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Research Article

MEASUREMENT OF SMALL QUANTITIES OF INSULIN-LIKE ACTIVITY USING RAT ADIPOSE TISSUE. II. EVALUATION OF PERFORMANCE*

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In the preceding paper (1), a biological assay method was proposed which utilized the increased production of CO_2 from glucose in response to minute quantities of insulin by epididymal adipose tissue of rats. Since the quality of an assay method is best revealed by its performance over a period of time, the present report will present the results obtained during 25 months when this assay procedure was applied to insulin solutions and was tested on more complex material such as human blood serum. The quantitative evaluation of the data was carried out to a large extent independently from the actual performance of the assays, although consultations between the statistical and the biological teams occurred at irregular intervals of a few months.

MATERIALS AND METHODS

Blood sera. Blood samples were collected by venipuncture and allowed to clot at room temperature for 1 to 2 hours. After centrifugation at room temperature, the sera were separated and immediately frozen after removing a small sample for glucose determination. The samples were then kept frozen until the evening preceding the assay when they were transferred to a cold room kept at 4° C. When repeated determinations were to be carried out on the same serum sample, aliquots were frozen separately in order to avoid repeated thawing and freezing.

Assay design. The technical details of the assay procedure have been described in the preceding paper (1). In a routine assay of unknowns, the standard insulin solution was always given at least at two concentrations: ≥ 31 μU and $\leq 1{,}000$ μU per ml. Four "unknowns" were usually assayed at single levels. The six "treatments" were each given to a tissue segment from each of three rats. Subsequent to the demonstration (1) of systematic differences between proximal, medial and distal segments,[1] a "balanced segment" design (illustrated in Table I) was adopted to eliminate these differences as well as differences between rats, from comparisons between treatments. The balanced segment design was also used in studies of the log dose-response curve (Table I).

Statistical methods. Except for some early analyses, the data were recorded as the logarithms (logs) of the counts per minute per milligram wet weight of adipose tissue (1). The calculations for an assay are illustrated in Figure 1. The log counts per minute per milligram were first subjected to an analysis of variance to derive the residual standard deviation (s) after removing the effect of differences between rats, between segments, and between treatments. The standard slope (b), calculated for each assay separately, was used to estimate, by accepted methods (2, 3), the logarithm (M′) of the potency of an "unknown" substance relative to the mean log dose ($\bar{x}_s$) of insulin standard. The logarithm (M) of the estimated insulin-like activity (ILA) in microunits of insulin per milliliter was calculated from M′ as shown in

TABLE I

Examples of design for segment balance over treatments within assays

Routine assay	Standard curve	Rat 1	Rat 2	Rat 3
	μU insulin			
Unknown 1	31	D*	P*	M*
Insulin, low dose	62	M	D	P
Unknown 2	125	P	M	D
Unknown 3	250	D	P	M
Insulin, high dose	500	M	D	P
Unknown 4	1,000	P	M	D

* D, P and M designate distal, proximal and medial adipose tissue segments, respectively.

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[1] The segment closest to the base (i.e., closest to the epididymis) is termed the "proximal" segment, followed by the "medial" and the "distal" segments.

Response Metameter: y = log cpm Assay Date 8-28-59

RESPONSE:

RAT	Standard: Lo: 31	Hi: 1000	U1	Unknowns: U2	U3	U4	Sum(R)	Segment	Sum(S)
1	M 1.25	M 1.88	D 1.16	P 1.60	D 1.89	P 1.76	9.54	D	8.75
2	D 0.70	D 1.48	P 0.96	M 1.36	P 1.34	M 1.46	7.30	M	9.09
3	P 1.07	P 1.78	M 1.34	D 1.56	M 1.80	D 1.96	9.51	P	8.51
Sum(T)	3.02	5.14	3.46	4.52	5.03	5.18	26.35 = (Σy)		
$\bar{y}$	1.007	1.713	1.153	1.507	1.677	1.727			

BASIC CALCULATIONS:

$\Sigma(y^2)$: 40.6751 $\Sigma(R^2)/6$: 39.1236

$(\Sigma y)^2/N$: 38.5735 $\Sigma(S^2)/2r$: 38.6018

$\Sigma(T^2)/r$: 40.0251

$b = \frac{\bar{y}_{Hi} - \bar{y}_{Lo}}{\bar{x}_{Hi} - \bar{x}_{Lo}} = \frac{0.706}{1.509} = 0.468$

$\lambda = s/b$: .203

ANALYSIS OF VARIANCE:

Source	d.f	SS	MS	F	P
Total	17	2.1016			
Rats	2	0.5501	.2750	30.56	<.001
Segments	2	0.0283	.0142	1.58	>.05
Treatments	5	1.4516			
Regr. (std)	(1)	0.7491	.7491	= SSR 83.23	<.001
Residual	8	0.0716	.0090	= s^2	
			.095	= s	

POTENCY ESTIMATE:

		U1	U2	U3	U4
	(1) $\bar{y}_u - \bar{y}_s$	−.207	+.147	+.317	+.367
$\bar{y}_s$: 1.360					
	(2) (1)/b = M'	−.442	+.314	+.677	+.784
$\bar{x}_s$: 2.246	(3) (2) + $\bar{x}_s$ = M	1.804	2.560	2.923	3.030
	(4) $\log^{-1}$(3) = R	64	363	838	1071

EXPANDED STANDARD ERROR OF M:

$t^2_{.05}$: 2.306 $t^2_{.05}$: 5.318

$g = s^2t^2/SSR$: .064

$1/(1-g)$: 1.068

$(N_s+N_u)/N_sN_u$: .5

(5) $[(1)^2/SSR][1/(1-g)]$	.061	.031	.143	.192
(6) $\left[\frac{1}{1-g}\right]\left[(5) + \frac{N_s+N_u}{N_sN_u}\right]$	.599	.567	.687	.739
(7) $\sqrt{(6)}$	.774	.753	.829	.860
(8) λ(7) "Expanded" SE_M	.157	.153	.168	.175

CONFIDENCE LIMITS:

(9)	t(8) = 1/2 L	.362	.353	.387	.404
(10)	[1/(1−g)](2)	−.472	+.335	+.723	+.837
(11)	(10) + $\bar{x}_s$	1.774	2.581	2.969	3.083
(12)	(11) − (9)	1.412	2.228	2.582	2.679
(13)	(11) + (9)	2.136	2.934	3.356	3.487
(14)	$\log^{-1}$(12) = R_{Lo}	26	169	382	478
(15)	$\log^{-1}$(13) = R_{Hi}	137	858	2270	3069

FIG. 1. THE STATISTICAL ANALYSIS OF A ROUTINE ASSAY.* This analysis form is designed for routine assays in which six "treatments," two doses of insulin standard and four "unknowns," each at one level, are given to the adipose tissue segments of each of three rats. Modifications in the form and the analysis must be made if there are one or more missing observations (8) or if there is a different arrangement of treatments, i.e., more than two dose levels of insulin or more than one dose level of an unknown.

