AN EXAMPLE OF THE USE OF EXTENDED CROSS-OVER DESIGNS IN THE COMPARISON OF NPH INSULIN MIXTURES

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INTRODUCTION

When a solution of protamine and a solution of zinc insulin are combined, a precipitate of protamine zinc insulin is produced. Suspensions of this type have been employed in the management of diabetes and have advantage over unmodified insulin in that they control the patient's blood sugar level over a relatively long period of time. Recently, a new type of long acting insulin, designated as NPH Insulin, has become available. NPH Insulin is a suspension of protamine zinc insulin such that the protamine content will not be less than, nor more than ten percent greater than, the quantity required for the isophane-ratio. The isophane-ratio is that ratio of insulin to protamine which results in equivalent amounts of insulin and protamine remaining in the supernatant as tested by nephelometric procedures. We were interested in determining whether it would be possible to detect a biological difference in a preparation in which the protamine content was five percent less than the quantity required by the isophane-ratio.

NPH Insulin is prepared from insulin which has been previously assayed and approved by two independent laboratories, as required by the Food and Drug Administration. For this reason, it is not anticipated that routine biological assays will be required to control the normal production of NPH Insulin.

EXPERIMENTAL METHOD

Two NPH Insulin mixtures were prepared; Mixture A (the "standard") was prepared to contain the isophane-ratio of protamine to insulin, Mixture B (the "unknown") was prepared to contain five percent

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less protamine than Mixture A. Each mixture contained a concentration of insulin equivalent to 40 units per ml.*

The experimental procedure employed was a minor modification of the simple cross-over assay for Globin Zinc Insulin Injection, official in the U.S. Pharmacopoeia XIV (1). This modification consisted only of limiting the observation period to six instead of nine hours and with four post-injection bleeding times equally spaced within the six-hour period of observation. The real difference between the two mixtures was expected to be small. For this reason, it was planned beforehand to extend the cross-over to include both a switchback and a double switchback design in an attempt to obtain more evidence upon which to base a decision. All three designs were analyzed separately and compared.

Twenty-two female rabbits, weighing from 2.5 to 3.5 kg, were distributed randomly between two equal groups. At weekly intervals, according to the scheme shown in Table I, single subcutaneous injections of either Mixture A or B were administered in volumes of 0.051 ml as measured from a micrometer syringe.

TABLE I
INJECTION SCHEDULE

Group	Period (Date)			
	1 (6-27-50)	2 (7-3-50)	3 (7-10-50)	4 (7-17-50)
I	A	B	A	B
II	B	A	B	A

Blood samples were obtained from each rabbit at 0 (initial level), 1.5, 3.0, 4.5, and 6.0 hours after injection. Blood sugar levels were determined for each sample. The bleeding times were spaced equally to facilitate computation of the results. The raw results for all animals at all time periods are presented in Table II.

COMPUTATIONAL PROCEDURE

Brandt (2) has thoroughly discussed the analysis of cross-over designs. Our data, however, include an additional sub-unit, bleeding times, and require an extension of Brandt's methods. Although Brandt discusses the use of covariance, he does not consider the case where the concomitant variable is common to all values in the sub-unit. In

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TABLE II

BLOOD SUGAR LEVELS (mg. percent)

