STUDIES ON THE GROWTH OF TISSUES *IN VITRO*

IV. ON THE MANNER IN WHICH GROWTH IS STIMULATED BY EXTRACTS OF EMBRYO TISSUES

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(With Eight Text-figures)

In a previous number of this *Journal* (8) a method was described for measuring the growth rates of tissues *in vitro*. The colonies of fibroblasts were grown in modified Carrel flasks and photographed from below at regular intervals, so that a continuous record was obtained of the behaviour of the more peripheral cells of the colony over long periods of time. Mitoses could be easily observed, and the total number of cells on the field fairly accurately estimated. In this way the percentage of cells dividing per hour, or "mitotic index", was calculated, and thereby a measure obtained of one of the more important factors concerned in the growth of tissues. By the use of this method it is possible to investigate the reaction of tissues to various growth-promoting or growth-inhibiting media, and to gain some insight into the general behaviour of growing tissues.

In the experiments to be described in this paper two types of tissues have been under investigation, and they have been treated with embryo extract, a medium of known growth-promoting power, in order to study the manner in which they react to the presence of growth-promoting substances. The tissues used have been periosteal fibroblasts, from the frontal bone of the 11-day chick embryo, and heart fibroblasts, also from the chick (10-day embryo). For making the embryo extract several 7-day chick embryos were taken and washed free from blood in Tyrode solution. They were then minced and forced through a special extract press (1) and the resulting *Brei* centrifuged. The clear, rather opalescent and viscous supernatant fluid was termed the first centrifugate and was used throughout as the standard embryo extract. For experimental purposes it was diluted with Tyrode solution in definite proportions. The residue after decanting the supernatant fluid from the original *Brei* was then diluted with Tyrode solution, re-extracted, and again centrifuged. This gave a second centrifugate, rather less potent than the first. A third centrifugate was obtained in a similar way. These second and third centrifugates were not used for experimental purposes but were useful for feeding the permanent strains of tissues from which the experimental cultures were obtained. With the

exception of one series of experiments on heart fibroblasts, all the experiments were performed on tissues which had been subcultured more than twelve times. This prolonged cultivation is found to lead to greater uniformity in behaviour of the tissues as a whole, although there is still considerable variation in behaviour among individual cells of such tissues. Presumably it allows the cells to become more acclimatised to life *in vitro*, and may actually weed out some of the more sensitive cell types. The assertion, however, that pure cultures of cells are produced in this way is not based on any very sound evidence, and except in one or two specific cases cannot be regarded as anything more than an hypothesis. Many of the experiments were done on sister cultures, *i.e.* tissues obtained by dividing a previous culture into two equal fragments. In general the behaviour of such sister cultures was reasonably similar.

It should be remembered that by this photographic method only a limited area of the culture can be examined, and the assumption has to be made that this particular area is typical of the growth zone of the culture as a whole, and moreover that the culture under investigation is behaving in a manner comparable to other cultures growing under similar conditions. These points can be to some extent checked by direct observations on control cultures made at the same time, and by repetition of the experiment. Another important point to notice is that since only a comparatively small number of cells can be conveniently photographed, generally between 80 and 400, errors in estimating percentage growth rates are naturally large, so that for this reason also several experiments have to be considered for each observation. The chief source of error is in the estimation of the total number of cells on the field. The mitoses can be counted with considerable accuracy, but since their total number is relatively small there is necessarily a great hourly variation. As far as the observations allow conclusions to be drawn, there is no indication of anything intrinsically rhythmic in the occurrence of mitoses, although, as will be shown later, periodic alterations in the medium lead to corresponding changes in division rate. Under constant conditions mitoses appear to occur at random.

The errors due to the limited numbers of divisions are minimised if the growth is considered over longer periods than 1 hour, that is to say over periods of 4 hours for example; but then any sudden variations in growth, which are in practice found to take place, are masked, and the growth curves obtained, though smoother, tend not to show as much as the more irregular curves plotted for hourly observations.

