Effect of pH on Biological Activity of Chorionic Gonadotropin

Abstract. Increasing the *p*H from 3.0 to 10.8 increased the uterine weight threefold and the number of positive vaginal smears from 0 to 78 percent in immature rats injected with chorionic gonadotropin in aqueous solution. Activity of a solution held at *p*H 2.6 and 5°C for 24 hours was restored by neutralization.

Recently this laboratory received for assay a commercial sample of chorionic gonadotropin which consisted of a vial containing the dry chorionic gonadotropin powder and a second vial containing an aqueous diluent in which was incorporated, among other things, 25 mg of thiamine hydrochloride per milliliter. In accordance with the usual practice, the powder was first dissolved in the diluent and was then further diluted with 10-percent alcohol in order to obtain the proper dosage range for the bioassay. For comparison, similar solutions of International Standard chorionic gonadotropin in 10-percent alcohol only were prepared. The sample failed to elicit a uterine weight response when injected into immature female rats in total doses of 0.3 and 0.6 international unit per rat, whereas the International Standard chorionic gonadotropin at these doses showed the activity expected. The injections were made subcutaneously in two equal daily injections for 3 days, with sacrifice of the animals on the 5th day.

The assay was repeated, but the sample diluent was omitted and the sample powder was dissolved directly in 10-percent alcohol. The powder now showed the full labeled potency.

When the International Standard chorionic gonadotropin was dissolved in the sample diluent or in 10-percent ethyl alcohol containing an equivalent amount of U.S.P. thiamine hydrochloride, the International Standard chorionic gonadotropin showed no activity at the usual doses administered. However, when a solution of the International Standard chorionic gonadotropin was injected at one site and the thiamine hydrochloride was injected at another site simultaneously, there was no inhibition of activity.

Since it has been reported that acid solutions of chorionic gonadotropin are unstable (*1*), International Standard chorionic gonadotropin in 10-percent alcohol containing hydrochloric acid equivalent to that supplied by the thiamine hydrochloride (*p*H 2.6) was injected, and there was no response. However, when the solution of chorionic gonadotropin so prepared was neutralized with NaOH after standing at 5°C for 24 hours, full activity was restored; this indicated that stability was not a factor.

Table 1 shows the results of two experiments in which the *p*H was varied from 3.9 to 10.8. In these experiments all vaginas were opened with a cotton swab on the evening of the 4th day and vaginal smears were made on the 5th day at 96 and 100 hours after the first injection; following this the animals were sacrificed and their uteri were weighed. In the first experiment the *p*H of the injection medium was varied by the addition of either dilute HCl or dilute NaOH, the final volume of each group being kept constant. In the second experiment the *p*H was adjusted by means of McIlvaine's phosphate-citric acid buffer (*2*), and with dilute NaOH.

As Table 1 demonstrates, not only is the activity of chorionic gonadotropin inhibited at a low *p*H but it is considerably enhanced at a high *p*H. These observations are important in connection with the development of an official method of bioassay for this drug. It is also of interest to know whether the effects noted here also obtain when the drug is used in human beings.

Recently Banik and Chakravarti (*3*) reported that the activity of human chorionic gonadotropin was inhibited when injected in the male toad in the same solution with quinine dihydrochloride, ergotoxine, ethanesulfonate, emetine hydrochloride, or atropine sulfate. It is possible that the inhibition noted was due to the low *p*H of the injected solutions rather than to an inhibitory action of the drugs themselves.

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Table 1. Effect of the *p*H of the injection medium on responses of immature rats to 0.6 units of International Standard chorionic gonadotropin.

*p*H	Buffer	Uterine weight (mean ± standard error)	Vaginal smear		
			Positive (No.)	Animals (No.)	Positive (%)
		Experiment A			
3.2	HCl	39.5 ± 4.5	0	11	0
6.0	None	60.2 ± 6.8	3	12	25
8.6	NaOH	71.5 ± 9.8	8	13	62
10.8	NaOH	98.6 ± 9.1	10	13	77
Control	None	42.0 ± 3.6	0	12	0
		Experiment B			
3.0	Phosphate-citric acid	33.5 ± 8.5	0	18	0
5.0	Phosphate-citric acid	49.2 ± 6.5	2	18	11
7.0	Phosphate-citric acid	73.4 ± 6.7	6	18	33
9.6	NaOH	78.1 ± 4.9	9	18	50
10.8	NaOH	109.2 ± 6.1	14	18	78

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17 February 1959

Male Sterility Induced in Tomato by Sodium 2,3-Dichloroisobutyrate

Abstract. Spraying tomatoes with 0.3 percent sodium 2,3-dichloroisobutyrate (FW-450) at anthesis induced male sterility for 12 days, beginning 12 days after treatment. Only 20 percent fewer fruits were set on treated plants hand-pollinated with pollen from unsprayed plants than were set on untreated plants. The flowers again showed normal fertility 37 days after treatment.

