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On a Method for Calculation and Analysis of Results in the McKenzie Assay for Thyrotropin (TSH).* (29057)

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In the elegant biological method reported by McKenzie(1) for assessing TSH activity, one measures in suitably prepared animals, the radioactivity of 2 samples of blood; at time zero, a first sample of blood (internal control) is withdrawn and TSH injected immediately thereafter; 2 hours later, a second sample of blood (experimental) is obtained. McKenzie reported that there is a (linear) relationship between the log of the dose of injected TSH and the difference in radioactivity counts between the 2 samples of blood. We have recently become interested in this assay in studying the neurohumoral control of the secretion of TSH(2). In this paper is proposed a new method for calculating and analyzing the results obtained in this technique which greatly improve its reliability and usefulness. Similar problems have been recently discussed regarding the ovarian ascorbic acid depletion test for LH(3,4). As in the case of the LH assay, we will introduce here for the TSH method the principle of covariance adjustment and analysis. A detailed discussion of this method of calculation, including its limitations as well as the mathematical requisites it necessitates has been published(3,4). The second principle regarding the TSH assay discussed here is transformation of the original data into metameters more amenable to classical mathematical analysis.

Materials, methods, results. 1. *Method to assess TSH activity.* This is essentially that reported by McKenzie(1) with minor modifications for practical convenience. Female SA mice (body weight 10-15 g) (Duterme Farms, Condé s/Huisne, France) are kept in temperature controlled rooms (22°C ± 2) on a low iodine diet (Nutritional Biochemicals, Cleveland, Ohio) with double (glass) distilled water as drinking fluid, for 10 days. They are then injected i.p. with 1.5 μc I^{131} carrier free, followed 5 hours later by 10 μg 1-thyroxine s.c.; 24 hours later they received a second injection of 5 μg 1-thyroxine s.c. and are used 48 hours after this last injection for bioassay of TSH. Under rapid ether anesthesia, 0.25 ml of blood is withdrawn from the jugular vein in a heparinized tuberculin syringe and the material to be tested (for TSH activity) is immediately injected by the same route in a total volume of 0.3 ml; 2 hours later, again under ether anesthesia, a second 0.25 ml blood sample is taken from the same or contralateral jugular vein. The radioactivity has been measured in a well scintillation counter followed by a manual scaler (Saphymo, Paris) and more recently in a fully automatic well-counter (Tracerlab) with pulse analyzer and autoprinter.

2. *Principle of covariance adjustment and*

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analysis in TSH assay. We will assume that the volume of both blood samples (before TSH and after TSH) is identical within the experimental error of well calibrated syringes. In carefully controlled experiments we have observed that the correction factor introduced by knowing the exact volumes or weights of the blood samples (rather than relying on the syringe reading) is so minimal that it does not justify the labor involved in the multiple weighings of tubes.

This being assumed, the quantity of radioactivity in any second blood sample (experimental) depends on: a) level of radioactivity of the corresponding first blood sample (internal control); b) TSH effect of the material injected after the first blood sampling. Use of this method shows that levels of radioactivity in the first samples are not similar even though the animals have been similarly pretreated. To compensate for this variability, McKenzie proposed expressing the variation of radioactivity between the two blood samples as percentage of the level of the first sample. Instead of this "proportional adjustment," we propose *covariance analysis:* values of the second blood samples *(dependent variable)* are adjusted by covariance to values of the first blood samples *(independent variable).*

3. *Principle of transformation of original data in TSH assay.* Percentage variations on radioactivity counts per minute are not continuous variables; they follow distributions which are not the normal distribution. Being discrete variables, they cannot be used as variates in the classical analysis of variance or in the standard statistics of bioassay, all designed for continuous variables with normal distributions. The best exemplification of these statements for our study is the diagram of Fig. 5 in McKenzie's original paper: that the results as calculated by his original method show heteroscedasticity is clearly observed, viz. the standard error of the means increases with increasing means. Application of the standard tests of significance in an analysis of variance requires that the experimental errors be independently and normally distributed(5-8); these conditions do not exist in the crude data obtained in the TSH assay as calculated according to McKenzie. One answer to these problems is *transformation* of the crude data so that the transformed data fit approximately in a normal distribution and so that means and variances become independent‡. Experiments have been calculated with logarithmic and square root transformations. The most consistent reduction of heteroscedasticity was obtained with the logarithmic transformation (Tables I, II).

4. *Proposed mode of calculation for TSH assay.* This new method embodies both principles of covariance analysis and transformation of data. The crude results (total counts/fixed time/fixed volume of blood) on both blood samples are transformed into their logarithms ($\log_{10}$). An analysis of covariance is then performed between the transformed second blood values and the transformed first blood values, after verification for each experiment of the necessary prerequisites to covariance analysis§ (see 2,3 for detailed discussion). Statistical significance between the various (adjusted) means is assessed by one or several of the available tests for multiple comparisons(2). In the case of bioassays (4- or 6-point assays as well as multiple 4- or 6-point assays with equally spaced doses of TSH standard and unknowns), the mathematical analysis follows that proposed by Bliss (10) or Finney(11).

