
STUDIES ON THE GROWTH OF TISSUES
IN VITRO

V. FURTHER OBSERVATIONS ON THE MANNER IN WHICH
CELL DIVISION OF CHICK FIBROBLASTS IS AFFECTED
BY EMBRYO TISSUE JUICE

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(With Seven Text-figures)

It has already been shown (1933, 1936) that chick fibroblasts (mesenchyme cells derived from the heart or from periosteal tissue) can be reduced to a state in which growth by cell division is almost at a standstill and that, by the addition to the medium of embryo tissue juice, the cells can be caused to divide again. The higher the concentration of embryo juice used, the greater is the number of cell divisions which occur. The addition of the embryo juice is followed within the first 2 hours by increased movement of the cells, which reaches its maximum in about 10 hours, but it is not until this latter time that cell divisions begin again.

During these experiments the embryo juice, once added, was left in contact with the culture. At the suggestion of Dr Pantin, experiments were undertaken to investigate whether this was a necessary part of the procedure, or whether it is possible to cause cells to divide by the application of embryo juice for only a limited time. The experiments were successful and the results throw interesting light on the process of cell division. There are reported also in this paper certain observations on the duration of the mitotic phases under different conditions and their relationship to cell movement as a whole, the latter being estimated by measuring the average migration rate of the cells during hourly intervals. Finally, some observations have been made on the size of cells during the metaphase of division under various conditions.

The experimental procedure was precisely the same as in the previous experiments in that cultures from strains of chick periosteal fibroblasts were grown in flasks in a medium of plasma and Tyrode solution for at least 48 hours, by which time cell divisions had practically ceased to occur. The Tyrode solution was then removed and replaced by embryo juice which remained in contact with the culture for varying lengths of time. Different concentrations of juice (i.e. neat embryo juice diluted with Tyrode solution) were tried and in this way graded responses were obtained from the tissues. When the embryo juice was removed after the required

time interval it was replaced directly by Tyrode solution, without washing, but allowing the juice to drain away from the surface of the coagulum. As before, the coagulum was composed of 0.05 c.c. of plasma and 0.15 c.c. of Tyrode solution. When the juice was removed some of it probably remained enclosed in the plasma coagulum, but the evidence shows (see p. 257) that the active substance in the juice was not specifically absorbed by or adsorbed on to the coagulum, but probably distributed itself evenly between the solid and fluid phases of the medium. It was thought advisable therefore to make no attempt to remove the juice from the coagulum itself, so that allowance could be made for the residual amount left behind in the solid part of the medium.

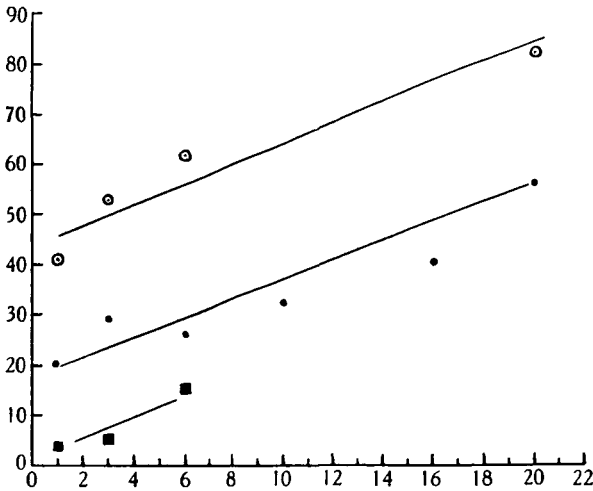


Fig. 1. Graph showing the relation between the duration of the application of embryo juice and the amount of growth which is produced. Ordinates: the aggregate percentage of cells which divide between the 10th and 20th hours after the application of the juice. (These figures therefore do not include any mitoses which may happen to occur in the latent period, mitoses which may be due to the fact that the previous growth had not quite ceased, and do not include any mitoses of the second crop (see page 260).) Abscissae: time in hours. \odot 40 per cent embryo juice; \bullet 15 per cent embryo juice; \blacksquare 5 per cent embryo juice.