The high cost of F_1 hybrid tomato seed has been one of the factors limiting commercial use of such seed. The use of male sterile mutants to eliminate the necessity for hand emasculation has been suggested as a means of reducing the cost of hybrid seed. Larson and Paur (*1*) described a functional male sterile mutant and suggested techniques of utilizing it. Many male sterile mutants have been identified, and some were listed by Rick (*2*). Before it would be possible to produce hybrid tomato seed commercially by means of these techniques, a breeding program of undetermined duration would probably be necessary, unless suitable male sterile mutants could be found by searching in large field populations, where, according to Rick (*3*), the normal incidence is about 0.05 percent. Alternatively, male sterility might be induced in one of the parents by irradiation, as discussed by Lesley and Lesley (*4*). If male sterility could be induced in tomatoes by means of a chemical, F_1 hybrid seed could be produced without hand emasculation and without

the delay required for breeding programs, and produced cheaply, as shown by Hafen and Stevenson (*5*), who reported a pollination cost of about $6 per pound of seed where male sterile mutants were used.

A selective gametocide, sodium 2,3-dichloroisobutyrate (*6*), induced male sterility in cotton, according to Eaton (*7*). The gametocide was tested on field tomatoes at Morden, Manitoba, Canada, in 1958. The varieties Early Chatham, Earlinorth, Monarch, Mustang, Cavalier, Early Lethbridge, Early Hybrid, Manitoba, Bounty, Scotia, and Harrow were used. Spray treatments consisted of applications of four concentrations (0.075, 0.15, 0.3, and 0.6 percent) of the chemical in water; each concentration was applied on three different dates (23 June and 11 and 29 July). There were two unsprayed controls. Each spray was applied to a single row which consisted of 55 plants, five plants of each of the 11 varieties. The tractor-mounted sprayer was operated at a pressure of 45 lb/in.^{2} to give an application rate of 80 gal/acre over the area actually sprayed. When first sprayed, the plants had an average spread of 16 in. and a height of 10 in.; at the second spraying they had a spread of 22 in. and a height of 13 in.; they were not sprayed to the point of run-off on either occasion.

The fertility of the blossoms was evaluated in weekly fruit counts. Figure 1 presents the results for the treatments of 23 June, when the first flowers were at anthesis. The curves represent the average number of fruits set per plant for the 11 varieties.

In the 14-day period between 21 and 35 days after treatment, the number of fruits set on the unsprayed controls averaged 9.8 per plant, in comparison with 10.0, 2.9, 0.9, and 0.2 fruits for the plants sprayed with the 0.075-, 0.15-, 0.3-, and 0.6-percent concentrations, respectively, of the gametocide. The 0.075-percent concentration did not induce sterility. The 0.15 percent concentration caused some reduction in fruit set, and the 0.3 and 0.6 percent concentrations almost prevented further set. In the following 7-day period, between 35 and 42 days after treatment, fruit set was 30.6 for the controls and 33.6, 25.8, 10.7, and 0.8 for the plants treated with the 0.075-, 0.15-, 0.3-, and 0.6-percent concentrations, respectively, of the gametocide. In this period the two low concentrations had little effect, the 0.3 percent concentration had an intermediate effect, and the 0.6 percent concentration practically prevented set. The finding of 1.0 fruit set in the 3-week period for plants treated with the 0.6-percent concentration of the gametocide was attributed to pretreatment fertilization and delayed expansion of the fruit. About 6

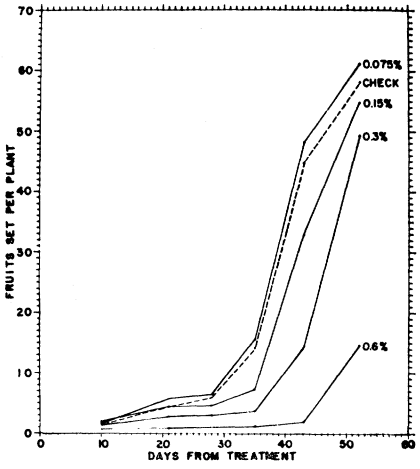

Fig. 1. Average number of fruits set per plant for 11 varieties of tomato sprayed 23 June with 2,3-dichloroisobutyrate in four concentrations (solid lines) compared with average number for unsprayed controls (dashed line).

days after anthesis, fruits were sufficiently expanded to be identified as pollinated and therefore to be counted as set.

