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Use of Tissue Culture Mediums Sterilized with Gamma Radiation from Cobalt-60.* (21029)

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Recent interest in tissue cell cultivation, resulting primarily from its successful application to problems in virology, has stimulated considerable effort towards simplification and improvement of methods and materials. In spite of the increased use, however, the greatest difficulty confronting the tissue culture worker continues to be the lack of a simple medium which can be easily prepared and handled. A synthetic medium, capable of sustaining cell proliferation, has not yet been developed and almost all tissue culture mediums contain serum and embryonic extract as major components. While these materials may be sterilized by filtration the procedure is both tedious and uncertain.

The present paper is a preliminary report of studies designed to test the feasibility of using tissue culture mediums sterilized with gamma radiation from Cobalt-60. Though ample evidence is available that such radiations will sterilize a variety of biological materials under appropriate conditions(1-3) it was necessary to establish the fact that the particular materials in question here, namely serum and embryonic extract, could be sterilized in the form in which they are commonly used for tissue culture work. A second and more critical point was to determine the suitability of such irradiated mediums for cell cultivation and was concerned with: a) possible destruction of growth promoting properties, b) production of toxic substances or materials capable of stimulating abnormal growth, c) alteration of other significant physical or chemical properties. Since plasma is frequently used in tissue culture work to provide an imbedding matrix, it was substituted for serum in these initial studies. In this way it was possible to determine the effect of irradiation on the clot forming ability as well as on growth promoting properties. The effect of gamma radiation on the ability of embryonic extract to induce clot formation was also studied.

Materials and methods. Irradiation for this study was carried out with the large Cobalt-60 source of the Michigan Memorial Phoenix Project(4). This source develops 2.2 x 10^5 rep/hr in the center well: samples were irradiated in the center well and received total dosages varying from 1 x 10^5 to 1.2 x 10^6 rep. In all experiments, control samples were kept at approximately 15°C, the temperature of the irradiation chamber, for the duration of the exposure. Tests were made with *chicken plasma* and *embryonic extract*: they were used in the form of commercially dehydrated products (Difco) and as freshly prepared fluid samples. The former were irradiated both in the dehydrated state and following

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reconstitution with distilled water. All samples were irradiated in 5 ml quantities that were tightly sealed in 10 ml glass bottles. Following irradiation the samples were refrigerated at 4°C until tests could be performed. Aqueous embryonic extract was stored at −20°C. Twenty four-hour broth cultures of *E. coli* I, *Staph. aureus* RK2, and a recently isolated coagulase positive *Staph. aureus* were used to determine the ability of gamma radiation to sterilize tissue culture fluids. Samples were contaminated with known numbers of viable organisms and then irradiated in either the aqueous state or following dehydration.‡ In fluid specimens the numbers of viable organisms per ml were determined by plating 0.1 ml of each 5 ml sample in brain heart infusion agar both before and after irradiation. Plate counts were made after 48 hours and again after 72 hours incubation at 35°C. For dehydrated samples, the numbers of organisms per ml prior to irradiation were determined from control samples of the same lot held at room temperature. *pH* was determined on fluid samples before and after irradiation. The pH of dehydrated samples exposed to gamma radiation was measured following reconstitution and was checked against that of reconstituted control samples. All pH measurements were made with a Beckman model G pH meter using a microadapter cup(5). The *clotting properties* of chicken plasma samples were tested by adding 2 drops of "standard" embryonic extract to 4 drops of plasma in a 10 x 75 mm tube. Both coagulation time and consistency of the clot were evaluated. In the case of dehydrated plasma, a non-irradiated sample from the same production lot served as a control. The ability of embryonic extract to induce clot formation of a "standard" plasma sample following irradiation was tested in the same way. For the study of growth promoting properties explants from the ventricle of 9- to 11-day chick embryos were embedded in a plasma clot on 11x22 mm coverglasses. The latter were inserted in Agarslant culture tubes§ and overlaid with a medium containing 40% horse serum or human ascitic fluid, 50% Hank's balanced salt solution, and 10% chick embryonic extract. These tubes were incubated in a horizontal position at 35°C. Increase in area of outgrowth was measured using a calibrated ocular micrometer. At the end of the observation period the coverglasses were removed, the outgrowth fixed with Bouin solution, and then stained with Harris haematoxylin. The mitotic activity of the cells in the outgrowth was determined by microscopic examination of these stained slides.

