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Maintenance of Fibroblasts in Artificial and Serumless Media.

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The importance of developing artificial media, which can be used in place of serum for maintaining the life of organs and tissues outside the body, hardly needs to be emphasized. Many of the studies for which the organ-culture technic[1] was invented, as well as others that can be carried out by the simpler methods of tissue-culture, depend for their success on the creation of suitable artificial media. These media are needed to reduce the cost of experimentation, to make possible extensive work with human organs and those of small animals from which serum in large quantity cannot be obtained, and for all studies in which the production of serum and other protein substances is to be investigated. For cultivating organs and for all work with tissues in which function rather than growth is the subject of study, it is important that these media maintain the cells without causing proliferation. All the artificial media previously reported have been designed to promote growth.[2,3] The media to be described in this paper were designed for maintenance. One of them is serumless. In the others, a very small amount of serum has been incorporated. The results obtained when these media were used to maintain a pure strain of fibroblasts in tissue-culture are described below. Experiments in which they were used for cultivating organs will be reported in another communication.[4]

The compositions of the media are as follows:

MEDIUM I.

Whole-blood digest to give either 30 or 60 mg % nitrogen
Serum, 2 or 3 %
Tyrode's solution

MEDIUM II.

Whole-blood digest to give either 30 or 60 mg % nitrogen

	per 100 cc	
Cysteine hydrochloride	9.0	mg
Insulin	0.1	unit

[1] Carrel, A., and Lindbergh, C. A., *Science*, 1935, **81**, 621; Lindbergh, C. A., *J. Exp. Med.*, 1935, **62**, 409; Carrel, A., *J. Exp. Med.*, 1937, **65**, 515.

[2] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1926, **44**, 503; 1928, **47**, 353, 371; 1928, **48**, 533; Baker, L. E., *J. Exp. Med.*, 1929, **49**, 163; *Science*, 1936, **83**, 605.

[3] Vogelaar, J. P. M., and Erlichman, E., *Am. J. Cancer*, 1933, **18**, 28.

[4] Some of this work on media for organ-cultivation has already been incorporated by Carrel, A., and Lindbergh, C. A., in *The Culture of Organs*, New York, Paul B. Hoeber, Inc., 1938.

Thyroxine	0.001	mg
Hemin	0.004	"
Vitamin A (containing some D)	100.0	units
Vitamin B_1	0.1	gamma
Vitamin B_2	3.4	"
Ascorbic acid	0.3	mg
Glutathione	1.2	"
Glucose	200.0	"
Potassium iodide	0.13	"
Salts as in Tyrode's solution		

To bring the vitamin A into solution, it was necessary to dissolve it at high concentration in serum and then use a small amount of this serum in the medium.[5] The concentration required proved to be only 0.07%.

Many of the substances in this medium were selected because they had previously been found by Baker,[2] or by Vogelaar and Erlichman,[3] to prolong the life of cells in artificial growth-promoting media. The concentrations of the individual constituents have been adjusted to those that seemed best suited to maintenance.

MEDIUM III.

This contained all the constituents listed under Medium II and in addition the following in 100 cc:

Tryptophane	5 to 10.0	mg
Witte's peptone to give	6.0	" N
Sodium glycerophosphate	57.5	"
Urea	2.4	"
Glycerine	0.2	cc
Thymus nucleic acid*	20.0	mg
Antuitrin	0.2	cc
Adrenalin chloride (1 to 1000)	0.1	"
Eschatin (suprarenal cortex hormone)	0.1	"
Pitressin (pituitary hormone)	0.1	"

This medium also contained some serum, about 0.07%, needed to dissolve the vitamin A.

MEDIUM IV.

This medium contained no vitamin A and no serum. Otherwise, its composition is the same as Medium III.

The blood digest was prepared from whole bovine blood, which was first incubated with chloroform to destroy the antienzyme, and then digested with pancreatin. All undigested proteins and the growth-promoting proteoses formed from them were removed with trichloracetic acid. Then the filtrate was boiled to destroy the remaining trichloracetic acid and drive off the chloroform. The resultant mixture was made isotonic and adjusted to pH 7.4. It was found on analysis to have a ratio of amino to total nitrogen of 0.45.

