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THE MULTIPLICATION OF FIBROBLASTS IN VITRO.

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It is well known that the life of tissues cultivated in the plasma of an adult animal lasts only for a certain period of time. 10 years ago, one of the writers attempted to prevent the death of the tissues living under these conditions.[1] It was supposed that if their waste products were removed by frequent washings, the cells would multiply indefinitely. Every few days, fragments of embryonic heart, cultivated in plasma, were washed in Ringer solution, and placed in a fresh medium. The duration of their life was very much increased, but death still occurred sooner or later. No tissue could be kept alive for more than 3 months.[2] It was evident that the adult plasma did not contain the substances necessary for the indefinite multiplication of fibroblasts. This fact was confirmed by Quagliariello,[3] who injected serum proteins into dogs, and found that they are not used as food by the tissue cells. In the more recent experiments of Kerr, Hurwitz, and Whipple,[4] there was no evidence that serum proteins may be considered as intermediary products between food proteins and parenchyma proteins. Although connective tissue cannot live indefinitely in plasma, it often displays a great activity for several days or weeks. What is the origin of the substances used by the cells in the process of multiplication? The opinion of Lewis,[5] and Ingebrigtsen[6] is that the tissues grow within the limits determined by the amount of food stored up in the body of each individual cell. Burrows[7] also believes that the food material of the tissues is not derived from the medium but from the cells within the fragment. The growth observed in the culture would be a simple transfer of material from the cells of the center of the fragment to those which have migrated into the medium. When growth ceases after a few transplanta-

[1] Carrel, A., *J. Am. Med. Assn.*, 1911, lvii, 1611.

[2] Carrel, A., *J. Exp. Med.*, 1912, xv, 516.

[3] Quagliariello, G., *Arch. fisiol.*, 1912, x, 150.

[4] Kerr, W. J., Hurwitz, S. H., and Whipple, G. H., *Am. J. Physiol.*, 1918–19, xlvii, 356.

[5] Lewis, M. R., and Lewis, W. H., *Anat. Rec.*, 1911, v, 277.

[6] Ingebrigtsen, R., *J. Exp. Med.*, 1912, xvi, 421.

[7] Burrows, M. T., *Anat. Rec.*, 1916–17, xi, 335. Burrows, M. T., and Neymann, C. A., *J. Exp. Med.*, 1917, xxv, 93.

tions, the sum of the total growth would be less than the original mass. Although no accurate measurements have been made, there is no doubt that a fragment of tissue cultivated in the plasma of an adult animal does not increase materially in size, in spite of the active proliferation of the cells. But the cause of this temporary activity has not as yet been ascertained.

It is also well known that the addition of embryonic tissue juice to the plasma of an adult animal activates the rate of cell division and brings about an immense increase in the mass of the tissue.[8] A strain of fibroblasts derived from a small fragment of embryonic heart has produced about 30,000 cultures in the past 9 years, and is as active today as at the beginning of its life. If this strain had been allowed to grow freely, the volume of tissue so produced would be very much larger than the earth. There is no doubt, therefore, that when embryonic juice is added to adult plasma new cells are made from the substances contained in the medium, and that this process can go on indefinitely.[9]

It then appears that adult plasma alone, and plasma mixed with embryonic juice, differ widely in their action on the growth of tissue. The reason for these differences is still incompletely known. It is certain that a mixture of embryonic juice and adult plasma had the power to increase the rate of cell division. But the part played respectively by the constituents of the medium in the multiplication of the cells is not thoroughly understood. The purpose of this article is to investigate whence come the substances used by the fibroblasts cultivated in adult plasma alone, and what constituents of the medium are responsible for the increase of the mass of the tissue when embryonic juices are added to the plasma.

I.

Although the mass of a tissue cultivated in the plasma of an adult animal does not increase, or increases very little, the building of the new cells requires some material which must come from the plasma or from the tissue itself. In order to determine the part played by the plasma, the action of its constituents, fibrin and serum, was separately studied. Connective tissue having been found to grow as extensively in fibrin fixed by formaldehyde as in normal fibrin, the rôle of fibrin must be considered as purely mechanical. In a first series of experi-

[8] Carrel, A., *J. Exp. Med.*, 1913, xvii, 14. Ebeling, A. H., *J. Exp. Med.*, 1913, xvii, 273. Carrel, A., *J. Exp. Med.*, 1913, xviii, 287; 1914, xx, 1.

[9] Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 531.

ments, the influence of serum was investigated by measuring the growth of connective tissue in media containing no serum, and serum of different concentrations. In a second series of experiments, the possible action of substances contained in the tissues themselves was examined.

1. Rate of Growth of Tissue in Media Containing Varied Dilutions of Serum.—Fragments of embryonic heart and of a 9 year old strain of fibroblasts were cultivated in media composed of fibrinogen suspension and of Tyrode solution, and containing no serum, or serum in varied dilutions. The serum was taken from the plasma of chickens which had fasted 24 hours. The animals were about 2 years old and in good health. The serum was preserved in paraffined or Pyrex tubes, and its hydrogen ion concentration was measured. Sometimes it was found slightly modified after a few days. The serum was diluted with Tyrode solution sterilized by filtration through a Berkefeld filter. The suspension of fibrinogen was prepared by a technique already known.[10] A medium composed of 10 per cent fibrinogen suspension and 90 per cent Tyrode solution gave a firm coagulum in which embryonic heart tissue could be cultivated without the occurrence of liquefaction. But if a fragment of old strain of fibroblasts was used, liquefaction took place. A little serum had to be added to the medium to prevent this accident. The other media were made of 10 per cent fibrinogen suspension and of mixtures of Tyrode solution and of serum, such that the concentration of serum varied from 2.37 to 90 per cent. The cultures were prepared and measured according to a method previously described.[11] The area of the original fragment was measured immediately after the preparation of the culture, and 48 hours later. The growth was expressed generally by the relative increase of the surface of the tissue; that is, the total area minus the area of the original fragment, divided by the area of the original fragment. The ratio of the relative increase of the experiment to the relative increase of the control permitted the comparison of the growth of cultures belonging to different experiments or groups of experiments. The absolute increase of the tissue was known by the

[10] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiii, 641.

