THE CULTIVATION OF TISSUES FROM CHICK EMBRYOS IN SOLUTIONS OF NaCl, CaCl₂, KCl AND NaHCO₃

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TWENTY-FOUR FIGURES

Having found that tissues from chick embryos grow readily in artificial media, nutrient agar and bouillon,¹ the next step was the attempt to cultivate such tissues in media all the constituents of which are known. In collaboration with Dr. Arthur Koelker we were able to obtain excellent growth in combinations of amido-acids and polypeptids of known constitution, in Locke's solution. Locke's solution with maltose and dextrose was then tried with such good results that Locke's solution was attempted with the very startling discovery that these embryonic tissues grow readily in the Locke's solution and various combinations of NaCl, CaCl₂, KCl, and NaH CO₃ as indicated in table 1.

So far the cultures in salt solutions have not grown as extensively as those in Locke's solution plus dextrose or maltose or the amido-acids and polypeptids, nor have these given as extensive growth as nutrient agar or bouillon, and these in turn have not been as fine or lived as long as those in plasma.

It is to be hoped that an artificial medium will be found as satisfactory as the plasma, for the advantages are obvious if one can work with a known medium in the investigation of the many new problems, which suggest themselves.

¹ On the growth of embryonic chick tissues in artificial media, nutrient agar and bouillon. Johns Hop. Hospital Bul., April, 1911.

The following account of the growth of tissues in salt solutions is fragmentary and work is being continued along various lines such as the differences in behavior of different organs in various solutions and the attempt to obtain an optimum solution for each tissue.

THE METHOD

Usually 10 cc. of each solution is made up from stock solutions. These are autoclaved and then poured into warm Petri dishes. Small pieces several millimeters in diameter are cut out of the various organs of the embryo and put directly in the solution. Each piece is then cut up into a number of much smaller pieces a fraction of a millimeter in diameter. With a capillary tube one or more are drawn up and transferred with a small drop of the solution onto a cover-glass and then inverted, to form a hanging drop, over a hollow ground slide on which has been placed a ring of stiff vaseline. The slides are kept at about 40° C.

The embryos are dissected under aseptic conditions. Instruments, slides, covers and capillary tubes are sterilized in a free flame.

Ordinary vaseline can be stiffened by the addition of paraffin.

The cultures are examined on a warm stage.

Permanent preparations are made by fixing the coverslips in osmic vapor and staining in Ehrlich's haematoxylin. The growths adhere to the coverslips and there is practically no distortion.

All photographs and drawings except those of the nerves were made from fixed preparations.

Eighty-five culture media were prepared at different times and either six or twelve cultures made from each, making in all about 600 slides, which were examined daily. Among the eighty-five are repetitions to test more carefully the medium or the growth of tissues from chicks of different ages. The earlier cultures contain only liver, the more recent ones usually have two or more tissues, such as liver, intestine, kidney, heart and spleen, in the same hanging drop.

It will be seen from the table that tissues did not grow in solutions without NaCl; on the other hand, tissues grow readily with-

TABLE 1

*Showing percentages of salts**L = liver, I = intestine, K = kidney, H = heart, S = spleen*

NaCl	CaCl ₂	KCl	NaHCO ₃	NUMBER OF CULTURES	NUMBER WITH GROWTH	AGE OF CHICK	ORGAN	DURATION OF GROWTH	EXTENT OF GROWTH
(1) H ₂ O.				6	0	10	L, I, K		
(2) 0.3375				6	0	8	L, I		
(3) 0.6525				6	0	8	L, I		
(4) 0.75				12	8	8	L	2	Slight
(5) 0.9				12	2	5	L	2	Extensive
(6) 1.575				6	0	8	L, I		
(7) 2.025				6	0	8	L, I		
(8)			0.005	6	0	10	L, I K		
(9)			0.015	6	0	10	L, I K		
(10)			0.05	6	0	10	L, I K		
(11) 0.75	0.02			12	0	8	L		
(12) 0.9	0.00625			6	0	13	L		
(13) 0.9	0.00625			6	5	8	L, I	3	Extensive
(14) 0.9	0.0125			6	0	13	L		
(15) 0.9	0.025			6	3	8	L, I	3	Extensive
(16) 0.9	0.125			6	6	8	L, I	3	Extensive
(17) 0.675		0.10		6	6	8	L, I H	5	Good
(18) 0.9			0.05	6	0	10	L, I K		
(19)	0.0625	0.10		6	0	8	I, I		
(20)	0.125	0.21		6	0	8	L, I		
(21)	0.25	0.42		6	0	8	L, I		
(22)		0.10	0.05	6	0	10	L, I K		
(23)	0.025	0.04	0.02	6	0	8	L, I		
(24)	0.075	0.12	0.02	6	0	8	L, I		
(25)	0.125	0.31	0.1	6	0	8	L, I		
(26) 0.657		0.10	0.05	6	6	8	L, I, H	2-3	Good
(27) 0.675	0.125		0.1	6	1	8	L, I	2	Slight
(28) 0.9	0.025		0.02	6	6	8	L, I	3-4	Extensive

