The effects of varying oxygen tensions on organ cultures

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KURZFASSUNG: **Die Wirkungen verschiedener Sauerstoffspannungen auf Organkulturen.** Die Sauerstoffbedürfnisse embryonaler und adulter Organe sind recht unterschiedlich. Erstere werden durch hohe Sauerstoffspannungen getötet, letztere überleben vielfach nicht bei niedrigen Spannungen. Im günstigsten Falle können nur dünne Scheiben adulter Organe lebend erhalten werden, selbst wenn die Luft im Kulturgefäß durch Sauerstoff ersetzt wird. Organkulturen wurden daher in einer Druckkammer durchgeführt unter Sauerstoffpartialdrucken zwischen 0,2 und 4,0 Atmosphären (absolut). Die höheren Drücke begünstigten das Überleben einiger Organe von adulter Ratte und Maus, z. B. Nebennierenrinde, Leber und braunes Fett, welche bis zu Drücken von 1,0 Atmosphären nicht gut gedeihen. Andererseits überlebten embryonale Organe und bestimmte adulte Organe – wie etwa Nebennierenmark – besser bei relativ niedrigen Sauerstoffdrücken und wurden durch höhere Drücke stark geschädigt. Im Falle der adulten Leber wurden QO_2-Bestimmungen und Studien der bei der Glucuronid-Synthese beteiligten Enzyme benutzt, um die herkömmlichen histologischen Kriterien für das Überlebensvermögen nach mehreren Kulturtagen zu ergänzen. Es wird die Schlußfolgerung gezogen, daß die Anwendung hoher Sauerstoffdrücke von beträchtlichem Wert sein könnte für Arbeiten mit Organkulturen.

INTRODUCTION

Everyone is familiar with the type of culture, usually known as tissue culture, but better called cell culture. A good example would be the growth of embryonic cells from the chick where thin sheets of cells grow well in the presence of air and in fact are often killed by oxygen. This type of de-differentiated growth, in which are found numerous mitotic figures, must be contrasted with the type of culture known as organ culture, where in the case of adult organs at least, oxygen is usually required for successful maintenance of the normal histological pattern of the organ. Merely replacing the air with oxygen is only partially successful as shown by TROWELL (1959, 1961).

Recent developments in the clinical field suggested the possibility of using oxygen under increased pressure to improve the technique of organ culture. The work of BOEREMA (1961) and of SMITH et al. (1961) on patients exposed to oxygen at increased pressures showed the value of this procedure, and at the same time the absence of signs of oxygen poisoning which might have been expected.

MATERIALS AND METHODS

For the present work an improvised pressure chamber was used consisting of a small horizontal autoclave heated by an immersion heater thermostatically controlled at 37°C. The air in the apparatus was replaced by an oxygen carbon dioxide mixture and thereafter experiments were carried out under oxygen pressures of 1.0, 2.0, 3.0 or 4.0 atmospheres absolute; i. e. when the reading on the pressure gauge was 15 lbs./sq. inch the pressure was taken as one atmosphere above normal atmospheric pressure, or 2 atmospheres absolute etc. Experiments were also conducted in air and in 60% oxygen at normal pressures, i. e. in oxygen at partial pressures of 0.2 and 0.6 atmospheres absolute.

The theoretical advantage of using high pressures of oxygen can be seen in the formula (GERARD 1931)

$$r = \sqrt{6\,CD/A}$$

where r in cm. is the limiting radius of a spherical organ culture in which the oxygen concentration at the centre just reaches zero, C is the external concentration of oxygen at the surface of the culture in atmospheres, D is the diffusion constant of oxygen and A is the oxygen consumption of the culture in ml./ml. tissue/min.

TROWELL (1961) succeeded in obtaining larger cultures (increasing the value of r) by using organs from the cow in place of the usual small laboratory animals. Since tissue QO_2 is proportional to body size this led to a reduction in the value of A in the above equation. TROWELL was less successful in attempting to increase the value of D by adding haemoglobin to the culture medium as this proved to be toxic. There remains the possibility of increasing the size of the cultures by raising the value of C. Obviously if the external concentration of oxygen is increased, this should increase the value of r by a factor which would equal the square root of the increase in atmospheres. In turn this should lead to larger cultures without central necrosis provided oxygen poisoning is not encountered.

