CL. THE EFFECT OF THE OXIDATION-REDUCTION POTENTIAL OF THE MEDIUM ON THE GROWTH OF TISSUE CULTURES.

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THE peculiarly high aerobic and anaerobic glycolysis of malignant cells suggests that they may be able to survive conditions of anoxaemia which are fatal to normal tissue cells. Warburg *et al.* [1924] have suggested the possibility of a connection between the increased power of glycolysis shown by tumour cells and their irregular and unrestrained growth. It becomes therefore of some importance to know how normal and malignant cells behave when given a poor oxygen supply while growing *in vitro*. The point has been studied by several workers, but their results have been conflicting.

Wind [1926] found that the Rous chicken sarcoma could grow under strictly anaerobic conditions which inhibited the growth of 10-day chick embryo heart tissue. Wright [1928] also found that malignant cells grew and divided at lower oxygen pressures than that in which normal embryonic tissue could grow; but he did not find that malignant cells would grow under strictly anaerobic conditions. He gives the lowest partial pressure of oxygen at which mitosis occurred in each tissue he examined, as follows:

			mm. O ₂
Embryo chick heart	...	...	12
Sarcoma (J.R.S.)	...	...	6
Carcinoma (M 2164)	...	...	3

Fischer [1928] came to the opposite conclusion and reports that malignant cells survived less well under low pressures of oxygen when compared with normal cells.

While our experiments were in progress Laser [1933] published a paper in which he stated that if a culture of normal embryonic tissue were first allowed to grow for 24 hours in a normal atmosphere, it would then continue to grow well for several days under strictly anaerobic conditions. One or two experiments made to test this, reported later in this paper, do not support his conclusions.

It occurred to us that possibly the growth and division of tissue cells in such experiments might be governed more directly by the oxidation-reduction potential of the culture medium than by the concentration of oxygen in the gas phase. That the oxidation-reduction potential of a culture medium might have a real biological significance is suggested by the studies of Knight and Fildes [1930]. They found that germination of the spores of tetanus bacilli occurs only on the negative side of a certain very definite oxidation-reduction potential of the culture medium ($E_h = 110$ mv., when the p_H of the medium = 7.6).

The E_h of the culture medium they employed fell spontaneously when a stream of purified nitrogen was bubbled through it. They were able to maintain the E_h at any required level by mixing with the purified nitrogen varying proportions of unpurified nitrogen, containing traces of oxygen. The partial pressure of oxygen which would be theoretically in reversible equilibrium with such a solution, $E_h = +110$ mv., would be about 10^{-44} atmospheres [Clark, 1928; Michaelis, 1930]. It is obvious therefore that the oxygen used in these experiments was not in reversible equilibrium with the oxidation-reduction systems of the medium. The definite and consistent results obtained by Knight and Fildes suggest that the germination of tetanus spores took place when a certain level of E_h was reached by the oxidation-reduction systems in the medium, rather than when a particular low concentration of oxygen was present. It is possible that at low oxygen pressures tissue cells also are affected more by the E_h of the culture medium in which they grow than by the oxygen pressure of the gas or of the solution. We have indeed no direct evidence that this is so; the possibility stimulated the performance of this series of experiments.

The purpose of this paper is to record how the growth and division of cells from the embryo chick heart may be correlated with the E_h of the medium in which it is suspended. In a later paper we hope to present a similar correlation between the growth of tumour cells and the E_h of the culture medium.

EXPERIMENTAL.

Preparation of medium and cultures.

In the earlier experiments the salt solution used as the basis of the medium was the phosphate solution recommended by Pannett and Compton [1924]. This fluid was chosen because if Tyrode solution containing bicarbonate were employed, then on passing a stream of nitrogen or air through the medium a considerable quantity of CO_2 would be removed, and the p_H would move too much towards the alkaline side. Media containing the correct concentration of Ca^{++} cannot however be adequately buffered by phosphate, on account of the precipitation of calcium phosphate. The addition of serum also complicates matters. In the later experiments, therefore, the usual Tyrode solution was used, and special gas mixtures containing 2.5 % carbon dioxide were employed. This amount of carbon dioxide kept the p_H of the medium at about 7.4, and in this respect much better control of the conditions was obtained. This change in the nature of the medium did not produce any apparent change in the behaviour of the cultures during an experiment.

