PHYSIOLOGICAL ONTOGENY.

A. Chicken Embryos.

IV. The Negative Acceleration of Growth with Age as Demonstrated by Tissue Cultures.

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It has been shown by weighing chicken embryos that the percentage rate of growth decreased with age (1). The experiments to be now reported were planned so as to determine whether the growth rate and the latent period of growth of fragments of tissue removed from the hearts of embryos varied with age in the same fashion. In other words, are functional changes that take place in the organism as a whole mirrored by similar changes in individual cells? Do the cells themselves undergo differentiations in form and function so that when removed from the organism they exhibit characteristics of life which vary in kind or quantity according to the age of the embryo from which they were derived? The rate of division is one character by which cells of different ages may be compared.

By implanting pieces of tissue of approximately equal mass from corresponding sections of the heart into similar culture media an answer to this question may be obtained. Here the independent variable is the incubation age of the chick. That the enlargement of the area which occurs under certain conditions of tissue culture is not simply the result of cell migration has been shown by Burrows (2) and Carrel (3). Accordingly we were led to believe that growth phenomena could be investigated by this method.

It soon became evident that values for growth rate comparable to those found in studies of the embryo as a whole could not be obtained by the tissue culture method without making certain arbitrary and

unjustifiable assumptions. It was found that the differences between the growth indices of tissues of different ages varied inversely with the length of the period of cultivation. For instance a fragment from a 4 day embryo after 24 hours had grown 15 times as much as a similar piece from a 16 day embryo; whereas after 4 days the growth area in the younger piece was only about 5 times that from the older section. Although some arbitrary time could have been chosen for measurement which would give values for growth rate change similar to the figures already described as having been obtained for the whole embryo, there were no facts to justify us in this or any other selection of a time interval. It was evident that for a positive result we could hope for a tissue culture growth curve which in its main characteristics only, duplicated the curve for the whole embryo.

Carrel has shown that the rate of growth of a standard 11 year old strain of fibroblasts was affected by the age of the hen from which the plasma culture medium was derived (4). The older the donor the lower was the growth index; that is to say, the slower the growth of the transplanted tissue.

The present investigation concerns itself with the other aspect of the problem; namely, the rate of growth of tissue from embryos of different ages in a standard plasma medium. There are numerous factors of error, some inherent in the material and others related to our tissue culture technique. For instance we have chosen arbitrarily the ventricular wall of the heart as the site for dissection and have cut pieces about 0.4 sq. cm. in size. As the embryo develops, however, the cardiac wall becomes more compact, the muscular nature of its structure becomes more marked, and the relative proportion of endo-, meso-, and pericardium is altered. The thickness of the wall also varies. For these reasons it is impossible to cut fragments of identical composition or size in embryos of different ages. In view of the fact, moreover, that the different organs of the body are developing at varying rates, the growth rate of cardiac muscle may be no criterion of general cellular behavior or the rate of aging in the whole embryo. This seems to be a theoretical objection, but may be irrelevant since the growth from these fragments consists mostly of fibroblasts, which are relatively undifferentiated and probably of a like nature, metabolic rate, etc., throughout the body. It was thought that the medium

chosen as a standard might have a differential effect on tissues of varying ages; in other words as this study is concerned with the change in rate of growth with age, there is an error inherent in the use of any medium other than the natural one. If the natural *milieu* were used, however, one could not differentiate cellular from environmental effects. One advantage of choosing the tissue rather than the plasma as a measure of age is that tissues can be easily obtained from a chick at an early period of embryonic life whereas it is not possible to collect plasma so early. The studies comprising this series are limited to the embryonal period. Since it is well known that the period of greatest negative growth acceleration occurs at the inception of life (5) this most important part of the cycle could not be included if the investigation were limited to the influences of plasma. Finally it is obvious that as soon as the tissue has been transplanted and has started to develop it is no longer identical with the tissue *in vivo*. It becomes dedifferentiated one might say. The significance of the experiment is necessarily limited by these conditions.

Method.

Our method was so similar to Dr. Carrel's that it would seem sufficient to give the publications of Carrel and Ebeling as reference (6). Dr. Carrel put at our disposal all his knowledge dealing with the technique of tissue culture. We should like to take this opportunity to express our appreciation of his assistance and goodwill.

As a review, however, and because in some ways the procedure was slightly modified, it would not be inappropriate to outline our technique for growing tissue fragments from the ventricular wall of embryo chicks of different ages. The method of collecting, handling, and incubating the eggs has been described elsewhere (7).

