ON THE BIOASSAY OF THYROTROPHIN IN PLASMA

By

Claus Rerup and Arne Melander

ABSTRACT

The $^{131}$I mouse blood level increase method of thyrotrophin (TSH) bioassay (McKenzie 1958) was investigated with regard to its practical efficiency and statistical validity. This method did not fulfil the requirements of a valid bioassay because of variance heterogeneity and curvilinearity of the logdose response line.

Studies on $^{131}$I elimination and thyroxine action time in mice yielded a biological half life of $^{131}$I of about 5 days, while the estimated period of action time of 20 µg of 1-thyroxine was 3 days.

The advantages of the method adopted here are briefly summarized as follows:

1) No pretreatment with low-iodine diet, no feeding of thyroid powder.
2) The response metameter of choice, preferable to three others, is the log cpm-ratio which gave linearity, homoscedasticity, precision, and ease of calculation.
3) The animals can be used from day 2 to day 6 inclusive.
4) Small samples of blood (50 µl) and constriction pipettes are preferable to larger samples and the use of syringes: higher precision, the same accuracy and repeated use of the same animals.
5) Cross-over design improved the index of precision from 0.23 to 0.15.
6) Evidence on specificity: no response to plasma from hypophysectomized rats and to some other substances, high values after PTU (propylthiouracil) and in some clinical conditions.

The method is not sufficiently sensitive (mean threshold dose was 0.017 mU of TSH) to detect TSH levels in normal human or mouse plasma, which thus appear to be lower than 0.05 mU per ml. In normal rats, the mean plasma TSH level was 0.4 mU per ml. Elevated plasma TSH was observed in patients with myxoedema and malignant exophthalmos (highest value recorded was 0.9 mU per ml), and in rats and mice after prolonged treatment with PTU. The plasma levels in mice after PTU treatment for 5 weeks were as high as 4.9 mU per ml.
Since the early work of Smith (1916) and Allen (1916) who described an adenohypophysial principle influencing thyroid gland activity, a large number of assay methods for thyrotrophin (TSH) has been described; yet methods sensitive enough to detect TSH in the plasma or serum of man or laboratory animals are few. Measurements of serum or plasma TSH have been performed mainly with the following methods:

1) The stasis tadpole method of D'Angelo & Gordon (1950), in which the effect of TSH on thyroid cell height of starved tadpoles is measured histometrically. The sensitivity of this method has been reported to be 0.1 mU (milliunit) of the International or U. S. P. standard TSH (McKenzie 1960).

2) The 131I blood level increase method of McKenzie (1958), which is a modification of the technique of Adams & Purves (1955) replacing guinea pigs by mice, and which has a reported sensitivity of 0.025 mU of TSH.

3) The thyroid slice weight increase method of Bakke et al. (1957), in which the weight increase of incubated ox thyroid slices constitutes the response to added TSH. The reported sensitivity is 0.01 mU of TSH.

4) The 131I release method of Bottari & Donovan (1958) modified by El Kabir (1962), in which the release of 131I from guinea pig thyroid slices incubated in an 131I containing medium is measured. The lower limit of TSH detection was reported to be 0.001 mU of TSH.

Among the methods listed that of Bottari & Donovan (1958) appears to be the most sensitive. However, Rerup et al. (1962) showed that the method gave a non-specific response to plasma from hypophysectomized rats, which was logdose dependent and parallel to those from normal rat plasma. The in vitro method of Bakke et al. (1957) and the amphibian method of D'Angelo & Gordon (1950) are both dependent on the continuous availability of fresh ox thyroid glands and tadpoles, respectively, and this precluded their use in our laboratory. For these reasons it was considered worth while to investigate the measurement of plasma TSH according to the main principles described by McKenzie (1958).

