EFFECT OF FREEZING AND THAWING AND OF DILUENT ON THE POTENCY OF HUMAN CHORIONIC GONADOTROPHIN IN THREE METHODS OF BIOASSAY

By

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ABSTRACT

In a study designed as a factorial experiment, the biological activity of standard solutions of human chorionic gonadotrophin in distilled water (A), saline (B), 1% bovine serum albumin (C), 0.5% gelatin (D), and borate buffer of pH 9 (E) was investigated under four different conditions of freezing and thawing, using the following three methods of bioassay: ovarian ascorbic acid depletion in rats (OAAD), uterine weight in mice (UW), and ovarian hyperaemia in rats (OH).

Repeated freezing and thawing and prolonged storage at -15°C did not affect the potency in any test. In the OAAD test, the potency was increased 4-5 fold by D, and 2-3 fold by C. In the OH test, E augmented the potency 2-3 fold.

These findings are of interest in the practice of bioassay, in studying mechanisms of response, and regarding administration for therapeutic purposes. Diluents which possess augmenting properties could be used to improve the sensitivity of a bioassay if standard and unknowns showed the same degree of augmentation.

The biological potency of gonadotrophin preparations can be destroyed, or reduced, or enhanced, by a variety of agents, including heating in solution, the pH of the injection medium, chemical reagents which attack specific groups in protein molecules, and unidentified contaminants present in extracts from tissues and body fluids. (See, for example, Bourillon et al. 1959 a, b; Albert

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1961, pp. 177, 179; and Bell & Loraine 1967, pp. 61, 166). The effect of some agents has been reported to vary, depending upon the nature of the gonadotrophin preparation and the response used in the bioassay.

The effect of freezing and thawing on the biological activity of gonadotrophin solutions has apparently not been investigated systematically. Bell and Louwerens (Bell & Loraine 1967, p. 312) have mentioned that a loss of potency of some 20 % may occur. This question has some practical importance since in many instances samples are frozen and thawed at least once before they are injected for bioassay.

The present factorial experiment was designed to study the effect of four different freezing conditions on the potency of standard solutions of human chorionic gonadotrophin (HCG) in five different diluents, as estimated by three methods of bioassay.

Material and Methods

Hormone and Diluents

One starting solution was made up for the whole experiment by dissolving the required amount of Second International Standard Preparation of human chorionic gonadotrophin (Bangham & Grab 1964) in distilled water. Five aliquots were then diluted with distilled water or concentrated diluent to obtain a concentration of 50 IU/ml in each of the following aqueous media: (A) distilled water; (B) sodium chloride (0.9%); (C) bovine serum albumin (1.0%); (D) gelatin (0.5%); and (E) borate buffer of pH 9, 0.1 M (0.62% H₃BO₃ + 0.2% NaOH) (all % as w/v). The five solutions were distributed into vials containing 1.0 ml each.

Freezing Conditions

For each diluent, the vials were divided into four batches.

Batch 1 and 3 were frozen once at -15°C or -72°C, respectively, and stored until assayed.

Batch 2 and 4 were frozen at -15°C or -72°C, respectively. After three days, the solutions were thawed in a water-bath (25°C), then frozen again at -15°C and -72°C within the hour. Thawing and freezing was done three times at intervals of two days, the third time four days before starting the first series of assays.

All batches were treated in this way on the same days, and all were stored at -15°C between and after treatments. Freezing was completed in 15 minutes at -15°C, and in 15-25 seconds at -72°C. Thawing was completed in 3 to 6 minutes.

Bioassays

Several hours or one day before starting an assay, the vials to be assayed were thawed and the solutions diluted as required, without changing the composition of the diluent. Thus, the five diluents listed were also the injection media. The solutions were kept at +4°C until injected.

Three methods of bioassay were used, viz. the tests based on ovarian ascorbic acid depletion (OAAD) in rats (Parlow in Albert 1961, p. 300), on uterine weight increase
(UW) in mice (Borth et al. 1959), and on ovarian hyperaemia (OH) in rats (Borth et al. 1957). Solutions were injected into the tail vein in the OAAD test, subcutaneously once daily for three days in the UW test, and intraperitoneally 16 h before autopsy in the OH test. Two or three dose levels were used for each solution, and 5 or 6 (OAAD, UW) or 10 (OH) animals at each dose level.