Only fresh blood is used. On the morning of the experiment a hen approximately 1 year old and bred from the same stock which provides the eggs is bled from the carotid artery through a paraffined cannula into iced paraffined tubes. These are centrifuged and the plasma which furnishes the culture medium for that day is removed by a pipette. The experiment is carried on under sterile precautions, the linen, gowns, etc., being sterilized by steam and the instruments and glassware by dry heat (at 145–155° for 1 hour). The glassware such as dishes, watch-glasses, pipettes, and test-tubes is all of Pyrex or other glass relatively free from alkali. This does not apply to the so called Gabritschewski dishes which do not come in contact with the cultures or medium at any time. The tissues are planted on mica cover-glasses.

The experiment commences with the preparation of extract. Two 10 day old embryos are cut up into small bits, 1 cc. of Ringer's solution is added, the whole is centrifuged for 10 minutes, and the supernatant fluid (avoiding a layer of tissue debris which is usually found floating on top) is pipetted off. Whenever tissue extract is mentioned it refers to this preparation.

The ages of the embryos range from 4 to 18 days of incubation. The hearts are removed and dissected in Ringer's solution on Pyrex glass slabs with an iridectomy scalpel. Under the glass dish is a piece of black coordinate paper which guides one in cutting sections of the proper size. Only those plates were included the fragments of which averaged between 0.310 and 0.490 sq. mm. The variations in size between these limits are rendered insignificant by the method of estimating growth. Ten small fragments are transferred from the dissecting dish to the mica cover-glass in a pipette, the excess of Ringer's solution removed, and 0.5 cc. of chicken plasma added. It is spread out so as to cover a circle 4.4 cm. in diameter etched on the mica. This provides for an equal number of pieces (ten) in an equal quantity of the medium (0.5 cc.) distributed over an equal surface (15.2 sq. cm.) and therefore of approximately equal thickness. The tip of the knife dipped as far as a certain point into the tissue extract preparation is used to stir the medium and distribute the fragments equally throughout the plasma. The minimal amount of tissue extract thus introduced has some effect in accelerating growth, but it is used principally to insure the formation of a good clot.

When clotting has occurred the mica is inverted over the Gabritschewski dish and sealed with paraffin and vaseline. All the fragments in one dish are of one age. In the case of the 4 and of the 5 day embryos two hearts are necessary to provide ten pieces. In the older chicks, on the other hand, two plates were occasionally planted with fragments from the same heart.

The fragment and the peripheral growth were measured every 24 hours until enlargement ceased by drawing the projected image of it, magnified 27 times, and measuring the area circumscribed in the drawing with a planimeter. As the depth of growth could not be accurately determined, development was estimated by comparing areas.

We do not know whether growth (enlargement of area) is a function of the area or of the circumference. Theoretical considerations might suggest the latter, since there is no limiting surface and the internal growth, that is to say the swelling, or cell division within the original fragment has little influence on the marginal radiations. Empirically we tried to settle the point in the following manner. Twelve plates were planted with similar technique. There were 110 pieces of tissue in all (six to ten fragments planted in each dish). The area of the fragments in each dish varied from 0.288 to 0.452 sq. mm. in size, the average size being 0.382 sq. mm. The incubation ages of the embryos from which the tissue was removed varied from 4 to 14 days.

Growth was measured in the usual fashion, and the index of growth was estimated by four different methods for each of the 110 fragments after 24 hours and then again after 48 hours (Fig. 1). The methods employed for finding a value for growth rate were: (1) subtracting from the total area of tissue after n days of cultivation (A_n) the area of the central portion after n days of cultivation (B_n) (which was not usually of the same size as the original fragment since the latter may

