DELETE       BEGINS THE DELETION OF A LINE OF DATA
                  YOU WILL BE PROMPTED FOR LINE NUMBER ON
                  THE FOLLOWING LINE
INSERT      BEGINS INSERTION OF A LINE.  YOU WILL
                  WILL BE PROMPTED FOR THE NEW LINE NUMBER
REPLACE     SAME AS DELETE FOLLOWED BY INSERT COMMANDS
PLOT        PLOT OF YVAR vs. MEAN
INPUT       BEGIN ENTERING DATA ON NEXT AVAILABLE LINE

ANY OTHER COMAND WILL RESULT IN MESSAGE
ILLEGAL COMMAND

COMMAND ? review
```

the "REVIEW" command is used to display the data you have already entered.

```
    1.    NAME OF THIS EXPERIMENT:  HOT AND COLD ANALOG I
    2.    COUNTING TIME (MIN) :  5.
    3.    VOLUME OF TUBE (ML.) :  .5
    4.    SPECIFIC ACTIVITY (UC/UG) :   1000.
    5.    MEAN TOTAL COUNTS :  65789.
    6.    HOT LIGAND # :  1
    7.    COLD LIGAND # :  1
    8.    MOL.WT., MEASURED LIGAND :  1130.
    9.    MOL.WT., COLD LIGAND :  1100.
   10.    DOSE UNITS :  NANOGRAMS
   11.    COUNTING EFFICIENCY :  .5
 VARIANCE(Y)=A0+A1*Y+A2*Y^2+A3*Y^A4
   12.    A0 :  0.
   13.    A1 :  0.
   14.    A2 :  .0001
   15.    A3 :  0.
   16.    A4 :  0.
```

```
 DOSE  COUNTS  COUNTS  COUNTS
   17.    0. 7997. 7891. 8081. 8265. 8262. 8213.
   18.    .05 7676. 7713.
   19.    .1 7244. 7716.
   20.    .2 7210. 6950.
   21.    .5 6451. 6268.
   22.    1. 6020. 6321.
   23.    3. 5477. 5317.
   24.    10. 4926. 5266.
   25.    30. 5250. 4943.
   26.    1000. 4931. 4724. 4479. 5072. 4385. 4694.

COMMAND ? INPUT
```

"INPUT" allows you to enter data in sequence starting where you left off last time.

```
 26           : ?  JUNK LINE

 27           : ?  <return>

COMMAND ? REP 26
```

This replaces the old line 26 with a new line.

```
 26           : JUNK

COMMAND ? DEL 26
```

This deletes line 26.

```
COMMAND ? FINISH
```

You have reviewed and accepted the data, so now you tell the computer to finish up its calculations. The computer stores the data set EXP3A in a form ready for the fitting program SCAFIT

```
 DO YOU WANT A GRAPH FILE MADE? [Y]
DOSE# TOTAL              BOUND                B/T
1     2.0980308E-11      2.5889074E-12        0.1233970
2     1.1188940E-10      1.3086275E-11        0.1169572
3     2.0279850E-10      2.3057545E-11        0.1136968
4     3.8461667E-10      4.1391203E-11        0.1076168
5     9.3007124E-10      8.9905423E-11        9.6665092E-02
6     1.8391622E-09      1.7249921E-10        9.3792275E-02
7     5.4755254E-09      4.4918469E-10        8.2034990E-02
8     1.8202799E-08      1.4099844E-09        7.7459753E-02
9     5.4566435E-08      4.2271173E-09        7.7467352E-02
10    1.8182028E-06      1.3028486E-07        7.1655847E-02
BYE
STOP:
helix%
```

4.1.2 Listing of output files

The following is a listing of EXP3A.BAS and EXP3A.CRV, the output files of SCAPRE in the foregoing session. They will be used as input files to SCAFIT, the main fitting program.

```
EXP3A.BAS  Listing

HOT AND COLD ANALOG I
5.
.5
1000.
65789.
1
1
1130.
1100.
NANOGRAMS
.5
0.
0.
.0001
0.
0.
0. 7997. 7891. 8081. 8265. 8262. 8213.
.05 7676. 7713.
.1 7244. 7716.
.2 7210. 6950.
.5 6451. 6268.
1. 6020. 6321.
3. 5477. 5317.
10. 4926. 5266.
30. 5250. 4943.
1000. 4931. 4724. 4479. 5072. 4385. 4694.
```

EXP3A.CRV Listing

```
"HOT AND COLD ANALOG I"
0             0             0.0001        0
0
1             1
2.09803E-11
2.09803E-11   2.58891E-12   6
1.11889E-10   1.30863E-11   2
2.02798E-10   2.30575E-11   2
3.84617E-10   4.13912E-11   2
9.30071E-10   8.99054E-11   2
1.83916E-9    1.72499E-10   2
5.47553E-9    4.49185E-10   2
1.82028E-8    1.40998E-9    2
5.45664E-8    4.22712E-9    2
1.81820E-6    1.30285E-7    6
```

Description of curve file EXP3A.CRV:

Line#	Description
1	Comments
2	Four weighing parameters a0,a1,a2,a3.
3	Fifth weighing parameter a4.
4	Varying ligand number, labeled ligand number.
5	Total hot ligand conc.[M].
6-end	Total varying ligand conc[M]; bound, labeled ligand conc[M], Replication weight.

4.1.3 Fit the data

After preparing the displacement data we are ready to fit them. Here we will fit a simple, one-site model followed by an attempt at a more complex two-site fit. We assume you are already logged on to the computer.

```
.scafit

                        L I G A N D:
     Curve fitting and data analysis for ligand binding data
                 National Institutes of Health

                Program: SCAFIT version 2.4MAGGI


FILE NAME FOR SESSION (NO EXTENSION) ? exp3a

NUMBER OF LIGANDS (HIGHEST NUMBER USED) ? 1
```

For the homologous experiment EXP3A, the highest ligand number entered is 1.

```
TYPE THE DATA FILE NAME(S) (NO EXTENSION)
SEPARATE WITH <CR> END WITH BLANK LINE
? exp3a
? <return>
```

A blank entry ends the list of input files. Later we will fit several data files simultaneously.

```
CURVE FILE   EXP3A.CRV
WEIGHTING PARAMETERS
         0.             0.      9.9999997E-05         0.
0.
 VARYING LIGAND:   1   LABELED LIGAND:   1
 NUMBER OF POINTS:           10
```

EXP3A has been correctly read in.

```
NUMBER OF SITES ? 1
```

We attempt a one-site fit first.

```
DO YOU WISH TO WEIGHT POINTS ACCORDING TO
NUMBER OF TUBES AT A GIVEN DOSE (Y/N) [N] ? <return>

WOULD YOU LIKE THE COOPERATIVITY MODEL (Y/N) [N] ?

WOULD YOU LIKE THE ADDITIONAL D PARAMETER(Y/N) [N] ?

THE FOLLOWING PARAMETERS WILL BE CONSIDERED:
 K11 R1  N1  C1
```

```
THE FOLLOWING PARAMETER GROUPS HAVE A SHARED VALUE:
```

There are no shared parameters. This option is not needed in this session.

```
DO YOU WANT THE SAME SHARED PARAMETERS AS
 BEFORE (Y/N) [Y] ? <return>
```

Generally, take the default answers on these two questions. They are useful in more complex situations.

```
LIST THE CONSTANT PARAMETERS (NO COMMA)
 [ C1 ] ? <return>

 C1  = [   1.00000     ] ? <return>
```

The value for C1 should always be a constant

```
DO YOU WANT AUTOMATIC INITIAL ESTIMATES (Y/N) [Y] ? <return>

AFTER ADJUSTING FOR NONSPECIFIC, THERE ARE    9 POINTS LEFT

 INITIAL ESTIMATE FOR K11   [ 6.126472E+07 ] ? <return>
 INITIAL ESTIMATE FOR R1    [ 7.976734E-10 ] ? <return>
 INITIAL ESTIMATE FOR N1    [ 6.946807E-02 ] ? <return>
```

The automatic initial estimates may be over- ridden if you choose, but this is seldom required.

```
THE INITIAL ESTIMATES ARE
 K11 =  6.126472E+07
 R1  =  7.976734E-10
 N1  =  6.946807E-02
 C1  =   1.00000

ARE THESE  VALUES OK (Y/N) ? Y
```

Last chance to modify the initial estimates before fitting the model.

```
  IT.         EPSILON        SUM OF SQUARES
  0                0.            999.466

TYPE C,R,P,D,S,G,X OR H FOR HELP ? H

 ***   HELP   ***

  TYPE "C" TO CONTINUE ITERATIONS
  TYPE "R" TO RESET PARAMETERS
  TYPE "P" TO SEE PARAMETER VALUES
  TYPE "D" TO VIEW DATA AND MODEL VALUES
  TYPE "G" TO GET PLOT OF DATA
  TYPE "X" TO EXIT WITHOUT FINAL ESTIMATES
  TYPE "S" TO GET SUMMARY

TYPE C,R,P,D,S,G,X OR H FOR HELP ? P
```

This prints out the current parameter values.

```
K11 =   6.1264720E+07
R1  =   7.9767337E-10
N1  =   6.9468066E-02
C1  =    1.000000


TYPE C,R,P,D,S,G,X OR H FOR HELP ? D
```

An abbreviated display of the data is given.

```
#       LOG(TOTAL)    B/T         B/T-PRED    DIFF        RESIDUAL
PLOT
CURVE: EXP3A
1       -10.678       0.1234      0.1058      0.0176       |*
2       -9.951        0.1170      0.1056      0.0114       |*
3       -9.693        0.1137      0.1054      0.0083       |*
4       -9.415        0.1076      0.1050      0.0026        *
5       -9.031        0.0967      0.1039      -0.0072     *|
6       -8.735        0.0938      0.1022      -0.0084     *|
7       -8.262        0.0820      0.0966      -0.0146     *|
8       -7.740        0.0775      0.0856      -0.0082     *|
9       -7.263        0.0775      0.0753      0.0022       *
10      -5.740        0.0717      0.0654      0.0063       |*
```

At this point, SCAFIT has not yet tried to minimize the sum of squares by adjusting your parameters. Type "C" to continue.

```
TYPE C,R,P,D,S,G,X OR H FOR HELP ? C
NUMBER OF ADDITIONAL ITERATIONS ?  12
```

Allow 5 or 10 iterations initially.

```
  IT.        EPSILON      SUM OF SQUARES
    1       9.999999E-03       904.852
```

The program succeeds in lowering the sum of squares on the first iteration, so we are probably on the road to convergence.

```
 IT.         EPSILON       SUM OF SQUARES
   2      9.999999E-03           839.463

 IT.         EPSILON       SUM OF SQUARES
   3      9.999999E-03           776.290

 IT.         EPSILON       SUM OF SQUARES
   4      9.999999E-03           714.767

 IT.         EPSILON       SUM OF SQUARES
   5      9.999999E-03           655.105

 IT.         EPSILON       SUM OF SQUARES
   6      9.999999E-03           597.432

 IT.         EPSILON       SUM OF SQUARES
   7      9.999999E-03           541.871
```

```
  IT.        EPSILON       SUM OF SQUARES
    8      9.999999E-04          540.985

  IT.        EPSILON       SUM OF SQUARES
    9      9.999999E-04          414.742

  IT.        EPSILON       SUM OF SQUARES
   10      9.999999E-05          328.390

  IT.        EPSILON       SUM OF SQUARES
   11      9.999999E-06           43.586

  IT.        EPSILON       SUM OF SQUARES
   12      9.999999E-07           43.348

TYPE C,R,P,D,S,G,X OR H FOR HELP ? C
NUMBER OF ADDITIONAL ITERATIONS ?  2
```

A few more iterations are required. The value of sum of squares should stabilize, indicating convergence.

```
CONVERGED, TYPE "S" TO GET FINAL RESULTS
```

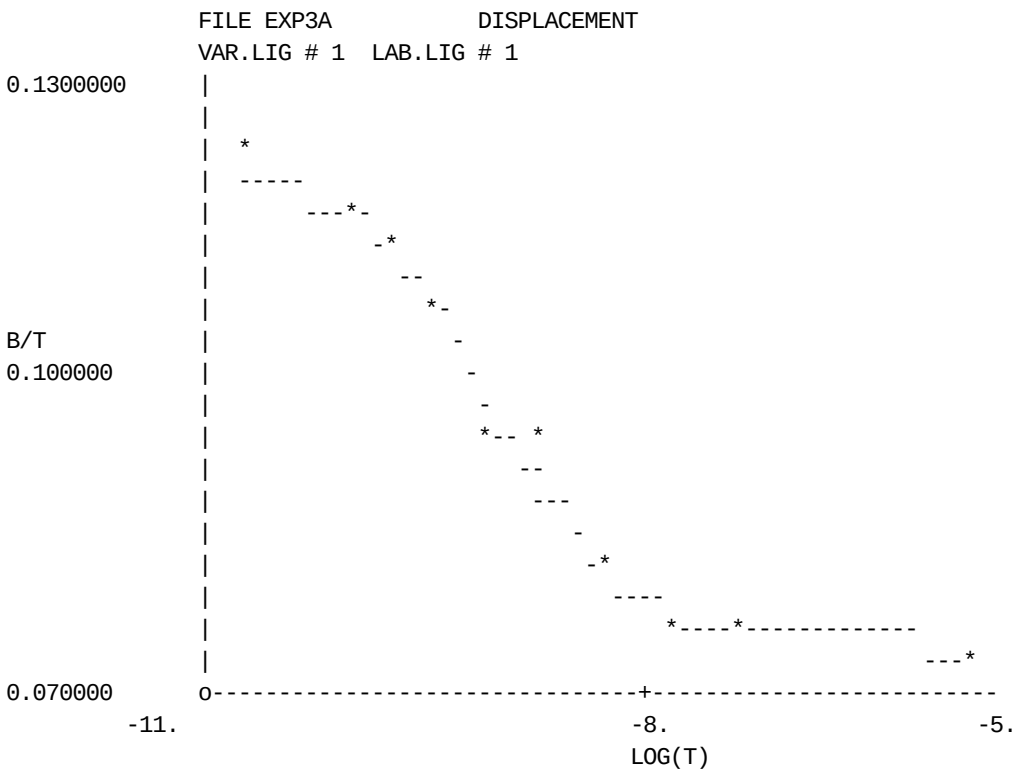
The program only needed one more iteration to achieve convergence.

```
TYPE C,R,P,D,S,G,X OR H FOR HELP ? G
```

Let's take a look at the graph. On the DEC-10, high quality plots are also available.

```
 type  L  to change GRAPH LIMITS
 type  G  to choose GRAPH TYPE [DISPLACEMENT]
 type  C  for CONFIDENCE LIMITS (+/- 2 SIGMA) [N]
 type  1  to graph the DISPLACEMENT CURVE EXP3A
 type  0  to END GRAPHING SECTION
```

```
CHOOSE AN OPTION ? 1
```




```
 type  L  to change GRAPH LIMITS
 type  G  to choose GRAPH TYPE [DISPLACEMENT]
 type  C  for CONFIDENCE LIMITS (+/- 2 SIGMA) [N]
 type  1  to graph the DISPLACEMENT CURVE EXP3A
 type  0  to END GRAPHING SECTION

CHOOSE AN OPTION ? 0
```

We jump out of graphics with a zero.

```
TYPE C,R,P,D,S,G,X OR H FOR HELP ? S
```

The program has previously reported convergence since it could no longer reduce the sum of squares. We now request a summary of the results.

```
L I G A N D: SCAFIT VERSION 2.4MAGGI

CAUTION: Results and interpretation of analysis depend upon
several important assumptions. See User's Guide for details
```

```
FINAL PARAMETER ESTIMATES, EXP3A FIT NO.   1

        VALUE        %CV     LOG-VALUE        Kd          K*R

K11    1.1913E+09    19.%     9.0760       8.3942E-10    0.0587
R1     4.9243E-11    17.%
N1     8.0601E-02     2.%
C1     1.0000E+00

K11*R1   0.0587            5.%
```

Parameter values have units of liters/mole for K11, moles/liter for R1 and Kd, the dissociation constant. The column labeled Kd is just 1/K for the affinity constant. N1 and C1 are unitless. The %CV is percentage coefficient of variations or standard error divided by the parameter value. LOG-VALUE is base 10 logarithm of the value. K11 × R1 is a unitless measure of the amount of binding of the (in this case) first ligand to the first receptor. Compare this value to N1 in order to get a qualitative impression of the importance or significance of the specific binding. In this case, specific binding is roughly equal to the non-specific binding.

```
WOULD YOU LIKE THE COVARIANCE-CORRELATION MATRIX  (Y/N) [N] ? Y


 COVARIANCE MATRIX, CORRELATIONS IN UPPER TRIANGLE

 K11              R1          N1

  5.242767E+16    -0.966913    0.498267

 -1.888072E-03    7.272809E-23      -0.627883

  151214.         -7.097065E-15     1.756702E-06
```

Notice the high negative correlation between K11 and R1 (-.96). See section 6.

```
CURVE      SUM OF        D.F.    MEAN       RUNS    RESIDUALS
           SQUARES               SQUARE

EXP3A      43.347        7.0     6.1924     4       O.K.+----++++-
TOTAL      43.347        7       6.1924             5    5
```

This table gives a summary of the fit. The columns are :

CURVE

The name of this curve. Here the only one.

SUM OF SQUARES

This is the weighted sum of squared residuals or differences between observed and model-predicted

values.

D.F.

Degrees of freedom or number of data points less the number of parameters.

MEAN SQUARE

Sum of squares divided by degrees of freedom.

RUNS

This figure gives the number of runs of either positive or negative residuals in the curve. A good fit should give a large number of runs. The message "OK", "NOK", "POOR" is an expression of the quality of the fit based on the runs test.

RESIDUALS

This is a series of signs of the residuals, which allows you to quickly inspect them for apparent patterns. No pattern means a satisfactory fit. Here there are 10 residuals, with 5 positive and 5 negative. Although there is some clustering of minus and plus residuals, this is not significant, but OK.

```
   GRAPHICS FILE NOW BEING WRITTEN  FOR FIT NO.   1

            ----------------------------

#       LOG(TOTAL)  B/T        B/T-PRED   DIFF         RESIDUAL PLOT
CURVE: EXP3A
1       -10.678     0.1234     0.1213     0.0021         |*
2       -9.951      0.1170     0.1175     -0.0005        *
3       -9.693      0.1137     0.1142     -0.0005        *
4       -9.415      0.1076     0.1089     -0.0013        *
5       -9.031      0.0967     0.0991     -0.0024       *|
6       -8.735      0.0938     0.0911     0.0027         |*
7       -8.262      0.0820     0.0817     0.0003         *
8       -7.740      0.0775     0.0770     0.0005         *
9       -7.263      0.0775     0.0754     0.0021         |*
10      -5.740      0.0717     0.0746     -0.0030      * |

ANOTHER FIT (Y/N) [N] ? Y

NUMBER OF SITES ? 2
```

We try a two-site fit to check for any marked improvement.

```
DO YOU WISH TO WEIGHT POINTS ACCORDING TO
NUMBER OF TUBES AT A GIVEN DOSE (Y/N) [N] ? <return>

WOULD YOU LIKE THE COOPERATIVITY MODEL (Y/N) [N] ?<return>
```

```
WOULD YOU LIKE THE ADDITIONAL D PARAMETER(Y/N) [N] ?<return>

THE FOLLOWING PARAMETERS WILL BE CONSIDERED:
 K11 K12 R1  R2  N1  C1
```

Two more parameters are considered, K12 and R2.

```
THE FOLLOWING PARAMETER GROUPS HAVE A SHARED VALUE:


DO YOU WANT THE SAME SHARED PARAMETERS AS
 BEFORE (Y/N) [Y] ? <return>

LIST THE CONSTANT PARAMETERS (NO COMMA)
 [ C1 ] ? <return>


 C1  = [   1.00000     ] ? <return>

DO YOU WANT AUTOMATIC INITIAL ESTIMATES (Y/N) [Y] ? <return>
```

Automatic initial estimates should work for the two-site model in most cases.

```
AFTER ADJUSTING FOR NONSPECIFIC, THERE ARE    9 POINTS LEFT

 INITIAL ESTIMATE FOR K11 [  1.894878E+09 ] ? <return>
 INITIAL ESTIMATE FOR K12 [  1.379443E+07 ] ? <return>
 INITIAL ESTIMATE FOR R1  [  2.757844E-11 ] ? <return>
 INITIAL ESTIMATE FOR R2  [  1.496268E-09 ] ? <return>
 INITIAL ESTIMATE FOR N1  [  6.946807E-02 ] ? <return>

THE INITIAL ESTIMATES ARE
 K11 =  1.894878E+09
 K12 =  1.379443E+07
 R1  =  2.757844E-11
 R2  =  1.496268E-09
 N1  =  6.946807E-02
 C1  =   1.00000

ARE THESE  VALUES OK (Y/N) ? Y
      IT.         EPSILON     SUM OF SQUARES
      0                0.               130.760

TYPE C,R,P,D,S,G,X OR H FOR HELP ? C
NUMBER OF ADDITIONAL ITERATIONS ?  27


      IT.         EPSILON     SUM OF SQUARES
      1           0.          31.179

      IT.         EPSILON     SUM OF SQUARES
      2           0.          27.336

      IT.         EPSILON     SUM OF SQUARES
```

```
      3           9.999999E-05      22.742

      IT.   EPSILON     SUM OF SQUARES
      4     9.999999E-05      18.858
```

Not all the iterations are shown here.

```
      IT.   EPSILON     SUM OF SQUARES
      22    9.999999E-05      18.225

      IT.   EPSILON     SUM OF SQUARES
      23    9.999999E-05      18.223

      IT.   EPSILON     SUM OF SQUARES
      24    9.999999E-04      18.221


CONVERGED, TYPE "S" TO GET FINAL RESULTS
TYPE C,R,P,D,S,G,X OR H FOR HELP ? S
```

With this particular example you will notice several Fortran errors after this step. They have been deleted from this session.

```
L I G A N D: SCAFIT VERSION 2.4MAGGI

CAUTION: Results and interpretation of analysis depend upon
several important assumptions. See User's Guide for details

 FINAL PARAMETER ESTIMATES,  exp3a FIT NO.   2
            VALUE      %CV      LOG-VALUE        KD        K*R
 K11       1.4294E+09  21.%       9.1552       6.9958E-10  0.0575
 K12       7.1780E+05  ****%      5.8560       1.3931E-06  0.0105
 R1        4.0216E-11  22.%
 R2        1.4654E-08  ****%
 N1        7.2424E-02  109.%
 C1        1.0000E+00
```

The asterisks (****) mean that the %CV is too large to print. Thus, the parameters for the second site K12 and R2 are indeterminate.

```
 K11*R1   0.0575            5.%

 K12*R2   0.0105          730.%

WOULD YOU LIKE THE COVARIANCE-CORRELATION MATRIX  (Y/N) [N] ?
<return>

CURVE      SUM OF        D.F.     MEAN        RUNS     RESIDUALS
           SQUARES                SQUARE

EXP3A      18.221        5.0      3.6442      6 O.K.   +----+--+-
TOTAL      18.221        5        3.6442               3     7
```

Compared with the previous fit, there are 2 more runs in the

residuals, indicating a somewhat better fit with two-sites.

```
FIT      SUM OF       D.F.     MEAN SQUARE   F
         SQUARES

1        43.348       7        6.1925        3.4500     (P=0.1140)
2        18.221       5        3.6442                   ---
```

Although the sum of squares was substantially lower for the second fit, more parameters had to be estimated. The F-test accounts for this extra complexity and determines that the second fit was not significantly better than the first. Nevertheless, there is some indication of the presence of a second site. To be significantly better, the p-value should be less than 0.05.

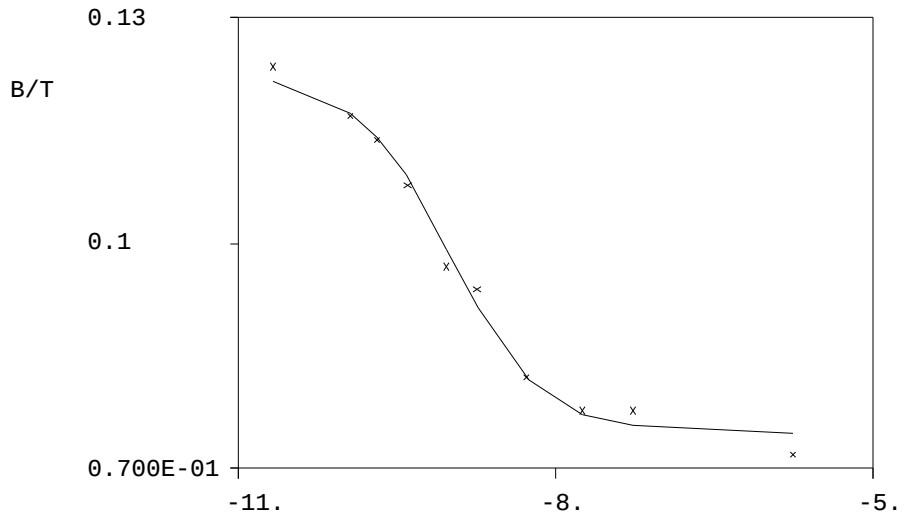
```
GRAPHICS FILE NOW BEING WRITTEN  FOR FIT NO.   2

          ------------------------------


#       LOG(TOTAL) B/T        B/T-PRED    DIFF       RESIDUAL PLOT
CURVE:EXP3A
1       -10.678    0.1234     0.1220      0.0014      |*
2       -9.951     0.1170     0.1176      -0.0007     *
3       -9.693     0.1137     0.1140      -0.0003     *
4       -9.415     0.1076     0.1083      -0.0007     *
5       -9.031     0.0967     0.0984      -0.0017    *|
6       -8.735     0.0938     0.0908      0.0030      |*
7       -8.262     0.0820     0.0825      -0.0005     *
8       -7.740     0.0775     0.0784      -0.0010    *|
9       -7.263     0.0775     0.0769      0.0005      *
10      -5.740     0.0717     0.0717      0.0000      *


ANOTHER FIT (Y/N) [N] ? N

DO YOU WANT TO REDEFINE THE INPUT SET (Y/N) [N] ? <return>
WOULD YOU LIKE TO SEE THE GRAPH (Y/N) [N] ? <return>
SESSION LOG IN exp3a.ses
GRAPHICS FILE IN exp3a.ses
helix%
```

4.1.4 Graph the data

At this point, we are ready to view the displacement curves on the Tektronix screen. To do so, execute the scagrf program by typing scagrf. Enter the graphics file name for the appropriate session, and choose the displacement curve option. As before, underlined entries indicate your responses to the computer's questions. Default responses are given inside square brackets.

