ation from EtOH it melted at 142~143°. *Anal.* Calcd. for $C_{11}H_{12}N_2$: C, 83.6; H, 4.92. Found: C, 83.47; H, 5.25.

Summary

(1) Condensation of the type of malonic ester synthesis between halogen compounds of aromatic heterocyclic series and active methylene compounds was examined. The success of the reaction in this field depends both upon the reactivity of halogen and that of methylene. The weaker the reactivity of halogen, the more active should be the methylene compounds.

(2) Two sets of sequence of reactivity, one for halogen compounds and the other for reactive methylene compounds, were obtained.

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52. Yosoji Ito, Bun-ichi Tamaoki, and Kunio Kurata: Studies on Insulin Assay. II. An Application of the Up-and-Down Method for Insulin Assay.

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Since the principle of the "Up-and-Down Method" was introduced in testing the sensitivity of explosives to shock[1], the applicability of this method for biological assay has been suggested by statisticians[1~4]. However, in these papers[1,2], they exemplified only the artificial data in order to introduce the computation. Recently, the original up-and-down method was ingeniously improved[5], and after the statistical examination[5], this method was found to have the following remarkable advantages theoretically as well as practically in comparison to the standard probit method which has been regarded as a routine procedure for the assessment of ED_{50}.

1) Arithmetical easiness, 2) better efficiency, and 3) constant existence of estimate even in small samples.

On the other hand, this up-and-down method has the disadvantage, in general, for biological assay which is intrinsic to this method, even though it is greatly improved by the ingenious modification[5], i. e., the condition of sequentiality, or, the response to the preceding test must be known, before each trial is started[1,5]. It is the purpose of this paper to apply this method practically to the estimation of ED_{50} of insulin, to compare the results obtained by this method with the ones by the probit analysis from the same data, and to discuss the biological significance of the ED_{50} estimated by this method.

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Experimental Methods and Materials[6]

1) Experimental Animals—Common white mice (male) weighing between 15 and 20 g.

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were used. 2) Colony Diet—Animals received wheat, barley, rice, dried fish meat, and green vegetable, *ad libitum*. 3) Fasting Period—Before the injection of insulin, animals were fasted for 2 hours. 4) Treatment of Mice during the Test—After the injection of insulin, animals were transferred into the incubater with air vents which was thermostatically controlled at 40°±0.5°. Or, in the sloped screen procedure[7,8], they were transferred onto a screen sloped at an angle of 60° at temperature of 20°±1°. 5) Determination of Animal Response—Both convulsion and collapse caused by insulin were used as criteria of response, while, in the sloped screen procedure, it was determined as being positive when the mice on the screen fell to the ground owing to the convulsion or collapse during the testing period after the injection. The others were considered to be negative. 6) Testing Period—We had already confirmed the normal distribution of the frequency of response to the time after the insulin injection at 30°[6], but as, in the up-and-down method, it is especially desirable to shorten the testing period, the period was determined as one hour, and for the compensation of this, the temperature during the test was raised up to 40°, while, in the sloped screen procedure, it was decided as one and half hours after the consideration of our previous experiments[9]. 7) Insulin, its Solvent, and Administration —In this experiment, the insulin powder, the potency of which was estimated as being 15.2 units per mg. according to the Japanese Pharmacopoeia VI was used. The solution of insulin in this experimentation was prepared by dissolving it into the solvent[10] so as to be 1 unit per cc. This solution was administered subcutaneously on the back of mice by the Micro-Meter Syringe*.

Experimental Results

Experiment I Application of the Up-And-Down Method for the Estimation of ED_{50} of Insulin. After the injection of insulin, animals were transferred to the incubater, and were observed for one hour.

Table I. Summarized Result of Experiment I

i	n_i		in_i	i^2n_i	$(i+1)^2n_i$
	+	−			
3	10	0	0	0	0
2	12	9	18	36	81
1	2	11	11	11	44
0	0	1	0	0	1
Total	24	$21=N$	$29=A$	$47=B$	$126=C$

Generally, N, A, B and C are respectively denoted as

$$N=\sum n_i,\quad A=\sum in_i,\quad B=\sum i^2n_i \text{ and } C=\sum(i+1)^2n_i$$

where the smaller one of the two totals (+) and (−) should be chosen as N[1].