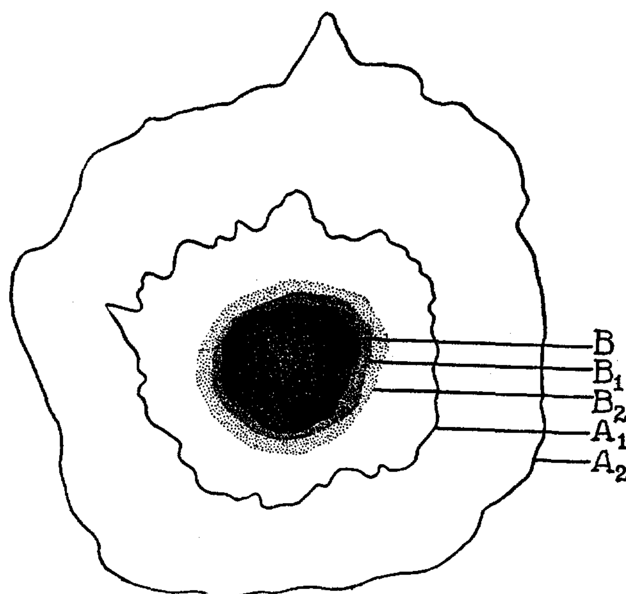

FIG. 1. A schema to show the enlargement of the implanted fragment (B) after 1 day (B_1) and after 2 days (B_2); and the margins of the new growth after 1 day (A_1) and after 2 days (A_2). (*Cf.* Table I.)

enlarge or in some cases contract), (2) dividing the growth area as just described ($A_n - B_n$) by the size of the original fragment (B), (3) dividing the growth area ($A_n - B_n$) by the area of the central fragment at the time of measurement (B_n), (4) dividing the growth area ($A_n - B_n$) by the square root of the central fragment (B_n).

The coefficient of variability[1] for each of these methods is given in

[1] The coefficient of variability is the standard deviation relative to the mean value; *i.e.*, $Cv = \frac{100\sigma}{M}$.

Table I. It can be seen that no method shows any conspicuous advantage over any other. We finally used as an index of growth the formula:

$$\frac{A_n - B_1}{B_1} \text{ or } \frac{A_n}{B_1} - 1$$

We determined the coefficient of variability at infrequent intervals in subsequent experiments, and found that as our technique improved the variations became less pronounced.

TABLE I.

The Coefficient of Variability for Various Methods of Measuring Tissue Culture Growths.

Method for obtaining growth index.	Coefficient of variability.*		
	24 hrs.	48 hrs.	Average.
1. Growth $(A_n - B_n)$	41.8	30.4	36.1
2. $\frac{\text{Growth}}{\text{Original fragment}}$ $\frac{(A_n - B_n)}{B}$	44.3	30.8	37.5
3. $\frac{\text{Growth}}{\text{Central area}}$ $\frac{(A_n - B_n)}{B_n}$	43.1	31.2	37.1
4. $\frac{\text{Growth}}{\sqrt{\text{Central area}}}$ $\frac{(A_n - B_n)}{\sqrt{B_n}}$	42.0	28.1	35.0

* The coefficient of variability is the standard deviation relative to the mean value; *i.e.*, $Cv = \frac{100\sigma}{M}$.

RESULTS.

The accompanying curve (Fig. 2) shows that the growth rate and the decline in the growth rate (*i.e.*, negative acceleration) after 48 hours of cultivation are greatest for the early days of life and, except during a short period when there is an apparent increase, decrease with age. We considered that our culture medium, namely plasma from a 1 year old hen, might exercise a differential effect on pieces of different ages since it was arbitrarily selected and did not represent the normal environment of the tissue. For instance it might be more suitable for the older than for the younger pieces. We performed a

few experiments with plasma from hens 2 and 3 years old and obtained comparable figures. This change in the age of the plasma apparently had no marked differential effect on growth rate. As plasma from young embryos cannot be obtained with facility the reproduction of a more natural environment was not attempted. We did perform a series of experiments in which the plasma was diluted with equal parts of Ringer's solution (Table II). This change seemed to increase markedly the rate of growth of the older fragments, but the curve was nevertheless of the same general shape (Fig. 3).

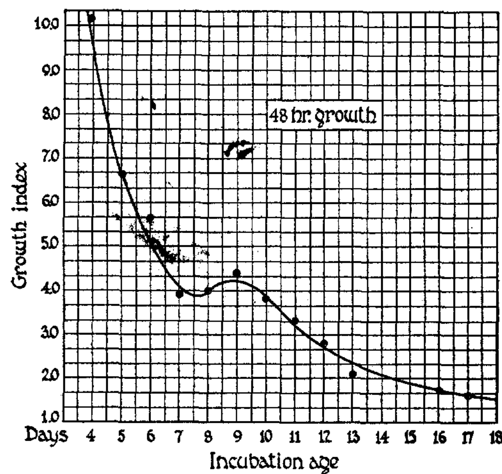

FIG. 2. The tissue culture growth rate, as approximately represented by the growth index after 48 hours—

$$\frac{\text{Total area (2 days)} - \text{area of original fragment after 1 day}}{\text{Area of original fragment after 1 day}} = \frac{A_2 - B_1}{B_1}$$

Plotted as a function of embryonic age. Abscissæ are the ages of the embryo from which the fragments were derived (Table II).