NOTATION: y = an individual response (log cpm/mg). x = log dose of insulin. $\bar{y}$, $\bar{x}$ = mean of y and x, respectively. D, M, P = distal, medial and proximal segment of fat pad, respectively. R = in the analysis of variance, sum of y for one rat. R, R_{Lo}, R_{Hi} in computation of potency and confidence limits, μU ILA. r = number of rats or of responses to one "treatment." T = sum of y for one "treatment." S = sum of y for a segment (D, M or P). N = 6r, total number of responses. N_s = 2r, number of responses to insulin standard. N_u = r, number of responses to a specific unknown. Σ = "the sum of." $t_{0.05}$, $t_{0.01}$ = value of t at the significance level selected (p = 0.05, p = 0.01) for the available degrees of freedom (d.f.) in the residual mean square (s^2). SS = sum of squares. MS = mean square (SS divided by d.f.). F = variance ratio (5), i.e., specific MS divided by residual MS.

* FORMULAS FOR THE ANALYSIS OF VARIANCE

Source	d.f.	SS	Example
Total	N-1	$\Sigma(y^2) - (\Sigma y)^2/N$	$(1.25^2 + \cdots + 1.96^2) - 26.35^2/18$
Rats	r-1	$\Sigma(R^2)/6 - (\Sigma y)^2/N$	$(9.54^2 + \cdots + 9.51^2)/6 - 26.35^2/18$
Segments	3-1	$\Sigma(S^2)/2r - (\Sigma y)^2/N$	$(8.75^2 + \cdots + 8.51^2)/6 - 26.35^2/18$
Treatments	6-1	$\Sigma(T^2)/r - (\Sigma y)^2/N$	$(3.02^2 + \cdots + 5.18^2)/3 - 26.35^2/18$
Regr. (std)	(1)	$(T_{Hi} - T_{Lo})^2/2r$	$(5.14 - 3.02)^2/6$
Residual	N-r-7	Total SS − (Rat + Segment + Treatment SS)	2.1016 − (0.5501 + 0.0283 + 1.4516)

Test for regression: F = regression MS/residual MS = 83.23, $p < 0.001$. If the regression F ratio is not significant, potency estimates cannot be made.

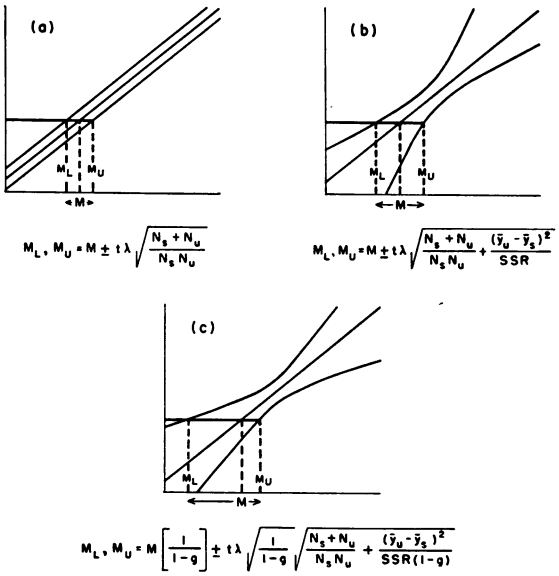

FIG. 2. SCHEMATIC ILLUSTRATION OF DIFFERENT METHODS USED IN CALCULATING CONFIDENCE LIMITS FOR M. The three diagrams show the same dose-response regression line, surrounded by confidence limits (for the line) that correspond to the formula illustrated. a. APPROXIMATE LIMITS WHICH NEGLECT ERROR IN SLOPE. b. WIDER APPROXIMATE LIMITS, BASED ON CALCULATIONS THAT IGNORE THE STATISTIC c. The confidence limits around the regression line are equally spaced in a horizontal direction from each point on the line, and M_L and M_U are symmetrically placed about M. c. ACCURATE LIMITS COMPUTED FROM THE RIGOROUS FORMULA (4), WHICH ALLOWS CORRECTLY FOR ERRORS IN THE ESTIMATES OF THE REGRESSION LINE AND IN THE RESPONSES. The confidence limits around the regression line are equally spaced in a vertical direction from each point on the line. M_L and M_U are asymmetrically placed about M. When the value of g is very small the limits in b and in c are practically identical.

the figure. The "expanded" variance [2] and the confidence limits (M_L and M_U) for M were calculated (Figure 1) according to the rigorous formula (4) which incorporates terms for the error in the regression line and for the difference between the mean response to unknown ($\bar{y}_u$) and to the reference standard ($\bar{y}_s$). A comparison between approximate formulas for the confidence limits and the rigorous formula is made schematically in Figure 2.

Log dose-response curves were analyzed by the usual regression methods (5). The "paired-data" t test was used to compare correlated slopes such as those in Table II. "Heterogeneity" of slopes in different assays or of M values from different assays for the same unknown was tested by the method of Cochran (6, 7), which is illustrated, for M values, by Bliss (3, 8).

Results reported as not significant had a probability (p) > 0.05 under the appropriate null hypothesis.

Log dose-response curves

A study of the response to graded doses of insulin in the range 31 to 1,000 μU per ml was made in a balanced segment experiment (Table I) performed on 5 consecutive assay days (Figure 3). A highly significant ($p < 0.001$) linear regression on log dose was observed on each day, and the five slopes did not vary significantly from each other. Curvature was not significant on any day, or in the pooled results. Comparable results were obtained in eight subsequent similar assays (Table II) except that in two instances significant deviations around the straight line were present.

