tion was certified as being most suitable for parallel line assay among the responses at every 24 hours, in respect to the lineality of its log dose-response curve and the quantity of its " λ ".

2) The relationship between the reduced responses and the log doses could be found as being linear in Experiment II.

3) The unreduced responses caused by estrone and estradiol at the 72nd hour after injection, were certified as being proportional to their log doses. (Received July 7, 1952)

366. Yosoji Ito, Bun-ichi Tamaoki, Masako Egusa, and Hideharu Sakamoto: Studies on Sexual Hormones. VIII. Estrogen Bioassay, based on the Weight of Mice Uterus, and its Application.

(3) The Application of this Method for Qualitative and Quantitative Assay.

(Pharmaceutical Institute, Medical Faculty, University of Tokyo)

As a means of qualitative assay of estrogen, the assay method on the uterine weight of mice has been reported in our previous papers^{1,2)} as being applicable for a routine method. Furthermore, the increase in the uterine weight had been certified as being respectively proportional to log dose of natural estrogens such as estrone and estradiol, and judging from these tendencies, "parallel line assay method" seemed to be applicable for quantitative assay, under the assumption that the log-dose response curves, due to standard and test preparation be parallel to each other.

The writers take this opportunity to express deep gratitude to Dr. D. J. Finney, Lecturer in the Design and Analysis of Scientific Experiment, University of Oxford, and to Dr. C. I. Bliss, the Connecticut Agricultural Experiment Station, who gave many valuable advices on the statistical computations.

Experimentals

1) Application for a qualitative assay—This assay method was utilized in order to test qualitatively the estrogenic activities of the following preparations. Immature female mice were divided at random into four groups, and were administered with following preparations in the same manner as that taken in the previous experiments¹⁾.

Abbreviations:

C: 0.1 cc. of the purified soy-bean oil, which was used to prepare the following preparations, per mouse.

WOP**: 0.1 cc. of the oil solution, containing 0.1 mg. of the phenolic fraction extracted from whale ovaries, per mouse.

WTP**: 0.1 cc. of the oil solution, containing 0.1 mg. of the phenolic fraction extracted from whale testis, per mouse.

HTP**: 0.1 cc. of the oil solution, containing 0.1 mg. of the phenolic fraction extracted from hog testis, per mouse.

At the 72nd hour after the injection, the mice were sacrificed and their uterine weight was measured, according to the same procedure as previously stated¹⁾.

As Table II shows, it was found that there was no evidence of heterogeneity of the variances caused by the above four preparations. Under this certified condition, the analysis of variance of Table I was practicable, as shown by Table III, and thereby, against the within-class variation, or $V_{R(T)}$, the between-class variation, or V_T , was certified as being significant (level of significance, 0.05). Then it was necessary for our purpose to point out which fraction or fractions had caused

1) Y. Ito, B. Tamaoki, M. Egusa, H. Sakamoto: This Journal **72**, (12) (1952).

2) Y. Ito, B. Tamaoki, M. Egusa, H. Sakamoto: Ibid., **72**, (1286) (1952).

** Details of chemical procedures for the extraction of these fractions will be described in other papers by the same authors.

Table I. Experimental data on the uterine weight.

	<i>T</i>			
	<i>C</i>	<i>WOP</i>	<i>WTP</i>	<i>HTP</i>
1	-5	30	-20	-5
2	-10	-5	-10	15
3	-30	0	-10	-10
4	-10	0	25	-5
5	-15	5	-15	-15
6	0	-5	-20	-20
7	-20	15	-10	-15
8	-10	0	-10	25
9	-10	0	-10	-15
10	5	0	-20	-5
Total	-105	40	-100	-50

The original uterine weight, x , was transformed to a linear relationship as follows: $y=10x-50$, where y denotes the number in the above Table.

the significant difference in response to the control.