Group	Rabbit No.	Period 1 Hours after Injection					Period 2 Hours after Injection					Period 3 Hours after Injection					Period 4 Hours after Injection				
		0.0	1.5	3.0	4.5	6.0	0.0	1.5	3.0	4.5	6.0	0.0	1.5	3.0	4.5	6.0	0.0	1.5	3.0	4.5	6.0
	1	77	52	35	56	64	90	47	52	68	90	85	52	35	39	60	94	60	60	77	94
	2	77	56	43	56	64	85	52	60	81	94	103	26	68	30	30	107	60	68	90	94
	3	77	43	64	39	64	90	30	30	47	60	99	39	39	73	90	103	60	60	90	99
	4	81	56	22	35	39	107	47	47	47	73	77	26	56	64	64	116	52	26	68	120
I	5	90	73	52	60	64	94	60	60	77	90	90	60	18	35	64	94	56	47	81	99
	6	103	47	22	60	99	90	47	30	43	68	85	56	56	77	94	111	73	85	103	111
	7	99	47	52	47	81	99	26	64	52	90	90	43	18	30	39	111	56	43	90	111
	8	90	35	22	26	22	94	8	30	26	43	103	39	22	39	94	90	18	26	18	22
	9	90	64	47	47	60	81	39	47	77	94	77	56	26	64	94	94	52	47	81	90
	10	90	35	12	26	68	81	26	35	30	85	81	56	60	81	103	90	22	52	85	90
	11	85	68	56	68	73	94	56	68	73	90	105	73	47	77	90	97	73	52	81	94
Subtotals		959	576	427	520	698	1005	438	523	621	877	995	526	445	609	822	1107	582	566	864	1024
	12	103	26	22	39	68	90	35	30	39	94	103	26	26	56	103	111	68	52	77	103
	13	85	68	56	64	81	94	52	43	47	56	85	43	22	68	103	111	52	99	103	120
	14	85	35	8	64	99	90	35	30	43	99	73	22	12	56	103	94	52	47	73	101
	15	85	35	47	77	68	85	39	52	52	64	90	12	43	85	99	92	52	73	99	107
II	16	85	35	39	85	77	85	47	56	77	81	90	43	35	64	81	85	52	52	77	99
	17	103	56	47	64	56	103	35	39	68	99	90	35	18	64	94	107	47	43	85	101
	18	90	52	35	35	39	90	43	30	47	77	77	56	64	81	99	111	60	68	107	111
	19	81	22	12	22	77	81	12	22	30	77	68	12	8	8	39	85	26	12	18	39
	20	85	30	22	52	94	90	30	26	52	90	85	39	22	56	90	105	68	64	81	81
	21	85	47	33	77	107	85	39	39	43	60	85	35	26	43	73	90	43	35	81	90
	22	94	39	35	52	85	94	26	30	39	81	90	22	26	73	99	103	47	35	64	94
Subtotals		981	445	356	631	851	987	393	397	537	878	936	345	302	654	983	1094	567	580	865	1046

this experiment, the initial blood sugar values are necessarily common to the blood sugar values for all other bleeding times.

The physiological relation between time and blood sugar levels, following insulin injection, is quite complex and is not adequately described by any simple function. Since the objective of the experiment was to determine the effect of a difference in protamine content, it was felt that the customary pharmacopoeial procedure, which specifies that the results be analyzed in terms of the *differences* in blood sugar levels between the two mixtures, should be adopted. The unit selected for analysis, therefore, was the difference in blood sugar levels produced by the two mixtures in the same group of animals at the various test periods. Brandt (2) has shown that in a design of this type the blood sugar differences are confounded with the periods $\times$ groups interaction when a simple cross-over (2 test periods) is involved, with the quadratic component of periods $\times$ groups when three test periods are involved and with the cubic component of periods $\times$ groups when there are four test periods. For all but two test periods, where only a simple subtraction of Period 1 $-$ Period 2 is necessary, the differences are most easily computed by means of polynomial coefficients, as given in Table VI and illustrated by example later.

For the purpose of this study, the only relevant factors that need be considered are the nature of the differences between preparations and the possible influence of the initial blood sugar values upon subsequent readings.

A. Analysis of Two-Period Differences

Table III lists under "hours after injection" the difference in blood sugar level between the first and second test periods at each bleeding time. These are readily computed from the raw values in Table II. Thus, for rabbit number 1, the difference at three hours after injection is,

$$P_1 - P_2 = 35 - 52 = -17$$

The "totals" column represents the algebraic sum of the differences at 1.5, 3.0, 4.5, and 6.0 hours after injection. Thus, for rabbit number 2,

$$4 - 17 - 25 - 30 = -68$$

The remaining columns list the linear, quadratic, and cubic components of the blood sugar differences for each animal. These were computed as the sums of the products of the differences at each bleeding time and the corresponding orthogonal polynomial coefficients for $n' = 4$ equally spaced bleeding times. The latter may be obtained from Fisher and Yates (3) and are reproduced for convenience in Table IV.