As pointed out in the earlier account of the method, when tissues are grown in a solid coagulum of plasma and Tyrode solution (0·05 c.c. plasma + 0·15 c.c. Tyrode) and the clot bathed with a supernatant fluid consisting of 1 c.c. of Tyrode, the growth rate declines steadily from the time when it is first possible to obtain photographs, namely after about the twentieth hour. The mitotic index reaches zero at some time between the fortieth and seventieth hours. In practice it is found that if cultures are grown in this way for 48 hours, by the end of that time the growth by cell division can be considered to have ceased. If the cultures remain undisturbed for a longer period only very occasional divisions occur, and it makes little or no difference if the supernatant fluid (Tyrode solution) be renewed or not. Indeed, renewing the Tyrode

is perhaps a little inhibitory. The cultures, although not actively growing, remain alive in such a medium for several days. The exact cause of the cessation of cell division is not known. Whether it is due to exhaustion of a growth-promoting substance or of food substances in general is uncertain. Evidence perhaps favours the former view, for the addition of serum from an adult cock, which presumably contains considerable nutriment but probably no growth-promoting substances, allows the cultures to survive for longer than in Tyrode solution but causes practically no increase in the mitotic index (Fig. 1), only an occasional mitosis being observed. This argument is, however, not conclusive, because serum from adult animals has been stated to be somewhat inhibitory to growth, and experiments to be described later show that this inhibition is a real phenomenon. The quiescent cells in plasma bathed with Tyrode solution are perfectly healthy, and, since their growth rate is zero, they are in a convenient condition for studying the effects of growth-promoting substances. Moreover, they are found to respond in a perfectly characteristic manner to the addition of such substances.

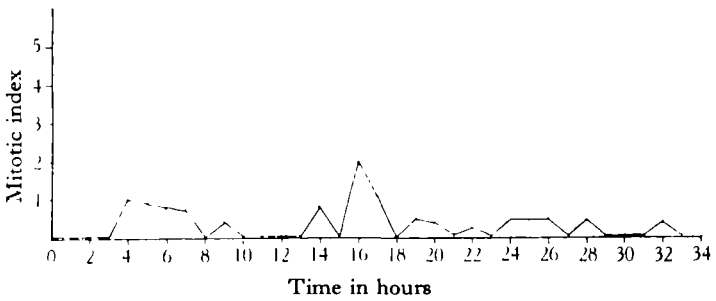

Fig. 1. Mitotic index of periosteal fibroblasts, when the Tyrode solution was replaced by serum at zero hours.

When a culture of heart fibroblasts is treated after 48 hours with embryo extract, it is once again stimulated to activity, but there is a latent period before any visible effects are produced. Part of this is presumably due to the re-establishment of steady conditions after the necessary disturbance of changing the fluid medium, and part of it must be due to the time taken for the active constituents of the extract to diffuse through the plasma coagulum and so to reach the tissues. It is not known how long either of these processes take, but, in point of fact, the tissues can be seen to show increased migratory activity after about 2 hours. The rate of movement of the cells can be calculated by observing the distance moved by the peripheral cells of the colony during hourly periods. Generally it is possible to observe about fifteen cells in this way and to take their mean velocity. It is found that the rate of migration begins to increase after about 2 hours, and goes on increasing steadily for several more hours, depending on the strength of the extract (see Figs. 6 and 7). The stronger the solution the quicker the cells reach their maximum velocity. During this period of renewed activity there are several coincident changes in the morphology of the culture and of its individual cells. At first the cells are spindle-shaped and closely packed together, so that the colony as a whole has a very definite boundary, but on

adding the extract the peripheral cells begin to scatter, at the same time becoming more triangular or fan-shaped, with the base of the triangle away from the central implant. From the base numerous pseudopodia project outwards into the medium. Increased movement of granules and vacuoles within the cells becomes noticeable at a very early stage. After 7 or 8 hours the cells become drawn out into the much narrower shapes characteristic of cells growing in embryo extract. Only the cells on the periphery are mechanically capable of undergoing these changes, and it is not all of those that can be seen in the same state at any one time, for the cells, as they migrate away, are constantly altering their shapes. But the changes are sufficiently marked to give quite a different appearance to the culture. It is probable that the changes in the morphology of the cells are correlated more or less directly with changes in the consistency of the medium, for it is known that in the presence of embryo extract liquefaction of the coagulum readily occurs in the immediate vicinity of the cells.