```
helix% scagrf


          *****  LIGAND SYSTEM GRAPHICS PROGRAM  *****
     Curve fitting and data analysis for ligand binding data
                 National Institutes of Health



               CONVEX SCAGRF   v1.1     March 1991

    Graphics are designed for Tektronix 4105 display terminal

GRAPHICS FILE NAME (NO EXTENSION) ? exp3a
DO YOU WANT INDIVIDUAL PLOTS?(Y/N)[N] ? Y

 CURVE HEADER:"exp3a  FIT NO.  1  N= 8.060111E-02"
 VARYING LIGAND #1   LABELED LIGAND #1

 DO YOU WANT TO PLOT THIS CURVE ? (Y/N) [Y]<return>
 SCATCHARD PLOT OR DISPLACEMENT CURVE (S/D) [S]? D

 WITH CONFIDENCE LIMITS (+/- 2 SIGMA) (Y/N)[N]<return>
 XMIN,XMAX,DELTAX       -11.0          -5.00           3.00
 YMIN,YMAX,DELTAY       0.700E-01      0.130          0.300E-01
 ARE THESE VALUES ACCEPTABLE [Y] ?<return>
```

```
exp3a.SES :  "exp3a  FIT NO.  1  N= 8.060111E-02"
VAR.LIG# 1 LAB.LIG# 1
```



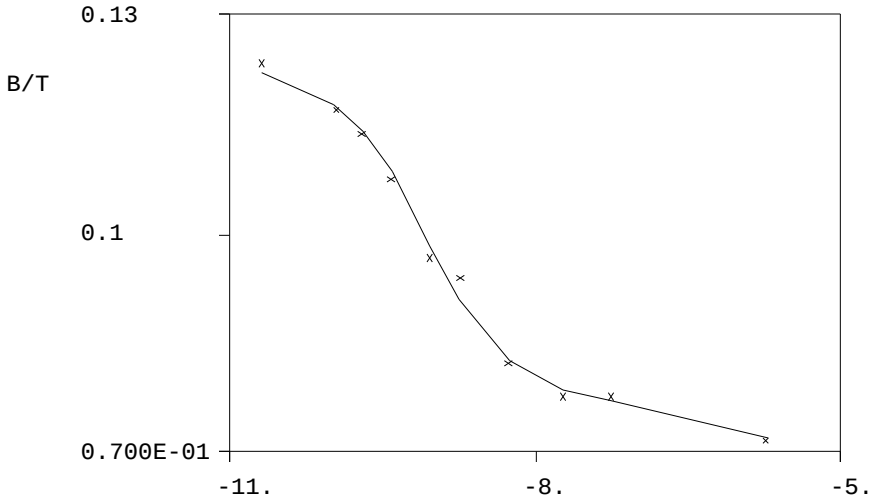
DO YOU WANT TO CHANGE THE AXIS PARAMETERS [N] ? <return>

CURVE HEADER:"exp3a FIT NO. 2 N= 7.242359E-02"
VARYING LIGAND #1   LABELED LIGAND #1

DO YOU WANT TO PLOT THIS CURVE ? (Y/N) [Y] <return>
SCATCHARD PLOT OR DISPLACEMENT CURVE (S/D) [S]? d

WITH CONFIDENCE LIMITS (+/- 2 SIGMA) (Y/N)[N] <return>
XMIN,XMAX,DELTAX -11.0 -5.00 3.00
YMIN,YMAX,DELTAY 0.700E-01 0.130 0.300E-01
ARE THESE VALUES ACCEPTABLE [Y] ? <return>

```
exp3a.SES :  "exp3a  FIT NO.  2  N= 7.242359E-02"
VAR.LIG# 1 LAB.LIG# 1
```




```
DO YOU WANT TO CHANGE THE AXIS PARAMETERS [N] ? <return>
DO YOU WANT A CONGLOMERATE PICTURE ?(Y/N) n
STOP:
helix%
```

4.2 Heterologous Displacement Experiments

In this experiment the labeled "tracer" reagent is biochemically distinct from the displacer compound, so that two distinct ligand numbers must be used (usually #1 and #2). Also, there are two competing chemical equilibrium reactions:

$H_1 + R_1 = H_1R_1 : K11$
$H_2 + R_1 = H_2R_1 : K21$

There is not enough information in a single displacement curve to solve for all the unknown parameters (K11,K21,R1) so we must make some assumptions or have additional data. If we know the Kd (1/K11) for the tracer, we may enter this information separately, as a constraint on K11. Alternately, we may fit a tracer self-displacement (homologous) curve along with the heterologous curve to establish a value for K11. This approach is taken in section 4.3. Failure to add appropriate constraints or more curves will ultimately cause SCAFIT to fail; there simply is not enough information available.

Remember that the inhibition constant K_i value depends not only on the ED_{50}, but also on the affinity of tracer and the amount of receptor present. Thus, K11 and R1 must be established before K21 may be estimated.

4.2.1 Prepare the data

We now enter the heterologous displacement data EXP4A, using SCAPRE. See section 4.1.1 for a fuller explanation of program SCAPRE operation

```
helix% scapre
                    ***   LIGAND SYSTEM   ***
                    PROGRAM SCAPRE  --  v2.6

            THIS PROGRAM WILL PRODUCE THE INPUT FILE
                    FOR THE SCAFIT PROGRAM
              (I.E. BOUND AMOUNT VS. TOTAL AMOUNT)


                 WRITTEN BY PETER MUNSON, DEC 1978



 TO SEE LIST OF COMMANDS -- ENTER HELP

 FILE NAME FOR STORING THIS DATA (NO EXTENSION) ? exp4a
```

```
CAN'T FIND FILE exp4a.dat
OPENING NEW FILE exp4a.bas

    1.    NAME OF THIS EXPERIMENT:      Hot analog I, cold GNRH
    2.    COUNTING TIME (MIN) :         5
    3.    VOLUME OF TUBE (ML.) :        .5
    4.    SPECIFIC ACTIVITY (UC/UG) :   1100
    5.    MEAN TOTAL COUNTS :           65789
    6.    HOT LIGAND # :                1
    7.    COLD LIGAND # :               2
    8.    MOL.WT., MEASURED LIGAND :    1130
    9.    MOL.WT., COLD LIGAND :        1200
   10.    DOSE UNITS :                  nanograms
   11.    COUNTING EFFICIENCY :         .5
 VARANCE(Y)=A0+A1*Y+A2*Y^2+A3*Y^A4
   12.    A0 :                          0
   13.    A1 :                          0
   14.    A2 :                          .0001
   15.    A3 :                          0
   16.    A4 :                          0
 DOSE  COUNTS  COUNTS  COUNTS
   17.    .05 7892 8144
   18.    .1 7906 7831
   19.    .2 7854
   20.    .5 7937
   21.    1 7253
   22.    3 6129 6445
   23.    10 5556 5997
   24.    30 5637 5984
   25.    100 5524 5275
   26.    30000 4479 4450 4444 4485 4449 4305
   27.

 COMMAND ? rev
    1.    NAME OF THIS EXPERIMENT:  HOT ANALOG I, COLD GNRH
    2.    COUNTING TIME (MIN) :  5.
    3.    VOLUME OF TUBE (ML.) :  .5
    4.    SPECIFIC ACTIVITY (UC/UG) :   1100.
    5.    MEAN TOTAL COUNTS :  65789.
    6.    HOT LIGAND # :  1
    7.    COLD LIGAND # :  2
```

Because of the displacing ligand (Analog I) differs chemically from the labeled ligand, it is given a seperate ligand number.

```
    8.    MOL.WT., MEASURED LIGAND :  1130.
    9.    MOL.WT., COLD LIGAND :  1200.
   10.    DOSE UNITS :  NANOGRAMS
   11.    COUNTING EFFICIENCY :  .5
 VARIANCE(Y)=A0+A1*Y+A2*Y^2+A3*Y^A4
   12.    A0 :  0.
   13.    A1 :  0.
   14.    A2 :  .0001
   15.    A3 :  0.
```

```
  16.    A4 :  0.
 DOSE  COUNTS  COUNTS  COUNTS
  17.    .05 7892. 8144.
  18.    .1 7906. 7831.
  19.    .2 7854.
  20.    .5 7937.
  21.    1. 7253.
  22.    3. 6129. 6445.
  23.    10. 5556. 5997.
  24.    30. 5637. 5984.
  25.    100. 5524. 5275.
  26.    30000. 4479. 4450. 4444. 4485. 4449. 4305.

 COMMAND ? fin
 DO YOU WANT A GRAPH FILE MADE? [Y] n
 DOSE#        TOTAL              BOUND             B/T
1           8.3333333E-11      2.3245125E-12      0.1218745
2           1.6666667E-10      2.2811709E-12      0.1196021
3           3.3333333E-10      2.2769670E-12      0.1193817
4           8.3333329E-10      2.3010298E-12      0.1206433
5           1.6666666E-09      2.1027301E-12      0.1102464
6           4.9999995E-09      1.8226755E-12      9.5563084E-02
7           1.6666666E-08      1.6746755E-12      8.7803431E-02
8           4.9999997E-08      1.6845324E-12      8.8320233E-02
9           1.6666665E-07      1.5653787E-12      8.2072988E-02
10          4.9999999E-05      1.2858553E-12      6.7417547E-02
BYE
STOP:
helix%
```

4.2.2 Listing of output files

EXP4A.BAS Listing

```
HOT ANALOG I, COLD GNRH
5.
.5
1100.
65789.
1
2
1130.
1200.
NANOGRAMS
.5
0.
0.
.0001
0.
0.
.05 7892. 8144.
.1 7906. 7831.
.2 7854.
.5 7937.
1. 7253.
3. 6129. 6445.
10. 5556. 5997.
30. 5637. 5984.
100. 5524. 5275.
30000. 4479. 4450. 4444. 4485. 4449. 4305.
```

EXP4A.CRV Listing

```
 HOT ANALOG I, COLD GNRH
       0.0000000        0.0000000        0.0001000
0.0000000
       0.0000000
     2           1
  1.9073007E-11
   0.8333334E-10     0.2324512E-11    2
   0.1666667E-09     0.2281171E-11    2
   0.3333333E-09     0.2276967E-11    1
   0.8333334E-09     0.2301030E-11    1
   0.1666667E-08     0.2102731E-11    1
   0.5000000E-08     0.1822676E-11    2
   0.1666667E-07     0.1674675E-11    2
   0.5000000E-07     0.1684532E-11    2
   0.1666667E-06     0.1565379E-11    2
   0.5000000E-04     0.1285855E-11    6
```

4.2.3 Fit the data

We now fit the heterologous curve using a single receptor model. We constrain K11 to the value obtained in section 4.1.3, which then allows us to estimate K21. Note that inhibition constant K_i = 1/K21 here.

```
Helix% scafit

                        L I G A N D:
      Curve fitting and data analysis for ligand binding data
                   National Institutes of Health

                Program: SCAFIT version 2.4MAGGI


FILE NAME FOR SESSION (NO EXTENSION) ? exp4a

NUMBER OF LIGANDS (HIGHEST NUMBER USED) ? 2

TYPE THE DATA FILE NAME(S) (NO EXTENSION)
SEPARATE WITH <CR> END WITH BLANK LINE
 ? EXP4A
 ? <return>

CURVE FILE   EXP4A.CRV
 WEIGHTING PARAMETERS
0.             0.      9.9999997E-05         0.             0.

VARYING LIGAND:   2   LABELED LIGAND:   1
NUMBER OF POINTS: 10


NUMBER OF SITES ? 1

DO YOU WISH TO WEIGHT POINTS ACCORDING TO
NUMBER OF TUBES AT A GIVEN DOSE (Y/N) [N] ? <return>

WOULD YOU LIKE THE COOPERATIVITY MODEL (Y/N) [N] ?

WOULD YOU LIKE THE ADDITIONAL D PARAMETER(Y/N) [N] ?

THE FOLLOWING PARAMETERS WILL BE CONSIDERED:
 K11 K21 R1  N1  N2  C1

THE FOLLOWING PARAMETER GROUPS HAVE A SHARED VALUE:


DO YOU WANT THE SAME SHARED PARAMETERS AS
BEFORE (Y/N) [Y] ? <return>
```

```
LIST THE CONSTANT PARAMETERS (NO COMMA)
 [ C1 ] ?
 C1 K11 N2

 K11 = [  0.       ] ? 1.19E9
```

K11 is fixed to the value obtained in the fit of EXP3A in section 4.1.3.

```
 N2  = [  0.       ] ? <return>
```

N2 is fixed at zero since the binding of ligand number 2 is not directly observable (it is unlabeled).

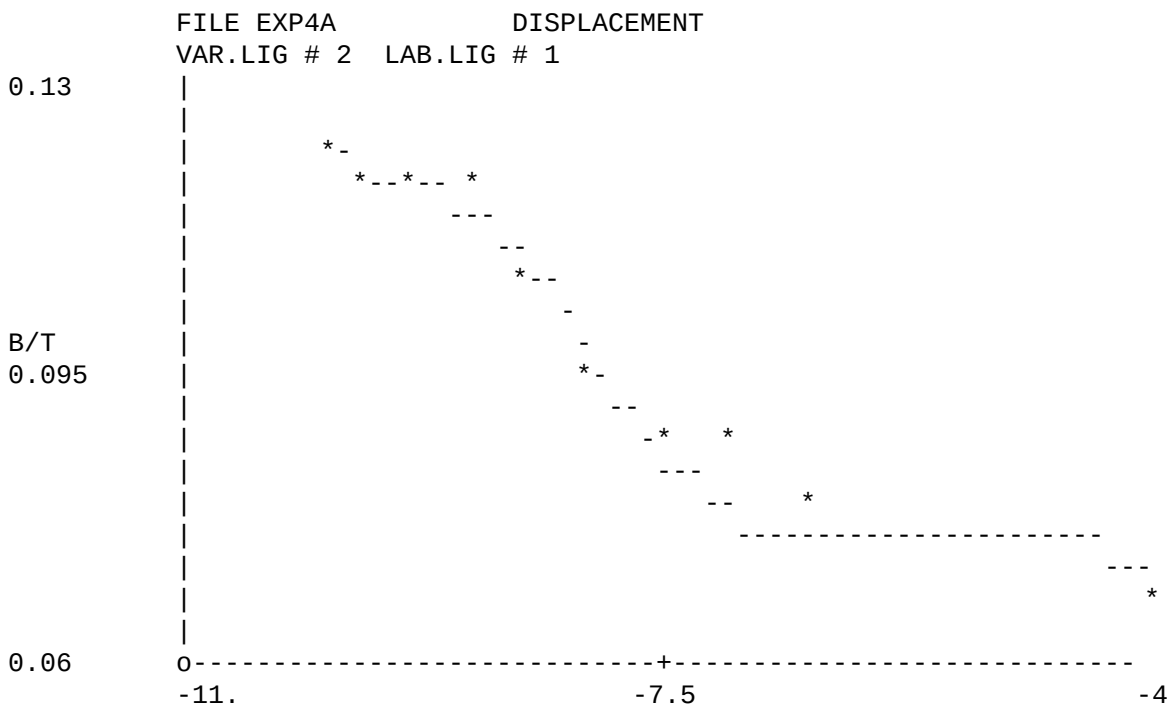
```
 C1  = [  1.00000  ] ? <return>

 INITIAL ESTIMATE FOR K21 [        0.     ] ? 1.19e9
 INITIAL ESTIMATE FOR R1  [        0.     ] ? 4.9e-11
 INITIAL ESTIMATE FOR N1  [        0.     ] ? 8.06e-2
```

Enter initial values obtained in fit of EXP3A in section 4.1.3. If these are not available, remember these rules-of-thumb:

$$K11 \times R1 + N1 = \text{initial B/T}$$
$$N1 = \text{final B/T}$$
$$K21 = K11$$

```
THE INITIAL ESTIMATES ARE
 K11 =  1.190000E+09
 K21 =  1.190000E+09
 R1  =  4.900000E-11
 N1  =  8.059999E-02
 N2  =        0.
 C1  =   1.00000

ARE THESE  VALUES OK (Y/N) ? Y

  IT.        EPSILON       SUM OF SQUARES
    0            0.             3539.048

TYPE C,R,P,D,S,X,G OR H FOR HELP ? c
NUMBER OF ADDITIONAL ITERATIONS ? 3
  IT.        EPSILON       SUM OF SQUARES
    1            0.             1413.330

  IT.        EPSILON       SUM OF SQUARES
    2            0.              320.379

  IT.        EPSILON       SUM OF SQUARES
    3            0.              300.270

  IT.        EPSILON       SUM OF SQUARES
    4      9.999999E-02          300.178

CONVERGED, TYPE "S" TO GET FINAL RESULTS
TYPE C,R,P,D,S,G,X OR H FOR HELP ? G
```

```
 type  T  to change TERMINAL TYPE [0]
 type  L  to change GRAPH LIMITS
 type  G  to choose GRAPH TYPE [DISPLACEMENT]
 type  C  for CONFIDENCE LIMITS (+/- 2 SIGMA) [N]
 type  1  to graph the DISPLACEMENT CURVE EXP4A
 type  0  to END GRAPHING SECTION

CHOOSE AN OPTION ? 1
```




```
 type  T  to change TERMINAL TYPE [0]
 type  L  to change GRAPH LIMITS
 type  G  to choose GRAPH TYPE [DISPLACEMENT]
 type  C  for CONFIDENCE LIMITS (+/- 2 SIGMA) [N]
 type  1  to graph the DISPLACEMENT CURVE EXP4A
 type  0  to END GRAPHING SECTION

CHOOSE AN OPTION ? 0

TYPE C,R,P,D,S,G,X OR H FOR HELP ? S
```

```
L I G A N D: SCAFIT VERSION 2.4MAGGI

 CAUTION: Results and interpretation of analysis depend upon
several important
 assumptions. See User's Guide for details

 FINAL PARAMETER ESTIMATES,  exp4a FIT NO.   1

      VALUE       %CV   LOG-VALUE   KD          K*R

 K11  1.1900E+09        9.0755      8.4034E-10  5.8415E-02
 K21  1.0055E+08  55.%  8.0024      9.9456E-09  0.0049
 R1   4.9088E-11  12.%
 N1   8.2671E-02  5.%
 N2   0.0000E+00
 C1   1.0000E+00


 K11*R1 0.0584    12.%
 K21*R1 0.0049    59.%
```

Parameter values have units of liters/mole for K11, moles/liter for R1 and Kd, the dissociation constant. The column labeled Kd is just 1/K for the affinity constant. N1 and C1 are unitless. The %CV is percentage coefficient of variations or standard error divided by the parameter value. LOG-VALUE is base 10 logarithm of the value. K11 × R1 is a unitless measure of the amount of binding of the (in this case) first ligand to the first receptor. Compare this value to N1 in order to get a qualitative impression of the importance or significance of the specific binding. In this case, specific binding is roughly equal to the non-specific binding. Note that the %CV for K11 is blank, indicating that K11 was set to a constant value by the user.

```
WOULD YOU LIKE THE COVARIANCE-CORRELATION MATRIX  (Y/N) [N] ?
<return>


 CURVE      SUM OF      D.F.  MEAN        RUNS        RESIDUALS
            SQUARES           SQUARE

 exp4a      300.178     7.0   42.8826     6  O.K.     +-++--+++-

 TOTAL      300.178     7     42.8826                  6   4
```

This table gives a summary of the fit. The columns are :

CURVE

The name of this curve. Here the only one.

SUM OF SQUARES

This is the weighted sum of squared residuals or differences between observed and model - predicted values.

D.F.

Degrees of freedom or number of data points less the number of parameters.

MEAN SQUARE

Sum of squares divided by degrees of freedom.

RUNS

This figure gives the number of runs of either positive or negative residuals in the curve. A good fit should give a large number of runs. The message "OK", "NOK","POOR" is an expression of the quality of the fit based on the runs test.

RESIDUALS

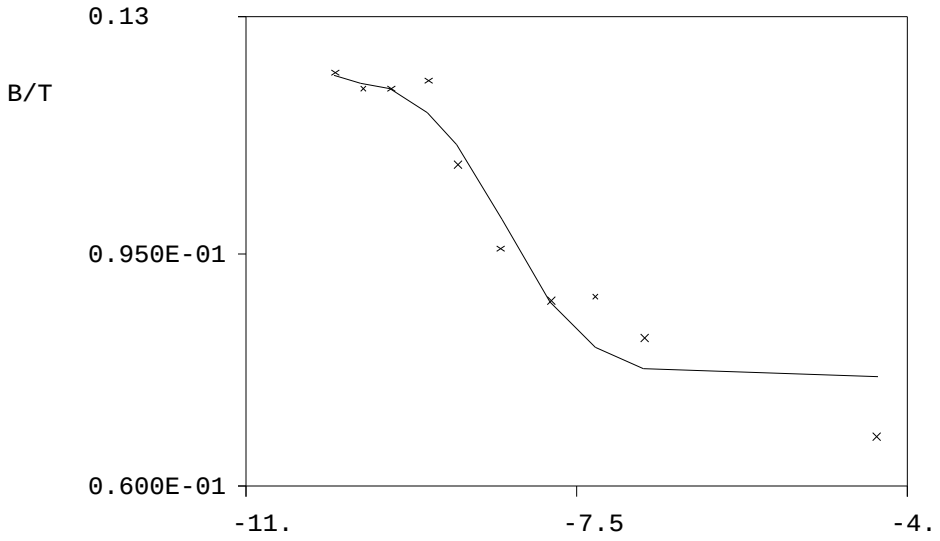
This is a series of signs of the residuals, which allows you to quickly inspect them for apparent patterns. No pattern means a satisfactory fit. There there are 10 residuals, with 6 positive and 4 negative.

```
GRAPHICS FILE NOW BEING WRITTEN  FOR FIT NO.   1

                  ----------------------------

#       LOG(TOTAL)    B/T        B/T-PRED   DIFF       RESIDUAL PLOT
CURVE:EXP4A
1       -10.079       0.1219     0.1212     0.0006      *
2       -9.778        0.1196     0.1206     -0.0010     *
3       -9.477        0.1194     0.1193     0.0001      *
4       -9.079        0.1206     0.1159     0.0048      |*
5       -8.778        0.1080     0.1113     -0.0032     *
6       -8.301        0.0956     0.1001     -0.0046    *|
7       -7.778        0.0878     0.0876     0.0002      *
8       -7.301        0.0883     0.0808     0.0075      |*
9       -6.778        0.0821     0.0778     0.0043      |*
10      -4.301        0.0674     0.0764     -0.0089    *|

ANOTHER FIT (Y/N) [N] ? <return>

DO YOU WANT TO REDEFINE THE INPUT SET (Y/N) [N] ? <return>
WOULD YOU LIKE TO SEE THE GRAPH (Y/N) [N] ? <return>
SESSION LOG IN exp4a.ses
GRAPHICS FILE IN exp4a.grf

Helix%
```

4.2.4 Graph the data

```
Helix% scagrf

          *****  LIGAND SYSTEM GRAPHICS PROGRAM  *****
      Curve fitting and data analysis for ligand binding data
                  National Institutes of Health


       CONVEX SCAGRF   v1.1     March 1991

       Graphics are designed for Tektronix 4105 display terminal

 GRAPHICS FILE NAME (NO EXTENSION) ? exp4a

 DO YOU WANT INDIVIDUAL PLOTS ?(Y/N) [Y] <return>


 CURVE HEADER:"exp4a  FIT NO.  1  N= 8.267135E-02"
 VARYING LIGAND #2   LABELED LIGAND #1

 DO YOU WANT TO PLOT THIS CURVE ? (Y/N) [Y] <return>
 SCATCHARD PLOT OR DISPLACEMENT CURVE (S/D) [S]? d

 WITH CONFIDENCE LIMITS (+/- 2 SIGMA) (Y/N)[N] <return>
XMIN,XMAX,DELTAX       -11.0          -4.00           3.50
YMIN,YMAX,DELTAY       0.600E-01      0.130          0.350E-01

ARE THESE VALUES ACCEPTABLE (Y/N) [Y]  ? Y
```

```
 exp4a.SES :  "exp4a  FIT NO.  1  N= 8.267135E-02"
 VAR.LIG# 2 LAB.LIG# 1
```




```
 DO YOU WANT TO CHANGE THE AXIS PARAMETERS [N] ?
 DO YOU WANT A CONGLOMERATE PICTURE ?(Y/N)
STOP:
helix%
```

4.3 Heterologous and Homologous Displacement

A second, perhaps better approach to estimating K_i from heterologous displacement curves is to fit both heterologous and homologous curves together. Here, we repeat the analysis of EXP4A, and instead of freezing the parameter K11, we use the homologous curve EXP3A which contains information about this parameter.

When two separate curves are fit together, the possibility of a scale factor between curves comes up. The parameter C2 allows us to scale EXP4A relative to EXP3A. Although not used here, this is sometimes appropriate when pooling data from replicate experiments.

4.3.1 Listing of output files

The two data files used in this section have previously been prepared in earlier sections. EXP3A.BAS and EXP3A.CRV from section 4.1.1, and EXP4A.BAS and EXP4A.CRV from section 4.2.1. Please refer to those sections for file listings.

4.3.2 Fit the data

```
                    L I G A N D:
     Curve fitting and data analysis for ligand binding data
                 National Institutes of Health

                  Program: SCAFIT version 2.4


FILE NAME FOR SESSION (NO EXTENSION) ? SEC432

NUMBER OF LIGANDS (HIGHEST NUMBER USED) ? 2

TYPE THE DATA FILE NAME(S) (NO EXTENSION)
SEPARATE WITH <CR> END WITH BLANK LINE
 ? EXP3A
 ? EXP4A
 ? <return>

CURVE FILE   EXP3A.CRV
WEIGHTING PARAMETERS
 0.0000000E+00, 0.0000000E+00, 1.0000000E-04, 0.0000000E+00,
0.0000000E+00
VARYING LIGAND:   1   LABELED LIGAND:   1
NUMBER OF POINTS: 10

CURVE FILE   EXP4A.CRV
WEIGHTING PARAMETERS
 0.0000000E+00, 0.0000000E+00, 1.0000000E-04, 0.0000000E+00,
 0.0000000E+00
VARYING LIGAND:   2   LABELED LIGAND:   1
NUMBER OF POINTS: 10


NUMBER OF SITES ? 1

DO YOU WISH TO WEIGHT POINTS ACCORDING TO
NUMBER OF TUBES AT A GIVEN DOSE (Y/N) [N] ? <return>

THE FOLLOWING PARAMETERS WILL BE CONSIDERED:
 K11 K21 R1  N1  N2  C1  C2

THE FOLLOWING PARAMETER GROUPS HAVE A SHARED VALUE:



DO YOU WANT THE SAME SHARED PARAMETERS AS
BEFORE (Y/N) [Y] ? <return>
LIST THE CONSTANT PARAMETERS (NO COMMA)
```

```
 [ C1 ] ?
```

We constrain C2=C1=1 since the scale factor is not to be used. Parameter N2 is fixed, since ligand number 2 is unavailable as a labeled species.

```
 C1 C2 N2

 N2  = [  0.000000E+00 ] ? <return>
 C1  = [   1.00000     ] ? <return>
 C2  = [   1.00000     ] ? <return>

DO YOU WANT AUTOMATIC INITIAL ESTIMATES (Y/N) [Y] ? <return>

AFTER ADJUSTING FOR NONSPECIFIC, THERE ARE  9 POINTS LEFT
AFTER ADJUSTING FOR NONSPECIFIC, THERE ARE  0 POINTS LEFT
```

These points are temporarily deleted when obtaining initial estimates. They are restored during the fit.

```
INITIAL ESTIMATE FOR K11 [  6.126521E+07 ] ? <return>
INITIAL ESTIMATE FOR K21 [  0.000000E+00 ] ? 491353
```

No initial estimate was obtained for K21. Enter a value close to your guess of 1/Ki, or close to K11.

```
INITIAL ESTIMATE FOR R1  [  7.976648E-10 ] ? <return>
INITIAL ESTIMATE FOR N1  [  6.946825E-02 ] ? <return>

THE INITIAL ESTIMATES ARE
 K11 =  6.126521E+07
 K21 =   491353
 R1  =  7.976648E-10
 N1  =  6.946825E-02
 N2  =  0.000000E+00
 C1  =   1.00000
 C2  =   1.00000

ARE THESE  VALUES OK (Y/N) ? Y

  IT.        EPSILON       SUM OF SQUARES
    0      0.000000E+00         2799.169

TYPE C,R,P,D,S,G,X OR H FOR HELP ? C
NUMBER OF ADDITIONAL ITERATIONS ?  5

  IT.        EPSILON       SUM OF SQUARES
    1      1.000000E-03         2306.104

  IT.        EPSILON       SUM OF SQUARES
    2      1.000000E-03         1599.734

  IT.        EPSILON       SUM OF SQUARES
    3      1.000000E-03         1104.726
```

```
  IT.        EPSILON       SUM OF SQUARES
    4      1.000000E-03          792.422

  IT.        EPSILON       SUM OF SQUARES
    5      1.000000E-03          574.403

TYPE C,R,P,D,S,G,X OR H FOR HELP ? C
NUMBER OF ADDITIONAL ITERATIONS ?  6

  IT.        EPSILON       SUM OF SQUARES
    6      1.000000E-03          445.384

  IT.        EPSILON       SUM OF SQUARES
    7      1.000000E-04          411.898

  IT.        EPSILON       SUM OF SQUARES
    8      1.000000E-05          355.762

  IT.        EPSILON       SUM OF SQUARES
    9      1.000000E-06          352.429

  IT.        EPSILON       SUM OF SQUARES
   10      1.000000E-07          352.219

CONVERGED, TYPE "S" TO GET FINAL RESULTS
TYPE C,R,P,D,S,G,X OR H FOR HELP ? S

L I G A N D: SCAFIT VERSION 2.4

CAUTION: Results and interpretation of analysis depend upon
several important assumptions. See User's Guide for details

FINAL PARAMETER ESTIMATES, SEC432 FIT NO.   1

        VALUE        %CV       LOG-VALUE       Kd           K*R

K11    1.2317E+09    30.%       9.0905      8.1187E-10    0.0580
K21    1.6436E+08    33.%       8.2158      6.0843E-09    0.0077
R1     4.7064E-11    28.%
N1     8.1222E-02     2.%
N2     0.0000E+00
C1     1.0000E+00
C2     1.0000E+00
```

Notice the relatively good agreement with the values given in section 4.2.3.

```
K11*R1  0.0580       6.%
K21*R1  0.0077      34.%

WOULD YOU LIKE THE COVARIANCE-CORRELATION MATRIX  (Y/N) [N] ?
<return>
```

```
CURVE      SUM OF        D.F.     MEAN         RUNS      RESIDUALS
           SQUARES                SQUARE

EXP3A      44.448        8.0      5.5561       4  O.K.   +----++++-
EXP4A      307.771       8.0      38.4713      6  O.K.   +-++---++-

TOTAL      352.219       16       22.0137                 10    10
```

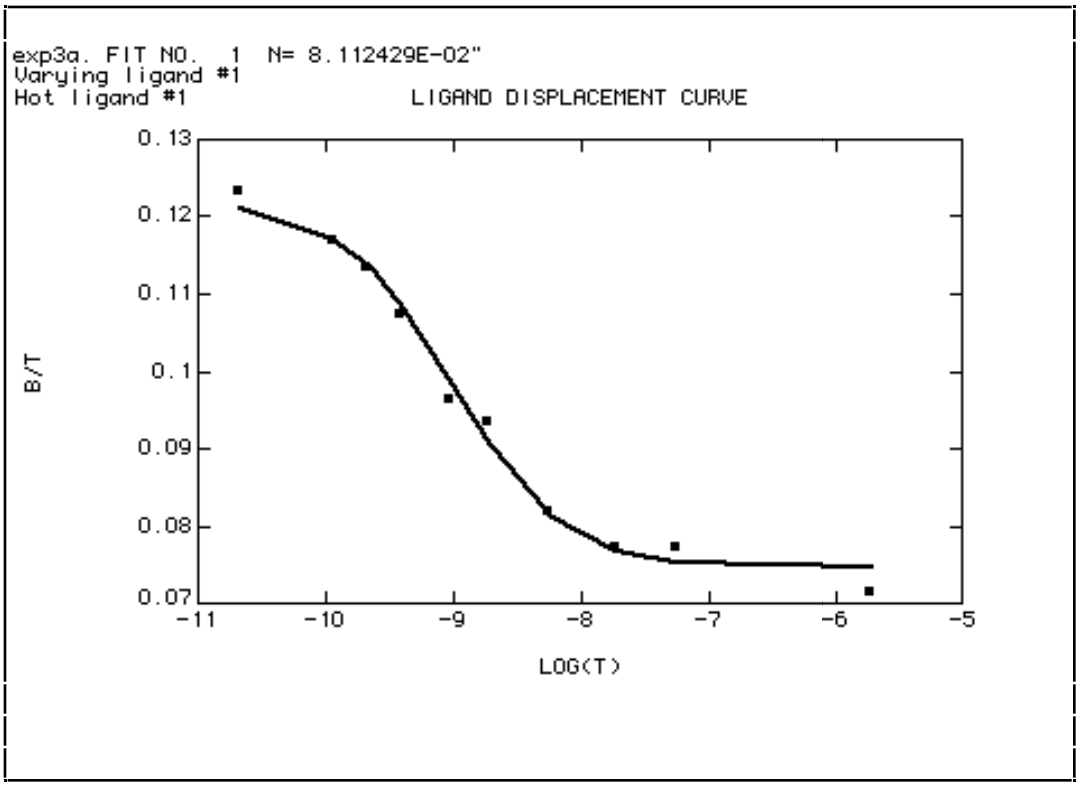
See section 4.2.3 for a description of the output here. Since there are now two curves, the residuals in each curve may be inspected.