TABLE III
DIFFERENCES AND TERMS FOR TWO-PERIOD ANALYSIS

Rabbit No.	Hours after Injection					Totals (y)	Sums of Products (y)		
	0 = x	1.5	3.0	4.5	6.0		Linear	Quadratic	Cubic
1	−13	5	−17	−12	−26	−50	−88	8	−46
2	−8	4	−17	−25	−30	−68	−110	16	−10
3	−13	13	34	−8	4	43	−69	−9	117
4	−26	9	−25	−12	−34	−62	−116	12	−82
5	−4	13	−8	−17	−26	−38	−126	12	−12
6	13	0	−8	17	31	40	−118	22	−44
7	0	21	−12	−5	−9	−5	−83	29	−51
8	−4	27	−8	0	−21	−2	−136	14	−72
9	9	25	0	−30	−34	−39	−207	21	31
10	9	9	−23	−4	−17	−35	−59	19	−83
11	−9	12	−12	−5	−17	−22	−80	12	−50
Subtotals	−46	138	−96	−101	−179	−238	−956	156	−302
12	13	−9	−8	0	−26	−43	−43	−27	−41
13	−9	16	13	17	25	71	31	11	−3
14	−5	0	−22	21	0	−1	43	1	−129
15	0	−4	−5	25	4	20	54	−20	−82
16	0	−12	−17	8	−4	−25	49	−7	−67
17	0	21	8	−4	−43	−18	−204	−26	−28
18	0	9	5	−12	−38	−36	−158	−22	4
19	0	10	−10	−8	0	−8	−28	28	−16
20	−5	0	−4	0	4	0	16	8	−8
21	0	8	−6	34	47	83	157	27	−81
22	0	13	5	13	4	35	−19	−1	−33
Subtotals	−6	52	−41	94	−27	78	−102	−28	−484
Differences between Subtotals	−40	86	−55	−195	−152	−316	−854	184	182

As an example, the sum of products for the linear component for rabbit number 3 would be derived from Tables III and IV as follows:

$$(13)(-3) + (34)(-1) + (-8)(+1) + (4)(+3) = -69$$

TABLE IV
ORTHOGONAL POLYNOMIAL COEFFICIENTS FOR $n' = 4$

Component	Coefficients			
Linear	−3	−1	+1	+3
Quadratic	+1	−1	−1	+1
Cubic	−1	+3	−3	+1

The differences between the sub-totals as shown in the last row of Table III represent the differences between the two insulin mixtures (Mixture A − Mixture B). The difference between the sub-totals in the "totals" column is a measure of the difference in the mean blood sugar levels for the two mixtures, while the differences between the sub-totals for the "sums of products" columns characterize the nature of the difference over the four bleeding times, or, in other words, serves as a means of comparing the blood sugar curves produced by the two insulin mixtures. This will be elaborated on further in the section on interpretation of results.

There now remains the problem of testing the mixture differences for significance and of determining the effect, if any, of the initial blood sugar levels upon the post-injection observations. The present official U.S. Pharmacopoeia (1) assays of long acting insulin preparations take no account of a possible effect of the initial blood sugar level. Earlier investigators (4, 5), however, have shown that this may be of considerable importance. Covariance analysis with the initial blood sugar level as the concomitant variable was employed in this investigation. The analysis is conveniently laid out in the form shown in Table V. There are four sources of variation (differences) relevant to the mixture comparison, each with a sum of squares representing the difference between mixtures and an error term with 19 degrees of freedom, after adjusting for covariance. The analysis is conducted on a whole-unit basis.