Thus, m, the estimate of the ED_{50} of the insulin in the metametric scale is calculated as follows :

$$m=y'+d\left(\frac{A}{N}\pm\frac{1}{2}\right)$$

where y' is the lowest dose metameter on which the less frequent event occurs, d is the interval between the successive dose metameters, and the plus sign is used when the

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* This instrument "Agla" was made by Burroughs and Welcome & Co. Ltd. of England and its accuracy is said to be ±0.00005.
** Accordingly, $C=\sum(i+1)^2n_i=\sum n_i+2\sum in_i+\sum i^2n_i=N+2A+B$
Thus the above equation is convenient to check the calculation in Table I[4], for example, in this analysis, $N+2A+B=21+2\times29+47=126\rightarrow C$

analysis is based on the negative response, while the minus one is used when it is based on the positive response. Therefore, in the case of this experiment, $y'=0$, $d=1$, and the plus sign was used as follows:

$$m=0+1\times\left(\frac{29}{21}+\frac{1}{2}\right)=1.881$$

The sample standard deviation, s, is computed as

$$s=1.620\,d\left(\frac{NB-A^2}{N^2}+0.029\right)$$
$$=1.620\times1\times\left(\frac{21\times47-29^2}{21^2}+0.029\right)$$
$$=0.583$$

In this computation, the estimate of σ or s is quite an accurate approximation, as $(NB-A^2)/N^2$ is more than 0.3[1,2]. Therefore, the standard error of m, s_m which is dependent not only on σ but also on the ratio d/σ is computed as

$$s_m=Gs/\sqrt{N}$$
$$=(1.1\times0.583)/\sqrt{21}$$
$$=0.140$$

where the coefficient G* in the above equation which depends on d/σ and the position of the mean relative to the interval can be approximately read from the graph[1]. Here, as the ratio of d to s is 1/0.58 or 1.72, the G can be obtained as 1.1 from the graph. Therefore, the fiducial limits were approximately computed as follows:

$$m\pm ts_m=1.881\pm1.96\times0.140$$
$$=1.607,\ 2.155$$

Accordingly, the ED_{50} of the insulin and its fiducial limits (P=0.95) were obtained as 9.2×10^{-3} cc., and, 7.6 and 11.1×10^{-3} cc. of the insulin solution (1 unit per cc.).

Experiment II Another Application of the Up-And-Down Method for the Longer Series of Trials. Experiment II was preformed separately under the same conditions as stated in Experiment I.

Table II. Summarized Result of Experiment II

i		4	3	2	1	0	Total
n_i	+	1	7	23	5	0	36
	−	0	1	7	22	5	35

By the same computing technique as stated in Expt. I, the ED_{50} and its fiducial limits were estimated as follows:

$m=1.614$
$(NB-A^2)/N^2=0.44$
$s=0.766$
$d/s=1.31$
$G=1.03$
$s_m=0.1334$

$m\pm ts_m=1.614\pm1.96\times0.133$
$=1.353,\ 1.875$
$ED_{50}=7.7\times10^{-3}$ cc.
Its Fiducial Limits; 6.4, 9.2×10^{-3} cc.

Combination of the Estimates obtained from Expts. I and II

	j	m_j	s_j	$1/s_j^2=w_j$
Expt. I	1	1.881	0.140	51.02
Expt. II	2	1.614	0.133	56.53

The combined estimate, m_c, can be computed as

$$m_c=\frac{w_1\times m_1+w_2\times m_2}{w_1+w_2}=1.740$$
$$\chi_0^2=\sum w_j m_j^2-(\sum w_j m_j)^2/Zw_j$$
$$=1.91$$

As the above χ^2-test shows, there is no evidence of the heterogeneity of these two

* Alternatively, G is approximately computed by the following formula[2]
$G=(0.9+d/8\sigma)$ where $0.2\leq d/\sigma\leq2.4$

estimates. Thus, the combined ED_{50} and its fiducial limits were approximately calculated as

$$m \pm t\sqrt{V(m_c)} = 1.740 \pm 0.182 = 1.562,\ 1.940$$

, where $V(m_c) = \dfrac{1}{w_1 + w_2} = 0.0964^2$

$ED_{50} = 8.4 \times 10^{-3}$ cc., Its Fiducial Limits; 7.4, 9.6×10^{-3} cc.