The original method which requires a special low-iodine diet before 131I injection and thereafter the addition to the diet of 0.066 per cent of thyroid powder (U. S. P.) in order to maintain an efficient blockade of endogenous TSH release, has a relatively low capacity for serial analyses, since blood sampling is performed by aspiration of free blood from a punctured tail vein by means of a heparinized tuberculin syringe. Furthermore, from a theoretical point of view, the method does not appear to meet all requirements of bioassay validity, since the variance of the response was found to be heterogeneous over the working part of the logdose response line (condition of heteroscedasticity) in our earlier observations (Rerup et al. 1962, unpublished) as well as those of Sakiz & Guillemin (1964); these latter authors suggested a log-transformation and covariance analysis of the assay data, in order to eliminate
the heteroscedasticity. The question of linearity or non-linearity of the log-
dose response line does not seem to have received particular attention, which
appears from McKenzie's (1958) statement: »Absolute increase (in blood $^{131}$I
level) with arithmetic dose and $\%$ increase with log-dose both gave linear
results«. This cannot be true, of course, in a large material, in which both the
above mentioned response parameters must yield the same (either linear or
higher) mathematical function of a given dose parameter, differing only with
regard to values of constants.

Since, in this bioassay, blood $^{131}$I level increases following TSH injection,
the time concentration pattern of blood $^{131}$I in controls and l-thyroxine treated
mice was considered to be of interest.

The main points to be investigated were thus:

1) The type of elimination of $^{131}$I from mouse blood, and the effect of
thyroxine on this elimination.

2) The number of days suitable for TSH assay.

3) The need for a low-iodine diet pretreatment and thyroid powder feeding.

4) The simplification of blood sampling, the increase of its precision, and
the reduction of the sample volume in order to use the same mouse repeatedly.

5) The regression of the response or its metameter on dose or its metameter.

6) The specificity, sensitivity, and precision of the method.

7) Plasma TSH findings in man, rat, and mouse.

METHOD

Female mice of the NMRI strain* weighing 16 ± 2 g at the time of their arrival were
used throughout. They were kept on a normal pellet diet for mice** and tap water
ad libitum. No dietary changes were made before or during the experimental period.
$^{131}$I was injected intraperitoneally (8 μc per mouse in 0.1 ml) followed immediately
by l-thyroxine given subcutaneously (20 μg per mouse in 0.1 ml). A second dose of
thyroxine was given 72 hours later. The mice were ready for TSH assay from two
to six days following $^{131}$I injection. Beginning on a Saturday we were thus able to
use the animals during the following week at any time between Monday morning and
Friday evening (Table 1).

Altogether 2500 mice were used. Standard TSH (U.S.P. thyrotropin reference
standard (tablets), 0.074 units per mg; or a substandard with a potency of 0.6
U.S.P. units per mg) was dissolved in 0.1 x acetic acid and diluted with 0.9% 
NaCl as required. In concentrations below 10 mU per ml, the diluent was 0.9% NaCl
containing 0.2% gelatine (U.S.P.). Dilutions of standard TSH or heparinized plasma
were injected into the tail vein at volumes between 0.1 and 0.4 ml. Blood sampling
was performed without anaesthesia or prewarming, by puncturing the orbital venous

* Laboratory Animal Breeding, Laven, Denmark.
** Ferrosan Ltd., Malmö, Sweden.
plexus (Pettit 1913; Riley 1960) with a 50 μl commercial constriction pipette*** which had been rinsed previously with heparin solution (100 units per ml). The content of the pipette was then plated on a stainless steel planchette and the blood 131I activity measured in a gas flow counter with end window (Nuclear Chicago, model 181B). The assay procedure was begun with the initial blood sampling and followed immediately by the intravenous injection of saline, standard TSH, or plasma. The final blood sampling was performed 120 minutes after the injection. An increase in blood 131I level over that of the controls following the injection of active material was taken as a positive TSH response, which can be expressed in different ways as is shown below. The number of mice per treatment group was never less than 5 and usually more. The blood sample volume (50 μl) was small enough to allow the daily use of the same mice over the five days suitable for assay, without impairment of their general condition. In the studies on 131I elimination from the blood up to 20 blood samples were taken at daily intervals from the same animals. The differences between the method described and that of McKenzie (1958) will appear stepwise under Results. The statistical treatment of the data was performed according to Bliss (1952), Finney (1952), and Gaddum (1958). Homogeneity of variances within an experiment was checked using the χ2-test of Bartlett (1937), in which

$$\chi^2 = 2.3026 (k-1) \left( n \cdot \log \bar{s}^2 - S \log s^2 \right) (1/C),$$

where $k =$ the number of observations per groups, $n =$ the number of groups, and

$C = 1 + \frac{n + 1}{3n(k-1)}$ (Snedecor 1946).

