Computer Program Sequence for Analysis and Summary of Radioimmunoassay Data*

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A sequence of three programs is described for efficient design and analysis of radio-immunoassays. The first program designs the assay; the second program analyzes all available data according to the organizational base provided by the first program; and the third program summarizes the results of several assays in either tabular or graphical form. The analysis program uses a logit response–log dose transformation to obtain a linear inhibition curve for all preparations assayed at multiple levels. The curves are subjected to a weighted, least squares, regression analysis. All curves are tested for linearity and parallelism with the curve chosen as standard, and a weighted mean potency estimate with error limits is computed for all preparations run at single or multiple dose levels with any degree of replication.

INTRODUCTION

The increasing use of computer technology for handling large amounts of radio-immunoassay data has been brought about by two considerations. First, the calculation process is a time-consuming chore. The burden of making the necessary calculations involved in sophisticated statistical tests for estimation of error or evaluation of curves virtually precludes the use of any manual system. The second evaluation of curves virtually precludes the use of any hand system. The second consideration is the need for convenient processing and summary of the additional data which may be produced as more efficient methods of calculation become available. The set of three programs presented here provides not only a reasonably sophisticated calculation structure, but also a total data processing system which facilitates the steps from assay planning to data summary analysis. It is designed for use in double antibody radioimmunoassays, but would be valid for other binding

assays employing radioisotopically labeled ligand in which the radioactivity of the bound fraction is counted, regardless of the isotope employed.

This system consists of three procedures: protocol of assay, calculation of results, and summary of data (see Fig. 1). The protocol program designs the assay, describes
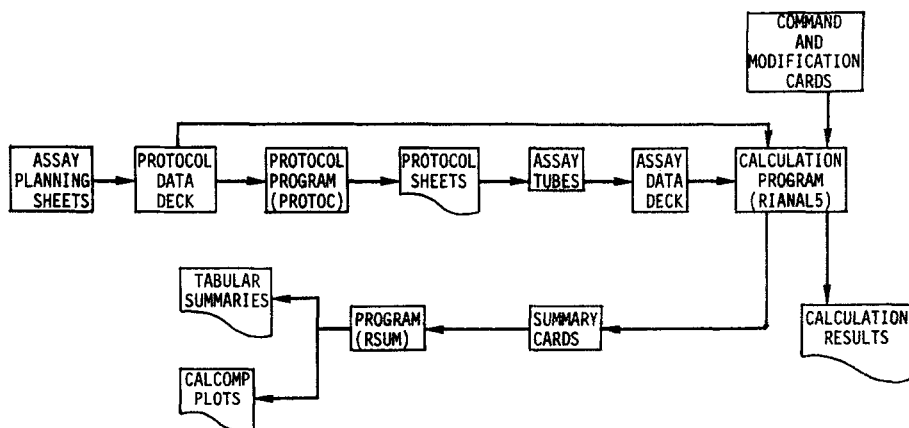

FIG. 1. Flow diagram of the total system; figure illustrates the relation between the inputs, outputs and programs.

all tubes in detail, determines the position of each tube in the assay, and serves as the organizational base upon which the other two programs are constructed. The calculation program analyzes the standards and unknowns, producing error estimates, a variety of statistical validity checks, and optional data summary cards. When appropriate data summary cards have been collected from one or more assays, they are used as input to the summary program. This third program produces either tabular or graphical summary results, or both, on single or multiple observations which are in single or multiple treatment groups.

DESCRIPTION OF PROGRAMS

I. The Protocol

By means of the assay planning sheets, the investigator indicates which standard and unknowns are to be run in the assay. This step has not proven as cumbersome as originally feared. Cards are keypunched from the assay planning sheets (see Fig. 2, listing of protocol deck). Parameters to be entered for each unknown include sample identification (up to 24 characters), sample volume, number of replications at each dose level, number of dose levels, dilution factor between dose levels if more than one are employed, and concentration of unknown.

Several considerations reduce the effort necessary to produce this deck of cards. For example, one protocol card can specify assay details for many tubes. If an

unknown is to be run in duplicate at ten dose levels, twenty tubes will be protocolled by a single protocol card. Further, several defaults have been built into the program. In many cases, only the sample identification must be keypunched for an unknown, and the remainder of the card may be left blank. The program then uses the specifications set for the previous protocol card to describe the new sample. The protocol cards serve as input data to the protocol program (PROTOC), which produces assay protocol sheets (see Fig. 3, output from protocol program).