The mean indices of precision ($\lambda$) were 0.38 (OAAD), 0.24 (UW), and 0.34 (OH). The mean ratios of upper to lower fiducial limit ($P = 0.05$) were 4.2 (OAAD), 2.7 (UW), and 4.6 (OH).

Results were expressed as relative potencies in terms of solution B-1 (saline, frozen once) which was used as the standard preparation in all assays.

Experimental Design

The four solutions in the same diluent were assayed together in a multiple assay. Three such assays were run simultaneously in the same week, one for each method of assay. The whole series of multiple assays was repeated several weeks later, in the same order. Assays with diluents A, B, C, D, E were started in weeks No. 1 and 8, 2 and 10, 3 and 13, 4 and 16, 6 and 21, respectively. Thus, the study represented a $4 \times 3 \times 5 \times 2$ factorial design including 4 freezing conditions, 3 methods of assay, 5 diluents, and 2 replicates in time. The 120 results were derived from 30 multiple assays. A total of 2350 rats and 850 mice were used.

After having been stored for eleven months at $-15^\circ$C, aliquots of the B-1 solution used as the standard in this study were assayed against a fresh saline solution of the international standard, which had not been frozen prior to the assay.

Statistical Methods

A range test (Dixon 1953; Diem 1962, p. 170) was used (at $\alpha = 0.05$) to justify the rejection of a small number (less than 4%) of outlying responses in the OAAD test.

The bioassays were evaluated by correct rather than approximate methods, using computer programmes written for the analysis of multiple parallel-line assays (Borth et al. 1969). Three statistics reflecting the internal error of assays were computed. The index of precision $\lambda$ summarizes the precision of a method under the working conditions prevailing in a particular place. The log fiducial range ($P = 0.05$) (or its antilogarithm, the ratio of upper to lower fiducial limit) reflects the precision of individual results as affected by the design and the number of animals used. The variance of log potency was used to assess the variation between assays in $\chi^2$ tests (Bliss 1952).

The effect of the experimental factors studied was evaluated by standard techniques of analysis of variance (Cochran & Cox 1950), using the variation between individual replicates as the error term, and computing (for $P = 0.05$) least significant differences between group means. All calculations were done after logarithmic transformation.

RESULTS

The mean relative potencies obtained with the various diluents (injection media) and freezing conditions are plotted in Fig. 1 a, b and c, for the OAAD, UW, and OH tests, respectively. The least significant difference (lsd) is also shown. In order to exceed random variation as defined by the variance of
Fig. 1 a.
Ovarian ascorbic acid depletion test in rats (OAAD).

Fig. 1 b.
Uterine weight test in mice (UW).

Fig. 1 c.
Ovarian hyperaemia test in rats (OH).

Lack of effect of freezing conditions (1, 2, 3, 4) on the biological potency of HCG dissolved in different diluents (A, B, C, D, E). RP = relative potency in terms of standard (B-1). Isd = least significant difference \( (P = 0.05) \) between any two points plotted, each of which represents the mean of two assays. (Details see text).

replicates, by the assumption of normalcy, and by \( P = 0.05 \), the difference between any two points plotted would have to be greater than the Isd.

The graphs illustrate, and the analysis of variance confirmed, that freezing the solutions once or four times, slowly or rapidly, did not produce any noticeable differences in biological potency in the three assay methods used. The
absence of any effect of freezing and storage was also evident when the saline solution B-1, after eleven months' storage at $-15^\circ$C, was assayed against a fresh solution of the same international standard preparation. The following relative potencies and limits were obtained for B-1: 0.8 (0.5–1.2) in the OAAD test; 0.9 (0.7–1.2) in the UW test; and 1.0 (0.6–1.6) in the OH test.

On the other hand, there were considerable differences between diluents regarding the biological response. This was shown in the analysis of variance where the mean squares for methods, diluents and their interaction all exceeded the replicate variation ($P < 0.005$, 0.005, and 0.05, respectively). In Fig. 2, the mean potencies are plotted for each method and diluent (injection medium), disregarding now the negligible differences between freezing conditions. The lsd is half that in the other figures since each plotted point is based on 8 observations rather than 2.