**RESULTS**

**Time concentration pattern of blood 131I in normal and thyroxine treated mice**

The elimination of 131I in mice followed, after a rapid fall during the first 24 hours, a first order reaction with an estimated biological half life of 117 hours (about 5 days). In thyroxine treated mice (20 μg) the initial sharp fall in blood 131I level continued to rather low values which remained low for three days. On the fourth day the blood 131I increased above the controls, and was subsequently cleared in parallel to the latter, with a calculated biological half life of 123 hours. When an additional 20 μg of thyroxine was given three

*** Bie & Berntsen, Copenhagen.
days after the first injection, the low blood $^{131}$I level was maintained for three additional days, after which time it increased above that of the controls and that of mice which had received a single thyroxine injection, and was subsequently eliminated in parallel to the other two groups, with a calculated biological half life of 122 hours (Fig. 1).

The differences between the calculated half lives were not significant. The mean body weight of the mice increased from 21.8 to 24.0 g during the 24 days of handling and blood withdrawal. Four repetitions of the experiment described in Fig. 1 and yielding 900 additional observations, fully confirmed the result.

**Number of days suitable for TSH assay**

According to Fig. 1 there was an efficient blockade of endogenous TSH secretion for six days in mice treated twice with 20 $\mu$g of l-thyroxine. Sufficiently stable blood $^{131}$I levels were present from day 2 to day 6, during which time the sensitivity of the mice to TSH and the regression of the TSH response on dose were investigated in four separate experiments of the following design: 30 mice pretreated with $^{131}$I and thyroxine (see method) were marked individually, divided into 5 groups of 6 mice each, and used daily for 5 days (day 2–6) in the TSH assay procedure. The doses were: 0.9 % NaCl,

![Graph](image)

*Fig. 1.*

Elimination pattern of $^{131}$I from mouse blood. Abscissa: Time in days. Ordinate: Blood $^{131}$I level, expressed in cpm in 50 $\mu$l. $^{131}$I dose: 8 $\mu$c, given intraperitoneally.

○ = controls; × = mice receiving 20 $\mu$g of l-thyroxine at day 0; • = mice receiving 20 $\mu$g of l-thyroxine at day 0 and day 3 (subcutaneously). 6 mice per point.
0.04, 0.12, 0.36, and 1.08 mU of TSH per mouse, respectively. The response was preliminarily expressed according to McKenzie (1958) as
\[
\frac{\text{final cpm}}{\text{initial cpm}} \times 100.
\]
The arrangement of doses, animal groups, and the mean responses obtained on the five consecutive days is given in Table 2.

Table 2.

Design (A) and mean responses (B) for estimating log dose response dependence in TSH bioassay using the same individuals on 5 consecutive days.

A.
Arrangement of doses for 5 groups of 6 mice each. \(S_1 = 0.04\), \(S_2 = 0.12\), \(S_3 = 0.36\), and \(S_4 = 1.08\) mU of TSH per mouse. The three pairs of triplets put into rectangles were used for testing the efficiency of a cross-over design.

<table>
<thead>
<tr>
<th>Day</th>
<th>Mouse group</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>NaCl</td>
</tr>
<tr>
<td>3</td>
<td>NaCl</td>
</tr>
<tr>
<td>4</td>
<td>Dose</td>
</tr>
<tr>
<td>5</td>
<td>NaCl</td>
</tr>
<tr>
<td>6</td>
<td>NaCl</td>
</tr>
</tbody>
</table>

B.
Mean responses, preliminary expressed as \(\frac{\text{final cpm}}{\text{initial cpm}} \times 100\) (McKenzie 1958), listed below according to dose. Mean body weight of the mice and test for variance heterogeneity included at right.