```
  GRAPHICS FILE NOW BEING WRITTEN  FOR FIT NO.   1

                  ------------------------------

#       LOG(TOTAL)    B/T        B/T-PRED    DIFF      RESIDUAL PLOT
CURVE: EXP3A
1       -10.678       0.1234     0.1212      0.0022     *
2       -9.951        0.1170     0.1173      -0.0004    *
3       -9.693        0.1137     0.1140      -0.0003    *
4       -9.415        0.1076     0.1087      -0.0011    *
5       -9.031        0.0967     0.0989      -0.0022    *
6       -8.735        0.0938     0.0911      0.0027     |*
7       -8.262        0.0820     0.0820      0.0001     *
8       -7.740        0.0775     0.0774      0.0001     *
9       -7.263        0.0775     0.0759      0.0016     *
10      -5.740        0.0717     0.0751      -0.0035   *|
CURVE: EXP4A
11      -10.079       0.1219     0.1207      0.0012     *
12      -9.778        0.1196     0.1201      -0.0005    *
13      -9.477        0.1194     0.1191      0.0003     *
14      -9.079        0.1206     0.1161      0.0046     |*
15      -8.778        0.1080     0.1119      -0.0039   *|
16      -8.301        0.0956     0.1013      -0.0058   *|
17      -7.778        0.0878     0.0881      -0.0003    *
18      -7.301        0.0883     0.0805      0.0079     | *
19      -6.778        0.0821     0.0769      0.0052     |*
20      -4.301        0.0674     0.0751      -0.0077  * |


ANOTHER FIT (Y/N) [N] ? <return>

DO YOU WANT TO REDEFINE THE INPUT SET (Y/N) [N] ? <return>
WOULD YOU LIKE TO SEE THE GRAPH (Y/N) [N] ? <return>
SESSION LOG IN SEC432.SES
GRAPHICS FILE IN SEC432.GRF

MON
```

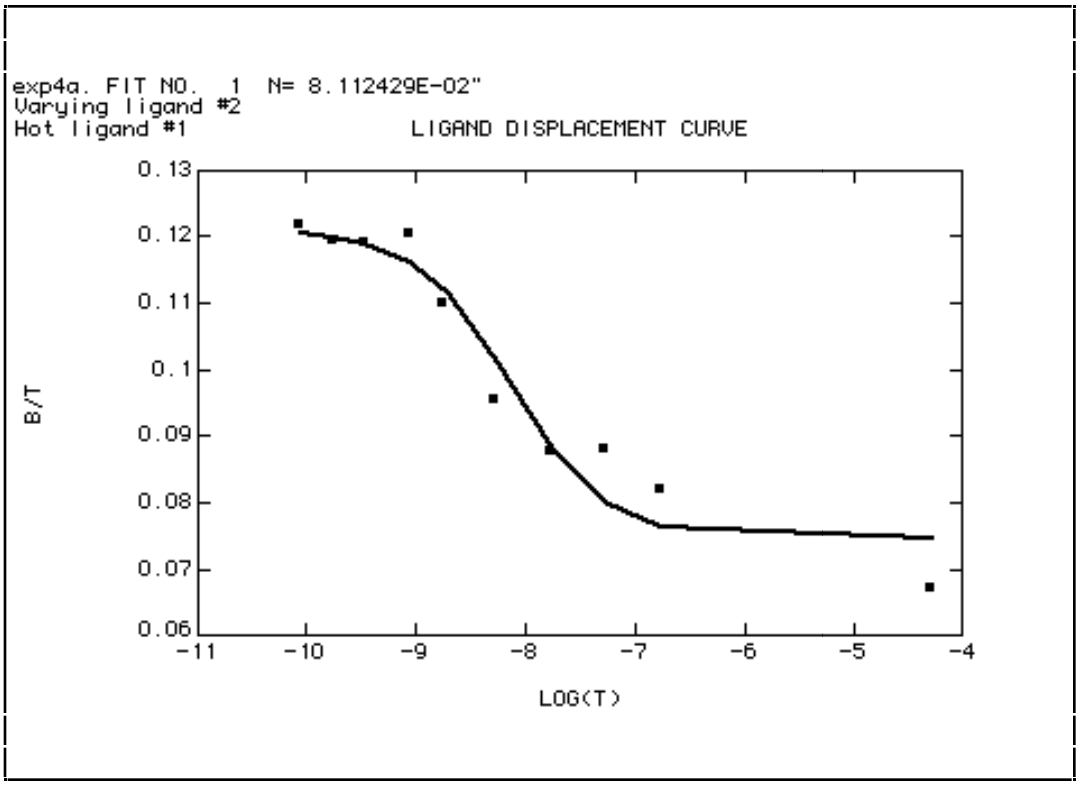
This gets you back to the monitor level.

4.3.3 Graph the data

```
.BASIC

READY, FOR HELP TYPE HELP
OLD SCAGRF

READY
RUN

SCAGRF        13:57         11-SEP-86


GRAPHICS FILE NAME (WITHOUT EXTENSION) ? SEC432
DO YOU WANT INDIVIDUAL PLOTS?(Y/N)[N] ? Y

CURVE HEADER: EXP3A  FIT NO.  1  N= 8.122249E-02
VARYING LIGAND NO.  1  LABELED LIGAND NO.  1

DO YOU WANT THIS CURVE PLOTTED (Y/N)[Y] ? <return>
SCATCHARD PLOT OR DISPLACEMENT CURVE (S/D)[S]? D
WITH CONFIDENCE LIMITS (+/- 2 SIGMA) (Y/N)[N] ? <return>
GRAPHIC TERMINAL #
TYPE IN THE NUMBER '4' FOR A TEKTRONIX T4006 OR T4010
 '6' FOR A TEKTRONIX T4014
 ? 4
MINX, MAXX, DELTAX: -11 -5    3
MINY, MAXY, DELTAY :  0.07  0.13   0.03
ARE THESE VALUES ACCEPTABLE (Y/N) [Y]  ? <return>
```




```
WOULD YOU LIKE TO MAKE A PLOT FILE OF WHAT YOU SEE NOW Y/N [N] ?
<return>
WOULD YOU LIKE TO CHANGE THE AXES PARAMETER (Y/N) [N] ? <return>

CURVE HEADER: EXP4A  FIT NO.  1  N= 8.122249E-02
VARYING LIGAND NO.  2  LABELED LIGAND NO.  1

DO YOU WANT THIS CURVE PLOTTED (Y/N)[Y] ? <return>
SCATCHARD PLOT OR DISPLACEMENT CURVE (S/D)[S] ? D
WITH CONFIDENCE LIMITS (+/- 2 SIGMA) (Y/N)[N] ? <return>

MINX, MAXX, DELTAX: -11  -4  3.5
MINY, MAXY, DELTAY :  0.06  0.13    0.035
ARE THESE VALUES ACCEPTABLE (Y/N) [Y] ? <return>
```




```
WOULD YOU LIKE TO MAKE A PLOT FILE OF WHAT YOU SEE NOW Y/N [N] ?
<return>
WOULD YOU LIKE TO CHANGE THE AXES PARAMETER (Y/N) [N] ? <return>
DO YOU WANT A CONGLOMERATE PICTURE (Y/N)[N] ? <return>

THATS ALL FOLKS

TIME:  3.57 SECS

READY

MON
```

Return to monitor mode.

4.4 Displacement experiments with multiple labeled ligands

The real power of computerized analysis using LIGAND becomes apparent when fitting multi-ligand multi-site models. In this section, we have self- and cross-displacement curves for two ligands (four curves) and are interested in testing one- and two-site models. As we will see, although two of the curves (EXP3A and EXP4A) did not show significant evidence for two-sites, such evidence was found when they were fit together with two additional curves (EXP1A and EXP2A). Thus, the suggestion of a two-site model found using EXP3A alone (sect. 4.1.3) is confirmed using multiple curves with multiple labeled ligands.

Many other models are possible (more sites, more ligands) in other situations, using the parameter sharing and parameter constraint options. Even allosteric or cooperative models may be tried with special versions of SCAFIT.

This section uses four data sets:

EXP1A.BAS
EXP2A.BAS
EXP3A.BAS
EXP4A.BAS

The preparation of the last two, EXP3A.BAS and EXP4A.BAS, is detailed earlier in this document. The preparation of the additional data files EXP1A.BAS and EXP2A.BAS should be done the same way. You will find listings for these files in the following section.

4.4.1 Listing of output files

EXP1A.BAS Listing

```
01000   "HOT AND COLD"
01010   "5."
01020   ".5"
01030   "1300"
01040   "60010."
01050   "2"
01060   "2"
01070   "1230."
01080   "1200."
01090   "NANOGRAMS"
01100   "0."
01110   "0."
01120   ".0001"
01130   "0."
01140   "0"
01150   "0. 5123. 5026. 5063. 4966."
01160   ".05 5185. 4956."
01170   "0.1 4993. 4847."
01180   "0.2 4762. 4246."
01190   "0.5 4366. 4537."
01200   "1. 4112. 4183."
01210   "3. 4081. 3890."
01220   "10. 3910. 4114."
01230   "30. 4009. 3900."
01240   "200. 3414. 3184."
01250   "300. 3198. 3009."
01260   "1000. 2605. 2635."
01270   "5000. 2253. 2473."
01280   "30000. 1987. 1884. 1974. 1861."
```

EXP1A.CRV Listing

```
01000   "HOT AND COLD"
01010    0              0              0.0001        0
01020    0
01030    2              2
01040    1.35242E-11
01050    1.35242E-11    1.13686E-12    4
01060    9.68576E-11    8.18391E-12    2
01070    1.80191E-10    1.47732E-11    2
01080    3.46858E-10    2.60331E-11    2
01090    8.46858E-10    6.28193E-11    2
01100    1.68019E-9     1.16124E-10    2
01110    5.01352E-9     3.32968E-10    2
01120    1.66802E-8     1.11516E-9     2
01130    5.00135E-8     3.29576E-9     2
01140    3.33347E-7     1.83255E-8     2
01150    5.00014E-7     2.58589E-8     2
01160    1.66668E-6     7.27662E-8     2
01170    8.33335E-6     3.28140E-7     2
01180    0.00005        1.60515E-6     4
```

EXP2A.BAS Listing

```
01000   "HOT GNRH COLD ANALOG I"
01010   "5"
01020   ".5"
01030   "1300"
01040   "60010"
01050   "2"
01060   "1"
01070   "1230"
01080   "1100"
01090   "NANOGRAM"
01100   "0"
01110   "0"
01120   "0.0001"
01130   "0"
01140   "0"
01150   "0.05 4721 4474"
01160   "0.1 4528 4127"
01170   "0.2 4320 4302"
01180   "0.5 3914 4098"
01190   "1 4071 3845"
01200   "3 4065 4016"
01210   "10 3775 4009"
```

EXP2A.CRV Listing

```
01000   "HOT GNRH COLD ANALOG I"
01010    0              0              0.0001        0
01020    0
01030    1              2
01040    1.35242E-11
01050    9.09091E-11    1.03612E-12    2
01060    1.81818E-10    9.75272E-13    2
01070    3.63636E-10    9.71553E-13    2
01080    9.09091E-10    9.02817E-13    2
01090    1.81818E-9     8.91999E-13    2
01100    5.45455E-9     9.10592E-13    2
01110    1.81818E-8     8.77125E-13    2
```

4.4.2 Fit the data

Here, we fit EXP3A and EXP4A, homologous and heterologous displacement curves with labeled ligand #1 together with two displacement curves which have labeled ligand #2 (EXP1A and EXP2A). Since the labeled ligands should "see" the same receptor, it should be possible to use a single model to fit all of this data simultaneously, and in so doing, improve our confidence in the underlying theoretical model.

The suggestion of two sites found in the EXP3A curve will be pursued, so we require up to a two-site, two-ligand model, or altogether four Ks, two Rs and two Ns, or 8 parameters. In addition, since we have four curves, there will be four scale factors C1, C2, C3, and C4. These are all fixed equal to 1, since all four curves came from the same receptor preparation.

```
                    L I G A N D:
     Curve fitting and data analysis for ligand binding data
                 National Institutes of Health

                  Program: SCAFIT version 2.4


FILE NAME FOR SESSION (NO EXTENSION) ? SAT

NUMBER OF LIGANDS (HIGHEST NUMBER USED) ? 2

TYPE THE DATA FILE NAME(S) (NO EXTENSION)
SEPARATE WITH <CR> END WITH BLANK LINE
 ? EXP1A
 ? EXP2A
 ? EXP3A
 ? EXP4A
 ? <return>

CURVE FILE   EXP1A.CRV

WEIGHTING PARAMETERS
 0.0000000E+00, 0.0000000E+00, 1.0000000E-04, 0.0000000E+00,
0.0000000E-08
VARYING LIGAND:   2   LABELED LIGAND:   2
NUMBER OF POINTS: 14

CURVE FILE   EXP2A.CRV


WEIGHTING PARAMETERS
 0.0000000E+00, 0.0000000E+00, 1.0000000E-04, 0.0000000E+00,
0.0000000E+00
```

```
VARYING LIGAND:   1   LABELED LIGAND:   2
NUMBER OF POINTS: 7

CURVE FILE   EXP3A.CRV
WEIGHTING PARAMETERS
 0.0000000E+00, 0.0000000E+00, 1.0000000E-04, 0.0000000E+00,
0.0000000E+00
VARYING LIGAND:   1   LABELED LIGAND:   1
NUMBER OF POINTS: 10

CURVE FILE   EXP4A.CRV
WEIGHTING PARAMETERS
 0.0000000E+00, 0.0000000E+00, 1.0000000E-04, 0.0000000E+00,
0.0000000E+00
VARYING LIGAND:   2   LABELED LIGAND:   1
NUMBER OF POINTS: 10
```

We now try four data sets to be fit simultaneously. These sets comprise a complete self- and cross- displacement study.

```
NUMBER OF SITES ? 1
```

We will first try the one binding site model. Since there are two ligands in the data, there will be two different affinity constants, one receptor capacity, and one non-specific binding parameter. Because there are four experiments, there will be four different correction parameters.

```
DO YOU WISH TO WEIGHT POINTS ACCORDING TO
NUMBER OF TUBES AT A GIVEN DOSE (Y/N) [N] ? <return>

THE FOLLOWING PARAMETERS WILL BE CONSIDERED:
 K11 K21 R1  N1  N2  C1  C2  C3  C4

THE FOLLOWING PARAMETER GROUPS HAVE A SHARED VALUE:


DO YOU WANT THE SAME SHARED PARAMETERS AS
BEFORE (Y/N) [Y] ? N

INPUT A GROUP OF SHARED PARAMETERS : C1 C2 C3 C4
```

But, since the four curves really came from the same experiment, we shall constrain the correction parameters for receptor concentration to share the same value.

```
INPUT A GROUP OF SHARED PARAMETERS : <return>
LIST THE CONSTANT PARAMETERS (NO COMMA)
 [ C1 ,C2 ,C3 ,C4 ] ?
 C1
```

As before at least one (and in this case all) of the correction parameters must be fixed at a given value.

```
 C1  = [   1.00000     ] ? <return>

DO YOU WANT AUTOMATIC INITIAL ESTIMATES (Y/N) [Y] ? <return>
```

```
AFTER ADJUSTING FOR NONSPECIFIC, THERE ARE    9 POINTS LEFT
AFTER ADJUSTING FOR NONSPECIFIC, THERE ARE   13 POINTS LEFT
```

In determining the initial estimates, the program temporarily deletes the "non-specific" points. These points are then restored for the complete fit.

```
INITIAL ESTIMATE FOR K11 [  6.126521E+07 ] ? <return>
INITIAL ESTIMATE FOR K21 [  0.000000E+00 ] ? 6E7
```

Initialize K21 to the same value as K11

```
INITIAL ESTIMATE FOR R1  [  7.976648E-10 ] ? <return>
INITIAL ESTIMATE FOR N1  [  6.946825E-02 ] ? <return>
INITIAL ESTIMATE FOR N2  [  2.985101E-02 ] ? <return>

THE INITIAL ESTIMATES ARE
 K11 =  6.126521E+07
 K21 =  6.000000E+07
 R1  =  7.976648E-10
 N1  =  6.946825E-02
 N2  =  2.985101E-02
 C1  =   1.00000
 C2  =   1.00000
 C3  =   1.00000
 C4  =   1.00000

ARE THESE  VALUES OK (Y/N) ? Y

  IT.        EPSILON       SUM OF SQUARES
    0      0.000000E+00        23501.211

TYPE C,R,P,D,S,G,X OR H FOR HELP ? C
NUMBER OF ADDITIONAL ITERATIONS ?  10
  IT.        EPSILON       SUM OF SQUARES
    1      0.000000E+00         9665.114

  IT.        EPSILON       SUM OF SQUARES
    2      0.000000E+00         4175.488
  IT.        EPSILON       SUM OF SQUARES
    3      0.000000E+00         4117.614

  IT.        EPSILON       SUM OF SQUARES
    4      0.000000E+00         4115.969

  IT.        EPSILON       SUM OF SQUARES
    5      0.000000E+00         4114.920

  IT.        EPSILON       SUM OF SQUARES
    6      0.000000E+00         4114.323

  IT.        EPSILON       SUM OF SQUARES
    7      1.000000E-03         4113.885

CONVERGED, TYPE "S" TO GET FINAL RESULTS
```

```
TYPE C,R,P,D,S,G,X OR H FOR HELP ? S


 L I G A N D: SCAFIT VERSION 2.4

CAUTION: Results and interpretation of analysis depend upon
several important
assumptions. See User's Guide for details

FINAL PARAMETER ESTIMATES,    SAT FIT NO.   1

       VALUE          %CV    LOG-VALUE      Kd            K*R

K11    5.2666E+07     40.%    7.7215     1.8987E-08     0.0532
K21    3.5091E+07     41.%    7.5452     2.8497E-08     0.0355
R1     1.0107E-09     41.%
N1     7.1686E-02      7.%
N2     4.6220E-02      5.%
C1     1.0000E+00
C2     1.0000E+00
C3     1.0000E+00
C4     1.0000E+00
```

Notice that the parameter values differ significantly from those given in section 4.3.2. This may indicate that the overall model is wrong.

```
K11*R1   0.0532     13.%
K21*R1   0.0355     10.%

WOULD YOU LIKE THE COVARIANCE-CORRELATION MATRIX  (Y/N) [N] ?
<return>

CURVE      SUM OF     D.F.     MEAN       RUNS        RESIDUALS
           SQUARES             SQUARE

EXP1A      2081.588   12.8     163.2618   4  N.O.K.   +++----+++---
EXP2A      279.535    5.8      48.6148    3  O.K.     +-----+
EXP3A      1129.109   8.8      129.0411   3  N.O.K.   +++------+
EXP4A      623.653    8.8      71.2746    3  N.O.K.   ++++---+++

TOTAL      4113.885   36       114.2746              20       21

          GRAPHICS FILE NOW BEING WRITTEN  FOR FIT NO. 1

                ------------------------------


#       LOG(TOTAL)  B/T       B/T-PRED     DIFF       RESIDUAL PLOT
CURVE: EXP1A
1       -10.869     0.0841    0.0755       0.0086       |*
2       -10.014     0.0845    0.0754       0.0091       |*
3       -9.744      0.0820    0.0753       0.0066       |*
4       -9.460      0.0751    0.0752       -0.0001      *
```

```
5       -9.072      0.0742    0.0747       -0.0005      *
6       -8.775      0.0691    0.0739       -0.0048     *|
7       -8.300      0.0664    0.0712       -0.0048     *|
8       -7.778      0.0669    0.0647       0.0022       *
9       -7.301      0.0659    0.0562       0.0097       | *
10      -6.477      0.0550    0.0468       0.0081       | *
11      -6.301      0.0517    0.0460       0.0057       |*
12      -5.778      0.0437    0.0447       -0.0011      *
13      -5.079      0.0394    0.0443       -0.0049     *|
14      -4.301      0.0321    0.0442       -0.0121    * |
```

Notice the apparent pattern in the residuals for EXP1A.

```
CURVE: EXP2A
15      -10.041     0.0766    0.0754       0.0012      *
16      -9.740      0.0721    0.0752       -0.0031     *
17      -9.439      0.0718    0.0750       -0.0032     *
18      -9.041      0.0668    0.0743       -0.0075    *|
19      -8.740      0.0660    0.0731       -0.0072    *|
20      -8.263      0.0673    0.0693       -0.0019     *
21      -7.740      0.0649    0.0612       0.0037      |*
CURVE: EXP3A
22      -10.678     0.1234    0.1110       0.0124       |*
23      -9.951      0.1170    0.1108       0.0061       |*
24      -9.693      0.1137    0.1107       0.0030       *
25      -9.415      0.1076    0.1103       -0.0027      *
26      -9.031      0.0967    0.1093       -0.0126     *|
27      -8.735      0.0938    0.1077       -0.0139     *|
28      -8.262      0.0820    0.1023       -0.0203    * |
29      -7.740      0.0775    0.0910       -0.0136     *|
30      -7.263      0.0775    0.0794       -0.0020      *
31      -5.740      0.0717    0.0674       0.0043       |*
CURVE: EXP4A
32      -10.079     0.1219    0.1109       0.0110      |*
33      -9.778      0.1196    0.1108       0.0088      |*
34      -9.477      0.1194    0.1106       0.0088      |*
35      -9.079      0.1206    0.1099       0.0107      |*
36      -8.778      0.1080    0.1088       -0.0008     *
37      -8.301      0.0956    0.1051       -0.0095    *|
38      -7.778      0.0878    0.0959       -0.0081    *|
39      -7.301      0.0883    0.0840       0.0043      *
40      -6.778      0.0821    0.0739       0.0082      |*
41      -4.301      0.0674    0.0669       0.0005      *


ANOTHER FIT (Y/N) [N] ? Y

NUMBER OF SITES ? 2
```

Now a two-site fit is attempted.

```
DO YOU WISH TO WEIGHT POINTS ACCORDING TO
NUMBER OF TUBES AT A GIVEN DOSE (Y/N) [N] ? <return>
```

```
THE FOLLOWING PARAMETERS WILL BE CONSIDERED:
 K11 K12 K21 K22 R1  R2  N1  N2  C1  C2  C3  C4

THE FOLLOWING PARAMETER GROUPS HAVE A SHARED VALUE:


DO YOU WANT THE SAME SHARED PARAMETERS AS
 BEFORE (Y/N) [Y] ? N

INPUT A GROUP OF SHARED PARAMETERS : C1 C2 C3 C4
INPUT A GROUP OF SHARED PARAMETERS : <return>

LIST THE CONSTANT PARAMETERS (NO COMMA)
 [ C1 ,C2 ,C3 ,C4 ] ?
 C1

 C1  = [   1.00000     ] ? <return>

DO YOU WANT AUTOMATIC INITIAL ESTIMATES (Y/N) [Y] ? <return>

AFTER ADJUSTING FOR NONSPECIFIC, THERE ARE    9 POINTS LEFT
AFTER ADJUSTING FOR NONSPECIFIC, THERE ARE   13 POINTS LEFT

INITIAL ESTIMATE FOR K11 [  1.894922E+09 ] ? <return>
INITIAL ESTIMATE FOR K12 [  1.379565E+07 ] ? <return>
INITIAL ESTIMATE FOR K21 [  0.000000E+00 ] ? 2E9
                          Initialize K21 to the same value as K11

INITIAL ESTIMATE FOR K22 [  0.000000E+00 ] ? 1E7
                          Initialize K22 to the same value as K12

INITIAL ESTIMATE FOR R1  [  2.757794E-11 ] ? <return>
INITIAL ESTIMATE FOR R2  [  1.496141E-09 ] ? <return>
INITIAL ESTIMATE FOR N1  [  6.946825E-02 ] ? <return>
INITIAL ESTIMATE FOR N2  [  2.985101E-02 ] ? <return>

THE INITIAL ESTIMATES ARE
K11   = 1.894922E+09
K12   = 1.379565E+07
K21   = 2.000000E+09
K22   = 1.000000E+07
R1    = 2.757794E-11
R2    = 1.496141E-09
N1    = 6.946825E-02
N2    = 2.985101E-02
C1    = 1.00000
C2    = 1.00000
C3    = 1.00000
C4    = 1.00000

ARE THESE VALUES OK (Y/N) ? Y

  IT.        EPSILON       SUM OF SQUARES
```

```
    0      0.000000E+00        30603.297

TYPE C,R,P,D,S,G,X OR H FOR HELP ? C
NUMBER OF ADDITIONAL ITERATIONS ?  10

  IT.        EPSILON       SUM OF SQUARES
    1      1.000000E-03         6108.195

  IT.        EPSILON       SUM OF SQUARES
    2      1.000000E-03         2041.514

  IT.        EPSILON       SUM OF SQUARES
    3      1.000000E-03          950.993

  IT.        EPSILON       SUM OF SQUARES
    4      1.000000E-03          711.638

  IT.        EPSILON       SUM OF SQUARES
    5      1.000000E-03          641.188
  IT.        EPSILON       SUM OF SQUARES
    6      1.000000E-03          617.687

  IT.        EPSILON       SUM OF SQUARES
    7      1.000000E-04          615.630

  IT.        EPSILON       SUM OF SQUARES
    8      1.000000E-05          601.930

  IT.        EPSILON       SUM OF SQUARES
    9      1.000000E-06          601.316

CONVERGED, TYPE "S" TO GET FINAL RESULTS
TYPE C,R,P,D,S,G,X OR H FOR HELP ? S


 L I G A N D: SCAFIT VERSION 2.4

CAUTION: Results and interpretation of analysis depend upon
several important assumptions. See User's Guide for details

FINAL PARAMETER ESTIMATES,    SAT FIT NO.   2

        VALUE        %CV     LOG-VALUE       Kd          K*R

K11    2.2553E+09    29.%     9.3532      4.4339E-10    0.0587
K12    6.8240E+05    32.%     5.8340      1.4654E-06    0.0174
K21    6.4368E+08    27.%     8.8087      1.5536E-09    0.0167
K22    1.3456E+06    24.%     6.1289      7.4316E-07    0.0344
R1     2.6017E-11    26.%
R2     2.5559E-08    24.%
N1     7.1125E-02     4.%
N2     3.4910E-02     4.%
C1     1.0000E+00
```

```
C2      1.0000E+00
C3      1.0000E+00
C4      1.0000E+00
```

These values are compatible with those from the two-site fit given in section 4.1.3. Thus, we have more confidence in the two-site model.

```
K11*R1  0.0587        7.%
K12*R2  0.0174       21.%
K21*R1  0.0167       14.%
K22*R2  0.0344        5.%

WOULD YOU LIKE THE COVARIANCE-CORRELATION MATRIX  (Y/N) [N] ?
<return>


CURVE      SUM OF     D.F.      MEAN       RUNS        RESIDUALS
           SQUARES              SQUARE

EXP1A      284.969    12.0      23.7474    6  O.K.     +++----++---+-
EXP2A      54.884     5.0       10.9768    2  N.O.K.   -----++
EXP3A      103.932    8.0       12.9915    3  N.O.K.   -----+----
EXP4A      157.532    8.0       19.6915    2  POOR     ---+++++++

TOTAL      601.316    33        18.2217                16          25


------------------------------

 FIT     SUM OF       D.F.     MEAN SQUARE       F
         SQUARES

  1     4113.885      36       114.2746       64.2600     (P= 0.0000)
  2      601.316      33        18.2217                    ---
```

The improvement of the fit for two-site is dramatic and significant ($p<.001$) as determined by the sum of squares F-test. Using only EXP3A, we were unable to obtain this result, yet were led to attempt the two-site model. Extra information in EXP1A and EXP2A seems to have made the difference.