In all these graphs there appears to be a deflection, or "hump," between the 7th and 11th days in an otherwise smooth curve. Speculation as to the nature and cause of this feature will be postponed until certain changes occurring at this age in the physiology of the heart have been investigated.

In Table III have been collected the average results for each plate with their deviations. If these are equated against age (Fig. 4) it

will be found that no better smooth curve can be drawn to describe them than the one derived from the formula $\frac{\frac{dw}{dt}}{W} = 3.6\, t$. The general shape of the tissue culture curve is accordingly seen to be similar to that found to be representative of growth changes in the embryo as a whole.

TABLE II.

The Growth of Tissue Cultures in Undiluted and Diluted Plasma as a Function of the Age of the Embryo from Which Fragments Were Removed.

Undiluted plasma medium.					Plasma medium diluted with equal parts Ringer's solution.		
1	2	3	4	5	6	7	8
Age of embryo.	No. of fragments.	B_1 Average area of original section after 1 day.	1 day. Growth index, $\frac{A_1 - B_1}{B_1}$.	2 days. Growth index, $\frac{A_2 - B_1}{B_1}$.	No. of fragments.	B_1 Average area of original sections after 1 day.	Growth index, $\frac{A_1 - B_1}{B_1}$
days		*sq. mm.*				*sq. mm.*	
4	95	0.377	3.11	10.2	12	0.316	2.74
5	100	0.411	1.34	6.6	10	0.362	1.40
6	115	0.342	1.19	5.6	7	0.322	1.53
7	111	0.372	0.90	3.9	35	0.417	1.46
8	67	0.487	0.80	4.0	8	0.410	1.71
9	118	0.369	0.95	4.4	47	0.367	1.49
10	96	0.372	0.76	3.8	26	0.388	1.19
11	57	0.362	0.51	3.3	25	0.367	1.22
12	98	0.391	0.45	2.8			
13	88	0.361	0.30	2.1	20	0.351	1.11
14	38	0.352	0.27	2.9	20	0.378	1.27
16	40	0.321	0.16	1.7	19	0.351	0.73
17	35	0.330	0.03	1.6			
18					7	0.315	0.61

Curves to show the change in percentage growth during the period of cultivation are shown in Fig. 5. The growth is expressed as the difference between the logarithm of the whole area and the logarithm of the original fragment $\left(\log \left[\frac{A_n}{B}\right]\right)$. All pieces may be started on the base line. Each point represents the average value of ten sections (Table IV). The slope of the line is equivalent to the percentage rate of growth. The curves are representative of others. It seems

that the maximum growth rate of the younger pieces is reached at the very commencement of their history, whereas the oldest fragments do not attain their greatest velocity until later; that is to say, after about $1\frac{1}{2}$ days. All the pieces stopped growing about the same time.

Latent Period.

The time intervening between the incubation of the cultures and the first appearance of cells protruding from the peripheral margin is

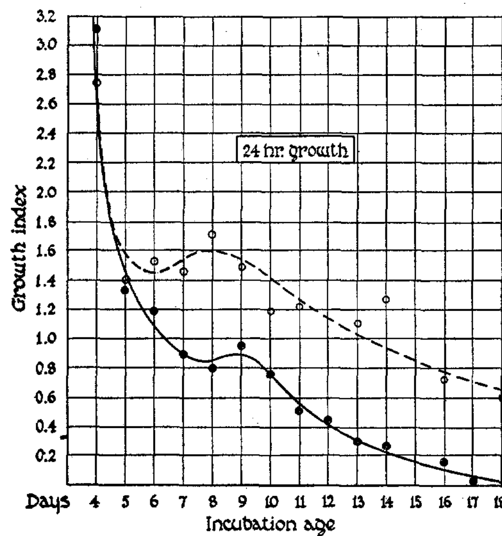

FIG. 3. The growth index after 24 hours in chicken plasma (⊙——⊙), and in plasma diluted with Ringer's solution (○---○) equated against embryonic age (Table II).

termed the latent period. A satisfactory interpretation of the processes involved during the latent period has never been rendered, either for tissue cultures or for analogous experiments, such as the transplantation of bacteria and yeast colonies into a new environment. In studying the growth of bacteria in fresh media it has been found that, unless special precautions are taken (8), soon after planting the number of viable organisms in the culture becomes diminished (9). It is only somewhat later that the growth and multiplication of the colonies commence. The latent period has been taken as the time

TABLE III.