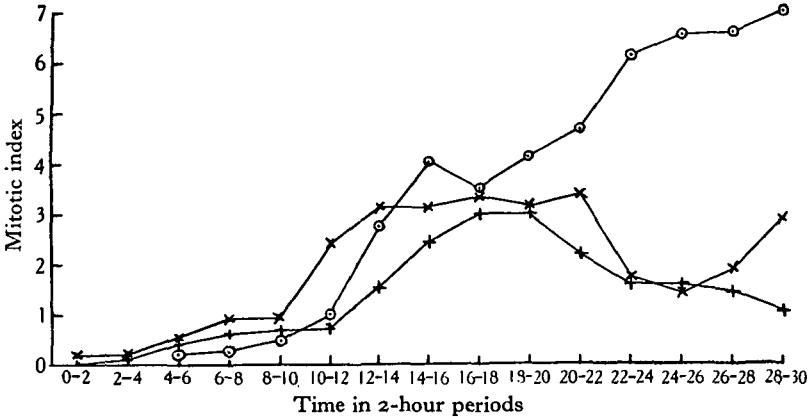
At first a series of observations was made using different concentrations of juice and applying them for different lengths of time. As a result of these it became obvious (see Fig. 1) that the concentration of the juice was a more important factor than the time for which it was allowed to act in determining the amount of growth obtained. Using 15 per cent embryo juice, there was found to be comparatively little difference between the effects produced by treatment for 1 hour and treatment for as long a period as 10 hours. On the other hand 15 per cent juice always produced a greater effect than 5 per cent, and 40 per cent juice than 15 per cent. Five per cent embryo juice seemed to be about the minimum concentration which produced any effect and this only if kept in contact with the culture for 3 hours, or more certainly for 6 hours. With low concentrations of the juice (5 per cent) the time factor became slightly more important than with higher concentrations (15 and 40 per cent) in the sense that almost no effect was obtained by applications lasting

for 3 hours or less, whereas the higher concentrations always produced a considerable effect even after application for 1 hour only. If it be assumed that the embryo juice distributes itself evenly between the fluid phase and the coagulum, then it is important to take into account that portion which is left in the clot after the supernatant fluid containing the bulk of the juice is changed for Tyrode solution. The relative volumes of solid and liquid phase used were such that if, after fresh Tyrode had been added, the juice again distributed itself evenly between solid and liquid, then 40 per cent juice would leave behind 8 per cent in the medium, 15 per cent would leave 3 per cent, and 5 per cent would leave 1 per cent. The last two figures are, in the light of what has already been stated about the 5 per cent juice, probably negligible. On the other hand 8 per cent is a factor of some importance and one which is reflected in the behaviour of the tissues. Cultures grown in 40 per cent juice, which was afterwards removed, showed an increased duration of the ensuing growth phase, which as will be seen later, is probably correlated with the residual 8 per cent. The facts that lower concentrations of juice did not give this prolonged growth, and that 8 per cent is about the minimum concentration which would be expected to show it, would seem to indicate that the juice was not absorbed by the clot to any greater extent than could be accounted for by an equal distribution between the solid and liquid phases.

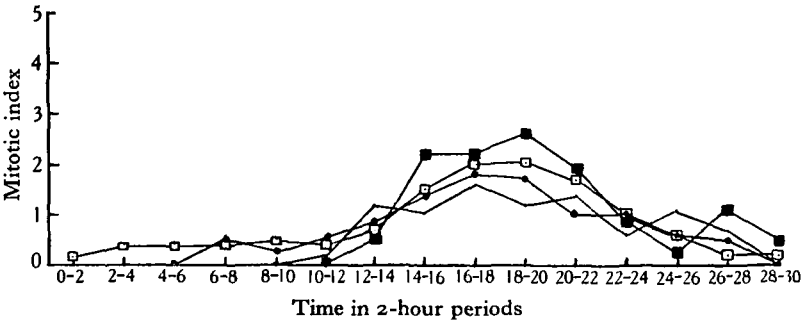
So far then, two points stand out as of importance. In the first place it is apparent that the concentration of the embryo juice is of more significance than the time for which it acts, and secondly that cells can divide when there is only a negligible quantity of embryo juice in the medium. The evidence points to the conception that the juice is utilized or picked up by the cells rapidly at first and then progressively more slowly. If the concentration of juice is low then only a few cells obtain sufficient to cause them to divide, whereas with stronger concentrations more cells reach the threshold level. In this connexion it is interesting to speculate as to whether cells can be divided into groups according to the concentration of juice which they require to cause them to divide, or whether the amount of juice required depends on the particular functional state of the cell and thus varies from time to time. If the embryo juice is simply picked up by the cells it does not appear to be lost from them into the Tyrode solution during the latent period, for the effects of a dose of 15 per cent juice lasting for 1 hour are not much less than those of a 10-hour dose of the same concentration. This leads to the second point, which is that the cells can divide when there is little or no embryo juice present in the medium surrounding them. The only necessity is that the cells shall have been treated with the juice some 10–12 hours previously, and then they can contain within themselves all the necessities for the performance of those metabolic processes which have their climax in cell division. This would seem to be a more reasonable point of view than that the cells steadily lose to the Tyrode solution the substances which they pick up in the first few hours in the embryo juice, and that only those cells divide which can retain sufficient to allow them to go through this process. If this were so, embryo juice applied for 10 hours should produce a far larger crop of mitoses than when the same concentration is applied for only