[11] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

width of the ring of new tissue around the original fragment, measured with a micrometer.

In ten experiments, embryonic heart tissue was cultivated in media containing from 0 to 90 per cent serum (Table I). The growth of every fragment in the experimental medium was compared to the growth of a control in plasma. The increase of the tissues was expressed by the width of the ring of fibroblasts. The experiments in which the layer of new tissue was of unequal thickness were discarded. The figures of Table I show that the tissues grew at least as well in Tyrode solution containing no serum as in plasma. The growth was larger in media, the serum concentration of which varied from 2.37 to 85 per cent, than in plasma. When the amount of growth obtained in a given concentration of serum was compared with that obtained, not in plasma, but in another concentration of serum, it was found that the action of a medium containing 2.37 per cent serum was as marked as that of a medium containing 90 per cent serum. The growth of embryonic heart appeared to be independent of the concentration of the serum as long as the amount of fibrin was not modified. This shows that the cells in their process of multiplication do not make use of the serum to an extent measurable by the present method.

In thirty experiments, an old strain of fibroblasts was used instead of embryonic heart (Table II). It did not grow in a medium composed only of fibrin and Tyrode solution, because the coagulum liquefied in a few hours. There was practically no difference between the amount of growth of the fragments cultivated in media the serum concentrations of which varied from 2.37 to 80 per cent. When fragments of the old strain were cultivated in 10 per cent serum and 50 per cent serum, and kept in the same media for several passages, no difference in the amount of new tissue was observed, even after four passages, as is shown by Text-fig. 1. In several experiments, two fragments of the old strain were studied comparatively in plasma and in 10 per cent serum. The tissues grew at about the same rate, in spite of the great difference in the composition of the medium. The rate of growth decreased rapidly and in about the same way in both media. Death occurred after the sixth or seventh passage. In Text-fig. 2 is shown a typical experiment. The decrease in the rate of growth is more rapid during the first passages in normal plasma

TABLE I.

Growth of Embryonic Heart Tissue in Plasma and Varied Dilutions of Serum, Expressed in Terms of Width of Ring.

Experiment No.	Culture No.	Composition of medium.																				Remarks.
		Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	
			0 per cent.		2.37 per cent.		8.3 per cent.		16.5 per cent.		20.5 per cent.		22 per cent.		30 per cent.		33 per cent.		50 per cent.		85 per cent.	
1	18585				1.5		1.8		1.1													
2	18656	0.2	0.4	0.1	0.2	0.2	0.1	0.05	0.2	0.2	1.0					0.2	2.0	0.1	2.0			
3	18700	0.5	Liquefied.	0.4	1.6	0.3	2.0	0.2	0.3													
4	18735	0.6	1.0	0.1	1.6	1.0	1.0	0.7	1.6			0.7	1.3	0.6	1.6							
5	18795	0.8	1.0	0.8	1.1	0.7	1.3	0.8	1.2			0.8	1.2	1.0	1.4							
6	18840	0.5	0.5	0.5	2.0	0.4	1.6	0.7	1.8			0.7	1.5	0.6	0.5							
7	18864	1.5	2.0	1.5	4.0	1.6	4.5	1.3	3.3			1.1	3.5	1.1	1.1							
8	18916	0.8	2.0	0.6	2.2	0.5	3.6	0.6	1.2			0.6	1.1	0.8	1.6					0.9	1.2	
9	18951							0.7	0.5													
10	18962	0.9	2.5	1.2	1.2	1.9	1.7															90 per cent serum control instead of plasma.
Average.		0.6	1.0	0.6	1.7	0.8	1.9	0.6	1.2	0.2	1.0	0.8	1.7	0.8	1.2	0.2	2.0	0.1	2.0	0.9	1.2	

The increase of the tissues is expressed by the width of the ring of fibroblasts which invaded the medium over a period of 48 hours. In the first nine experiments, the control was cultivated in adult plasma. In the tenth experiment, the control was cultivated in 90 per cent serum instead of plasma.

TABLE II.