<table>
<thead>
<tr>
<th>Day</th>
<th>NaCl</th>
<th>Dose</th>
<th>Mean body weight</th>
<th>Heterogeneity of variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>88</td>
<td>147</td>
<td>366</td>
<td>535</td>
</tr>
<tr>
<td>3</td>
<td>94</td>
<td>134</td>
<td>320</td>
<td>617</td>
</tr>
<tr>
<td>4</td>
<td>95</td>
<td>139</td>
<td>317</td>
<td>402</td>
</tr>
<tr>
<td>5</td>
<td>117</td>
<td>219</td>
<td>237</td>
<td>309</td>
</tr>
<tr>
<td>6</td>
<td>90</td>
<td>173</td>
<td>235</td>
<td>347</td>
</tr>
</tbody>
</table>

Total number of responses: 150.
Test for heterogeneity of variances from the total of responses: \(\chi^2 = 82.4; P < 0.001\).
Fig. 2 shows the relationship of percentage final cpm over initial cpm to logdose.

From Table 2 and Fig. 2 it is seen that on all 5 days there was a clear monotonous logdose response dependence and a higher mean response ($P < 0.001$) at the 0.04 mU dose level than after saline injection. On the first assay day (day 2), the control responses were clearly below 100 ($P < 0.01$), and there was a slightly decreased response at the higher dose levels with time. Statistical analysis showed that the variance of the response increased significantly with increasing mean response on all days. Departure from linearity of the logdose response line could not, under these conditions, be tested in a valid way. The *upwards concave* shape of the logdose response line, as well as significant heteroscedasticity, however, were observed in each of the three repetitions of the above experiment yielding an additional 450 observations. The elimination of this bias is dealt with below.

**Omission of pretreatment with low-iodine diet and thyroid powder feeding**

At the beginning of this investigation the mice were pretreated with a low-iodine diet and thyroid powder was fed during the assay period according to
McKenzie (1958). The original regimen was then changed stepwise with regard to one single component as follows:

1) Replacement of low-iodine diet by normal diet, to which no salt or fish-meal had been added.

2) Replacement of thyroid powder feeding by subcutaneous l-thyroxine injection twice at double dosage (see method).

3) Replacement of the salt- and fish-meal-free diet by normal standard diet. Before and after each of the above changes careful investigations were performed with regard to $^{131}$I elimination, sensitivity to TSH, and regression of the response on dose.

Comparison of the results obtained from at least 130 mice for each change in design (a total of about 520 mice), showed no difference with regard to elimination pattern, sensitivity, and regression. These findings led to the abandonment of low-iodine diet pretreatment and thyroid powder feeding.

**Blood sampling improvement**

a) **Sampling speed**

As mentioned by McKenzie (1962) to one of us (C.R.) blood sampling by means of puncture of the orbital venous plexus appeared to have advantages. This sampling technique, described as early as 1913 (Pettit), proved of great value for the performance of larger serial analyses. Commercial constriction pipettes were found to be suitable, enabling the operator to obtain the desired blood sample (50–200 µl) within a few seconds without after-bleeding and from mice not necessarily prewarmed. Sampling plus intravenous injection of test material is easily performed within 90 seconds, so that 50–60 TSH responses can conveniently be obtained in a single experiment.

b) **Sampling accuracy**

The importance of sampling accuracy appears from Fig. 3, which shows that the blood $^{131}$I measurement is dependent on the blood volume delivered to the sample pan, and that no stage of »infinite thickness« of the sample layer had been obtained in a sample volume up to 1500 µl.

Sampling accuracy was higher when pipettes were used instead of syringes as shown in Table 3.

c) **Sampling volume reduction**

The originally used $2 \times 0.1$ ml of blood represent a blood loss of about 10 per cent in a 20 g mouse. A possible multiple use of the same animals from day to day necessitated a reduction in blood sample volume. Sampling of 50 instead of 100 µl was studied in three experiments (45 mice), in which 50 and 100 µl were taken almost simultaneously from the same mice, saline or TSH solution injected, and two hours later 50 and 100 µl taken again. Analysis of the response (Table 4) showed excellent correlation and equal precision was obtained from 50 µl and 100 µl samples.
Working with a sample volume of 50 µl it was possible to use the mice daily for 5 days (see also Fig. 2). Initially there was a slight decrease in their body weight (about 0.5 g). On the last day of assay, however, a net increase in body weight was usually observed (Table 2).