Cultures were carried out in small petri dishes, and apart from the introduction of oxygen under pressure, the technique was similar to that of TROWELL (1959). Each petri dish contained a stainless steel grid platform on which was placed a piece of lens paper. Medium was added until it just reached and wetted the lens paper, the medium consisting of serum 20%, Parker's 199 80%. For all animal tissue horse serum was employed, human serum being used in the case of the cultures of human foetal organs. Since Parker's 199 is balanced to be in equilibrium with 5% CO_2, it was necessary to adjust the gas mixture so that at different pressures the partial pressure of CO_2 remained constant, thus maintaining the correct pH. The tissue was placed, in most experiments, on the surface of the lens paper so that it would be as close to the oxygen as possible. In a few instances, however, the tissue was submerged, particularly when high pressures were being used.

RESULTS

Various organs and tissues from a 13-week human foetus of 90 mm. crown-rump length were cultivated for 3 days at 4 different partial pressures of oxygen. Short segments of small intestine after 3 days in the presence of air (0.2 atmospheres oxygen) showed good preservation of the serous and muscular coats (Fig. 1*a*). The parts of the culture farthest from the supply of oxygen, however, lost their characteristic structure, the villi of the mucous coat being replaced by a mass of proliferating de-differentiated cells which obliterated the lumen of the gut. When the partial pressure of oxygen was raised to 0.6 (Fig. 1*b*) or to 1.0 atmosphere (Fig. 1*c*) there was remarkable preservation of the entire thickness of the intestine, there being little to choose between these pressures. On the other hand, when the pressure was further raised to 2.0 atmospheres (Fig. 1*d*) there was widespread degeneration of all parts of the culture. In the same way human foetal skin was well preserved at 0.2 atmospheres (Fig. 1*e*) whereas at 2.0 atmospheres (Fig. 1*f*) the epidermis separated from the corium, and the cells of the basal layer of the epithelium showed numerous pyknotic nuclei. It would, therefore, appear that foetal tissue survives best in the lower ranges of oxygen tension.

When organs or organ slices from adult laboratory animals are considered, however, the results of culture at varying oxygen tensions are somewhat different. Slices of rat adrenal cultivated at 0.2 atmospheres oxygen quickly showed (Fig. 2*a*) complete necrosis of all the deeper parts of the tissue, only the capsule and the surface cells in the zona glomerulosa surviving. When the partial pressure of oxygen was raised to 1.0 atmosphere a considerable improvement resulted. After 10 days there was still survival of zona glomerulosa, zona reticularis and the medulla, but the intermediate zona fasciculata had completely degenerated (Fig. 2*b*). This has been the finding of other workers, and it might be said to represent only a very partial success so far as the adrenal cortex is concerned. In the case of the adrenal medulla, however, cultivation at 1.0 atmosphere can yield useful results. After 7 days the chromaffin reaction is still positive (Fig. 2*d*) and potassium iodate continues to react with the medullary cells. After cultivation for 10 days a modified Giemsa stain (Fig. 2*c*) suggests that the medulla is continuing to produce or store pressor amines. Direct assay is now being used to confirm the presence of both adrenaline and noradrenaline indicated by these histochemical tests.

When slices of rat adrenal were cultivated at 2.0 atmospheres a completely different pattern of preservation emerged (Fig. 2*e*). At this pressure the medulla quickly died, but the entire cortex including the zona fasciculata survived (Fig. 2*f*). It is,