3 hours, whereas in fact the difference is very small. The point is of some importance in the light of the results which were obtained when two applications of embryo juice were made to the cultures, or when the latter were treated for 16 or 20 hours.

While it was found to make comparatively little difference whether the juice was applied for 1 or 10 hours, there was on the other hand a great divergence between the results of these experiments and those of the earlier ones in which



Duration of application of embryo tissue juice: 16-30 hours.



Duration of application of embryo tissue juice: 1-10 hours.

Fig. 2. Mean curves showing the effect on the mitotic index of applying embryo tissue juice (15 per cent in Tyrode solution) to cultures of periosteal fibroblasts for different lengths of time, from 1 to 30 hours. ○—○ 30 hours; ×—× 20 hours; +—+ 16 hours; ■—■ 10 hours; ●—● 6 hours; □—□ 3 hours; — 1 hour.

the embryo juice was applied for the whole duration of the experiment, that is for 30 hours. Fig. 2 shows clearly that when the duration of application of 15 per cent embryo juice lasted for 10 hours or less the curves of growth obtained are all of the same type. They all rise to a maximum at about the 16th hour and then fall away to zero by about the 28th hour. On the other hand the curve for the continuous application rises fairly steadily after the 16th hour. Some interesting light is thrown on this point by plotting the figures in another way. The results are shown in Figs. 3 and 4. In Fig. 3 it is supposed that each of the cultures originally contained 100 cells and as cells divide the total population follows the summation curves,

which are obtained by integrating from the hourly mitotic indices. Now when these summation curves are plotted logarithmically, Fig. 4 is obtained and it becomes fairly clear that the points of each curve fall into three distinct groups. The slopes of the curves are small during the latent period, they increase during the second 10 hours, and then at the 20th hour there is another inflexion. The uppermost

