

DR. CARREL'S IMMORTAL CELLS*

by

J. A. WITKOWSKI**

1. INTRODUCTION

BETWEEN 22 October and 12 November 1910, Alexis Carrel and Montrose T. Burrows presented no less than seven papers to the Société de Biologie (Paris), reporting their experiences of culturing adult mammalian tissue outside the body.¹ They described the general features of the technique that Burrows had adapted from that of Ross G. Harrison, and went on to describe cultures of kidney, bone marrow and spleen, thyroid, Rous sarcoma, and a human sarcoma. Carrel and Burrows claimed that: "nous avons tenté d'établir une méthode générale qui permette de cultiver, comme des microbes, tous les tissus et organes adultes des animaux supérieurs et de l'homme."²

It was unlikely that such a bold assertion would go unchallenged and one reaction to this series of papers was frank disbelief, publicly expressed by J. Jolly in an article published on 26 November 1910.³ Based on his own experiences of maintaining leucocytes *in vitro*,⁴ Jolly denied that Carrel and Burrows had yet achieved true tissue culture and he suggested that they were mistaken in the interpretation of their results. Jolly claimed that "certaines de leurs observations semblent se rapporter à des phénomènes de mort"⁵ and that "il s'agit là d'un phénomène de dissociation mécanique de nécrobiotique, et non d'un bourgeonnement".⁶ Jolly demanded evidence of cell division in culture although he pointed out that even this might not be

* This paper is dedicated to Michael Abercrombie, F.R.S., 1912-1979.

** J. A. Witkowski, Ph.D., Jerry Lewis Muscle Research Centre, Hammersmith Hospital, London W12.

I am indebted to Dr. Ralph Buchsbaum for the description of his visit to the Rockefeller Institute, and to the late Dr. Michael Abercrombie, F.R.S., for his interest and encouragement. Mrs. Ruth Sternfeld of the Archive Office at the Rockefeller Institute very kindly searched the Archive's files for press articles on Carrel. I am very grateful to Dr. Helen Morton, Dr. Margaret Murray, Professor K. R. Porter, and Dr. Charity Waymouth for their invaluable comments on the manuscript.

¹ (a) A. Carrel and M. T. Burrows, 'La culture des tissus adultes en dehors de l'organisme', *C.r. Soc. Biol., Paris*, 1910, 69: 293-294; (b) *idem*, 'Culture de substance rénale en dehors de l'organisme', *ibid.*, 298-299; (c) *idem*, 'Culture de moelle osseuse et de rate', *ibid.*, 299-301; (d) *idem*, 'Cultures primaires, secondaires et tertiaires de glande thyroïde et culture de péritoine', *ibid.*, 328-331; (e) *idem*, 'Culture de sarcome en dehors de l'organisme', *ibid.*, 332-334; (f) *idem*, 'Seconde génération de cellules thyroïdiennes', *ibid.*, 365-366; (g) *idem*, 'Culture "in vitro" d'un sarcome humain', *ibid.*, 367-368.

² Carrel and Burrows, *op. cit.*, note 1(a) above.

³ J. Jolly, 'A propos des communications de M. M. Alexis Carrel et Montrose T. Burrows sur "la culture des tissus"', *C.r. Soc. Biol., Paris*, 1910, 69: 470-473. For a fuller discussion of the reception accorded the work of Carrel and Burrows, see J. A. Witkowski, 'Alexis Carrel and the mysticism of tissue culture', *Med. Hist.*, 1979, 23: 279-296.

⁴ Jolly had studied the behaviour of salamander leucocytes in serum *in vitro* as early as 1903 and returned to the same subject in 1910. J. Jolly, 'Sur la durée de la vie et de la multiplication des cellules animales en dehors de l'organisme', *C.r. Soc. Biol., Paris*, 1903, 55: 1266-1268; *idem*, 'Sur la survie des cellules en dehors de l'organisme', *ibid.*, 1910, 69: 86-88; *idem*, 'Sur la survie des leucocytes', *ibid.*, 295.

⁵ Jolly, *op. cit.*, note 3 above, p. 471.