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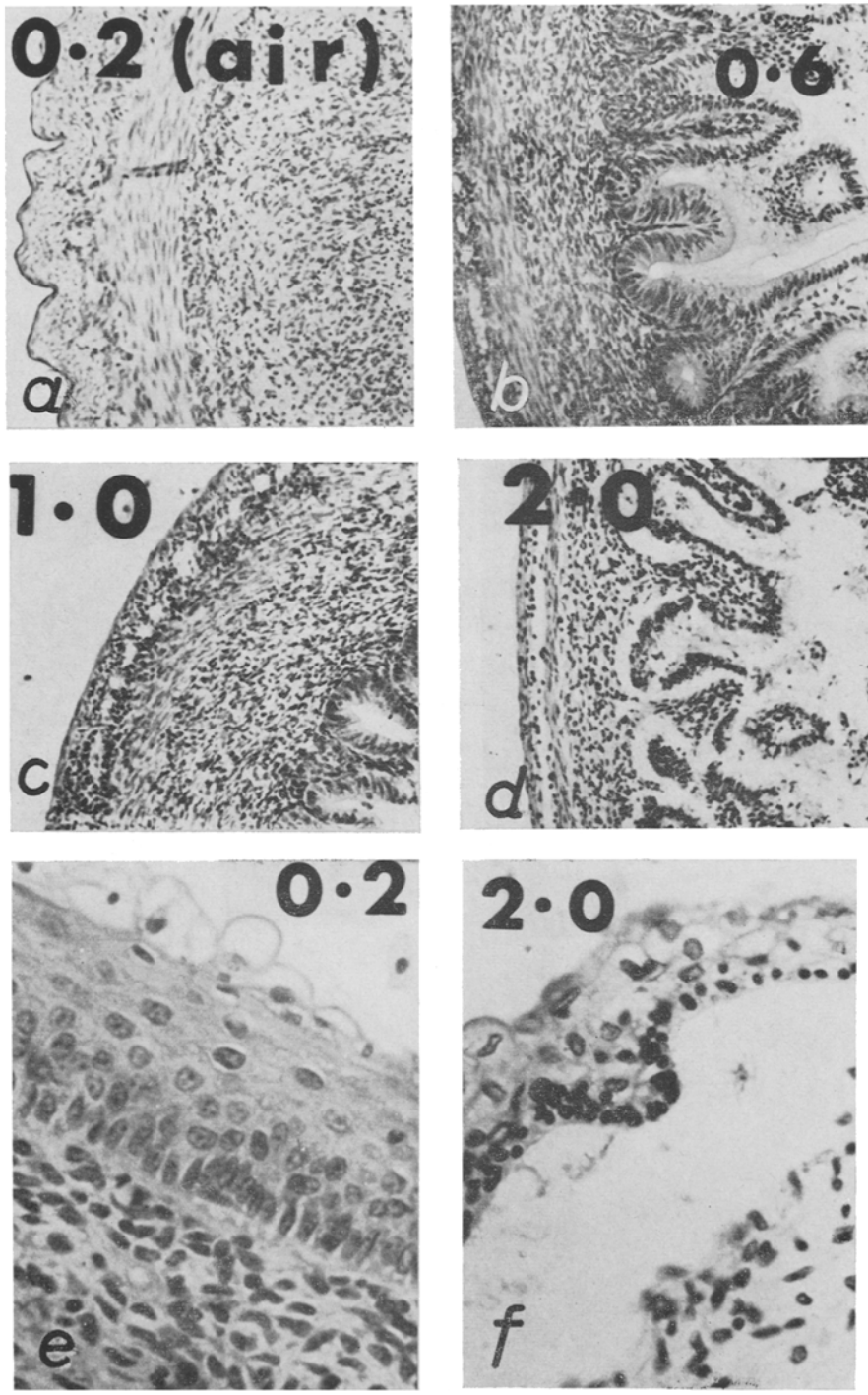
Fig. 1: *a* Small intestine from 13-week human foetus cultivated in air for 3 days. Haematoxylin and eosin × 115. The numbers in this and the following illustrations indicate the partial pressure of oxygen in atmospheres. *b* Small intestine from 13-week human foetus cultivated in 60 % O_2 for 3 days. Haematoxylin and eosin × 115. *c* Small intestine from 13-week human foetus cultivated in 95 % O_2 (virtually 1.0 atmosphere) for 3 days. Haematoxylin