The potencies of the saline solutions (B), that is, the results obtained by assaying the standard against itself, were all close to 1.0, and the analysis of variance confirmed the absence of any main effects and interactions in this group. The response in the UW test was not noticeably affected by the injection medium. In the OAAD test, the mean potencies of the borate, albumin and gelatin solutions were 1.9, 2.5 and 4.4, respectively. The borate solution showed a mean potency of 2.3 in the OH test. These values differ from unity by amounts too great to be easily accounted for by random variation.

When tested individually in $\chi^2$ tests, most pairs of replicate assays could be

![Fig. 2.](image)

Effect of injection medium on the biological potency of HCG. Same representation as in Fig. 1. Each point plotted represents the mean of eight assays. NaCl = saline, $H_2O$ = distilled water, Bor. = borate buffer pH 9, Alb. = 1% bovine serum albumin, Gel. = 0.5% gelatin.
regarded as homogeneous \( P > 0.05 \), but the inter-assay variation as a whole exceeded the intra-assay error. Both the difference between replicates and the fiducial range varied erratically, and no dependence on time, diluent, or freezing was apparent. The mean standard deviations of log potency as calculated from the variation between replicates and from the intra-assay variation were, respectively, 0.22 and 0.15 in the OAAD test, 0.26 and 0.10 in the UW test, and 0.30 and 0.16 in the OH test. The corresponding variance ratios are too great to be attributed to chance. Most interactions of two and three factors in the analysis of variance were smaller than the variation between replicates. For these reasons, the latter was used as the error term in the comparison of means described in preceding paragraphs.

**DISCUSSION**

It seems that solutions of HCG in various vehicles currently used in bioassay may be frozen and thawed repeatedly and stored for months in the frozen state without change in biological activity. The widespread useful practice of keeping frozen aliquots of standard solutions until needed seems to be as safe a procedure as has hitherto been assumed as self-evident, apparently without much published evidence.

It is a matter of conjecture whether a similar study on HCG in body fluids would produce the same result. This may seem doubtful in view of the unexpectedly large variations in potency found in a recent collaborative study on the estimation of HCG in frozen serum samples using different methods in different laboratories (Borth in Bell & Loraine 1967, p. 77; Baechler et al. 1969).

The presence of albumin and of gelatin in the injection medium increased the potency in the OAAD test 2–3fold and 4–5fold, respectively, while no effect was noted in the OH and UW tests. It seems reasonable to assume that these findings result from the combined action of two factors: (i) the time changes in the concentration of HCG available at the target site, and (ii) the dependence of the response on the dynamics of local hormone supply (see Borth et al. 1959, p. 201). An investigation of these factors would be of some interest regarding the mechanism of action of the hormone as well as its administration for therapeutic purposes. It is an intriguing question why the »potentiating« effect was noted with the assay method using intravenous injection and having the shortest time interval between injection and observation of response. If the effect of albumin and gelatin is merely to »decrease the rate of inactivation« (Katzman et al. 1943), this could most simply be attributed to the dilution of the hormone with the other proteins, in the metabolizing enzyme systems of the test animal. The change thus produced in Factor (i) could plausibly account for the findings.
The enhancing effect of borate buffer (pH 9) observed in the OH test, and perhaps also present in the OAAD test, may be the same phenomenon as the augmenting effect of alkaline pH reported for the UW, vaginal smear, and prostate weight tests in rats injected twice daily (Umberger & Gass 1959; Diczfalusy in Albert 1961, p. 177), although the absence of this effect in our UW tests in mice injected once daily must also be noted. The mechanism of the phenomenon is a matter of speculation.

The various diluents produced no consistent changes in the precision of assays as assessed either by the internal error or by the variation between replicates. The sensitivity of the OAAD assay could be improved by a factor of 4 or more by using 0.5% gelatin as the injection medium, provided that the enhancing effect found here with the international standard preparation of HCG could be shown to occur consistently with the standard and unknown preparations being assayed. Similarly, the use of an alkaline buffer as the diluent in the OH assay may also be of interest in this respect.

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