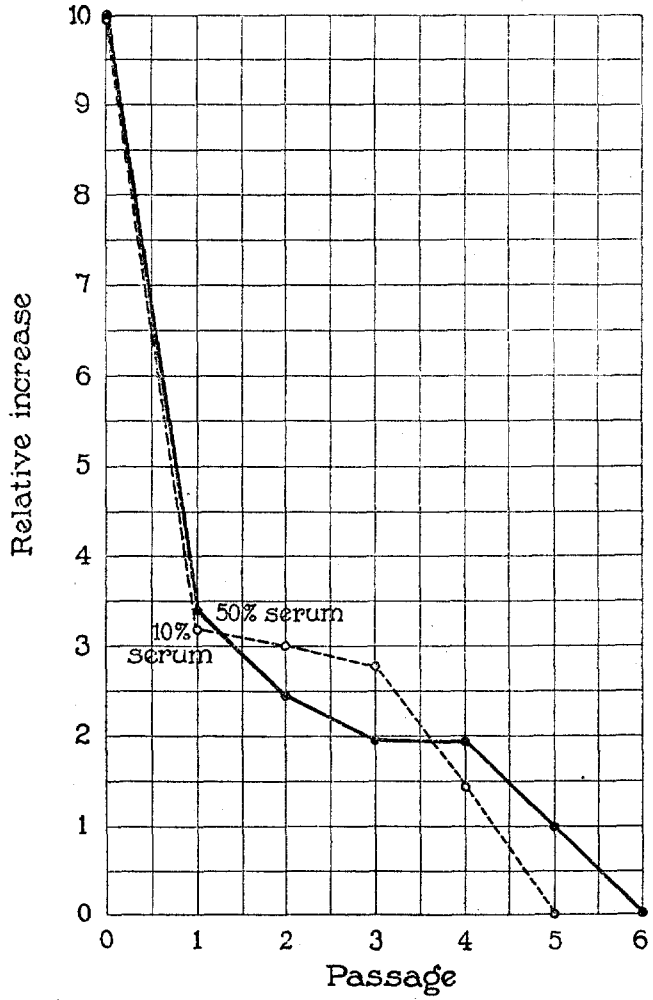
Growth of an Old Strain of Fibroblasts in Plasma and Varied Dilutions of Serum.

Experiment No.	Culture No.	Date.	Composition of medium.															
			Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, serum.	Experiment, serum.	Control, serum.	Experiment, serum.	Control, serum.	Experiment, serum.	Control, serum.	Experiment, serum.
				2.37 per cent.		8.3 per cent.		16.5 per cent.	90 per cent.	22 per cent.	90 per cent.	30 per cent.	10 per cent.	50 per cent.	10 per cent.	80 per cent.
		1920														
1	18651	Dec. 13			1.0	1.2										
2	18684	" 20	0.1	0.2	(After 24 hrs.)											
3	18784	" 20	2.5	1.5												
4	18785	" 20	2.0	1.2												
5	18786	" 20			2.2	2.2										
6	18787	" 20			1.6	1.8										
7	18861	" 23	1.7	1.5												
8	18862	" 23	Average,	Average,	1.7	1.5										
9	18863	" 23	2.07	1.6	1.5	1.5										
10	18951	" 28			Average,	Average,	1.1	1.2								
		1921			1.6	1.64										
11	19046	Jan. 3							0.5	0.9						
12	19047	" 3							0.7	1.0						
13	19048	" 3							0.6	0.8						

14	19049	Jan. 3	Control serum.								0.8	0.9				
15	19050	" 3	90 per cent.								0.75	1.0				
16	19051	" 3									0.7	0.8				
17	19067	" 5	Few cells.	1.6	Control serum.											
18	19068	" 5	" "	1.6												
19	19069	" 5	" "	1.9	91 per cent.											
20	19098	" 8		Average,	0.1	1.5										
21	19233	" 17		1.7	0.5	1.1										
22	19234	" 17			0.6	1.0										
23	19235	" 17			0.4	1.3										
24	19237	" 17			0.5	1.3										
25	19364	" 28			Average,	Average,										
26	19365	" 28			0.42	1.22										
27	19366	" 28														
28	19385	" 30											2.0	2.0	1.0	1.0
29	19386	" 30											2.0	2.2	1.1	1.2
30	19387	" 30											2.2	2.2	1.0	1.2
Average							1.1	1.2	0.6	0.9	0.75	0.9	2.07	2.13	1.03	1.13

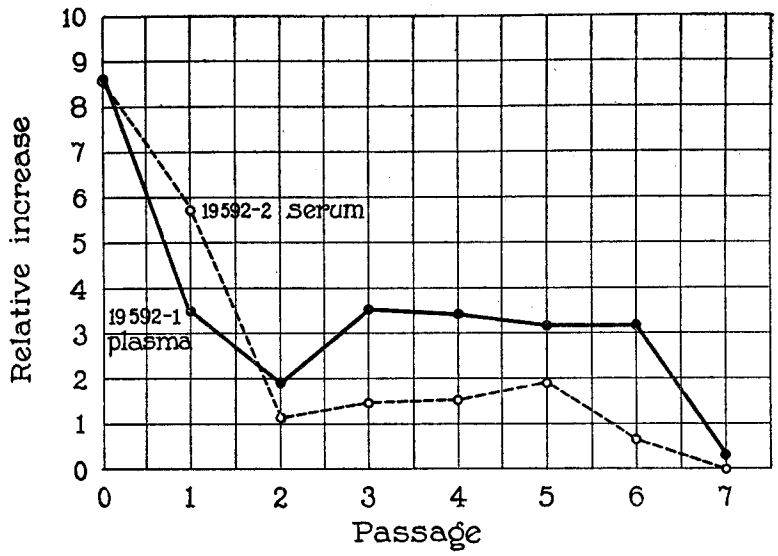
The increase of the tissue is expressed in terms of the width of the ring of new tissue produced in 48 hours.

than in a medium containing only 10 per cent serum. Afterwards, there are only small differences in the amount of tissue produced in both media, and death occurs at the same time. This completes the



TEXT-FIG. 1. Comparison of the rate of growth of two fragments of an old strain of fibroblasts in media containing 10 and 50 per cent serum, in the course of five and six passages (Experiment 19364).

demonstration that the fibroblasts in adult plasma or serum do not make use of the serum proteins.