Serum was obtained by allowing blood taken from the wing vein of a fowl to clot and the clot to contract and express the serum at 38° . The serum was centrifuged before use. Embryo extract was prepared from a fine mince of two 11-day or three 10-day chick embryos by the addition of 10 ml. Tyrode solution followed by centrifuging. The tissue used was taken from the apex of the heart of a 10–11-day chick embryo. Small fragments were explanted on to $\frac{3}{4}$ in. coverslips in a thin film of a mixture of equal parts of fowl plasma and embryo extract, and this mixture was allowed to clot. 15 ml. of a solution made up of 25 % embryo extract, 10 % serum and 65 % Tyrode solution were placed in a small beaker 6.5 cm. high and with a diameter of 3.5 cm. Three of the coverslip cultures were then placed in the beaker, standing on their edges on the bottom and leaning against the sides in an almost vertical position. The beaker was then closed by the rubber bung carrying the electrodes, gas

inlet and outlet tubes and agar bridge. The whole of the above process was carried out with sterile fluids and apparatus and under strictly aseptic conditions. The bung with its appurtenances was autoclaved while supported in the mouth of a boiling-tube half-filled with distilled water. Just before placing the bung in the culture vessel the tip of the tapered end of the agar bridge, which had been sealed, was broken off so that the KCl-agar might make contact with the medium in the beaker. The outer ends of the gas inlet and outlet tubes were tightly plugged with cotton wool (Fig. 1).

Fig. 1.

Preparation of the gas stream.

Nitrogen containing 2.5 % carbon dioxide was obtained from the British Oxygen Co. This mixture contained about 0.2 % of oxygen, which was removed by passing the gas through two sintered glass wash-bottles containing a solution of chromous chloride. This has the advantage of absorbing the last traces of oxygen without affecting the carbon dioxide [Warburg and Christian, 1931]. Unpurified nitrogen and 2.4 % carbon dioxide from another cylinder were added to the stream of purified gas as required for purposes of poisoning the potential. A mixture of air with 2.5 % carbon dioxide was passed through the control vessel. With this amount of carbon dioxide the p_H of the media remained at about 7.4.

Both gas mixtures were passed through wash-bottles containing 0.9 % NaCl, which were kept at body temperature in the incubator. The gas was thus saturated with water vapour, and all danger of evaporation of the media was avoided. An open side-tube, at a point just before the gas entered the experimental vessel, dipped into water and served as an escape valve. The pressure of gas passing through the culture vessel could be nicely adjusted by varying the depth of the water. Rubber connections were reduced to a minimum and made where necessary with short pieces of pressure tubing.

Electrical measurements.

The E_h of the culture medium was measured by determining the potential set up on metal electrodes immersed in the solution. The circuit was completed by a half-cell formed by a bright platinum electrode immersed in $N/10$ hydrochloric acid saturated with quinhydrone; the half-cell was connected to the culture vessel by a saturated KCl-agar bridge. The quinhydrone $N/10$ HCl half-cell was used because it is easy to prepare and it will give a potential which is reproducible with considerable accuracy [Morgan *et al.*, 1931]. The potential of this half-cell was taken to be +623 mv. [Clark, 1928, p. 420]. The use of a saturated KCl-agar bridge introduced unavoidable errors of 2–3 mv. [MacLagan, 1929], but this is not serious for experiments involving differences of the order of 10 mv. The metal electrodes were carefully cleaned with hot chromic acid before each experiment by the method described by Morgan *et al.* [1931]. This was very necessary to ensure consistent behaviour of the electrodes in such poorly poised solutions as the media used. It is important to avoid taking any current from the metal electrodes, which are very easily polarised especially in poorly poised solutions. For this reason the use of a static electrometer is essential. The Lindemann electrometer proved to be very convenient and satisfactory. It was set up in the form used by Kerridge [1926] for measurements with the glass electrode. Two or more independent metal electrodes were used in each vessel. An individual electrode, apparently perfect, will sometimes be very inaccurate in these poorly poised solutions. It then has to be discarded.