According to the statistical procedure in our previous paper³⁾, the following inequality must be satisfied, if D is the significant difference between two totals of responses.

$$\frac{L^2}{10V_{R(T)}+10V_{R(T)}} > F_{36}^1(0.05)=4.11 \quad \therefore D > 111$$

It was then found in this experiment that *WOP* had a significant estrogenic activity, in comparison with the control, while *WTP* and *HTP* had not. On the weight of ovaries and adrenal glands: At the same time, the weight of the ovaries and adrenal glands were analyzed by the similar statistical method to find no significant difference among them.

2) Application as quantitative assay.

- (1) 3,4-*bis*(*p*-hydroxyphenyl)-3-hexene, or Diethylstilbestrol.
- (2) 3,4-*bis*(*m*-methyl-*p*-hydroxyphenyl)-hexane, Meprane or Dimethyl Hexestrol.
- (3) 3,4-*bis*(*p*-hydroxyphenyl)-hexane, or Hexestrol.
- (4) 3,4-*bis*(*p*-hydroxyphenyl)-heptane.
- (5) 3,4-*bis*(*p*-hydroxyphenyl)-pentane.

The estrogenic activity of the following synthetic estrogens were quantitatively assayed by this method, using the 4-point assay method^{4,5,6)}. The experimental procedures, stated previously^{1,2)} were also applied in this experiment.

The statistical procedures, by which the potency ratios were calculated in this paper, followed Finney's method⁴⁾. However, before the estimations of potency ratios, the homogeneity of the variation, due to the following 10 treatments, needed to be testified.

As shown by Table V, their heterogeneity was certified among the 10 treatments. After further analysis, it was found that there was no evidence of heterogeneity of variances among the 8 treatments (2)-(5). Then it became possible to apply the following analysis and estimate the potency ratio among (2)-(5). *Computation of the relative potencies.* For example, when (2) and (3) is "Standard" and "Unknown" preparation, respectively, the relative potency (3) to (2) could be calculated as follows:

Table II. Test of homogeneity of the variances of responses, due to the four treatments.

Factor	<i>SS</i>	<i>V</i>	$\log V$	<i>f</i>
<i>C</i>	872	97	1.9868	9
<i>WOP</i>	1040	116	2.0645	9
<i>WTP</i>	1550	172	2.2355	9
<i>HTP</i>	1850	206	2.3139	9
<i>n</i> =4		591	8.6007 = $S \log V$	

Mean variance $\bar{s}^2=591/4=148$

$\log \bar{s}^2=2.1703$

$n \log \bar{s}^2 - S \log V = 8.6812 - 8.6007 = 0.0805$

$\chi_0^2 = 2.3026 \times 9 \times 0.0805 = 1.6682$

Correction Factor = $1 + \frac{n+1}{3n \cdot f} = 1.046$

Corrected $\chi_0^2 = 1.6682/1.046 = 1.5948$

(cf. χ^2 (d.f.=3)=7.815 ($\alpha=0.05$)).

Table III. Analysis of variance of Table I.

Factor	<i>SS</i>	<i>f</i>	<i>V</i>	<i>F</i> ₀
<i>T</i>	1358	3	453	3.06*
<i>R(T)</i>	5312	36	148	
Total	6670	39		

cf. $F_{36}^3(0.05)=2.85$

3) Y. Ito, B. Tamaoki, M. Egusa: This Journal **72**, 172 (1952).

4) J. H. Burn, D. J. Finney, L. G. Goodwin: "Biological Standardization" p. 75 (Rev. Ed.). England (1950).

5) "The Short-Cut Method of 4-Point Assay" C. I. Bliss: Sci., **100**, 577 (1944); C. I. Bliss: J. Am. Statist. Assoc. **39**, 479 (1944).

6) Also, its short-cut method; L. Knudsen: Ann. New York Acad. Sci., **52**, Art. 6,889 (1950).

Table IV. Experimental data of the mice's uterine weight.