```
GRAPHICS FILE NOW BEING WRITTEN  FOR FIT NO.   2

                  ------------------------------

#         LOG(TOTAL)      B/T          B/T-PRED       DIFF        RESIDUAL
                                                                  PLOT
CURVE: EXP1A
1         -10.869     0.0841       0.0791       0.0049          |*
2         -10.014     0.0845       0.0785       0.0060          | *
3         -9.744      0.0820       0.0779       0.0041          |*
4         -9.460      0.0751       0.0768       -0.0017        *|
5         -9.072      0.0742       0.0744       -0.0002         *
```

```
6       -8.775    0.0691      0.0720      -0.0029     *|
7       -8.300    0.0664      0.0683      -0.0019     *|
8       -7.778    0.0669      0.0655      0.0013       *
9       -7.301    0.0659      0.0635      0.0024       |*
10      -6.477    0.0550      0.0558      -0.0009      *
11      -6.301    0.0517      0.0530      -0.0013     *|
12      -5.778    0.0437      0.0439      -0.0002      *
13      -5.079    0.0394      0.0364      0.0029       | *
14      -4.301    0.0321      0.0342      -0.0021     *|
```

Note the lack of pattern now in the residuals for EXP1A.

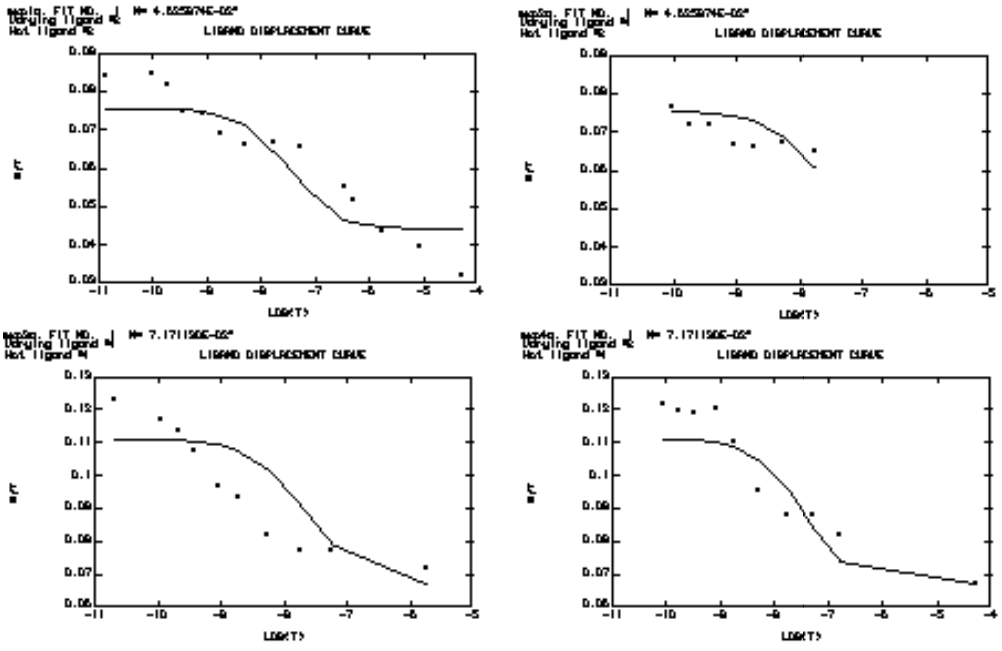
```
CURVE: EXP2A
15    -10.041     0.0766      0.0770      -0.0004      *
16    -9.740      0.0721      0.0754      -0.0033     *|
17    -9.439      0.0718      0.0732      -0.0013      *
18    -9.041      0.0668      0.0699      -0.0031     *|
19    -8.740      0.0660      0.0679      -0.0019     *|
20    -8.263      0.0673      0.0659      0.0014       |*
21    -7.740      0.0649      0.0649      0.0000       *
CURVE: EXP3A
22    -10.678     0.1234      0.1266      -0.0032     *|
23    -9.951      0.1170      0.1202      -0.0032     *|
24    -9.693      0.1137      0.1153      -0.0016      *
25    -9.415      0.1076      0.1085      -0.0008      *
26    -9.031      0.0967      0.0982      -0.0015      *
27    -8.735      0.0938      0.0916      0.0022       |*
28    -8.262      0.0820      0.0853      -0.0033     *|
29    -7.740      0.0775      0.0825      -0.0050     *|
30    -7.263      0.0775      0.0813      -0.0038     *|
31    -5.740      0.0717      0.0734      -0.0018     *|
CURVE: EXP4A
32    -10.079     0.1219      0.1248      -0.0029     *|
33    -9.778      0.1196      0.1229      -0.0033     *|
34    -9.477      0.1194      0.1197      -0.0004      *
35    -9.079      0.1206      0.1125      0.0081       | *
36    -8.778      0.1080      0.1050      0.0030       |*
37    -8.301      0.0956      0.0934      0.0022       |*
38    -7.778      0.0878      0.0855      0.0023       |*
39    -7.301      0.0883      0.0821      0.0062       | *
40    -6.778      0.0821      0.0793      0.0028       |*
41    -4.301      0.0674      0.0666      0.0008       *


ANOTHER FIT (Y/N) [N] ? <return>

DO YOU WANT TO REDEFINE THE INPUT SET (Y/N) [N] ? <return>
WOULD YOU LIKE TO SEE THE GRAPH (Y/N) [N] ? <return>
SESSION LOG IN SAT.SES
GRAPHICS FILE IN SAT.GRF

MON
```

This gets you back to the monitor level.

4.4.3 Graph the data

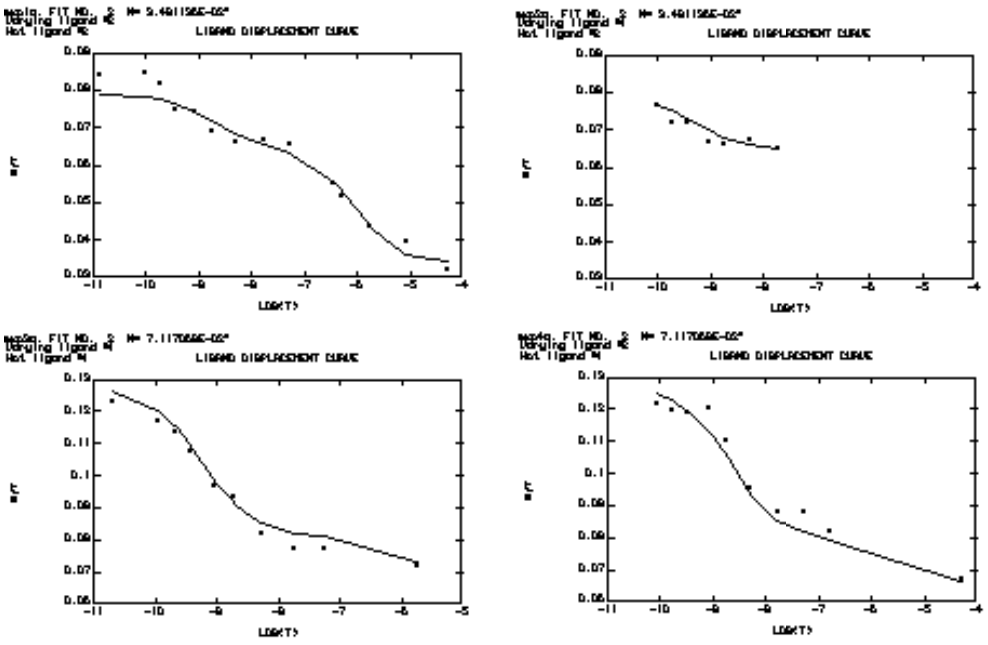
For brevity, we show a composite of the output for EXP1A-EXP4A. For operational instructions for SCAGRF, see section 4.1.1 or 3.4. Note the significant lack of fit in all four panels.

One Site Fit



This fit is substantially better than the first fit, as seen by the reduced departure of the data from the curves. A small but suggestive departure still remains in the fourth panel, not explained by a two- site fit.

Two Site Fit



5. Data from more than one experiment -- Correction factors

In many situations, you may have data collected in different experiments where the binding proteins were prepared under slightly different conditions. However, you may wish to analyze all of the data simultaneously, since the values of the binding affinities should be the same for each experiment. In this case we can assume that the only differences between experiments arise due to fluctuations in receptor or binding protein concentration from experiment to experiment.

To account for these fluctuations in R, we have introduced a correction parameter Ck, which may be fitted to the data, and which adjusts or scales the values of Rj for that particular experiment (k). Thus, if the second curve (experiment #2) has three times the number of receptors present as the first curve, then C2 will be 3.0 and C1 should be 1.0. Generally, we make all of the correction factors relative to the first curve, i.e. we fix C1 = 1. Thus, all of the other experiments are adjusted to the first one.

If there is more than one class of receptor sites, then each class is scaled by the same correction within each experiment. Thus, the ratio of R values R1:R2:R3 is constrained to be the same for each experiment. Further, the correction also scales the non-specific value, Ni. If the specific binding for experiment 2 is assumed to be twice that for experiment (curve) 1 (C2=2.0), then non-specific binding is also assumed to be twice as high for curve 2. Under certain circumstances, non-specific binding should be separately corrected for each experiment. This may be done with the optional D-parameter (See program swiches).

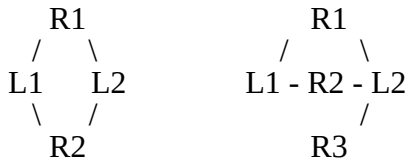
Note that we may have several curves per experiment by constraining several C's to be shared. For example, we may require the following: C1=C2=C3, C4=C5, C6=C7=C8.

Experience has shown that, at least with membrane preparations, there is sufficient variability between even well controlled experiments to require the use of correction factors. Failure to do so results in a totally inadequate fit. Correction factors may also be fixed at measured protein or DNA concentrations. This will cause the R values to be scaled by protein units, i.e., moles of binding sites per mole of protein.

6. Interpreting the results

Statistical Model, Hypothesis Testing, Adequacy of the fit

The program is capable of answering a number of well-posed statistical questions about your data, usually of the form "Is Model A a better representation of the data than the alternative Model B?" For example, "Does the data provide evidence of a second class of receptors, or does a single class explain the data sufficiently well, given the experimental noise?" With multiple ligands, more complex questions can be posed, involving which receptor classes bind to each ligand. It is often helpful in thinking about the problem to specify alternative models in the form of diagrams such as these:



Model A Model B

After each model has been fit and its parameter values estimated, one chooses between models of different complexity (different number of free parameters) using an F-test criterion on the residual variances. Essentially, we first assume that the simpler (smaller number of free parameters) model is correct and try to justify the added complexity in terms of the increase in "goodness-of-fit" of the second model. Specifically, define

$$F = \frac{(SSa-SSb) / (dfa-dfb)}{(SSb / dfb)}$$

where SSa is the residual sum of squares for Model A, SSb is the residual sum of squares for Model B, and dfa, dfb are the degrees of freedom for Models A and B, respectively. Then, if F is greater than the critical value for the F statistic with (dfa-dfb) and dfb degrees of freedom, at the P=.05 level, we can say that the second, more complex model provides a significantly better fit to the data. This is the "extra sum of squares" F-test mentioned by Draper and Smith in their book Applied Regression Analysis (Wiley, New York, 1981).

One should note at this point that the choice of variance model parameters may have a strong influence on the relative fits of Model A and Model B. This is especially true if the reason one model is chosen over the other depends on a few extreme points in one of the curves. In such a situation, a reasonable approach is to fit the data both with and without these questionable points. If the results are the same, then the weighting model is not an issue. However, if the results do change, then the choice of weighting model should be carefully re-validated (Get expert statistical advice in this situation).

In some cases, the parameters of the more complex model may not be well-determined, and the program may not converge. We may still be able to conclude that it is the better model if we can find some set of parameter values which provide a significantly better fit than the simpler model. Thus, even though some parameters may have large uncertainties (greater than 100%), the overall model may still provide a better fit to the data.

In addition to comparing the relative goodness of fit of two models, one can gauge the appropriateness of a certain model to the data by using the RUNS TEST (see Bennett and Franklin, Statistical Analysis in Chemistry and the Chemical Industry,New York, Wiley and Sons, 1954). This test assumes that the order of the signs of the residuals (differences between data point and fitted curve) should be random. Thus, a pattern of signs such as ++++---+++++ (3 runs in 12 residuals) would be unlikely to occur by chance, and might signify a poor fit of the model to the data. The level of significance of such patterns is computed by the program.

Confidence Intervals and Standard Errors.

The standard errors (actually %CV is printed out) given by the program are approximations to the truth. They assume normal gaussian errors in the data, and are asymptotically correct (when number of data points is large). Thus, they are best treated only as indications of the reliability of the parameters. One should note that there is often an extremely high covariance between a given pair of parameters (e.g. K11 and R1) such that the standard errors for the two parameters are misleadingly small. That is, considering each parameter independently may lead to confusion. A remedy for this is to make a "K vs R" plot giving the confidence ellipse around the K11 and R1 estimates (see Munson and Rodbard, 1984). This usually shows a large negative covariance between these two parameters. Increasing the K estimate by a factor of 2 and decreasing the R estimate by 2 may not materially affect the goodness of fit to the data.

Further, when computing confidence intervals for the parameters, one finds that by first taking logarithms of the parameters, one can get more reasonable results, especially in the case of large standard errors. This is because the parameters are more nearly log-normally distributed. Thus, to calculate the 95% confidence interval for the parameter K11, first convert K11 and its standard error to log units. Then find the conventional confidence interval using the Students t statistic, then convert back to the original units. The number t is roughly 2.0 for a 95% confidence.

$$
\begin{aligned}
K11' &= \ln(K11) \\
CV &= \text{s.e.}(K11) / K11 \\
\text{lower bound } (K11') &= K11' - t \times CV \\
\text{upper bound } (K11') &= K11' + t \times CV \\
\text{lower bound } (K11) &= \exp(\text{lower bound } (K11')) \\
\text{upper bound } (K11) &= \exp(\text{upper bound } (K11'))
\end{aligned}
$$

Notice that logarithms are taken to the base e, although, you may use base 10, if you introduce the correction factor, 2.303, thus

$$\ln(K11) = 2.303 \times \mathrm{Log10}(K11)$$

Weighting Models

The choice of weighting model may have a large influence over the results of the fit. With inappropriate weighting, the program may not even converge, whereas correct or nearly correct weighting may allow convergence in only a few iterations. Further, weighting affects the statistical interpretations of the results. Specifically, before comparing two models, make sure that both runs use the same variance model coefficients. Program LIGAND does this automatically if the comparison is done in a single session.

Wildly different values for the residual variance can result if different weighing coefficients are used, thus masking any true differences in the appropriateness of the models in question. Weighting models also affect the size of the calculated %CV for the parameters.

The weighting model used in SCAFIT is a general one. It models the variance as either a quadratic or exponential function of the response level (y). The default values for the parameters (A0=A1=A3=A4=0 and A2=.0001) are really quite reasonable for ligand binding experiments, since they correspond to a constant one percent error in [Bound]. This model may underestimate the variance for very low values of y, however. Thus, you might set A0 to some small, non-zero value. Remember, that A2 and A4 are unitless, A1 has units of the original data (e.g. femtomolar), A0 has [original units] squared and A3 has [units] raised to the (2-A4) power. These values must be set accordingly.

If you wish to improve the values for the weighting model parameters, make a plot of variance (Y) vs average Y value for a set of tubes which have been run in replicate. You need at least 50 tubes run in duplicate to get reliable estimates for your particular variance model. After making the plot, do a linear and quadratic regression on the points (or take logs on both axes and then do linear regression to get values for A3 and A4). For a more complete explanation see (Rodbard D, et al, Clin Chem 22, 350-358(1976).).

7. PERSONAL COMPUTER OPERATION

REQUIREMENTS

The LIGAND system will run on most IBM-PC compatible machines including the IBM PC AT, i386 and i486 machines. The programs will run with or without numeric coprocessor, although program operation is dramatically improved with a coprocessor. The SCAGRF program requires a graphics adapter. See the end of this section for a list of machines that run the LIGAND system. It may prove helpful to read through the beginning of this manual and also use the CONVEX sessions throughout the manual for additional assistance.

GETTING STARTED

Before you use the LIGAND system you should make a backup copy of the distribution disk for regular use using one of the following procedures.

Two floppy system :

1) place LIGAND disk in drive A:
2) place blank formatted disk in drive B:
3) at the A> prompt type :
   COPY *.* B:
   and hit the enter key.

Hard disk system :

1) place LIGAND disk in drive A:
2) from the DOS subdirectory on the hard disk type :
   DISKCOPY A: B:
   and hit the enter key.
3) follow the instructions given by the DISKCOPY program, using a blank disk as your target diskette.

This creates a backup copy of the LIGAND system. You are now ready to start using the programs. Remember to toggle the caps lock key on before running any of the programs because some of the input must be in capital letters.

OPERATION

Two floppy system:

You will probably want to maintain a seperate diskette just for your data files. This data diskette can go in the B drive. The only files on the disk in the A: drive are the LIGAND system files. With the LIGAND system disk in the A: drive and a data disk in the B drive, you are ready to begin. To run any of the programs, first type B: to make the B drive the default drive. Now type A:<program name> and hit the enter key. <program name> refers to any of the LIGAND system programs - SCAPRE, SCAHOT, SCAFIT, or SCAGRF.

Also, if you would like to run any of the LIGAND system programs from either the A: drive or the B: drive without including the drive specification, you can use the DOS path statement to set a path to your A: drive.

Hard Disk system:

You will probably want to create a subdirectory for the LIGAND system on your hard disk. The procedure for setting up a subdirectory follows.

1) From the C> prompt type 'MD LIGAND' and hit the enter/return key. This creates the subdirectory. You only need to do this once.

2) Now type 'CD LIGAND' and hit the enter/return key. This makes LIGAND the current directory.

3) With the LIGAND system disk in the A: drive, type
'COPY A:*.*'
and hit the enter/return key. This copies all the files to the hard disk.

4) To begin any of the programs, all you need to do is type the name of the program (without the extension) at the C> prompt and hit the enter/return key.

The next time you want to use the LIGAND system, make sure you are in the LIGAND subdirectory before trying to run any of the programs. You can do this by typing 'CD LIGAND' from the root directory of your hard disk. Once you are in the ligand subdirectory you can run any of the programs by simply typing the name of the program and hitting the enter/return key.

HARDWARE

The LIGAND system has been tested and run on the following personal computers:

IBM PC, PC/XT, PC/AT
ATT 6300
Zenith Z-150 series
Compaq Plus Portable

All of the above computers were tested with an IBM compatible colors graphics adapter (CGA). SCAGRF will also work with a HERCULES, EGA, VGA, or MCGA graphics boards. Some versions of LIGAND may require a 80287 coprocessor on the IBM PC/AT.

DOS COMMAND LINE SWITCHES

The SCAFIT program (v 3.0) supports four command line switches. These allow you to involve special options within the program.

SWITCH DEFINITIONS:

C - Cooperativity option. This option enables the cooperativity parameters B1, B2, etc. The value measures the degree of cooperativity of the corresponding site. A value of 1 is equivalent to no cooperativity, > 1 is "positive cooperativity" and < 1 is "negative cooperativity". The underlying model supposes that each receptor is actually a bivalent pair which binds the second ligand molecule with greater or less affinity than the first. For a full explanation of the meaning of the parameter, see Munson, P.J. and Rodbard, D. Computerized analysis of ligand binding data: Basic principles and recent developments, in Computers in Endocrinology, Rodbard, D. and Forti, G. Eds, Raven Press, New York, 117-145 (1984).

D - A seperate set of correction factors the D-factor, which are just like the C-factors, adjusts for varying nonspecific binding in seperate curves. In this option, C multiplies the R parameters and D multiplies the N parameters.

N - Session file option. By default, the program creates a session file. If this option is present in the command line, then the session file will not be created.

W - This option causes the program to read the third column in the .CRV file. These numbers (number of replicates which were averaged to get the B and T values) are then used as weights inside the program. These weights are in addition to the weights determined by the variance model parameters. These weights are used to give twice as much emphasis to the mean of duplicates as for a single observation, for example. Not using the /WEIGHT option is equivalent to setting column 3 to all ones.

DEFAULT VALUES:

COOPERATIVITY	= OFF
SESSION FILE	= ON
WEIGHTED FIT	= OFF
D-CORRECTION FACTORS	= OFF

EXAMPLES:

`C : \SCAFIT/C`	This will turn on the cooperativity feature (B parameters)
`C : \SCAFIT/N`	This will suppress creation of a session file
`C : \SCAFIT/W`	This will cause the parameters to be weighted
`C : \SCAFIT/D`	This will turn on the D-parameter feature

7.1 Preparing the data - SCAPRE

The following section demonstrates the use of the SCAPRE program. It assumes you have a two drive system with the program disk in the A: drive and the data disk in the B: drive.

```
B> A:SCAPRE
```

Start the program from the DOS prompt.

```
                    ***   LIGAND SYSTEM   ***
     Curve fitting and data analysis for ligand binding data
                 National Institutes of Health

                PROGRAM SCAPRE V2.1  --  AUG. 1985

FILE NAME FOR STORING THIS DATA (NO EXTENSION) ? EXP3A
```

After you enter the file name here and hit the enter key, the screen will clear and you will be prompted in the upper left corner to start entering data.

```
 1 NAME OF THIS EXPERIMENT: HOT AND COLD ANALOG I
```

After you enter the name of the experiment and hit return, the next prompt will appear directly below this one.

```
 2 COUNTING TIME(MIN): 5.0
```

Continue entering responses to the prompts. After you input line 10, the next prompt will appear in the upper right of your screen. There will be 5 values to enter. The following prompt will appear on the left side of the screen again, below line 10. Enter the counts starting at line 16 and continue until they are all entered. Line 38 is the last line of input allowed. When you have entered the last line of data, or at any other time during the input phase, just hit the return key in response to the prompt. This gets you into the COMMAND MODE. If you type HELP, you will see this message on your screen.

```
THE LEGAL COMMANDS ARE:
'INPUT'     BEGIN ENTERING THE DATA AT THE NEXT AVAILABLE LINE
'HELP'      TYPE THIS MESSAGE
'FINISH'    PRESENTS AND STORES CALCULATED DATA INFILE AND
            ON THE SCREEN
'DELETE'    BEGINS THE DELETION OF A LINE OF DATA YOU WILL BE
            PROMPTED FOR LINE NUMBER ON THE FOLLOWING LINE
'INSERT'    BEGINS INSERTION OF A LINE YOU WILLBE PROMPTED
            FOR THE NEW LINE NUMBER

ANY OTHER COMMAND WILL RESULT IN THE MESSAGE 'ILLEGAL COMMAND'

HIT ANY KEY TO CONTINUE   <spacebar>
```

Strike any key to return to the COMMAND MODE. The screen clears and your input data is displayed.

```
 1 NAME OF THIS EXPERIMENT: HOT AND COLD ANALOG I
```

```
 2 COUNTING TIME(MIN): 5.
 3 VOLUME OF TUBE(ML.): .5
VARIANCE(Y)=A0+A1×Y+A2×Y2+A3×Y A4
 4 SPECIFIC ACTIVITY (UC/UG): 1000      11 A0: 0
 5 MEAN TOTAL COUNTS: 65789             12 A1: 0
 6 HOT LIGAND #: 1                      13 A2: 0.0001
 7 COLD LIGAND #: 1                     14 A3: 0
 8 MOL.WT., MEASURED LIGAND: 1300       15 A4: 0
 9 MOL.WT., COLD LIGAND: 1100
 10 DOSE UNITS: NANOGRAM
DOSE  COUNTS  COUNTS   COUNTS
 16 : 0. 7997. 7891. 8081. 8265. 8262. 8213.
 17 : 0.05 7676. 7713.
 18 : 0.1 7244. 7716.
 19 : 0.2 7210. 6950.
 20 : 0.5 6451. 6268.
 21 : 1. 6020. 6321.
 22 : 3. 5477. 5317.
 23 : 10. 4926. 5266.
 24 : 30. 5250. 4943.
 25 : 1000. 4931. 4724. 4479. 5072. 4385. 4694.

COMMAND ? FINISH
```

Now that all the data is entered we will demonstrate some of the editing features.

INPUT

At the command prompt type INPUT.You will be prompted for input at the next line number following your last line of input.

REPLACE

Replace is used to replace an existing line of input with a new one. At the command prompt type REPLACE followed by the line number you want to replace. (Ex. REPLACE 10) The cursor will move up to the line number you chose and the old value for that line will be erased. You can input the new value for that line.

DELETE

Delete is used to remove a line of input from the data set. Type DELETE followed by a line number (ex. DELETE 17). In this example line 17 will be deleted and all lines after it will move up one line.

INSERT

Insert is used to add an additional line of input between two existing lines. Issue the command INSERT followed by the line number you wish to add.

When you reach the point where you are satisfied with your

input data, you will want to exit the program and continue. Do this by issuing the command FINISH. This saves your data file and also creates a curve file (.CRV). After this the screen will clear and you will see the following table.

LINE	DOSE (NANOGRAM)	TOTAL COUNTS	AVG OF BOUND COUNTS	TOTAL CONC [M]	BOUND CONC[M]	B/T
16	0	65789	8118.167	2.10E-11	2.59E-12	.1234
17	.05	65789	7694.5	1.12E-10	1.31E-11	.1170
18	.1	65789	7480	2.03E-10	2.31E-11	.1137
19	.2	65789	7080	3.85E-10	4.14E-11	.1076
20	.5	65789	6359.5	9.30E-10	8.99E-11	.0967
21	1	65789	6170.5	1.84E-09	1.72E-10	.0938
22	3	65789	5397	5.48E-09	4.49E-10	.0820
23	10	65789	5096	1.82E-08	1.41E-09	.0775
24	30	65789	5096.5	5.46E-08	4.23E-09	.0775
25	1000	65789	4714.167	1.82E-06	1.30E-07	.0717

DO YOU WISH TO CONTINUE WITH THIS PROGRAM ? N

Answer 'N' to exit the program, or 'Y' to edit or enter more data.

7.2 Preparing the data - SCAHOT

The data entry program SCAHOT operates the same as SCAPRE. There are a few different parameters, which you can see by comparing the SCAHOT screen below with the SCAPRE screen in the documented SCAPRE session (section 7.1). Use the SCAPRE session as a guide to using the SCAHOT program, keeping in mind the differences between the two.

Here is the SCAHOT screen after recalling the data file SUZ1.BAS. This data set will be used in the following section for the fitting program.

```
 1 NAME OF THIS EXPERIMENT: SUZ1
 2 COUNTING TIME(MIN): 1
 3 VOLUME OF TUBE(ML.): .5      VARIANCE(Y)=A0+A1×Y+A2×Y 2+A3×Y A4
 4 SPEC. ACTIVITY (DPM/MOLE): 46.398E15 11 A0: 0
 5 COUNTING EFFICIENCY (CPM/DPM): .43   12 A1: 0
 6 LIGAND #: 1                          13 A2: 0.0001
 7 BINDABILITY FRACTION (1. IF 100%): 1 14 A3: 0
 8 MOL.WT.: 584.64                      15 A4: 0
 9 BACKGROUND COUNTS [0]: 55
10 DOSE UNITS OF COLD LIGAND: NANOMOLES
COLD TOTAL-COUNTS   COUNTS  COUNTS     COLD TOTAL-COUNTS  COUNTS
 16 : 0 380666 2780 3076                28 : 0 9563 301 210
 17 : 50 380666 655 651                 29 : 50 9563 90 86
 18 : 0 287380 2878 2418                30 : 0 4871 127 140
 19 : 50 287380 616 622                 31 : 50 4871 67
 20 : 0 182300 1422 1530                32 : 0 2262 81 98
 21 : 50 182300 403 340                 33 : 50 2262 57 62
 22 : 0 74406 896 865
 23 : 50 74406 209 250
 24 : 0 38073 631 517
 25 : 50 38073 128 172
 26 : 0 19249 453 344
 27 : 50 19249 103 127
```

7.3 Fitting the data - SCAFIT

The SCAFIT program on the IBM/PC behaves nearly identically to SCAFIT on the DEC-10. Compare this session with section 3.3.

```
B> A:SCAFIT
```

Start the SCAFIT program.

```
                  ***   LIGAND SYSTEM   ***
          Curve fitting and data analysis for binding data
                    National Institutes of Health


              PROGRAM SCAFIT  --  FORTRAN VERSION 2.3.2


FILE NAME FOR GRAPHICS (WITHOUT EXTENSION) ? SUZ1
NUMBER OF LIGANDS (HIGHEST NUMBER USED) ? 1

TYPE THE DATA FILE NAME(S) (WITHOUT EXTENSION)
SEPARATE WITH <CR> END WITH BLANK LINE
? SUZ1
? <return>

CURVE FILE  SUZ1.CRV
WEIGHTING PARAMETERS
VARYING LIGAND:   1   LABELED LIGAND:   1
NUMBER OF POINTS:       18

NUMBER OF SITES ? 1

DO YOU WISH TO WEIGHT POINTS ACCORDING TO
NUMBER OF TUBES AT A GIVEN DOSE (Y/N) [N] ? <return>

THE FOLLOWING PARAMETERS WILL BE CONSIDERED:
K11 R1  N1  C1

THE FOLLOWING PARAMETER GROUPS HAVE A SHARED VALUE:


DO YOU WANT THE SAME SHARED PARAMETERS AS
BEFORE (Y/N) [Y] ? <return>

LIST THE CONSTANT PARAMETERS (NO COMMA)
[C1 ] <return>

C1  = [   1.00000     ] ? <return>
```

```
DO YOU WANT AUTOMATIC INITIAL ESTIMATES (Y/N) [Y] ? <return>

AFTER ADJUSTING FOR NONSPECIFIC, THERE ARE    9 POINTS LEFT

INITIAL ESTIMATE FOR K11 [  5.075841E+07 ] ? <return>
INITIAL ESTIMATE FOR R1  [  3.003147E-10 ] ? <return>
INITIAL ESTIMATE FOR N1  [  2.129737E-03 ] ? <return>

THE INITIAL ESTIMATES ARE
K11 =   50758410.0000000
R1  =   3.003147E-010
N1  =   2.129737E-003
C1  =          1.0000000

ARE THESE VALUES OK (Y/N) ? Y

IT.       EPSILON      SUM OF SQUARES
  0      .000000             9881.72300

TYPE C,R,P,D,G,X,S OR H FOR HELP ? C
NUMBER OF ADDITIONAL ITERATIONS ? 3

IT.       EPSILON      SUM OF SQUARES
  1      .000000             7544.50600

IT.       EPSILON      SUM OF SQUARES
  2      .000000             7375.60800

CONVERGED, TYPE "S" TO GET FINAL RESULTS
TYPE C,R,P,D,G,X,S OR H FOR HELP ? S


FINAL PARAMETER ESTIMATES, SUZ1.GRF     FIT NO.   1

       VALUE          %CV     LOG-VALUE   Kd           K*R

K11    6.3188E+07     38.%    7.8006      1.5826E-08   1.5688E-02
R1     2.4827E-10     31.%
N1     2.3679E-03     7.%
C1     1.0000E+00

K11*R  1.5688E-02     13.%

WOULD YOU LIKE THE COVARIANCE-CORRELATION MATRIX  (Y/N) [N] ?
<return>


CURVE      SUM OF        D.F.     MEAN         RUNS     RESIDUALS
           SQUARES                SQUARE

SUZ1       7375.608      15.0     491.7072     8        O.K.  +------+++++-+--
TOTAL      7375.608      15       491.7072              8     10
```

```
                GRAPHICS FILE NOW BEING WRITTEN  FOR FIT NO.   1

                        ------------------------------

#       LOG(TOTAL)      B/T         B/T-PRED    DIFF         RESIDUAL PLOT
CURVE: SUZ1.CRV
1       -7.418          .0075       .0069       .0006          *
2       -4.000          .0016       .0024       -.0008        *|
3       -7.541          .0090       .0079       .0011          |*
4       -4.000          .0020       .0024       -.0004        *|
5       -7.738          .0078       .0096       -.0018        *|
6       -4.000          .0017       .0024       -.0006        *|
7       -8.128          .0111       .0129       -.0018        *|
8       -4.000          .0023       .0024       .0000          *
9       -8.419          .0137       .0148       -.0012         *
10      -4.000          .0025       .0024       .0001          *
11      -8.716          .0179       .0161       .0018          *
12      -4.000          .0031       .0024       .0008          |*
13      -9.021          .0211       .0169       .0042          |*
14      -4.000          .0035       .0024       .0011          |*
15      -9.316          .0163       .0173       -.0010         *
16      -4.000          .0025       .0024       .0001          *
17      -9.655          .0156       .0175       -.0019         *
18      -4.000          .0020       .0024       -.0003        *|


WOULD YOU LIKE TO SEE YOUR GRAPH NOW [N] ? <return>
ANOTHER FIT (Y/N) [N] ? <return>
DO YOU WANT TO REDEFINE THE INPUT SET (Y/N) [N] ? <return>
GRAPHICS FILE IN SUZ1.GRF
```

7.4 Graph the data - SCAGRF

The SCAGRF program is an IBM-PC based program designed to assist users in plotting the results obtained from LIGAND. It uses as its input the graph files (.GRF) created by the SCAFIT program.

SCAGRF has been designed to work on machines with the following graphics cards.

Hercules
CGA
EGA
VGA
MCGA

The type of video card is automatically detected by the program.

SCAGRF now supports laser printers that use the picture drawing language PostScript. The best hard copy plots are achieved using a laser printer. Dot matrix printers are still supported. Previously, the plots were produced at screen resolution. The software has been modified to take advantage of the higher resolution available on 9 pin dot matrix printers. SCAGRF uses a printing protocol which works on most dot matrix printers.

In order to support still more printers, SCAGRF is capable of producing a Lotus 123 .PIC file. This file can be printed using the print graph program distributed with Lotus 123 version 2.

SCAGRF Operation

The SCAGRF program exits as a single executable file. Your may run it from a floppy drive or copy it to your hard disk.

Start the program by typing scagrf at the DOS prompt.