In the early experiments both gold and platinum electrodes were used in each vessel. Dixon and Quastel [1923] have pointed out that in some sulphur-containing oxidation-reduction systems gold and platinum electrodes may behave differently. In our experiments the gold and the platinum electrodes behaved alike showing that the readings were not complicated by the presence of the anomalous systems described by Dixon and Quastel. In the later experiments only platinum electrodes were used. The electrodes were formed of thin platinum foil, 2 cm.² in area, which were welded on to a short piece of platinum wire. The platinum wire was fused on to a piece of copper wire and was then fused into a glass holder with sealing-in glass.

In the early experiments a glass electrode (Haber's bulb form) was also immersed in the culture medium. The bulb of the electrode was blown from the special glass recommended by MacInnes and Dole [1930]. It was obtained from the Corning Glass Co., New York (No. 015). The glass electrode contained $N/10$ HCl and was fitted with a $N/10$ HCl quinhydrone half-cell. Special precautions were taken to ensure perfect insulation. By this means it was possible to determine whether there was any gross change of reaction of the culture medium during the experiment. If bacterial infection developed the medium turned rapidly acid (at the same time the E_h would be rapidly lowered), but in an uninfected culture the p_H remained practically constant for the duration of the experiment. Therefore, for the sake of simplicity, the glass electrode was discarded in the later experiments. The p_H of the medium was determined at the end of each experiment. In an uninfected experiment, in which the 2.5 % carbon dioxide mixtures were used, the p_H was always about 7.4. In an infected culture it had often fallen to below 7.0.

On passing the oxygen-free nitrogen and carbon dioxide mixture through the culture medium, the potential of the metal electrode, and therefore the E_h of the solution, fell more or less rapidly. If the unpurified nitrogen mixture was added to the gas stream as the required potential was approached the fall in E_h

was checked. By varying the rates of flow of the oxygen-free and the unpurified mixtures the E_h could be poised at the required level. The culture medium in this respect behaved like the media used by Knight and Fildes [1930].

Experimental procedure.

The cultures and electrode vessels, having been assembled, were placed in the incubator, and the electrical connections were made to the electrometer leads and to the standard quinhydrone half-cell. After allowing 30 mins. to 1 hour for temperature equilibration, the first potential measurements were made. The gas flow was then started; through the experimental vessel O_2 -free nitrogen with 2.5 % CO_2 ; through the control vessel air with 2.5 % CO_2 .

It was found that violent bubbling of gas through the fluid medium had for some unknown reason a deleterious effect upon the cultures. The reason for this is not clear, but it may be related to the fact that the growth-promoting power of embryo extract [Carrel, 1913] and of serum [Carrel and Ebeling, 1922] is said to be diminished by shaking. Whether this is an oxidation or merely a mechanical inactivation is not known. It was necessary, however, because of this phenomenon, to allow only a short spell of rapid bubbling to remove oxygen from the fluid and space above it, and then to cut down the rate of flow until the amount of agitation of the fluid was quite small. Throughout the experiment care was taken that the rates of flow of gas through experimental and control vessels were, as far as could be judged, the same. When the potentials registered by the electrodes in the experimental vessel had fallen almost to the desired level, unpurified N_2 , i.e. N_2 containing a small quantity of oxygen, was introduced into the gas stream, and the rates of flow of purified and unpurified gas were adjusted so that the potentials came to and remained at this level. The experiment was continued for 40–48 hours, the gas flow in the experimental vessel being adjusted when necessary to maintain the constant potential. After 40–48 hours the coverslips were taken out of the vessels and the cultures, experimental and control, fixed in Bouin's fluid, stained with Mayer's haemalum, and examined. The p_H of the fluid medium was determined, at 38°, with the aid of a glass electrode of the Stadie type [Stadie *et al.*, 1931].

Results.