The second application of the gametocide was made 11 July, when about four fruits were set per plant (see Fig. 2). In the 17-day period between 17 and 34 days after treatment, fruit set was 44.0 fruits per plant for the controls and 28.9, 21.9, 5.9, and 2.6 fruits for plants treated with the 0.075-, 0.15-, 0.3-, and 0.6-percent concentrations, respectively, of the gametocide. The rate of fruit set was substantially reduced by treatment with the 0.3-percent gametocide, and set was almost inhibited by treatment with the 0.6-percent concentration. In the following 7-day period only 2.3 fruits were set

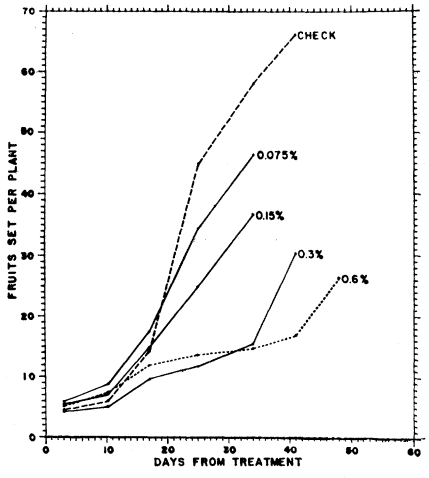

Fig. 2. Average number of fruits set per plant for 11 varieties of tomato sprayed 11 July wtih 2,3-dichloroisobutyrate in four concentrations compared with average number for unsprayed controls.

on the plants that had been sprayed with the 0.6-percent concentration; however, 14.9 fruits were set on these treated with the 0.3-percent concentration—a finding that indicated return to fertility.

Gametocide applications made on 29 July (for which data are not presented) to plants with an average set of 16 fruits produced a pattern of fruit set similar to that for the two earlier treatments. In all three instances application of a 0.6-percent concentration of the gametocide caused foliage to yellow within several days, reduced the growth rate, and inhibited the production of pollen almost immediately.

The male fertility of treated plants was further evaluated by recording the presence or absence of pollen and by testing the viability of any pollen produced by applying it to emasculated flowers of unsprayed plants. The female fertility of treated plants was evaluated by pollinating the flowers with normal viable pollen obtained from unsprayed plants of the same variety. It was found that male sterile flowers could be identified by the lighter yellow color of the staminal tube. Results of this part of the experiment for the 23 June and 11 July applications showed no male or female sterility attributable to treatment with the 0.075-percent concentrations. The 0.15-percent concentration induced a high degree of male sterility for about 13 days, beginning 15 days after treatment, but complete absence of pollen was not observed to result from treatment at this concentration. A slight reduction in female fertility was apparent. The 0.3-percent concentration caused complete absence of pollen for about 12 days, beginning 12 days after treatment. During this period, about 30 percent of the flowers on plants sprayed with the 0.3 percent concentration, when pollinated with normal pollen, set fruit, as compared with about 50 percent of the flowers on unsprayed control plants; this finding was interpreted as indicating reduced female fertility in the treated plants. The return to normal pollen production was gradual. By 37 days after treatment, pollen production was normal. The 0.6 percent concentration induced complete male sterility (absence of pollen) for a period of about 19 days, beginning 12 days after application. Pollen production was normal 37 days after treatment. No fruits were set when normal pollen was applied between 15 and 22 days after treatment to the flowers of plants treated with this 0.6-percent concentration; this was taken to indicate female sterility. Female fertility was again normal 37 days after treatment.

The 11 varieties of tomato tested did not differ in their response to the gametocide with respect to fruit setting. The

variety Early Lethbridge showed slightly more yellowing of foliage than the others, and the reduction in rate of growth was slightly greater in this variety.

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12 January 1959

Ionizing Energy as an Aid in Exchange Tritium Labeling

Abstract. The tritium labeling of organic compounds by the Wilzbach technique—that is, by simple exposure of the compound to tritium gas—is greatly accelerated by the simultaneous exposure of the system to a silent electrical discharge. The incorporation of tritium into benzene was increased by a factor of about 10^4 without undue decomposition. Cobalt-60 γ-rays were found to be far less useful for increasing the tritium incorporation.

The method described by Wilzbach (*1*) for the labeling of organic compounds by exposure to tritium gas has come into wide use. The compound to be labeled is exposed to subatmospheric pressures of tritium gas at room temperature for a few days. The radioactive gas is then removed, and the exposed compound is rigorously purified by recrystallization, distillation, or chromatography. By this technique, labeled organic compounds with specific activities of the order of 10 mc/g may be obtained.

It has been shown that reactions with recoiling tritons from the labeling gas are not the principal process by which the organic substrate becomes labeled (*2*). The most likely process appears to be reactions between ionized or excited organic molecules and the tritium gas, a supposition furthered by the work of Ahrens *et al.* (*3*), in which was measured the product distribution obtained by exposing hydrocarbons to tritium-labeled hydrogen and to γ-radiation. It therefore seemed to us that the exchange labeling could be greatly speeded up by providing the system with an external source of ionizing energy. For this purpose we applied, separately, a silent electrical discharge and γ-rays from a Co^{60} source to a benzene-HT system (*4*). The electrical-discharge work recalls a recent publication by Wolfgang *et al.* (*5*), in which organic compounds were irradiated with T^+ and T^+_2 ions accelerated with a d-c voltage. In our experiments we used a high-voltage alternating current whose sole purpose was to provide a greater number of excited molecules; no ion accelerations were involved.