and eosin × 115. *d* Small intestine from 13-week human foetus cultivated in O_2 at 2.0 atmospheres for 3 days. Haematoxylin and eosin × 115. *e* Skin from 13-week human foetus cultivated in air for 3 days. Haematoxylin and eosin × 390. *f* Skin from 13-week human foetus cultivated in O_2 at 2.0 atmospheres for 3 days. Haematoxylin and eosin × 390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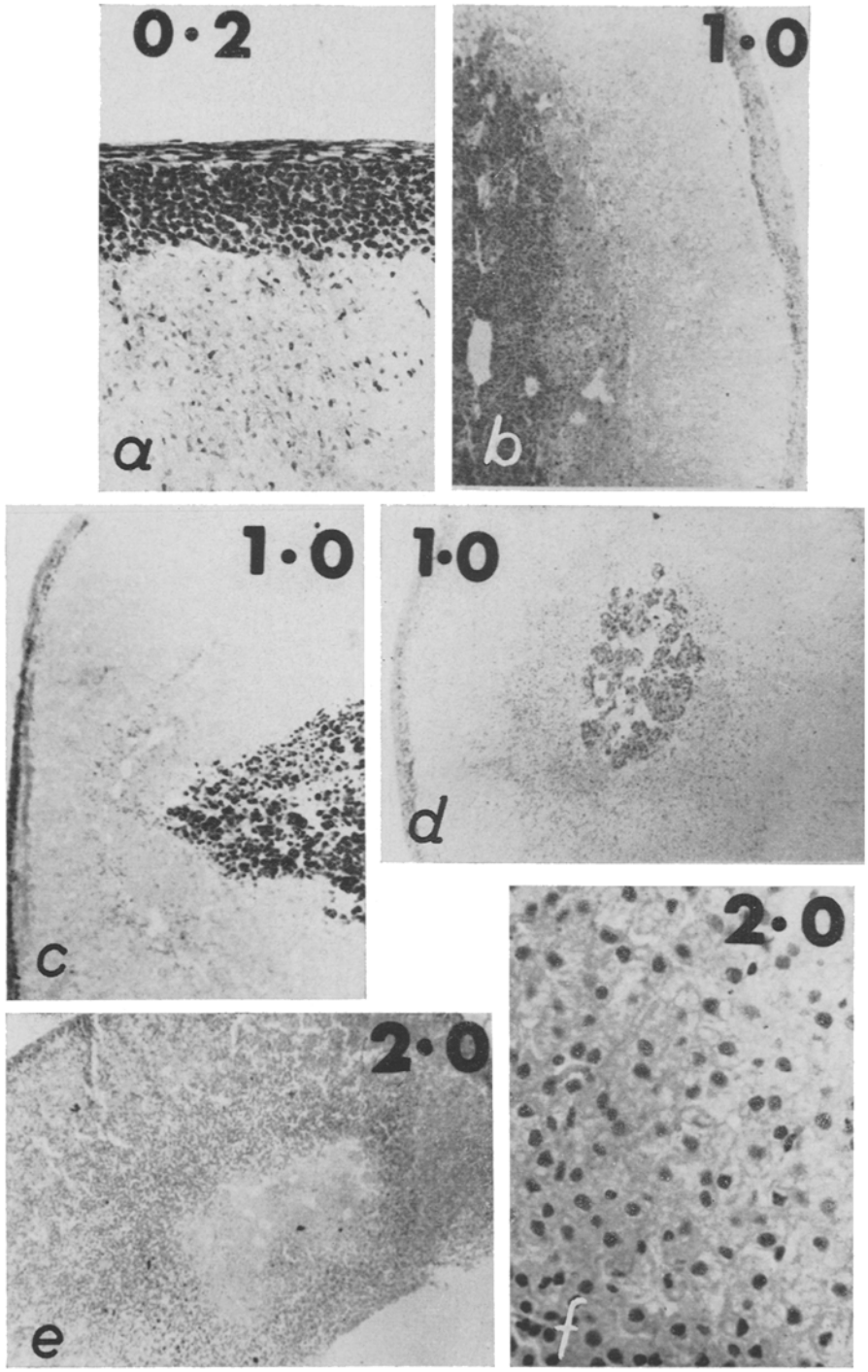
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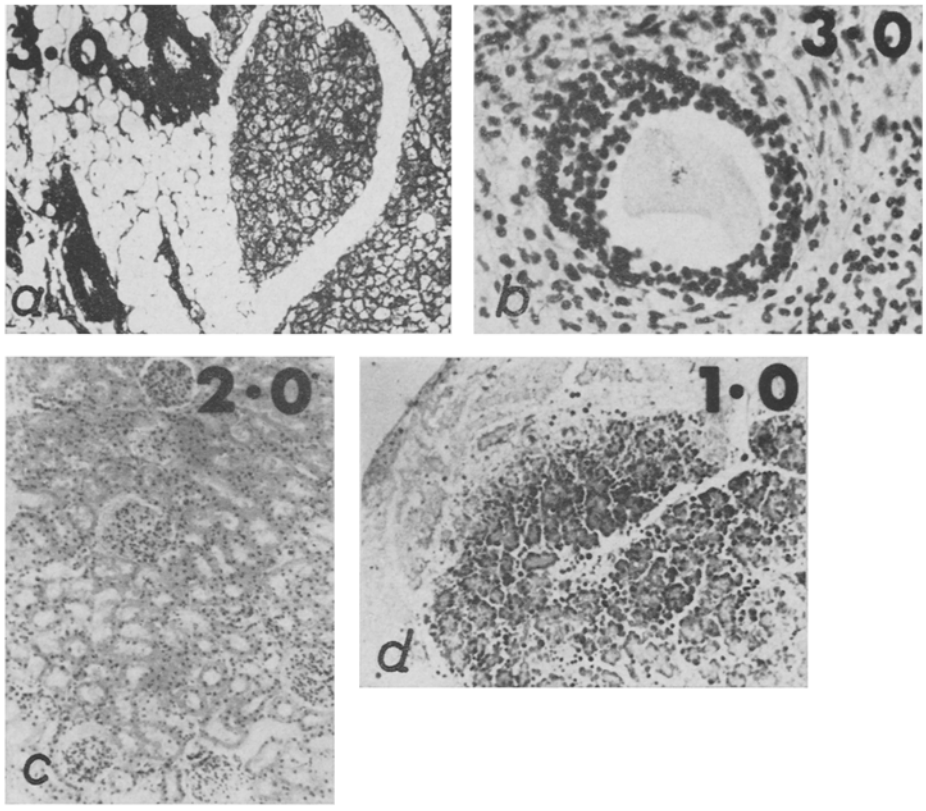

