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SECTION MEETINGS

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Separation of Growth Promoting Activity from Horse Serum by Dialysis.* (25838)

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Although a few tissue culture cell lines have been propagated serially in protein free medium(1), serum protein is required for serial propagation of most mammalian cell lines. It has been shown to play a role in the adhesion of cells to glass(2) and may also act as a carrier of as yet unidentified growth factors which are bound to protein and slowly released into the medium(3). In a study directed toward elucidation of some of the factors in serum required for cell growth it was observed that when serum was dialyzed against a synthetic medium or a salt solution under certain conditions, the dialysate supported growth of cells in cultures when it was used as a substitute for the normal serum supplement in synthetic medium. A method for separation of this fraction from serum and initial studies on its behavior will be presented.

Dialysis procedures. ¾ inch cellulose dialyzer tubing is inserted into a 6 oz. prescription bottle, the moderne oval model (E. H. Sargent Co.), so that about 1 inch of the open end overhangs the lip. Ten to 15 ml of double glass distilled water is placed in the bag and bottle is lightly capped with the plastic cap. The apparatus is sterilized by autoclaving at 121°C for 15 minutes. The water in the bag is replaced with serum and an equal amount of the dialyzing fluid is placed in the outer chamber. The excess dialyzer tubing is folded against the outside edge of the lip and the apparatus is tightly sealed with a rubber stopper. The apparatus is placed on its side in a 37°C incubator so that the sac comes in contact with the fluid.

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Test for protein. The dialysate was tested for protein contamination with 2.5% sulfosalicylic acid(4). The sulfosalicylic acid-dialysate mixture was allowed to stand for 2 hours at room temperature and was then examined in a narrow beam light source. This procedure was sensitive enough to detect protein corresponding to a 1:100,000 dilution of whole serum. *Tissue culture technic.* Cell strains used in these studies were the Mox strain (obtained from Dr. E. A. McCulloch, Univ Toronto), clone cultures derived from the Mox strain(5), HeLa. J-96, LLMC #1 (obtained from Dr. D. P. Gustafson, Purdue Univ), HEP 2 and Giardi human heart (obtained from Pitman Moore Co., Indianapolis, Ind.). These cells are normally maintained in a medium consisting of 20% horse serum and 80% synthetic medium CMRL 1066(6) with no added antibiotics. Dialysate medium was prepared by substituting undiluted dialysate for the serum portion of the medium. Five day cultures of cells grown in serum supplemented medium were washed in Hanks' basic salt solution(7) 3 times and suspended in dialysate medium so that final concentration was 50,000 cells per ml. One to 2 ml of this suspension was inoculated into tubes supplied with a 5% CO_2 atmosphere and incubated at 37°C. Total cell numbers were determined after 5 to 7 days incubation by counting in a hemocytometer and later, when it became available, in a Coulter electronic particle counter(8). Controls consisted of cells grown in serum supplemented medium and unsupplemented medium 1066. The Mox strain can be maintained in unsupplemented medium 1066 for a period of 10 to 15 days. During this time there is a slow, progressive degeneration of the cells with little evidence of multiplication. Periodic changes of the medium do not alter this picture and subcultures under these conditions have been unsuccessful.

Results. Initially, dialysis of horse serum was carried out at 4°C and 37°C for 144 hours against medium 1066, and the dialysate was tested for its ability to substitute for serum in supporting growth of cells. Increase in cell numbers in response to the 4°C and 37°C dialysates amounted to 2% and 81% respectively of total response of the cells to serum supplemented medium. The difference in activities of the dialysates suggested that the higher temperature may have caused release of growth promoting activity in the serum. Accordingly, whole serum was heated at 37°C for 7 days or longer, then dialyzed against medium 1066. A preparation was obtained under these conditions after 48 hours of dialysis which supported growth of the cells to the same extent as serum supplemented medium.

Sixty-four dialysates have thus far been prepared from 5 different lots of horse serum using this technic with either 1066 or Hanks basic salt solution as the dialyzing fluid, and all supported growth of cells (Table I). No detectable reaction was observed when these dialysates were tested with sulfosalicylic acid.