Because many of these results showed an apparent, though nonsignificant, flattening at the upper part of the curve, the regressions were recalculated for the dose range 31 to 500 μU per ml. This calculation resulted in an increased slope and a slightly lower index of precision ($\lambda = s/b$) in 11 of the 13 cases (Table II). The difference in the two sets of slopes was highly significant ($p < 0.001$). Deviations from the regression line were now significant in only one assay. The combined findings, therefore, suggested that the 1,000 μU per ml concentration might be beyond the linear portion of the dose-response curve although individual assays with three rats were not, in general, sufficiently powerful to detect this phenomenon.

Routine assays

In the period March 1958 through March 1960, several modifications were introduced in the design of the assays and in the doses of standard insulin used. The assays

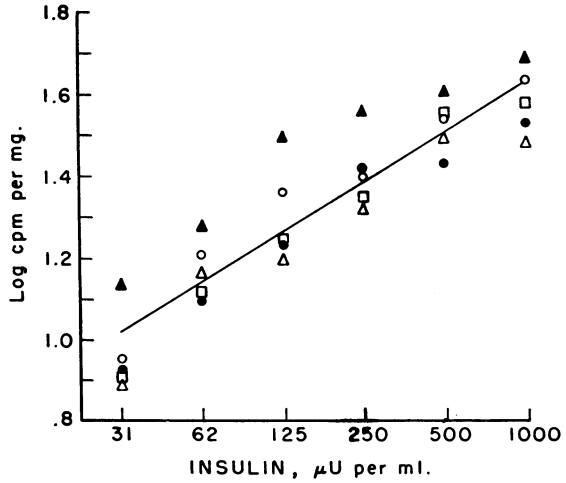

FIG. 3. DOSE–RESPONSE CURVES OBTAINED ON FIVE DIFFERENT DAYS. Each point shows the mean log counts per minute per milligram of tissue from three rats; the line is the calculated mean log dose-response curve.

[2] This "expanded" variance is identical with Bliss's (3) use of $L^2/4t^2$ where L is the confidence interval for an estimate.

TABLE II

Summary statistics showing response to log dose of standard insulin *

Assay date	Residual standard deviation (s)	Response to 6 doses (range 31–1,000 μU) b	Response to 6 doses (range 31–1,000 μU) λ	Response to 5 doses (range 31–500 μU) b	Response to 5 doses (range 31–500 μU) λ
5 Day experiment					
4/6/59	0.083	0.405	0.205	0.399	0.208
4/7/59	0.095	0.450	0.211	0.503	0.189
4/8/59	0.081	0.415	0.195	0.448	0.181
4/9/59	0.149	0.388	0.384	0.455	0.327
4/14/59	0.094	0.357	0.263	0.404	0.233
Subsequent assays					
5/13/59	0.075	0.415	0.181	0.469	0.160
8/5/59	0.115	0.377	0.305	0.430	0.267
8/27/59	0.094	0.331	0.284	0.325	0.289
9/25/59	0.071	0.502†	0.141	0.581†	0.122
9/28/59	0.109	0.425	0.256	0.486	0.224
9/30/59	0.133	0.477	0.279	0.518	0.257
10/2/59	0.145	0.424	0.342	0.492	0.295
10/5/59	0.062	0.375†	0.165	0.448	0.138
Mean‡	0.100	0.406	0.264	0.448	0.231
Standard deviation‡	0.028	0.041	0.063	0.053	0.057

* Each line is based on responses, in log cpm per mg, from three rats.
† Significant deviations from linearity ($p < 0.05$).
‡ In the calculations for mean and standard deviation, the assays with significant curvature were omitted.

were divided, according to these modifications, into the four series shown in Table III and Figure 4. To study the characteristics of the method under routine laboratory conditions, a "routine assay" was defined as one which: *a*) was intended for the study of the response to substances other than solutions of standard insulin; *b*) conformed to the design of its period; and *c*) included three responses to each of at least two dose levels of standard insulin.

TABLE III

Summary of 147 routine assays for which the log counts per minute were analyzed

Series	I	II	III	IV
Dates	3/5/58–4/3/59	4/13/59–5/27/59	6/10/59–10/19/59	11/6/59–3/31/60
Doses of insulin standard (μU/ml)	62–1,000	62–1,000	31–1,000	31–500
Balanced segment design	No	Yes	Yes	Yes
No. of assays included	45	16	31	55
Mean ± observed standard deviation:				
s	0.085 ± 0.031	0.103 ± 0.026	0.106 ± 0.032	0.102 ± 0.039
b	0.262 ± 0.071*	0.318 ± 0.090	0.356 ± 0.082	0.446 ± 0.068
λ	0.340 ± 0.143*	0.349 ± 0.117	0.313 ± 0.126	0.232 ± 0.092
Number of assays with significant† rat variation	39	8	20	31
Proximal segment giving lowest response:				
No. of assays		8	27	49
No. of assays in which difference was significant†		1	9	23

* Does not include 5 assays with nonsignificant slope on standard.
† $p < 0.05$.

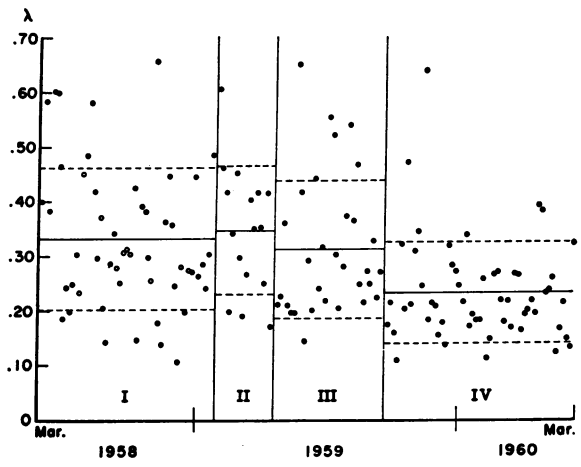

FIG. 4. VALUES OF λ OBTAINED IN ROUTINE ASSAYS WITH SIGNIFICANT SLOPES PERFORMED DURING THE PERIOD MARCH, 1958 THROUGH MARCH, 1960. Assays analyzed by $\sqrt{cpm}$ have been included and their λ values shown by open circles. In each part of the figure, the solid line represents the mean λ and the broken lines indicate one standard deviation on each side of the mean. Roman numerals refer to series identified in Table III.