⁶ *Ibid.*, p. 472.

conclusive if the tissue contained dividing cells before it was explanted.⁷

It was also believed that cells in Carrel's cultures did not utilize nutrients present in the culture medium, but simply used food stores in themselves or the products released by dying cells.⁸ Eventually when these stores were exhausted the cultures died. Carrel however believed that the death of cells in culture was due to the accumulation of inhibitory substances in the plasma clot around the cells, and it was these that limited the lifespan of a culture to about fifteen days. Carrel was able to extend the period of growth by regularly washing the cultures in saline before re-explanting in fresh plasma.⁹ These results led Carrel to declare that: "la senescence et la mort sont un phénomène contingent et non nécessaire."¹⁰

Both of these criticisms – that his cultures did not contain growing cells and that these cells did not utilize nutrients in the medium – could be answered by growing cultures for long periods of time. The cells would have to divide and use extracellular nutrients if the cultures were to survive. This was the vowed intention of Carrel's paper: 'On the permanent life of tissues outside of the organism',¹¹ and he declared: "The purpose of experiments described in this article was to determine the conditions under which the active life of a tissue outside of the organism could be prolonged indefinitely."¹²

It was in this paper that he described the establishment of a series of chick embryonic heart cultures, one of which (number 725) fulfilled all Carrel's expectations and was to become world-famous as the "immortal" cell strain.¹³ It was grown for thirty-four years and played an important part in the development of theories on cell ageing.¹⁴ However, in 1961, L. Hayflick and P. S. Moorhead¹⁵ demonstrated that normal

⁷ Carrel and Burrows showed photographs of dividing cells to a meeting of the Société de Biologie on 7 January 1911 (A. Carrel and M. T. Burrows, 'A propos des cultures "in vitro" des mammifères', *C.r. Soc. Biol., Paris*, 1911, 70: 3-4), but Jolly was not satisfied. He had himself observed mitotic figures in tissues *in vitro* but he believed that these were cells that had been dividing *in vivo* and died on explanting or were continuing to divide "plus ou moins lentement, ou avortent". He conceded that some cells of some tissues might be able to divide *in vitro* but these were "moins importante". J. Jolly, 'Sur la signification des figures de mitose que l'on observe dans les tissus séparés du corps', *C.r. Soc. Biol., Paris*, 1910, 69: 608-610.

⁸ M. T. Burrows, 'Some factors regulating growth', *Anat. Rec.*, 1916-1917, 11: 335-339; A. Fischer, *Tissue culture*, Copenhagen, Levin & Munksgaard, 1925, p. 23; M. R. Lewis and W. H. Lewis, 'The cultivation of tissues from chick embryos in solutions of NaCl, CaCl₂, KCl and NaHCO₃', *Anat. Rec.*, 1911, 5: 277-293.

⁹ A. Carrel, 'Le rajeunissement artificiel des cultures de tissus', *C.r. Soc. Biol., Paris*, 1911, 71: 401-402.

¹⁰ *Ibid.*, p. 402.

¹¹ A. Carrel, 'On the permanent life of tissues outside of the organism', *J.exp. Med.*, 1912, 15: 516-528. This paper was published in the year that Carrel won the Nobel Prize for medicine for his surgical studies. He became a celebrity, and although newspaper reports concentrated on his surgery of blood vessels and organ transplantation, a number referred to his tissue culture studies. For example, the *New York Times* published a long article entitled 'Dr. Carrel's miracles in surgery' on 13 October 1912, that included a report that Carrel had succeeded in maintaining cultures of chick heart cells for 120 days. The *New York Times* quoted at some length from Carrel's paper but other newspapers were less accurate and many believed that Carrel had kept an intact chicken heart living *in vitro*. The *Rural Weekly*, (St Paul, Minnesota), headlined its article on 24 October 1912: 'He keeps hearts alive in test tube and wins \$39,000 Nobel Prize!'

¹² Carrel, *op. cit.*, note 11 above.

¹³ See, for example, four articles, separated by sixty-eight years that appeared in *Scientific American*: G. Grandcourt, 'The "immortality" of tissues', *Scient. Amer.*, 1912, 107: 344, 354-355; B. M. Newman, 'Making tissues ageless', *ibid.*, 1940, 162: 284-285; L. Hayflick, 'Human cells and aging', *ibid.*, 1968, 218: 32-37; *idem*, 'The cell biology of human aging', *ibid.*, 1980, 242: 42-49.

¹⁴ B. L. Strehler, *Time, cells and aging*, New York, Academic Press, 1977, pp. 37-42.

¹⁵ L. Hayflick and P. S. Moorhead, 'The serial cultivation of human diploid cell strains', *Exp. Cell Res.*, 1961, 25: 585-621.

Dr. Carrel's immortal cells

human embryonic diploid fibroblasts had a limited lifespan in culture; such cells did not survive in culture for more than about fifty cell doublings or thirty weeks calendar time. Similar studies on chick embryonic fibroblasts have shown that their lifespan in culture is even shorter – about twenty-five cell doublings or sixty to eighty days.¹⁶ The phenomenon of *in vitro* cell senescence is now firmly established, but no satisfactory explanation of Carrel's "immortal" chick fibroblasts has been proposed.