The assay protocol sheets contain all information necessary to set up the assay. The heading area describes parameters pertinent to the assay as a whole. Subsequently, each tube in the assay is described in detail. Identification and concentration

```
CARD TYPE              INPUT

Assay Number           D136-30
Type of Assay          OFSHHFSHHFSH
Purpose of Assay       DETERMINE FSH CONCENTRATION IN PREPARATIONS
Diluent                PBS-1%E.W.
Anti-serum             ANTI-OFSH 1:1800
Excess Anti-body       NONE
Labled Prep            B144   I327
Anti-RGG               #9  1:12
Incubation             24+24+72
Standard               STND     B56          200.3.  9.  .5  80. MI
Unknown                B518                  100.2.  5.      0.1 ML
   "                   B519                  200.            1.0 UG
   "                   R1429-2 B564                  2.  .1  7.7
   "                   R1429-3 B565                          15.2
```

FIG. 2. Input to protocol program; card type explains the information on each line. The input is in formatted card image form, as it would be submitted to the computer.

of the sample, its tube number in the assay, and the amount of sample and buffer solution are indicated. Tubes described by the protocol program include the standard and unknowns protocolled by the investigator, as well as tubes common to all assays. Common tubes which are automatically protocolled include total count tubes (containing only labeled ligand), background tubes (containing nonimmune serum in place of antiserum) and buffer control tubes (containing antiserum and buffer, but no competing unlabeled ligand).

The order of the tubes in the assay is determined by the program. A standard curve is placed at the beginning and end of the assay—if the standard is to be run in triplicate, the third one goes in the center—which serves as a check on the assay's continuity. With this consistent, unambiguous sheet in hand, the technician is ready to set up the assay. The cost of running the program for a 300 tube assay at the University of Michigan Computing Center is roughly 75¢.

II. *The Calculations*

Inputs to the calculation program (RIANAL5) are three. First is the protocol data deck which was previously used as input to the protocol program. RIANAL5 must have identification for all data produced by the radioisotope counter. The protocol deck is the medium by which this is accomplished.

```
ASSAY #          D136-30
DATE PLANNED     03-17-71
TYPE             OFSHHFSHHFSH
STANDARD         B56
PURPOSE          DETERMINE FSH CONCENTRATION IN PREPARATIONS
DILUENT          PBS-1% E.W.
ANTISERUM        ANTI-HFSH 1:1800
XS ANTIBODY      NONE
LABELED PREP     B144 I327
ANTI-RGG         #9  1:12
INCUBATION       24+24+7
```

TUBE #	CONC	LABEL	TUBE #	SAMPLE UL/TUBE	BUFFER UL/TUBE
1	0.0 /ML	TOTAL COUNTS	1	0.0	0.0
2	0.0 /ML	TOTAL COUNTS	2	0.0	0.0
3	0.0 /ML	TOTAL COUNTS	3	0.0	0.0
4	0.0 /ML	NRS(BACKGROUND)	4	0.0	500.0
5	0.0 /ML	NRS(BACKGROUND)	5	0.0	500.0
6	0.0 /ML	NRS(BACKGROUND)	6	0.0	500.0
7	0.0 /ML	BUFFER CONTROL	7	0.0	500.0
8	0.0 /ML	BUFFER CONTROL	8	0.0	500.0
9	0.0 /ML	BUFFER CONTROL	9	0.0	500.0
10	80.000MI/ML B56	-STD	10	200.0	300.0
11	80.000MI/ML B56	-STD	11	100.0	400.0
12	80.000MI/ML B56	-STD	12	50.0	450.0
13	80.000MI/ML B56	-STD	13	25.0	475.0
14	80.000MI/ML B56	-STD	14	12.5	487.5
15	80.000MI/ML B56	-STD	15	6.2	493.8
16	80.000MI/ML B56	-STD	16	3.1	496.9
17	80.000MI/ML B56	-STD	17	1.6	498.4
18	80.000MI/ML B56	-STD	18	0.8	499.2
19	0.100ML/ML B518		19	100.0	400.0
20	0.100ML/ML B518		20	100.0	400.0
21	0.100ML/ML B518		21	50.0	450.0
22	0.100ML/ML B518		22	50.0	450.0
23	0.100ML/ML B518		23	25.0	475.0
24	0.100ML/ML B518		24	25.0	475.0
25	0.100ML/ML B518		25	12.5	487.5
26	0.100ML/ML B518		26	12.5	487.5
27	0.100ML/ML B518		27	6.2	493.8
28	0.100ML/ML B518		28	6.2	493.8
29	1.000UG/ML B519		29	200.0	300.0
30	1.000UG/ML B519		30	200.0	300.0
⋮					
⋮					
⋮					
69	80.000MI/ML B56	-STD	69	1.6	498.8
70	80.000MI/ML B56	-STD	70	0.8	499.2

FIG. 3. Output from protocol program: the heading information is echoed from input. Selected output lines are shown, including total count and background tubes at the front of the assay, and a standard curve at the beginning and end.