TEXT-FIG. 2. Comparison of the rate of growth of an old strain of fibroblasts in adult plasma and 10 per cent serum, in the course of seven passages (Experiment 19592).

2. Rate of Growth of Tissue in Plasma.—Fragments of embryonic heart and of an old strain of fibroblasts were cultivated in adult plasma, transferred into new medium every 48 hours, and studied comparatively. The heart was taken from 10 to 14 day old chick embryos, cut in small fragments in a little Ringer solution, and imbedded in plasma. Coagulation always occurred spontaneously after a few minutes, and the growth was measured at the end of 48 hours. During several passages, the tissues displayed great activity, and the surface of the original fragment increased, as well as the width of the ring of new tissue. The rate of growth reached its maximum after a few passages. Then it progressively decreased and death occurred after from ten to thirty passages. The individual differences in the length of life of the cultures were probably due to the nature of the plasma and to the technique used in the transfer of the tissue. The details of two typical experiments are given as an illustration in Table III and Text-fig. 3. The surface of the original fragment, the surface of the new tissue, and the relative increment of the fragment at

each passage were calculated. In the first experiment, the value of the relative increment increased as far as the fourth passage. At the same time, the area of the fragment became larger and reached its maximum at the fifth passage. Later, it progressively decreased and death occurred after the twelfth passage. In the second experiment there was also an increase in the rate of growth of the fragment and

TABLE III.

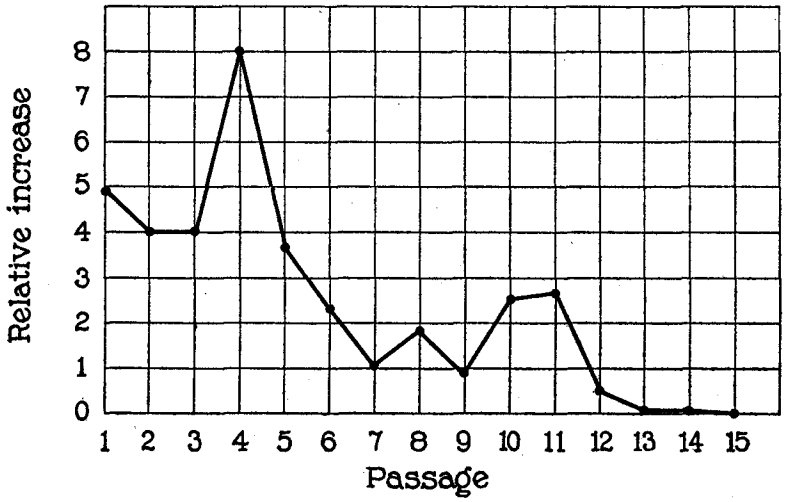
Growth of Embryonic Heart Tissue in Adult Plasma during Fifteen and Eleven Passages (Experiments 18947 and 18948).

Experiment 1.						Experiment 2.					
Passage No.	Culture. No.	Date.	Area of fragment.	Area of growth.	Relative increase.	Passage No.	Culture No.	Date.	Area of fragment.	Area of growth.	Relative increase.
		1920						*1920*			
1	18947	Dec. 29	1.6	9.1	5.69	1	18948	Dec. 28	1.0	4.9	4.9
2	19006	" 30	1.8	5.7	3.2	2	19007	" 30	1.5	6.0	4.0
		1921						*1921*			
3	19028	Jan. 1	2.1	12.5	5.92	3	19029	Jan. 1	2.5	10.0	4.0
4	19044	" 3	2.5	19.3	7.7	4	19045	" 3	2.1	16.9	8.0
5	19065	" 5	5.1	20.1	4.0	5	19066	" 5	2.6	9.5	3.65
6	19091	" 7	3.5	11.5	3.85	6	19092	" 7	3.3	7.5	2.29
7	19122	" 10	4.4	4.3	1.0	7	19123	" 10	2.8	3.0	1.07
8	19152	" 12	2.7	2.8	1.0	8	19153	" 12	2.3	4.2	1.8
9	19189	" 14	1.2	0.7	0.06	9	19190	" 14	1.9	1.6	0.89
10	19214	" 15	1.5	5.2	3.45	10	19215	" 15	1.3	3.25	2.5
11	19231	" 17	2.1	Few cells.	0	11	19232	" 17	1.7	4.5	2.65
						12	19255	" 19	1.3	0.7	0.5
						13	19280	" 21	0.7	0.4	0.06
						14	19294	" 22	0.5	0.6	0.1
						15	19314	" 24	0.4	0	0

in their size, the maximum of which was reached at the sixth passage. It was impossible to know whether the active multiplication of the cells and the increase in the area of the original fragment during the first passages meant an increase in the volume of the fragment. This point could be ascertained if the tissues were weighed or the cells counted, but the precision of the method which was used is not sufficient to detect a slight and temporary increase of the mass. It

was obvious, however, that the activity of the cultures reached a maximum after a few passages, then decreased until death occurred. It seemed that in the beginning of its life *in vitro* the growth was activated by something which progressively disappeared.

