XCVIII. STABILITY OF THE INTERNATIONAL STANDARD OF THE OESTRUS-PRODUCING HORMONE IN ALCOHOLIC SOLUTION.

BY IDWAL WYN ROWLANDS AND ROBERT KENNETH CALLOW.

From the National Institute for Medical Research, London, N.W. 3.

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THE International Standard of the oestrus-producing hormone consists of the hydroxyketonic compound known in this country as ketohydroxyoestrin or oestrone. Since it is desirable, for some purposes, to prepare and store alcoholic solutions of the standard substance, it has been considered advisable to investigate its stability when kept in this form for various periods and at different temperatures. Butenandt [1930] reported that the stability of oestrone in alcoholic solution was small, a decrease of activity of at least one-third taking place in 2 months.

Technique.

Preparation of alcoholic solution. For biological assay a sample of the International Standard was dissolved in the proportion of 2 mg. in 100 ml. of absolute alcohol (i.e. 0.002 %); for spectrographic examination the concentration was 0.0125 %. Solution in the cold was slow, and was completed by heating to 50° for 10 minutes on a water-bath. The solution was cooled, transferred to dry sterile ampoules of non-alkaline resistance glass and immediately sealed.

Storage. Each set of ampoules was divided into batches. Those for biological assay were stored at -2°, at room temperature in the dark and at 37°, respectively. Those for spectrographic examination were divided into four batches stored (a) at -2°, (b) at room temperature in the dark, (c) at room temperature, in diffused daylight, (d) at 37°. Batches (a) and (d) were occasionally exposed to artificial light but otherwise kept in the dark. The absorption spectra were measured on the instrument described by Philpot and Schuster [1933].

Biological assay. The method of assay consisted in the determination, by means of a dose-response curve, of the dose necessary to cause oestrus in 50 % of a group of ovariectomised mice. It followed closely that employed by Deanesly and Parkes in some work on the suitability of the International Standard, a report of which has been submitted to the Permanent Commission on Biological Standardisation of the League of Nations Health Organisation, but not yet published.

Test animals. 111 immature albino mice about 4 weeks old were ovariectomised. After a period of about one month, control vaginal smears were taken. The mice were divided into four groups, the number in each being shown in Table I. The same group was injected with the same batch of hormone at each test, except in the last test, where four animals from group 1 were removed to group 4. A fortnight before each test all the mice were "primed" with 1 γ of hormone contained in 0.5 ml. 10 % alcohol. The smears were stained in methylene blue. A mixture of nucleated and cornified epithelial cells, or cornified cells alone in the absence of leucocytes, was considered as a positive reaction.

Table I.

Number of mice per group for each test.

Group	Solution injected kept at	For standard curve	Number of animals		
			After 3 months	After 6 months	After 12 months
1	37°	30	27	27	17
2	-2°	28	28	26	20
3	Room temp.	25	23	23	18
4	Control	28	24	18	18

Biological assay.

Preliminary experiment. An experiment on the freshly made alcoholic solution containing 1 mg. of crystalline hormone in 50 ml. absolute alcohol was made to verify the curve obtained previously by Deanesly and Parkes. Four aqueous solutions were made from the alcoholic solution containing known amounts of hormone as shown in Table II.

Table II.

Solution	Volume of alcoholic solution in 50 ml. H ₂ O ml.	Crystalline substance in 0.4 ml. of the aqueous solution γ	Percentage positive reactions
A	1.0	0.16	100
B	0.6	0.096	90
C	0.35	0.056	60
D	0.20	0.032	29

0.4 ml. of each of the solutions A-D was injected subcutaneously over 36 hours into the groups 1-4 shown in Table I. The times of injection were as follows: 1st day, 9.30 a.m., 9.30 p.m.; 2nd day, 9.30 a.m., 9.30 p.m.; and smears were taken: 3rd day, 4 p.m., 11.30 p.m.; 4th day, 9.30 a.m., 4 p.m., 11.30 p.m.; 5th day, 9.30 a.m. The percentage number of positive reactions is shown in Table II and plotted in Fig. 1.

The curve conforms in shape very closely to that obtained by Deanesly and Parkes, but it is displaced somewhat to the left so that the weight giving a 50 % response is 0.047 γ (see Table III) as compared with 0.061 γ for the previous curve.