A statistical analysis of the log counts per minute per milligram of tissue was made of all 30 routine assays performed in the three month period, May to July, 1959, and all of the 55 routine assays performed in the five month period, November 1959 through March 1960. The other routine assays summarized in Table III were chosen for analysis for a variety of reasons, mainly that of deriving estimates of ILA for certain human sera and pancreatic extracts.

Five of the assays analyzed, all in Series I, were nonvalid because the responses to standard insulin failed to show a significant slope. Where the slope was significant, the values of b and of λ in the assays of the first three series were similar, but an appreciable and statistically significant ($p < 0.01$) change may be observed in the statistics from Series IV assays (Figure 4).

A detailed tabulation of results in consecutive assays from Series III and IV is shown in Table IV. In each part of the table, the variation in the mean log counts per minute observed with the low dose of insulin was comparable to the variation in the mean log counts per minute with the high dose. This finding stands in contrast to the unequal variation (heteroscedasticity) that is observed in the counts per minute themselves at different levels of response (1).

The responses to the 31 μU per ml concentration of standard insulin were considerably lower ($p < 0.001$) in the Series IV assays in Table IV than in the Series III assays in the same table. Subsequent assays of series IV have maintained approximately the same level of response. It is possible that the relatively high slopes obtained in Series IV are due primarily to this phenomenon. Such differences between large groups of animals may represent seasonal or other differences in the metabolic state of the animals used, differences occurring despite the precautions employed in the effort to prevent them.

TABLE IV

Data from two groups of consecutive routine assays *

Assay date	Mean response to standard insulin: Low dose	High dose	s†	b	λ
A. 18 consecutive assays from Series III					
Standard insulin (μU/ml)	31	1000			
6/10/59	0.723	1.217	0.069	0.327	0.211
6/15	0.823	1.410	0.088	0.389	0.226
6/17	0.933	1.503	0.136	0.378	0.360
6/18	0.907	1.520	0.085	0.406	0.209
6/24	1.057	1.743	0.089	0.455	0.196
6/26	0.987	1.747	0.099	0.504	0.196
7/1	1.060	1.494	0.186	0.287	0.648
7/6	0.963	1.380	0.115	0.276	0.417
7/7	0.940	1.410	0.045	0.312	0.144
7/9	0.750	1.303	0.107	0.367	0.292
7/10	1.035	1.630	0.079	0.395	0.200
7/14	1.057	1.513	0.134	0.303	0.442
7/15	1.197	1.687	0.078	0.325	0.240
7/16	0.983	1.563	0.122	0.384	0.318
7/23	1.000	1.687	0.099	0.455	0.218
7/27	1.103	1.550	0.163	0.296	0.551
7/29	1.060	1.576	0.177	0.341	0.519
7/30	1.023	1.513	0.098	0.325	0.302
Mean	0.978	1.525	0.109	0.362	0.316
Standard deviation	0.120	0.145	0.037	0.064	0.144
B. 21 consecutive assays from Series IV					
Standard insulin (μU/ml)	31	500			
11/6/59	0.747	1.317	0.084	0.472	0.177
11/10	0.823	1.263	0.077	0.358	0.216
11/17	0.477	1.197	0.095	0.597	0.159
11/18	0.853	1.450	0.055	0.494	0.111
11/19	0.840	1.353	0.137	0.425	0.322
11/20	0.717	1.220	0.084	0.414	0.202
11/27	0.813	1.433	0.246	0.522	0.472
11/30	0.723	1.167	0.077	0.369	0.210
12/2	0.763	1.257	0.126	0.408	0.310
12/9	0.883	1.350	0.134	0.385	0.348
12/10	0.963	1.447	0.097	0.398	0.245
12/11	0.587	1.083	0.262	0.412	0.637
12/14	0.540	1.097	0.084	0.462	0.182
12/16	0.503	1.123	0.111	0.513	0.216
12/17	0.633	1.110	0.082	0.395	0.209
12/18	0.548	1.150	0.077	0.500	0.155
12/21	0.787	1.260	0.070	0.393	0.178
12/22	0.447	0.933	0.055	0.404	0.136
12/28	0.567	1.030	0.122	0.384	0.318
12/29	0.720	1.023	0.071	0.252	0.280
12/30	0.667	1.113	0.100	0.370	0.270
Mean	0.695	1.208	0.107	0.425	0.255
Standard deviation	0.146	0.146	0.054	0.074	0.122

* Each line is based on responses, in cpm per mg, from three rats.

† d.f = 8 except in assays 6/18 and 7/27 where d.f. = 7.

TABLE V

Minimum 95 per cent confidence ratio for potency estimates in two standard insulin dose ranges for an assay with six responses to standard insulin and three responses to a single level of an unknown serum

λ	Approximate ratio which ignores g	Accurate estimates of minimum ratio in two standard insulin dose ranges	
		31–1,000 μU	31–500 μU or 62–1,000 μU
0.10	2.1	2.1	2.1
0.15	3.1	3.2	3.2
0.20	4.5	4.7	4.9
0.25	6.5	7.2	7.7
0.30	9.5	11.3	12.8
0.35	13.8	18.6	23.0
0.40	20.2	32.0	46.5
0.45	29.3	59.4	114.7
0.50	42.8	122.0	401.0
0.55	62.1	292.0	3,072.0

Effectiveness of assay design. Variation between the tissues of different rats was significant and often very large in 67 per cent of the routine assays reported here. In these cases, the procedure of making all comparisons within rats reduced the residual error term by a factor of 2 to 10 (9).

The difference between segments was less marked and less consistent. The proximal segments gave lower responses in 5 of the 13 standard curves in Table II; the difference was significant at $p < 0.05$ in 2 of these. Similarly, the proximal segments gave lower values in 84 out of the 102 routine assays (significantly lower in 33) in Series II through IV. The overall finding of lower values in 84 out of 102 assays is highly significant ($p < 0.001$) by a χ^2 test (5).