The second characteristic occurrence on the addition of the extract is that the cells tend to accumulate fat droplets, and the amount of fat which they contain

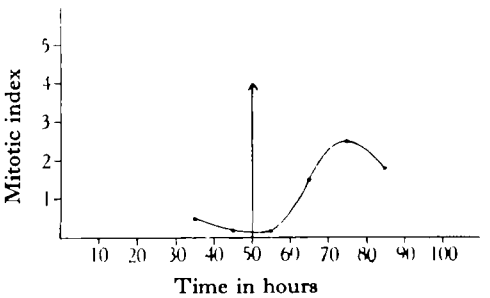

Fig. 2. Average curve for a series of experiments on fresh chick heart tissues. Culture started in plasma and Tyrode solution at zero hours. Embryo extract (about 10 %) added at 50 hours. Smoothed curve.

seems to vary in proportion to the concentration of the extract added. Heart fibroblasts accumulate more than periosteal fibroblasts.

After about 10 or 12 hours cell divisions begin to occur and they become increasingly numerous during a period of about another 12 hours, after which time they again slowly decrease in number. Fig. 2 shows the effect of adding embryo extract to fresh explants of chick heart tissue. It is an average curve for a series of experiments, in which the extract used was made in a rather different way, by extracting one 10-day chick embryo with 5 c.c. of Tyrode solution. Such extract corresponds in activity to the normal extract used in other experiments diluted about ten times. It was found that the age of the chick and the duration of culture in plasma and Tyrode solution previous to the addition of embryo extract had little or no effect on the length of the latent period before the cell proliferation started. In one instance the "starvation" period was increased up to 72 hours without producing any noticeable effect on the length of the latent period. There is thus a period of about 8 hours between the time when the extract can be assumed to have

reached the tissues, in that increased migration is evident, and the time when cell division commences.

When the same experiment was performed upon old strains of periosteal fibroblasts, but using different concentrations of extract, similar results were obtained with regard to the latent period, and these experiments showed that it became noticeably longer when the concentration of extract was low (10 per cent. first centrifugate, 90 per cent. Tyrode). Above a certain limit (15 per cent. extract) the latent period was not significantly affected by increasing the amount of extract present (Figs. 3, 4, 5 and 6).

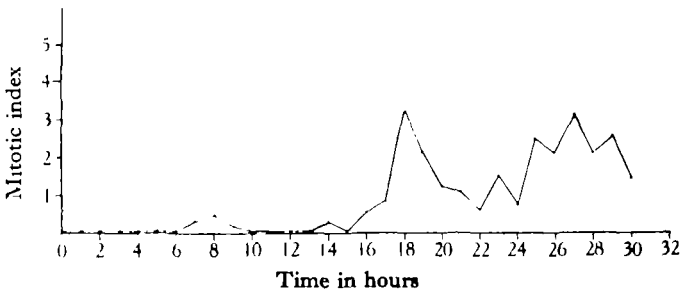

Fig. 3. Growth of periosteal fibroblast. 10 % embryo extract added at zero hours.

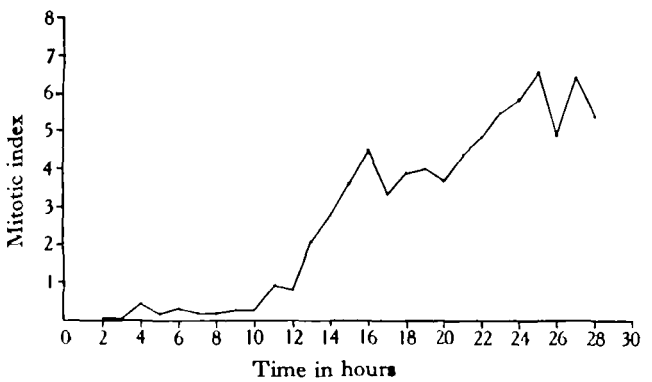

Fig. 4. Growth of periosteal fibroblast. 15 % embryo extract added at zero hours.