Stability tests. After 3 months one ampoule from each of the batches kept at different temperatures was opened and from each 0.45 ml. of the alcoholic solution was taken and made up to 50 ml. with distilled water. 0.4 ml., of the aqueous solutions was injected over 36 hours into each mouse, which thus received 0.072 γ . As shown in Table I these aqueous solutions made from the alcoholic solutions kept at 37°, -2° and room temperature were injected into groups 1, 2 and 3 respectively.

From Fig. 1 a 70-80 % response was to be expected from a fresh solution and, allowing for a slight loss in activity during the 3 months, approximately a 50 % response was aimed at. From the 100 % positive result obtained it is not possible to calculate the unit in any of the groups. This high response can only be explained by variation in the sensitivity of the mice, a fact which emphasises the need for a control group in the tests.

After 6 and 12 months the test was repeated. In both instances 0.35 ml. of the original alcoholic solution was made up to 50 ml. with water. A similar

amount of the aqueous solution (0.4 ml.) was injected into the same groups of mice. The amount of hormone injected into each mouse in both of these tests was reduced to 0.056 γ . After 6 months the units for the three batches and the control shown in Table III are uniformly higher than expected. The difference

Fig. 1. International Standard of the oestrus-producing hormone; curve showing percentage positive response of vaginal smears to various doses of the hormone. The weight giving 50 % response is 0.047 γ (0.47 International Unit) and is considered as one mouse unit.

between the units is insignificant. The group receiving the hormone kept at 37° gave the highest response, which further shows that no loss in activity had occurred. On account of this a period of another 6 months was allowed to elapse before making a further test. After 12 months the units for the three solutions are almost identical and show very little difference from the control unit of 0.044 γ . No loss in activity has occurred.

Table III.

Weight of hormone in γ required to cause 50 % response.

Time from start of exp. at which the solutions were tested	Kept in alcoholic solution at			Control solution
	-2°	Room temp.	37°	
—	—	—	—	0.047
3 months	—	—	—	—
6 months	0.067	0.080	0.066	0.077
12 months	0.047	0.046	0.048	0.044

Simultaneously with each test a similar volume of a freshly made alcoholic solution (1 mg. in 50 ml. of absolute alcohol) of the International Standard was injected into group 4 so that each animal received the same amount of hormone. The results, which are shown in Table III, are expressed in terms of the amount of hormone required to induce a 50 % response (1 M.U.), the amounts being calculated from the curve in Fig. 1.

Spectrographic examination.

Fresh solutions of hormone. The absorption spectrum measured in 0.0125 % alcoholic solution was in fair agreement with that recorded by Dingemanse *et al.* [1931] showing a maximum value of $E_{1\text{ cm.}}^{1\%} = 81$ (as compared with 82.5) at $281.5m\mu$, but the small subsidiary hump which they record at $291m\mu$ was, in our measurements, visible only as an inflection in the curve. The curve is plotted in Fig. 2, in terms of

$$\epsilon = \frac{1}{d} \cdot \log_{10} \frac{I_0}{I} - \epsilon_0.$$

Stability measurements. Measurements were taken of the contents of one ampoule from each batch at intervals of 3, 9 and 12 months after the original sealing.

In batches (a), (b) and (c) no change in the absorption outside the range of the experimental error was detected. In batch (d) (kept at 37°) as shown in Fig. 2, there was a slight increase of doubtful significance in the absorption at

Fig. 2. Absorption spectra of solutions of the International Standard of the oestrus-producing hormone in 0.0125 % alcoholic solution.

— Fresh solution.

- - - - Solution kept 12 months at 37° .

the peak at $281.5m\mu$, but at the minimum at $250m\mu$ there was a progressive increase of absorption, amounting in all to a change in the value of ϵ from 0.06 to 0.31. This change is not sufficiently large or characteristic for definite conclusions to be drawn as to its exact cause. It may be due to decomposition of oestrone, but the retention of the shape of the absorption curve as a whole makes it extremely unlikely that, if such decomposition actually occurs, it does so to any considerable extent.

SUMMARY.

Alcoholic solutions of the International Standard of the oestrus-producing hormone stored in sterile sealed ampoules at -2° , room temperature or 37° , for 12 months show neither decline in activity as measured by the vaginal cornification test, nor any change in ultra-violet absorption, with the exception of a small increase in general absorption when stored at 37° .

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