Maximum precision of estimates. A useful measure of the precision of an assay is the "95 per cent confidence ratio" (10–12) defined as the ratio of the upper limit of the 95 per cent confidence interval of a potency estimate to the lower limit of the same interval. This ratio is equal to the antilogarithm ($\log^{-1}$) of $L = M_U - M_L$. Using the notation of Figure 1, the rigorous calculations yield:

$$L = 2t\lambda \sqrt{\frac{1}{1-g}} \sqrt{\frac{N_s+N_u}{N_sN_u} + \frac{(\bar{y}_u-\bar{y}_s)^2}{SSR\,(1-g)}}. \quad [1]$$

When $\bar{y}_u = \bar{y}_s$, Equation 1 reduces to the minimum L where

$$\min L = 2t\lambda \sqrt{\frac{1}{1-g}} \sqrt{\frac{N_s+N_u}{N_sN_u}}. \quad [2]$$

The antilogarithm of Equation 2 is the minimum confidence ratio attainable in a particular assay. The statistic g in Equations 1 and 2 varies with the error in the estimate of slope. When this error is sufficiently small to make $g < 0.05$, it may be omitted from the calculations (2, 13). Under these conditions Equation 2 reduces to the formula of Figure 2a, namely,

$$Q = 2t\lambda \sqrt{\frac{N_s+N_u}{N_sN_u}}. \quad [3]$$

The value of t in these formulas depends on the level of confidence chosen (95 per cent) and on the degrees of freedom (d.f.) for s^2. In the present design there are 8 d.f. giving $t = 2.306$. The value of $\frac{N_s+N_u}{N_sN_u}$ depends on the number of responses contributing to an individual potency estimate, and in the routine assay design it is equal to 0.5. If these constants are substituted in Equations 2 and 3, the formulas reduce, in this routine design, to:

$$\min L = 3.26\lambda \sqrt{\frac{1}{1-g}} \quad [4]$$

$$\text{and } Q = 3.26\lambda. \quad [5]$$

The statistic g is related to the index λ as is shown by the algebraic identities:

$$g = \frac{s^2t^2}{SSR} = \frac{s^2t^2}{b^2r\Sigma(x-\bar{x})^2} = \frac{\lambda^2t^2}{r\Sigma(x-\bar{x})^2}. \quad [6]$$

In the routine assay design, $t^2/r\Sigma(x-\bar{x})^2$ depends only on the doses of standard insulin given. For the dose ranges used in these assays, the following values may be calculated:

Standard insulin per ml	$3\Sigma(x-\bar{x})^2$	g
31 μU—1,000 μU	3.414	$1.558\,\lambda^2$
31 μU— 500 μU or 62 μU—1,000 μU	2.187	$2.434\,\lambda^2$

The minimum L to be expected for values of λ ranging from 0.10 to 0.55 was calculated by inserting the appropriate values of 1/(1-g) into Equation 4. The corresponding minimum confidence ratios are shown in Table V where they may be compared with the approximations given by Equation 5. It is clear that in small assays of this type, use of the approximate formula will lead to appreciable underestimation of the minimum error of potency estimates unless λ is < 0.20. The small number of responses per assay produced the phe-

TABLE VI

Results of repeated assays of same sera

Subject	Date when sample was obtained	Assay date	Potency estimate of ILA (μU/ml)	Log potency (M)	Expanded variance predicted for M	Observed variance	p Value in test for heterogeneity
L.G.	9/8/59	9/10	582	2.765	0.1005	0.0420	>0.30
		9/14	335	2.525	0.1756		
		9/16	597	2.776	0.0400		
		10/14	224	2.350	0.0276		
J.K.	9/15/59	9/17	336	2.526	0.0259	0.0147	>0.50
		10/6	212	2.326	0.0342		
		10/14	351	2.545	0.0289		
G.C.	11/–/58	12/1	26	1.407	0.0134	0.0535	>0.10
		12/15	54	1.734	0.0269		
	8/20/59	8/28	64	1.804	0.0240	0.0892	>0.50
		9/2	32	1.511	0.0600		
		9/3	25	1.391	0.1399		
		9/8	21	1.318	0.5227		
		9/21	31	1.490	0.0581		

nomenon, notable in Table V, of a rapid expansion in the minimum confidence ratio with an increase in the value of λ.

The confidence ratios may be reduced by increasing the number of responses per assay. Depending on the design (2) such an increase could: reduce the value of t by its effect on the d.f.; reduce the magnitude of $(N_s + N_u)/N_sN_u$; increase the value of $r\Sigma(x\text{-}\bar{x})^2$; and reduce the size of g. For example, if six rats were used in the routine assay of four unknowns with no other change in the design, the 95 per cent minimum confidence ratios for an assay where $\lambda = 0.30$ would be 6.6 for the approximate ratio, and 7.0 and 7.2 for the accurate estimates in the two dose ranges shown in Table V, a decrease of approximately 40 per cent in the accurate ratios.

Interassay variation. The foregoing section refers to the reproducibility of assay results as estimated from the statistics within individual assays. Accumulated experience, however, permits direct comparisons of results in different assays and, therefore, comparisons between predicted and observed reproducibility (14).

The slopes of the different assays within Series I and Series IV did not vary significantly more than expected for estimates based on two responses per rat. In the other two series the observed variance between slopes was approximately double the predicted variance ($p < 0.05$). This common phenomenon is of no practical significance when standard slopes are run in each assay. The acid test of interassay variation is the reproducibility of M values obtained in different assays of the same unknown. Estimates from two to five independent assays of four different sera are shown in Table VI together with the "expanded" variance of M calculated in each assay, and with the variance calculated from the observed M values. It is clear both from the p values in the test for heterogeneity and from the comparison of observed with predicted variances that the interassay variation among these M values was not greater than predicted.

RESULTS

Tests of "normal" human sera

Technically valid estimates [3] of ILA were available for 50 undiluted serum samples obtained from 30 fasting, apparently normal human subjects. The 95 per cent confidence ratios were > 100 for 12 of these estimates obtained in a total of eight different assays. In two assays there were fewer than 15 usable responses; in the other instances large "expanded" standard errors of M were produced by a combination of λ values > 0.40 with mean responses for the unknowns that were at one of the extremes of the standard curve.

The remaining 38 estimates of ILA involving 23 subjects are shown in Table VII as microunits of insulin per milliliter of serum, together with

[3] These were derived from assays with significant slopes.