**Regression of the response on dose, validity of assay**

For reasons given below the following response parameters were investigated over a dose range between 0.025 and 1.08 mU of TSH with regard to homoscedasticity and linear regression on dose or logdose:

- \( y_1: \frac{\text{final cpm}}{\text{initial cpm}} \times 100 \) (McKenzie 1958)
- \( y_2: \log \text{final cpm} \)
- \( y_3: \log \text{final cpm} - \log \text{initial cpm} + 2 (= \log y_1) \)
- \( y_4: \log \text{final cpm} \) covariance adjusted for \( \log \text{initial cpm} \) (Sakiz & Guillemin 1964).

800 measurements at three or four dose levels were analyzed in order to find valid and optimal assay conditions. It was found that none of the above parameters yielded valid conditions with absolute dose. When logdose was used...
Table 3.
Assessment of sampling error in a) sampling by means of a syringe and b) sampling by means of a pipette. From a heparinized blood pool containing $^{131}$I 10 replicate samples were taken with a 0.5 ml tuberculin syringe and constriction pipettes, respectively. Sample volumes: 50 and 100 µl.

<table>
<thead>
<tr>
<th>Blood $^{131}$I, cpm</th>
<th>Syringe sampling</th>
<th>Pipette sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>121</td>
<td>199</td>
<td>114</td>
</tr>
<tr>
<td>108</td>
<td>192</td>
<td>114</td>
</tr>
<tr>
<td>112</td>
<td>204</td>
<td>114</td>
</tr>
<tr>
<td>104</td>
<td>187</td>
<td>119</td>
</tr>
<tr>
<td>114</td>
<td>187</td>
<td>112</td>
</tr>
<tr>
<td>106</td>
<td>196</td>
<td>110</td>
</tr>
<tr>
<td>106</td>
<td>179</td>
<td>111</td>
</tr>
<tr>
<td>106</td>
<td>184</td>
<td>115</td>
</tr>
<tr>
<td>102</td>
<td>182</td>
<td>110</td>
</tr>
<tr>
<td>110</td>
<td>189</td>
<td>112</td>
</tr>
<tr>
<td>Mean cpm</td>
<td>109</td>
<td>190</td>
</tr>
<tr>
<td>Standard deviation$^a$</td>
<td>± 5.59</td>
<td>± 7.87</td>
</tr>
<tr>
<td>Per cent error</td>
<td>5.1</td>
<td>4.1</td>
</tr>
</tbody>
</table>

$^a$) of single observation.

$y_1$ was associated with the previous mentioned curvilinear regression (upwards concave) together with highly significant heteroscedasticity (Fig. 2, Table 2). Working with three dose levels and a design in which the doses were distributed so that each mouse received each of the doses at one occasion (Table 5) we obtained the same result (Fig. 4).

In this situation the logarithmic transformation was indicated for two reasons: 1) It would tend to straighten the regression line, and 2) it would diminish or eliminate the heterogeneity of the variance about the line. When the three chosen transformations $y_2$, $y_3$, and $y_4$ were tried it was found that the pronounced departure from linearity of the regression line had disappeared, that the conditions of variance homogeneity were fulfilled, but that the precision of the line varied depending on which transformation was chosen. The metamerter $y_2$ (log final cpm) gave a low precision (mean index of precision, $\lambda = 0.52$) pointing to the importance of a correction for initial blood $^{131}$I level, whereas $y_3$ and $y_4$ were about equivalent, yielding an average index of precision ($\lambda$) of 0.23. The effect of the logarithmic transformation of $y_1$, originally used in Fig. 2 and Fig. 4 is given in Fig. 5 a, b.

186
Table 4.
Correlation of responses from 100 µl and 50 µl blood samples, respectively, in the in vivo $^{131}$I release method of TSH assay. Responses preliminary expressed as $\frac{\text{final cpm}}{\text{initial cpm}} \times 100$ (McKenzie 1958).