Average Growth Index Figures with Standard Deviations for Each Age.

Days	4	5	6	7	8	9	10	11	12	13	14	16	17
Average growth index for each culture plate* after 48 hrs.	10.0	6.2	7.4	3.8	5.6	5.7	3.5	3.7	3.4	3.8	2.5	1.3	1.2
	9.0	4.2	5.3	5.8	4.4	1.9	5.2	2.7	3.7	2.3	2.6	1.0	2.2
	12.0	6.7	5.5	4.5	3.8	7.0	4.8	3.9	3.8	2.0	3.8	1.7	2.2
	8.6	4.9	3.9	3.9	3.3	3.4	5.4	2.9	3.3	1.9	2.8	1.5	0.7
	8.1	3.2	6.0	4.5	3.8	1.9	4.3	2.8	3.5	3.4		4.0	
	8.0	5.4	5.1	2.1	4.5	5.3	3.7	3.6	2.1	1.0			
	11.2	12.6	4.5	1.9	3.3	4.7	5.6	3.4	3.7	2.0			
	13.7	7.7	4.3	3.9		5.4	3.3		2.1	1.0			
	14.2	8.5	5.6	3.7		4.7	2.5		2.1	3.4			
	7.4	4.7	4.8	4.7		5.7	2.0		4.3				
	8.4	6.3	4.8	2.5		7.1			2.0				
			8.1	3.2		4.4			2.4				
						1.9							
						2.9							
Average†	10.0 ±1.5	6.4 ±0.8	5.4 ±0.8	3.7 ±0.7	4.1 ±0.5	4.9 ±1.2	4.0 ±0.8	3.3 ±0.3	3.0 ±0.5	2.3 ±0.7	2.9 ±0.2	1.9 ±0.7	1.6 ±0.4

* Usually ten fragments in each plate.

† The averages are given ± the standard deviation.

intervening between the plantation of the culture and that moment at which the original number of living bacteria is again estimated to be present. In the case of tissue cultures we had no quantitative method for estimating cellular death after transplantation and therefore this phase of negative growth which has been clearly demonstrated for bacteria is not apparent. At present we shall simply show how

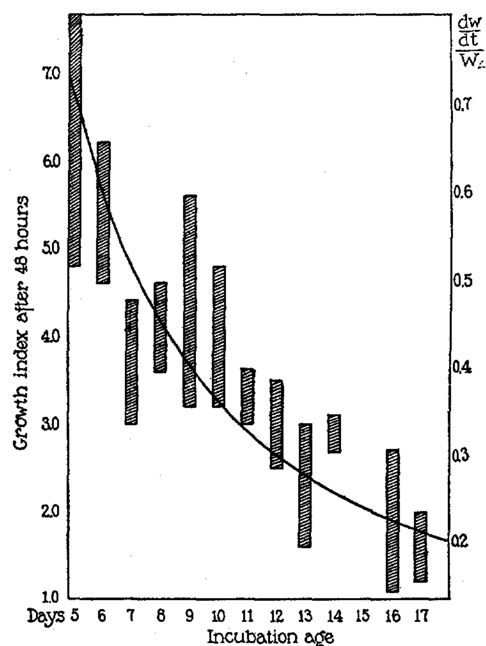

FIG. 4. The growth index ± the standard deviation (Table III) plotted against the age of the embryo. The smooth curve corresponds to the ordinates on the right hand side, which are for $\frac{\frac{dw}{dt}}{W}$; *i.e.*, the percentage growth rate of the embryo as a whole over the same period (*cf.* Murray (1)).

the latent period is related to the age of the tissue. It should be compared with the curve showing the growth index as a function of age. The fact that these two curves are dissimilar would seem to indicate that the phenomena in question are of a different nature. The latent period as measured in these experiments corresponds roughly to the term reactivity or irritability, the stimulus being the