Fig. 2 shows the potentials which were observed in a typical experiment. Each curve relates to a single electrode, and in this experiment the gas flow was adjusted to maintain the E_h indicated by the electrodes in the experimental vessel at about 20 mv. It will be noted that the variation between the potentials of the three electrodes in the experimental vessel covers a range of about 20–30 mv., which is sufficiently small for the purposes of this work. In the control vessels the agreement is not quite so good. This might be expected where a considerable amount of oxygen is present. During the first 24 hours of the experiment there is a gradual drift of the potential to a more positive value. These characteristics were observed so consistently in all the experiments that in many of the later ones it was not considered necessary to make potential measurements in the control vessel.

Experiments were discarded in any of the following circumstances. (1) When there was marked disagreement between the behaviour of the electrodes in the same vessel. (2) When the E_h during the course of the experiment showed wide fluctuations owing to imperfect adjustment of the gas stream. (3) When the culture medium showed evidence of bacterial infection. (4) If for any unknown reason the control cultures did not grow well.

The following experiments we consider to be free from these defects: two in which the E_h was maintained at +120 mv., one in which it was maintained at +20 mv., one at -30 mv., and two at -100 mv. An indication of the activity of the cultures may be obtained by reference to Table I. To obtain the

Fig. 2. Continuous line, experimental vessel. Broken line, control vessel.
●—●, Platinum electrodes. ○—○, Gold electrodes.

Table I.

No. of exp.	E_h of medium (mv.)	Areas in mm. ²			Mean area of outgrowth of exp. culture as % of that of controls
		Area of explant A_1	Area of whole culture A_2	Area of outgrowth $A_2 - A_1$	
1	+120	Exp. { 0.27	1.87	1.60	34
		0.34	2.38	2.04	
		Contr. { 0.42	5.57	5.15	
		0.37	5.85	5.48	
2	+120	Exp. { 0.58	2.63	2.05	43
		0.67	2.73	2.06	
		0.57	2.23	1.66	
		Contr. { 0.33	4.78	4.45	
3	+20	0.57	5.04	4.47	12
		Exp. { 0.44	1.07	0.63	
		0.45	1.91	1.33	
		Contr. 0.54	7.62	7.08	
4	-30	Exp. { 0.64	1.06	0.42	2.3
		0.69	0.81	0.12	
		Contr. { 2.34	15.25	12.91	
		1.95	13.05	11.10	
5	-100	Exp. Only a few cells have migrated out			
		Contr. { 0.93	5.93	5.00	
		0.97	4.72	3.75	
		0.86	4.96	4.10	
6	-100	0.58	3.38	2.80	
		Exp. Only a few cells have migrated out			
		Contr. Cultures infected			

figures recorded in this table the fixed and stained cultures were placed under the microscope, and with the aid of a drawing eye-piece the outlines of the central fragment and of the whole culture were traced on to a sheet of paper. The areas outlined on the paper were then measured with a planimeter, and knowing the magnification, the actual areas of the cultures were calculated. These are given in the table in mm.² For the purpose of comparing the growth of experimental and control cultures the area of the outgrowth has been used. This is given by the difference between the area of the whole culture, A_2 , and that of the central fragment, A_1 . It was thought that this would be a truer representation of the activity than the fraction A_2/A_1 which is often used. A_1 in the latter expression ought really to be the original area of the explant, but the area of the explant when measured at the end of the experiment is sometimes, especially in well-growing cultures, considerably greater than its initial value. This is very noticeable in Exp. 4 in the table. When the difference $A_2 - A_1$ is considered, however, the error due to this uncertainty about the true original area is much diminished, because A_1 is small compared with A_2 . The final column of the table shows the average outgrowth of the experimental cultures expressed as a percentage of that of the control. It should be pointed out that in addition to diminution in the area of outgrowth in the series, there is at the same time a very marked decrease in the density of cell population in the outgrowth. This factor cannot conveniently be gauged numerically, but because of it the nett effect of reducing the E_h of the medium is really much more marked than the figures in the table suggest.

The apparent E_h in the control vessels was always of the order of +280 to +360 mv. When the E_h was maintained at +120 mv., the activity of the cultures was already markedly inhibited but was still considerable. In the two experiments the areas of outgrowth were 43 and 34 % respectively of those of the control cultures. Mitoses were still occurring at this E_h , as many as 19 being observed in a single culture. Examples can be seen in Plate VI, fig. 1. The appearance of the cells was not different from that of the control seen in Plate VI, fig. 2, though the number of mitoses was much smaller.