Sample	(1)		(2)		(3)		(4)		(5)	
	H	L	H	L	H	L	H	L	H	L
1	16	0	4	0	7	0	2	0	-2	-2
2	13	0	4	0	2	-3	2	-3	1	-3
3	16	0	6	-1	4	2	2	-2	-2	-2
4	10	4	5	0	0	-2	10	-2	-1	-3
5	7	3	3	0	3	6	2	-2	-4	-1
6	6	-1	-1	4	6	0	6	-2	3	-6
7	2	5	7	3	0	3	2	0	3	-5
8	5	3	3	-2	3	-2	7	-2	4	-3
Total	75	14	31	4	25	4	33	-13	2	-25

The original weight x was transformed to a linear relationship as follows: $y=2x-14$, where y denotes the number in the above Table. H and L in Table IV denote high and low dose of the respective preparations; 4 and 1 γ /0.1 cc. of the purified soybean oil.

Table V. Test of homogeneity of variance.

		SS	f	V	log V
(1)	H	192	7	27	1.43136
	L	35	7	5	0.69897
(2)	H	41	7	6	0.77815
	L	28	7	4	0.60206
(3)	H	45	7	6	0.77815
	L	64	7	9	0.95424
(4)	H	69	7	10	1.00000
	L	8	7	1	0.00000
(5)	H	59	7	8	0.90309
	L	19	7	3	0.47712
n=10				79	7.62314=S log V

$\bar{s}^2=79/10=7.9$ $\log \bar{s}^2=0.90309$
 $n \log \bar{s}^2 - S \log V = 9.0309 - 7.6231 = 1.4078$
 $\chi_0^2 = 2.3026 \times 7 \times 1.4078 = 22.69$
cf. $\chi^2(f=9, \alpha=0.05) = 16.9190$
Test of homogeneity of Variance (2)~(5)
 $\bar{s}^2=6, n \log \bar{s}^2 - S \log V = 6.22520 - 5.49281$
 $= 0.73239$
 $\chi_0^2 = 2.3026 \times 7 \times 0.73239 = 11.80$
Corrected $\chi_0^2 = 11.805/1.05 = 11.24$
cf. $\chi^2(f=7, \alpha=0.05) = 14.07$

At first, in order to compute the variance of "error", the analysis of variance was necessary under the condition of the homogeneity of variance which had been certified, as shown by Table V.

$$S_{\text{preq.}} = \frac{\{(UH+UL)-(SH+SL)\}^2}{32} = \frac{(35-29)^2}{32} = 1.13$$
$$S_{\text{Reg.}} = \frac{\{(UH+SH)-(UL+SL)\}^2}{32} = \frac{(56-8)^2}{32} = 72.00$$

Table VI. Experimental data of the uterine weight of mice caused by (1) and (2). (from Table IV).

Dose	Unknown (2)		Standard (3)	
	High	Low	High	Low
1	4	0	7	0
2	4	0	2	-3
3	6	-1	4	2
4	5	0	0	-2
5	3	0	3	6
6	-1	4	6	0
7	7	3	0	3
8	3	-2	3	-2
Total	31	4	25	4
	UH	UL	SH	SL

Table VII. Analysis of variance of Table VI.

Factor	SS	f	V
Preparation	1.13	1	1.13
Regression	72.00	1	72.00
Parallelism	1.13	1	1.13
Between Doses	74.26	3	
Error	177.74	28	6.35= s^2
	252.00	31	

$$S_{\text{Para.}} = \frac{\{(UH+SL) - (SH+UL)\}^2}{32} = \frac{(35-29)^2}{32} = 1.13$$

$$S_{\text{Doses}} = S_{\text{Prep.}} + S_{\text{Reg.}} + S_{\text{Para.}}$$

$$S_{\text{Total}} = \sum x_i^2 - (\sum x_i)^2 / 32 = 4^2 + 4^2 + \dots + 3^2 + (-2)^2 - 64^2 / 32 = 252.00$$

$$S_{\text{Error}} = S_{\text{Total}} - S_{\text{Doses}}$$

$$\text{Test of parallelism: } F_0 = V_{\text{Para.}} / V_{\text{Error}} = 1.13 / 6.35 < 1$$

Then, the assumption of parallelism could be certified, and the following calculations could be made.