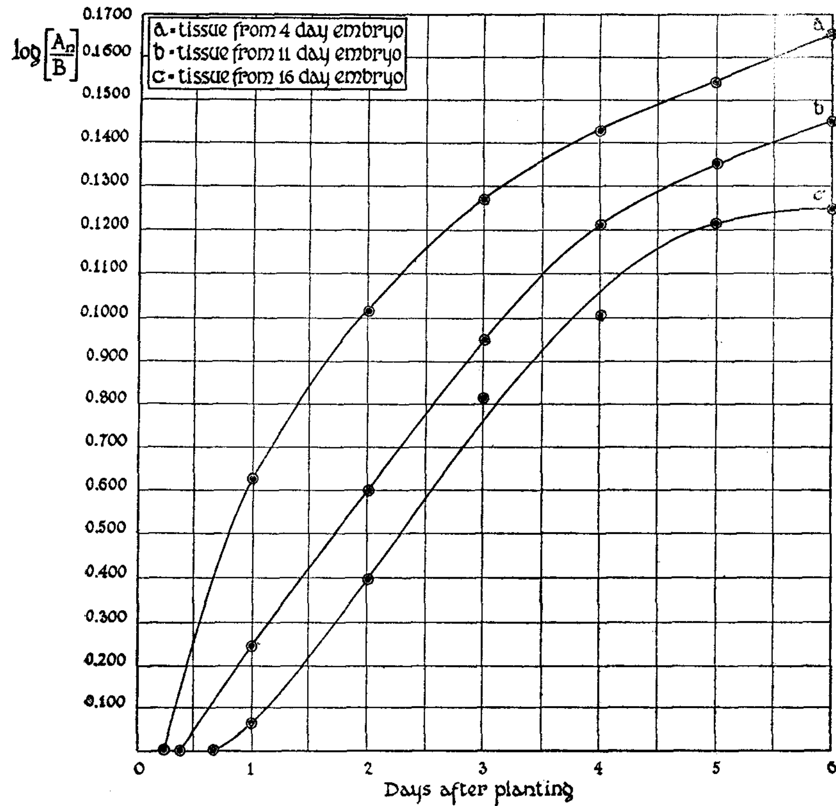

FIG. 5. The growth in culture of tissues from embryos of 4, 11, and 16 days of age respectively over a 6 day period. The ordinates are the logarithms of the percentage increase in area; *i.e.*, $\log\left[\frac{A_n}{B}\right]$. The abscissæ represent the duration of cultivation in days (Table IV).

TABLE IV.

Percentage Growth of Tissue Cultures for the 6 Day Period.

No. of plate.	Age of embryo.	Log percentage growth (*i.e.* $\log\left[\frac{A_n}{B_1}\right]$; *i.e.*, $\log A_n - \log B_1$).					
		Days after planting.					
		1	2	3	4	5	6
	days						
R 113	4	0.622	1.004	1.262	1.418	1.531	1.646
R 120	11	0.238	0.592	0.943	1.205	1.348	1.438
M 139	16	0.062	0.386	0.804	0.996	1.205	1.237

incision and the response being the initial growth of cells from the periphery. In another paper it will be seen that the latent period seems to be correlated to the chemical form or constitution of the tissues rather than to the rate of growth.

The technique employed was the same as that used in the growth experiments.

Within about 5 minutes of planting the plate was placed, mica side up, in a Freas electric oven where the temperature was kept at about 38°C., a rather narrow temperature fluctuation being maintained by the use of an electric fan in the incubator. The plates were then regularly observed at varying intervals under

TABLE V.

Latent Period of Tissue Cultures.

Undiluted plasma.			Plasma diluted with Ringer's solution.		
Age of embryo.	No. of fragments.	Average time.	Age of embryo.	No. of fragments.	Average time.
days		*hrs.*	*days*		*hrs.*
4	52	5.5	4	20	4.9
5	90	5.7	5	30	5.3
6	111	5.8	6	29	4.9
7	88	5.9	7	40	5.6
8	56	6.0	8	50	5.5
9	83	6.7	11	30	6.9
10	60	7.7	13	10	7.8
11	50	9.3	14	20	9.3
12	71	10.4	18	10	11.0
13	69	11.7			
16	42	16.1			

low and high power magnification in an observation incubator. The arbitrary point chosen as terminating the latent period was the moment when at least three young cells could be clearly identified sprouting out at different points on the periphery. The average time interval in hours (for the ten fragments in each plate) before this amount of growth was noted was taken as the latent period for an embryo of the age involved (Table V). Several plates were prepared for each age.