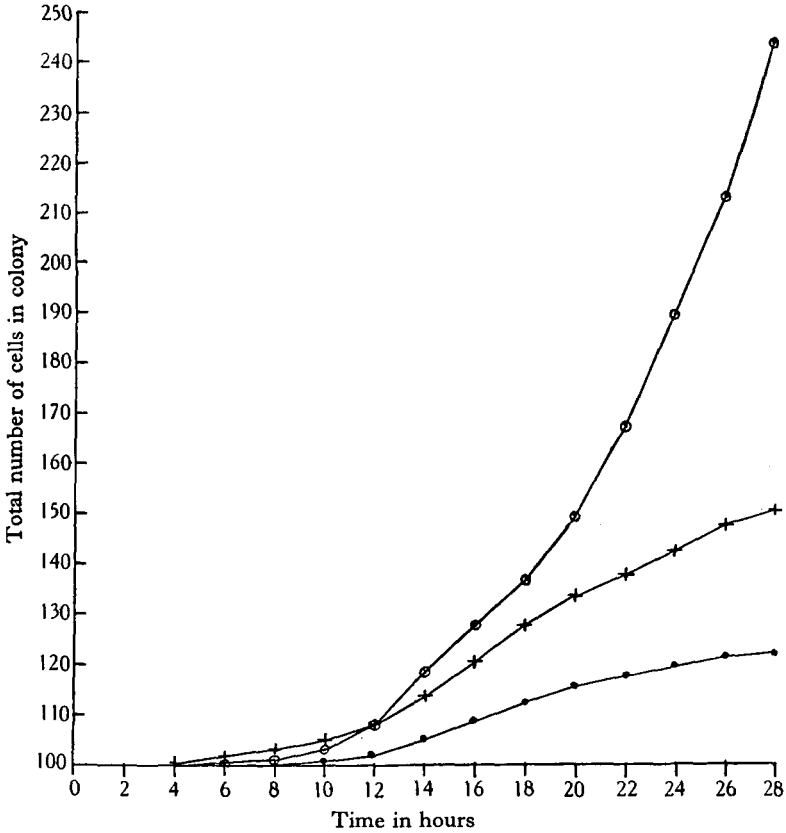


Fig. 3. Summation curves of the total number of cells in hypothetical colonies each of which started with 100 cells, calculated from the mitotic indices. \odot — \odot 15 per cent embryo juice in contact with culture throughout; +—+ 15 per cent embryo juice in contact with culture for 16 hours; \bullet — \bullet 15 per cent embryo juice in contact with culture for 6 hours.

curve plotted for experiments in which the cultures were continuously treated with embryo juice gives an upward turn, where all the others start to descend. This would seem to indicate that in the "continuous application culture" the growth proceeds in two distinct phases, whereas in the others the second phase is apparently absent or greatly reduced. This would fit in with the idea that when the embryo juice is applied for a period of 10 hours or less the cells receive sufficient of the active substance to allow them to divide once, and if the juice is in contact with the culture throughout the experiment the daughter cells of the first divisions can divide again. Experiments in which the juice was kept on the culture for 16 or

20 hours show intermediate curves (Fig. 2). In short, application of embryo juice for from 1 to 10 hours produces a single crop of mitoses; applications for periods of longer than 10 hours produce progressively a larger and larger second crop of

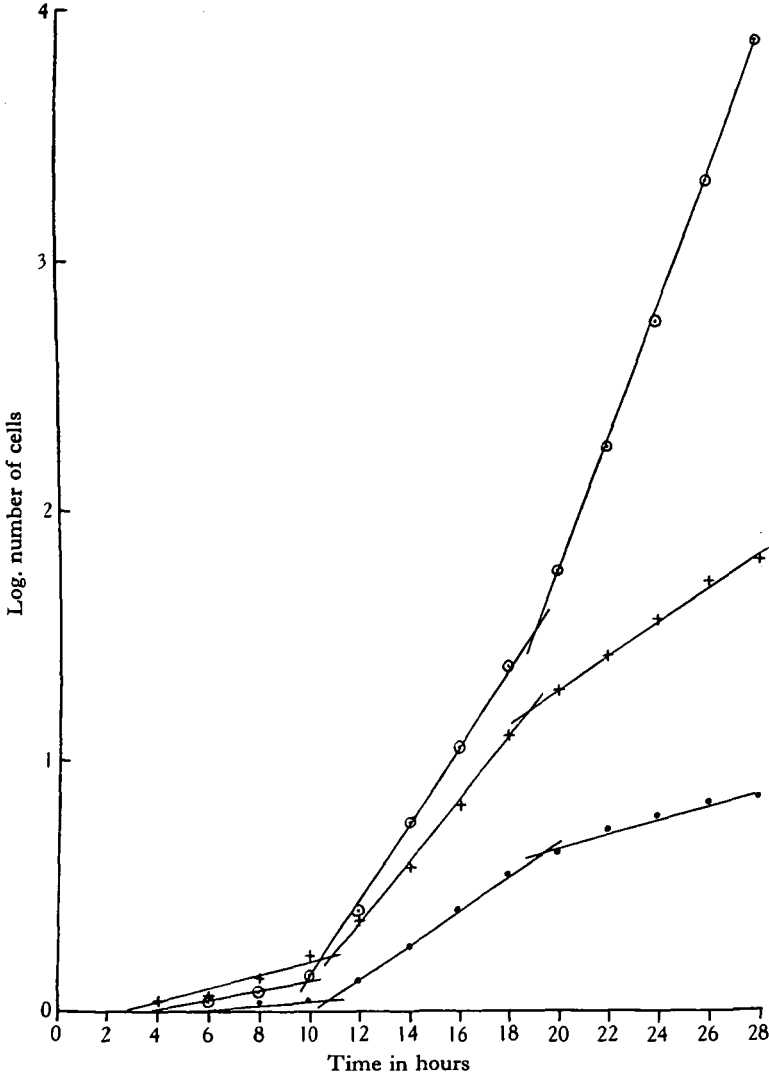


Fig. 4. The same curves as in the preceding figure but plotted on a logarithmic scale. Note the points of inflexion between the 10th and 12th hours and between the 18th and 20th hours.