It is the purpose of this article to describe the history of Carrel's culture and to suggest a new solution of tissue culture's oldest mystery.

2. HISTORY

The first mention of these cultures is in Carrel's paper 'On the permanent life of tissues outside of the organism',¹⁷ received for publication by the *Journal of experimental Medicine* on 15 March 1912. Carrel reported establishing cultures of embryonic chick heart on 17 January 1912, but he described in detail only experiment 720. A. Ebeling reported later that sixteen cultures of chick heart were set up on that day, and as the "immortal" strain was culture 725 it seems a safe assumption that cultures 720 and 725 were parts of the same series.¹⁸

Small fragments of chicken heart were placed on a coverslip in a drop of hypotonic plasma (three parts plasma with one or two parts of distilled water) that was allowed to clot before the coverslip was inverted over a hollow-ground slide. The cultures were incubated at 39°C until sufficient growth had occurred to necessitate subculturing. Portions of the cultures were removed with a cataract knife and immersed in Ringer's saline solution for several minutes before re-explanting in fresh hypotonic plasma. With minor variations this remained the basic method for handling the cultures.

On 1 June 1912, the cultures became the responsibility of Albert Ebeling, who wrote the first of a series of papers describing progress of the cultures.¹⁹ He reported that the series of sixteen cultures had become reduced to five by March 1912, but these recovered and subculturing led to twenty-five to thirty cultures at the time Ebeling took charge. The detailed account²⁰ shows that this increase in growth was due to the addition of chick embryo extract to the medium beginning on 13 March 1912. In 1907 Carrel had been studying wound repair and had found that pulped tissue laid in the wound increased the rate of healing.²¹ He applied this technique to tissue culture,

¹⁶ R. J. Hay and B. L. Strehler, 'The limited growth span of cell strains isolated from the chick embryo', *Exp. Geront.*, 1967, 2: 123-135.

¹⁷ Carrel, *op. cit.*, note 11 above.

¹⁸ A. H. Ebeling, 'The permanent life of connective tissue outside of the organism', *J. exp. Med.*, 1913, 17: 273-285.

¹⁹ *Ibid.* It is ironic that the "immortal" cells became so closely associated with Carrel, who grew them for six months, rather than with Ebeling who grew them for thirty-four years. It was, of course, the result of Carrel's self-aggrandizement.

²⁰ Ebeling listed each subculture of the cell strain 725 between 17 January 1912 and 15 January 1913. The cells were subcultured on 129 occasions and Ebeling described in detail the growth and culture conditions of each subculture. *Ibid.*, pp. 275-285.

²¹ A. Carrel, 'Artificial activation of the growth in vitro of connective tissue', *J. exp. Med.* 1913, 17: 14-19. Carrel's claim that he could accelerate the rate of repair of tissues using extracts caused a sensation in the United States press. Carrel wrote that if the rate of repair could be accelerated "ten times only . . . a fracture of the leg could be cured in four or five days", and the newspaper reports implied that this advance was imminent. For example on 16 January 1913 *The Star* (Philadelphia) ran an article headlined 'Carrel's incomprehensible compound will bring joy to many'. It justified its wild speculations with the sentence "while the good doctor doesn't come right out and say so, he leads us to believe that in future we will be quite exempt from all bodily injuries".

testing extracts of various tissues for their growth-promoting properties and found that chick embryo extract was the most potent.²² It became a standard constituent of the culture medium.

Despite this recovery, technical problems and infections reduced the number of cultures to a single one on 25 September 1912. This was derived from an area of non-contracting connective tissue that was small but growing actively, and by January 1913 the number of cultures had increased to thirty. On 3 February 1913, the culture had been subcultured on 138 occasions and was similar in appearance to the original culture.²³

Carrel published the next two papers on the cultures when they had been growing for sixteen and twenty-eight months respectively. In the first of these papers²⁴ he described experiments concerned with the effects of medium on cell growth and the ways in which cells modified their medium. Among the conclusions reached by Carrel was that these cultures “definitely demonstrate(s) that the tissues were not in a state of survival, as was the case in certain earlier experiments, but in a condition of real life, since the cells of which they were composed, like microorganisms, multiplied indefinitely in the culture medium”.²⁵ This was also his conclusion when he reported that the cells were still growing at least as actively after twenty-eight months in culture as they were in the original culture.²⁶

The next description of the cultures was given by Ebeling in 1919, after the cells had been growing for seven years.²⁷ They had been subcultured on 1,390 occasions but had not changed their morphology and were still growing actively. Ebeling described the current technique in detail but this was the same as that used originally except that the period of washing in Ringer’s solution was reduced to forty-five seconds, and chick embryo extract was regularly included in the culture medium. The cells appeared to be growing more rapidly than previously but Ebeling thought that this might be due simply to improvements in technique and not to a change in the cells’ characteristics.