TABLE 1—Continued

	NaCl	CaCl ₂	KCl	NaHCO ₃	NUMBER OF CULTURES	NUMBER WITH GROWTH	AGE OF CHICK	ORGAN	DURATION OF GROWTH	EXTENT OF GROWTH
(29)	1.35	0.05		0.04	6	6	8	L, I	3-4	Extensive
(30)	0.9	0.00625	0.021		6	3	13	L	2-3	Good
(31)	0.9	0.00625	0.042		6	3	13	L	2-3	Fair
(32)	0.9	0.0125	0.021		6	4	13	L	2-3	Slight
(33)	0.9	0.0125	0.04		6	6	13	L	3-4	Good
(34)	0.9	0.025	0.021		6	0	13	L		
(35)	0.9	0.025	0.042		6	2	13	L, H	2	Fair
(36)	0.9	0.0125	0.021	0.01	6	4	13	L, I	2-3	Good
(37)	0.9	0.0125	0.021	0.02	6	4	13	L, I	2-3	Extensive
(38)	0.9	0.025	0.021	0.01	6	6	13	L, S	3-4	Good
(39)	0.9	0.025	0.021	0.02	6	6	13	L, I	3-4	Good
(40)	0.9	0.025	0.042	0.01	6	6	13	L, I	2-3	Good
(41)	0.9	0.025	0.042	0.02	6	4	13	L, I	2-3	Good
(42)	0.9	0.025	0.042	0.02	6	6	8	L, I	4	Good
(43)	0.9	0.025	0.042	0.02	12	12	7	L	6	Extensive
(44)	0.9	0.025	0.042	0.02	6	4	9	L	4	Slight
(45)	0.9	0.025	0.042	0.02	6	5	5	L	3	Extensive
<i>Sodium decreased</i>										
(46)	0.675	0.025	0.042	0.02	6	5	8	L, I	2-3	Extensive
(47)	0.45	0.025	0.042	0.02	6	4	8	L, I	2	Good
(48)	0.225	0.025	0.042	0.02	6	0	8	L, I		
(49)	0.675	0.0625	0.105	0.05	6	6	8	L, I, H	1-2	Good
(50)	0.675	0.125	0.21	0.1	6	6	8	L, I, H	4-5	Extensive
(51)	0.675	0.125	0.21	0.1	12	12	7	L	2	Extensive
(52)	0.225	0.150	0.252	0.12	6	6	8	L, I	3	Extensive
<i>Sodium increased</i>										
(53)	1.	0.025	0.042	0.02	12	8	4	L	2-3	Extensive
(54)	1.1	0.025	0.042	0.02	14	12	7	L	3-4	Extensive

TABLE 1—Continued

	NaCl	CaCl ₂	KCl	NaHCO ₃	NUMBER OF CULTURES	NUMBER WITH GROWTH	AGE OF CHICK	ORGAN	DURATION OF GROWTH	EXTENT OF GROWTH
(55)	1.2	0.025	0.042	0.02	12	11	7	L	3-5	Extensive
(56)	1.125	0.025	0.042	0.02	6	6	7	L	2	Fair
(57)	1.23	0.025	0.042	0.02	6	2	7	L	1-2	Slight
(58)	1.2375	0.025	0.042	0.02	6	6	8	L, I	8-9	Extensive
(59)	1.3	0.025	0.042	0.02	12	5	8	L	2	Extensive
(60)	1.35	0.025	0.042	0.02	6	5	7	L	2-3	Extensive
(61)	1.45	0.025	0.042	0.02	12	0	8	L		
(62)	1.4625	0.25	0.042	0.02	6	6	8	L, I	8-9	Extensive
(63)	1.46	0.025	0.042	0.02	6	3	9	L	3	Extensive
(64)	1.5	0.025	0.042	0.02	12	0	8	L		
(65)	1.57	0.025	0.042	0.02	6	0	9	L		
(66)	1.575	0.025	0.042	0.02	6	0	15	L		
(67)	1.585	0.025	0.042	0.02	6	6	8	L,H,I	8-9	Extensive
(68)	1.6975	0.025	0.042	0.02	6	1	8	L,H,I	3	Slight
(69)	1.7	0.025	0.042	0.02	12	0	7	L		
<i>Calcium decreased</i>										
(70)	0.9	0.00625	0.042	0.02	6	4	13	L, I	2-3	Good
(71)	0.9	0.0125	0.042	0.02	6	3	13	L,I,S	3	Good
<i>Calcium increased</i>										
(72)	0.9	0.035	0.042	0.02	6	2	15	L	2-3	Good
(73)	0.9	0.04	0.042	0.02	6	2	15	L	2-3	Slight
(74)	0.9	0.045	0.042	0.02	6	1	15	L	2-3	Extensive
(75)	1.575	0.05	0.042	0.02	6	0	15	L		
(76)	0.9	0.06	0.042	0.02	6	2	15	L	2-3	Good
(77)	0.9	0.075	0.042	0.02	6	2	15	L	2-3	Fair
(78)	0.9	0.225	0.042	0.02	6	0	15	L		
(79)	0.45	0.0125	0.021	0.01	12	6	8	L	3	Good
(80)	0.225	0.00625	0.010	0.005	12	0	8	L	0	