Fig. 3: *a* Retroperitoneal white and brown adipose tissue from the rat cultivated in oxygen at 3.0 atmospheres for 7 days. Mallory × 95. *b* Rat ovary slice cultivated in oxygen at 3.0 atmospheres for 7 days. Haematoxylin and eosin × 350. *c* Rat kidney slice cultivated in oxygen at 2.0 atmospheres for 3 days. Haematoxylin and eosin × 95. *d* Rat pancreas slice cultivated in oxygen at 1.0 atmosphere for 24 hours. Surface digested. Haematoxylin and eosin × 95

therefore, now possible to maintain different zones of the adrenal cortex by choosing different partial pressures of oxygen; thus 0.2 atmospheres supports only zona glomerulosa, 1.0 atmosphere maintains zona glomerulosa and zona reticularis while 2.0 atmospheres will in addition preserve zona fasciculata. The possible value of such cultures is obvious and a start has been made on the estimation of steroid hormones in the culture media in which adrenals have been maintained at different pressures.

Fig. 2: *a* Rat adrenal slice cultivated in air for 4 days. Haematoxylin and eosin × 160. *b* Rat adrenal slice cultivated in oxygen at 1.0 atmosphere for 10 days. Haematoxylin and eosin × 40. *c* Rat adrenal slice cultivated in oxygen at 1.0 atmosphere for 10 days. Modified Giemsa stain × 40. *d* Rat adrenal slice cultivated in oxygen at 1.0 atmosphere for 7 days. Formalin-dichromate × 40. *e* Rat adrenal slice cultivated in oxygen at 2.0 atmospheres for 6 days. Haematoxylin and eosin × 40. *f* Rat adrenal slice cultivated in oxygen at 2.0 atmospheres for 6 days. Cells of zona fasciculata. Haematoxylin and eosin × 370