TABLE VII

Estimates of ILA of 38 undiluted serum samples from 23 fasting normal individuals *

Subject	Sex	Age	ILA in μU/ml serum† Estimate	95% Confidence limits
P.B.	M	37	510	200–1,600
H.B.	M	24	35	7– 92
R.B.	M	26	140	38– 390
Z.C.	M	38	310	94–1,200
A.F.	M	25	300	130– 790
R.H.	M	29	440	150–1,600
S.M.	M	25	840	380–2,300
G.Z.	M	33	440	170–1,600
D.C.	F	24	810	260–4,210
U.D.	F	22	130	28– 430
A.E.	F	23	360	170– 830
V.S.	F	39	350	180– 690
G.C.	M	33	33‡	21– 53
			43‡	26– 72
Y.D.	M	33	190	73– 460
			300	100– 920
			100	24– 290
			510	230–1,300
			130	10– 570
P.D.	M	?	44	32– 110
			180	100– 300
B.J.	M	28	89	15– 270
			270	64–1,400
J.K.	M	34	310	110–1,000
			300‡	190– 480
B.L.	M	30	390	130–1,600
			35	6– 94
H.M.	M	29	100	22– 320
			290	170– 490
U.M.	M	27	360	150– 970
			450	210–1,100
A.R.	M	35	89	40– 170
			300	60–1,800
			940	220–1,200
F.W.	M	31	150	37– 460
			230	48–1,100
L.G.	F	28	150	78– 440
			360‡	210– 610

* Repeated samples from an individual were taken on separate occasions.
† Rounded to two significant digits.
‡ Derived from weighted means of multiple estimates in Table VI.

their 95 per cent confidence limits. The estimates ranged between 33 and 940 μU per ml.

ILA of serum following infusions of glucose and of mannose [4]

Samples of blood were collected from nondiabetic fasting subjects in apparent good health, just before an intravenous infusion of either glucose or mannose (0.5 g per kg body weight in a 50 per cent solution given over 3 minutes) and also 10, 20 and 60 minutes after the infusion. All four samples from any one subject were tested together in one assay.

Serum glucose levels after the infusion of glucose showed the expected pattern, the mean values for the subjects in Figure 5 being 75, 275, 212 and 82 mg per 100 ml in the four time periods. Following mannose infusion, glucose values varied randomly within normal limits, while the mean serum mannose dropped from a peak of 216 mg per 100 ml at 10 minutes to 90 mg per 100 ml at 1 hour.

The sera from seven subjects in the glucose experiments were tested in eight assays, two of which failed to show a significant slope in the response to standard insulin. Potency estimates could be calculated for four subjects from one assay each; for a fifth subject (G.C.) estimates of ILA were derived from weighted mean M's from two assays (15).

Five subjects were given mannose infusions. In the assays of their sera, mannose was added to all the media in a concentration equal to the mean mannose content of the sera tested (200 to 230 mg per 100 ml). The usual response to standard insulin, with significant slopes, was seen in all five assays.[5] Estimates of ILA were calculated from these assays in the usual way, for comparative purposes, although they were not included in Table VII.

Twenty minutes after the infusion of glucose, the ILA of the serum of each subject was in-

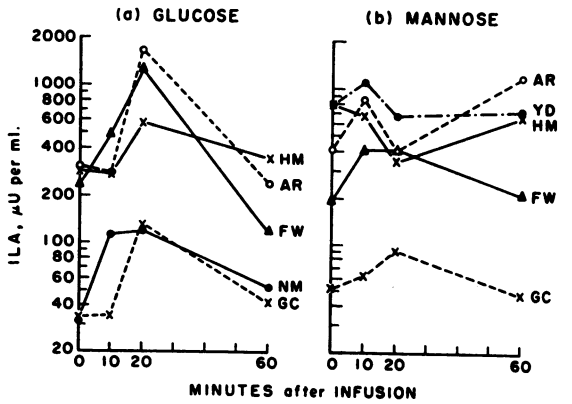

FIG. 5. ESTIMATES OF ILA IN HUMAN SERA PLOTTED ON A LOGARITHMIC SCALE: a. FOLLOWING INFUSION OF GLUCOSE; b. FOLLOWING INFUSION OF MANNOSE.

[4] The mannose infusions were carried out in collaboration with Drs. Francis C. Wood, Jr. and George F. Cahill, Jr.

[5] Not included in the assay summary because of the added mannose.

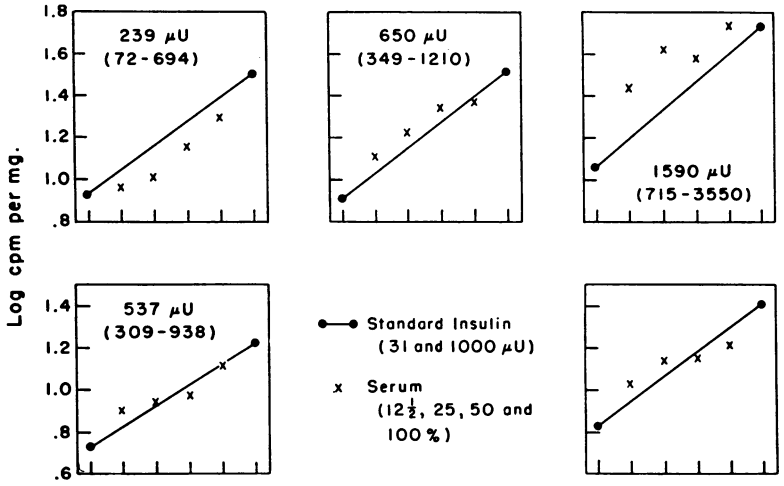

FIG. 6. THE RESPONSE OF ADIPOSE TISSUE TO SERIAL DILUTIONS OF HUMAN SERUM AND TO STANDARD INSULIN. The dose of insulin or serum is shown on the abscissa. The estimates of ILA with 95 per cent confidence limits in microunits per milliliter, as calculated from all the data, are shown where there was no significant nonparallelism.

creased (Figure 5) by a factor which varied from 100 to 440 per cent. The increase was significant (at $p < 0.05$) in all cases but one (N. M.). In contrast, after the infusion of mannose the ILA varied in a random fashion (Figure 5) and none of the differences reached statistical significance.

To form an approximate estimate of the power of these assays to detect changes in ILA, the statistics in each assay were used to calculate the smallest difference between two means of three responses each that would be significant at $p < 0.05$ by a one-sided t test. For each "least significant difference," the corresponding increase in potency was calculated, and ranged from 40 to 330 per cent (i.e., a potency 1.4 to 4.3 times the pre-infusion value). A least significant difference was also calculated for samples tested in separate assays (15). The calculations, which were based on the *minimum* "expanded" variance of an M from each assay, appreciably underestimated the differences which would actually have been required for statistical significance. Even these underestimates indicated that, to be detected, samples compared in different assays would have to show at least twice as much change as could be detected within an assay.