TABLE 1—Continued

Isotonic solutions

	NaCl	CaCl ₂	KCl	NaHCO ₃	NUMBER OF CULTURES	NUMBER WITH GROWTH	AGE OF CHICK	ORGAN	DURATION OF GROWTH	EXTENT OF GROWTH
(81).....	1.167	0.025	0.042	0.02	6	5	9	L, I	3	Extensive 1 or 2 cells
(82)	0.0	1.575	0.042	0.02	6	?2	9	L, I		
(83)	0.032	1.532	0.042	0.02	6	0	9	L, I		
(84)	0.0	0.025	1.545	0.02	6	0	9	L, I		
(85)	0.03	0.025	1.503	0.02	6	0	9	L, I		

out either CaCl₂ or KCl, or NaHCO₃. The percentage of NaCl in the various solutions may vary from 0.225 to 1.6975. The cultures seem to live longest in the higher percentages. The percentage of CaCl₂ may vary from 0 to 0.15 and even a higher percentage may allow growth, for the growth with 0.15 per cent was quite extensive, showing that the limit was not reached. The percentage of KCl may vary from 0 to 0.252 but here likewise the upper limit has not been determined. The percentage for NaHCO₃ may vary from 0 to 0.12.

Growth can take place in NaCl alone, in NaCl in combination with CaCl₂ or KCl or both, in NaCl and NaHCO₃ with either CaCl₂ or KCl or both. Only one combination of NaCl and NaHCO₃ was tried, no growth occurred but there are probably combinations in which growth will take place.

NaCl is apparently the most essential salt and although the others are not necessary the maximum growth was attained when all four were used. With the 1.585 per cent growth did not begin until the second day, with 1.6975 per cent it did not appear until the third day.

THE CHARACTER OF GROWTH

Much uncertainty exists as to exactly which kind of cells give rise to the various types of growth. Most organs have a variety of cells any one of which may behave in a number of ways. There is considerable variation in the character of the growth and in the character of the individual cells both in different cultures and from the same piece.

The table is far from a complete one and many more combinations remain to be tried out. Many more cultures should also be made under as similar conditions as possible with some of the solutions already tried, before correct conclusions can be drawn as to the effect of various combinations on growth. Two pieces of tissue taken from the same organ of the same embryo, cut into small pieces in the same dish and transplanted into the same drops will show sometimes marked differences in growth. The main difference in the preparation consists in the cutting up of the pieces with the scissors. There are also differences in the size and thickness of the drop.

These salt solutions can hardly be considered as nutrient media but correspond more to what Loeb designates as 'protective media.' It is quite remarkable that cells will grow in such widely different solutions where the osmotic pressure must vary considerably. It will be interesting to determine the osmotic pressures of these solutions.

Such growth as does take place will probably be limited by the amount of stored up food in the cells as well as by the medium. In most of the solutions growth seems to be accelerated during the first twenty-four or forty-eight hours as compared with that in plasma. In some of the solutions, however, as has been noted, growth is retarded.

The small pieces, which are to be planted are often kept for an hour or more in 10 cc. of solution before transplanting. The plasma, blood and lymph are thus well washed out, and so the later growth of cells into the solution is not to be explained by the influence of escaped plasma about the piece.

It is difficult to distinguish between growth and the migration of cells from the transplanted piece. Active growth is indicated

by membrane formation of all types and by the extensive radiation and reticular formations especially when mitosis is found here and there as it is in the stained preparations. Likewise, the outgrowth of the nerve fibers undoubtedly shows that growth occurs. What proportion of the cells merely wander outward from the piece without cell-division cannot be readily determined. Both mitotic and amitotic cell division take place.

The liver from the older embryos does not seem to grow nearly as well in the various solutions as that taken from younger ones. The intestine on the other hand grows about equally well whether it be taken from eight or thirteen-day embryos.

In many of the solutions maximum growth occurred during the first twenty-four hours but in Locke's solution with the higher percentage of NaCl growth is delayed and prolonged.