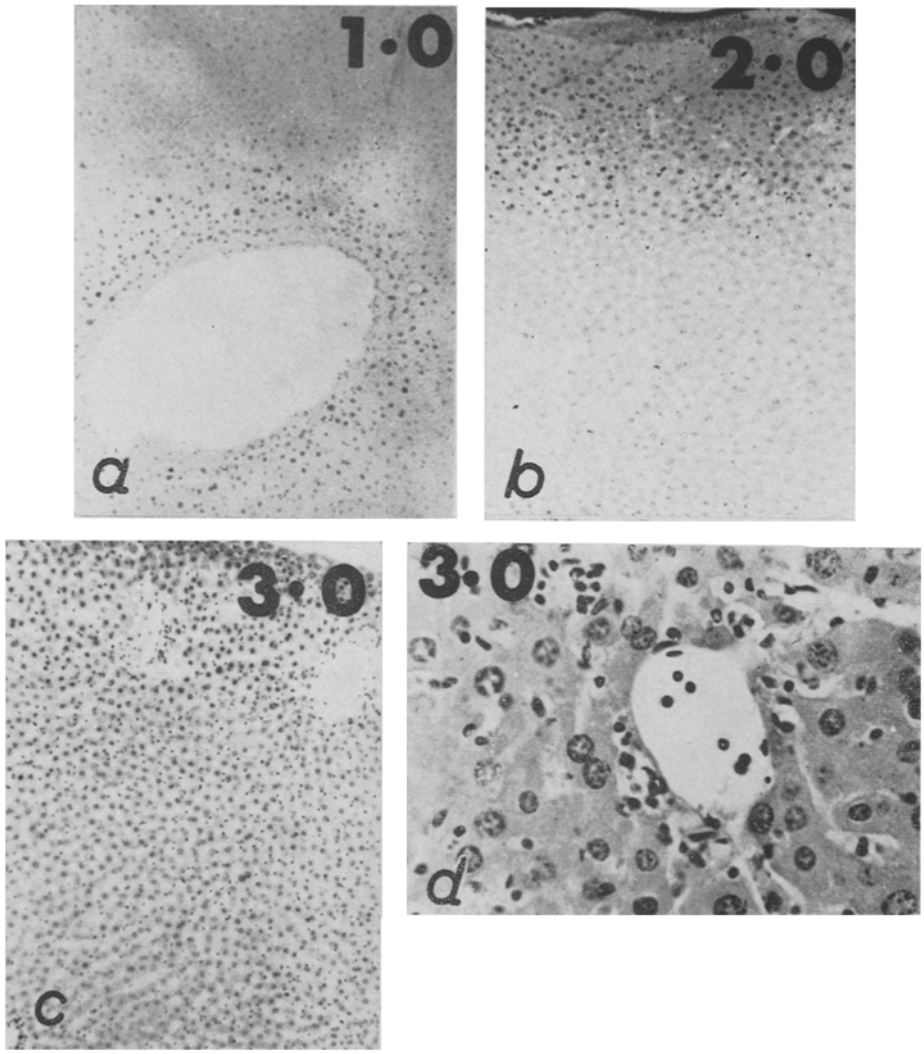

Fig. 4: *a* Rat liver slice cultivated in oxygen at 1.0 atmosphere for 2 days. Haematoxylin and eosin × 110. *b* Rat liver slice cultivated in oxygen at 2.0 atmospheres for 3 days. The upper surface (nearest the oxygen supply) is marked with Indian ink. Haematoxylin and eosin × 110. *c* Rat liver slice cultivated in oxygen at 3.0 atmospheres for 6 days. At such high pressures it is possible to submerge the tissue in the medium as was done in this case. Haematoxylin and eosin × 110. *d* Mouse liver slice cultivated in oxygen at 3.0 atmospheres for 7 days. Haematoxylin and eosin × 370

While ordinary white fat is well maintained by a pressure of 1.0 atmosphere, brown fat survives badly. Trowell (1959) drew attention to the fact that the cells of brown fat are very similar in appearance to those of the adrenal cortex and that they may secrete similar hormones (Selye & Timiras 1949). It may, therefore, be signifi-

cant that brown fat like the zona fasciculata of the adrenal cortex seems to require higher pressures of oxygen for survival. After 7 days at 3.0 atmospheres (Fig. 3*a*) brown fat continues to show a normal histological appearance.

The ovary of the mature rat was another organ found by TROWELL (1959) to be unsatisfactory in culture at normal pressures. At 2.0 atmospheres, however, the general architecture is well preserved for at least a week. The mature ova degenerate, but the developing follicles retain their characteristic appearance. At 3.0 atmospheres (Fig. 3*b*) ova continue to degenerate and the follicular cells are somewhat loosely packed.

The kidney (Fig. 3*c*) is yet another organ which benefits from higher oxygen tensions, thicker slices surviving than at lower pressures. The rat pancreas appears to digest itself *in vitro* at 1.0 atmosphere (Fig. 3*d*). As was to be expected, raising the oxygen pressure did not prevent this, but neither did a simple siphon designed to wash away the trypsin as it was released and activated.

Slices of rat liver cultivated at 1.0 atmosphere did not survive well. Only a thin layer of cells near the surface was maintained, and within two days the central part of the culture was completely necrosed, apart from a few surviving cells near large empty blood vessels which presumably had conveyed oxygen to the interior (Fig. 4*a*). Considerable improvement occurred when the pressure of oxygen was raised to 2.0 atmospheres (Fig. 4*b*) and still more when the pressure was further raised to 3.0 atmospheres (Fig. 4*c*). The increase in thickness of the layer of surviving cells was approximately equal to the value predictable from the equation given above. A few experiments conducted at 4.0 atmospheres suggested that possibly this was approaching the useful upper limit, since there were rather more pyknotic nuclei near the surface than could be accounted for by the initial trauma of slicing. Further work will be required to confirm this.