Fragments of a 9 year old strain of fibroblasts were also grown in adult plasma. Previous to the experiment, the strain was kept in a condition of great activity in a mixture of plasma and embryonic juice. Its relative increase in 48 hours was generally from 6 to 10. The fragments were divided and put into plasma. Coagulation of



TEXT-FIG. 3. Growth of embryonic heart in adult plasma during fifteen passages (Experiment 18948).

the medium was obtained by a small piece of fibrin, because fibroblasts living *in vitro* cannot bring about the clotting of the plasma, as embryonic tissues do. Whenever the tissue was cultivated in adult plasma alone, the rate of growth became very much slower. According to the condition of the adult plasma, the decrease in the rate of growth and in the size of the fragment was more or less rapid. In several cases, death occurred after two or three passages, as shown in Table IV and Text-fig. 4. Before the experiment, the relative increase after 48 hours was 10.5. After the first, second, and third passages in adult plasma alone, the relative increase became respec-

tively 1.3, 0.8, and 0. In other experiments, the life of the old strain in adult plasma was longer, and death occurred after six or ten passages (Text-fig. 2). The variations were due to the degree of activity of the strain previous to the experiment, and to the condition of the plasma.

There was a striking difference between the growth of the fragment of embryonic heart and the strain of fibroblasts. As soon as the strain of fibroblasts was placed in plasma alone, the rate of multiplication of its cells decreased (Text-figs. 2 and 4), while under the same conditions the activity of a fragment of embryonic heart increased for several days (Text-fig. 3). The life of the heart was much longer than that of the strain of fibroblasts. These phenomena could not be

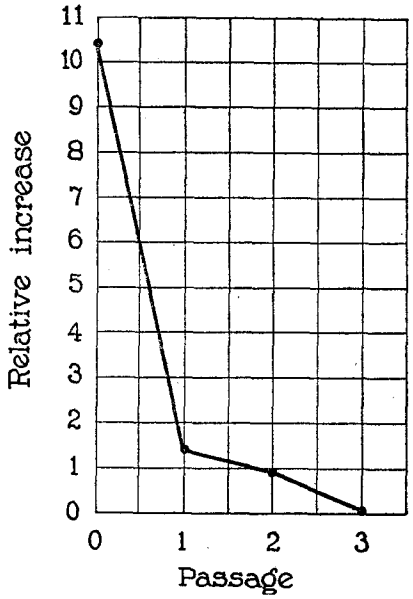
TABLE IV.

Growth of an Old Strain of Fibroblasts in Adult Plasma during Three Passages (Experiment 18907–2).

Passage No.	Culture No.	Date.	Area of fragment.	Area of growth.	Relative increase.
		1920			
1	18907–2	Dec. 26	5.7	59.3	10.4
	18947	" 28	5.0	6.8	1.36
		1921			
2	19006	Jan. 1	5.3	4.7	0.89
3	19028	" 3	5.3	0	0

attributed to a greater original activity of the embryonic heart, because the rate of proliferation of the fibroblasts of the 9 year old strain is at least as rapid as that of the connective tissue cells of a 10 or 12 day old embryonic heart. They might be due to substances contained in the tissue itself and more abundant in the embryonic tissue than in the strain of fibroblasts. It is known that tissue juices which activate the rate of cell division have also the power of coagulating fluid plasma. Chicken plasma is rapidly transformed into a solid coagulum by a small fragment of embryonic heart, even though the latter has been thoroughly washed in Ringer solution. On the contrary, a fragment of the old strain of fibroblasts does not bring about the coagulation of the plasma. It is, then, possible that embryonic heart contains some of the tissue juice which was found by

one of the writers to be capable of greatly accelerating the rate of cell multiplication,[1] while the strain of fibroblasts lacks it. During the first passages of embryonic heart, the small amount of juice contained in the tissue is probably responsible for the greater activity of the cells. When, after a few days, the original fragment has become surrounded by a dense reticulum of fibroblasts, the rate of growth de-



TEXT-FIG. 4. Growth of an old strain of fibroblasts in adult plasma alone during three passages (Experiment 18907–2).

creases just as it does in a culture of the old strain. It was observed frequently that if a cut were made through the original fragment, after growth had almost ceased, there was a resumption of activity, possibly due to the setting free of some embryonic juice. The activity displayed in adult plasma by both embryonic heart and old strain may be caused by the substances stored within the cells of the tissue, which have the power of increasing the rate of cell division, as is already known.

II.

The indefinite multiplication of fibroblasts, which occurs as soon as embryonic tissue juice is added to adult plasma, may be explained by two different hypotheses: the embryonic juice renders possible the utilization by the cells of substances contained in the serum or the fibrin; or it supplies itself the material required by the cells for their proliferation.