The final paper in this series was published by Ebeling in 1922 when the cultures were ten years old and had been subcultured on 1,860 occasions.²⁸ He concluded that the continued growth of these cells proved conclusively that the cells utilized nutrients in the culture medium and pointed out that if the cultures had been kept their mass would have been greater than that of the sun!²⁹ A more appropriate image was used in a New York newspaper at this time; the cells would have formed a “rooster . . . big enough today to cross the Atlantic in a stride; it would also be so monstrous that when perched on this mundane sphere, the World, it would look like a weathercock”!³⁰

²² *Ibid.*, p. 16.

²³ Ebeling, *op. cit.*, note 18 above, p. 274.

²⁴ A. Carrel, ‘Contributions to the study of the mechanism of the growth of connective tissue’ *J. exp. Med.*, 1913, **18**: 287-298.

²⁵ *Ibid.*, p. 298.

²⁶ A. Carrel, ‘Present condition of a strain of connective tissue twenty-eight months old’, *J. exp. Med.*, 1914, **20**: 1-2.

²⁷ A. H. Ebeling, ‘A strain of connective tissue seven years old’, *ibid.*, 1919, **30**: 531-537.

²⁸ A. H. Ebeling, ‘A ten-year old strain of fibroblasts’, *ibid.*, 1922, **35**: 755-759.

²⁹ *Ibid.*, p. 755.

³⁰ ‘Scientists may now watch living connective tissue reproduce its ultimate cells’, *The World* (New York), 12 June 1921. The article described time-lapse cinematography of the “immortal” cells carried out by Alessandro Fabbri, a New York amateur scientist.

Dr. Carrel's immortal cells

No scientific article greeted the twelfth birthday of the strain in 1924, but the *New York Tribune* published a celebratory article on 17 January.³¹ This article was remarkably accurate and comprehensive and it seems certain that it was written by a scientist familiar with tissue culture research. It pointed out that popular interest had been aroused by the apparent immortality of the cells and went on to discuss the relevance of cellular immortality to *in vivo* ageing.

On 17 January 1940, the *New York World Telegram*³² reported that all Carrel's experimental work at the Rockefeller Institute had been discontinued when he returned to France in July 1939, and that the immortal strain of cells was dead. However the obituary was premature and the next day the paper reported that the cells had been taken from the Institute and were being grown in a private laboratory.³³ The Rockefeller Institute refused to name either scientist or laboratory, but it was Albert Ebeling who had left the Rockefeller after Carrel's departure. Ebeling was now established at the Lederle laboratories of the American Cyanamid Company where he had set up a tissue culture laboratory for the testing of drugs. In 1942 Ebeling wrote a popular account of the cultures under the title: 'Dr. Carrel's immortal chick heart. Present authentic facts about this oft falsified scientific celebrity'.³⁴ Among the "fantastic legends" that Ebeling corrected were: ". . . Dr. Carrel's original tiny fragment of chick embryo heart-tissue has grown into a large, pulsating chicken heart; or pieces have to be "snipped off" from time to time to hold it in bounds; or it is being kept in a glass jar or on a white marble slab, with the added setting of a group of scientists crowded around intently watching and tending it constantly, day and night!"³⁵

An example of such a legend appeared in *Collier's Magazine* of 24 October 1936.³⁶ The article described the perfusion apparatus designed by Charles Lindbergh and Carrel,³⁷ but the author referred to the "immortal" strain: ". . . he [Carrel] found that

³¹ 'Isolated tissue holds life 12 years in test', *New York Tribune*, 6 January 1924.

³² 'That chicken heart of Dr. Carrel's is dead at 28', *New York World Telegram*, 17 January 1940. According to a report that appeared in *Newsweek* on 29 January 1940, the *New York World Telegram* had enquired about the state of the "immortal" cell strain on 17 January of every year since the culture was established.

³³ 'Cancel that obituary on the chicken heart', *New York World Telegram*, 18 January 1940.

³⁴ A. H. Ebeling, 'Dr. Carrel's immortal chicken heart', *Scient. Amer.*, 1942, 166: 22-24.

³⁵ *Ibid.*, p. 22. The last fallacy has an element of truth. When Carrel and Burrows first began to grow cells at the Rockefeller Institute, they visited the laboratory throughout the night to ensure that the incubators were at the correct temperature. (Carrel quoted in A. Fischer, *Biology of tissue cells*, Cambridge University Press, 1946, see p. 225.)

