Studies on a Serum Substitute for Mammalian Cells in Culture
I. Biological Efficacy of Whole and Fractionated Peptone Dialysate (36086)

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(Introduced by W. E. Heston)

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A renewed investigation of defined substitutes for serum in tissue culture medium is a necessity. The use of a chemically defined culture medium supplemented with serum or other biologics is an expedient but less than satisfactory practice. Serum is known to contain enzymes, hormones, "natural" antibodies, and cytotoxic factors; it can be the source of contaminants such as mycoplasma and adventitious viruses. Moreover, the present lack of large volumes of high quality, standardized sera emphasizes the need for a biologically efficacious replacement. Paradoxically, most, if not all, currently available diploid cell strains require serum for their growth and survival, and the cultivation of established lines is more difficult in its absence. The isolation and characterization of the serum component(s) responsible for its *in vitro* activity is a highly complicated and incomplete task, and when attempted the fractions fail to support cell growth to the same degree as whole serum.

Bacto-peptone, a heat stable enzymatic digest of animal tissue, has been used widely (1–5) as a serum substitute in cell culture media. Pumper and co-workers (6) reported that the components(s) responsible for the biological activity of crude Bacto-peptone was dialyzable but did not reside in the free amino acids present. The objective of the present work was to further separate and biologically analyze this dialyzable mixture.

Materials and Methods. Cell cultures. Cells of a rabbit myocardium line, designated RH-PD, were used. These cells grow readily in peptone dialysate supplemented MEM with a population doubling time of 29.8 hr, are heteroploid, and have undergone several hundred passages *in vitro* in the absence of serum. Stock cultures were propagated in Modern Oval prescription bottles (Foster-Forbes Glass Co., Marion, IN). When confluent (5–6 days), monolayers were dispersed by gentle scraping with a platinum wire loop, and fresh cultures were initiated with approximately 2.0×10^5 cells/ml of culture medium. Sodium bicarbonate buffered Eagle's minimum essential medium (Grand Island Biological Co., Grand Island, NY) was supplemented with peptone dialysate, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 1 mg/ml of dextrose.

Preparation of whole peptone dialysate. Bacto-peptone powder (Difco, Inc., Detroit, MI) was reconstituted as a 10% (w/v) solution in triple-distilled water. Peptone dialysate (PD) was prepared by the method of Pumper *et al.* (6), pooled, and evaporated on a Buchler all-glass, rotary flash evaporator. For cell culture purposes, dried PD was reconstituted in triple-distilled water and autoclaved.

Gel filtration of peptone dialysate. The low molecular weight components of PD were separated further by Sephadex G-10 chromatography. PD was eluted with sterile, triple-distilled water, the effluent was monitored at 265 mμ and tested for Lowry reactive material (7). Each UV-absorbing peak was separately pooled and flash evaporated.

Biological efficacy of whole and fractionated peptone dialysate. To quantitate the growth of RH-PD cells in whole PD, several PD concentrations were prepared by dilu-

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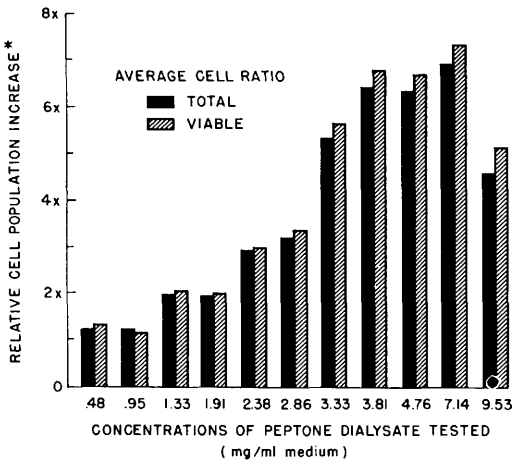

FIG. 1. Growth of RH-PD cells in MEM supplemented with various concentrations of peptone dialysate. The relative cell population increase (*) represents the ratio of the cell number after 4 days incubation to the original seed inoculum.

tion from a 200 mg/ml stock solution. For analysis, 0.2 ml of the appropriate PD dilution was mixed with a 4.0 ml aliquot of RH-PD cells in MEM. The seed inoculum was approximately 1.6×10^5 cells/ml. All cultures were incubated at 37°, and after incubation the total and viable cell counts visually enumerated for each sample. The results were expressed as a ratio of the cell numbers after incubation to that originally added to the culture flask (relative cell population increase).