```
>scagrf
```

You will be prompted for a .grf file name.

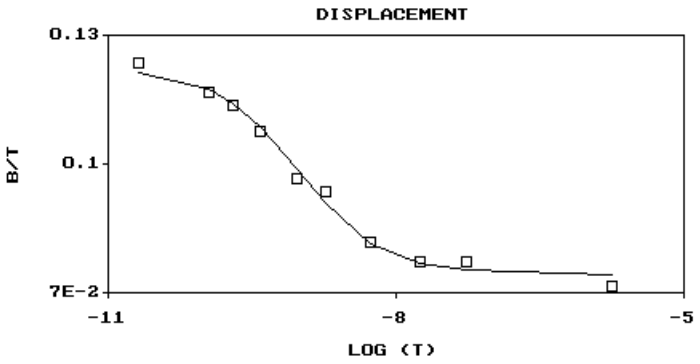
```
Graphics file name (no extension) ? scagrf
```

(Don't enter the .grf file extension, it is assumed.)
The file scagrf.grf, found on the Ligand distribution diskette, will be used to demonstrate SCAGRF operation.

SCAGRF produces three types of plots: displacement, scatchard and conglomerate. When the program starts the plot displayed will be displacement by default.

The following displacement plot will be displayed on the screen.

```
A:SCAGRF.SES :  EXP3A.CRV    FIT NO.  1  N= 8.060675E-02
VAR.LIG # 1   LAB.LIG #  1
```




```
C:Change  S:Scatchard     N:Next  F:First  P:Print  R:Restart  Q:Quit
```

The line at the bottom of the screen contains 7 different commands...

```
C:Change S:Scatchard N:Next F:First P:Print R:Restart Q:Quit
```

A command is entered by typing the first letter of the command. No return is necessary. These commands are only active while a plot is on the screen and the commands are displayed at the bottom of the screen.

Change Command

Type the letter 'C', the following screen will be displayed.

```
Graph Parameters Set Up Screen

  Label 1: SCAGRF.ESE: EXP3A.CRV   FIT NO. 1 N = 8.0606
  Label 2: VAR.LIG # 1  LAB.LIG # 1
  Title:   DISPLACEMENT
  X label: LOG (T)
  Y label: B/T

  x min:    -1.1000E+01       x max:      -5.0000E+00
  y min:     7.0000E-02       y max:       1.3000E-01
  Recompute axis limits (Y/N): Y

  X divisions (1-15): 2         Y divisions (1-15):  2
  Symbol Scaling Factor (1-10):  3
  Use confidence limits (+/-2 sigma) (Y/N) : N
  Nonspecific binding value for correcting scatchard: 0.08060675

  Plot conglomerate (Y/N) : N
  Varying Ligand (1,1): 1
  Labeled Ligand (1,1): 1
  Fit Number (1,1): 1


F2:Accept new values  Esc:Cancel
```

You can change the plot labels, title and x and y labels. The program keeps separate titles for the scatchard and displacement plots. In order to change the axis limits you must answer 'N' to Recompute axis limits (Y/N):, else the limits will be recomputed and your changes will not be reflected in the plot. The X and Y divisions are used to alter the number of tick marks on the respective axis.

The symbol scaling factor alters the size of the symbol used to plot a point.

To produce a conglomerate plot answer 'Y' to 'Plot conglomerate (Y/N):'. When a conglomerate plot is produced the file is searched for all plots that have the same varying ligand, labeled ligand and fit number. The data from these plots is pooled to produce a single displacement or scatchard plot.

Strike the Esc key to ignore your changes and return to the plot. Hiting 'F2' will register your changes and display an updated plot.

Scatchard Command

This command is only active when a displacement plot is being displayed. Striking the letter 'S' will result in the display of a scatchard type plot.

Displacement Command

This command is only active when a scatchard plot is being displayed. Striking the letter 'D' will result in the display of a displacement type plot.

Next Command

To display the next plot in the file, type the letter 'N'. The graph file will be read and the next plot will be displayed on the screen. If no more plots are in the file a message to this effect will be displayed. If the current plot is a scatchard type plot then the next plot will also be displayed as a scatchard plot.

First Command

You can display the first plot in the file at any time by typing the letter 'F'.

Print Command

Plot may be printed on a dot matrix printer or a postscript file may be created which can be downloaded to a laser printer which supports postscript. (You will need to have special hardware and software to download the postscript file.)

Enter 'P' to print the plot. The following screen will be displayed...

```
Printer Set Up Screen

These coordinates alter the size and position of the
plot box.
The lower left hand corner of the page is (0,0).

     Upper Left x page coordinate   (inches):   2.00
     Upper Left Y page coordinate   (inches):   9.00
     Lower Right x page coordinate  (inches):   7.00
     Lower Right y page coordinate  (inches):   6.00

     Post Script File (.ps will be appended):   scagrf


F1:Postscript F2:Save F3:Dot Matrix F4:Lotus 123 Esc:Cancel
```

Entering F1 will result in the creation (or replacement) of the file scagrf.ps. The default postscript file name is the prefix of the .grf file. You may enter your own file name instead of using the default. To print this file you will have to exit this program (i.e. hit escape and then Quit), and then download the postscript file scagrf.ps to your laser printer.

Creating a PostScript File

Entering F1 will result in the creation (or replacement) of the postscript file gsample.ps. The default postscript file name is the prefix of the .grf file. You may enter your own file name instead of using the default. To print this file you will have to exit this program (i.e. hit escape and then Quit), and then download the PostScript file gsample.ps to your laser printer. The page coordinates apply to PostScript file.

Dot Matrix Printing

Enter F3 to print the plot on a dot matrix printer hooked up to printer port LPT1. Once printing has started you may interrupt it by striking any key. SCAGRF supports 9 pin dot matrix printers which emulate the Epson FX series of printers. This method creates a plot at a much higer resolution than available with screen dumps. You may alter the size of the plot using the page coordinates.

Creating Lotus 123 Format .PIC Files

Entering F4 will result in the creation of the Lotus 123 .PIC file gsample.pic. (You may enter a different file name. .PIC is always appended.) This .PIC file may be printed using the Lotus 123 program Printgraph which is supplied with version 2 of Lotus 123. This enables SCAGRF to indirectly support a wide range of printers. Note, the page coordinates do not apply to this method.

F1, F2, F3 or F4 will result in your changes being saved. If you make changes in this screen and then enter Esc, without having entered F1, F2 or F3, then your changes will not be saved.

Restart Command

The restart command is used to change the .grf file.

Quit Command

Enter 'Q' to exit the program. Again, this command is only active when a plot is displayed.

Prt-Screen Keyboard Key

The prt-scr key is active only when a plot is being displayed. Screen dumps are supported in Hercules, CGA, EGA and VGA video modes. You do not have to have the MS-DOS graphics driver loaded to use this function as SCAGRF has its own print screen routine. In general, however, the Print command will result in better looking dot matrix plots.

8. MACINTOSH OPERATION

REQUIREMENTS

MacLIGAND will run on just about any Macintosh computer running system 6 or system 7. While MacLigand will run on most Macs with 32 bit addressing turned on, it is probably a good idea to turn 32 bit addressing off and reboot your machine before running the program. For Macintosh Quadra and Centris users please note that you must turn off the cache. A cache switch has been included in the software distribution that will allow you to do this.

LIGAND requires at least 600k of available RAM at execution. If SCAFIT is running on a one megabyte machine then some of the facilities that are loaded into resident memory (for example: MacBugs, Scrapbook pictures, RAM cache, etc...) might have to be removed. The LIGAND system has been tested on the following Apple Computers:

Macintosh plus	Macintosh SE
Macintosh SE/30	Macintosh II
Macintosh IIx	Macintosh IIcx
Macintosh IIci	Macintosh IIfx

Macintosh LIGAND was ported from the FORTRAN version which runs on the DEC-10 and the IBM-PC. The program was compiled using the Microsoft FORTRAN compiler (version 2.4) and the Macintosh interface was developed with the FACEIT 4.0 interface package (Faceware, 1310 N. Broadway, Urbana, IL 61801).

GETTING STARTED

You will find two files on the distribution diskette, a Read Me file and a self expanding archive file. The Read Me file will contain information regarding the latest version of the program. To install the LIGAND system, double click on the Ligand.SEA file. You will be asked where you want the programs placed.

The following files are being distributed:

MacSCAFIT vx.x
MacSCAPRE vx.x
exp1 data
exp1 data crv
exp2a data
exp2a data crv
exp3a data
exp3a data crv
exp4a data
exp4a data crv
help.txt

Program Descriptions
suz1 data
suz2 data crv
topics.txt
cache switch

If the files loaded onto your hard drive differ, please refer to the Read Me file on the distribution diskette.

The MacLIGAND system on the Macintosh consists of two executable files, MacSCAPRE and MacSCAFIT. Once the programs have been copied to your hard drive you can run MacSCAPRE or MacSCAFIT (the curve fitting program) by double clicking on them.

MAC-LIGAND INTRODUCTION

We have tried to develope MacLigand to follow the standard user-interface philosophy used on all Macintosh software. All software developed on the MacIntosh is menu-driven and controlled with a mouse, you must tell MacLigand what to do next by selecting an option from one of the menus.

For IBM-PC users of LIGAND the Mac version is somewhat different. The SCAHOT and SCAPRE programs from the IBM-PC version have been combined into the single program MacSCAPRE. The graphics have been integrated into the MacSCAFIT program which is nice because now you could for example perform a single iteration and then examine the plot. Also saturation plots have been added to the Mac version that are not available on the IBM-PC version. On line help is also available by clicking on the HELP button in the About Dialog.

Generally the logical first step to take when executing a MacIntosh program is to Open a file. This option is available under the File menu. In SCAPRE when Get Data File is selected from the File menu, information is automatically put into the Counts spreadsheet, and the View/Edit Data dialog box. This information can then be edited and reprocessed. In MacLigand when Open .CRV File... is selected, the file is displayed in the session window, and the data is read into the program internally to be fitted at a later time. In both programs, the menu selecting process should continue in a logical fashion until all data has been processed and fully examined.

The MacLigand program makes use of three window types:

1) An editor window
2) A spreadsheet window
3) A graph window

The SCAPRE program uses two editor windows (Curve file, and Data file), and two spreadsheet windows (Counts and Final Results). The editor windows are used to display the data file and curve file, and should NOT be edited. The spreadsheet windows are used to enter Counts data, and view the final results (much like the IBM-PC version when Finish is entered). In SCAFIT, there is no spreadsheet window, but there is one editor window and one graph window. The editor window is used to record the current session, and the graph window is used to plot the current fit.

The remainder of this chapter describes the menus of SCAPRE and SCAFIT, and runs through an example session of the data file EXP1a.

PREPARING THE DATA - MacSCAPRE

This section first describes the different Menus and then runs through a typical MacSCAPRE session. In the session text all menu references will be in bold type.

SCAPRE MENUS

THE APPLE MENU

The Apple menu gives you access to the About finder window and any desk accessories installed on your MacIntosh. (In addition, with Multi-finder on, you can use the Apple menu to move among open applications. For details, see chapter 10 of the Macintosh System Software User's Guide.)

About Scapre Displays program version number and program credits.

Clipboard The clipboard is used to capture any text or picture that is either cut or copied from any available text editor window or graphics window. The amount of RAM used by the clipboard, text editors, and pictures is also displayed.

Desk Accessories The SCAFIT program allows you to access any of the available desk accessories which are located under the apple menu of the finder.

THE FILE MENU

Open... Allows you to open a data file that was previously created by the program MacSCAPRE. Upon selection, data will be loaded into both the the Counts window, and the Experimental Parameters dialog (which can be found under the SCAPRE menu).

Import... Allows you to open a data file that was created from an application other than MacSCAPRE.

Save Used to update a file that already exists. Use this when you are modifying an existing raw data file.

Save As... Used to save the data, in the front window, to a new file.

Save Settings... Can be used to customize the appearance of your windows, and control which windows will be shown at launch time.

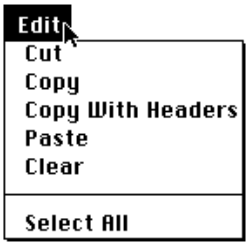
Page Setup Used to set up the page defaults to be used when printing.

Print... Sends the text or selected picture to the printer.

Transfer... Will quit the MacSCAPRE application and transfer to another application stored on disk.

Quit After selecting quit you will be prompted to save any files that you were working on.

EDIT MENU



Cut Will remove selected text or pictures from window or spreadsheet and place them into the clipboard for temporary storage. Any text or picture in the clipboard can then be pasted into another window, spreadsheet, or even into the scrapbook to be manipulated by other programs. (MacDraw, SuperPaint, etc.)

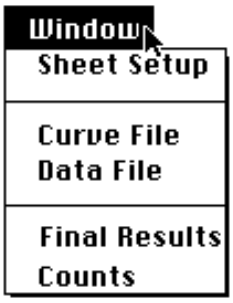
Copy Same as Cut, except that the text or pictures will remain in the window or spreadsheet.

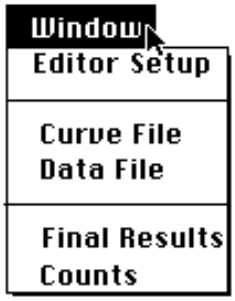
Copy With Headers Same as Copy, except that this option can only be used when the spreadsheet is the front window. This allows you to copy the spreadsheet headers along with the selected data in the spreadsheet.

Paste Once text or pictures have been either cut or copied, paste can be used to place them back in a window, spreadsheet, or into the scrapbook.

Clear Will clear any selected text or picture(s) from a window or spreadsheet. This information will also be removed from memory.

Select All Will select all of the text or pictures in the current (front) window or spreadsheet.





WINDOW MENU

The first menu item will be Sheet Setup if a spreadsheet is the front window. Otherwise, if an editor window is the front window then Editor Setup will be displayed.

Sheet Setup Used to set the format of the data in the current spreadsheet.

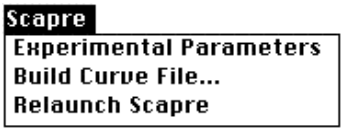
Editor Setup Used to set certain text and file handling options. To save these options each time the program is executed select **Save Settings...** from the file menu before quitting the program.

Curve File, Data File, Final Results, Counts Brings selected windows into view as the front window.

SCAPRE MENU

Experimental Parameters Displays a dialog box with experimental parameters such as specific activity, volume of tube, counting time, dose units etc.

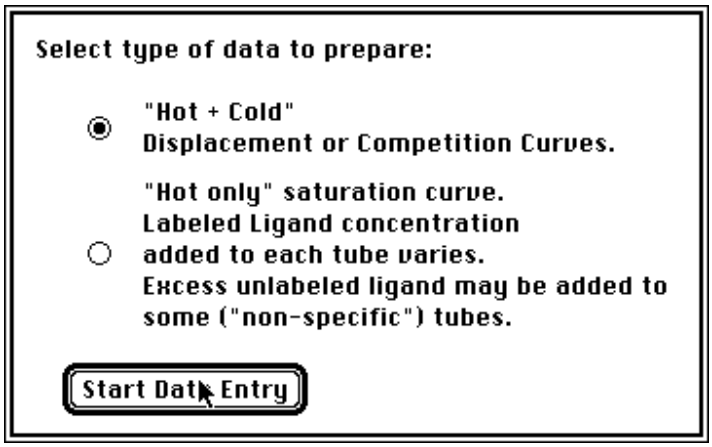
Build Curve File... This item will perform the calculations on the counts data and display final results in the final results spreadsheet. You will be prompted automatically to save and if you wish give the curve file a different name. After processing the data and creating the curve file you are ready to go on to the MacSCAFIT program.



Relaunch Scapre Will re-launch the program MacSCAPRE. (Displays a warning alert first.)

SCAPRE SESSION

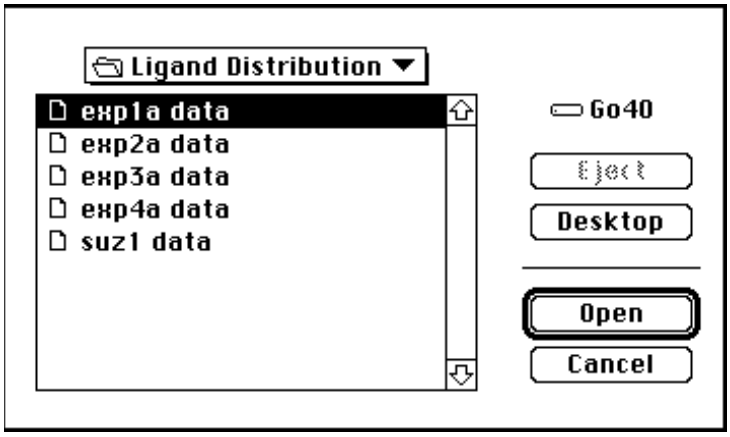
Launch the MacSCAPRE program by double-clicking on the SCAPRE application. Once the program has started, an introduction alert will be displayed (Click on the alert once to remove it). When the introduction alert disappears from the screen, the following dialog box will appear:



At this point select the type of data you would like to process ("Hot + Cold" data was selected in this example).

New data can be entered from the keyboard by filling in the counts spreadsheet. Next select **Experimental Parameters** from the SCAPRE menu. The counting time, volume of tube, specific activity etc. are entered into the Experimental Parameters Dialog box.

In this sample session we will open a sample data file, exp3a data, that is distributed with the programs. To read the 'exp1A data' file, select the menu item **Open ...** from the File menu. This will display the standard MacIntosh Open dialog which will allow you to open only those files that have been created/saved using the MacSCAPRE program.

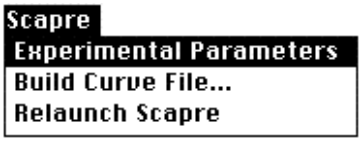


Once a data file is selected from this dialog, the counts sheet, and the Experimental Parameters dialog box will be filled with 'exp3a data'.

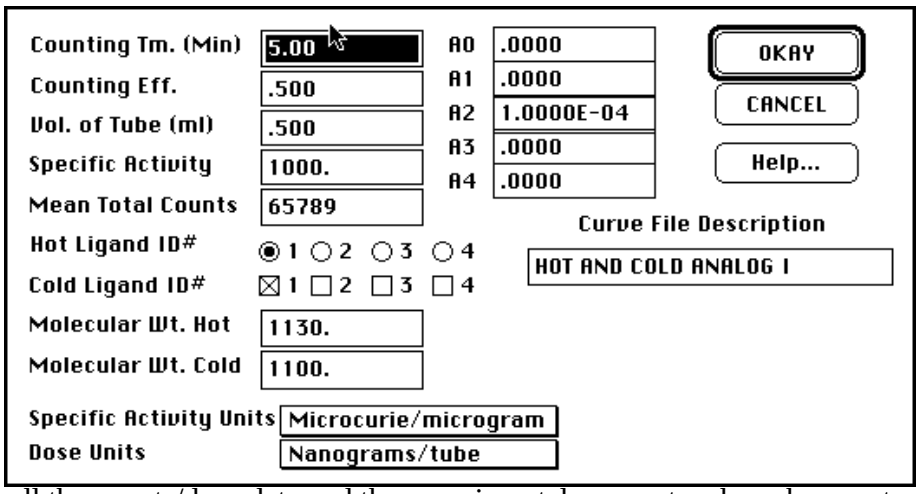
Counts (exp3a data)

	Dose 1	count 1	count 2	count 3	count 4	count 5
1	0.000	7997.000	7891.000	8081.000	8265.000	8262.000
2	0.050	7676.000	7713.000	0.000	0.000	0.000
3	0.100	7244.000	7716.000	0.000	0.000	0.000
4	0.200	7210.000	6950.000	0.000	0.000	0.000
5	0.500	6451.000	6268.000	0.000	0.000	0.000
6	1.000	6020.000	6321.000	0.000	0.000	0.000
7	3.000	5477.000	5317.000	0.000	0.000	0.000
8	10.000	4926.000	5266.000	0.000	0.000	0.000
9	30.000	5250.000	4943.000	0.000	0.000	0.000
10	1000.000	4931.000	4724.000	4479.000	5072.000	4385.000
11	0.000	0.000	0.000	0.000	0.000	0.000
12	0.000	0.000	0.000	0.000	0.000	0.000

Select **Experimental Parameters** from the Scapre menu:



The following dialog will be displayed. Note: If you are entering 'hot only' data the dialog will differ slightly.



Once all the counts/dose data and the experimental parameters have been entered, then the curve file can be built. To Build the curve file, select the menu item **Build**

Curve File from the Scapre menu. If the curve file build is successfull, you will be prompted with a dialog allowing you to save the curve file on the hard disk. You can accept the default name or rename it before saving.

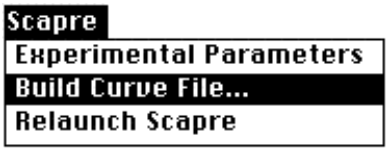
The final results spreadsheet is shown below:

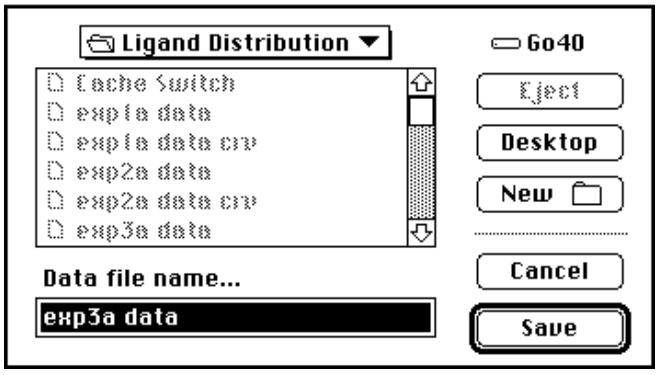
Final Results (exp1a.dat)

	Dose	Total Conc	Amount Bound	B/T
1	0.00000e+0	1.35242e-11	1.13686e-12	8.40610e-2
2	5.00000e-2	9.68575e-11	8.18391e-12	8.44942e-2
3	1.00000e-1	1.80191e-10	1.47732e-11	8.19863e-2
4	2.00000e-1	3.46858e-10	2.60331e-11	7.50542e-2
5	5.00000e-1	8.46858e-10	6.28193e-11	7.41793e-2
6	1.00000e+0	1.68019e-9	1.16124e-10	6.91135e-2
7	3.00000e+0	5.01352e-9	3.32968e-10	6.64139e-2

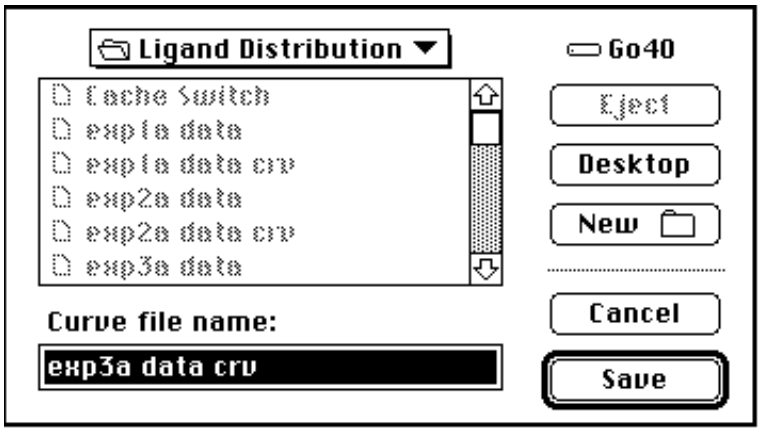
This spreadsheet is simply a view-only display which cannot be edited from the keyboard. You will be prompted automatically to save the curve file.

To save the raw data file (your counts and experimental parameters) first click on the counts spreadsheet. This will bring the counts spreadsheet to the front. Select the File Menu. If you are working with a file that had already been saved on disk then **Save...** as well as **Save As...** will appear in the File Menu. **Save...** is used to update an existing file while **Save As...** is used to create a new file. If you launch the MacSCAPRE program and are entering new data for the first time, then only **Save As...** will appear in the File Menu.





When Save is chosen the program goes on to ask for the name for the curve file. The default is the name of the data file read in with 'crv' tagged on . You can change the name, as well as the location where the file will be saved before clicking the save button.



Scapre is now finished. Select the menu item **Quit** to finish this session. At this point you will be given another opportunity to save your files it you have not already done so.

FITTING THE DATA - MacSCAFIT

SCAFIT MENUS

APPLE MENU

About Scafit APL Displays the MacSCAFIT program introduction alert box which contains the program version number and program credits. The HELP button in this alert box is used to obtain on line help.

Clipboard The clipboard is used to capture any text or picture that is either cut or copied from any available text editor window or graphics window. The memory used by the clipboard, text editors, and pictures is also displayed.

Desk Accessories The MacSCAFIT program allows the user to access any of the available desk accessories which are located under the apple menu of the finder.

FILE MENU

Open... This will most likely be the first menu item used when running the MacSCAFIT program. This option allows the opening of any existing curve file (a file created by either MacSCAPRE). Once a file is selected, a dialog box appears which prompts the user for another curve file. When all files have been selected, click on the button labeled NO in the dialog box.

Save As... Used to save any text or picture selected from a window. If the session window is the current window, then the information will be stored as text and can be accessed using any word processor. Alternately, if the graph window is the current window, then any picture (graph) will be stored on disk as a PICT file, which can be read and manipulated by MacDraw.

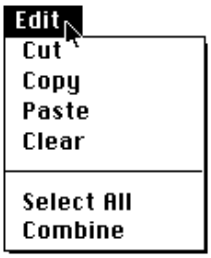
Save Settings... Can be used to customize the appearance of your windows, and control which windows will be shown at launch time.

Page Setup... Used to set up the page defaults to be used when printing.

Print... Works in the same fashion as **Save As...**, except that any text or picture selected is sent to the printer rather than a file on disk.

Transfer... Will quit the current application and transfer to a selected application stored on disk.

Quit Quits MacSCAFIT and saves the session window automatically,.(unless switch number five under the **Switch Settings** menu is unchecked)



EDIT MENU

Different selections will appear in the **Edit** menu depending on which window is the front window.

Cut Will remove text or pictures from window and place them into the clipboard for temporary storage. Any text or picture in the clipboard can then be pasted into another window, or even into the scrapbook to be manipulated by other programs. (MacDraw,SuperPaint, etc.)

Copy Same as Cut, except that the text or pictures will remain in the window.

Paste Once text or pictures have been either cut or copied, paste can be used to place them back in the window, or into the scrapbook.

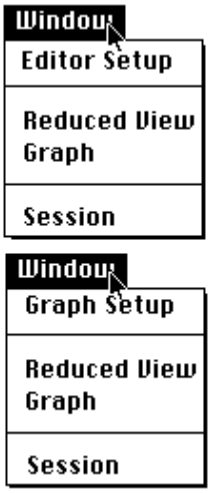
Clear Will clear any selected text or picture(s) from both the window and memory.

Select All Will select all of the text or pictures in the current (front) window.

Tabs to Spaces Is displayed only if the session window is the front window. This option will replace all spaces in selected text with a tab. This is useful for numeric data which can be selected and stored to be later read into a spreadsheet.

Combine Is displayed only if the graph window is the front window. Select two or more graph frames (hold down the shift key and click on each frame with the mouse), then choose **Combine** to combine them into a single frame so they may be printed together on a single page.

WINDOW MENU



A different Setup menu appears depending on which window is in front. These menu items should be used by advanced users only. Use the default settings the first few times you use the programs.

Graph Setup Allows you to set up defaults for the graph window. If you would like to use these defaults each time the program is executed, then select **Save Settings...** from the file menu before Quitting the program.

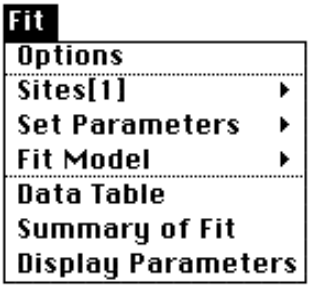
Editor Setup Used to set certain text and file handling options. To save these options each time the program is executed select **Save Settings...** from the file menu before quitting the program.

Reduced View, Graph, Session When selected will bring that window into view as the front window. You can place more than one plot on a page. The reduced view window provides a display of what the entire page will look like when it is printed out.

SCAFIT MENU

This is the main menu for the SCAFIT program itself. Fitting a binding model requires that you first specify the number of sites, initial parameter values, and parameter constraints if any. The model is fit iteratively until the program converges. Then a summary and graph can be obtained.

Options Displays a dialog box which allows for the setting of five program switches.



Sites Number of classes of binding sites to be used.

Set Parameters Will list all parameters used during a particular fit. Each parameter value can then be changed, set constant, or shared with other parameters.

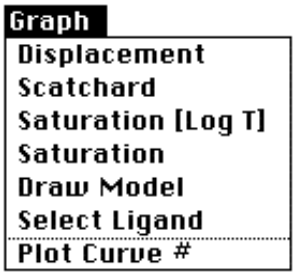
Fit Model A submenu which allows for one, five, ten, or twenty iterations of the curve fitting routine .

Data Table Will display the values LOG(T), B/T, B/T(predicted), B/T - B/T(predicted), and give a Residual plot of the data.

Summary of Fit Lists current parameter values and other parameter estimates.

Display Parameters **Writes the current parameter values to the session window.**

GRAPH MENU



Displacement Results in the creation of a displacement type plot; Bound/Total vs Log(Total). The axis limits will be set automatically. To alter the plot select Plot Curve #, a plot set up dialog will be displayed. Selecting this menu item will always reset the plots axis limits.

Scatchard Results in the creation of a scatchard type plot; Bound/Free vs Bound. The axis limits will be set automatically. Note that the non specific value displayed at the top of the plot is subtracted from each data point. You may alter the non specific to be subrtracted by selecting the **Plot Curve #** menu item.

Saturation [Log T] Results in the creation of a saturation type plot using Log Total for the X axis. The axis limits will be set automatically.

Saturation Results in the creation of a saturation type plot; Bound vs Total. The axis limits will be set automatically.

Draw Model Draws a picture of the model along with the parameter values. You can place this information anywhere on the plot.

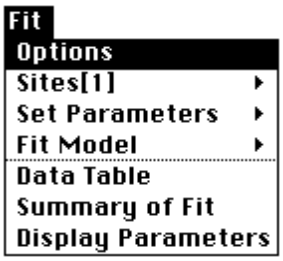
Plot Curve #? Choose this selection to modify the plot. If you want to change the plot type, for example change from a displacement to a scatchard plot you will first have to select the **Scatchard** menu item. This will result in the plot being redrawn. Then select the **Plot Curve #**, you will then be presented with a scatchard plot set up dialog.

Conglomerate Plot This Menu item will exist only if you have opened more than one curve file.

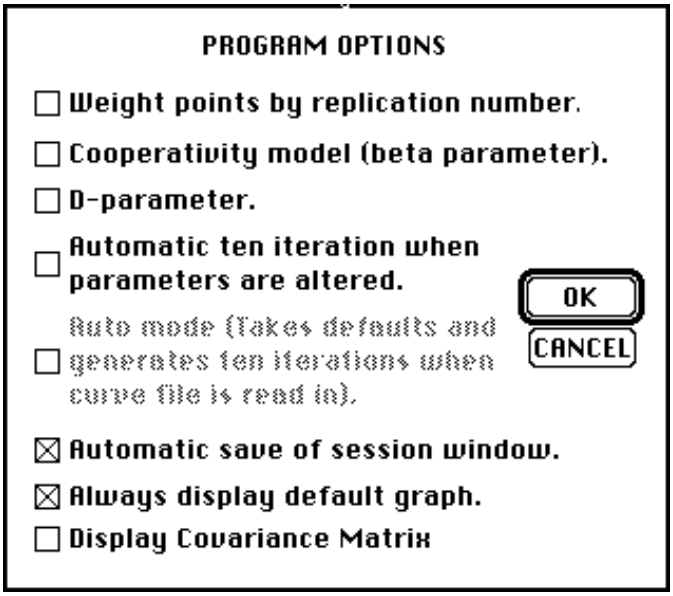
SCAFIT SESSION

We now run through a sample session of SCAFIT. The program output is presented in the output window and is shown here, interspersed with comments in italics.