Estimation of the ED_{50} from the Combined Results On the assumption that Expt. II had been performed successively after Expt. I*, the estimate of ED_{50} of the insulin might be computed from the combined data of Expts. I and II, instead of the combination of the two estimates as stated above.

Table III. Summarized Result of Experiments I and II

i		4	3	2	1	0	Total
n_i	+	1	17	35	7	0	60
	−	0	1	16	33	6	56

$m = 1.714$ $s_m = 0.1016$

$(NB - A^2)/N^2 = 0.4184$ $m \pm ts_m = 1.714 \pm 1.96 \times 0.1016$

$s = 0.7241$ $= 1.515,\ 1.913$

$d/s = 1.38$ $ED_{50} = 8.2 \times 10^{-3}$ cc.

$G = 1.05$ Its Fiducial Limits; 7.2, 9.4×10^{-3} cc.

Application of the Probit Analysis for the Combined Result The principle of the probit analysis was applied for the same data as shown in Table III for the estimation of ED_{50} of the insulin as follows :

Table IV. Probit Analysis of the Combined Result of Experiments I and II

Dose-metameter x	n	(+) r	r/n p	Emp. probit	Exp. probit Y	Work. probit y	Weight. coefficient w
4	1	1	1.000	—	7.9	8.213	0.0190
3	18	17	0.944	6.589	6.7	6.536	0.2077
2	51	35	0.686	5.485	5.4	5.467	0.6005
1	40	7	0.175	4.065	4.1	4.085	0.4714
0	6	0	0	—	2.9	2.494	0.1103

$\chi^2 = Snwy^2 - S^2nwxy/Snwx^2$
$= 0.384$

$b = Snwxy/Snwx = 1.319$

$g = \dfrac{t^2}{b^2 Snwx^2} = 0.108$

$ED_{50} = 8.0 \times 10^{-3}$ cc.

Its Fiducial Limits; 6.9, 9.3×10^{-3} cc.

Experiment III. Application of the Up-And-Down Method for the Sloped Screen Procedure in the Insulin Assay In this experiment, animals were transferred onto the sloped screen as previously stated. The experimental data were summarized as shown in Table V and were respectively analyzed by the two methods.

Table V. Summarized Result of Experiment III

i		4	3	2	1	0	Total
n_i	+	4	16	17	6	0	43
	−	0	4	15	16	6	41

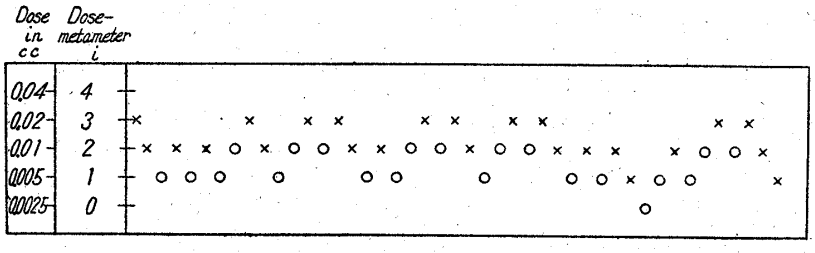

Fig. 1.
Results of 45 Tests

* In fact, there were 2 days' interval between Expts. I and II.

1) The estimate of the ED_{50} could be obtained using the same technique as stated in Expts. I and II.

$m=1.915$, $s=1.231$, $d/s=0.812$, $G=0.98$, $s_m=0.188$

Then, the ED_{50} and its fiducial limits could be estimated as

9.4×10^{-3} cc. and 7.4, 12.2×10^{-3} cc.

2) For the same data, the probit analysis[2] was also applied, and the ED_{50} was estimated as follows;

$\chi_0^2=0.3102$, $b=0.8479$, $g=0.145$

$ED_{50}=9.0\times10^{-3}$ cc.

Its Fiducial Limits; 6.9, 12.2×10^{-3} cc.

Discussion

The up-and-down method had not yet been used in the field of pharmacology and toxicology, despite the fact that this method has some remarkable theoretical and practical advantages in comparison to the standard probit method. Besides both the advantage and disadvantage of this method mentioned in the paper[1,5], it is practically necessary to consider the biological response in detail which is going to be used in the assay, such as the type of the response, the testing period, the preparation before the test, etc., because these characteristics become very important, sometimes decisive, factors for the choice of the method. In this paper, this method was applied for the estimation of ED_{50} of insulin, though this assay method required considerably long time. It is most desirable for the up-and-down method that each trial needs a short testing period, such as the explosive to shock which was originally used in this method[1].