While liver slices cultivated at 3.0 atmospheres retain reasonably normal histology for 10–14 days, there is then a progressive deterioration. This might be due to leaching out of some essential material from the liver cells and its continued loss each time the medium is renewed, i. e. every 3–4 days. Attempts were, therefore, made to replace this hypothetical loss, and two methods were tried. In the first, "feeder" cultures of fresh liver slices were placed beside the original slice each time the medium was renewed. In the second method, a simple extract of fresh liver in Medium 199 was incorporated in the medium at each renewal. Unfortunately neither method was found to be of value, and after 28 days only a few areas of surviving cells were found, no significant difference being observed between control and experimental cultures.

It might be said that liver cultures, even under pressure, are very slowly dying, and it was, therefore, of interest to know what changes might be taking place in the early stages when the histological appearance of the tissue was still virtually normal. It was soon apparent that the glycogen content of the cells as shown by the PAS reaction fell to very low levels within the first 24 hours of culture, and thereafter none could be detected by this technique.

Next, the oxygen consumption of liver slices was investigated after various periods of culture under pressure. This was done, using the microrespirometer devised by CRUICKSHANK (1954) and the results are shown in Table 1. It will be seen that there appears to be no significant change in the Q_{O_2} values over a period of 6 days.

Table 1

Rat liver slices cultivated in the presence of oxygen at 3.0 atmospheres. Oxygen uptake is expressed as μl. O_2/mg. dry wt. tissue/hr., glycogen content in terms of the intensity of the PAS reaction

	QO_2	Glycogen
Fresh liver slice	4.6	PAS +++
1 day	5.2	PAS +
3 days	3.5	PAS —
6 days	4.5	PAS —
6 days – last 3 days with additional glucose. 5% 1 : 9	4.3	PAS —

The Table also shows that the addition of extra glucose to the culture medium (1:9 of glucose 5% w/v) had no effect on either Q_{O_2} or glycogen content.

Further work was done on mouse liver, which after 7 days at 3.0 atmospheres (Fig. 4*d*) has a relatively normal appearance, apart from peripheral clumping of the chromatin in the nuclei. Mouse liver was chosen as it was convenient for investigating the effect of culture under pressure on the enzymes involved in glucuronide synthesis. Figure 5 shows the results obtained so far, using different substrates. It will be seen that although activity does fall off, it does so much less rapidly than would be the case with the ordinary slice technique of the biochemist. Since these enzymes are said to be associated with the endoplasmic reticulum of the cell, electron microscope

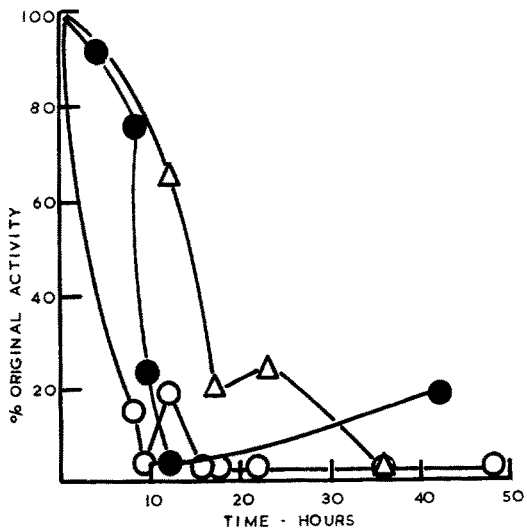

Fig. 5: Levels of UDP glucuronate glucuronyl transferase in organ cultures of mouse liver; *o* – aminophenol as substrate: ○; *p* – nitrophenol as substrate: △; overall glucuronide synthesis with *o* – aminophenol as substrate: ●

studies are now in progress to see whether the fall off in activity of the enzymes in cultured liver cells coincides with any change in the fine structure of the cytoplasm.

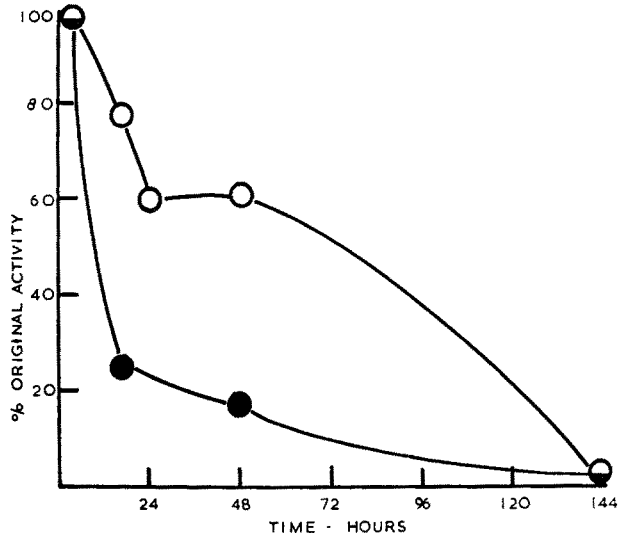

Fig. 6: Levels of *o* – aminophenyl glucuronide formation in guinea-pig kidney slices cultured; with additional glucose: ○; without additional glucose: ●

The addition of extra glucose to the medium had only a small effect on the maintenance of glucuronide synthesis in the case of liver. This was in contrast to the results obtained with guinea-pig kidney (Fig. 6) where glucose had a marked effect in prolonging this activity in organ cultures maintained at 3.0 atmospheres.