Linear regression coefficient of weight of uterus on dose:

$$b = \frac{(UH+SH) - (UL+SL)}{32} = 48/32 = 1.500$$

Relative potency in $1/2 \log$ (High dose/Low dose) scale.

$$M = \frac{\bar{y}_u - \bar{y}_s}{b} = \frac{2\{(UH+UL) - (SH+SL)\}}{(UH+SH) - (UL+SL)} = 0.250$$

where $\bar{y}_s$ and $\bar{y}_u$ denote the mean responses of the "Standard" and "Unknown" preparations, respectively.

$$"g"-criterion; g = \frac{s^2 t^2}{b^2 S_{xx}} = \frac{6.35(2.05)^2}{(1.500)^2 \cdot 32} = 0.371 > 0.1$$

The fiducial limits of the relative potency ($P=0.05$) in $1/2 \log 4$;

$$(\bar{x}_s - \bar{x}_u) + \frac{M - \bar{x}_s + \bar{x}_u}{1-g} \pm \frac{st}{b(1-g)} \sqrt{(1-g) \left(\frac{1}{N_s} + \frac{1}{N_u} \right) + \frac{(M - \bar{x}_s + \bar{x}_u)^2}{S_{xx}}} \quad (1)$$

where $t = t$ value for the number of the degree of freedom of s^2 , from t^2 -table. ($P=0.05$); N_s and N_u denote the number of the animals which are administered "Standard" and "Unknown" preparations; $\bar{x}_s$ and $\bar{x}_u$ denote the mean dose of the "Standard" and "Unknown" preparations, respectively.

In this assay, x_s are designated to be equal to $\bar{x}_u$, and then the formula (1) can be simplified into the following form.

$$\frac{M}{1-g} \pm \frac{st}{b(1-g)} \sqrt{(1-g) \left(\frac{1}{N_s} + \frac{1}{N_u} \right) + \frac{M^2}{S_{xx}}} = 0.397 \pm 1.552$$

Relative potency in common logarithm: $\log R = 1/2 \log 4 \times 0.250 = 0.075$

Potency ratio in normal scale: $R = 1.20$

Fiducial limits of R : Upper limit = 3.87

Lower limit = 0.45

The above calculations were applied for other cases, and the calculated results are summarized in Table VIII.

Table VIII Summarized results of the calculation of potency ratios: (2)~(5).

Standard	Unknown	$V_{\text{prep.}}$	$V_{\text{reg.}}$	$V_{\text{para.}}$	V_{Error}	g	Potency ratio	Fiducial limits ($P=0.05$)
(3)	(2)	1.13	72.00	1.13	6.35	0.371	1.20	0.45~3.87
(5)	(2)	105.13	91.13	0.00	5.26	0.242	4.43	2.05~24.8
(5)	(3)	84.50	72.00	1.13	6.69	0.390	4.49	1.76~78.1
(2)	(4)	7.03	166.53	11.28	5.20	0.131	0.75	0.42~1.25
(5)	(4)	57.78	166.53	11.28	5.54	0.140	2.26	1.33~5.01
(4)	(3)	2.53	140.28	19.53	6.63	0.199	1.20	0.63~2.54

Discussion; Four-point assay, which was applied in this experiment, is a simple type of "parallel line assay" and from the analysis of variance in this design, it was not possible to obtain any information about the lineality of the dose-response curve of the respective preparations. Therefore, the assumption that the responses to the above-mentioned synthetic estrogens be proportional to the logarithm of their doses was underlying in our experiment, though the responses to natural estrogens, such as estrone and estradiol, had previously been found to be proportional to their log doses. Besides their lineality, the parallelism of the log dose-response curves of the standard and unknown preparation was one of the important assumptions of "parallel line assay"^{4,5}. Test of departure of parallelism between the two curves which was included in the analysis of variance was carried out and it was found that there was no evidence of "non-parallelism".