‡ Preparation of dehydrated samples contaminated with known numbers of test organisms was kindly carried out by Dr. C. W. Christensen, Difco Laboratories.

TABLE I. Viable Bacterial Count as a Function of Radiation Dosage.

Dosage (rep) × 10^5	Coagulase positive *Staph. aureus* In balanced salt sol.	Coagulase positive *Staph. aureus* In aqueous EE*	*Staph. aureus* RK2 In dehydrated plasma	*E. coli* I In dehydrated plasma
0	1.1×10^6	4×10^6	390	230
1.0	21	125	0	0
1.5	—	11	—	—
2.0	—	0	0	0
2.5	0	0	—	—
3.0	—	0	0	0

* Embryonic extr.

Results. Table I shows that *E. coli* I and *Staph. aureus* RK2 in dehydrated chicken plasma were killed by 1 x 10^5 rep of gamma radiation when the starting inoculum was 2 x 10^2-4 x 10^2 organisms per ml. Aqueous embryonic extract contaminated with 4 x 10^6 *Staph. aureus* per ml was sterilized at 2 x 10^5 rep. In other experiments these organisms were destroyed by equivalent dosages when suspended in liquid plasma. It will be noted that the killing appears to be essentially the same for the organisms in embryonic extract or plasma as for a saline suspension of the

TABLE II. Clotting Time of Dehydrated Chicken Plasma following Irradiation.

Dosage (rep)	Duration, hr	Clotting time in min. Dehydrated	Clotting time in min. Dehydrated and reconstituted
0	Control	.8	1.0
2.2 × 10^5	1	.8	1.3
4.4 "	2	—	2.0
6.6 "	3	—	4.3
8.8 "	4	.8	5.0
1.8 × 10^6	8	1.3	—
2.2 "	10	1.3	—

§ Fisher Scientific Supply Co. (modified form).

TABLE III. Clot Induction Time of Aqueous and Dehydrated Chick Embryonic Extract after Irradiation.

Dosage (rep)	Duration, hr	Clotting time in min. Aqueous	Dehydrated
0	Control	1.5	.25–.33
2.2×10^5	1	1.5	.25–.33
4.4 "	2	1.5	—
6.6 "	3	—	.25–.33
8.8 "	4	1.5	.25–.33
1.3×10^6	6	1.5	—
1.8 "	8	1.5	—

organism.

Dehydrated plasma from one commercial lot was used in studying the effect of radiation on clotting properties and samples were irradiated both in the dehydrated state and following reconstitution with distilled water. The data in Table II show that as much as 8.8 x 10^5 rep was tolerated by plasma in the dehydrated state with no alteration of clotting time or consistency of the clot while samples irradiated in the liquid state showed a significant increase in clotting time following 4.4 x 10^5 rep exposure. Clotting time for one sample of fresh chicken plasma was increased from .5 to 1.3 minutes following exposure to 8.8 x 10^5 rep.

Irradiated samples were stored at 4°C for several weeks to check the possibility of changes occurring some time after irradiation, due to delayed chemical reactions. After 3 to 4 weeks storage the clotting time of these samples was determined and compared with that observed before and immediately following exposure to gamma radiation. In no instance was a significant alteration of clotting time observed under these conditions.

Both dehydrated and aqueous chick embryonic extracts were irradiated and subsequently tested for their ability to induce clotting of chicken plasma as shown in Table III. Repeated tests failed to show a change in clot induction time following exposure to doses of gamma radiation as high as 8.8 x 10^5 rep. One series of experiments failed to show any change in aqueous embryonic extract following irradiation of 1.8 x 10^6 rep. Measurement of pH of samples following irradiation in the dehydrated state showed a maximum change over the controls of 0.3 pH unit. The maximum change in fluid samples as determined by measurement before and after irradiation was 0.2 pH unit.

The ability of irradiated medium components to support normal growth of tissue cells was not impaired as shown by the data in Tables IV, V, VI. It will be noted in Table IV that embedding explants in irradiated plasma does not adversely influence growth of chick heart tissue as determined either by increase in area of outgrowth or by mitotic configuration. In fact, from these data, there would appear to be a stimulation of growth. This finding has not been a consistent one, however, and continuous growth for 6 weeks in clots formed from plasma exposed to 1.8 x 10^6 rep failed to alter the rate of growth or the mitotic configurations of chick heart explants (Table V). These cultures were carried through 4 transplantations.