The response to diluted sera

The effect of diluting human sera was tested in five assays. In each instance a sample of serum, taken from a fasting normal volunteer, was tested full strength and also after dilution with buffer to 50, 25 and 12.5 per cent strength. The results are shown in Figure 6. The slope of the response to standard insulin was highly significant ($p < 0.01$) in each assay. In one assay, there was significant ($p < 0.05$) nonparallelism between the slope on standard insulin and on serum. In the other four assays estimates of ILA and of 95 per cent confidence limits (Figure 6) were calculated from all the data with a "combined" estimate of the slope based on the responses to both the serum and the standard insulin (2, 8).

The values for λ, calculated from the combined slope, were 0.36, 0.22, 0.22 and 0.23, in the order shown in Figure 6. The 95 per cent confidence ratios, calculated according to Equation 1, were: 9.6, 3.5, 5.0 and 3.0. These ratios were smaller than the minimal ratios (Table V) corresponding to similar λ values, both because all 18 responses in an assay contributed to the estimate of slope (thus diminishing the value of g), and because 12 responses for a test serum were available for each estimate of potency.

On the other hand, a question must be raised about the effect of dilution on the serum. Although there was no significant nonparallelism in four of the assays, the slope of the response to serum was flatter than the standard slope in each instance. This result in the five cases combined

was significant ($p < 0.05$). Estimates of the relative potency of the 12.5 per cent dilution computed for the five examples in Figure 6 were, in the order shown, 110, 170, 170, 170 and 270 per cent of the potency of the same serum before dilution.

DISCUSSION

This procedure meets the first major requirement for a quantitative biological assay method by exhibiting a relatively reproducible linear response to the logarithm of increasing doses of standard insulin. The results of experiments with multiple doses of standard insulin have been confirmed by the observations that over a period of 25 months significant regressions occurred in all but 5 of the 147 routine assays analyzed. The suggestion that the linear portion of the dose-response curve lies below the concentration 1,000 μU per ml, has been supported by the finding that the assays in Series IV (31 to 500 μU of insulin) had significantly steeper slopes and better λ values than the earlier series. The further observation that the variation in the response to high and low doses was of similar magnitude shows that the log counts per minute were a satisfactory response metameter for these assays.

Although potency estimates derived from these assays have a large error, the precision can be increased not only by increasing the size of individual assays, but also (Table VII) by reassaying an unknown on several days. The evidence available to date regarding the reproducibility of independent estimates of the potency of a sample of serum from assay to assay does not indicate the presence of important interassay error. The precision of the method compares favorably with that of other methods used to measure ILA in blood. For the rat diaphragm method, reported values (16, 17) of λ have been in the range 0.20 to 0.40. Willebrands, Groen and van der Geld (12, 18) have estimated a 95 per cent confidence ratio of 9.0 in assays with five responses each at single levels of standard insulin and of diluted serum. Since their calculations omit both the term $1/(1\text{-}g)$ and the term for $\bar{y}_u - \bar{y}_s$ in Equation 1, this estimate is comparable to the approximate minimum ratio calculated from Equation 3. From the data on the standard response presented (18) by these authors it may be calculated that the value of λ was approximately 0.50 to 0.60.

The use of epididymal adipose tissue offers the important advantage that all estimates for unknowns can be based on a response to two or more dose levels of standard insulin obtained from tissues of the same rats at the same time. It is now generally accepted that estimates made in this way are more reliable than those calculated from an independent, previously obtained, estimate of standard slope. The importance of this feature is indicated by the variation observed between the slopes of assays in Series II and III.

The possibility of giving a number of different treatments to the tissue from each rat has several additional advantages. One set of responses to standard insulin provides the basis for potency estimates of several unknowns, thereby producing more efficient utilization of animals and laboratory time. Moreover, this opportunity to compare several sera or other unknowns within an assay adds considerably to the power of the method. A difference between two samples tested in one assay is subject only to the experimental variation affecting the responses to the two samples. Two potency estimates from two separate assays are each subject to experimental variation affecting a mean response to the unknown and to standard insulin, and to variation affecting an estimate of slope. As a result, a "between-assay" comparison of two potency estimates is subject to variation affecting the responses both to the unknowns, and to two separate sets of standard insulin doses. With a given degree of precision per assay, therefore, and no interassay variation, within-assay comparisons can detect smaller differences than can between-assay comparisons.

The effect on ILA of diluting complex samples such as sera, has important practical implications for the use of this assay method, in addition to its physiological interest and its relevance to the fundamental validity of the assay method (2). Although in pharmacology nonparallel behavior of the responses to unknowns and to the standards suggests nonidentity, in complex biological samples other interpretations have to be considered. For example, the activity may be present in different chemical or physicochemical states (such as "bound" and "free") related to each other by equilibria sensitive to dilution. Still, unless serum does produce responses that are parallel to the reference standard, potency estimates derived from

diluted serum and even from undiluted serum must be interpreted with special caution.

The preliminary results reported here suggest that if dilution of serum does produce a relative increase in potency as shown by the response of epididymal adipose tissue, this effect is considerably less pronounced than the increase observed in assays utilizing the rat diaphragm. For example, from the data presented by Randle (19), it may be calculated that dilution of serum to 25 per cent altered ILA to 80, 320, 330, 400 and 660 per cent of that observed before dilution. Willebrands, van der Geld and Groen (18) reported estimates for sera diluted to 10 per cent which were 350 to 1,300 per cent of estimates made from more concentrated preparations of the same sera. In the light of the smaller discrepancies (110 to 270 per cent ILA in diluted sera) observed in this laboratory, further study is being given to the feasibility of estimating the total ILA of undiluted serum from assays performed on diluted serum. If this procedure should prove possible, the scope of the assay method would be appreciably increased.