**Experiment A**

<table>
<thead>
<tr>
<th>Dose, mU TSH</th>
<th>Mouse No.</th>
<th>Response obtained from 100 µl sample</th>
<th>50 µl sample</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>1</td>
<td>93.9</td>
<td>91.1</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>80.2</td>
<td>81.8</td>
<td>-1.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>127.2</td>
<td>134.6</td>
<td>-7.4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>91.9</td>
<td>109.1</td>
<td>-17.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>97.5</td>
<td>106.4</td>
<td>-8.9</td>
</tr>
<tr>
<td>Mean</td>
<td>104.6</td>
<td>98.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

|                | 6         | 113.6                                | 103.2        | 10.4       |
|                | 7         | 176.3                                | 147.7        | 28.6       |
| 0.04           | 8         | 167.6                                | 174.0        | -6.4       |
|                | 9         | 170.9                                | 169.4        | 1.5        |
|                | 10        | 142.4                                | 152.0        | -9.6       |
| Mean           | 149.3     | 154.2                                |              |            |

|                | 11        | 259.2                                | 278.8        | -19.6      |
|                | 12        | 313.2                                | 304.7        | 8.5        |
| 0.12           | 13        | 270.5                                | 250.0        | 20.5       |
|                | 14        | 295.7                                | 290.4        | 5.3        |
|                | 15        | 265.0                                | 273.5        | -8.5       |
| Mean           | 279.5     | 280.7                                |              |            |

**Total** 2665.1 2666.7 - 1.6  
**Mean difference in response** - 0.1  
**Correlation coefficient, $r = 0.987$; $P < 0.001$**

Table 5.
Experimental design for testing the regression of the response on logdose using three point determinations on each of 4 consecutive days in the same individuals. $S_1 = 0.025$, $S_2 = 0.10$, and $S_3 = 0.40$ µu of TSH.

<table>
<thead>
<tr>
<th>Day</th>
<th>Mouse group and dose</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>NaCl</td>
<td>$S_1$</td>
<td>$S_2$</td>
<td>$S_3$</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>$S_3$ NaCl</td>
<td>$S_1$</td>
<td>$S_2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>$S_2$ $S_3$ NaCl</td>
<td>$S_1$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>$S_1$ $S_2$ $S_3$</td>
<td>NaCl</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Graph showing mean log dose response line using three dose levels and the design given in Table 5.

The response parameter $y_3$ was chosen for further work because it is easy to calculate and because parameter $y_4$ was frequently associated with a high variation of the regression of log final cpm on the concomitant variate (log initial cpm). A frequent tendency of the covariance regression coefficient to decrease with increasing response sometimes precluded the proper use of covariance analysis as shown in Table 6.

**Specificity of the response**

We compared the effect of plasma from normal and hypophysectomized rats with saline. As can be seen from Fig. 6 the effect of plasma from hypophysectomized rats was indistinguishable from the effect of saline, whereas normal rat plasma and standard TSH gave the usual response.

Corticotrophin (synthetic tetraicosapeptide*), lysine vasopressin (synthetic**), histamine, insulin, oxytocin, and noradrenaline, all given at higher than maximal physiological doses, failed to evoke a positive response.

---

* Ciba Ltd., Basle, Switzerland.
** Sandoz Ltd., Basle, Switzerland.
Effect of logarithmic transformation of McKenzie's (1958) original response parameter $y_1$ (upper curve) to $\log y_1 = y_3$: Greatly reduced curvature and heteroscedasticity (lower curve).

a) applied to the data of Table 2 and Fig. 2,
b) applied to the data of Fig. 4.

Fig. 5.
Table 6.
Analysis of the experiment from Fig. 4 and 5b with regard to the covariance regression at different dose levels (or response levels). Independent variate, $v = \log$ initial cpm.
Dependent variate, $y = \log$ final cpm. 72 observations.