```
                              LIGAND
     Curve fitting and data analysis for ligand binding data
                  National Institute of Health
                      SCAFIT Version 4.93
------------------------------------------------------------
```



In this program version, various program options (switches) are set. To display this dialog, select OPTIONS from the Scafit menu. Note, typically you don't need to change any of the default switch setting.



WEIGHT POINTS BY REPLICATION NUMBER: Causes each data point to be given a weight proportional to the number of replicate that were present in the data set input from SCAPRE or SCAHOT.

COOPERATIVITY MODEL; (BETA PARAMETER): *I*nvokes the bivalent cooperativity model. (See Munson,P.J. and Rodbard,D. Computerized Analysis of Ligand Binding Data: Basic Principles and Recent Development, in Computers in Endocrinology, Rodbard,D. and Forti,G., eds., Raven Press, New York, 117-145 (1984).*)*

D-PARAMETER: Adds additional D parameters to the fit. This is useful for separate adjustment of the nonspecific binding parameter for each experiment.

AUTOMATIC ten iterations when parameters are altered Automatically performs ten iterations after you exit the K R & N parameters dialog set up dialog.

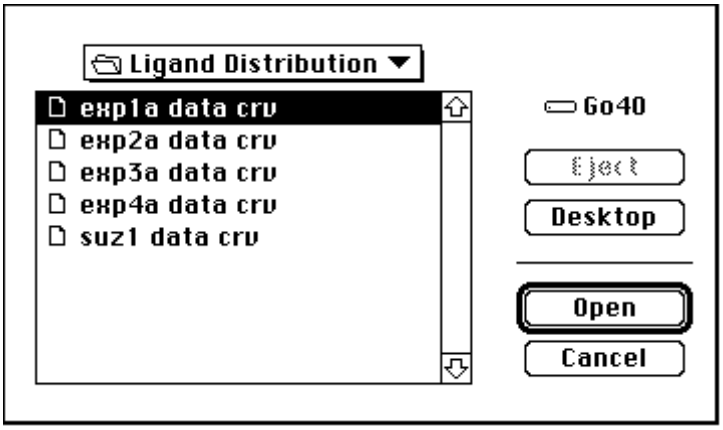
AUTOMATIC SAVE OF SESSION WINDOW*:* Saves session window automatically at quitting time.

ALWAYS DISPLAY DEFAULT GRAPH This option is not being used currently.

Display Covariance Matrix: Writes the covariance matrix to the session window.

```
Switch Settings:
      Weighing parameters     = OFF
      Cooperativity           = OFF
      D Parameter             = OFF
      Auto .GRF save          = OFF
      Auto Session save       = ON
```

Once the switches are set, the user can then select a curve file. Go to the File menu and select **Open...** *. At this point the standard open dialog is displayed, and the user can open one .CRV file at a time.*




```
THE FILE exp1a.crv IS BEING READ IN...
Exp1a.dat
 .0000 .0000 1.0000E-04 .0000 .0000
 2 2
 1.3524E-11
 1.3524E-11 1.1369E-12 4.000
 9.6858E-11 8.1839E-12 2.000
 1.8019E-10 1.4773E-11 2.000
 3.4686E-10 2.6033E-11 2.000
 8.4686E-10 6.2819E-11 2.000
 1.6802E-09 1.1612E-10 2.000
 5.0135E-09 3.3297E-10 2.000
 1.6680E-08 1.1152E-09 2.000
 5.0014E-08 3.2958E-09 2.000
 3.3335E-07 1.8325E-08 2.000
 5.0001E-07 2.5859E-08 2.000
 1.6667E-06 7.2766E-08 2.000
 8.3333E-06 3.2814E-07 2.000
 5.0000E-05 1.6051E-06 4.000
```

To select another curve file, select Yes from the following dialog box.

Follow this process until all files are entered. Remember, the maximum number of curves allowed is 20.

```
THE FILE exp2a.crv IS BEING READ IN...
EXP2A.DAT
.0000 .0000 1.0000E-04 .0000 .0000
 1 2
 1.3524E-11
 9.0909E-11 1.0361E-12 2.000
1.8182E-10 9.7527E-13 2.000
 3.6364E-10 9.7155E-13 2.000
 9.0909E-10 9.0282E-13 2.000
 1.8182E-09 8.9200E-13 2.000
 5.4545E-09 9.1059E-13 2.000
 1.8182E-08 8.7712E-13 2.000

THE FILE exp3a.crv IS BEING READ IN...
Sample Data
 .0000 .0000 1.0000E-04 .0000 .0000
 1 1
 2.0980E-11
 2.0980E-11 2.5889E-12 6.000
 1.1189E-10 1.3086E-11 2.000
 2.0280E-10 2.3058E-11 2.000
 3.8462E-10 4.1391E-11 2.000
 9.3007E-10 8.9905E-11 2.000
 1.8392E-09 1.7250E-10 2.000
 5.4755E-09 4.4918E-10 2.000
 1.8203E-08 1.4100E-09 2.000
 5.4566E-08 4.2271E-09 2.000
 1.8182E-06 1.3028E-07 6.000


THE FILE exp4a.crv IS BEING READ IN...
Exp4a.dat
 .0000 .0000 1.0000E-04 .0000 .0000
 2 1
 1.9073E-11
 8.3333E-11 2.3245E-12 2.000
 1.6667E-10 2.2812E-12 2.000
 3.3333E-10 2.2770E-12 1.000
 8.3333E-10 2.3010E-12 1.000
```

```
1.6667E-09 2.1027E-12 1.000
5.0000E-09 1.8227E-12 2.000
1.6667E-08 1.6747E-12 2.000
5.0000E-08 1.6845E-12 2.000
1.6667E-07 1.5654E-12 2.000
5.0000E-05 1.2859E-12 6.000
```

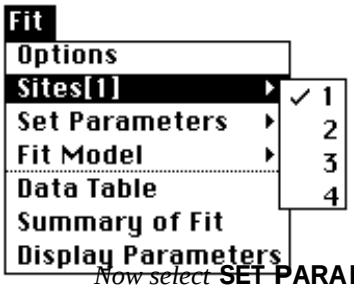
At this point, all curve files are entered, and some information pertaining to each file is displayed.

```
CURVE FILE    exp1a.crv
WEIGHTING PARAMETERS
     .00000         .00000         .00010         .00000         .00000
VARYING LIGAND:   2   LABELED LIGAND:   2
NUMBER OF POINTS:   14

CURVE FILE  exp2a.crv
WEIGHTING PARAMETERS
     .00000         .00000         .00010         .00000         .00000
VARYING LIGAND:   1   LABELED LIGAND:   2
NUMBER OF POINTS:    7

CURVE FILE  exp3a.crv
WEIGHTING PARAMETERS
     .00000         .00000         .00010         .00000         .00000
VARYING LIGAND:   1   LABELED LIGAND:   1
NUMBER OF POINTS:   10

CURVE FILE  exp4a.crv
WEIGHTING PARAMETERS
     .00000         .00000         .00010         .00000         .00000
VARYING LIGAND:   2   LABELED LIGAND:   1
NUMBER OF POINTS:   10
```

The maximum number of ligands is calculated automatically and displayed here.

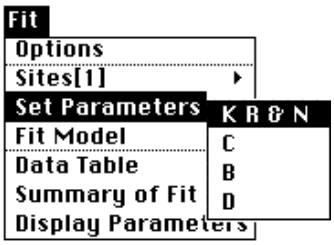
```
NUMBER OF LIGANDS (HIGHEST NUMBER USED) = 2
```

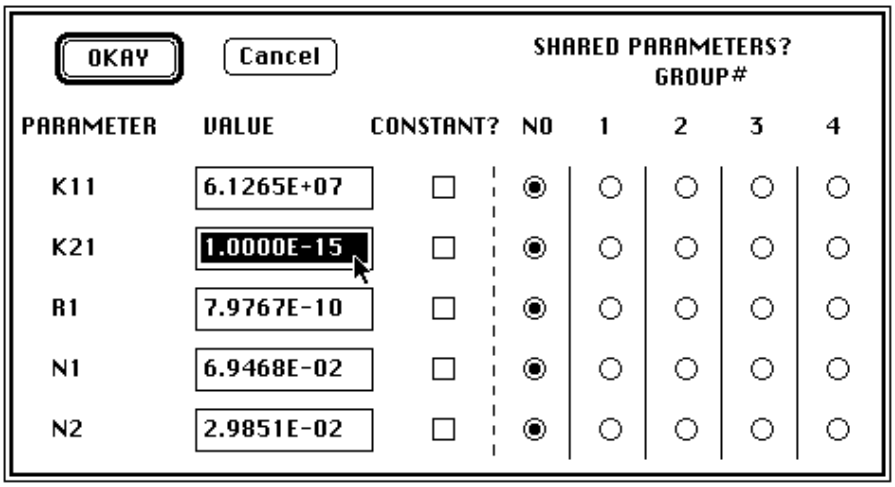
The number of sites defaults to 1. If you wanted to change the number of sites you select sites in the SCAPRE menu. A sub menu will be displayed choose, 1,2,3 or 4. In this example we will use 1 site.
Whenever you change the number of sites, information regarding the number of parameters will be written to the session window.




```
THE FOLLOWING PARAMETERS WILL BE CONSIDERED:
K11 K21 R1  N1  N2  C1  C2  C3  C4
```

Now select **SET PARAMETERS K R & N** *from the Scafit menu.*

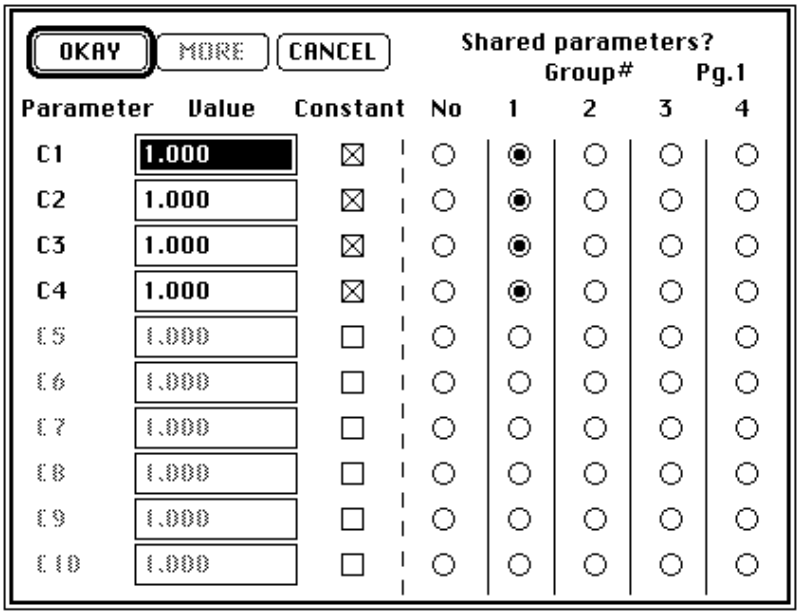




Automatic initial estimates are usually generated for most parameters. In this case, the value for K21 is not generated. (This is apparent because all parameter values that equal zero are changed to 1.0E-15 to avoid any divide by zero possibilities.) Thus, change the value of K21 from 1.0E-15 to 6E7.. Whenever MacSCAFIT is unable to determine initial estimates you can enter the values

1e7 for any K param, 4e-10 for any R param and 2e-2 for an N parameter. These values will usually be good enough to allow the algorithm to begin performing iterations.

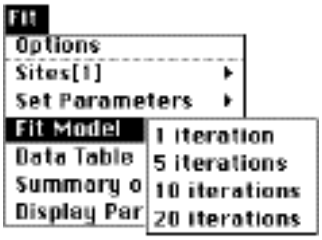
Once these parameters are entered, the C parameter values should be entered:



In this test case, the parameters C1:C4 are going to be set constant to the value one.Incidentally, they are all made to share the same value, although this is extraneous since they are all constant.

```
 THE FOLLOWING PARAMETER GROUPS HAVE A SHARED VALUE:
(C1 ,C2 ,C3 ,C4 )

THE CONSTANT PARAMETERS ARE:
 C1 C2 C3 C4

THE INITIAL ESTIMATES ARE
 K11 =  6.126500E+07
 K21 =  6.000000E+07
 R1  =  7.976700E-10
 N1  =  6.946800E-02
 N2  =  2.985100E-02
 C1  =   1.00000
 C2  =   1.00000
 C3  =   1.00000
 C4  =   1.00000
```

Next, select Iterate and specify **10 iterations** *from the Scafit menu. This example will converge after seven iterations.*




```
 IT.         EPSILON        SUM OF SQUARES
   0        .000000             23529.371

 IT.         EPSILON        SUM OF SQUARES
   1        .000000              9742.490

 IT.         EPSILON        SUM OF SQUARES
   2        .000000              4182.336

 IT.         EPSILON        SUM OF SQUARES
   3        .000000              4114.384

 IT.         EPSILON        SUM OF SQUARES
   4        .000000              4112.633

 IT.         EPSILON        SUM OF SQUARES
   5        .000000              4111.491

 IT.         EPSILON        SUM OF SQUARES
   6        .000000              4110.866

 IT.         EPSILON        SUM OF SQUARES
   7      9.999999E-04           4110.403


CONVERGED
```

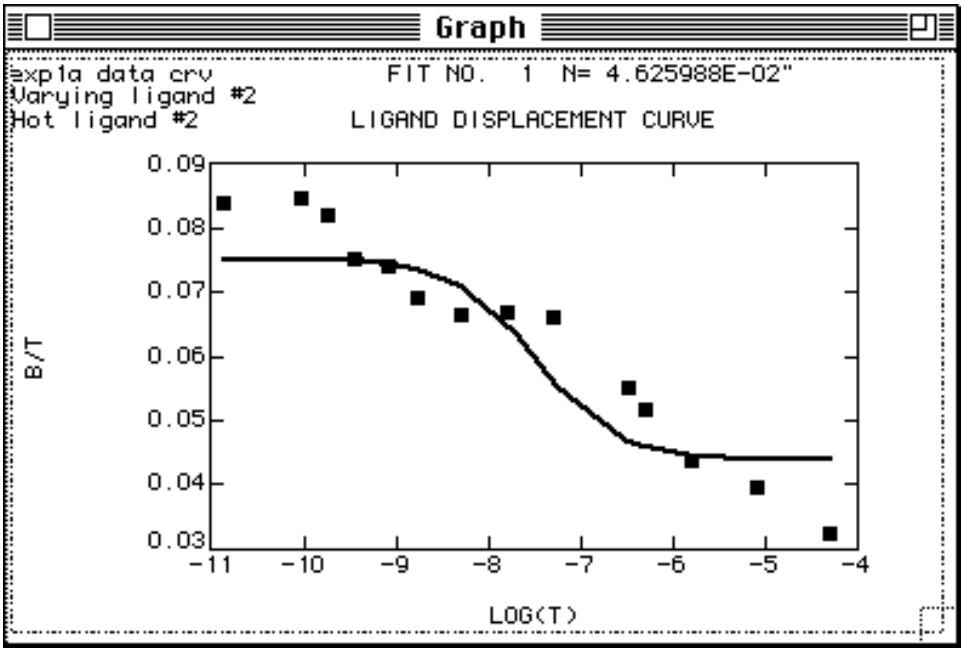
To Summarize the data, select **Summary of Fit** *from the Scafit menu.*

```
L I G A N D: Scafit Version 3.2

CAUTION: Results and interpretation of analysis depend upon several important
assumptions. See User's Guide for details

CONVERGED

 FINAL PARAMETER ESTIMATES, SCAFIT    FIT NO.   1
           VALUE           %CV      LOG-VALUE        KD              K*R
 K11       5.3384E+07      40.%       7.7274       1.8732E-08      .0535
 K21       3.5390E+07      41.%       7.5489       2.8256E-08      .0355
 R1        1.0018E-09      41.%
 N1        7.1693E-02       7.%
 N2        4.6252E-02       5.%
 C1        1.0000E+00
 C2        1.0000E+00
 C3        1.0000E+00
 C4        1.0000E+00
 K11*R1    .0535           13.%
 K21*R1    .0355           10.%


 CURVE       SUM OF       D.F.       MEAN        RUNS      RESIDUALS
             SQUARES                 SQUARE

 exp1a.crv 2085.799      12.7      163.5921      4  N.O.   +++---++++---
 exp2a.crv  280.825       5.8       48.8391      3  O.K.   +-----+
 exp3a.crv 1129.623       8.8      129.0998      3  N.O.   +++------+
 exp4a.crv  614.156       8.8       70.1892      3  N.O.   +++++--+++

 TOTAL     4110.403      36        114.1779               21  20
```

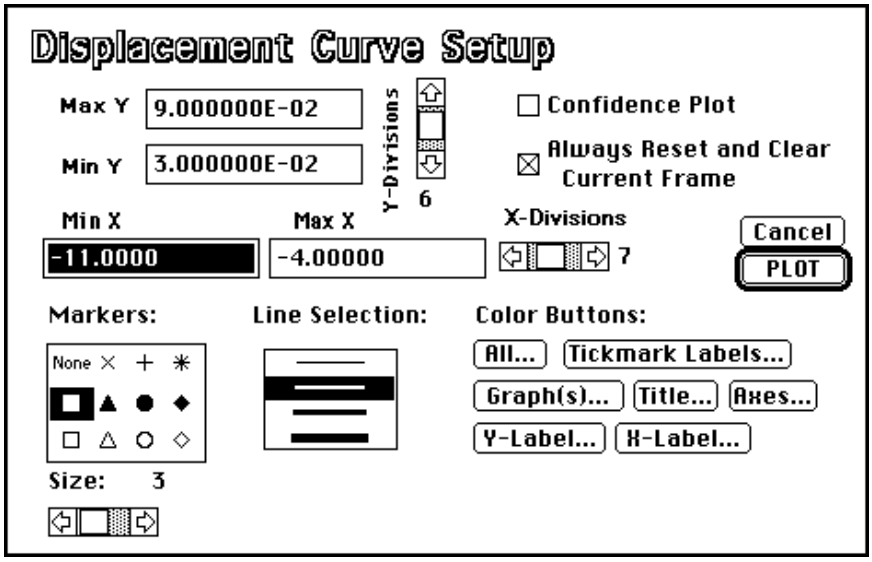
To display the data table select **Data Table** *from the Scafit menu.*

```
 #   LOG(TOTAL)   B/T      B/T-PRED    DIFF     RESIDUAL PLOT
CURVE: exp1a.crv
  1    -10.869      .0841      .0755      .0085       |*
  2    -10.014      .0845      .0754      .0091       |*
  3     -9.744      .0820      .0754      .0066       |*
  4     -9.460      .0751      .0752     -.0001        *
  5     -9.072      .0742      .0747     -.0005        *
  6     -8.775      .0691      .0740     -.0048       *|
  7     -8.300      .0664      .0712     -.0048       *|
  8     -7.778      .0669      .0646      .0022        *
  9     -7.301      .0659      .0562      .0097        | *
 10     -6.477      .0550      .0468      .0081        | *
 11     -6.301      .0517      .0460      .0057        |*
 12     -5.778      .0437      .0448     -.0011        *
 13     -5.079      .0394      .0443     -.0049       *|
 14     -4.301      .0321      .0442     -.0121    *  |

CURVE: exp2a.crv
 15    -10.041      .0766      .0754      .0012        *
 16     -9.740      .0721      .0753     -.0031        *
 17     -9.439      .0718      .0750     -.0032        *
 18     -9.041      .0668      .0743     -.0075       *|
 19     -8.740      .0660      .0731     -.0071       *|
 20     -8.263      .0673      .0692     -.0019        *
 21     -7.740      .0649      .0611      .0037        |*

CURVE: exp3a.crv
 22    -10.678      .1234      .1112      .0122        |*
 23     -9.951      .1170      .1110      .0059        *
 24     -9.693      .1137      .1108      .0029        *
 25     -9.415      .1076      .1105     -.0029        *
 26     -9.031      .0967      .1095     -.0128       *|
 27     -8.735      .0938      .1078     -.0140       *|
 28     -8.262      .0820      .1024     -.0203      * |
 29     -7.740      .0775      .0910     -.0135       *|
 30     -7.263      .0775      .0794     -.0019        *
 31     -5.740      .0717      .0674      .0043        |*