CONCLUSION

It would appear from the work described that whereas foetal or embryonic tissue survives best in organ culture when the oxygen tension is low, certain adult organs do better when the pressure of oxygen is high, and some do not survive at all unless the oxygen pressure is raised. On the other hand, the adrenal medulla and possibly the ovarian follicular cells (both epithelial tissues) are killed or damaged by moderately high oxygen pressures. Further work will be necessary to investigate the possibility that those tissues in which the cells are separated by spaces of the order of 200 Å (c. f. SMART 1963) are more sensitive to oxygen than tissues with large extra-cellular spaces.

The method of organ culture described, using varying oxygen tensions, may have considerable possibilities. By selecting the appropriate pressure, various tissues can either be supported or suppressed, so that the functions of individual portions of an organ may be capable of more detailed investigation.

SUMMARY

1. Human embryonic organ slices survived better in organ culture when the oxygen tension was relatively low. Necrosis, presumably due to oxygen poisoning was seen at an oxygen pressure of 2.0 atmospheres.
2. Slices of organs from adult laboratory animals varied in their reponse to high oxygen pressures *in vitro*.
3. In some organs, e. g. liver and kidney, considerably thicker slices survived in culture at pressures of 2.0 and 3.0 atmospheres than at 1.0 atmosphere.
4. Some tissues, zona fasciculata of the adrenal cortex and brown fat, which do not survive at 1.0 atmosphere were found to do well at raised pressures of oxygen.
5. Other tissues, notably the adrenal medulla, survived well at 1.0 atmosphere, but were destroyed by high oxygen pressure.
6. Rat liver slices, cultivated under high oxygen pressure maintained a reasonably normal histological appearance for 10–14 days, but rapidly lost their glycogen content. Their oxygen consumption remained essentially the same for at least 6 days. Attempts to prolong the survival of liver slices by the use of "feeder" cultures and by extracts of liver were unsuccessful.
7. Glucuronide synthesis rapidly fell off in slices of mouse liver maintained at 3.0 atmospheres. A similar result was obtained with guinea-pig kidney, but in this case, the addition of extra glucose to the medium had a marked effect in delaying the fall.

ACKNOWLEDGMENTS

It is a pleasure to record my indebtedness to Miss Morag C. McKean who carried out the cultures in the "feeder" and liver extract experiments, and to Mr. W. S. Myles and Dr. G. J. Dutton for conducting the biochemical work involving glucuronide formation. This work has been supported by a grant from The Medical Research Council.

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Discussion following the paper by MACDOUGALL

HEUSNER: Wir haben in Zusammenarbeit mit Dr. E. PETROVIC dieselben Schwierigkeiten der Sauerstoffzufuhr angetroffen. Doch haben wir nicht mit hohen Drucken gearbeitet, sondern die Diffusionsbedingungen durch eine kontinuierliche Sauerstoffzufuhr durch das flüssige Medium verbessert. Wir konnten so Leber, Braunes Fett, Schilddrüse und Milz längere Zeit kultivieren.

MACDOUGALL: Dr. HEUSNER explained his own method of culture, and I would like to thank him for drawing my attention to his elegant technique.

SMITH: Does one find an optimal level of pO_2 which might correlate with the capacity for terminal electron transport as may be represented by the numerical density of mitochondrial units in the respective tissues?

MACDOUGALL: The optimum pressure varies with the tissue or organ. In the case of liver, 4.0 atmospheres would appear to be approaching the upper useful limit, since at this pressure the number of pyknotic nuclei seen near the surface of the slice was rather greater than might be expected due to the trauma of slicing. However, not enough work has been done at this pressure to be certain. Apart from the pancreas, which as Dr. SMITH said, is rather a special case, it might appear that cells rich in mitochondria do better at relatively high pressures. This aspect of the problem is being borne in mind in further work which is now proceeding.