‡ See various chapters on transformation in (5,7, 8,9) and Bartlett(6).

§ These consist essentially in assessing absence of heterogeneity of slopes (between dependent and independent variables) and of variances between the various groups. In several hundreds of experiments in which these parameters were studied we have only accidentally encountered heterogeneity of the results after log transformation. In each case, such evidence of heterogeneity could be correlated with some unusual or abnormal circumstance. Thus, in view of the lengthy calculations involved here, one may consider running them only in selected experiments for checking purposes after observation of the results has shown unusual values. On the other hand, if all these operations are done with electronic computers (see below), it is highly recommended that calculation of these parameters be included in the routine programmation.

TABLE I. Four-point Assays of Unknowns vs USP Reference Standard for Thyrotropin—Various Parameters of the Statistical Analysis on Original Data, or After Square Root and Log Transformation.

Protocol No.	Heterogeneity of slopes (F)			Heterogeneity of variances (χ^2)			F regression			Confidence limits		
	Original data	$\sqrt{}$	$\log_{10}$	Original data	$\sqrt{}$	$\log_{10}$	Original data	$\sqrt{}$	$\log_{10}$	Original data	$\sqrt{}$	$\log_{10}$
4103 (a)	4.49*	1.31	.19	7.66	5.08	3.67	40.81***	74.33***	99.16***	.64- 1.44	.76- 1.36	.85- 1.39
4103 (b)	2.14	1.22	.47	1.01	.85	2.48	49.73***	63.61***	66.91***	.63- 1.25	.61- 1.13	.56- 1.03
4106 (a)	4.94*	2.65	1.12	5.07	4.05	3.56	36.39***	59.62***	99.33***	.61- 1.42	.63- 1.20	.65- 1.05
4106 (b)	3.81	1.87	.85	3.55	4.29	5.62	5.70*	8.97*	10.62**	.57- 9.09	.66- 3.33	.69- 2.66
4118 (b)	2.02	2.07	2.05	5.37	3.32	2.04	11.48**	13.18**	14.47**	.45- 2.72	.38- 1.94	.29- 1.45
4119 (b)	.02	.05	.56	2.57	1.15	.66	14.90**	18.14**	18.77**	1.99-31.55	2.07-20.62	1.98-17.27
4126 (a)	.59	.21	.16	1.96	.88	.58	16.55**	20.14***	20.43***	1.30- 7.17	1.46- 6.97	1.54- 7.87
4126 (b)	2.04	.96	.40	9.84*	8.06*	6.80	13.99**	18.86**	23.33***	.72- 3.97	.83- 3.35	.93- 3.16
4127 (a)	1.73	1.78	1.87	8.58*	6.68	5.25	15.22**	18.68**	22.81***	.44- 1.98	.66- 2.45	.88- 2.99
4137 (c)	.37	.17	.22	12.76**	7.95*	4.05	15.19**	22.53***	28.44***	.54- 2.20	.68- 1.98	.79- 1.99
4137 (b)	9.38**	3.30	.70	5.88	3.10	1.29	10.47**	19.33**	29.06***	1.38-26.18	1.72- 9.84	2.03- 7.88
4250 (b)	.21	.10	.18	10.00*	6.76	4.31	6.22*	9.54*	12.31**	.47-34.54	.77- 9.67	.88- 6.65
4259 (a)	6.27*	3.13	1.45	3.69	3.74	4.48	10.26**	14.29**	18.09**	.03- .78	.09- .79	.15- .81
4269 (b)	2.15	1.76	1.59	9.42	7.67	6.16	14.49**	18.62**	21.64***	.92- 5.09	1.07- 4.68	1.21- 4.87
4275 (a)	.44	.18	.21	.55	.91	1.41	8.60*	10.32**	11.34**	.27- 2.81	.35- 2.52	.39- 2.38

Levels of statistical significance: * .05; ** .01; *** .001; absence of asterisk: not significant.