<table>
<thead>
<tr>
<th>Day</th>
<th>Covariance regression coefficient $b = [vy]/[v^2]$ at dose level</th>
<th>Difference due to</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$S_1$</td>
<td>$S_2$</td>
<td>$S_3$</td>
<td>Days</td>
</tr>
<tr>
<td>3</td>
<td>1.1305</td>
<td>0.8388</td>
<td>-0.0523</td>
<td>1.835</td>
</tr>
<tr>
<td>4</td>
<td>1.2344</td>
<td>0.5802</td>
<td>0.0240</td>
<td>22.44</td>
</tr>
<tr>
<td>5</td>
<td>0.8219</td>
<td>0.0918</td>
<td>0.0222</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.7281</td>
<td>0.4237</td>
<td>0.0682</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.9787</td>
<td>0.4836</td>
<td>0.0155</td>
<td></td>
</tr>
</tbody>
</table>

* Covariance analysis biased.

Fig. 6.
Graph showing specificity of the thyroxine blocked mouse for TSH in rat plasma.

**Precision and sensitivity**

Following transformation of parameter $y_1$ of the data in Table 2 into $y_3$ the five equations of the logdose response lines yielded a mean index of precision
of 0.229 and a mean threshold dose of 0.017 mU TSH (the latter being obtained by solving the equation for \( y = 2.00 \)). The mean index of precision in cross-over tests was clearly more favourable than in the usual assay design (Table 7).

Table 7.
Some relevant data from the statistical analysis of the experiment from Table 2 (150 responses). Original response transformed to its logarithm.

<table>
<thead>
<tr>
<th>Day</th>
<th>Slope of line ( b )</th>
<th>Variance of response ( s^2 )</th>
<th>Index of precision ( \frac{s}{b} = \lambda )</th>
<th>( L = \frac{b}{s} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.527</td>
<td>0.0174</td>
<td>0.250</td>
<td>4.00</td>
</tr>
<tr>
<td>3</td>
<td>0.669</td>
<td>0.0170</td>
<td>0.195</td>
<td>5.13</td>
</tr>
<tr>
<td>4</td>
<td>0.475</td>
<td>0.0118</td>
<td>0.229</td>
<td>4.37</td>
</tr>
<tr>
<td>5</td>
<td>0.278</td>
<td>0.0057</td>
<td>0.270</td>
<td>3.70</td>
</tr>
<tr>
<td>6</td>
<td>0.434</td>
<td>0.0089</td>
<td>0.217</td>
<td>4.61</td>
</tr>
</tbody>
</table>

No significant departure from linearity at any day.
No significant heteroscedasticity at any day.
Mean index of precision (reciprocal of mean \( L \)) = 0.229.
Weighted mean threshold dose = 0.0165 mU TSH.

Efficiency of cross-over principle

<table>
<thead>
<tr>
<th>Days</th>
<th>between mice variance</th>
<th>within mice variance ( \frac{2.37}{1.75} )</th>
<th>Index of precision in cross-over test ( \frac{0.142}{0.159} )</th>
<th>( L )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 – 3</td>
<td>2.37</td>
<td>1.75</td>
<td>0.142</td>
<td>7.04</td>
</tr>
<tr>
<td>3 – 4</td>
<td>1.75</td>
<td>2.42</td>
<td>0.159</td>
<td>6.29</td>
</tr>
<tr>
<td>5 – 6</td>
<td>2.42</td>
<td>2.42</td>
<td>0.154</td>
<td>6.494</td>
</tr>
</tbody>
</table>

Mean index of precision (reciprocal of mean \( L \)) = 0.151.

Plasma TSH, preliminary findings in man, rat, and mouse

Plasma from normal adult men or women failed to give positive responses indicating that the level of TSH in the normal adult human is less than 0.05 mU per ml. Elevated plasma TSH was found most frequently in patients with myxoedema. The highest level observed was 0.9 mU per ml from a patient with malignant exophthalmos.

In Sprague-Dawley rats the mean plasma TSH level was 0.4 mU per ml. The variation between individual rats was, however, very large and in exceptional cases we did not obtain a positive response at all from 0.4 ml of
undiluted plasma. According to expectation treatment with propylthiouracil (PTU) was invariably followed by an increase, and treatment with thyroxine, by a complete disappearance of plasma TSH.