As the up-and-down method has already been discussed statistically,[1,2,5] the biological significance of the ED_{50} estimated by the method will be discussed here, comparing with the one by the standard technique of the probit method. In the up-and-down method, the response of n animals to the stimulus has been sequentially observed for the longer period, say, $a+np$*, when a and p are respectively the time for preparation before injection and the testing period after injection for a single trial, while, in the standard probit method, all animals are simultaneously observed just for the period $a+p$. Though it is not desirable to continue the experiment for a long period, the estimate obtained by this method naturally includes the different meaning from the one by the routine probit method done for the shorter period. In the experiment for the estimation of ED_{50} by the standard probit method, it is not possible to get any information whether this process is stationary or not, but in the up-and-down method, it is quite possible to do so when several sets of experimental series are simultaneously paralleled. Expressed in another way, the small variation of the estimate by the original up-and-down method includes not only the small variation of the individual sensitivity under the experimental conditions but also the stationary process of the sensitivity, at least, for the period.

In this paper, both the computing techniques of the up-and-down method and of the probit analysis were used to estimate the ED_{50} for the same results, but it is not right to discuss the two just from the results calculated by them. Because, the reason why we could concentrate almost all animals around the ED_{50} is not only the fact that our guess for ED_{50} is right but also that we actually used the up-and-down procedure as our experimental scheme. Nevertheless, it could be mentioned that the estimates by

* In the case when the preparation before test needs longer time than the test does, such as the experiment in this paper, this statement is at least true, but under the condition that the preparation is not necessary, the period would probably be a $2np/3$[5]. This difference, however, is not important for further discussion here.

the up-and-down method are approximate but closely similiar to the one by the probit analysis, and the computation in the former is, in fact, far less laborious than the one in the latter.

Therefore, the up-and-down method can be recommended as a pilot test[5], when the ED_{50} of the stimulus in question is entirely unknown, and each test does not need long periods. Thus, after sufficient experimental data are obtained, the way to analyse the results is just up to the experimenter's choice, depending on his practical purpose. Thereafter, he may sequentially perform the up-and-down procedure to the pilot test until he will get the desirable fiducial interval, or on the other hand, he may plan the standard probit method for the estimation, based upon the results obtained by the previous pilot test, or he may take the modified up-and-down method by the simultaneous performance of several short up-and-down series[5], if each test needs considerably long period.

Summary

The original up-and-down method was applied for the mouse method of insulin assay and the results were compared to the one analyzed by the probit analysis. The biological significance of ED_{50} obtained by the up-and-down method was discussed in comparison with the one by the standard probit method.

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53. Tyunosin Ukita and Satoshi Nakazawa : Santonin Analogs. III.* On Monocarboxylic Acid obtained from Dihydroalantolactone.

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An interesting observation during the catalytic hydrogenation of dihydroalantolactone (I) was reported in the 1st paper of this series[1]. Thus, although, dihydroisoalantolactone (III) quantitatively yielded tetrahydroalantolactone (II) after consumption of one mole of hydrogen, smaller yield of (II) was obtained in the case of dihydroalantolactone (I) after an exceeded consumption of hydrogen.

This paper describes the isolation and identification of a monocarboxylic acid from a mixture of catalytic hydrogenation products of (I).

The hydrogenation of (I) with both Adams' platinum oxide in glacial acetic acid and 6% palladium-charcoal in ethanol produced a saturated monocarboxylic acid (IV) in the yields of 65.7% and 16.6%, respectively. (II) was the other component in these reaction mixtures.

(IV) was recrystallized from aqueous methanol to give small cubelets, $C_{15}H_{26}O_2$, m.p. 65~67°, $[\alpha]_D^{17}$: +36.1°, and this saturated acid was converted to an acid amide (V), $C_{15}H_{27}ON$, m.p. 138~140°. Methylation of (IV) with diazomethane gave a methyl ester (VI), $C_{16}H_{28}O_2$, $b.p_{0.05}$ 91°, which was reduced to a carbinol (VII), $C_{15}H_{28}O$, $b.p_{0.1}$ 116°, by lithium aluminum hydride. (IV) was recovered again from (VII) by oxidation with dichromate.

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