The second source of input is a set of modification cards, all optional describing changes to the assay as protocolled which the investigator may wish to make because of whim or circumstance. These options include the ability to:

1. Describe tubes which were added to and counted at the end of the assay, in addition to those originally protocolled.

2. Delete all reference in the output to any unknown sample which was not set up or counted properly, e.g., one which was dropped or for which insufficient sample was available. While a single standard tube may not be eliminated, any of the multiple standard curves may be ignored.

3. Change the concentration or sample volume of any unknown which was not run with the protocolled value for either or both of these parameters.

4. Indicate a delay in the counting procedure. RIANAL5 is designed to correct raw counts for half-life decay (the user supplies the decay factor for the isotope to be used) from the time the assay begins counting. Exercising this option thus allows for any discontinuity in the counting process.

5. Use any other preparation run in duplicate at multiple dose levels as the standard in place of the original standard. This is quite convenient for measuring the relative potency and parallelism of several preparations against each other.

The third input is a deck containing the results produced by the counting device. Each card contains a tube number, counting time, and number of counts. Any combination of preset time or preset counts may be used to obtain any desired counting error within the desired period of time. These cards may be produced automatically by any one of several available devices connecting counter and keypunch. The order of the cards in the deck corresponds to the tube sequence and is exactly the same as indicated on the protocol sheet.

Calculations and output—RIANAL5 (see Fig. 4, flow sheet for RIANAL5). RIANAL5 first reads the protocol deck and internally reproduces the protocol, so that it can identify each count card as that deck is read in. Modifications to the protocol, if any, are made according to the modification cards. The count cards are then read in as indicated by the modified protocol.

Background is calculated and all counts are converted to counts per minute corrected for background and isotopic decay. The default decay correction is based on the elapsed time of the assay and the half-life of ^{131}I. The elapsed time is the sum of the counting time plus the time required to change samples. The sample changing time employed is 25 seconds, but the program could be easily modified to use a different figure. If an isotope other than ^{131}I is counted, the appropriate half-life may be substituted by means of a protocol option card.

Having completed the above calculations, RIANAL5 checks the IGNORE option. If the option to ignore one standard curve has been exercised, the curve is expunged, as are individual unknown tubes, if so specified. Total count and background tubes are analyzed and the results printed out (see Fig. 5, the beginning of RIANAL5 output).

Next, the buffer control tubes are analyzed. These tubes serve as the 100% point, the point against which all other tubes are measured for calculation of percent bound. Three buffer control tubes are run with each standard curve, resulting in six or nine of these tubes, depending on the number of standard curves.

READ IN PROTOCOL CARDS → WRITE OUT HEADING INFO. → RE-PROTOCOL ASSAY → READ IN MODIFICATION CARD → MODIFY PROTOCOL ACCORDINGLY → READ IN COUNT CARDS PER MODIFIED PROTOCOL

CALCULATE & PRINT TOTAL COUNTS AND BACKGROUND → CALCULATE COUNTS/MIN FOR ALL SAMPLES CORRECTED FOR BACKGROUND AND DECAY → CALCULATE BUFFER CONTROL & ASSOCIATED VARIANCES; REJECT REPLICATES EXCEEDING VARIANCE LIMIT → PRINT BUFFER RESULTS AND STATISTICS

SET UP DUPLICATE STANDARD CURVE TABLE ①

CALCULATE VARIANCE RATIOS FOR ALL UNKNOWNS. STORE REFERENCE TO ALL REJECTED REPLICATES → SHUFFLE STANDARD CURVE ARRAYS IF NEW STANDARD REQUESTED → TWO OR THREE STANDARD CURVES? — two

three → SET UP TRIPLICATE STANDARD CURVE TABLE ②

① DETERMINE VARIANCE RATIOS FOR REJECTION OF STANDARDS → MAKE INITIAL ESTIMATE OF SLOPE & INTERCEPT

② DETERMINE VARIANCE RATIOS FOR REJECTION OF STANDARDS → MAKE INITIAL ESTIMATE OF SLOPE & INTERCEPT

PRINT VARIANCE RATIOS FOR ENTIRE ASSAY → MODIFY ORIGINAL ESTIMATE OF SLOPE BY ITERATIVE WEIGHTING PROCEDURE (TEN TIMES)

MAKE INITIAL UNWEIGHTED ESTIMATE OF SLOPE OF UNKNOWN CURVE OF PREVIOUS REPLICATE SET ⑤

PRINT STANDARD CURVE TABLE AND STATISTICS → BEGIN CALCULATIONS OF UNKNOWN VALUES → IS UNKNOWN FIRST OF A NEW REPLICATE SET? — YES