To test the growth promoting activity of fractionated PD, stock solutions of the individual fractions were prepared and tested at final concentrations of 1.0 and 2.5 mg/ml. All other procedures employed were identical to those described for whole PD.

Results. Biological efficacy of whole peptone dialysate. Replicate cultures of RH-PD cells were grown in MEM supplemented with different concentrations of whole PD. As shown in Fig. 1, PD is efficacious over a broad concentration range. While cell populations are maintained at low concentrations, increasingly greater populations were observed with increasing PD concentrations, a plateau being reached at, and above, 3.8 mg PD/ml of culture medium. The reduced cell numbers at the highest PD concentration tested, 9.5 mg/ml, suggested a toxic response.

Gel filtration of peptone dialysate. A typi-

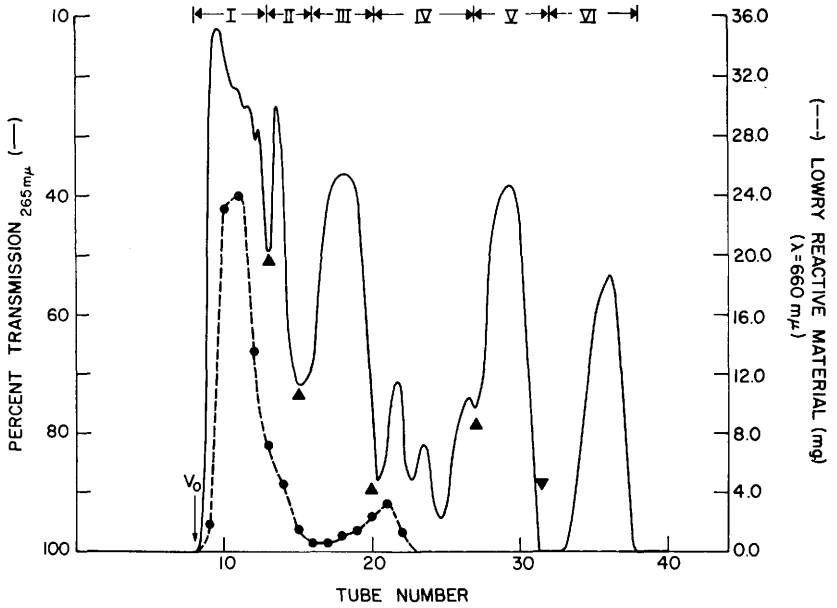

FIG. 2. Sephadex G-10 chromatography of 150 mg of sterile, reconstituted (aqueous) peptone dialysate (PD) on a 1.2 × 88 cm column: PD was eluted with sterile, triple-distilled water at 20 ml/hr; and the effluent from each UV-absorbing peak (delineated by arrowheads) was pooled and evaporated to dryness.