Tritium was kept in the form of uranium tritide, UT_3, from which the tritium gas could be obtained by heating the tritide to 450°C. The vacuum line and associated apparatus used to transfer the tritium to the organic substrate were similar to those recently described in *Nucleonics* (*6*).

The effect of the silent electrical discharge was determined in the following way: Benzene (600 μl) was exposed to 40 mc of tritium (partial pressure of $H_2 + HT$, 210 mm) in the apparatus shown in Fig. 1. The volume of the space occupied by the benzene vapor was about 60 cm^3; therefore, only 4 percent of the benzene was in vapor form and the remainder was in liquid form at the bottom of the tube. A silent discharge (alternating current, 20 kv, 1 ma) was passed through the system for 1 hour. The hydrogen-tritium gas was removed, and the irradiated benzene sample was purified to constant specific activity by vapor-phase chromatography. Two passes through a 5-ft paraffin column were enough to accomplish this; further passes through paraffin, Silicone, Carbowax, or Ucon columns led to no change in the specific activity. After the paraffin-column purification, 0.67 mc was found to have been incorporated into 500 μl of the purified benzene. Very little decomposition of the benzene was evident in this experiment. The isotopic percentage of the tritium used was only 0.1, and under these conditions the Wilzbach technique alone (that is, without the accompanying electrical discharge) would have given a total activity of only about 4×10^{-5} mc (or 0.04 μc) in the 500 μl of benzene. It is apparent that, with more tritium in the gas, very high specific activities can be obtained by the electric discharge method.

In a second experiment (with the entire assembly in a horizontal position to spread out the liquid benzene) passage of the current for 5 hours (46.5 mc, 256 mm partial pressure of the H_2-HT gas) resulted in the incorporation of 5.56 mc into a 200-μl aliquot portion of the purified benzene. However, in this experiment only 200 μl of the original 600 μl of the benzene was recovered. Extensive decomposition (roughly 50 percent) was observed to have taken place. Consequently, the conditions of the first experiment, with a shorter time of exposure to the electric discharge, are to be recommended.

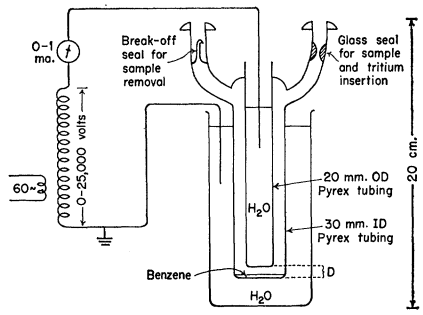

Fig. 1. Electrical discharge apparatus.

Further experiments were performed to test whether a change in the distance *D* (Fig. 1) through the liquid benzene would alter the amount of radioactivity incorporated under conditions otherwise identical. No significant differences were observed between an experiment performed with $D = 5$ mm and another with $D = 25$ mm.

To determine the effect of γ-radiation on the incorporation of tritium into benzene, the following experiment was performed: In each of two similar glass vessels (40-ml volume) were placed 600 μl of benzene and a mixture of H_2 and HT (specific activity 2.014 mc/cm^3 at standard temperature and pressure; total activity 25 mc). The total pressure in each vessel was 330 mm (benzene, 80 mm; H_2-HT, 250 mm). The vessels were then glass-sealed. One vessel was allowed to stand for 24 hours at room temperature. The total energy expended in this vessel by the tritium was 4.7×10^{17} ev, and the energy absorbed by the gases would be almost equal to this amount (*7*). The other vessel was irradiated for 24 hours at room temperature with γ-rays from a Co^{60} source. The total dose delivered to the gases was 2.7×10^{19} ev. The vessels were then opened, through break-off seals, on a vacuum line. The hydrogen-tritium gas was removed, and the irradiated benzene samples were purified to constant specific activity by vapor-phase chromatography. In the "control" benzene a total of 0.565 μc was incorporated; in the γ-irradiated sample a total of 3.85 μc was incorporated. The tritium labeling in the irradiated sample was therefore greater by a factor of 6.8.

A second experiment was performed in exactly the same way as the first except that in both vessels the partial pressure of the H_2-HT was 70 instead of 250 mm, and the total activity was 7 instead of 25 mc. As before, the partial pressure of the benzene was 80 mm. The total energy expended in (and absorbed by) the control vessel was 1.3×10^{17} ev. The gases in the γ-irradiated vessel received