NO ④

READ OFF STANDARD CURVE. STORE LOGIT & LOG FOR LATER ESTIMATE OF MEAN POTENCY ⑥

⑥ PRINT INDIVIDUAL VALUE ← LOOK AT NEXT UNKNOWN

ONE DOSE LEVEL. PRINT MEAN WITH NO STATISTICS

⑤ ZERO SLOPE? — YES

NO

SUMMARY CARD? — YES → PUNCH SUMMARY CARD ⑦

NO ⑦

USE ITERATIVE WEIGHTING PROCEDURE AS WITH STD. TEST FOR LINEARITY → TEST FOR SIGNIFICANCE OF SLOPE. CALCULATE COMBINED CURVE. TEST FOR LINEARITY OF COMBINED CURVE. CALCULATE MEAN AND STATISTICS → PRINT MEAN AND STATISTICS
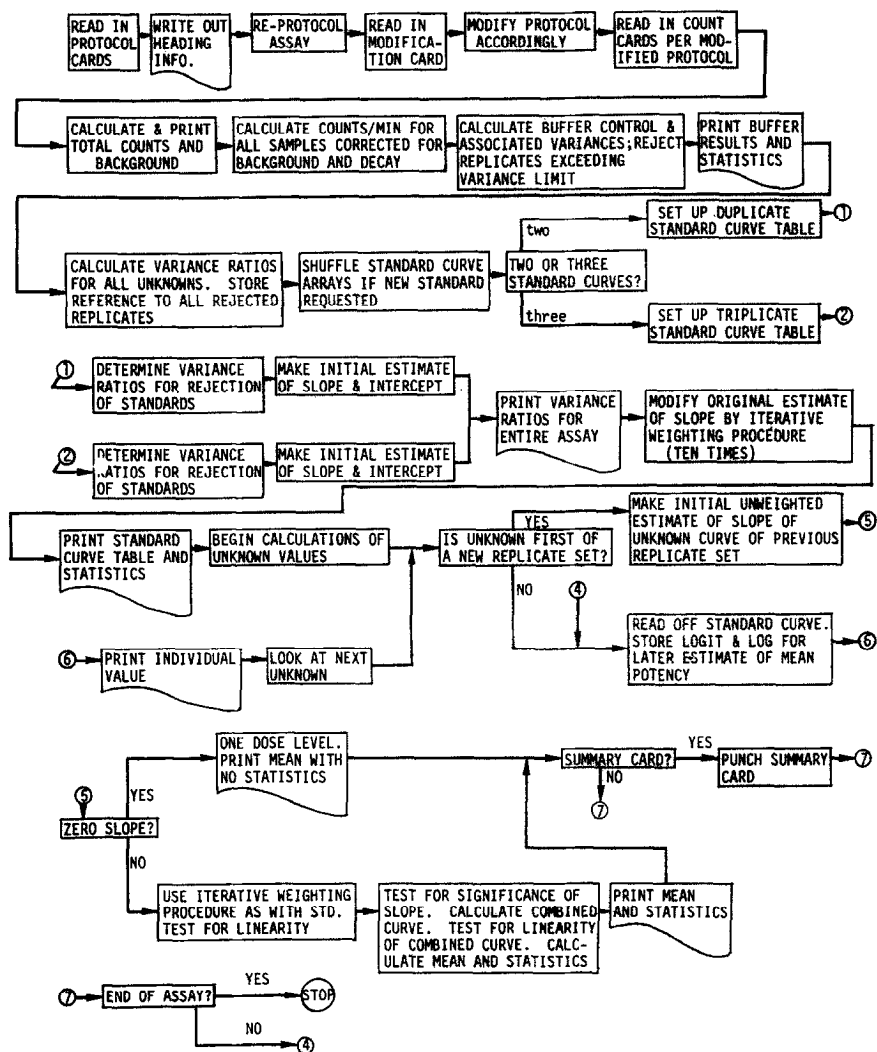
⑦ END OF ASSAY? — YES → STOP

NO ④

FIG. 4. Flow chart of RIANAL5: the internal logic of the calculation program is shown schematically at a low level of detail.