CURVE: exp4a.crv
 32    -10.079      .1219      .1111      .0108        |*
 33     -9.778      .1196      .1110      .0086        |*
 34     -9.477      .1194      .1108      .0086        |*
 35     -9.079      .1206      .1101      .0106        |*
 36     -8.778      .1102      .1090      .0012        *
 37     -8.301      .0956      .1052     -.0097       *|
 38     -7.778      .0878      .0960     -.0082       *|
 39     -7.301      .0883      .0840      .0043        *
 40     -6.778      .0821      .0739      .0082        |*
 41     -4.301      .0674      .0669      .0005        *
```

If you would like to see the graph at this point select the type of plot from the **GRAPH** *MENU. Selecting* **Displacement** *will result in the display of a displacement type plot.*



II you would like to modify the displayed plot, then select **Plot Curve #1** *from the* **GRAPH** *menu, the Displacement Curve Setup dialog will be displayed.*



This setup dialog allows you to set:

1. *Minimum and maximum values for the X and Y axis.*

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LIGAND: A Versatile Computerized Approach for Characterization of Ligand-Binding Systems

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We have developed a general strategy and a versatile computer program for analysis of data from ligand-binding experiments (e.g., radioreceptor assay systems for hormones, neurotransmitters, drugs). This method provides optimal (weighted least squares) estimates of "binding parameters" (affinity constants, binding capacities, nonspecific binding) for any number of ligands reacting simultaneously with any number of receptors. This approach provides two major advantages compared with other available methods: (i): It uses an exact mathematical model of the ligand-binding system, thereby avoiding the possible biases introduced by several commonly used approximations. (ii) It uses a statistically valid, appropriately weighted least-squares curve-fitting algorithm with objective measurement of goodness of fit, thereby avoiding the subjective graphical or simplified statistical methods which may introduce bias. Additional important features include the following. (i) The level of nonspecific binding is regarded as an unknown parameter, subject to uncertainty, which must be estimated simultaneously with other parameters of the system by appropriate statistical methods. This approach provides a more accurate and precise estimate of the parameters and their standard errors. (ii) Selected parameters can be forced to share a common value, or be fixed at any desired constant value. This feature facilitates hypothesis testing by appropriate statistical methods e.g., testing whether a particular experimental manipulation results in a change in affinity (K), binding capacity (R), or both parameters. (iii) One can combine results from multiple experiments by introduction of explicit scaling or "correction" factors which compensate for the commonly observed large degree of between-experiment variation of the overall binding capacity (B_{max}) while other properties of the system (e.g., K values, relative binding capacities for high- and low-affinity sites) are highly reproducible. (iv) One can characterize complex cross-reacting systems involving any number of ligands reacting simultaneously with any number of binding sites. This enables one to pool results from several curves obtained using several different ligands.

Studies of ligand binding to receptors (or to other macromolecules) have assumed increasing importance in a large number of scientific disciplines, e.g., endocrinology, immunology, enzymology, neurobiology, and of course, protein physical chemistry. Despite the enormous importance of ligand-binding experiments in a wide range of biochemical applications, most analysis has been performed by simple manual, graphical, and subjective methods often based on approximations of dubious validity. In the past, the use of such approximations was justifiable, if not unavoidable, due to lack of a suitable alternative. Today, however, with the ubiquitous presence of relatively economical computing resources, a more exact analytical method, involving an exact mathematical model and weighted least-squares curve fitting, is available. Unfortunately, most of the computerized-fitting methods developed to date have been severely restrictive: most available programs only consider one ligand binding to one, two, or at most three classes of sites (1–4). This restriction forces one to assume that

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labeled and unlabeled ligand have the same affinity for all classes of sites, and limits experimental designs to the use of only a single ligand. Further, many implementations of such programs have been cumbersome and frankly erroneous. Use of simplistic least-squares programs in terms of the Scatchard plot coordinate system often yield results which are inferior to those obtained by graphical analyses. In large measure, these inferior results have been due to the inappropriate choice of independent and dependent variables, and to the failure to account for the severe, systematic nonuniformity of variance of the dependent variable.

We now report the development of a general nonlinear and curve-fitting program which will analyze the results of ligand-binding studies using an exact physical chemical model with appropriate statistical methodology. It is based on the mathematical theory describing any number (n) of ligands reacting with any number of classes (m) of binding sites, ("n by m" model) developed by Feldman (5,6). The present program offers the following features.

(i) It allows for the simultaneous analysis of several displacement curves obtained for different unlabeled ligands, within an experiment. By combining the information from several unlabeled ligands, we obtain a more precise and accurate estimate of the parameters shared in common, e.g., binding capacity of the receptor(s) and nonspecific binding.

(ii) It permits analysis of curves (or families of curves) obtained using different labeled ligands.

(iii) It enables us to combine results from multiple experiments, while compensating for the frequently occurring problem that the total binding capacity and the nonspecific binding may vary systematically between experiments even after one has made corrections for different cell number, protein, or DNA concentration.

(iv) It provides direct and explicit statistical testing for alternative models or hypotheses. The program allows parameters to be fixed at any desired constant value. Thus, we may search for a significant second class of binding sites by fitting a model where R_2 (the capacity of the second site) is adjusted to its best value and comparing this fit to the one obtained when R_2 is forced equal to zero. This feature is also useful in reducing the complexity of difficult problems in preliminary attempts at curve fitting. Groups of parameters can also be forced to assume the same value, e.g., we can constrain the labeled and unlabeled ligands to share the same affinities for all classes of sites, or we may force the binding capacity to be the same for two different ligands. The appropriateness of these constraints can be tested by objective statistical criteria.

(v) It provides a variety of statistical methods for evaluating the "goodness of fit" for a given model, both parametric and nonparametric. It also provides a convenient graphical output, either in terms of dose–response curves or Scatchard plots, corrected or uncorrected for nonspecific binding. This allows visual inspection of the adequacy of fit.

(vi) It utilizes Total ligand concentration as the independent variable because this concentration is usually known precisely.

(vii) Weighting is provided to compensate for the nonuniformity of variance of the dependent variable, viz. Bound ligand concentration. Weights are based on the *predicted* rather than *observed* variances, and are refined after each iteration.

(viii) The parameters (K's, R's, N's)[1] are forced to assume physically plausible values (i.e., impossible negative values are excluded). When a given parameter approaches zero, the model is automatically reparameterized in terms of log (K) or log (R), so that negative values cannot occur.

[1] Throughout this paper, the symbol K denotes the equilibrium binding constant of association (i.e., K_a) with units (concentration)$^{-1}$. R denotes receptor site concentration. N denotes ratio of nonspecifically bound to free ligand.

(ix) Simplicity of operation: the program requests information from the user as needed: it operates in an interactive, conversational mode. During the curve-fitting procedure, the program pauses periodically and asks the user for advice: should it continue, stop, start over with new initial estimates, or redefine the model to employ more (or fewer) parameters, classes of sites, etc.

(x) The program is written in BASIC[2] and should be readily adaptable to minicomputers and larger desk-top calculators, and can be readily converted into other languages.

We shall illustrate the program and demonstrate its capabilities by means of a series of examples of increasing complexity.

CASE 1: LABELED LIGAND ONLY: ONE CLASS OF SITES

The simplest case involves only a single, radioactively labeled ligand binding to (presumably) a single class of receptors. Estradiol binding to mammary tumor cells is often regarded as representative of this case. Such a binding study is performed by adding increasing amounts of radioactively labeled hormone or ligand to several tubes, each of which also contains a small amount of the tissue or cell preparation containing the specific receptors. After equilibration, the Bound ligand is separated from the Free, and the concentration of the Bound ligand is then measured by counting radioactive disintegrations. Because much of the hormone may be nonspecifically bound to nonreceptor proteins, it is common to also include a set of tubes to which a large amount (e.g., a 100-fold excess) of unlabeled hormone has been added, thereby presumably saturating the specific receptor. Any Bound radioactivity in these tubes is assumed to represent binding to nonspecific or nonsaturable proteins. By subtracting this nonspecific binding from the total binding measured with the first set of tubes, one obtains an estimate of the amount of hormone bound to the receptor of interest (specific binding).

Usually, the analysis of such a binding study proceeds with the construction of a Scatchard plot, i.e., the plot of the ratio of Bound to Free ligand vs Bound ligand concentration (abbreviated B/F vs B). From this plot, one determines the affinity of binding by fitting a straight line to the data using the relationship: $K = -1 \times \text{slope}$. Further, the intercept with the Bound axis is an estimate of the binding capacity, designated R (or R_1 and R_2, if there are two classes of binding sites).

Figure 1A (closed circles) shows the Bound concentration (B) versus the Total concentration of ligand added to each tube for a computer simulation of a representative case[3] similar to data found in estrogen binding to mammary tissue. The triangles represent the radioactive ligand bound in the presence of a 100-fold excess of cold ligand i.e., nonspecific binding (NSB).[4] The squares represent specific binding obtained by subtraction of NSB from B. Figure 1B shows the specific binding data displayed in the Scatchard coordinates. Here one may approximate the data with a straight line (either with linear regression or by eye) and obtain esti-

[2] Listings of the program system "LIGAND," previously known as "SCAFIT," are available on request. The program is available in standard BASIC or in Super-BASIC, an extended BASIC language. Super-BASIC was developed by one of us (P.J.M.) for use on DEC-10 equipment having a SAIL compiler. The graphics segments of the program use the OMNIGRAPH system, and would need appropriate revision for most other (non-DEC-10) systems.

A version is available for the HP9845 desktop computer.

[3] The data for Figs. 1–3 were artificially generated on the computer. The values used represent the result of adding small, random numbers or "noise" to the exact values of [Bound]. The random numbers obey a Gaussian distribution, with a standard deviation proportional to the value of [Bound]. Thus, in these examples, the data are said to have a constant percentage error. This error structure represents a good approximation to what is often obtained experimentally.

[4] Abbreviations used: NSB, nonspecific binding; rms, root mean square; GABA, γ-aminobutyric acid; GnRH, gonadotrophin-releasing hormone; TBu, tertiary butyl.

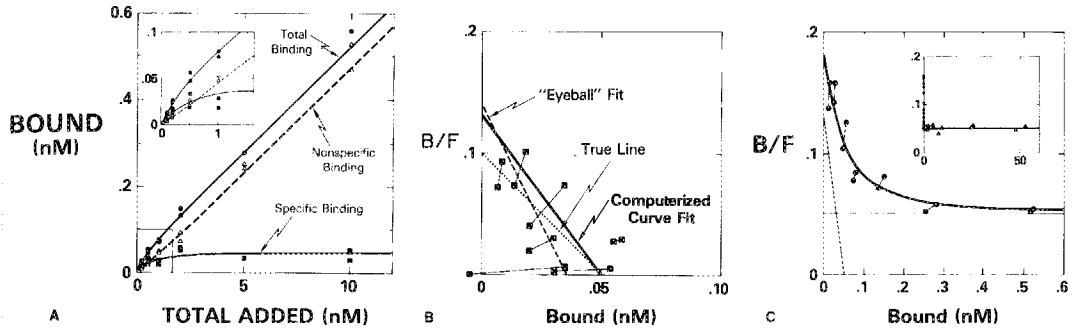

FIG. 1. (A) Bound labeled ligand concentration (nM) vs Total labeled ligand concentration, with (△) and without (●) the addition of a 100-fold excess of unlabeled ligand. Specific binding (■) is calculated difference between binding in presence and absence of excess unlabeled ligand. Smooth curves are computer-generated fits to the data. (B) Scatchard plot of specific binding data from A. Bound to Free ligand ratio vs Bound ligand concentration (nM). Duplicate determinations are joined by lines, indicating the unusual nature of the statistical errors. The dashed line indicates a manual fit to the data. Other investigators may choose other lines to represent the data. The nonlinear regression program LIGAND chose the solid line. The dotted line represents the true underlying model used to generate the data. (C) Scatchard plot of Total binding data in A, uncorrected for nonspecific binding. Horizontal line at $B/F = N = 0.05$ is the computer fit value for nonspecific binding. Diagonal line represents the estimated specific binding component. The additional points (▲, inset) arise by converting the nonspecific binding data (A) to Bound (labeled plus unlabeled) vs Total (labeled plus 100-fold excess of unlabeled) ligand concentration, and then plotting this data on the Scatchard plot. Inset shows 28 points, 14 of which are scattered around horizontal line.

mates of the binding affinity K, and binding capacity, R.

The measurement errors in the original duplicate points have been magnified by these transformations. It is not uncommon for one or more points to have a physically impossible negative value for Bound ligand concentration. However, in most reasonably precise experiments, these graphical techniques will often lead to adequate approximations of the parameters of interest.

In contrast to the above graphical approach, a computerized nonlinear curve-fitting analysis often results in improved estimates of the parameters and offers protection from the inaccuracies and subjectivity of graphical methods.

The first step is to reexpress the data in terms of Bound (B) versus Total (T) ligand added, either labeled or unlabeled. Thus, if the first tube contains 0.1 nM ligand, when we add a 100-fold excess of cold, the corresponding "nonspecific" tube would contain 10.1 nM ligand, assuming that the labeled and unlabeled ligand are chemically identical. Considered in this way, the data span a much larger concentration range (0.1–1010 nM) than originally (0.1–10 nM). While this range is difficult to plot meaningfully on a graph of Bound vs Total, it is an easy task for the computer to fit the binding model which predicts the Bound concentration as a function of Total hormone concentration, given the affinity K, the capacity R, and the nonspecific binding N. The computer adjusts the parameters K, R, and N until the model approximates the actual data points as closely as possible (Fig. 1A, upper curve). The final values for K, R, and N are now the best estimates (in the "least-squares" sense) for the true affinity, binding capacity, and level of nonspecific binding for this system. Although the fitting was done in the Bound vs Total coordinate system, the resulting curve may be displayed in the Scatchard coordinate system, corrected (Fig. 1B, solid line) or uncorrected (Fig. 1C, solid curve) for nonspecific binding. In the uncorrected Scatchard (Fig. 1C), the curve is clearly nonlinear, asymptotically approaching a horizontal line.

Since these data were artificially generated,

TABLE 1

COMPARISON OF PARAMETER ESTIMATION PROCEDURES FOR ONE-LIGAND, ONE-BINDING SITE DATA

Method	K_1 (nM^{-1})	R_1 (nM)	N	rms (%)
Manual Scatchard plot	3.9	0.036	—	—
Linear regression				
B/F on B	0.6 ± 0.5	0.09 ± 0.05	—	—
B on B/F	5.2 ± 3.7	0.035 ± 0.007	—	—
LIGAND program	2.6 ± 0.7	0.050 ± 0.009	0.050 ± 0.003	9.17
True	2.00	0.050	0.050	9.00

we know the true values for the binding parameters K, R, and N. Table 1 indicates that weighted least-squares curve fitting of the binding parameters using program "LIGAND" gave more accurate values than the other methods. The values obtained by the program are objective, reproducible, and nearly optimal from a statistical viewpoint. They avoid the subjectivity of the simple graphical approach. If simple linear regression is used on the Scatchard plot directly, the values obtained for K and R are even further from the true values than the graphical estimates, due to the correlated, nonuniform errors in both of the coordinates of the Scatchard plot (Table 1). The LIGAND computer program also calculates appropriate standard errors for the parameters, providing an objective and meaningful estimate of their precision.

In Fig. 1C, the B/F ratio of the horizontal asymptote to the curve corresponds to the value of the nonspecific binding, N. The 12 points with the highest dose levels lie extremely close to this horizontal straight line in a region of the curve which yields little information about the two major parameters of interest (K and R). Eliminating many of these points from the experiment would thus not substantially affect the precision of the estimates of K and R. Alternately, by redistributing the same 28 data points one could improve the precision of the estimates of K and R, while keeping the uncertainty in N within acceptable limits.

CASE 2: LABELED LIGAND ONLY: TWO CLASSES OF SITES

In many situations, a single binding site is not sufficient to explain the binding data (Fig. 2). Initially, we use LIGAND to fit a model with one class of binding sites, with parameters K, R, and N. A reasonable fit is obtained with relatively small standard errors for the parameters, and the average deviation of the observed Bound concentration to the fitted curve (root mean square or rms error) equal to 8.5% (Table 2). However, the Scatchard plot suggests departure from linearity, i.e., the one-site model may not be an adequate explanation for the data. This suggestion was investigated quantitatively by fitting a two-site binding model to

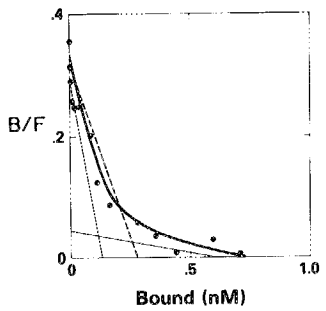

FIG. 2. Scatchard plot of simulated one-ligand, two-binding site data, corrected for nonspecific binding. Dashed line indicates the best fitting one-site binding model. Note that data points are not randomly scattered about this line. Solid curve is computerized best fit for a two-site model. The two binding components are indicated by the solid straight lines (Table 2).

TABLE 2

NONLINEAR REGRESSION PARAMETER ESTIMATES

	K_1 (nM^{-1})	R_1 (nM)	K_2 (nM^{-1})	R_2 (nM)	N	rms (%)	df	Sum of squares
One-site model	1.1 ± 0.2	0.28 ± 0.04	----	—	0.055 ± 0.002	8.5	15	1084
Two-site model	2.1 ± 0.7	0.14 ± 0.05	0.074 ± 0.077	0.62 ± 0.34	0.051 ± 0.003	6.3	13	516
True values	2.3	0.11	0.11	0.55	0.051	7.0	17	833

the same data, estimating the affinity and capacity for the first, high-affinity site (K_1 and R_1), the affinity and capacity for the second site (K_2 and R_2), and nonspecific binding (N) (Table 2). The fitted curve for this model is also displayed in Fig. 2 (curved line) and gives a perceptibly improved fit to the data, with the rms error now reduced to 6.3%. Next we proceed to test whether the model 2 provides a statistically significantly better fit than model 1, using an F ratio test (see Appendixes). The calculated F value is 6.96, greater than the critical level (6.7, $P < 0.01$), indicating that the reduction in the rms is highly significant. In general, this test can provide definitive answers to well-posed questions (e.g., Is there a second or third class of binding sites? Did the affinity constant change as a result of treatment?). Notice that K_1 for the one-site model is intermediate between K_1 and K_2 for the two-site model, but much closer to the high-affinity value (Table 2). The two-site model fit yields parameter values close to the true values. Thus, failure to detect and account for a possible second binding site may yield a biased underestimate of the affinity for the high-affinity site and an overestimate of its binding capacity. A similar problem occurs whenever an inappropriate model is fit to data: biased and possibly meaningless estimates of the parameters may be obtained.

The estimated precision of the parameters for the two-site model is much worse than for the one-site model. K_2 has greater than 100% coefficient of variation, whereas all the parameters of the one-site model were determined to better than 15%. Even so, the existence of the second site is judged statistically significant by the reliable F test. The larger uncertainty in the parameters of the true but more complex model results in part from the high-degree of interdependence of the five parameters in the two-site model. Many sets of values for K_1, R_1, K_2, R_2, and N will give rise to almost equally good fits to the data. As a simplified alternative to the F test described above, many investigators might be tempted to apply a Student's t test to the parameter R_2 to determine if it were significantly different from zero, and thus determine if the second site was needed in the model. This might be done by calculating.

$$t = \frac{R_2 - 0}{\text{SE of } R_2}, \qquad [1]$$

where the degrees of freedom for t will be taken as number of data points minus number of parameters = 18 − 5 = 13. In this case the result would be

$$t = .617 \text{ nM}/.339 \text{ nM} = 1.82, \qquad [2]$$

which would *not* be significant at the $P = 0.01$ (one-tailed) level ($t^* = 2.6$) contrary to the F ratio result given in the previous section. This discrepancy arises because the distribution of the estimate of R_2 is highly skewed, and does not follow a Gaussian distribution, as required by the t test. R_2 is

more nearly log-normally distributed. The F test depends directly on the distribution of the errors in the data points, rather than on the distribution of the calculated parameters. In this situation, where nonlinear curve fitting is used, the F test is decidedly preferable.

CASE 3: ONE LABELED LIGAND, SEVERAL UNLABELED LIGANDS, ONE CLASS OF SITES

A common experimental design in endocrinological and pharmacological studies is the use of multiple displacement curves. In this situation, several drugs or ligands are used to displace a small amount of the labeled compound from the putative drug receptor. One seeks to measure the binding affinities of each of the ligands relative to the affinity of the labeled ligand. The dose of unlabeled ligand yielding 50% displacement (ED_{50}) of the labeled ligand is roughly inversely proportional to the binding affinity of the unlabeled ligand. The ratio of two ED_{50} values provides a *crude* measure of the relative binding affinity of the two different ligands.

In Fig. 3A, Bound counts for the radioactivity labeled ligand (L_1^*) are plotted vs log of the dose of any of three unlabeled ligands, L_1, L_2, or L_3. For a given response value, it is possible to estimate a corresponding dose for each one of the curves, using graphical techniques or by fitting multiple simultaneous logistic curves with a computer program (e.g., ALLFIT, Ref. (7)).

One may now calculate the relative binding affinities for L_1 and L_2 using

$$K_2/K_1 \cong h_1/h_2, \qquad [3]$$

where h_1 is the dose of L_1 yielding the identical response (often, but not necessarily, the 50% response) as a dose h_2 of L_2. Similarly, one may calculate K_3/K_1. However, Eq. [3] is an approximation which is correct only when the B/F ratio is extremely small, e.g., less than 1%. Thus, the values obtained using Eq. [3] (Table 3, row 1) deviate significantly from the true values.

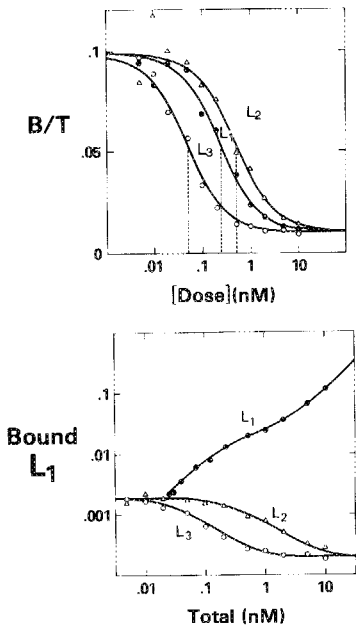

FIG. 3. (A) Displacement of labeled ligand L_1^* by increasing doses of unlabeled ligand L_1 (●), ligand L_2 (△), or ligand L_3 (○). A smooth sigmoidal curve was drawn through each curve, constraining the upper and lower plateaus to be identical, thus aiding the determination of the ED_{50} values (vertical dashed lines) of 0.24 nM for L_1, 0.51 nM for L_2, and 0.048 nM for L_3. These values are then used to calculate the approximate relative binding affinities for the three drugs. (B) Data from A transformed to [Bound L_1] vs [Total ligand] coordinate system, suitable for computerized curve fitting using program LIGAND. The solid lines represent the best-fitting curves. The curve for ligand L_1 is ascending, reflecting the fact that increasing Total L_1 concentration results in increasing Bound L_1 concentration. For L_2 and L_2, Bound L_1 concentration decreases with increasing Total concentration. The parameter values are given in Table 3. Note: the log–log scale is for convenience of data presentation only: the program considers the data in an arithmetic scale.

Another popular rule for relating the affinity to the ED_{50} is known as the Cheng–Prusoff correction (8):

$$1/K_2 \cong \frac{ED_{50}}{1 + p^*K_1}, \qquad [4]$$

where p^* is the concentration of labeled ligand, L_1 in this case; ED_{50} is the dose of L_2 which displaces 50% of the bound L_1, and K_1 is the previously determined affinity of L_1. If K_1 is not known, the same expres-

TABLE 3

RELATIVE BINDING AFFINITY ESTIMATES USING VARIOUS TECHNIQUES

	K_1 (nM^{-1})	K_2 (nM^{-1})	K_3 (nM^{-1})	R (nM)	N_1	rms (%)	K_2/K_1	K_3/K_1
Relative ED_{50} method	4.17	1.96	21.	—	—	—	0.474	5.05
Cheng–Prusoff	4.54	2.12	23.7	—	—	—	0.471	5.21
Rodbard–Lewald	—	—	—	—	—	—	0.448	6.33
LIGAND curve fitting	5.7 ± 0.6	2.5 ± 0.2	37.2 ± 3.8	0.020 ± 0.002	0.01 ± 0.001	7.42	0.431	6.53
True values	5.5	2.3	40.	0.02	0.01	7	0.418	7.27

Note. ED_{50} for the labeled ligand, L_1, is 0.24 nM, for L_2 is 0.51 nM, and for L_3 is 0.048 nM. The B/F ratio at the ED_{50} is 0.05. The concentration of labeled ligand, p^*, is 0.02 nM. The constraint ($N_1 = N_2 = N_3$) was used during the LIGAND curve-fitting, since neither N_2 nor N_3 may be determined from these data. A similar assumption is implicit in the first three approximate methods.

sion may be used to find K_1 as a function of the ED_{50} for unlabeled L_1 displacing itself; by replace K_2 with K_1 and solve the resulting equation. The Cheng–Prusoff correction provides a better estimates of K_2/K_1 and K_3/K_1 than does the simpler Eq. [3]. However, in the present example, the value of K_3/K_1 is still substantially different from the true value (Table 3, rows 2 and 5). In general whenever the affinity of the labeled ligand is less than the affinity of the unlabeled ligand: incorrect estimates of the relative affinities may be obtained.

An exact rule for a single homogenous class of binding sites is

$$\frac{K_2}{K_1} = \frac{h_1}{h_2 + h_2(B/F) + h_1(B/F)}, \quad [5]$$

where B/F is the bound to free ratio of the labeled ligand at the chosen response point (9). Again, we may also calculate K_3/K_1 by appropriate substitution of h_3 for h_2. When this rule was applied to the data in Fig. 3A, the results were in good agreement with the parameter values used to generate the data. In particular, the value of K_3/K_1 is closer to the true value (Table 3, rows 3, 5) than the result based on the Cheng–Prusoff approximation.

Many workers attempt to infer the binding affinity K for the labeled ligand from a single displacement curve. Of course, this could be done by plotting the data in the Scatchard coordinate system and finding the slope of the best fitting line. However, a number of approximate rules have been developed which relate the K value directly to the ED_{50}. When labeled ligand concentration is small, the ED_{50} in the B/F vs Total coordinate system is given by

$$ED_{50} = 1/K + R/2 \quad [6]$$

where R is the concentration of binding sites for this ligand (2). R may be ignored if the receptor concentration is sufficiently dilute relative to $1/K$. When the total concentration of labeled ligand (p^*) is large enough to saturate the all of the binding sites, then

$$ED_{50} = p^*, \quad [7]$$

and ED_{50} is no longer a measure of K. In general, when only a single ligand and single class of binding sites are involved, the ED_{50} is related to K, R, and p^* by a quadratic equation which can be solved analytically (2). When more than one ligand is involved, a set of quadratic equations arises, which require a computer program to calculate the numerical solution in all but the simplest of cases.

An alternative approach, which avoids any approximations, is to fit the exact binding equations to the entire range of data, and thus find direct estimates of the binding parameters involved. Such an approach is implemented in program LIGAND. In this example, one fits the parameters of a three-ligand, one-binding site (3 by 1) model directly to the binding data. When the specific activity of the labeled ligand is known, we may transform the data given in Fig. 3A to the coordinate system [Bound L_1] vs [Total] for L_1, L_2, or L_3 (Fig. 3B). We may assume the three-ligand, one-binding site (3 by 1) model is appropriate. The parameters to be fit are $K_1, K_2, K_3, R, N_1, N_2$, and N_3, i.e., binding affinity for L_1, L_2, and L_3, receptor concentration, and nonspecific binding for each ligand. Although there are now seven parameters to be determined, all of them except N_2 and N_3 may be estimated directly from the data by the curve fitting program. The nonspecific binding for L_2 and L_3 is often assumed to be equal to N_1, or to be negligible. Such an assumption is also implicit in all of the simpler approximations involving the ED_{50}. Fortunately, the values assumed for N_2 or N_3 usually have relatively little influence on the estimated value for K_1 and only a minor influence on K_2 or K_3.

The LIGAND program estimates were uniformly closer to the true values of the parameters than any of the above-mentioned approximations. The LIGAND program offers several other advantages. It provides a unified analytical approach, with a quantitative measure of goodness of fit, and can thus detect when the model itself is inadequate, e.g. if there were a second class of sites. Estimates of the precision of the parameter estimates is provided reflecting the statistical variations in the original data. Finally, the program is valid for all concentrations of p^*, all receptor occupancy levels, all initial binding or B/F levels, does not rely on intermediate analysis steps, but rather estimates the binding parameters directly from the data.

CASE 4: EVALUATING THE EFFECT OF EXPERIMENTAL MANIPULATIONS ON BINDING PARAMETERS

Often the investigator is primarily interested in evaluating whether a certain treatment of the receptor preparation affects the receptor concentration, the affinity constant, or both. When the binding curve shows an apparent change, one must ask: Is this change significant? Which parameter is changing? These questions can only be answered when the size and nature of the random fluctuations in the data are taken into account. The present program provides answers to these questions by allowing one to compare the goodness of fit of a model with and without a set of constraints.

The effects of the detergent Triton X-100 on the binding of ligands to GABA receptors in rat brain membranes have been previously noted (10). Whether pretreatment with detergent affects the binding affinity, the binding capacity, or both is investigated in the following example (data kindly provided by Dr. T. Costa). In the first experiment, increasing amounts of muscimol, along with a constant, small amount of [^{3}H]muscimol, were added to the preparations. These data are converted to Scatchard coordinates (open circles, Fig. 4). The second experiment was identical, except that small concentrations of Triton X-100 were added prior to incubation with the ligand (closed circles, Fig. 4).

The Scatchard plots of the two experiments, after correction for nonspecific binding, show a somewhat ambiguous result. In addition to the obvious change in slope, or K value, there is a minor, less obvious shift in the Bound axis intercept corresponding to the receptor capacity. Thus it appears that Triton X-100 may modulate the receptor number slightly, in addition to its profound effect on affinity.

Testing the significance of the effect on receptor concentration requires that we formulate alternative models or hypotheses. First, we consider the possibility that the

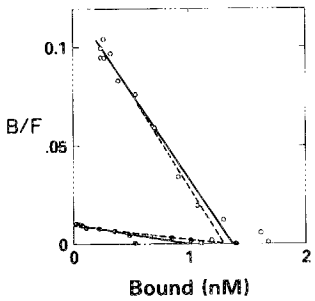