The sums of squares and products in all four sections are obtained from the terms in the second and the last four columns of Table III, labeled (x) and (y), respectively. The method of computation is the same for all four sections and is illustrated below for the linear term:

$[x^2]$

$$\text{Mixtures} = (-40)^2/44 = 36.36$$

where $44 = (2)\ (22)$ = sum of the squares of the coefficients for obtaining

TABLE V
ANALYSIS OF COVARIANCE (TWO-PERIOD DATA)

Sources of Variation		D.F.	Sums of Squares and Products			D.F.	Residuals		
			$[x^2]$	$[xy]$	$[y^2]$		S.S.	M.S.	F
Totals	Mixtures	1	36.36	287.27	2269.45	1	2067.66	2067.66	2.53
	Error	20	813.18	156.64	15565.73	19	15535.56	817.66	
	Total	21	849.54	443.91	17835.18	20	17603.22		
Linear	Mixtures	1	36.36	776.36	16575.36	1	17180.06	17180.06	4.00
	Error	20	813.18	−704.73	82275.55	19	81664.81	4298.15	
	Total	21	849.54	71.63	98850.91	20	98844.87		
Quadratic	Mixtures	1	36.36	−167.27	769.45	1	766.16	766.16	5.95*
	Error	20	813.18	74.55	2455.18	19	2448.35	128.86	
	Total	21	849.54	−92.72	3224.63	20	3214.51		
Cubic	Mixtures	1	36.36	−165.45	752.82	1	724.11	724.11	0.53
	Error	20	813.18	9.00	25785.36	19	25785.26	1357.12	
	Total	21	849.54	−156.45	26538.18	20	26509.37		

*Significant ($P < 0.05$)

the two-period differences times the number of rabbits. The former may be obtained directly from Table VI.

$$\text{Error} = \frac{(-13)^2 + \cdots + (0)^2 - [(-46)^2 + (-6)^2]/11}{2}$$
$$= 813.18$$

where 11 = number of rabbits per group

2 = sum of the squares of the coefficients for obtaining the two-period differences and may be obtained directly from Table VI.

$[xy]$

$$\text{Mixtures} = (-40)(-854)/44 = 776.36$$

Error

$$= \frac{(-13)(-88)+\cdots+(0)(-19)-[(-46)(-956)+(-6)(-102)]/11}{2}$$
$$= -704.73$$

$[y^2]$

$$\text{Mixtures} = (-854)^2/44 = 16575.36$$

$$\text{Error} = \frac{(-88)^2 + \cdots + (-19)^2 - [(-956)^2 + (-102)^2]/11}{2}$$
$$= 82275.55$$

The residual total sum of squares and that for error are obtained in the usual way from

$$[y^2] - [xy]^2/[x^2]$$

and the adjusted sum of squares for mixtures is obtained as the difference of these two residuals.

The interpretation of this analysis and that for three and four test periods is deferred to the section on interpretation of the results.

The coefficients for obtaining the required "differences" for two, three, and four test periods and their sums of squares are shown in Table VI.

B. Analysis for Three-Period Differences

Tables VII and VIII show the required terms and the analysis for three-period "differences".

The "differences" is columns 2 to 6 are computed from the values in Table II and the coefficients for three test periods in Table VI. Thus,

TABLE VI
COEFFICIENTS FOR OBTAINING "DIFFERENCES"

Test Periods	Coefficients	Sums of Squares
2	+1, −1	2
3	+1, −2, +1	6
4*	+1, −3, +3, −1	20

*The coefficients for four test periods normally would be −1, +3, −3, +1. The signs were reversed in order to make the results comparable with the two and three test period results.

for Rabbit 1, at 1.5 hours after injection, the required "difference" is obtained as follows:

$$(+1)(52) + (-2)(47) + (+1)(52) = 10$$

The other columns are computed in the same manner as for Table III.

The method of computation for the analysis of variance is identical with that for Table V, except for a change in the divisors. The divisor for mixtures becomes 132 ($=6 \times 22$), while the divisor for error becomes 6. The latter is obtained from Table VI.

C. Analysis for Four-Period Differences

Tables IX and X show the required terms and the analysis for four-period "differences". Computational procedure is as described above.