A mean counts per minute value is calculated for each set of triplicate buffer controls, and the individual values converted to a percent of their respective means. The variance of these percent terms, of each set for triplicates, is calculated and compared against the rejection criterion (see Error Analysis below). Following the rejection of replicates, if any, an overall mean count per minute value is calculated. This serves as the 100% point. The variances are stored for later inclusion with those of all other replicates, and all buffer control information is printed.

```
ASSAY #              D136-30
DATE ANALYZED        04-02-71
TYPE                 OFSHHFSHHFSH
PURPOSE              DETERMINE FSH CONCENTRATION IN PREPARATIONS
DILUENT              PBS-1%E.W.
ANTISERUM            ANTI-OFSH 1:1800
XS ANTIBODY          NONE
LABELED PREP         B144  I327
ANTI-RGG             #9  1:12
INCUBATION           24+24+72
STANDARD             STND   B56        -STD   80.00  MI/ML

TOTAL COUNTS PER MINUTE  17864. CPM  %SD =      .072
    COR CPM 17863. 17864. 17865.

AVERAGE BACKGROUND          319. CPM  % OF TOTAL  1.8
    COR CPM   305.   284.   368.

BUFFER CONTROL             5457. CPM  % OF TOTAL 30.5  % SD 2.17
    COR CPM 5274. 5370. 5469. V. RATIO      0.0332
    COR CPM 5576. 5475. 5577. V. RATIO      0.0114
    COR CPM 5429. 5507. 5434. V. RATIO      0.0223

VARIANCE RATIOS FOR ASSAY REPLICATES
    0.0      0.0      0.0      0.0001   0.0010   0.0013   0.0014
    0.0015   0.0016   0.0017   0.0025   0.0025   0.0036   0.0041
    0.0053   0.0063   0.0067   0.0068   0.0076   0.0078   0.0089
    0.0122   0.0126   0.0144   0.0161   0.0175   0.0184   0.0204
    0.0243   0.0246   0.0267   0.0294   0.0295   0.0318   0.0328
    0.0348   0.0358   0.0361   0.0370   0.0397   0.0408   0.0415
    0.0445   0.0473   0.0567   0.0580   0.0581   0.0797   0.0822
    0.1091   0.1135   0.1361   0.1509   2.3000   3.3350   7.6437
   53.7689

MEDIAN VARIANCE RATIO          0.0204
VARIANCE RATIO OF BUF. CON.    0.0223
```

FIG. 5. Output from RIANAL5: first page of output from the calculation program echoes the heading information from the protocol, details the total counts, background and buffer control tubes and establishes means for each. The variance ratio for each set of replicates in the assay is printed.

Error analysis for entire assay. A linear relationship between the variance of a replicate set s^2) and the percent bound (p) at which this set is read over most of the radioimmunoassay inhibition curve has been previously established (*1, 2*). RIANAL5 determines this relationship for each assay as it is calculated. Once computed, the relationship permits determination of a theoretical variance for each individual tube, which is later used as a weighting factor in the calculation of a mean potency estimate.

Since p vs. s^2 is approximately linear, the "variance ratio" ($r = s^2/p$) is essentially constant at all points. If s^2 were normally distributed, the slope (r) of the line p vs. s^2 would be the mean of all variance ratios in the assay. Since s^2 is not normally distributed, we have chosen the median variance ratio R = median r. Experience has provided statistical support for this technique; from over approximately 100 assays tested, the median variance ratio predicted by the entire assay has proved to be not significantly different from the variance ration of the buffer control tubes (the 100% point on the curve).

Replicate samples are rejected when the variance ratio exceeds 1.12. This constant

would be exceeded if two tubes containing buffer gave responses equal to or further apart than 92.5 and 107.5%. This rejection criterion is without theoretical basis, representing the level of disagreement between estimates which is unacceptable to our laboratory. In over 47 assays, tested in a variety of assay systems, 117 of 3935 replicate sets were rejected—roughly a 3% rejection rate.

Analysis of standard curve. An equation to describe the standard curve is obtained according to the methods of Rodbard (2) and Finney (3). An initial estimate of slope and intercept of the logit p vs. log potency standard curve is made by unweighted linear regression on those points between 10% and 90%. Modification of the initial estimate is accomplished by calculating a new series of responses—the working logit. The weight given each point for the weighted least-squares regression is the reciprocal of the variance of the logit of (2) p:

$$\text{variance logit } (p) = \frac{\text{var } (p)}{p^2(100\text{-}p)^2}$$

The variance of p is calculated from the error relationship established earlier. Thus,

$$\text{var}(p) = p \cdot R$$

where R is the median variance ratio. Substituting

$$\text{wt} = \frac{1}{\text{variance logit } (p)} \approx \frac{p^2(100\text{-}p)^2}{p \cdot R}.$$

The process of weighting and calculating a new slope using all points between 0 and 100% is repeated ten times. Experience has shown that differences between subsequent estimations of the slope to the third decimal place disappear after three or four such iterations.

The final estimation of the curve is then checked for linearity by determining if the scatter of mean values about the curve is significantly different from that predicted from within group variation. This check, and further statistical calculations noted for standard and unknowns, are from Finney (3) and Bliss (4). Following the linearity check, a table of results for the standard curve is printed (see Fig. 6, p. 2 of RIANAL5 results). The table includes the doses, responses, means of the responses, and the theoretical response of each dose read off the final estimate of the standard curve.