Plasma from normal mice did not evoke a positive response indicating that their plasma TSH level is lower than in the rat. PTU treatment for 5 weeks (0.1 per cent in the drinking water) led to high concentrations, i.e. 4.9 mU per ml, which is in accordance with blood transfusion experiments in mice (Rerup 1965).

DISCUSSION

The biological half life of $^{131}$I has been estimated to be 5.5 days in the rat (Mayberry & Astwood 1961) which in view of our results in mice, indicates a similar rate of iodine elimination in both species. The above results (Fig. 1) indicate further that during the stage of inhibited endogenous TSH release by exogenous thyroxine, iodine is «immobilized» in the thyroid and thus preserved from elimination, since following cessation of thyroxine action, it was cleared at a higher level than in the controls.

It is interesting to note that one week of low-iodine diet pretreatment was completely unnecessary under our working conditions. We do not, however, recommend an uncontrolled abandonment of this pretreatment, especially if the diet of the breeder or the normal laboratory content diet have a relatively high iodine content.

The replacement of thyroid powder feeding by thyroxine injection is in our hands an advantage for several reasons: 1) It is more convenient to deal with a single diet only. 2) The general condition of the animals was better with injection treatment. Thyroid powder feeding often led to symptoms of overdosage such as restlessness, loss in body weight, and profuse sweating. Moreover, since it is not known with certainty how complete the endogenous TSH release is blocked by thyroid hormone, we feel that injection treatment involving exact dosage gives a more homogenous degree of TSH release inhibition.

The number of mice to be pretreated with $^{131}$I and I-thyroxine is dependent on whether TSH in plasma or from extracts (standard TSH etc.) is to be assayed. In the latter case, one animal can be used at least five times and probably more under continued thyroxine treatment, unless the initial blood $^{131}$I level becomes too low to be measured accurately. When rat plasma and particularly human plasma were assayed, the general condition of the mice deteriorated so that we did not use them more than once. Haematuria was normally observed after the injection of 0.4 ml of human plasma.

The above results clearly show the necessity of metametric transformation of
the original parameter. The square root transformation, also tried by Sakiz & Guillemin (1964), was less efficient in diminishing heteroscedasticity than the log transformation. Since the variance of the original response \( (y_1) \) was not linearly related to the response itself, at least two components responsible for the observed variance heterogeneity can be distinguished: 1) The increase of the variance proportional to the increase in mean response due to the Poisson distribution of radioactivity measurement; 2) an additional mechanism enhancing variability of the response with increasing TSH doses, most probably a tolerance distribution. For this reason it seemed a priori unlikely that a total elimination of heteroscedasticity will be obtained by using the logtransformed response. In fact, usually a tendency towards variance heterogeneity remained after transformation (Fig. 5b), which was, however, never significant. The frequently observed phenomenon (Table 6) of decreasing regression of log final cpm on log initial cpm with increasing dose (or response) points to the need of checking the regression coefficients in the covariance analysis separately. If they differ significantly, covariance analysis is not appropriate. Sakiz & Guillemin (1964) have not observed heterogeneity of slopes in the covariance analysis as was found here, but these authors mainly worked with two dose levels (the logdose interval was not mentioned), which precludes direct comparison of the findings. It appears surprising that the above authors in a paper on metameric transformation did not consider the question of linearity or non-linearity of the logdose response line, though it is as important for assay validity as homoscedasticity. The findings of this investigation do not leave any doubt about the curvature of the original logdose response line, so that the logtransformation eliminated two phenomena of assay validity concurrently.

The assay procedure is one of the most simple known in bioassay: Sampling and plating is followed immediately by intravenous injection, and two hours later the final sampling and plating is performed. No biochemical analyses are involved and the computational work compares favourably with that of other methods.

ACKNOWLEDGEMENT

Grateful acknowledgement is made to Miss Siw Erman for her skilful technical assistance.

REFERENCES

Bottari P. M. & Donovan B. T.: J. Physiol. (Lond.) 140 (1958) 36P.
Rerup C.: Acta endocr. (Kbh.) 49 (1965) 159.

Received on March 22nd, 1965.