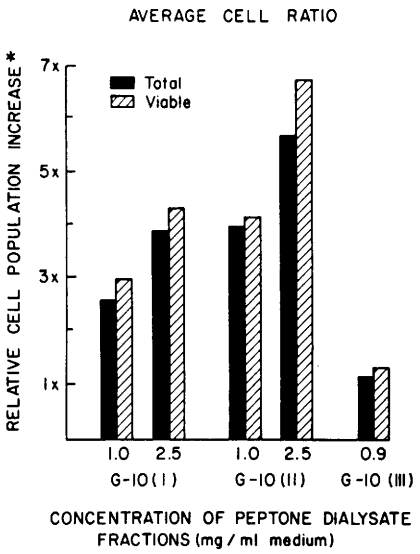

FIG. 3. Growth of RH-PD cells after 6 days incubation in MEM supplemented with fractionated peptone dialysate. The relative cell population increase (*) represents the ratio of the cell number on day 6 to the original seed inoculum.

cal elution pattern depicting Sephadex G-10 chromatography of 150 mg of PD is shown in Fig. 2. The major UV-absorbing peaks [delineated by arrowheads and termed G-10(I)–G-10(VI)] were collected, pooled, and flash evaporated. Greater than 70% of the starting material was poorly retarded by the column and eluted in fraction G-10(I). An additional 15% chromatographed as fraction G-10(II), and of the remaining fractions only G-10(III) yielded sufficient material for biological analysis. The Lowry reactive components were found predominantly in fractions G-10(I) and G-10(II). Reducing the amount of PD chromatographed or using 0.02 *M* ammonium bicarbonate failed to improve the separation procedure.

Biological efficacy of fractionated peptone dialysate. Fractions G-10(I), G-10(II), and G-10(III) were reconstituted and tested for their growth promoting activity in cultures of RH-PD cells. As shown in Fig. 3, fraction G-10(II) had the greatest activity; 1.0 mg/ml of G-10(II) supported the same degree of growth as 2.5 mg/ml of fraction G-10(I). This may be due to a higher concentration of some component(s) chromatographing principally in fraction G-10(II), or the presence in fraction G-10(I) of a low molecular weight entity(s), which partially binds the active component(s) or partially inhibits their biological effect. Fraction G-10(I) supported approximately the same degree of growth as whole PD (Fig. 1), while fraction G-10(III) maintained RH-PD cells at the original inoculum number.

Continuous growth of RH-PD cells in fraction G-10(I) supplemented MEM. RH-PD cells were subcultured through five successive transfers in MEM supplemented with fraction G-10(I) only. Confluent monolayers formed in 5 to 6 days; and no apparent differences between these and control cultures were observed. The low yield of fraction G-10(II) prevented a similar analysis.

Discussion. Peptone dialysate (PD) is a mixture of heat stable, low molecular weight entities isolated from Bacto–peptone. Sephadex G-10 chromatography of whole PD yielded two fractions which supported active cell growth, but to varying degrees. Fraction G-10(II) exhibited a greater growth promoting activity for RH-PD cells than did fraction G-10(I), and the latter fraction supported approximately the same degree of growth as did whole PD.

In an analogous series of experiments, Amborski and Moskowitz (8) separated crude, undialyzed Bacto-peptone, proteose peptone, and Bacto-protone into high and low molecular weight fractions by Sephadex G-15 chromatography. These fractions were tested on several cell lines which were routinely subcultured and preattached in serum containing MEM. Growth stimulation was observed in cultures supplemented with the low molecular weight fraction. Though not strictly comparable, this low molecular weight fraction was, at the minimum, a composite of fractions G-10(I) through G-10(VI), and its activity confirmed the earlier report of Pumper *et al.* (6) that the low molecular weight, dialyzable components of such tissue hydrolysates were biologically most active. In agreement is our observation that the nondialyzable components of Bacto-peptone were not capable of sustaining cell growth and survival (Pumper, unpublished data).

Summary. A biological titration of whole peptone dialysate (PD) demonstrated a broad concentration range over which large cell populations were obtained. Sephadex G-10 chromatography of PD resulted in six UV-absorbing fractions, three of which were recovered in sufficient quantities for biological analysis. Fractions G-10(I) and G-10(II) supported proliferation and survival, whereas fraction G-10(III) maintained cell populations at their seed inoculum level. Greater cell populations were observed in cultures supplemented with fraction G-10(II) than in those to which fraction G-10(I) was added.

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