mitoses; i.e. if the juice remains on the culture for more than 10 hours it is possible for the cells produced at the first division to give rise to a second generation. This points to the conclusion that if embryo juice is present in the medium when the cell divides, the cell can divide again, if not, then the cell has very little chance of undergoing a second division, unless more juice is subsequently added. There are

two possibilities; either the cell loses the stimulating factor to the medium during division, or it uses it up in the process of division. In the first case, if the medium contains embryo juice, very little will be lost by the cells, because as much will get in as will come out if the concentrations inside and outside the cell are the same. On the other hand, if the medium contains no juice, the cell will lose nearly all its supply and be unable to divide again, without a further application of embryo juice. In the second case the substance must stimulate the cells at once and the evidence indicates that it is not actually used until division occurs. Also the cells must only be able to pick up sufficient to carry them through one division and be incapable of picking up more until the supply they contain is used; all of which seems a little unlikely. The evidence is not yet sufficient, however, to allow a decision to be made as to which of these two processes is the one which occurs.

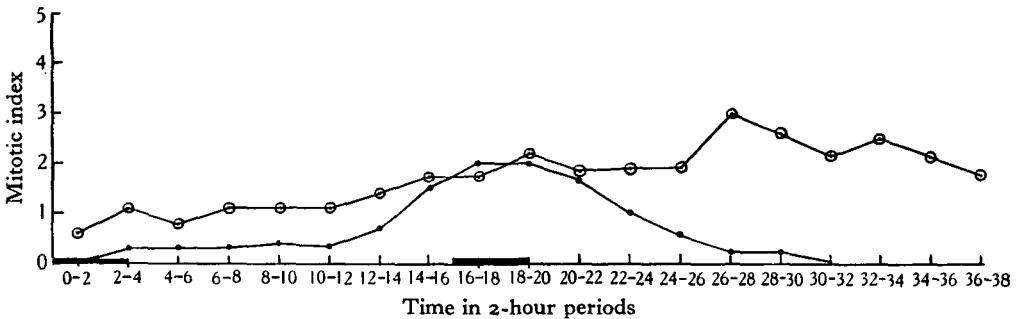


Fig. 5. Mean curves showing the effect on the growth index of cultures receiving respectively one and two 3-hour applications of 15 per cent embryo juice. The second application was made at the time when the first was presumed to be having its maximum effect. — Indicates the presence of embryo tissue juice. ⊙—⊙ mitotic index in cultures having two doses of 15 per cent embryo tissue juice; ●—● mitotic index in cultures having only the first dose of 15 per cent embryo tissue juice.

N.B. The abnormally high values for the mitotic index during the first 12 hours in the cultures receiving the double dose are probably explicable on the grounds that the strain of cells used in these experiments had a higher residual growth energy than the others, and that growth had not entirely ceased at the time when the first dose of juice was added.

The curves of Fig. 5, which record the effect of two 3-hour doses of embryo juice separated by a period of 16 hours, show quite clearly that the time spacing of the doses is far more important than mere duration up to 16 hours. The two separate applications produce a definite second outburst of mitoses, so that it is safe to assume that the cells have again become sensitive to the active substance after 16 hours, and that at this time the greatest number of cells are undergoing their first division. The cells are therefore again susceptible to a second dose of embryo juice either during or immediately after division.

It should be pointed out that in all experiments in which the medium is frequently changed the resulting growth is somewhat less than might be expected, but this can probably be explained partly by the general disturbance to the culture and partly by the removal of certain necessary substances from the plasma part of the medium. In comparing, for example, the curves for experiments in which the embryo juice was applied throughout the whole 30 hours with those in which two

separate doses were given, it is necessary to take into account the fact that in the former case the cultures are undisturbed throughout, while in the latter the medium is changed three times. Secondly, it is necessary to remember that there is also the time factor concerned, which, although of less importance than the concentration, is nevertheless not completely negligible, and the longer the application of embryo juice the more cells are caused to divide.