The most common form of growth consists of radiating and anastomosing cells, some of these may become entirely free in the surrounding area either singly or in groups. All gradations are found between such radiations and loose reticular formations and a continuous syncytial membrane one layer thick. The reticular formation may consist of rather broad cells with numerous processes or the cells may be exceedingly thin, the nucleus bulging out on either side. Such cells often have processes many times the length of the cell.

There is a striking similarity between the growth of normal tissues of embryo chicks in salt solutions, bouillon, nutrient-agar, and plasma and those pictured by Lambert and Hanes² for the growth of rat and mouse sarcoma and carcinoma in plasma.

The growing cells in salt solutions differ from those in plasma in one very noticeable feature, namely, the absence of the accumulation of fat or oil globules within the cell body which is so characteristic of plasma cultures.

In addition to these forms of growth the intestine often gives rise to a compact membrane with small nuclei. The cell borders can often be seen in the living specimens. They remind one very much of peritoneal mesothelium. The spleen, on the other

² Jour. Exp. Med., May, 1911.

hand, usually gives rise to many migrating cells, which move outward in a band.

In one culture of an eight-day embryo the outgrowth of sympathetic nerve fibers was followed. When first observed 18 hours after the culture was made they had already grown out quite extensively. During the next twenty-four hours there was a great increase in the number and length of the fibers. The process of growth in this solution (29) was essentially the same as that previously observed in a plasma medium. The growing fibers seem to make and break anastomoses with other fibers or to pass by each other without anastomosing. During the third day bead-like degeneration began.

Growth apparently takes place as well in fluid media as in the more solid lymph, plasma or agar media. The fibrin threads are not necessary either for the outgrowth of nerve fibers or the various types of cells. The outgrowing cells do, however, often creep along on the coverglass and the nerve fibers may have done so as well. In the hanging drop cultures the long radiating fibers go upward to the underside of the coverglass while in Petri dish cultures they often grow down to the bottom of the dish and anchor the piece of tissue quite firmly.

The adhesion of the growth to the cover slip probably explains why one can get such good permanent preparations, that are neither washed off nor distorted in the staining and dehydrating.

Fig. 1 From series 71 (see table). Twenty-hour culture of intestine, showing compact peritoneal? membrane and connective tissue? or syncytial membrane. $\times 60$ diameters.

Fig. 2 From series 39. Twenty-hour culture of intestine, showing a similar membrane and connective tissue ? growth. $\times 60$ diameters.

Fig. 3 From series 42. Eighteen-hour culture of intestine, showing compact membrane only. $\times 60$ diameters.

Fig. 4 From series 58. Two-day culture of liver, showing radiating strands of connective tissue. $\times 60$ diameters.

Fig. 5 From series 37. Twenty-hour culture of intestine. $\times 60$ diameters.

Fig. 6 From series 76. Three-day culture of liver, showing anastomosing islands of new cells. $\times 60$ diameters.

Fig. 7 From series 67. Three-day culture of liver, showing both radiating strands and syncytial formation. $\times 60$ diameters.

Fig. 8 From series 45. Twenty-four hour culture of liver, showing a different type of radiation with patches of syncytium. $\times 60$ diameters.

Fig. 9 From series 62. Two-day culture of liver, showing extensive radiation and syncytium. $\times 60$ diameters.

Fig. 10 From series 51. Twenty-hour culture of liver. $\times 60$ diameters.

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Fig. 11 From series 39. Forty-four-hour culture of liver showing syncytial membrane and migrating cells. $\times 60$ diameters.

Fig. 12 From series 55. Twenty-four-hour culture of liver. $\times 60$ diameters.

Fig. 13 From series 42. Enlarged drawing of the edge of the compact membrane of fig. 3, showing the thin protoplasmic margin. $\times 425$ diameters.

Fig. 14 From series 71, (see fig. 1, 14). Enlarged drawing of the junction of the compact membrane with the syncytial membrane of fig. 1. $\times 425$ diameters.

Figs. 15-18 From series 37 (see fig. 5). Enlarged drawings from fig. 5, 15 of the syncytial membrane with nuclei full of chromatin granules; 16, syncytial membrane; 17, branched spindle cells; 18, loose syncytial net. $\times 425$ diameters.

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Fig. 19 From series 65. Three-day culture showing syncytial membrane. $\times 325$ diameters.

Fig. 20 From series 45, (see fig. 8, 20). Enlarged drawing of fig. 8, showing the reticular formation. $\times 325$ diameters.

Figs. 21-24 From series 29. Four drawings of the outgrowth of sympathetic nerve fibers made 18, 19, 20 and 41 hours after the piece was transplanted; 22 shows only a small area. $\times 300$ diameters.

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