As seen in Table VI, exposure of either aqueous or dehydrated embryonic extract to a gamma radiation dosage of 8.8 x 10^5 rep failed to interfere with growth or significantly alter the mitotic pattern of chick heart explants when the extract was used to induce clot formation and was added to the liquid phase of the medium in a concentration of 20%.

Discussion. The results of the preliminary

TABLE IV. Growth of 11-Day Chick Embryonic Heart in Irradiated Plasma.

Dosage (rep)	% increased area in 48 hr	Mitotic configuration (%) Resting	Separated nucleoli	Splitting nucleus	Prophase	Metaphase	Anaphase	Telaphase
0	100	39	52	7	2	0	0	0
2.2×10^5	423	47	45	6	1	1	0	0
4.4 "	412	44	43	8	4	0	1	0
8.8 "	830	48	47	2	1	2	0	1
1.3×10^6	1140	50	45	3	2	0	0	0
1.8 "	528	50	41	4	4	1	0	0
2.0 "	685	50	46	1	1	0	1	1

TABLE V. Continuous Growth of 11-Day Chick Embryonic Heart in Plasma Exposed to 2×10^6 Rep of Gamma Radiation.

Dosage (rep)	Mitotic configuration (%)						
	Resting	Separated nucleoli	Splitting nucleus	Prophase	Metaphase	Anaphase	Telaphase
0	67	23	9	0	0	0	0
2×10^6	74	24	0	0	0	0	2

TABLE VI. Growth of 9-Day Chick Embryonic Heart in Medium Containing Irradiated Embryonic Extract.

Sample	Dosage (rep) $\times 10^5$	% increased area, 48 hr	Mitotic configuration (%)						
			Resting	Separated nucleoli	Splitting nucleus	Prophase	Metaphase	Anaphase	Telaphase
Control	0	228	53	35	10	0	2	0	0
Aqueous	4.4	280	45	42	12	0	1	0	0
"	8.8	324	55	32	9	2	2	0	0
Dehydrated	8.8	139	54	38	7	1	0	0	0
"	8.8	249	29	60	8	1	2	0	0

studies presented here indicate that either fluid or dehydrated plasma and embryonic extract contaminated with non-sporeforming bacteria may be sterilized by gamma radiation from Cobalt-60 in dosages of 2 x 10^5 rep or less. Furthermore, irradiation of either dehydrated or liquid samples at this level does not alter the clot forming properties of plasma, the clot inducing capacity of embryonic extract, or the growth promoting properties of serum, plasma, or embryonic extract for embryonic tissues.

While the clotting time of plasma was significantly increased by exposure to 4.4 x 10^5 rep when in the fluid state, samples treated in the dehydrated form showed no change at 8.8 x 10^5 rep. This apparent protection was not due to complete absence of water as the commercially dehydrated samples used contained approximately 10% residual moisture. Dehydrated plasma exposed to 2 x 10^6 rep was still suitable for explantation of chick tissues and did not alter the growth pattern of chick embryonic heart tissue when cultures were carried in it for a period of 6 weeks.

As shown by Proctor and Goldblith(1), Lawrence, Brownell, and Graikoski(2), and others, the dosage of gamma radiation required to kill bacterial spores is considerably higher than that needed for vegetative cells. For several species of spore forming bacteria this is in the neighborhood of 2 x 10^6 rep when the organisms are suspended in physiological salt solution. Studies are in progress at the present time to determine the dosage required to destroy spores in serum, plasma and embryonic extract. However, it will be noted from the data presented in Tables IV and V that irradiation of plasma at 2 x 10^6 rep does not appear to make it unsuitable for cell cultivation. Recent studies, to be published later, indicate that such dosages may, indeed, be well tolerated.

Summary. Plasma, serum, and embryonic extract contaminated with gram negative and non-sporeforming gram positive bacteria have been shown to be sterilized by a dosage of 2 x 10^5 rep of gamma radiation from Cobalt-60. Such treatment failed to alter the properties of these materials as related to their use in tissue culture work. These studies are being extended to cover dosages necessary to kill bacterial spores and efforts are being made to put the work on a more quantitative basis by utilizing established strains of tissue culture cells for assay procedures.

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