INTERPRETATION OF RESULTS

The mean difference between the two insulin mixtures for the two-period analysis is the simple difference between observations in the first and second periods. Using subscripts to denote the period, we have

$$\frac{A_1 - B_2}{11} = \text{mean difference for Group I}$$

$$\frac{B_1 - A_2}{11} = \text{mean difference for Group II}$$

$$\frac{A_1 - B_2}{11} - \frac{B_1 - A_2}{11} = \text{difference of means}$$

$$= \frac{A_1 - B_2 - B_1 + A_2}{11}$$

$$= \frac{A_1 + A_2}{11} - \frac{B_1 + B_2}{11}$$

TABLE VII
DIFFERENCES AND TERMS FOR THREE-PERIOD ANALYSIS

Rabbit No.	Hours after Injection					Totals (y)	Sums of Products (y)		
	0 = x	1.5	3.0	4.5	6.0		Linear	Quadratic	Cubic
1	−18	10	−34	−41	−56	−121	−205	29	−45
2	10	−22	−9	−76	−94	−201	−283	−31	129
3	−4	22	43	18	34	117	11	−5	87
4	−56	−12	−16	5	−43	−66	−72	−44	−94
5	−8	13	−50	−59	−52	−148	−204	70	−38
6	8	9	18	51	57	135	177	−3	−51
7	−9	38	−58	−27	−60	−107	−263	63	−191
8	5	58	−16	13	30	85	−55	91	−115
9	5	42	−21	−43	−34	−56	−250	72	−10
10	9	39	2	47	1	89	−69	−9	−173
11	2	29	−33	−1	−17	−22	−106	46	−142
Subtotals	−56	226	−174	−113	−234	−295	−1319	279	−643
12	26	−18	−12	17	−17	−30	32	−40	−86
13	−18	7	−8	38	72	109	241	49	−73
14	−22	−13	−40	34	4	−15	125	−3	−205
15	5	−31	−14	58	39	52	282	−36	−146
16	5	−16	−38	−5	−4	−63	69	23	−87
17	−13	21	−13	−8	−48	−48	−202	−6	−84
18	−13	22	39	22	−16	67	−131	−55	13
19	−13	10	−24	−30	−38	−82	−150	26	−30
20	−10	9	−8	4	4	9	− 3	17	−41
21	0	4	−19	34	60	79	221	49	−103
22	−4	9	1	47	22	79	85	−17	−125
Subtotals	−57	4	−136	211	78	157	569	7	−967
Differences	1	222	−38	−324	−312	−452	−1888	272	324

The average differences at each of the four bleeding times after injection are obtained, therefore, from the differences in the last row of Table III after division by 22 (= 11 × 2).

Similarly, it can be shown that the weighted difference of means for the three-period analysis is

$$\frac{A_1 + 2A_2 + A_3}{11} - \frac{B_1 + 2B_2 + B_3}{11}$$

TABLE VIII
ANALYSIS OF COVARIANCE (THREE-PERIOD DATA)

Source of Variation		D.F.	Sums of Squares and Products			D.F.	Residuals		
			$[x^2]$	$[xy]$	$[y^2]$		S.S.	M.S.	F
Totals	Mixtures	1	0.01	−3.42	1547.76	1	1551.46	1551.46	1.00
	Error	20	916.09	496.45	29839.64	19	29570.60	1556.35	
	Total	21	916.10	493.03	31387.40	20	31122.06		
Linear	Mixtures	1	0.01	−14.30	27004.12	1	27057.64	27057.64	7.02*
	Error	20	916.09	1720.42	76502.85	19	73271.90	3856.42	
	Total	21	916.10	1706.12	103506.97	20	100329.54		
Quadratic	Mixtures	1	0.01	2.06	560.48	1	560.80	560.80	1.89
	Error	20	916.09	−72.73	5628.85	19	5623.08	295.95	
	Total	21	916.10	−70.67	6189.33	20	6183.88		
Cubic	Mixtures	1	0.01	2.45	795.27	1	788.63	788.63	0.71
	Error	20	916.09	1243.12	22682.61	19	20995.72	1105.04	
	Total	21	916.10	1245.57	23477.88	20	21784.35		

*Significant ($P < 0.05$)