FIG. 4. Scatchard plot of [^{3}H]muscimol binding to rat brain membranes. Open circles, binding with pretreatment by Triton X-100. Closed circles, binding without pretreatment. Solid lines are computer fit allowing adjustable binding capacity for each experiment (Table 4, Fit 1). Constraining the binding capacity to be equal for both experiments (dashed line) does not significantly degrade the fit (Table 4, Fit 2).

affinity, K, the binding capacity, R, and the nonspecific binding, N might change after treatment with Triton. Thus, we need the parameters $\{K_1, R_1, \text{and } N_1\}$ to describe the system before treatment, and a separate set $\{K_2, R_2, \text{and } N_2\}$ to describe the situation after treatment. When we allow these parameters to assume their most favorable values (i.e., the values where the rms error is minimized), we obtain the values given as Fit 1 in Table 4, with an rms error of 4.8%.

Second, we consider the hypothesis that there is no change of R after treatment, i.e., that all the change is in the binding affinity, K, or nonspecific binding, N. Again we fit a model to the data, this time with the imposed constraint $\{R_1 = R_2\}$. The rms value increased to 5.0% (Fit 2, Table 4), indicating that the overall performance of the second model is not significantly worse ($F = 3.0$, $P > 0.05$).

Since the constraint $\{R_1 = R_2\}$ is consistent with the data, we may conclude that the binding capacity did not change as a result of treatment. In other cases, we might also test if $\{K_1 = K_2\}$ or $\{N_1 = N_2\}$, etc., even in cases where we have multiple classes of sites.

CASE 5: COMBINATION OF RESULTS FROM MULTIPLE EXPERIMENTS (FOR CASE OF ONE LIGAND, TWO BINDING SITES)

One of the most important features of the present program is its ability to enable us to "adjust," pool, and fit data from multiple experiments. Figure 5A shows the three individual Scatchard plots for aldosterone binding to A-6 epithelial cells derived from toad kidney in continuous culture for three separate experiments. Nonspecific binding has been subtracted and binding has been normalized per milligram DNA for each individual point. (These data were kindly provided by Drs. C. Watlington and J. Handler (11).) The Scatchard plot appears to be nonlinear, even after the nonspecific binding (for 10^{-6} M) has been subtracted. However, there was insufficient information within any one experiment (with only 10 points, including the "nonspecific" tube) to fit five parameters of a two-classes of sites model. The estimates of the parameters were essentially indeterminate (Table 5, rows 1–3). A model

TABLE 4

MUSCIMOL BINDING DATA: EFFECT OF CONSTRAINING THE BINDING CAPACITIES TO BE THE SAME ON THE GOODNESS OF FIT

	K_1 (mM^{-1})	K_2 (mM^{-1})	R_1 (nM)	R_2 (nM)	N_1	N_2	rms (%)	df	F
Fit 1	89. ± 6.	10.3 ± 1.6	1.4 ± 0.1	1.0 ± 0.2	0.0041 ± 0.0002	0.0050 ± 0.0002	4.8	20	—
Fit 2 $\{R_1 = R_2\}$	92. ± 6.	7.9 ± 0.4	1.3 ± 0.1		0.0042 ± 0.0002	0.0047 ± 0.0002	5.03	21	3.0 (nonsignificant)

involving a only single class of sites could be fit, but was clearly inconsistent with the data (cf. Case 2, above). Plotting the results superimposed on one graph, we see that all three curves have a similar shape (Fig. 5A). However, the three differ in terms of their intercepts with both the horizontal and the vertical axes. This pattern of congruent curves would be expected if K_1, K_2, and the ratios R_1:R_2:N were the same for all three experiments, suggesting that the three experiments were qualitatively similar, and differ only in the total amount of receptor present. Corrections by cell number, protein or DNA content are frequently used and often helpful, but only rarely will completely eliminate the large variability between experiments. A better approach is to calculate a correction factor (C) for each experiment relative to the first experiment, employing a least-squares procedure, at the same time that the five parameters of the mass action model are estimated. This forces all three curves to have exactly the same shape, and provides the best scaling factors. The results, obtained using program "LIGAND," are shown in Fig. 5B and Table 5, row 4. A satisfactory fit is obtained; the overall curve appears to be an adequate description of all three experiments, and no systematic departure from the model was detectable using the "number of runs" test ($P > 0.05$). The average scatter of a point around the fitted line corresponds to a ±19% error in the value of [Bound] for any given value of [Total]. The K and R values for the high-affinity class of site have about ±40 and ±25% errors, respectively. The K and R for the second class of sites have essentially 100% error; they are very ill determined. The overall value for N is very well determined (0.020 ± 0.003) and so are the correction factors for experiments 2 and 3 (0.54 ± 0.05 and 0.69 ± 0.06, respectively).

Now, we can examine the following questions.

(i) Is the fit involving two classes of sites significantly better than a fit involving only a single class of sites? Using the "extra sum of squares" principle (cf. Appendix B), we find that use of only a single class of sites increases the rms from 18.9 to 24.5% (Table 5, row 5) with a corresponding F value of 9.39 ($df = 2, 23$, $P < 0.001$). Hence, we conclude that a one-class of sites model is seriously inadequate, and a two-classes of sites model is statistically significant better. Even though we do not have sufficient information to precisely characterize K_2 and R_2 individually, the overall contribution of the second class of sites is significantly greater than zero.

(ii) Do the three experiments have the same K_1 and K_2 and ratios R_1:R_2:N? In

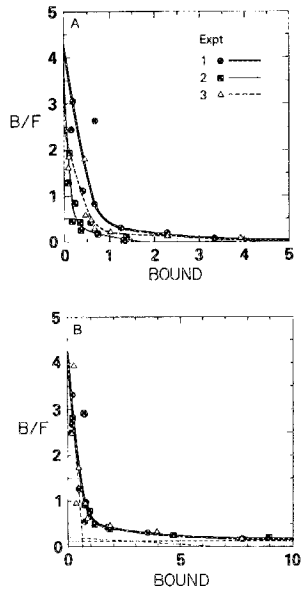

FIG. 5. Scatchard plots for nuclear binding of [^{3}H]aldosterone to A-6 toad kidney epithelium in tissue culture, corrected for DNA content, from three separate experiments. Units for Bound are nmol/mg DNA, for B/F are (mg DNA)$^{-1}$. (A) Values corrected for nonspecific binding. Curves correspond to rows 1–3 of Table 5. Note systematic change in overall binding between experiments, but similarity in shape of the curves. (B) "Best fit" per row 4 of Table 5. Dashed lines show individual components corresponding to the high- and low-affinity classes of sites and nonspecific binding. Results for experiments 2 and 3 have been scaled by the best estimate for C_2 and C_3. Note: points from all three experiments are randomly distributed around the fitted curve.

TABLE 5

ONE LIGAND, TWO RECEPTORS, THREE EXPERIMENTS, SIX FITS

	Experiment	K_1 (nM^{-1})	R_1 (nmol/mg DNA)	K_2 (nM^{-1})	R_2 (nmol/mg DNA)	N	C_2	C_3	Points	Parameters	df	SS	rms (%)
(1)	1	6.3 ± 5.0	0.70 ± 0.30	0.027 ± 0.066	6.4 ± 11.3	0.020 ± 0.006	—	—	10	5	5	2698	23.2
(2)	2	18.6 ± 21.5	0.18 ± 0.09	0.01 ± 0.11	2.0 ± 1.2	0.013 ± 0.002	—	—	10	5	5	1334	16.3
(3)	3	3.9 ± 1.8	0.62 ± 0.18	0.002 ± 0.020	81.0 ± 1150.	0.001 ± 0.100	—	—	10	5	5	1514	17.4
Total									30	15	15	5546	19.2
(4)	1–3	6.1 ± 2.4	0.64 ± 0.16	0.033 ± 0.033	6.4 ± 4.3	0.020 ±0.003	0.54 ± 0.05	0.69 ± 0.06	30	7	23	8300	18.9
(5)	1–3	3.0 ± 0.8	1.06 ± 0.18	—	—	0.030 ± 0.004	0.55 ± 0.07	0.70 ± 0.09	30	5	25	15000	24.5
(6)	1–3	5.0 ± 3.0	0.53 ± 0.20	0.024 ± 0.045	5.8 ± 5.8	0.014 ± 0.003	1.0	1.0	30	5	25	24580	31.3

other words, are we justified in forcing all three experiments to share these features? We can answer this by fitting each of the curves individually, using the values from the common fit as initial estimates, while holding the correction factors (C_2 and C_3) constant (Table 5, rows 1–3). The values for K_1 range from 3.9 to 18.6 nM^{-1}, and R_1 ranges from 0.18 to 0.7 nmol/mg DNA. However, the error for each of these values is large. Even allowing each curve to have its own 5 parameters (and hence 15 parameters in toto) does not provide a better overall fit than the use of the common 5 parameters and two correction factors. In fact, the average rms for Table 5, rows 1–3 combined (19.2%), is larger than the rms for row 4 (18.9%). Thus, the three experiments showed similar properties, and were indistinguishable in the face of the experimental errors. The use of common parameters was justifiable.

(iii) How much do we gain by the ability to "adjust" experiments 2 and 3 relative to experiment 1, over and above the correction for DNA content on individual flasks? We can answer this by constraining the values of $C_2 = C_3 = 1$ (i.e., not using the correction factors), and refitting the data (Table 5, row 6 vs row 4). The absence of the correction factors results in a striking increase of the rms from 18.9 to 31.3%, while the degrees of freedom only increase from 23 to 25. This is a highly significant increase ($F(2,23) = 22.62$) with a $P < 0.001$. We conclude that the use of the correction factors was justifiable and necessary. When $C_2 = C_3 = 1$, there are severe and systematic departures of the points from the fitted curves (as in Fig. 5A), readily detectable by the "number of runs" test ($P < 0.01$). In this case, the results of the F test analysis agree well with graphical inspection (Fig. 5A).

Use of the program LIGAND and the model fitting strategy employed here provides the best way to combine data from multiple experiments available to date. This approach was essential to even begin to get an objective initial description of data. The expenditure in effort to perform the computer analysis was small compared with the overall cost of the experiment.

Rather than having 10 observations in each of three experiments, the experimentalist would obtain estimates of parameters with about the same degree of precision with about 28 points in one

experiment. Unfortunately, it was not possible to obtain 28 points in one experiment using the technology available at the time these data were collected. With revised experimental technique, using filtration rather than centrifugation, and larger experiments, more precise estimates of parameters have already become available.

CASE 6: THREE LIGANDS, TWO CLASSES OF SITES, AND NONSPECIFIC BINDING, MULTIPLE EXPERIMENTS

Studies of pituitary plasma membrane gonadotrophin-releasing hormone (GnRH) receptors have been severely hampered by the binding of GnRH to the relatively large amount of low-affinity binding sites present in crude membrane preparations. These sites tend to mask the high-affinity "physiological" receptor binding, which is of primary interest to the endocrinologist. Further, GnRH is apparently degraded by endogenous peptidase enzymes which thus may alter the total ligand concentration. Clayton *et al*. have utilized analogs of GnRH substituted at positions 6 and 10 of the native decapeptide ([D-Ser(TBu)^6]des-Gly ^{10}-GnRH ethylamide or "D-Ser^6 analog," [D-Ala^6]-des-Gly^{10}-GnRH ethylamide or "D-Ala^6 analog") which appear to resolve both of these problems (12,13). The analogs do not appear to bind to low-affinity sites, yet maintain a high affinity for the true receptor and further, are not inactivated by the tissue preparations. These analogs are thus extremely desirable as probes of the GnRH receptor.

In order to verify that the two analogs bind to the same receptor as GnRH, a series of binding experiments was performed involving combinations of the ligands, e.g., labeled GnRH displaced by D-Ser^6 analog and labeled D-Ser^6 analog displaced by GnRH. These experiments were replicated in two different laboratories using 23 separate membrane preparations (data kindly provided by Drs. R. N., Clayton and J. Marshall).

To properly analyze the data from this study one must consider the interaction of three ligands with two or more classes of receptor sites. Since different experiments used membranes from different individual animals, prepared on different days and in different laboratories, we expected (and found) large variability in receptor concentrations between experiments. We utilized a three-ligand, two-site binding model with provision for nonspecific binding and for adjustable "correction factors" to allow pooling of data over experiments.

The data analysis was performed in five stages. In Stage I, the labeled GnRH, unlabeled GnRH (homologous) displacement curves were analyzed. All data for Bound and Total were expressed in molar concentrations, using the specific activity, counting time, counting efficiency for the particular gamma counter, etc. Because the displacement curves for GnRH showed an obvious two-step form, a two-site model was fiit to each of the curve. Finally, using the averaged values of the parameters as starting estimates, all the homologous GnRH displacement curves from both laboratories were analyzed simultaneously (Fig. 6A). This process involved estimating binding affinities (K_{11},K_{12}), receptor capacities (R_1,R_2), nonspecific binding (N_1), and also the correction factors (C_1,C_2, etc.) which adjust the receptor concentration for each experiments relative to the first one (Table 6, row 1).

In Stage II, all the data for homologous D-Ser^6 analog curves were analyzed as in Stage I, except that a one-site model was fit since the Scatchard plot for each D-Ser^6 analog experiment appeared to be linear. To test the possibility that D-Ser^6 analog might also bind to a second, low-affinity site, a two-site model was also fit to each individual experiment, and then to all experiments simultaneously (Table 6, row 2, Fig. 6B). Although each individual D-Ser^6 analog binding curve was adequately fit by a single class of binding sites, there was a strong indication of the existence of a second, low-affinity class when the five curves were

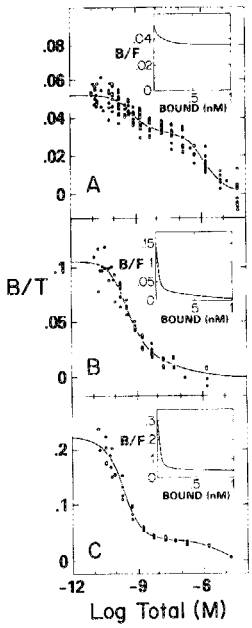

FIG. 6. Binding of GnRH and two analogs to rat pituitary membranes. Computer fit displacement curves (solid line) for homologous data, corrected for nonspecific binding. Inset is same curve transformed to Scatchard plot. (A) GnRH, simultaneous fit to 14 experiments (167 points). Data are adjusted by optimal corrected factors. The high degree of scatter includes between-experiment and between-laboratory components. Individual experiments show a much smaller scatter. (B) D-Ser[6] analog, simultaneous fit to five experiments (52 points). (C) D-Ala[6] analog, simultaneous fit to four experiments (43 points).

analyzed simultaneously. However, the presence of a second binding site for the D-Ser[6] analog depended almost entirely on a very few points in the high-dose region of the homologous analog displacement curves. If these points were deleted, a different conclusion would have been obtained. Additional data in this region of the displacement curve are needed to verify the existence of this second class.

In Stage III, the displacement curves involving only D-Ala[6] analog were analyzed using the techniques developed in Stage II for D-Ser[6] analog. Again, we found that a model involving a second class of binding sites provided a better overall description of the data (Fig. 6C, Table 6, row 3).

Stages I, II, and III established the existence of high- and low-affinity binding sites for each ligand. However, the question of whether or not GnRH and the analogs were in fact combining with the identical set of receptors was not yet considered. Conceivably each of the three ligands could interact with a different set of receptors. To reject this alternative, it was necessary to demonstrate that GnRH could completely displace bound D-Ser[6] analog, and vice versa, by making use of the heterologous displacement curves. In Stage II we measured high affinity binding site capacity, $R_1 = 2.6 \times 10^{-11}$ M for D-Ser[6] analog, which was consistently higher than $R_1 = 1.4 \times 10^{-11}$ M for GnRH measured in Stage I. Thus, we must also consider the possibility that D-Ser[6] analog might react with a class of binding sites in addition to the GnRH binding sites.

All the data involving GnRH and the D-Ser[6] analog were then analyzed simultaneously in Stage IV. Using the program LIGAND, we attempted to fit models A, B, C, and D (Fig. 7) to the data and then to choose the most appropriate one. Model A, which allows only one class of sites for the D-Ser[6] analog, was more or less ruled out in favor of model B in Stage II. Reanalysis with the added heterologous displacement curve data gave an even stronger indication (rms for A, 7.1%; rms for B, 6.1%; $F = 86$; with 2 and 246 df, $P < 0.01$) that model A was inappropriate. We may rule out model C on qualitative grounds alone. Since GnRH is observed to displace labeled D-Ser[6] analog (Fig. 8D) and D-Ser[6] analog completely displaces labeled GnRH (Fig. 8B), the specific receptors for these two ligand must be identical. If model C were correct, then Figs. 8B and D should show horizontal lines; no displacement should be observed.

A more subtle issue is the comparison of model B with model D. These two models are almost identical except that model D allows for an increased binding capacity for the D-Ser[6] analog. This feature was introduced to allow for the higher value for R_1 observed using the D-Ser[6] analog alone (see R_1 in Table 6). In model D, the binding

TABLE 6

GnRH, D-Ser[6] Analog, and D-Ala[6] Analog Binding Parameters

	K_1 (nM^{-1})	K_2 (mM^{-1})	R_1 (pM)	R_2 (nM)	N	rms (%)	df	Number of experiments
GnRH (L_1)	1.4 ± 0.4	0.68 ± 0.11	14. ± 4.	44. ± 7.	0.036 ± 0.001	7.0	150	14
D-Ser[6] (L_2)	3.1 ± 0.7	20. ± 19.	26. ± 6.	0.75 ± 0.66	0.070 ± 0.003	5.6	43	5
D-Ala[6] (L_3)	6.4 ± 0.9	0.20 ± 0.12	37. ± 4.	190. ± 150.	0.023 ± 0.007	8.3	35	4
GnRH	0.31 ± 0.02	0.66 ± 0.09	42.1 ± 3.2	47. ± 6.5	0.036 ± 0.001	8.1	409	42
D-Ser[6]	2.0 ± 0.2	0.29 ± 0.06	42.1 ± 3.2	47. ± 6.5	0.066 ± 0.003			
D-Ala[6]	6.0 ± 0.6	0.70 ± 0.10	42.1 ± 3.2	47. ± 6.5	0.029 ± 0.002			

Note. Rows 1–3: data from homologous displacement curves. Rows 4–6: simultaneous analysis of all data (homologous and heterologous displacement curves) from the three ligands.

site R_3 is assumed to be "hidden" from GnRH, but accessible to D-Ser[6] analog by using the constraint $\{K_{13} = 0\}$. Further, the affinity of D-Ser[6] analog for R_3 was assumed to be identical to its affinity for R_2, i.e., we added the constraint $\{K_{21} = K_{23}\}$. Using the values of the parameters based on model B as starting estimates, we attempted to adjust the binding capacity, R_3, to obtain a better fit to the data. The computer program indicated that $R_3 = 0$ was optimal, thus effectively rejecting model D in favor of model B. Since GnRH can completely displace the labeled D-Ser[6] analog from its receptors (Fig. 8D), there could be no site exclusively available to D-Ser[6] analog. Thus, by using a formal statistical comparison of models A, B, C, and D, we find that the most appropriate choice is model B.

Stage V: Having established the suitable overall model, we then estimated values for the parameters of a three-ligand, two-binding site model using data from all the experiments, involving all three ligands simultaneously (Table 6, rows 4–6). The full 3 by 2 model allows the D-Ser[6] and D-Ala[6] analogs to bind to both a high- and a low-

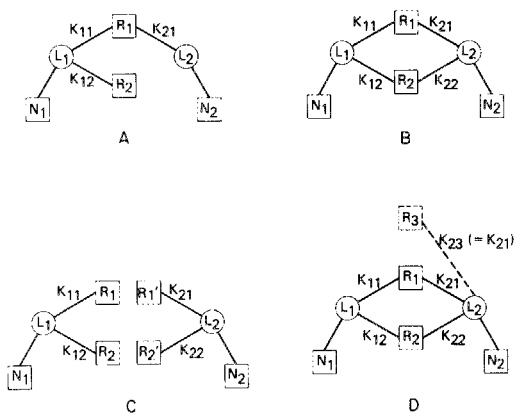

FIG. 7. Schematic binding models for GnRH binding study. Circles represent ligands (L_1, L_2, etc); squares represent classes of binding sites, either specific (R_1,R_2, etc.) or nonspecific (N_1,N_2, etc). (A) Allows only one binding site for the D-Ser[6] analog (L_2). (B) Allows two sites for both GnRH (L_1) and D-Ser[6] analog (L_2). (C) Requires completely separate binding sites for both ligands. (D) Allows extra binding site for D-Ser[6] analog, not available to GnRH. The affinity of this extra site is constrained to be the same as for the other high-affinity site $\{K_{23} = K_{21}\}$.

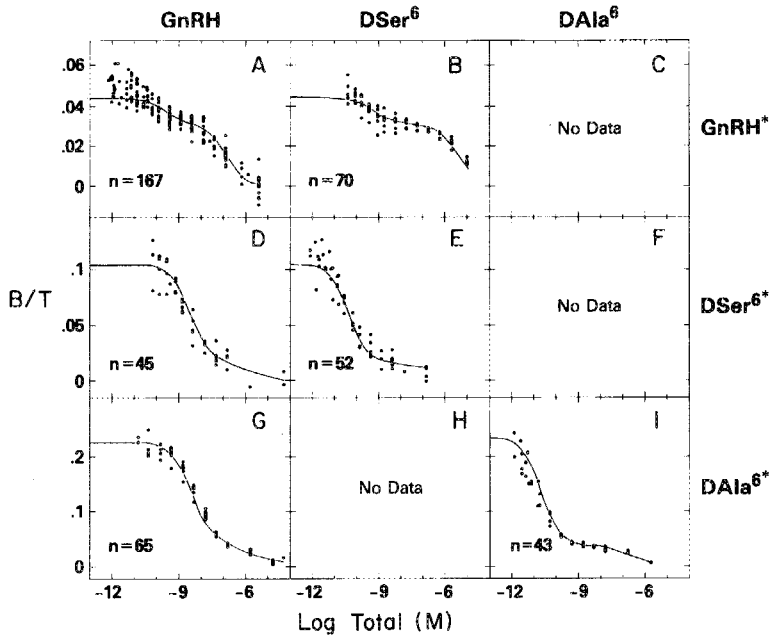

FIG. 8. GnRH and analog binding data, displacement curves, simultaneous fit to all experiments. Six of the nine possible displacement curves involving three ligands are arranged in a three by three array. The labeled ligand is indicated in the right margin, and the unlabeled (varying) ligand along the heading. Homologous curves lie along the main diagonal. The solid lines represent the simultaneous fit to all the data. The relatively large degree of scatter in some panels is due to the inclusion of components of between experiment and between laboratory errors. The B/T values have been corrected for nonspecific binding, and corrected for variable receptor concentration between experiments. Number of data points is indicated in each panel.

affinity site as distinguished from the original observation of a linear Scatchard plot (only one binding site for the analogs). The reason for this incorrect preliminary observation becomes clear in retrospect. Due to the nearly 10,000-fold difference between the affinities of the two binding sites for the D-Ser^6 and the D-Ala^6 analogs, and to the small number of points in the high-dose region, the low-affinity component was only a minor feature of the displacement curve (Figs. 6B, C), and was virtually indistinguishable from the presence of an increased level of nonspecific binding. The Scatchard plot for GnRH showed a definite curvature, since there was only a 500-fold difference between the binding affinities for the two sites. By virtue of analyzing the data simultaneously, the uncertainty of the estimates of K_{22} for D-Ser^6 analog has been reduced from $\pm 100\%$ to less than $\pm 20\%$, similarly for the D-Ala^6 analog. The simultaneous fit of the "3 by 2" model to all the data shows a reasonable rms error (8.1%, Table 6) which is compatible with the values obtained when fitting the data from each ligand individually. The systematic lack of fit in the low-dose region of the homologous GnRH curves (Fig. 8A) was judged to be minor relative to the overall fit of the full model. However, the exact cause of this problem would require further investigation.

DISCUSSION

We have described the development of a generally applicable analysis method and computer program designed to analyze data from a wide variety of ligand-binding experiments. Applications of earlier versions

of this program to specific studies have recently been reported (13–15). In the present paper, we have attempted to demonstrate the technical superiority of the method as well as the wide range of possibilities for its further application. The present method represents an improvement over most of the commonly used simplified or graphical approaches in that (i) the exact mathematical model is fit directly to the data without employing any simplifications or approximations; and (ii) an appropriate, objective criterion for the goodness of fit of the model is used in drawing conclusions from a given set of data.

We have applied this method to a series of examples using both synthetic data simulating representative cases and data collected in the laboratory. These examples show that the computerized method performs well and is generally superior to currently available noncomputerized approaches. Although graphical methods often perform adequately in the simpler cases (e.g., linear Scatchard plots), in the more complex cases a computerized nonlinear least-squares analysis is preferable, and in some cases, mandatory. Once the generalized computer program has been set up for the analysis of a complex binding experiment, all future experiments (even those involving only a single ligand) may be routinely analyzed with little or no increase in time and effort over what is required for noncomputerized means.

This approach enables one to utilize all the information contained in the data. The data points which were formerly discarded because they gave rise to negative values in the Scatchard plot are now retained in the analysis of *uncorrected* Bound vs Total ligand concentration and contribute to an improved, unbiased estimate of N. More reliable confidence limits become available for all parameters. Of course, the computerized method may yield larger confidence limits than previous subjective or ad hoc methods. This is because many previous methods ignore important sources of error, e.g., the uncertainty in estimates of N. Having appropriate confidence limits available, one can test the effect of experimental design on the precision of the estimates. For instance, one can optimize the choice of ligand concentrations when the total number of assay tubes is limited, in order to obtain the best estimate of the high-affinity component, K_1. By providing objective measures of goodness of fit, the program provides the investigator with an improved ability to discriminate between alternative hypotheses or models.

While an investment of time and effort is required to set up a computer program for routine analysis of ligand-binding data, this is small relative to the total cost of the experimental studies and is compensated by the potential benefits of using statistically valid techniques for data analysis.

APPENDIX A

In order to calculate Bound ligand concentration as a function of the total ligand concentrations for each ligand in the system, the computer program uses a standard numerical technique applied to equations describing the binding reactions for each ligand binding to each receptor class. We have modified the original derivation given by Feldman (5,6) to include new parameters for nonspecific binding and for correction factors to account for between-experiment variability of receptor concentration. The mathematical model describes the reactions of n ligands, L_i, binding to m classes of binding sites, R_j.

$$L_i + R_j \rightleftharpoons L_i R_j,$$

$$i = 1, \ldots, n, \quad j = 1, \ldots, m,$$

each with an affinity constant:

$$K_{ij} = \frac{B_{ij}}{F_i \cdot E_j} \qquad [A1]$$

where F_i represents the free concentration of ligand i, E_j is the concentration of empty receptor j, and B_{ij} is the concentration of ligand i bound to receptor j. Empty receptor,

E_j, is defined by the conservation of mass equation for receptors:

$$R_j = E_j + \sum_i B_{ij}$$

for

$$j = 1, \ldots, m, \qquad [A2]$$

where R_j is the total concentration of receptor j. Free ligand concentration, F_i, is defined by the equation for conservation of ligand:

$$L_i = F_i + \sum_j B_{ij}$$

for

$$i = 1, \ldots, n. \qquad [A3]$$

Here, L_i is the total concentration of ligand i. L_i may also stand for the name of the ith ligand (e.g., L_1, L_2, etc.). Similarly, R_j may also stand for the name of the jth receptor. Solving Eq. [A1] for B_{ij} and substituting for B_{ij} into Eqs. [A2] and [A3] yields

$$R_j = E_j + \sum_i K_{ij} E_j F_i$$

for

$$j = 1, \ldots, m, \qquad [A4]$$

$$L_i = F_i + \sum_j K_{ij} E_j F_i$$

for

$$i = 1, \ldots, n. \qquad [A5]$$

Solving Eq. [A4] for E_j yields

$$E_j = \frac{R_j}{1 + \sum_i K_{ij} F_i}$$

for

$$j = 1, \ldots, m, \qquad [A6]$$

which may then be substituted into Eq. [A5] to give

$$L_i = F_i + \sum_j \frac{K_{ij} R_j F_i}{1 + \sum_a K_{aj} F_a}$$

for

$$i = 1, \ldots, n. \qquad [A7]$$

Equation [A7] may now be solved numerically (e.g., using a Newton–Raphson technique) for F_i, given L_i, R_j, and the matrix of values K_{ij}. In turn, the solution for F_i may be substituted into Eq. [A6] to find E_j, and then, using Eq. [A1], we may calculate B_{ij}. Thus, we can calculate the bound ligand concentrations as a function of the total ligand concentrations for the ligand, $B_{ij}(L_1, L_2, _{in}, L_n)$ given the values of the binding parameters, K_{ij}, and R_j.

We have added two new parameters to the basic model as originally formulated, namely N_i for nonspecific binding and C_k which corrects for experiment to experiment variability in receptor concentration. Nonspecific or nonsaturable binding is incorporated into the model by adding a new parameter, N_i, to Eq. [A3]:

$$L_i = F_i + \sum_j B_{ij} + N_i F_i$$

for

$$i = 1, \ldots, n. \qquad [A8]$$

There is a nonspecific parameter, N_i, for each ligand in the system. Each N_i may be interpreted as the limiting B/F ratio of ligand L_i as F_i is made extremely large.

Correction factors, C_k, which adjust the values of R_j and N_i for the kth experiment may be added by replacing Eq. [A8] with

$$L_i = F_i + C_k(\sum_j B_{ij} + N_i F)$$

for

$$i = 1, \ldots, n. \qquad [A9]$$

In order to perform Newton–Gauss minimization of the sum of squared deviations of observed Bound from predicted Bound, it is necessary to compute the partial derivatives of the vector valued function, $\bar{B}(\bar{L})$ with respect to the parameter vectors $\bar{K}$, $\bar{R}$, $\bar{N}$, and $\bar{C}$. Evaluating these derivatives requires a straightforward application of the chain rule, once Eq. [A7] (or its generalization) has been solved numerically for $\bar{F}$.

APPENDIX B

Testing for Significance of Extra Parameters

When additional parameters are added to a model, the goodness of fit will tend to

improve, i.e., the residual sum of squares will decrease, simply because of the added flexibility of the model. Ultimately, if we were to add as many parameters as we have data points it is theoretically possible to make the curve run exactly through each point. We must compromise between adding new parameters and improving the goodness of fit.

There is a statistical test of whether the increase of goodness of fit for a model with additional parameters is significantly more than we would expect on the basis of chance alone. It is based on the "extra sum of squares" principle (16):

$$F = \frac{(SS_1 - SS_2)/(df_1 - df_2)}{SS_2/df_2}. \quad [B1]$$

Here, SS_1 and SS_2 are the residual sums of squares of the deviations of the points to the fitted curve and df_1, df_2 are the associated degrees of freedom (number of data points minus number of parameters) for the original model and the model with additional parameters, respectively. The calculated F ratio is then compared to the tabulated value for the F statistic with $(df_1 - df_2)$ and df_2 degrees of freedom. For example, in Case 2, above, we compare the fit of a one-site binding model with that of a two-site model, which has two new parameters (K_2 and R_2). In this case, the calculated F value is 6.96 (see Table 2), with 2 and 13 degrees of freedom. This value exceeds the tabulated value of 6.7 at the 1% probability level. Thus the two-site model significantly improves the goodness of fit, and is preferable to the simpler model.

Weighted Sum of Squares, rms

In order to determine the best model or the best values for the parameters the program evaluates the total squared deviations of the data points from the predicted values ($\hat{Y}_i$ for the i^{th} data point):

$$SS = \sum (Y_i - \hat{Y}_i)^2 \quad [B2]$$

and finds parameter values which minimize SS, i.e., make the curve run as near to the data points as possible.

When some data points are measured more reliably than others, one must introduce weights, w_i, to offset the tendency for an unreliable point to unduly influence the location of the curve. Thus, one minimizes

$$SS = \sum w_i(Y_i - \hat{Y}_i)^2. \quad [B3]$$

The values for w_i are chosen inversely proportional to the variance of Y_i so that points with smaller variance (more precise) get larger weights.

When the variance of Y_i is proportional to Y_i^2 (constant percent error) then w_i may be set to $1/Y_i^2$ or to $1/\hat{Y}_i^2$. Under these circumstances, the rms error $(=(SS/df)^{1/2})$ when multiplied by 100, may be interpreted as the average scatter, expressed as a percentage, of the data points around the predicted curve. Throughout this paper constant percentage error in the dependent variable ([Bound]) is assumed, although other models for the variance are possible and sometimes preferable. A general, empirical model for the variance is given by

$$\mathrm{Var}(Y) = a_0 Y^J, \quad [B4]$$

or alternatively

$$\mathrm{Var}(Y) = a_0 + a_1 Y + a_2 Y^2. \quad [B5]$$

For a more detailed discussion of weighting, see Refs. (17,18).

The numerical process for finding the values of the parameters which minimize SS uses a Newton–Gauss–Marquardt–Levenberg algorithm (19,20) with a constraint that all parameters take on only nonnegative values. This constraint is implemented by reparameterizing in the logarithm of any parameter which tends to become negative during any iteration. The program allows the user to interrupt the algorithm after each iteration and readjust the parameters, redefine the model, or fix various parameters, thereby reducing the numerical

difficulty of the calculations. With intelligent intervention by the user, it is possible to avoid the slow convergence and occasional numerical instability experienced with the ordinary Newton–Gauss algorithm. Reasonably good (order of magnitude) initial estimates of the parameters are required, but are generally available through the use of simple, graphical methods.

Runs Test

To determine if there is a systematic departure of the data points from the fitted curve, one may look at the signs of the residuals, $(Y_i - \hat{Y}_i)$, to determine if there is an element of nonrandomness in their sequence. One sort of nonrandomness is suggested by having too few or too many runs (or sign changes). Thus, ++++−−−−−++++ is a sequence of signs which seems nonrandom because it has too few (in this case, three) runs. The theoretical distribution of the number of runs in a sequence has been calculated (21) assuming that each sign is an independent random event. This test is applied by the present program, to check for evidence of significant lack of fit.

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