All the above experiments refer to the frequency of cell division. The records are also interesting in another way, namely that they throw light on the duration of mitosis itself and on its correlation with the general activity of the cells. The foregoing results show that embryo juice shortens the intermitotic period. Does it also affect the mitotic period? It was observed that when the cultures were in a medium in which growth was proceeding very slowly there was a tendency for cells to reach the metaphase of mitosis and stay in that rounded-up condition for long periods, often extending to an hour or more. At the end of that time they would sometimes divide, but often they would degenerate completely. Also, it was noticeable that in cultures where the concentration of embryo juice was low, there was a tendency for the mitoses which proceeded normally to take a much longer time to complete the process. To investigate this, actual measurements were made of the duration of the process in different concentrations of embryo juice. Since the photographs were only taken every 6 min. and the mitotic process is nearly always complete within an hour, no very accurate estimate can be obtained of the actual duration of any one division, but by counting the number of pictures upon which any one mitosis was visible, and then taking the average of a large number of such observations, it was possible to observe a marked difference between the duration in 40 per cent juice and that in 5 per cent juice. In 5 per cent juice the divisions were not very frequent so that the degree of accuracy is not high but, as shown in Fig. 6, there is a consistent difference between the duration in the three concentrations used. Probably there is no significant difference between the durations of mitoses when the juice was applied for different lengths of time, with the possible exception of those for the experiments in which it was applied throughout the whole duration of the experiment. In these there was some evidence that the mitoses occurring in the second outburst were slightly quicker than those in the first, but the method is not accurate enough to allow any definite conclusion to be drawn on this point. It is interesting that the duration of mitosis is much more affected by changing the concentration of embryo juice from 5 to 15 per cent than by changing it from 15 to 40 per cent. The curves suggest that there is a limit to the speed at which mitosis can proceed and that the minimum duration is somewhere in the neighbourhood of 40 min.

It has previously been shown (1936) that the migration rate of the cells is also directly affected by the concentration of the juice and it is interesting to notice (Fig. 7) that there is some degree of correlation between the duration of the phases of mitosis and the migration rate. The correlation is closer for the latter half of the process, the anaphase and telophase, than it is for the prophase and metaphase. This may be partly an experimental discrepancy owing to the difficulty of deciding

just when the prophase begins, or it may be that the actual movements of the cytoplasm characteristic of the later phases of cell division are closely similar to the ordinary migratory movements of the cells.

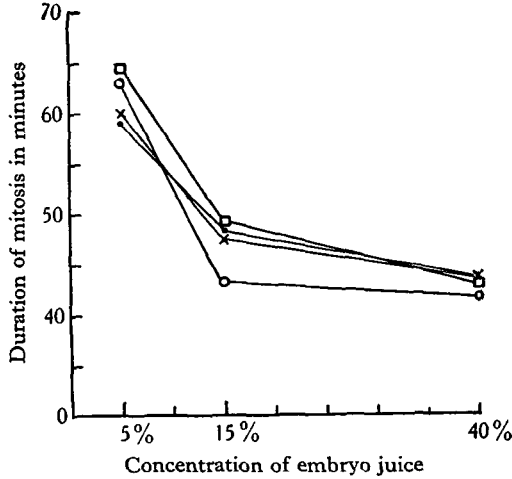


Fig. 6. Relation between the duration of mitosis and the concentration of embryo tissue juice. x—x embryo juice applied for 1 hour; □—□ embryo juice applied for 3 hours; •—• embryo juice applied for 6 hours; o—o embryo juice applied throughout.