TABLE IX
DIFFERENCES AND TERMS FOR FOUR-PERIOD ANALYSIS

Rabbit No.	Hours after Injection					Totals (y)	Sums of Products (y)		
	0 = x	1.5	3.0	4.5	6.0		Linear	Quadratic	Cubic
1	−32	7	−76	−108	−120	−297	−413	71	−31
2	24	−82	−1	−187	−222	−492	−606	−116	418
3	1	10	31	27	55	123	131	7	57
4	−125	−59	23	18	−108	−126	−152	−208	−34
5	−16	17	−121	−147	−113	−364	−416	172	−52
6	−23	1	15	59	66	141	239	−7	−67
7	−39	42	−129	−109	−183	−379	−655	97	−285
8	27	110	−28	47	153	282	204	244	−182
9	−16	63	−63	−73	−30	−103	−289	169	−63
10	0	103	35	94	32	264	−154	6	−248
11	21	46	−59	−1	−21	−35	−143	85	−241
Subtotals	−178	258	−373	−380	−491	−986	−2254	520	−728
12	31	−69	−42	13	−8	−106	238	−48	−104
13	−53	−11	−106	24	102	9	469	173	−277
14	−60	−56	−93	30	10	−109	321	17	−303
15	8	−98	−53	77	66	−8	622	−56	−226
16	15	−29	−76	−31	−22	−158	66	56	−128
17	−43	9	−59	−33	−60	−143	−181	41	−147
18	−60	31	69	30	−6	124	−150	−74	80
19	−43	−4	−42	−62	−76	−184	−236	24	−12
20	−35	−11	−54	−17	13	−69	109	73	−87
21	−5	−8	−41	−4	56	3	229	93	−47
22	−21	−20	−12	90	45	103	297	−53	−241
Subtotals	−266	−266	−509	117	120	−538	1784	246	−1492
Differences	88	524	136	−497	−611	−448	−4038	274	764

and the average differences are obtained from the differences in the last row of Table VII after division by 44 (= 11 × 4).

Finally, the weighted difference of means for the four-period analysis is

$$\frac{A_1 + 3A_2 + 3A_3 + A_4}{11} - \frac{B_1 + 3B_2 + 3B_3 + B_4}{11}$$

TABLE X
ANALYSIS OF COVARIANCE (FOUR-PERIOD DATA)

Source of Variation		D.F.	Sums of Squares and Products			D.F.	Residuals		
			$[x^2]$	$[xy]$	$[y^2]$		S.S.	M.S.	F
Totals	Mixtures	1	17.60	−89.60	456.15	1	568.03	568.03	0.26
	Error	20	1414.66	884.70	41847.07	19	41293.80	2173.36	
	Total	21	1432.26	795.10	42303.22	20	41861.83		
Linear	Mixtures	1	17.60	−807.60	37057.83	1	39082.57	39082.57	9.15**
	Error	20	1414.66	2163.18	84487.53	19	81179.78	4272.62	
	Total	21	1432.26	1355.58	121545.36	20	120262.35		
Quadratic	Mixtures	1	17.60	54.80	170.63	1	91.67	91.67	0.17
	Error	20	1414.66	1156.26	11472.04	19	10526.98	554.05	
	Total	21	1432.26	1211.06	11642.67	20	10618.65		
Cubic	Mixtures	1	17.60	152.80	1326.58	1	1223.44	1223.44	0.90
	Error	20	1414.66	413.97	25873.11	19	25751.97	1355.37	
	Total	21	1432.26	566.77	27199.69	20	26975.41		

**Significant ($P < 0.01$)

and the average differences are obtained from the differences in the last row of Table IX after division by 88 (= 11 × 8).

The average differences between the two insulin mixtures at the four bleeding times after injection and after two, three, and four periods are given in Table XI and shown graphically in Figure I.

TABLE XI
WEIGHTED AVERAGE DIFFERENCES (A MINUS B) IN MG. PERCENT OF BLOOD SUGAR

Hours After Injection	Number of Test Periods		
	2	3	4
1.5	3.9	5.0	6.0
3.0	−2.5	−0.9	1.5
4.5	−8.9	−7.4	−5.6
6.0	−6.9	−7.1	−6.9

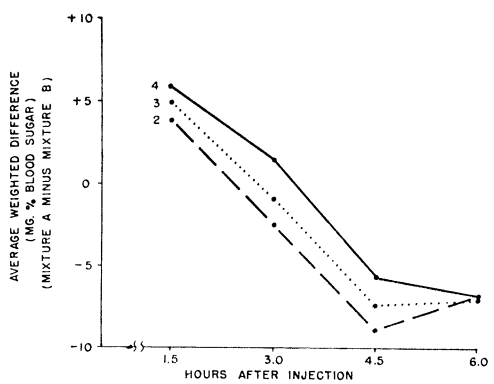

FIGURE I. CURVES OF AVERAGE WEIGHTED DIFFERENCES AFTER TWO TEST PERIODS (— — —), THREE TEST PERIODS (. . . .), AND FOUR TEST PERIODS (————).