³⁶ J. D. Ratcliff, 'The glass heart', *Collier's Magazine*, 24 October 1938.

³⁷ Lindbergh became interested in medical problems in 1930 when a relative became ill with pneumonia and "lesions on the heart". He raised the possibility of constructing an artificial heart and was introduced to Carrel who had been unsuccessfully trying to develop a perfusion pump. (Carrel's early studies on maintaining organs *in vitro* even inspired a poem entitled 'The heart in the jar' by Percy Mackaye in the *New York Times Book Review*, 8 December 1912.) A major difficulty was to maintain a sepsis in the apparatus, but after five years' work, Lindbergh developed an all-glass, one-piece pump that successfully maintained organs such as heart, thyroid, and ovaries for up to one week. As Corner has pointed out, the development of a pump to maintain whole organs coincided with a period when there was increasing concern with cellular and subcellular events rather than with the functioning of whole organs. The Lindbergh pump was expensive to maintain and the experiments required considerable efforts to perform at all. It was used only in a very few laboratories and rapidly fell into disuse. G. W. Corner, *A history of the Rockefeller Institute, 1901-1953*, New York, Rockefeller Institute Press, 1964, pp. 232-236. A sensational account will be found in Ratcliff, *op. cit.*, note 36 above.

occasional trimming kept it from literally growing out of the laboratory”!

The cells had now been growing for more than thirty years, and their accumulated volume would have been greater than that of the solar system! Ebeling described his culture methods in some detail and a photograph shows that he faithfully followed the methods employed at the Rockefeller Institute;³⁸ indeed, two of Carrel's technicians went with Ebeling to Lederle. Ebeling emphasized the importance of these cultures for the cytotoxic assay of drugs and claimed that the cell strain “has already thus ‘earned its keep’ over and over again”. The cultures were eventually discarded in 1946.

3. CURRENT VIEWS OF CELL AGEING *IN VITRO*

Carrel and his colleagues had shown that fibroblasts from chick embryo heart could be grown indefinitely in culture and their observations were confirmed subsequently by the establishment of many cell lines that apparently also grew indefinitely.³⁹ Nevertheless, there were frequent failures to obtain indefinite growth of cells and these failures were attributed to inadequacies of technique. R. Pearl for example believed that there was “abundant evidence” that cells could live indefinitely except “for the purely accidental intervention of lethal circumstances”.⁴⁰ Haff and Swim described the phenomenon of cell ageing *in vitro* in 1956, but attributed their failure to obtain continued growth of cells to deficiencies in the culture medium.⁴¹ They did derive three cell lines that grew indefinitely but they believed these were mutant cells selected for by the medium.

However, Hayflick and Moorhead showed that embryonic human lung fibroblasts that did not undergo spontaneous transformation and remained diploid had a limited lifespan of 50 ± 10 doublings *in vitro*.⁴² In a later paper Hayflick showed that cells frozen in liquid nitrogen did not begin to age until actively growing; cells frozen for as long as fourteen years still underwent approximately fifty cell doublings.⁴³ The possibilities that the rapid decline in growth was due to the release of inhibitory substances from a small proportion of ageing cells or to the release of lethal viruses were excluded by experiments in which young and old cells were grown in the same culture.⁴⁴ By using cells with a nuclear marker it was shown that each type of cell, young and old, completed its expected number of divisions independently of the other cells.⁴⁵

Other experiments suggest that cell senescence is not an artefact of tissue culture but may have some relevance to ageing *in vivo*. It might be expected that there would be a correlation between the age of the donor of cells and the number of doublings the cells

³⁸ Ebeling, *op. cit.*, note 34 above, p. 23.

³⁹ I. Macpherson, ‘Transformation of animal cells’, *Adv. Cancer Res.*, 1972, 13: 169-215.

⁴⁰ R. Pearl, ‘The biology of death; II. Conditions of cellular immortality’, *Scient. Mthly*, 1921, 12: 321-335, p. 326.

⁴¹ R. F. Haff and H. E. Swim, ‘Serial propagation of 3 strains of rabbit fibroblasts; their susceptibility to infection with vaccinia virus’, *Proc. Soc. exp. Biol. Med.*, 1956, 93: 200-204.

⁴² Hayflick and Moorhead, *op. cit.*, note 15 above. A comprehensive review of data on cell ageing *in vitro* will be found in L. Hayflick, ‘The cellular basis for biological aging’, in C. E. Finch and L. Hayflick (editors), *The biology of aging*, New York, Van Nostrand, 1976, pp. 159-186.