It should be noted that the "linearity" check does not test for systematic curvature and thus this check often indicates "non-linearity" when only random departure from linearity is present. If the curve is linear, the data are suitable for logit analysis. In addition, the investigator may check the fit by examining the table provided (Fig. 6). The difference between the mean response at each dose level and the theoretical value read from the logit fit should be small and random. In most cases, these differences are less than, or roughly the same as, the range of the responses which make up each mean response.

Calculations of unknowns. Each unknown is treated in the same manner as the standard curve. If the unknown is run at a single dose level, the first estimate of the

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OFSHHFSHHFSH D136-30    04-02-71   PAGE 2
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STANDARD DOSE-RESPONSE CURVE

DOSE		CURVE1	CURVE2	CURVE 3	RANGE	MEAN	LOGIT	DIFF
16.0000	MI	8.9%	10.2%	8.7%	1.5	9.3%	9.2%	-0.1
8.0000	MI	20.0%	22.0%	18.2%	3.8	20.0%	21.3%	1.2
4.0000	MI	38.7%	44.7%	49.9%	11.2	44.4%	41.8%	-2.7
2.0000	MI	66.2%	61.5%	65.7%	4.7	64.5%	65.6%	1.1
1.0000	MI	81.3%	80.5%	82.1%	1.6	81.3%	83.5%	2.2
500.0000	UI	90.7%	97.3%	97.3%	6.6	95.1%	93.1%	-2.0
250.0000	UI	94.1%	98.9%	95.0%	4.8	96.0%	97.3%	1.3
125.0000	UI	99.1%	97.0%	98.6%	2.0	98.2%	99.0%	0.7
62.5000	UI	98.7%	96.1%	100.3%	4.2	98.4%	99.6%	1.3

EST	SLOPE	S.E. SLOPE
1	-3.117	0.0
10	-3.243	0.097
11	-3.243	0.097

```
SLOPE OF STANDARD CURVE          -3.24
INTERCEPT OF STANDARD CURVE       1.62
RESIDUAL VARIANCE               0.0340
LAMBDA                         -0.0105
OBSERVED F OF LINEARITY         1.1374
SIGNIFICANCE LEVEL              0.3839
STANDARD CURVE IS LINEAR
```

DOSES ON THEORETICAL CURVE

```
95.0%    390.3921 UI/TUBE
90.0%    663.6316 UI/TUBE
85.0%    921.7031 UI/TUBE
80.0%      1.1803 MI/TUBE
70.0%      1.7307 MI/TUBE
50.0%      3.1587 MI/TUBE
30.0%      5.7650 MI/TUBE
20.0%      8.4530 MI/TUBE
10.0%     15.0344 MI/TUBE
```

```
100%-1 S.E. OF B.C.              74.0114 UI (99.5%)
       (LIMIT OF DETECTION)

100%-1 S.D. OF ASSAY           247.8509 UI (97.3%)
       (DERIVED FROM MED. VAR. RATIO)

100%-2 S.D. OF B.C.            274.7368 UI (97.0%)
```

FIG. 6. Output from RIANAL5: second page of output is a description of the standard curve. "RANGE" is difference between high and low values at a dose level. "MEAN" is the mean of values at a dose level. "LOGIT" is the value obtained by reading dose off theoretical standard curve. "DIFF" is difference between mean and logit. "EST" is n-th estimate of slope in the iterations to establish the standard curve. "B.C." stands for "buffer control tubes". Three different methods for determining assay "sensitivity" are provided.