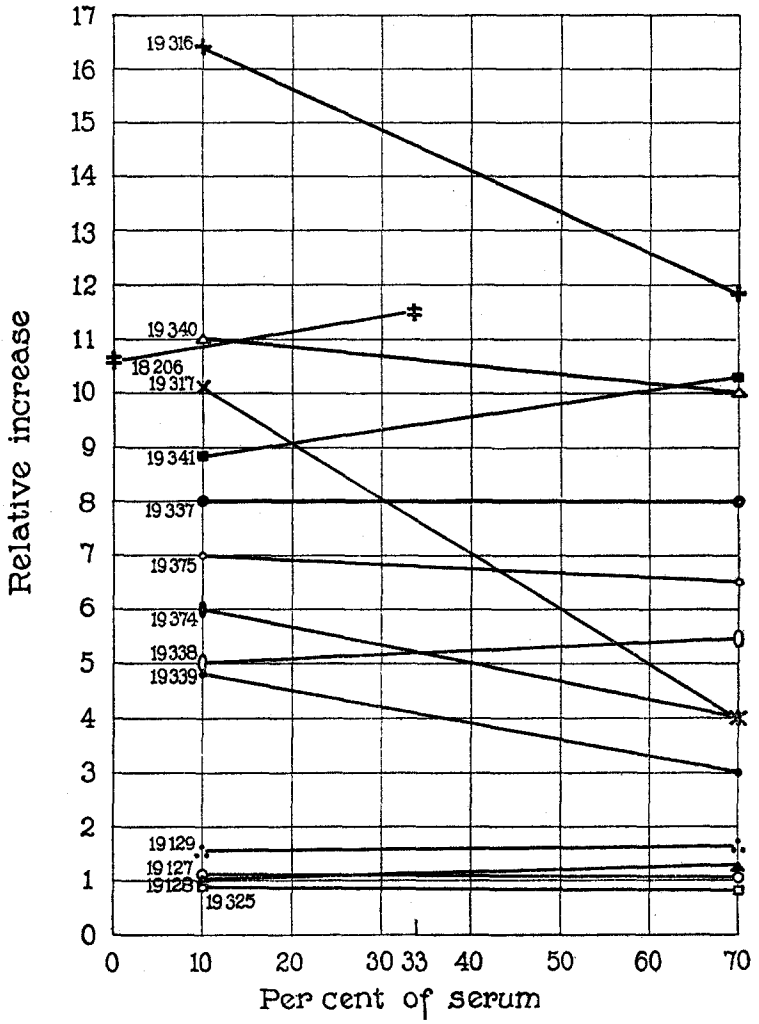
TABLE V.

Growth of an Old Strain of Fibroblasts in a Medium Composed of a Constant Amount of Tissue Juice and 10 and 70 Per Cent Serum.

Experiment No.	Culture No.	Date.	Relative increase.			
			Composition of medium.			
			Control.	Experiment.	Control.	Experiment.
			33 per cent.	0 per cent.	10 per cent.	70 per cent.
		1921				
1	18206		11.5	10.75		
2	19316	Jan. 24			16.4	11.8
3	19317	" 24			10.1	4.0
4	19325	" 25			0.9	0.8
5	19337	" 26			8.0	8.0
6	19338	" 26			5.0	5.4
7	19339	" 26			4.8	3.0
8	19340	" 26			11.0	10.0
9	19341	" 26			8.8	10.3
10	19127	" 10			1.1	1.0
11	19128	" 10			1.0	1.2
12	19129	" 10			1.6	1.6
13	19374	" 27			6.0	4.0
14	19375	" 27			7.0	6.5

Influence of Serum.—In a first group of experiments, it was attempted to find whether serum was used by the cells in the presence of embryonic juice. Tissue juices were obtained by centrifugation of a pulp made of 10 or 12 day old chick embryos. The media were composed of a constant amount of fibrinogen suspension and embryonic tissue juice, and of varied quantities of serum and Tyrode solution. Fragments of the 9 year old strain of fibroblasts were cultivated in these media. The coagula made of fibrin, embryonic juice, and Tyrode

solution liquefied after a few hours in all the experiments but one. In this experiment, the amount of new tissue was practically as large in



TEXT-FIG. 5. Growth of an old strain of fibroblasts in media containing a constant amount of tissue juice and varied dilutions of serum.

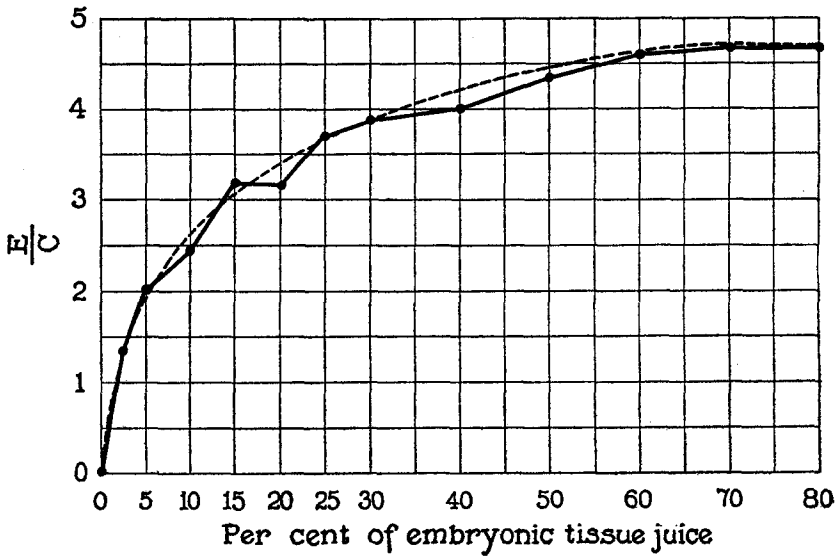
the medium containing no serum as in the medium containing 33 per cent serum. In thirteen experiments the media contained 10

and 70 per cent serum, and their action on the growing tissue was about identical, as is shown by the figures of Table V and by Text-fig. 5. The complete lack of serum, or its presence in low or high concentrations, had no influence on the rate of growth. This fact demonstrated that even in the presence of embryonic juices there was no utilization of the serum by the cells to a measurable extent.