The data on serum ILA which have been presented are intended to illustrate the possible applicability of the procedure to complex biological samples. This in no way implies that the activity measured in serum was indeed insulin. Studies related to the nature of the ILA of serum will be published after they have been completed, although it may be pointed out at this time that the response to glucose (and not to mannose) suggests an insulin component. Similarly, serum ILA measured by this procedure is higher in pancreatic and portal venous blood than it is in the general circulation (20), and is frequently elevated in serum obtained from patients with tumors of pancreatic islets with hypoglycemia (21). ILA in serum is also increased after the intravenous administration of insulin and recoveries of insulin added to serum are satisfactory.[6] A major portion of serum ILA is destroyed by incubation with reduced glutathione.[6] However, serum ILA does not appear to be reduced below the normal range in pancreatectomized dogs (22) or cats.[7] Accordingly, considerable work with isolation, extraction and characterization procedures will be required before a definitive statement on the nature of ILA in serum can be made.

[6] Steinke, J., Dagenais, Y. M., and Renold, A. E. Unpublished observations.

[7] Steinke, J., Lukens, F. D. W., and Renold, A. E. Unpublished observations.

SUMMARY AND CONCLUSIONS

Experiments and routine assays performed over a period of two years and involving over 500 rats indicate that it is feasible to measure minute concentrations of insulin (or of "insulin-like activity") by utilizing measurements of the $C^{14}O_2$ produced from glucose-1-C^{14} by epididymal adipose tissue of rats. The tissue response is recorded as the logarithm of the counts per minute of CO_2 produced per milligram of adipose tissue (wet weight). After incubation with standard insulin in concentrations of 31 to 500 μU per ml, the log counts per minute increase linearly with the log dose of insulin.

The utilization of epididymal adipose tissue permits assays to be so designed that simultaneous comparisons between standard insulin and several "unknowns" may be made while eliminating differences between rats and between the several segments of each epididymal fat pad. Six different combinations of substances and concentrations are tested in each experiment or assay. The design for a routine assay was presented together with the method of analyzing the results and of calculating accurate confidence limits.

Data from 147 routine assays were summarized. Significant slopes in the response to standard insulin were found in all 102 analyzed assays performed with the balanced segment design. The last series of 55 assays, in which standard insulin was given in concentrations of 31 and 500 μU per ml, had a mean index of precision (λ) of 0.23. With small assays, this value of λ still implies a rather large error, although the error may be decreased by modifying the design of an assay. The precision is at least comparable to that of other methods presently in use.

Within individual assays, each utilizing tissues from three rats, the method was sufficiently precise (or powerful) to detect an increase in serum insulin-like activity 20 minutes after the intravenous infusion of glucose.

Preliminary data suggest that potency estimates are reproducible from assay to assay, within the error of the method. Other preliminary experi-

ments suggest that, by this method, there may be less discrepancy in insulin-like activity following dilution of complex samples such as serum than has been observed in the rat diaphragm assay procedure.

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REFERENCES

1. Renold, A. E., Martin, D. B., Dagenais, Y. M., Steinke, J., Nickerson, R. J., and Sheps, M. C. Measurement of small quantities of insulin-like activity using rat adipose tissue. I. A proposed procedure. J. clin. Invest. 1960, **39**, 1487.
2. Finney, D. J. Statistical Method in Biological Assay. New York, Hafner Publishing, 1952.
3. Bliss, C. I. Analysis of the biological assays in U.S.P. XV. Drug Stand. 1956, **24**, 33.
4. Fieller, E. C. The biological standardization of insulin. Suppl. J. Roy. Stat. Soc. 1940–41, **7**, 1.
5. Snedecor, G. W. Statistical Methods Applied to Experiments in Agriculture and Biology, 5th ed. Ames, Iowa, The Iowa State College Press, 1956.
6. Cochran, W. G. Problems arising in the analysis of a series of similar experiments. Suppl. J. Roy. Stat. Soc. 1937, **4**, 102.
7. Cochran, W. G. The combination of estimates from different experiments. Biometrics 1954, **10**, 101.
8. Bliss, C. I. The Statistics of Bioassay. New York, Academic Press, 1952.
9. Cochran, W. G., and Cox, G. M. Experimental Designs, 2nd ed. New York, John Wiley and Sons, 1957, p. 112.
10. Bliss, C. I. Confidence limits for measuring the precision of bioassays. Biometrics 1956, **12**, 491.
11. Munson, P. L., and Sheps, M. C. An improved procedure for the biological assay of androgens by direct application to the combs of baby chicks. Endocrinology 1958, **62**, 173.
12. Willebrands, A. F., and Groen, J. Insulin bioassays *in vitro:* Using isolated rat diaphragm *in* Hormones in Human Plasma, H. N. Antoniades, Ed. Boston, Little, Brown and Co. In press.
13. Sheps, M. C., and Hendrie, K. H. A minimum error table for parallel line biological assays. J. Pharmacol. exp. Ther. 1958, **124**, 94.
14. Sheps, M. C., and Munson, P. L. The error of replicated potency estimates in a biological assay method of the parallel line type. Biometrics 1957, **13**, 131.
15. Sheps, M. C., and Moore, E. A. Methods for combining the results of two biological assays. J. Pharmacol. exp. Ther. 1960, **128**, 99.
16. Randle, P. J. Assay of plasma insulin activity by the rat-diaphragm method. Brit. med. J. 1954, 1, 1237.
17. Takeuchi, S., Ohashi, S., and Kobayashi, Y. Quantitative assay of insulin in blood plasma in normal and alloxanized dogs by rat diaphragm method. J. Pharmacol. exp. Ther. 1957, **119**, 436.
18. Willebrands, A. F., van der Geld, H. and Groen, J. Determination of serum insulin using the isolated rat diaphragm, the effect of serum dilution. Diabetes 1958, **7**, 119.
19. Randle, P. J. Insulin in blood. Ciba Found. Coll. Endocr. 1957, vol. XI, p. 115.
20. Pfeiffer, E. F., Renold, A. E., Martin, D. B., Dagenais, Y., Meakin, J. W., Nelson, D. H., Shoemaker, G., and Thorn, G. W. Untersuchungen über die Rolle des Pankreas im Wirkungsmechanismus blutzuckersenkender Sulfonylharnstoffe. Proceedings of the Third Congress, International Diabetes Federation, Stuttgart, Thieme, 1959, p. 298.
21. Dagenais, Y. M., Renold, A. E., Martin, D. B., and Lauris, V. Serum insulin-like activity in patients with pancreatic islet-cell tumors. Endocrinology 1959, **64**, 847.
22. Leonards, J. R. Insulin-like activity of blood, what is it? Fed. Proc. 1959, **18**, 272.