Parker (6) has indicated that different tissues behave differently towards embryo extract in respect of their ability to grow in this medium. Growth of periosteal fibroblasts has been stated to be optimal in 15 per cent. embryo extract, and to decrease with higher concentrations. The accompanying figures seem to show that this is not the case, but there are complicating factors, and the explanation for the apparent discrepancy is probably as follows. Previous methods of measuring growth have mostly depended on measuring the area occupied by the colonies and by subculturing the tissues. In other words the growth of a colony as a whole, the "Wachstumsbilanz" (E. Mayer (3)), has been measured, and not only the proliferation of its cells. Consequently anything which disturbs the colony as a whole will be regarded

as inhibiting growth. Now in the present series of experiments it has been found that the growth of periosteal fibroblasts, when the whole culture is considered, is again probably optimal in 15 per cent. extract, for the growth is then very uniform and there is little or no tendency for the coagulum to liquefy and so to cause the culture to break away and contract upon itself. In higher concentrations of extract on the

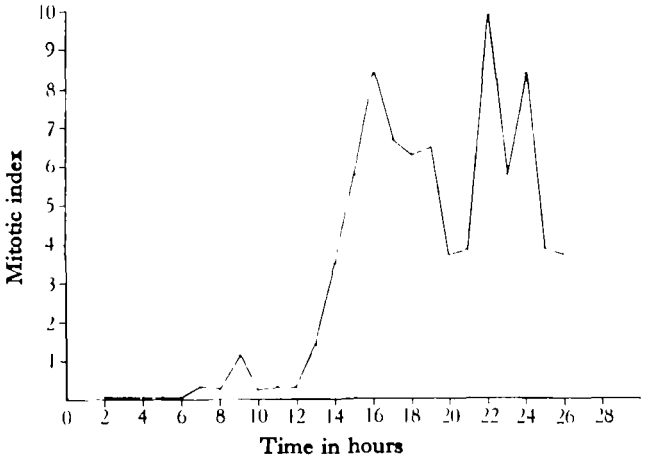

Fig. 5. Growth of periosteal fibroblast. 40 % embryo extract added at zero hours.

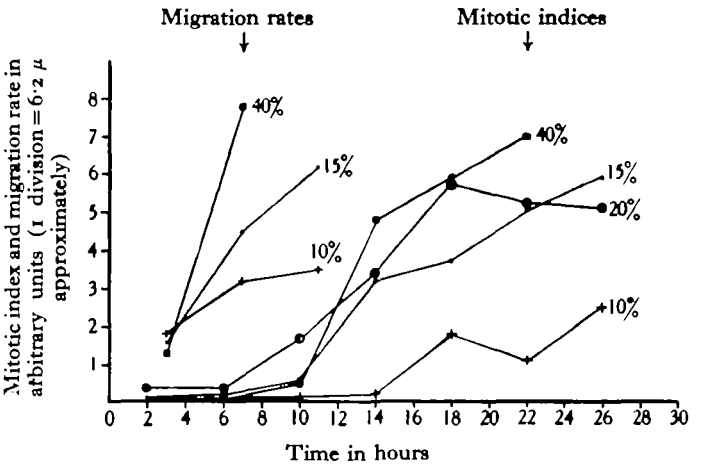

Fig. 6. Mitotic index and migration rate of periosteal fibroblasts in different concentrations of embryo extract (4-hour periods). Embryo extract added at zero hours.

other hand the delicate coagulum necessary in these experiments nearly always liquefies and breaks up, especially where it is in contact with the tissues. The tension exerted by the migrating cells seems to aid this process. This liquefaction makes the measurement of growth rather more difficult and the conditions are of course not so uniform, but it is significant that there is no lessening of the mitotic index, but rather

the reverse. It is probable, therefore, that the reduced growth rates found by previous workers to occur in the higher concentrations of extract are explicable on the grounds of this liquefaction interfering with the colony growth rather than on an actual inhibition of the cell divisions. The figures obtained in the present series of experiments in 20 and 40 per cent. extract are irregular. This may in part be due to the liquefaction of the coagulum in the peripheral region of the cultures which makes photography difficult, and often those cells which can be photographed are isolated from the central implant, so that the conditions are perhaps not always quite comparable. But it is significant that the majority of the figures are higher than those for 15 per cent. extract. On previous views, and also because of the abnormal surroundings of the cells when thus cut off from the central implant, it might have been expected that the mitotic indices in the higher concentrations of extract would show a decrease.