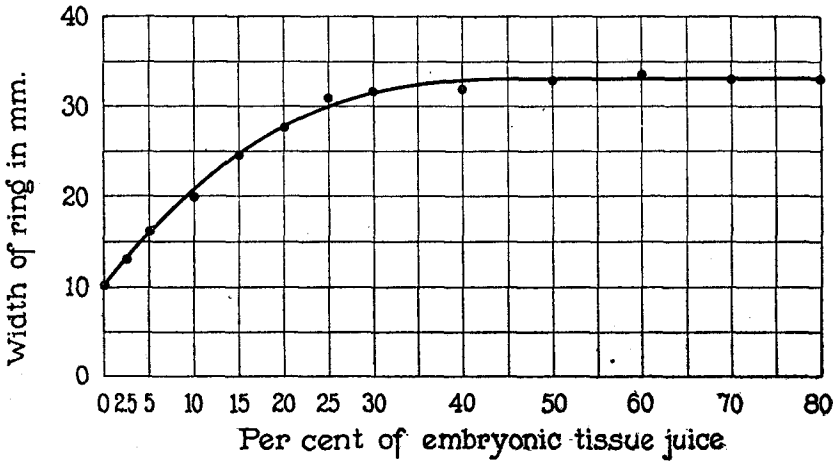
Influence of Fibrin.—In a second group of experiments, the action of the fibrin was studied. Plasma was coagulated by a little tissue extract at the surface of a cover-glass, and the coagulum was fixed in 2 per cent formaldehyde solution in Ringer solution for 1 hour. It became slightly bluish, but remained transparent. Then it was washed for 24 hours in distilled water, and for 24 hours in Ringer solution. It was then placed for half an hour in embryonic juice. The excess of embryonic juice was removed with blotting paper, and fragments of embryonic heart and of an old strain of fibroblasts were cultivated in the clot. The growth assumed the same characteristics and extent as in normal fibrin. In 48 hours, the width of the ring of reticulated fibroblasts was from 5 to 6, which is the same as in normal fibrin and embryonic juice. Since the growth in normal fibrin and in fibrin fixed by formaldehyde is the same, it is practically certain that fibrin plays only a mechanical rôle, and is not used by the cells.

Influence of Embryonic Juice.—In a third group of experiments, the embryonic juice was added to the medium in progressively increasing concentrations, while the amounts of fibrin and serum were kept constant. The tissues responded by considerable differences in the extent of growth, and these differences were the function of the concentration of the juice.

Fifty-five experiments were made. The action of the medium was ascertained by measurement of both the relative and the absolute increase of the tissue (Table VI). The ratios of relative increases of the experiments and of the control tissues were plotted in ordinates, while the concentrations of the juice present in the medium were plotted in abscissæ. The curve (Text-fig. 6) shows that the cells responded to the addition of small amounts of embryonic juice to the medium by a rapid increase of the rate of multiplication. Increases in concentration from 0 to 10 per cent brought about a greater change in the activity of the cells than increases from 10 to 80 per cent. After



TEXT-FIG. 6. Relation between the ratios $\left(\frac{E}{C}\right)$ of the relative increases of the tissue in experiments and controls, and the concentration of embryonic juice in the medium.



TEXT-FIG. 7. Relation between absolute increase of the tissue and the concentration of the embryonic juice in the medium.

TABLE VI.

Relation between the Amount of Growth Expressed in Terms of Relative Increase, Ratios of Relative Increases of Control and Experiments, and of Absolute Increase, and the Concentration of the Embryonic Juice in the Medium.

2.5 per cent tissue juice.						5 per cent tissue juice.						10 per cent tissue juice.						15 per cent tissue juice.					
Experiment No.	Relative increase.		$\frac{E}{C}$, Ratio.	Width of ring.		Experiment No.	Relative increase.		$\frac{E}{C}$, Ratio.	Width of ring.		Experiment No.	Relative increase.		$\frac{E}{C}$, Ratio.	Width of ring.		Experiment No.	Relative increase.		$\frac{E}{C}$, Ratio.	Width of ring.	
	Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.
19403	4.5	5.4	1.17	9	13	19418	2.85	4.59	1.6	7	15	19437	2.0	5.32	2.66	7	13	19470	2.5	8.92	3.57	13	18
19404	4.47	6.48	1.44	10	14	19419	3.71	9.46	2.58	7	16	19438	2.0	5.61	2.8	7	19	19471	2.30	9.66	4.2	11	25
19405	4.7	8.13	1.73	9	13	20043	2.47	5.93	2.41	10	16	20060	2.87	6.81	2.15	11	22	20084	3.02	8.55	2.84	11	27
20040	2.66	3.83	1.39	10	12	20044	2.21	4.20	1.9	10	18	20061	2.58	5.53	2.14	13	23	20085	4.78	11.21	2.34	12	28
20041	3.27	4.18	1.27	10	13	20045	2.9	5.71	1.97	10	16	20062	2.58	6.21	2.42	12	22	20086	3.05	10.02	3.28	10	24
20042	4.61	5.27	1.14	9	13																		
Average.....			1.36	9.5	13	Average.....			2.09	8.8	16.2	Average.....			2.43	10	19.8	Average.....			3.25	11.4	24.4