The averages for the latent period are shown graphically in Fig. 6. The effect of Ringer's solution as a plasma diluent in diminishing the latent period of the older pieces is clearly shown. This action is not enough, however, to account for the increase in the growth rate

shown to occur in previous experiments when Ringer's solution was added to the culture medium.

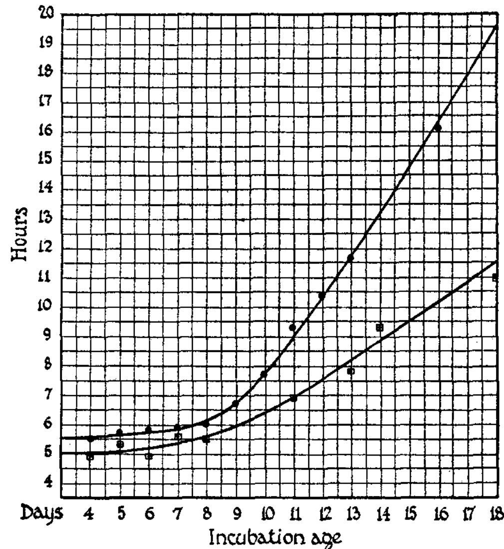

FIG. 6. The latent period of growth of fragments of heart muscle from chicken embryos of different ages when implanted in plasma (⊙) and in plasma diluted with Ringer's solution (⊡) (Table V). The ordinates represent the latent period in hours and the abscissæ the age in days.

DISCUSSION.

The results summarized in this paper, based upon the statistical average of over 1,000 heart muscle fragments of different ages planted and incubated under similar conditions, indicate that there is an increase in the latent period with age, most marked in the latter half of the embryonic cycle, and a decrease in the growth index, most marked in the beginning of life. In its general configuration the curve for the growth rate of tissue cultures resembles the curve for the organism as a whole except that there is a rise between the 7th and 11th days, for which we have at present no explanation. It may be due to the greater susceptibility to a foreign medium of the younger pieces or the result of factors specific to the heart.

From these observations the inference may be drawn that in a

multicellular organism the divisional velocity of the individual cells decreases progressively with age. It is not yet apparent how the separate components, that is to say the divisional rate (increase in number) and simple growth rate (increase in mass), are related to one another. The organization of the body as a whole becomes more defined with time, imposing upon cells certain habits which are in a sense acquired characters. Of these habits the one that concerns us at present is that which involves the rate of division and increase in mass. The habit that is acquired and inherited is one of constantly decreasing growth rate. When older tissue, as for instance a heart fragment from a 16 day old chicken embryo, is placed in a fresh environment, it does not assume immediately a divisional velocity typical of younger tissue. Under the conditions of our experiment the previous growth rate was not approached for 36 hours. In other words a certain number of cell divisions (*i.e.* generations) were required for it to lose the habit of slow growth previously imposed upon it by its organized environment. The happening is an expression of a process of rejuvenescence or dedifferentiation. The tissue soon assumes a growth rate determined by its environment, and as long as the environment can be kept relatively stable, that is to say of uniform composition, presumably there will be no further change in divisional velocity.

It is clear that the organism as a whole and probably each and every part, fixed and wandering cells and circulating fluids, all show variations with age which act to condition growth rate. The changes in the plasma, however, are obviously the results of changes in cellular activity throughout the body. For, do not nutritive substances, whence the body derives its energy, depend upon body cells for their absorption and preparation? And is not the concentration in the blood of various chemical substances, such as hormones, a function of the activity of various gland cells? And does not the elimination of injurious substances depend upon the viability of specific excretory tissues? Thus, it is that the *milieu intérieur* is determined by cellular dynamics and *vice versa*. The relationship is reciprocal.

Whatever be the chemical nature of the various products derived from body tissues and fluids which modify the growth activity of cells under experimental conditions their concentration in the body is

certainly a function of the metabolic activity of the living protoplasm. If these substances become decreased or increased with age or if new substances develop in the course of time, the conditions for the variation must be sought for in the tissues themselves.

SUMMARY.

1. Observations on tissue cultures of heart muscle from chicken embryos indicate that in general the growth rate of the individual cells decreases with age. The negative acceleration of growth is greatest at the beginning of life as in the intact embryo.

2. The latent period before growth commences increases with the age of the implanted tissue, but in a different fashion. The acceleration of the latent period is greatest near the *end* of the incubation period, thus demonstrating that the factors determining the *initiation* of growth and those determining the *extent* of growth are not similar.

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