At an E_h of +20 mv. a much greater inhibition of activity was evident. The area of outgrowth was only 12 % of that of the controls and was more sparsely populated with cells. The condition of the cells was still fairly good, and there were one and four mitoses respectively in the two cultures (Plate VI, fig. 3). One or two of these appeared somewhat abnormal.

At -30 mv. the outgrowth was even smaller, only 2.3 % of that of the controls, but it cannot be said that activity was completely inhibited. There were no mitoses; the cells were heavily vacuolated and appeared to be on the verge of degeneration (Plate VI, fig. 4).

At -100 mv. only a few cells had migrated from the explant and it seems possible that this migration had occurred in the early stages, before the strongly reducing character of the environment had had time to overcome some innate capacity of the cells for resisting. Nevertheless the condition of the cells in the experimental cultures (Plate VI, fig. 5) appeared to be better than that shown in Plate VI, fig. 4. The reason for this is at present obscure.

The general result was therefore that when the potential indicated by the electrode was +20 mv. or more positive a considerable amount of activity was permitted. Even at a potential of +120 mv. there was a marked inhibition of growth, and this inhibition had reached a maximum at -30 mv. There was little difference, if any, between the amount of activity at -30 mv. and that at -100 mv. Even at these low levels some of the cells retained a limited power of

migration for a few hours at any rate, and did not appear to be killed within the time of the experiment.

Inhibition of mitosis appears to be complete at a potential which is rather more positive than that necessary for maximum inhibition of migration. Mitosis has almost completely ceased at +20 mv., and was not observed at -30 mv.

In some of the experiments at potential levels below +120 mv., the cells of the experimental cultures showed heavy vacuolation and distortion of nuclei suggesting imminent degeneration. These appearances were not however sufficiently regular to be related to the potential level. They must at present be ascribed to some unknown and as yet uncontrolled variable in the environment of the cultures.

It has been mentioned that Laser [1933] has recently stated that chick embryo tissue can grow equally well under aerobic or anaerobic conditions, but only when it has first been allowed to grow under normal conditions for 24 hours. Two experiments have been made to test this. The experiments were carried out in the same way as those described above, but the vessels with the cultures were left in the incubator undisturbed for 1-2 days before the flow of nitrogen was commenced. The experimental cultures were then allowed to grow for a further similar period of time at $E_h + 50$ mv. The two experiments produced identical results. Fixing and staining of the cultures showed that the growth of the experimental cultures had been markedly inhibited, and the cells were much more heavily vacuolated than were the controls. There were several mitoses, but far fewer than in the controls. In many the mitotic figure was distorted in a striking fashion by the large vacuoles (probably fat) in the cytoplasm (Plate VI, fig. 6). In spite of the preliminary growth under aerobic conditions therefore the effects observed fit very well into the series of experiments already described and correspond with what was expected at that level of potential. Thus these results do not support Laser's conclusions.

DISCUSSION.

In the experiments described above mitosis was observed when the oxygen pressure must have been considerably lower than Wright's minimum figure for the same type of tissue, *i.e.* 12 mm. Hg. This follows from the fact that the oxygen pressure in the unpurified nitrogen was only about 2 mm. Hg, and this was diluted by oxygen-free nitrogen before reaching the cultures. Mitosis therefore occurred when the oxygen pressure was much below 2 mm. Hg.

In considering these experiments, it is important to realise that the oxidation-reduction systems which determine the electrode potentials cannot be in reversible equilibrium with the oxygen of the gas phase. The arguments which apply to the experiments of Knight and Fildes were supported by our experience. An E_h of +100 mv. corresponds thermodynamically to an oxygen pressure of about 10^{-45} atmospheres. To maintain this E_h it was necessary to pass in a certain proportion of unpurified nitrogen containing about 0.2 vol. % of oxygen. Although the final composition of the gas phase was not determined it must, of course, have been far in excess of 10^{-45} atmospheres.