Thirteen serial passages of the Mox strain and 5 clone cultures derived from the Mox strain, and 8 passages of LLMC #1 have thus far been made in dialysate medium with no diminution in growth activity of the cells. Cells grown in dialysate medium are well flattened and possess extremely clear cytoplasm with smooth unbroken margins. They cling tenaciously to glass and are quite fragile. The 4 other cell strains: HeLa, J-96, HEP 2 and Giardi human heart grew in dialysate medium with the characteristic growth patterns observed in serum supplemented medium, but no quantitative determinations have as yet been made with these strains.

Discussion. All that can be said about the chemical nature of the growth-promoting activity is that it can pass through a cellulose dialyzer membrane, it does not react with sensitive protein precipitants and remains stable for several months at 37°C. Studies on its chemical nature are in progress.

Although the data in Table I clearly indicate that the dialysate is capable of supporting growth of cells to the same extent as serum, some of the dialysates were not as effective as serum in this respect, and individual

TABLE I. Growth of Mox Cells in Medium 1066 Supplemented with Serum, with Dialysate and Unsupplemented in 5 Serum Samples.

No. of dialysates prepared	Dialyzing fluid	Inoculum $\times 10^{-3}$	Cell numbers at 7 days $\times 10^{-3}$		
			Serum supplemented	Dialysate supplemented	Unsupplemented
6	1066	100	1222	991-728*	110
9	"	"	950	624-574	126
10	"	50	715	778-718	>10
6	Hanks	"	630	165-115	45
8	"	"	665	630-490	25
2	"	"	710	622-600	52
2	1066	"	710	670-587	52
2	Hanks	100	606	341-291	120
2	1066	"	606	560-470	120

* Maximum and minimum growth.

dialysates prepared from a given lot of serum varied in amount of growth response elicited. This variability may have been due to factors in the dialysis procedure which were not controlled or to the condition of the glassware at the time tests were carried out. During these experiments difficulties were encountered in washing of our glassware due to a change in the water suply. For example, when cells were placed in medium 1066 alone they would usually settle onto the glass and appear healthy for at least one week, but when this change in glassware was noted the cells were destroyed overnight. Cells grown in dialysate medium did not fare as well in this glassware as they did previously, but little or no effect was observed with cells in serum supplemented medium. In addition to promoting growth, serum probably contains factors that serve to detoxify glass surfaces. Serum dialysate apparently does not contain these factors and the condition of the glassware is thus more critical when cells are grown in dialysate supplemented medium than in serum supplemented medium.

Occasionally a dialysate was obtained which gave a reaction in the test for protein. This was probably due to some defect in the dialyzer membrane and these dialysates were discarded.

Summary. A method for separation of a fraction from horse serum which does not react in tests for protein and which is capable of substituting for whole serum in tissue culture medium has been described. When this fraction was used as a supplement in synthetic medium, cell growth was obtained which compared favorably with controls grown in serum supplemented medium.

1. Evans, V. J., Bryant, J. C., Fioramonti, M. C., McQuilken, W. T., Sanford, K. K., Earle, W. R., *Cancer Research,* 1956, v16, 77.

2. Fisher, H. W., Puck, T. T., Sato, G., *Proc. Nat. Acad. Sci., U. S.,* 1958, v44, 4.

3. Eagle, H., *Science,* 1959, v130, 432.

4. Layne, E., *Methods of Enzymology,* edited by S. P. Colowick and A. K. Kaplan, vIII, 1957.

5. Schenck, D., Moskowitz, M., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 30.

6. Parker, R. C., Castor, L. N., McCulloch, E. A., *Spec. Publ. N. Y. Acad. Sci.,* 1957, v5, 303.

7. Hanks, J. H., Wallace, R. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 196.

8. Richar, W. J., Breakell, E. S., *A. J. Clin. Path.,* 1959, v31, 384.

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