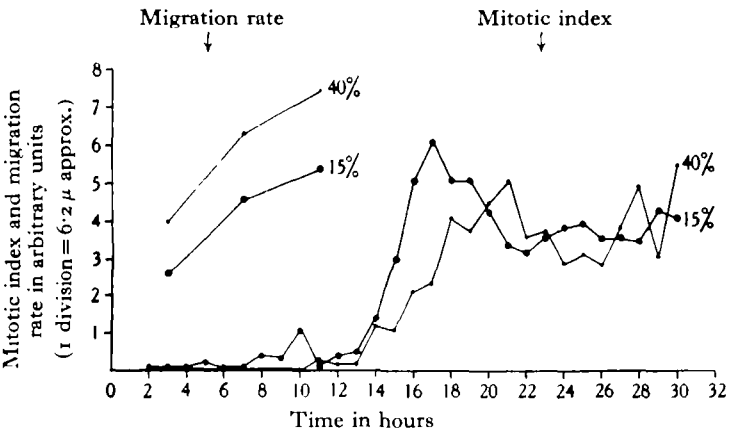

Fig. 7. Mitotic index and migration rate of heart fibroblasts in different concentrations of embryo extract. Embryo extract added at zero hours.

Further experiments performed upon so-called pure strains of heart fibroblasts gave interesting results in connection with the effects of embryo extract on growth. In the past these cells have been found to be not so adversely affected by high concentrations of extract as periosteal fibroblasts (6). In fact their growth has been found to be maximal in 40 per cent. extract. In the present experiments there was found to be no significant difference between growth in 15 per cent. and that in 40 per cent. extract in respect of the mitotic index, but the migration rate is higher in 40 per cent. than in 15 per cent. (Fig. 7). This is very marked at first but decreases later. It is probably sufficient to account for the apparent increase in growth of colonies in 40 per cent. over those in 15 per cent. extract, so that, by the area method of measurement, the growth rate would appear higher in 40 per cent. extract. The present experiments show however that the actual increase in cell number is practically unchanged.

There is one distinct point of difference between heart and periosteal fibroblasts. Liquefaction of the coagulum does not occur in cultures of heart fibroblasts until the

concentration of extract reaches 60 per cent. With periosteal fibroblasts it occurs with increasing ease in concentrations of 20 per cent. and above. It may be that the liquefaction is in some way correlated with growth rate, for the growth rate of periosteal fibroblasts is noticeably higher than that of heart fibroblasts, and they liquefy the medium much more readily (6). The method by which the liquefaction takes place in these cultures is interesting. Besides the main layer of cells near the glass there is often a subsidiary layer (Nebenschicht (4)) which grows out in the substance of the medium. After some hours this forms a ring of cells of ever-increasing radius, which weakens the coagulum within its circumference, and "explosion" of the colony occurs by a breaking of the coagulum and the retraction of the cells within the ring. This seems to be a special case of the type of liquefaction which will be described by Jacoby in a separate paper (2).

Several other points of interest have come to light during the course of these experiments, or by the use of the same method. In the first place, mitoses occur quite frequently among cells apparently quite isolated from the rest of the colony. Secondly, cells often round up as though about to divide and then spread out again as single cells. Several times they have been observed to go further and to form two round daughter cells, which instead of spreading out again and separating, reunite to form a single cell once more. Direct splitting of cells as observed by Parker (7) has also been noticed in these cultures, but it is impossible to say whether his assertion that the process is concerned with the formation of wandering cells is correct, since this photographic method is not a satisfactory one for distinguishing cell types. However, it was often found that cells became more or less star-shaped and acquired the characteristic appearance of wandering cells with broad membrane-like pseudopodia, so that possibly there is a connection. This appearance often occurred in cells growing on the glass surface after the clot had disappeared owing to liquefaction. It may be connected with the process of liquefaction itself.

The addition of proteose solutions (1 per cent.) to cells growing in plasma only has not so far been found to cause any increase in the growth rate.

The addition of a dialysate (48 hours) from embryo extract into Tyrode solution caused a very slight but definite increase in the growth index after the usual latent period.