Dorfman^{7,8}) had published the experimental results of the activities of natural and synthetic

7) R. I. Dorfman, A. S. Dorfman: Endocrinol, **42**, 85 (1948).

8) R. I. Dorfman, A. S. Dorfman, D. Grim: Ibid., **42**, 98 (1948).

estrogens which were evaluated by the simplified 4-point assay⁴⁾, but the following assumptions existed, although he did not refer to it, i.e. the homogeneity of the variances of responses at the respective dosage level, the lineality of log dose-response curve, the linear relationship between the oviduct weight and body weight of a chick, and the quantities of "g" in *g*-criterion⁴⁾ were less than 0.1. Dorfman also attempted to calculate indirectly the potency ratio of two estrogens whose response curves did not seem to be parallel, from their respective relative potencies to other estrogens, but he showed neither the validity of his calculations nor the method of calculating the standard error of the indirect estimate, which we will discuss statistically in detail in one of our papers.

The expenses for the works on the estrogen assay (VI~VIII) was partly defrayed by the Scientific Research Fund for the year of 1951 from the Ministry of Welfare, and for the year of 1952 from the Ministry of Education, for which our thanks are due. We are also grateful to Mr. S. Tanabe who kindly supplied the synthetic estrogens for our use.

Summary

The bioassay, based on the uterine weight of mice, was applied as a qualitative assay to test the estrogenic activities of the extracts from whale ovaries, whale testis, and hog testis, and it was found in this assay that only the extract from whale ovaries possessed a significant estrogenic activity, while the others had not. The estrogenic activities of several synthetic estrogens were quantitatively assayed, using the 4-point assay design, to calculate their relative potencies, which were tabulated.

(Received July 7, 1952)

367. Yoshio Sakurai, and Keishi Yoshino: Studies on Cancerocidal Substances.

I. Effect of Pterins on the Yoshida Sarcoma.*

(Iatrochemical Institute of Pharmacological Research Foundation, Inc.**)

At the present, it seems to be proper and convenient to use Yoshida sarcoma, a transplantable tumor fluid in the peritoneal cavity of a rat, as a screening tumor in the chemotherapeutic study of cancer.

We have already reported¹⁾ with our collaborators on the effect of some compounds upon this tumor, e.g. some chemotherapeutics, stibine compounds, and antibiotics.

Although many experiments have already been published about cancerocidal activity of pterins, their effect upon Yoshida sarcoma is yet unknown.

We synthesized an aminopterin and some kinds of comparatively simple derivatives of 2-amino-4-hydroxy- and 2,4-diamino-pteridine by the method of Seeger²⁾, Caine³⁾, and Daniel and Karrer⁴⁾. The method of animal screening test we adopted is as follows:

- 1) Determination of M.T.D. (maximum tolerated dose)
- 2) Intraperitoneal injection in rats (about 100 g. body weight), bearing 3-4 day-old Yoshida sarcoma, of a dose close to M.T.D.
- 3) Examination of the tumor-ascites at 1, 3, 6, 12, and 24 hours after injection.

Microscopic examination of Giemsa-stained smear preparation of ascites.

Evaluation of cellular changes produced by the treatment, in nucleus, protoplasm, and chromosome.

* M. Ishidate, Y. Sakurai: Studies on Cancerocidal Substances. I.

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1) T. Yoshida, M. Ishidate, Y. Sakurai et al.: *Gann*, **41**, 93 (1950).

2) D. Seeger: *J. Am. Chem. Soc.*, **71**, 1753 (1949).

3) C. K. Caine: *J. Am. Chem. Soc.*, **71**, 893 (1949).

4) P. Karrer: *Helv. Chim. Acta*, **32**, 1041 (1949).