curve produces a mean log potency and a slope of zero. No further estimates of the slope are attempted and the anti-log of the mean log potency is the mean potency estimate. Since a curve does not exist, none of the statistics associated with a curve is calculated or printed.

```
B518
   19 100.0UL/T      1157.CPM    21.%   8.031MI   803.12 MI/ML
   20 100.0UL/T      1217.CPM    22.%   7.851MI   785.14 MI/ML
   21  50.0UL/T      2396.CPM    44.%   3.787MI   757.57 MI/ML
   22  50.0UL/T      2248.CPM    41.%   4.069MI   813.83 MI/ML
   23  25.0UL/T      3536.CPM    65.%   2.064MI   825.46 MI/ML
   24  25.0UL/T      3400.CPM    62.%   2.201MI   880.45 MI/ML
   25  12.5UL/T      4186.CPM    77.%   1.326MI     1.06 I/ML
   26  12.5UL/T      4387.CPM    80.%   1.169MI   935.21 MI/ML
   27   6.2UL/T      4966.CPM    91.%   0.609MI   973.60 MI/ML
   28   6.2UL/T      4879.CPM    89.%   0.704MI     1.13 I/ML
         VAR      SLP    LAMBDA OBS F LIN SIG  LIN   CRIT F OBS F PARALLEL
UNKN 0.0262   -2.950 -0.009  2.240  0.202  YES    4.13   3.40  YES
COMB 0.0006   -3.153 -0.008  2.022  0.063  YES
B518                          0.100ML/ML   815.54(787.12-845.07)MI/ML N=10

B519
   29 200.0UL/T      1643.CPM    30.%   5.670MI    28.35 MI/UG
   30 200.0UL/T      1817.CPM    33.%   5.188MI    25.94 MI/UG
   31 100.0UL/T      3078.CPM    56.%   2.691MI    26.91 MI/UG
   32 100.0UL/T      3085.CPM    56.%   2.675MI    26.75 MI/UG
   33  50.0UL/T      3934.CPM    72.%   1.642MI    32.83 MI/UG
   34  50.0UL/T      4027.CPM    74.%   1.507MI    30.14 MI/UG
   35  25.0UL/T      4786.CPM    88.%   0.762MI    30.46 MI/UG
   36  25.0UL/T      4546.CPM    83.%   1.035MI    41.41 MI/UG
   37  12.5UL/T      4911.CPM    90.%   0.667MI    53.35 MI/UG
   38  12.5UL/T      4906.CPM    90.%   0.678MI    54.22 MI/UG
         VAR      SLP    LAMBDA OBS F LIN SIG  LIN   CRIT F OBS F PARALLEL
UNKN 0.0406   -2.789 -0.015  3.066  0.130  YES    4.13   5.91  NO
COMB 0.0007   -3.131 -0.008  2.759  0.014  NO
B519                          1.000UG/ML    27.85( 26.80- 28.93)MI/UG N=10

R1429-2 B564
    :
    :
    :
```

FIG. 7. Output from RIANAL5: third and successive pages show calculation of unknowns. Printed for each tube are volume of unknown, resulting counts per minute, percent of buffer control, amount of unknown per tube, and concentration of unknown in sample. When multiple dose levels are run, printed for both the unknown and combined curves are the residual variance, slope, lambda, F test for linearity, the probability value for the F test, and whether it is significant at the .05 level. For the unknown curve alone, an F test is used to check parallelism with the standard. Finally, the name and concentration of the unknown, the mean and one standard error confidence limits, and the number of individual values used in the calculation of the mean are printed. "COMB" lists values for the combined curve. A test for homogeneity of variance for the standard and unknown has not been included.

For an unknown run at multiple dose levels, standard parallel line bioassay-type statistics are employed (3, 4). After the linearity of the unknown curve and the significance of its slope are established, the curve is tested against the standard for parallelism. A mean potency estimate is then calculated using the slope of the combined curve of the standard and unknown. If the curves are not parallel, the mean potency is still calculated, but the investigator must use his knowledge of his own assay system in deciding whether such a result is meaningful.

Printout for each unknown sample includes tube number, sample volume, corrected counts per minute, percent bound, amount of measured ligand in the tube, and concentration of measured hormone in the unknown (see Fig. 7, p. 3 of

RIANAL5 output). A mean potency estimate with estimates of error is printed for each unknown. These error estimates represent one standard error on either side of the mean. Conversion of log units to antilogs results in unequal intervals between the mean and the upper and lower values. If the unknown was run at multiple dose levels, a table of statistics is printed which pertains to both the resulting and unknown combined curves. Values include the residual variance, slope, lambda, F-tests for linearity and parallelism, and additional comment if the slope is not significant.

If requested, a summary card is punched specifying essentially the same information available on the printed line containing the mean potency estimate. The exercise of this option produces cards for input to the summary program. The cost of running RIANAL5 with a 300 tube assay at the University of Michigan Computing Center is about $3.00.

III. *The Summary*

Machine specificity. Several routines in the summary program (RSUM)—particularly those used to produce the plots—are specific to the University of Michigan computer facility. Substantial reprogramming would be required to make RSUM run at any other facility. Consequently, discussion of RSUM will be more brief and theoretical to facilitate communication of the principles underlying the program.

Overview. The summary program (RSUM) is designed to summarize observations made over time. The relation of values to each other through a defined time sequence is the assumed basis of the experiment to be summarized. Measurement of hormones in menstrual cycles is an example of the type of problem which the program is designed to process.

The analysis technique employed is to choose a time segment of a defined number of days, hours, or minutes, and scan across all the data for a specific number of such segments. Segments may contain one or more data values, or indeed, none at all, depending on the sequence of observations along the time line. A mean and variance are calculated for each segment. These are printed, together with the number of values they represent. The same technique is used whether the data scanned represent a single individual, multiple individuals combined in one treatment group, or a combination of treatment groups.