Finally, the mitotic indices of two colonies of periosteal fibroblasts have been followed consecutively for a period of 10 days. The fluid phase in the first culture was 10 per cent. embryo extract in Tyrode solution, and, in the second, 10 per cent. extract and 20 per cent. serum in Tyrode solution. These fluid media were added as soon as clotting of the usual plasma coagulum had occurred. The fluid medium was renewed every 2 days (at the points marked by arrows in Fig. 8). Two main conclusions can be drawn from the results obtained. First, the serum appears to have a definite influence in decreasing the effect of embryo extract on the occurrence of cell divisions, and secondly, the growth is not constant but depends on the renewal of the extract. Following each change of the medium there is a rise in the value of the mitotic index, and as the culture gets older and the growth-stimulating substances more exhausted, this temporary outburst of growth becomes more and more marked.

Again there is a latent period before the increase in growth shows itself, and after reaching a maximum in about 12 hours, the growth rate steadily declines again till the next addition of extract. After the cultures had been growing for about 6 days, cell degeneration began to occur rapidly, and on the ninth and tenth days the number of healthy cells on the field remained almost constant, for although cells were still dividing, just as many were degenerating. Often the cells would apparently start to divide, but when they had reached the spherical form characteristic of metaphase of mitosis they remained in that condition for a long period and then eventually disintegrated. It seems probable therefore that the healthy growth of a culture in a flask is only possible for a period of 8–10 days, that is, when the medium is constituted as in the experiments outlined above. For continued active growth the culture must

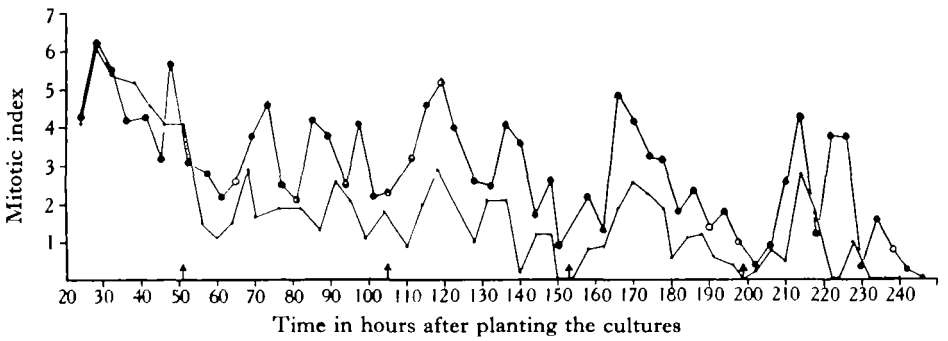

Fig. 8. Growth of periosteal fibroblasts in: 10 % embryo extract, 90 % Tyrode solution, ⊙—○—⊙; 10 % embryo extract, 20 % serum, 70 % Tyrode solution, •—•—•. ↑ indicates the renewal of the fluid phase of the medium.

be transferred to fresh plasma after that time, otherwise either the plasma is exhausted of its beneficial properties, or more likely becomes too contaminated with metabolites, so that the growth, although it may continue for longer periods, is slower and always to some extent counterbalanced by cell degeneration. Several other cultures were treated in the same way as the above, although not followed photographically but only by measurement of their areas. They showed similar behaviour. In some of the cultures liquefaction of the coagulum occurred towards the end of the experiment, and this phenomenon was found to happen more readily in those cultures to which serum had not been added. In other words serum besides checking the division of cells also checks the liquefaction of the coagulum, and it is interesting to speculate as to whether the two processes are connected.

DISCUSSION

The results reported in this paper are of interest in two ways. In the first place they throw light on the manner in which a non-growing system of cells can be converted into one in which cell proliferation is occurring. Secondly they show how cells of different origin can be compared in regard to their capacity for cell multiplication.

Cells living in plasma bathed with Tyrode solution alone cease to divide after 48 hours, but are capable of being stimulated to renewed activity. Only certain specific substances, however, of which embryo extract is one, seem capable of producing this activity. Serum and proteoses, both of which might be expected to provide abundant food for the cells, do not significantly bring about their proliferation. The original cessation of growth is probably due therefore to lack of growth-stimulating substance, rather than to lack of food substances in general. Possibly cells so reduced in activity are comparable to the cells in the adult animal which have ceased to grow, for these obviously do not lack food substances as a whole, but may be short of one or two substances in particular which are necessary for cell multiplication. This suggestion, however, takes no account of the process of differentiation which has been undergone by adult tissues and which may itself check cell division.