⁴³ L. Hayflick, ‘The limited *in vitro* lifetime of human diploid cell strains’, *Exp. Cell Res.*, 1965, 37: 614-636, p. 615.

⁴⁴ *Ibid.*, p. 624.

⁴⁵ *Ibid.*, p. 623.

Dr. Carrel's immortal cells

undergo *in vitro*. Martin and his colleagues⁴⁶ found that the number of doublings decreased by 0.2 doublings for every ten years of donor age, although Strehler believes that this correlation is not statistically significant.⁴⁷ There is also some indication of a correlation between the mean maximum lifespan of a species and the doublings undergone by the cells *in vitro*.⁴⁸ For example, mouse embryonic fibroblasts undergo fourteen to twenty-eight doublings and the mean maximum lifespan of a mouse is about 3.5 years; the corresponding figures for a man are forty to sixty doublings and 110 years. Figures are available for the Galapagos tortoise (90 to 125 doublings; 175 years)⁴⁹ but these may be unreliable because of the small sample! Skin fibroblasts from patients with progeria and Werner's syndrome that appear to involve premature ageing only undergo two and nine doublings respectively.⁵⁰

Various models have been proposed to account for the phenomenon of *in vitro* senescence, and Orgel suggested that increased errors in protein synthesis in ageing cells could provide the biochemical basis of cellular senescence;⁵¹ this suggestion has been made the basis of a more general "error theory".⁵² A large number of biochemical studies have been made of ageing cells⁵³ and some claim to have obtained data consistent with an "error theory".⁵⁴ Which, if any, of the biochemical changes found in old cells can be regarded as primary is unknown.

It has been suggested by Holliday and his colleagues that diploid cells are potentially immortal and that cell death in "old" cultures is not an ageing phenomenon.⁵⁵

⁴⁶ J. M. Martin, C. A. Sprague, and C. J. Epstein, 'Replicative lifespan of cultured human cells', *Lab. Invest.*, 1970, 23: 86-92.

⁴⁷ Strehler, *op. cit.*, note 14 above, p. 41.

⁴⁸ Hayflick, *op. cit.*, note 42 above, p. 163.

⁴⁹ S. Goldstein, 'Ageing *in vitro*; growth of cultured cells from the Galapagos tortoise', *Exp. Cell Res.*, 1974, 83: 297-302.

⁵⁰ Progeria is a disorder that appears to involve accelerated ageing with atherosclerosis of the major vessels developing as early as nine years of age. Other changes that occur are early greying and hair loss. In addition to the shortened *in vitro* lifespan of cultured cells, decreased mitotic activity, cloning efficiencies, and DNA synthesis have been reported for these cells. Hayflick *op. cit.*, note 42 above, p. 165. S. Goldstein, 'Lifespan of cultured cells in progeria', *Lancet*, 1969, i: 424; R. Holliday, J. S. Porterfield, and D. D. Gibbs, 'Werner's syndrome - premature aging *in vivo* and *in vitro*', *Nature, Lond.*, 1974, 248: 762-763.

⁵¹ L. E. Orgel, 'The maintenance of the accuracy of protein synthesis and its relevance to ageing', *Proc. nat. Acad. Sci., U.S.A.*, 1963, 49: 517-521.

⁵² L. E. Orgel, 'Ageing of clones of mammalian cells', *Nature, Lond.*, 1973, 234: 441-445.

⁵³ Hayflick, *op. cit.*, note 42 above, pp. 169-171.

⁵⁴ Orgel's theory predicts that increasing quantities of abnormal proteins will be synthesized as cells age, and data from Holliday's laboratory have supported this prediction. For example the heat lability of the enzyme glucose-6-phosphate dehydrogenase increases with increasing age of human embryonic diploid fibroblasts in culture (R. Holliday and G. M. Tarrant, 'Altered enzymes in ageing human fibroblasts', *Nature, Lond.*, 1972, 238: 26-30). When the change in enzyme activity was examined in individual cells using a histochemical technique, it was found that the frequency of cells with abnormal enzyme rose from 0.59×10^{-4} (at subculture 0 determined by extrapolation of regression lines) to 1.81×10^{-4} at subculture 20 and 5.52×10^{-4} at subculture 40. (S. J. Fulder and R. Holliday, 'A rapid rise in cell variants during the senescence of populations of human fibroblasts', *Cell*, 1975, 6: 67-73.)