Three time points may be defined in the data—the beginning, the end, and a variable point of interest between the extremes to be specified by the investigator. A peak level for a subject in question would be an example of such a point. The scan across the data can originate at any of the three points, permitting data on multiple individuals to be combined and synchronized in four different ways. That is, four separate scans are possible—from the beginning forward, from the end backward, and from the center in either direction.

Either CALCOMP (computed controlled free-moving pen which draws figures) or printer (computed generated images put out on the line printer) plots are possible.

Plots of the segment means may be obtained for both individuals and treatment groups. In addition, up to five individuals or treatment groups may be plotted
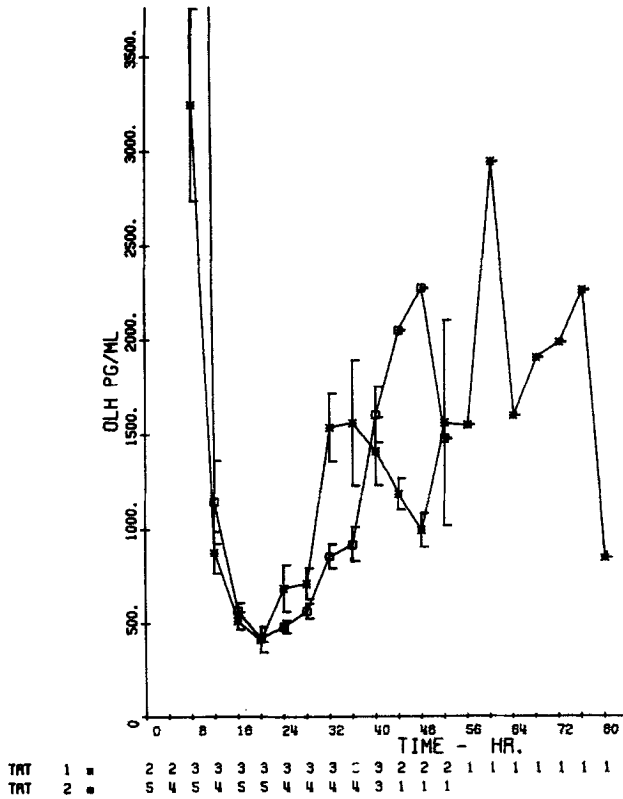
FIG. 8. An example of data analyzed and CALCOMP plotted by RSUM. Observations from two treatment groups have been analyzed at 4 hour intervals prior to an "event," the occurrence of an LH surge on the day of estrus in sheep. Vertical bars represent one standard error limits of the mean of the number of observations in each time interval as shown beneath the abscissa.

together on the same CALCOMP graph. If the individuals or groups are plotted separately, either CALCOMP or printer plots or both may be obtained. The CALCOMP plots are more involved and expensive to produce, making it advisable to obtain printer plots first for an approximate display of the data (Fig. 8).

Input. The input to RSUM is summary cards which may be produced by RIANAL5. Information on the summary card includes sample identification, time (year, month, day, hour, minute, or whatever portion of those numbers is appropriate), treatment number (if multiple treatment groups are to be included), and the value together with its unitage.

Calculations. The greater part of RSUM is concerned with setting up the framework of time segments into which data are sorted, as described in the overview. The calculations as such are done on two levels: by individual and by treatment group. Time segment means and variances may be calculated at both levels. At the

individual level, data values are aggregated into time segments and segment means and variances produced. Where multiple individuals are to be combined, the time segment means for such treatment groups are a weighted average—the average of the individuals' means weighted by their particular variances.

USE OF RIANAL5 FOR ASSAY QUALITY CONTROL

It is planned to modify RIANAL5 to provide, on a routine basis, a single punched card containing selected assay quality control parameters. By comparing these parameters with those from previous assays of the same type (stored on previously punched cards), it will be possible to determine with a separate program which parameters are outside 95% and 99% confidence limits as directly determined from analysis of all prior experiences with that assay. The results of this analysis can then be displayed in both summary tabular form and as batch plot scattergrams similar to those recommended by Rodbard et al. (5). Current experience suggests that useful parameters will include the assay median variance ratio, the percent of total labeled ligand bound in buffer control tubes and in "background" tubes, the slope of the standard curve, the dose of standard at the 50% intercept, and the assay results on two or three standard sera.

ACKNOWLEDGMENT

Note Added in Proof: The modifications suggested above for quality control analysis have now been incorporated into the latest version of the program, RIANAL6. Copies of these programs can be obtained by writing A.R.M.

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