In the two-period and three-period tests Mixture B (which was prepared to contain five percent less protamine) gave lower blood sugar values than Mixture A at one and one-half hours after injection, but thereafter permitted a more rapid recovery to normal blood sugar levels. In the four-period test, more rapid recovery occurred four and one-half hours after injection. Apparently, the five percent decrease in protamine content of Mixture B resulted in a more drastic initial

reduction of blood sugar and a less prolonged effect, which is what would be expected on the basis of experience with protamine-insulin mixtures.

The F-ratios obtained after two, three, and four periods are summarized in Table XII.

TABLE XII
F-RATIOS AFTER TWO, THREE, AND FOUR TEST PERIODS

Source of Variation	Number of Test Periods		
	2	3	4
Totals	2.53	1.00	0.26
Linear	4.00	7.02*	9.15**
Quadratic	5.95*	1.89	0.17
Cubic	0.53	0.71	0.90

*Significant ($P < 0.05$)
**Significant ($P < 0.01$)

The "Totals" is a measure of the mean differences between the two insulin mixtures averaged over all four bleeding times after injection. That none of the "Totals" are significant was not surprising since the initial differences were positive and the subsequent differences negative. In general, varying the protamine content of a long acting insulin mixture does not displace the blood sugar curve, but simply alters its shape. Because of this, mean differences are often of little value in comparing insulin preparations, whereas differences in the components of regression are of the greatest importance.

The linear, quadratic and cubic terms under "Source of Variation" in Table XII are a measure of the differences in the blood sugar curves resulting from the two insulin mixtures. Practice is necessary to obtain facility in interpreting the "difference" curve (Fig. I) without simultaneous reference to the actual blood sugar curves for each mixture. Brief consideration, however, will clarify the general principles involved. A horizontal, straight line at the "0" ordinate would indicate identical response to the two mixtures. A horizontal, straight line at any ordinate other than 0 would indicate parallel response, or a simple curve displacement. Any "difference" curve with a significant slope, or significant curvature, indicates fundamental differences in the time-response curves of the test preparations.

For the two-period analysis the quadratic component was significant

at the five percent level. The linear component was significant at the five percent level for the three-period analysis and at the one percent level for the four-period analysis. The reason for this is readily apparent from Figure I. The curve of differences for the four bleeding times tends overall, toward linearity as the number of test periods increases from two to four. This probably was due to the rabbits becoming less sensitive to insulin in the last two periods of the experiment. It will be noted from Table II that the blood sugar levels for the fourth period consistently were higher than those for the other periods.

Adjustment for initial blood sugar levels by covariance had no effect on the final results and in no case was there a significant reduction in error due to covariance. This was to be expected since a preliminary analysis of the initial values had shown that although there were significant period differences, the comparative levels were consistent for the two groups of rabbits from period to period.

The real difference between the two mixtures was small. It may be fortuitous that this was found to be significant in the simple cross-over experiment. On the other hand, significant differences persisted as the experiment was prolonged to include switchback and double switchback designs, despite the apparent decrease in sensitivity of the rabbits.

The extended cross-over designs yielded essentially the same results as the simple cross-over experiment. At all states of the investigation, there was evidence of a more rapid recovery of blood sugar levels in animals receiving the mixture with less protamine. The gain in discriminatory power resulting from the switchback designs does not seem to be commensurate with the additional cost, and danger of animal loss associated with these extended tests. Replicated simple cross-over designs would be more efficient from the standpoint of economies and might be expected to yield information of equal, or better, statistical validity.

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