20 per cent tissue juice.						25 per cent tissue juice.						30 per cent tissue juice.						40 per cent tissue juice.					
Experiment No.	Relative increase.		$\frac{E}{C}$, Ratio.	Width of ring.		Experiment No.	Relative increase.		$\frac{E}{C}$, Ratio.	Width of ring.		Experiment No.	Relative increase.		$\frac{E}{C}$, Ratio.	Width of ring.		Experiment No.	Relative increase.		$\frac{E}{C}$, Ratio.	Width of ring.	
	Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.
19484	2.34	5.89	2.53	9	17	19505	2.6	9.6	3.7	10	28	19511	3.66	15.0	4.07	10	31	19528	2.3	9.2	4.0	10	30
19485	3.59	8.52	2.47	12	20	19506	2.83	11.34	4.0	12	27	19512	3.94	15.0	3.8	10	32	19529	2.64	10.3	3.9	9	30
19509	4.2	16.09	3.81	11	33	19507	3.39	13.3	3.9	11	31	20001	2.33	9.08	3.9	10	33	19530	2.23	9.6	4.3	10	30
19510	4.18	15.68	3.80	11	35	20087	2.63	8.93	3.4	12	35	20002	3.5	11.79	4.17	9	32	19883	2.74	11.67	4.26	9	35
19998	3.0	10.97	3.65	12	31	20088	2.56	9.18	3.57	10	33	20003	2.9	10.0	3.45	14	30	19884	3.54	12.71	3.6	10	34
19999	3.17	9.52	3.02	11	27																		
20000	2.43	7.08	2.9	11	30	Average.....			3.7	11	30.8	Average.....			3.88	10.6	31.6	Average.....			4.01	9.6	31.8
Average.....			3.17	11	27.6																		

50 per cent tissue juice.						60 per cent tissue juice.						70 per cent tissue juice.						80 per cent tissue juice.					
Experiment No.	Relative increase.		$\frac{E}{C}$ Ratio.	Width of ring.		Experiment No.	Relative increase.		$\frac{E}{C}$ Ratio.	Width of ring.		Experiment No.	Relative increase.		$\frac{E}{C}$ Ratio.	Width of ring.		Experiment No.	Relative increase.		$\frac{E}{C}$ Ratio.	Width of ring.	
	Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.
19531	2.36	9.20	3.9	10	26	19544	2.52	11.6	4.6	12	33	19546	2.47	11.36	4.6	9	33	19620	2.33	10.62	4.58	13	33
19533	2.74	13.2	4.8	10	31	19545	2.85	13.3	4.66	7	34	19547	2.82	13.4	4.75	10	33	19621	1.93	9.08	4.7	12	33
19886	2.17	9.77	4.5	14	36																		
19885	2.49	9.95	4.0	13	34	Average.....			4.6	9.5	33.5	Average.....			4.65	9.5	33	Average.....			4.64	12.5	33
20150	2.76	12.91	4.67	10	35																		
20151	2.10	8.74	4.16	11	34																		
Average.....			4.37	11.2	32.7																		

the concentration of the juice in the medium had reached 40 per cent, no further increase was observed when larger amounts were added. The results were checked by measurement of the width of the ring of the new tissue; that is, by comparing the absolute instead of the relative increases (Table VI). The arithmetic mean of the width of the ring of new tissue of several experiments, made with each dilution of juice, was plotted in ordinates, and the concentration of the juice in abscissæ (Text-fig. 7). The curve (Text-fig. 6) showed the same rapid increase of the rate of growth when a small amount of juice was added to the medium. There was no doubt that the substances used by the fibroblasts came from the embryonic juice and that the activity of the fibroblasts was a function of the concentration of the embryonic juice in the medium.

III.

SUMMARY.

The results of the investigation of the cause of the multiplication of fibroblasts *in vitro* may be summarized as follows: Although the life of fibroblasts in the plasma of an adult chicken which has fasted for 24 hours is not permanent, their proliferation is very active for some time. Are the substances used by the cells in their multiplication supplied by the plasma or by the tissue itself? In media composed of a constant amount of fibrin and of a mixture of Tyrode solution and serum in varied concentrations, the amount of growth appeared to be independent of the concentration, and even of the presence of serum. Serum was evidently not used by the cells. It was also found that fibrin is not utilized. This fact explains the results of the experiments of Lewis, who showed long ago that embryonic tissue can grow extensively in Locke solution. Then, the material from which the new cells are built must come from the tissue itself, as was previously supposed by Lewis, Ingebrigtsen, and Burrows. A comparative study of the growth in adult plasma of embryonic heart and of a 9 year old strain of fibroblasts led to the hypothesis that traces of embryonic juice stored in the original fragments are responsible for the activity manifested by the tissues during their temporary life.

When embryonic juice is added to the plasma of an adult chicken, the rate of multiplication of the fibroblasts increases and their life *in vitro* becomes permanent. Does the presence of embryonic juice determine the use by the cells of substances contained in adult plasma? The tissues were cultivated in media containing a constant amount of fibrin and embryonic juice, and varied concentrations of serum. The rate of growth was found to be independent of the amount of serum contained in the medium. It was also observed that the rate of growth in fibrin fixed in formaldehyde solution did not differ from that in normal fibrin. This fact demonstrated that embryonic juice does not give to the cells the power of using the constituents of plasma. When fragments of the 9 year old strain of fibroblasts were cultivated in media containing a constant amount of serum and fibrin, and varied concentrations of embryo juice, the rate of growth was found to be a function of the concentration of the embryonic juice in the medium. It was, therefore, evident that the material employed by the fibroblasts in their indefinite multiplication *in vitro* was supplied by the embryonic juice.

IV.

CONCLUSIONS.

1. It may be concluded that, under the conditions of the experiments and within the limits of accuracy of the method, the temporary multiplication of fibroblasts cultivated in the plasma of an adult animal is not due to the serum. It may be attributed to the presence of a small amount of embryonic juice within the tissue itself.

2. The indefinite multiplication of fibroblasts in a medium composed of adult plasma and of embryonic juice is due neither to the serum nor to the fibrin. It depends entirely on substances contained in the embryonic juice.

3. There is a definite relation between the rate of growth and the concentration of the embryonic juice in the medium.