The procedure used in testing these media for their ability to maintain fibroblasts was as follows: Cultures from a pure strain of

[5] Baker, L. E., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 124.

* Dr. P. A. Levene of The Rockefeller Institute kindly prepared and furnished this substance.

chicken-heart fibroblasts were embedded in Carrel flasks (D-3) in 1 cc of coagulum containing 25% plasma. To get rid of the serum in this coagulum, the cultures were washed on the following day and every 2 days thereafter for 2 hours at 37°C with a large volume of the medium. Then this wash fluid was withdrawn and 0.5 cc fresh medium was supplied. Under this treatment, the serum originally present in the coagulum disappeared in about 2 weeks. The washing was continued, nevertheless, throughout the entire period of cultivation. To ascertain the effect of the various media, the cells were examined microscopically at frequent intervals. At the end of the cultivation-period, which extended from 42 to 56 days, the vitality of the cells and their ability to proliferate were tested by transplanting them into a growth-promoting medium. A sister colony was cultivated in each case in some control medium, the nature of which is indicated below.

Medium I, when tested in this manner, was found to be an excellent maintenance-medium. Fibroblasts cultivated in it were maintained in excellent condition for 42 days. During the first few days, *i. e.*, before all the serum had been washed out of the coagulum, the cells proliferated at a very slow rate. After the serum of the coagulum was removed, they were maintained with little or no proliferation. Then, when growth-promoting substances were added at the end of cultivation, they proliferated again. Control cultures kept in blood digest and Tyrode's solution died soon after all the serum had been removed from the coagulum.

Chicken-heart fibroblasts cultivated in Medium II lived for 50 days. They remained in better condition and outlived sister cultures kept in blood digest alone. But toward the end of the experiment, the cells in the experimental medium became scattered and began to look starved. As it seemed probable that longer cultivation in this medium would not be feasible, the colonies were transferred to a new coagulum and given growth-promoting nutrients. Active proliferation ensued. It would seem, therefore, that this medium can maintain the cells for a considerable time, but not indefinitely.

Medium III was devised in an attempt to improve Medium II. Four of the 10 new constituents added, antuitrin, peptone, tryptophane, and sodium glycerophosphate, when tested separately were found to improve the nutritive and maintenance values of Medium II. The other 6 gave indications of being beneficial, but the magnitude of the results obtained, when each was tested separately, was too small to constitute definite proof that they were essential. A comparison of Medium II and Medium III made on sister cultures of fibroblasts

showed that the cells cultivated in Medium III invariably remained in better condition and also outlived those cultivated in Medium II. When transferred after 56 days of cultivation to a growth-promoting medium, all the colonies cultivated in Medium III proliferated again, while only 25% of those cultivated in the simpler medium were found to be capable of renewed growth. In one experiment, some colonies that had been cultivated for a month by the procedure outlined above were left for another month without change of fluid. At the end of this time, the cells were still found to be in good condition and able to proliferate.

But Medium III is not serumless. In order to incorporate some vitamin A in it, 0.07% of serum had to be used. Vitamin A was added to this medium because it is always present in serum and has also been found a necessary constituent of artificial growth-promoting media. But no evidence that it was essential for maintenance has been obtained. Hence, to ascertain if this vitamin and the serum used to dissolve it could be eliminated, sister colonies of fibroblasts were cultivated in Medium III, with and without vitamin A. All the differences observed were in favor of the serumless medium. The cells in this medium seemed to be a little clearer and in better condition throughout the entire period of their cultivation. After 62 days, the colonies that had been carried in the serumless medium were transferred to a new coagulum and given growth-promoting nutrients. They responded by growing actively.

To summarize: Four media have been described in which fibroblasts in pure strain have been maintained in vital condition and with little or no proliferation for periods varying from 42 to 56 days. The first of these media is simple, inexpensive, and easy to prepare. The last is serumless.

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Simultaneous Distemper and Lymphocytic Choriomeningitis in Dog Spleen and the Sparing Effect on Poliomyelitis.

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While continuing the study of canine distemper in monkeys, evidence accumulated which indicated that our virus-source material