⁵⁵ Holliday and his colleagues have proposed a model to account for the limited *in vitro* lifespan of normal cells that takes account of the peculiarities of the culture system. They suggest that dividing cells have a certain probability of giving rise to cells that are committed to senescence. Depending in part on the rate at which subcultures are performed, the number of committed cells in the population rises and eventually the number of uncommitted cells reaches a critical lower limit so that they are lost by dilution. At this point the population of cells appears to die even though there are actively dividing cells present. (T. B. L. Kirkwood and R. Holliday, 'Commitment to senescence: a model for the finite and infinite growth of diploid and transformed human fibroblasts in culture', *J. theor. Biol.*, 1975, 53: 481-496.) There is some experimental

Nevertheless, for whatever reason, normal cells have a limited lifespan in culture and we must try to account for the fact that the cultures established by Carrel lived for thirty-four years.

4. CARREL'S IMMORTAL STRAIN REASSESSED

I shall first discuss two explanations for Carrel's results that have already been proposed, and then suggest a third explanation based on new information about culture techniques in Carrel's laboratory.

(a) Cell transformation theory

Although there were many failures in establishing "immortal" cell strains, it was found that some cultures gave rise to cells that grew indefinitely. However, it was realized that these cells differed from the cells of the tissue from which they were derived and had undergone a process called transformation.⁵⁶ The principal characteristics of transformed cells are that they are karyologically abnormal and can grow indefinitely *in vitro*. Transformation occurs spontaneously, particularly in murine cell cultures,⁵⁷ and can be induced by oncogenic viruses⁵⁸ or by chemical carcinogens.⁵⁹ For example the muscle cell line L6 was induced with methylcholanthracene⁶⁰ and the normal human embryonic cell strain WI-38 was transformed with the oncogenic virus SV-40.⁶¹ Although there have been reports of karyologically normal cells growing indefinitely these reports have not been confirmed and Hayflick considers that the criteria used to assess these cells as normal were inadequate.⁶² Could the "immortal" cells have been a spontaneously transformed cell line?

This is unlikely. Transformation is usually accompanied by changes in cell morphology and behaviour; for example they tend to pile up and form multilayers. However, the "immortal" strain fibroblasts were repeatedly described as being unchanged in appearance. Furthermore, spontaneous transformation is extremely rare in chick cells in contrast to rodent cells where it is generally the rule. Ponten grew sixteen strains of chick fibroblast for between twenty and twenty-seven cell doublings and found no examples of spontaneous transformation.⁶³ It is therefore improbable that the indefinite growth of Carrel's cells was due to the development of a cell line.

evidence in support of this hypothesis. (R. Holliday, L. I. Huschtscha, G. M. Tarrant, and T. B. L. Kirkwood, 'Testing the commitment theory of cellular aging', *Science*, 1977, 198: 366-372.)

⁵⁶ Macpherson, *op. cit.*, note 39 above.

⁵⁷ G. J. Todaro and H. Green, 'Quantitative studies of the growth of mouse embryo cells in culture and their development into established cell lines', *J. Cell Biol.*, 1963, 17: 299-313.

⁵⁸ J. Sambrook, 'Transformation by polyoma and simian virus 40', *Adv. Cancer Res.*, 1972, 16: 141-180.

⁵⁹ N. K. Mishra and G. DiMayorca, 'In vitro transformation of cells by chemical carcinogens', *Biochim. Biophys. Acta*, 1974, 355: 204-219.

⁶⁰ D. Yaffe, 'Retention of differentiation potentialities during prolonged cultivation of myogenic cells', *Proc. natl. Acad. Sci., U.S.A.*, 1968, 61: 477-483.

⁶¹ A. J. Girardi, F. C. Jensen, and H. Koprowski, 'SV-40 induced transformation of human diploid cells: crisis and recovery', *J. cell. comp. Physiol.*, 1965, 65: 69-83.

⁶² Hayflick, *op. cit.*, note 42 above, pp. 174-176.

⁶³ J. Ponten, 'The growth capacity of normal and Rous-transformed chick fibroblasts in vitro', *Int. J. Cancer*, 1970, 6: 323-332.

Dr. Carrel's immortal cells

(b) *Cell contamination theory*

Although Carrel's cultures were initially grown in a clot of hypotonic chicken plasma, chick embryo extract was later included. Carrel did this because of the growth stimulating effects of chick embryo extract that he described in a paper sent for publication in June 1912.⁶⁴ Addition of chick embryo extract to plasma in a ratio of 1:3 or 4 caused a marked increase in cell growth.