Other points in connection with the onset of cell proliferation concern the cause of the latent period, the shape of the growth curve, and the beginning of cell migration. The addition of serum or proteoses by themselves does not produce increased cell migration in the same way that embryo extract does, so that it may be justifiable to assume that the factor which increases cell migration is similar in character to, if not identical with, that which stimulates cell division. Otherwise it is necessary to postulate two factors peculiar to embryo extract. Now the migration of cells begins quite quickly after the addition of the extract, but cell division does not start for another 8 or 10 hours. The active substance, if identical with the growth-producing substance, can be presumed to reach the cells quickly, that is to say within an hour or two at the most, but a long time has to elapse before the effects of its presence on the occurrence of cell division become visible. Now it is noticeable that when cell divisions begin, they occur with increasing frequency for the next 10 or 12 hours. There is, however, some suggestion on all the curves shown, in some more marked than in others, that there is a peak about 16–18 hours after adding the extract, followed by a second wave of less intensity about 10 hours later. These peaks are masked when indices are calculated over 4-hour periods, in which case the growth curves assume fairly regular slopes showing a more steady rise in growth intensity (see Fig. 6). A consideration of the length of the latent period and the shape of the curve leads to some interesting speculations as to the manner in which the extract stimulates the growth of cells. The long latent period suggests that before cell division can occur there has to be a prolonged building-up process. If it is assumed that some substance necessary for the formation of new cell structures is missing from the plasma-Tyrode medium, then after the cells have divided in that medium they will not be able to synthesise new protoplasm, or in some other way prepare for the next division, and will remain at the beginning of the growth period. When embryo extract is again added synthesis recommences and, after the necessary interval, divisions start again. If it is assumed that the cells all take approximately the same time to reach the division point, then there should be a sudden outburst of divisions at that time after adding the extract. On the other hand the growth-promoting substance probably does not disappear from all the cells in the plasma-Tyrode medium at the same time, so that they will not all be at quite the same stage

of the growth process when the extract is added. Moreover, it is not likely that all the cells in the culture synthesise at the same rate. Possibly in a genuine pure line of cells this might be so, but in the ordinary tissue culture it has been shown (5) that there is great variation in the length of the intermitotic period, and it should not be expected therefore that all the cells would start to divide at once. A progressive increase in the number of divisions over a considerable period of time would be more likely, but this might show a peak when a time equivalent to the mean intermitotic period had elapsed since the cessation of divisions. There would, however, be some scatter round this peak, since the intermitotic period is very variable and the cells did not all stop dividing at the same time. On these lines it is difficult to see why the curve should continue to rise after the twentieth hour. Can it be that the cells which divided first are again beginning to divide and overlapping the slower ones in their first division? This would, however, imply that the intermitotic period had been somewhat shortened. But the data are insufficient to make the further discussion profitable on these lines, and the only conclusion which may be drawn is that embryo extract starts off a synthesis in the cells (or some other similar process) which culminates in cell division, but it does not actually stimulate the onset of the process of cell division itself.

SUMMARY

1. A method is described for testing the growth-promoting properties of various substances on tissues *in vitro*.

2. When growth of cells (chick fibroblasts) has ceased in a medium of plasma and Tyrode solution, it may be restarted again by the addition of embryo extract.

3. There is a latent period of from 2 to 3 hours before a greatly increased migration of cells occurs.

4. There is a latent period of from 10 to 12 hours before cell division starts again. After that time the percentage of cells dividing increases for the next 12 hours and then declines.

5. For low concentrations of extract the growth is proportional to the concentration.

6. Cell division of neither heart fibroblasts nor of periosteal fibroblasts is inhibited by concentrations of extract higher than 15 per cent. in Tyrode solution, but cultures of periosteal fibroblasts in such concentrations cause a "liquefaction" of the plasma medium. With heart fibroblasts, "liquefaction" of the medium does not readily occur, within the duration of these experiments, until the concentration of extract reaches 60 per cent.

7. The growth of two cultures in embryo extract, and embryo extract and serum respectively, has been followed for 10 consecutive days. The rate of cell division in the medium containing serum was less rapid. Cell degeneration commenced in both cultures after about 6 days, so that after about 8 or 10 days there was no further increase in the number of cells on the field. The addition of fresh fluid medium to the cultures was clearly reflected in corresponding increases in the values of the mitotic indices.

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