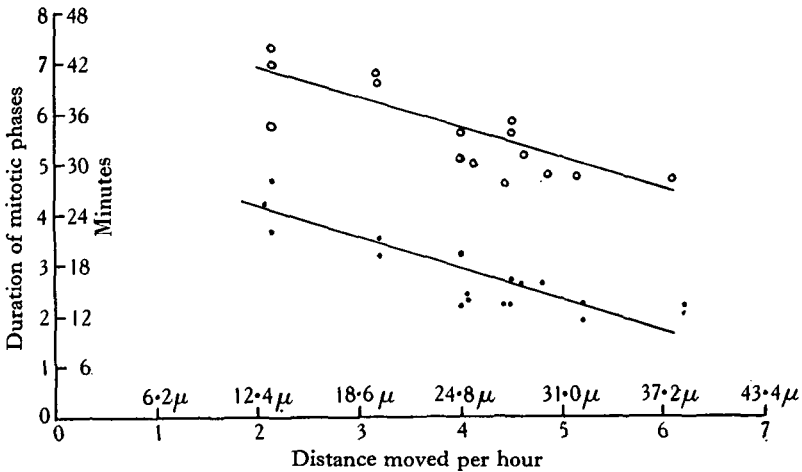


Fig. 7. Correlation between migration rate and the duration of mitotic phases. o duration of prophase plus metaphase; • duration of anaphase plus telophase. Ordinates: number of "frames" during which phase was evident and actual time taken. Abscissae: millimetres moved on photographs, and actual distance moved.

Finally, measurements of the sizes of the cells during metaphase of mitosis have produced some evidence which may be of use in deciding the vexed question as to whether a cell divides because it reaches a certain size or because it is stimulated to divide as distinct from being stimulated to increase in size. Owing to the limitations of the method the evidence cannot be taken as anything more than

suggestive. It is quite out of the question to measure on these photographs of cells in culture the size of the individual cells in the resting condition, since their boundaries are often ill-defined and there is no indication of their thickness. In mitosis, however, the fine processes are withdrawn and the cells become much more globular. In this condition measurements of their area give a somewhat more exact measure of their volume, but still there is the difficulty of their unknown thickness. If it be assumed that embryo juice causes divisions in which the cells round up to about the same extent, then measurements of their areas reflect fairly closely the volumes of the cells. In point of fact (Table I), there was no significant difference in the average area of cells in metaphase when the cells were growing in 5, 10, 15 or 40 per cent juice, so that it seems fairly safe to assume that in embryo juice the cells do on the average round up to about the same extent. At any rate the process depends on other factors than the juice, because for some yet unknown reason different cultures do show very different average sizes of cells. It will be noticed that in these measurements the average area has been taken and this is important, since there is again a very considerable (from 1 to 16 units of area) variation in the size of the cells, not only from culture to culture, but also among the cells of the same culture. This in some cases can possibly be correlated with the amount of fat and vacuoles in the cells, but this is not always a sufficient explanation. The accuracy of the method is naturally limited by this variation and by the rather small number of cells available, but the results are given for what they are worth. The variation in the size of the cells is in itself interesting in that it means that size alone is not the determining factor in causing division, if the culture can be considered as a uniform population. But further and perhaps stronger evidence for this conclusion was obtained by measurements of the areas of the cells at different times during the life of the same culture. It was found (Tables I and II) that when, as in these experiments, the cells had been subjected to a period of life in plasma alone until divisions had ceased, then, when they were stimulated to divide again, the average size of the cells dividing in the first crop (from the 12th to the 22nd hour) was about 15 per cent larger than that of the cells dividing in the second crop (from the 26th to the 36th hour). Now it is well known that cells can obtain all their necessary food substances from plasma alone, but that, although they remain alive and healthy for very long periods in such a medium, they do not divide or only divide at a very slow rate. It is interesting to notice, therefore, that the cells under these conditions get larger, and that after the addition of embryo juice which causes them to divide, the daughter cells seem to remain smaller in size. The difference in size may actually be rather larger than it appears in these experiments, for the cells in the diluted plasma used are generally clear, transparent and free from fat, whereas when embryo juice is added to the medium they quickly develop a certain number of fat droplets in their cytoplasm which may reasonably be expected to increase the volume of the cell. In these experiments, as the results already described in this paper show, the cells of the second crop of mitoses are in all probability derived from those of the first crop, so that in this case there is evidence that the daughter cells divide before they reach the same size