These considerations, together with the fact that the electrode potential can be raised or lowered by corresponding changes in the oxygen content of the solution, suggest that we may be dealing with a complex system somewhat as follows. We suggest, firstly, that the medium contains several reversible oxidation-reduction systems of an unknown nature. Each system is able to poise the potential, in the range of E_h about its own E_0 value, to an extent dependent upon the amount of the particular system present. The range of E_h covered by

all the systems together is such that some degree of poisoning can be manifested at any potential between, at any rate, +200 mv. and -100 mv. Secondly, the medium contains a reducing substance, or substances, which in the absence of oxygen can reduce the oxidised component of any of the reversible systems which determine the electrode potentials. In the absence of oxygen therefore this reduction takes place and the potential falls to a very low figure. Thirdly, in the experiments described, the medium contains oxygen. This, while not itself establishing equilibrium with the electrodes, tends to oxidise those systems which are in such equilibrium and therefore to raise the potential. A combination of these three factors might be expected to produce the observed results. The potential is set up by a reversible oxidation-reduction system effective at that point in the E_h scale. The condition of the reversible system is determined by the balance between oxidation of the system by oxygen and reduction of the system by a more strongly reducing substance in the medium.

The vigour of the reducing substances appears to vary in different samples of serum and embryo extract. In one experiment the E_h fell rapidly in the absence of oxygen to below -400 mv. In another experiment in which the same precautions were taken to eliminate traces of oxygen, the E_h did not fall below -20 mv. These results suggest that perhaps other factors besides oxygen pressure should be taken into account when considering the growth of tissues in low concentrations of oxygen. It is possible that the growth may be affected by the E_h more than by the oxygen pressure. The question, which of these two is the more important, cannot yet be regarded as settled. The possibility still remains that, whatever may be the factors governing the E_h , the growth of tissue is after all determined directly by the concentration of oxygen. More work upon the growth of cells under limiting conditions of oxygen pressure and of E_h is needed before this point can be decided.

It is of interest to consider these results in relation with those obtained by Needham and Needham [1925] and by Cohen *et al.* [1928], who studied the E_h of the protoplasm within amoebae by the micro-injection of oxidation-reduction dyes. From the results given by Needham and Needham the E_h within an amoeba appears to be maintained at about +100 mv. The experiments recorded above show that embryonic tissue cells, at any rate, can not only migrate but show mitosis when the medium surrounding them has E_h as low as +20 mv. It would be surprising if cells were able to live and multiply in a medium with E_h 80 mv. more negative than that within the cell itself. Our results are more in accord with those of Cohen *et al.*, which suggest that the E_h of the interior of the cell is below -130 mv. With E_h -100 mv. we find that embryonic cells of the chick are still able to survive to some extent, though there is no mitosis and little or no migration.

SUMMARY.

1. On passing pure nitrogen through a culture medium containing serum and embryo extract the E_h of the medium fell rapidly.
2. By adding different small amounts of oxygen the E_h could be maintained at any desired level between +300 mv. and -100 mv.
3. The growth and division of cells from embryonic chick heart tissue have been studied when the E_h of the medium has been maintained at different levels. In general the lower the E_h the poorer has been the growth of the tissue culture.
4. All mitosis ceased between E_h + 20 mv. and -30 mv. Migration of cells had almost entirely ceased at an E_h of -30 mv.
5. The possible significance of these results is discussed.

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DESCRIPTION OF PLATE VI.

- Fig. 1. Part of culture in medium at $E_h = 120$ mv., showing mitosis. $\times 400$.
 Fig. 2. Part of control to Fig. 1. $\times 400$.
 Fig. 3. Culture grown with medium at $E_h = 20$ mv., showing mitosis. $\times 400$.
 Fig. 4. Culture grown with medium at $E_h = -30$ mv. $\times 400$.
 Fig. 5. Culture grown with medium at $E_h = -100$ mv. $\times 400$.
 Fig. 6. Culture grown in air for 36 hours, then at $E_h = 50$ mv. for 48 hours. $\times 400$.

Note distorted mitotic figure in the centre of the field. The equatorial plate of chromosomes is pushed towards one end of the cell and is no longer lying at right angles to the long axis of the cell, as the result of the presence of large vacuoles, probably representing fat.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.