Examples. *Table I* shows a series of experiments which are classical 4-point assays of various unknowns *vs* the USP Reference Standard for Thyrotropin. They all have been calculated by covariance analysis, using successively the original data, the same original data now transformed in square roots and also in $\log_{10}$. The first chapter under the heading "heterogeneity of slopes" shows the F value in the Fisher test which precedes the covariance adjustment, to ascertain that the slopes between the dependent variable (counts on the second blood sample) and the independent variable (counts on the first blood sample) are not heterogeneous in the various experimental groups. Such heterogeneity occurs sometimes (original data); either of the 2 transformations leads to disappearance of such heterogeneity. The chapter under the heading "heterogeneity of variances" shows χ^2 values, corrected for small samples by the corrections factor C introduced by Bartlett(14); the χ^2 value will ascertain that the *reduced variances* between the various groups are not heterogeneous. The log transformation removes consistently any heterogeneity that may exist in the original data, whereas the square root transformation does not always do so, even though it gives lower χ^2 values, *i.e.* diminishes the tendency to scattering (of the original data). The last two chapters report the values for the variance ratio F of the regression (effect over log dose) in calculation of the validity of the bioassays and the confidence limits of the potency (antilog M) of unknown *vs* standard in each experiment. These figures show the benefits obtained by using the adequate transformation: the disappearance of the heteroscedasticity leads to higher F values for the regression (of effect/dose) and to narrower confidence limits of the calculated potency.

In *Table II* are reported experiments which are not 4-point assays of one unknown *vs* a standard; several unknowns are studied at 1 or 2 dose levels and compared to responses obtained in the control group receiving NaCl. Two known doses of TSH are regularly included in these experiments to assess the re-

TABLE II. Various Parameters of Statistical Analysis on Original Data or After Square Root and Log Transformation in Experiments Comparing Responses to NaCl Controls.

Protocol No.	Heterogeneity of slopes (F)			Heterogeneity of variances (χ^2)			F treatments			Test of Dunnett‡			
	Original data	$\sqrt{}$	$\log_{10}$	Original data	$\sqrt{}$	$\log_{10}$	Original data	$\sqrt{}$	$\log_{10}$	Treatment	Original data	$\sqrt{}$	$\log_{10}$
4301	2.19	1.27	.99	14.61	10.31	9.93	5.13***	5.98***	6.58***	NaCl			
										TSH .1 mu	–	–	*
										TSH .3 mu	–	**	**
										A_1	–	–	**
										A_2	**	**	**
										B_1	–	–	**
										B_2	**	**	**
										C_1	–	*	**
										C_2	*	**	**
										D_1	–	–	**
										D_2	–	**	**
4255†	.87	.53	.44	6.34	5.19	4.84	1.87	2.43	3.03*	NaCl			
										TSH 1.0 mu	–	–	–
										TSH 3.0 mu	–	*	*
										T-40	–	–	–
										T-43	–	–	*
										T-52	–	–	*
										T-55	–	–	–
4256	1.00	.47	.25	13.48	9.82	9.28	9.35***	11.27***	11.13***	NaCl			
										TSH .1 mu	–	–	–
										TSH .3 mu	**	**	**
										A	–	–	*
										B	**	**	**
										C	*	**	**
										D	**	**	**
										E	–	–	–
										F	–	–	–
										G	–	–	*

† Experiment in rats for assessing TRF activity; protocol for TRF assay described in detail elsewhere (*Experientia*, 1963, v19, 480).

‡ Multiple-Comparison Test of Dunnett (see text); levels of significance of various responses are calculated in comparison to that obtained, in each experiment, for NaCl control group. * .05; ** .01; *** .001; absence of asterisk: not significant.

sponse of the animals of that particular shipment. No efforts are thus made to calculate "potency" of the test materials in terms of the TSH standard; it is intended to test their TSH- or in some cases TRF-activity (as in protocol No. 4255), to localize for instance a zone of activity in the effluent of some separation method. The comparisons are made by the multiple comparison test of Dunnett(12) (see also 3,4). The chapter headed "*F* treatments" shows the variance ratio *F* of the overall experiment for the source of variation "treatments," in the analysis of covariance. The chapters "heterogeneity of slopes" and "heterogeneity of variances" are similar to those of *Table I.* It can be seen again that in this type of experimental design the log transformation is highly beneficial: it always decreases the *F* value of the station "heterogeneity of slopes"; it always decreases the χ^2 value of the station "heterogeneity of variances"; thus, it improves the *F* value for the variation due to "treatments."

Conclusions. Two simple and classical modifications in statistical treatment of the data obtained by the method of McKenzie for assessing TSH activity have removed some mathematical inconsistencies inherent in the original proposal; this has also increased considerably the precision and reliability of the method.

Transformation of original data followed by covariance analysis represents a tedious task. Thus the security procured by the correct and complete mathematical analysis is obtained only through considerable work. We have reported(13) on the use of electronic computers to perform these lengthy calculations, as is now routinely done in this laboratory.

Summary. A new method to calculate and analyze results in the McKenzie assay for TSH is presented. It is proposed: 1) to transform the original data (radioactivity counts/fixed volume/fixed time) in their logarithms ($\log_{10}$); this applies to both first (control) and second (after TSH) blood samples; 2) a covariance analysis is then applied on the transformed data, the second blood values (dependent variable) being adjusted to the values of the first blood sample (independent variable). This method removes the heteroscedasticity inherent in the original calculations as proposed by McKenzie, thus increasing precision and reliability of the experiments.

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