Table I

No. of exp.	Concentration of embryo juice and duration of its application	1st period*		2nd period†		Percentage difference between areas in first and second periods
		No. of cells	Average area of cell	No. of cells	Average area of cell	
110 D	5% for 1 hour	3	8.0	—	—	—
104 C	5% for 3 hours	1	8.0	5	7.6	- 5.0
104 D	5% for 3 hours	11	7.9	—	—	—
108 A	5% for 6 hours	10	7.6	—	—	—
108 B	5% for 6 hours	13	10.5	—	—	—
81 B	10% continuous	27	8.37	17	6.3	-24.8
85 Aa	15% continuous	98	5.04	61	3.78	-25.0
86 Ba	15% continuous	32	8.15	36	7.4	- 9.2
86 Bb	15% continuous	135	4.34	90	3.98	- 8.3
123 F	15% in two 3-hour doses	46	4.2	74	3.53	-16.0
135 D	15% in two 3-hour doses	38	5.0	113	4.3	-14.0
135 E	15% in two 3-hour doses	37	4.9	70	4.15	-15.3
106 A	40% for 1 hour	20	8.05	20	7.75	- 3.71
106 B	40% for 1 hour	27	7.22	16	8.7	+17.01
105 D	40% for 3 hours	56	5.84	48	5.21	-10.81
105 E	40% for 3 hours	40	7.50	7	7.45	- 0.61
84 A.	40% continuous	68	5.66	48	4.85	-14.3
87 C	40% continuous	100	6.21	8	5.0	-19.5

* For 5, 10 and 15 per cent experiments from 12th to 22nd hour. In 40 per cent experiments the growth periods tend to occur slightly earlier, and for that concentration the first period was taken from 10th to 20th hour.

† For 5, 10 and 15 per cent experiments from 26th to 36th hour. For 40 per cent experiments from 20th to 30th hour, or till such time as measurement became unreliable owing to liquefaction of the plasma medium.

‡ The mitoses in the second period here may be in part due to the delayed action of the 8 per cent embryo juice which remains in the medium after the removal of the main bulk, so that these figures may not be strictly comparable with the others.

Table II. Summary of experiments in which it may be confidently assumed that the majority of cells which divide during the second period are derived from those which divided in the first period

Concentration of embryo juice and duration of its application	No. of dividing cells in first period	No. of dividing cells in second period	Percentage difference in size
40% continuous	168	56	16.9
15% continuous	265	187	14.2
15% in two 3-hour doses	121	257	15.1

Mean difference in size 15.4%.

as the parent cell, which is an indication that cells divide, not because they are full-fed, but because some other process, set in action by substances present in the embryo juice, brings about the division. It seems reasonable to conclude therefore that there is present in embryo juice some particular factor responsible for setting in action a process which culminates after some 10-12 hours in the visible process of cell division. Unfortunately the present method can give no

information with regard to the size of the nuclei of these cells, or of the behaviour of the nuclear constituents, but only of the total cell volume, for it is on the nuclear material that one might expect the embryo juice to exert its effect, possibly by allowing the synthesis of some essential nuclear constituent.

One thing is clear; the embryo juice appears to increase many of the activities of the cells. It stimulates their movement; it causes them to divide; it accelerates the actual process of mitosis. Are all these activities the effects of one substance, or does embryo juice contain several active principles? Lastly, it should perhaps be emphasized that all the above experiments and observations have been carried out on periosteal fibroblasts and that the results may not be the same with other tissues, for it is well known that all tissues do not respond equally to treatment with embryo tissue juice.

SUMMARY

1. Embryo juice initiates in cells growing in plasma alone a process which after a latent period of some 10–12 hours culminates in cell division, and it is not necessary for the embryo juice to be present in any appreciable amount during the actual division process.

2. The approximate minimum effective dose is 5 per cent embryo juice in Tyrode solution acting for 3 hours. 15 and 40 per cent juice produce marked effects when applied for only 1 hour.

3. The concentration of embryo juice is a more important factor in determining the number of mitoses produced than is the time for which it acts.

4. Unless the embryo juice remain in contact with the culture for more than 10 hours, only one crop of mitoses occurs; but if it is present in the medium during or after the occurrence of the first divisions, then a second crop of divisions takes place. Evidence is adduced that it is the daughter cells produced during the first crop of mitoses which divide to produce the second crop.

5. The duration of mitosis is correlated with the concentration of the embryo juice. It approaches a minimum of about 40 min.

6. The duration of mitosis, particularly that of the ana- and telophases, is correlated with the rate of migration of the cells.

7. When a second crop of mitoses occurs in these cultures which have passed through a period in plasma alone, there is evidence that the size of the cells in metaphase of mitosis is significantly less than that of the cells of the first crop.

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