Embryo extract was first used with the "immortal strain" on 1 February 1912, and Ebeling reported that it was followed by active growth, although before and after this subculture growth was "slow" when plasma alone was used.⁶⁵ During the period from 13 March to 11 April 1912, when embryo extract was used, all the cultures grew very well.⁶⁶ It is not clear from Ebeling's account if extract was always included in the culture medium because he describes the cells being "cultivated in the same medium" without making clear what the "same medium" was. It may not have included embryo extract because he occasionally notes the addition of extract, for example on 12 July and 15 July 1912, although the "same medium" was employed before and after these days.⁶⁷ Extract was again specified as a constituent of the medium on 1 October 1912 and this led to a period of "excellent" growth.⁶⁸

Hayflick has pointed out that the periods of good cell growth correspond to the occasions on which embryo extract was incorporated in the culture medium.⁶⁹ He has suggested that the embryo extract contained living cells and it was these cells that grew and gave the appearance that the extract was stimulating the original cells of the culture. Could Carrel have been replenishing his cultures with "young" cells?

It is difficult to decide from Ebeling's reports if there was such a strict correlation between cell growth and the use of embryo extract, and if there was, if this was due to addition of cells or to some growth stimulatory effect of chick embryo extract. Despite the development of complex, fully-defined media, embryo extract is still used for certain types of cell culture, e.g. muscle,⁷⁰ to stimulate growth. The growth stimulation observed by Carrel might have been due, as he believed, to substances present in the extract.⁷¹

It is difficult to determine if cells might have been present in the embryo extract. In his paper of 1913 Carrel reported that extract prepared from embryonic tissue that was

⁶⁴ Carrel, *op. cit.*, note 21 above.

⁶⁵ Ebeling, *op. cit.*, note 18 above, p. 275.

⁶⁶ *Ibid.*, p. 276.

⁶⁷ *Ibid.*, p. 279.

⁶⁸ *Ibid.*, p. 281.

⁶⁹ Hayflick, *op. cit.*, note 43 above, pp. 627-628.

⁷⁰ C. R. Slater, 'Control of myogenesis in vitro by chick embryo extract', *Dev. Biol.*, 1976, **50**: 264-284.

⁷¹ Many attempts were made to isolate the active ingredient present in chick embryo extract. Fischer described experiments using ammonium sulphate, carbon dioxide, and alcohol precipitation to prepare fractions of extract that were tested for their growth promoting activity. None of these was successful, and, in keeping with biochemical thought of the time, Fischer believed that the "colloidal" properties of all proteins in the extract was important and that no single protein was responsible for growth stimulation. Fischer, *op. cit.*, note 8 above, pp. 44-71.

Willmer wrote in 1935: "How the extract produces these effects [growth stimulation] is still wrapped in the mists of obscurity, which, although they frequently appear to be lifting, come down again as thick as ever". E. N. Willmer, *Tissue culture*, London, Methuen, 1935, p. 22. Willmer gives a thorough discussion of the supposed activities of embryo extract, *ibid.*, pp. 43-64.

frozen after mincing was very active,⁷² and it is unlikely that cells would have survived this procedure. However, freezing was not included in the standard method described by Ebeling in 1917.⁷³ Embryos were minced in saline with scissors and the resulting pulp centrifuged for ten minutes to give a clear supernatant. Ebeling did not give the centrifugal force but Parker in 1938 recommended centrifuging at 2400 rpm.⁷⁴ This is more than sufficient to sediment cells and it is probable that the force used by Ebeling was also sufficient. Dr. K. R. Porter who worked at the Rockefeller Institute but in a different laboratory, recalls that embryo extract prepared in this way was highly viscous, and he believes it possible that some cells may not have been sedimented by centrifugation.⁷⁵

There is a further difficulty that cells present in embryo extract added to the plasma clot would have been evenly distributed over the area of the clot. There is no evidence to suggest that such a uniform pattern of cell growth was observed and only the peripheral halo of cells was used for subcultures. It is unlikely that sufficient cells could have been added to have so markedly improved growth in this area of the culture without forming a monolayer of cells over the whole area covered by the clot.

Without knowledge of the exact conditions used in Carrel's laboratory to prepare chick embryo extract it is not possible to determine if the "immortal" culture could have been repeatedly and accidentally "rejuvenated" by young cells in the embryo extract. The possibility remains that such contamination may have occurred and certainly by 1938 workers in Carrel's laboratory were aware of the possibility. Parker in his book on tissue culture methods mentioned the danger of transferring cells in the chick embryo extract and recommended freezing and thawing the extract to kill these cells.⁷⁶ Parker gives no indication of when the practice of freezing and thawing became routine in the preparation of embryo extract in Carrel's laboratory, but A. H. Drew as early as 1924 recommended the same procedure to "disintegrate the cells" present in the extract.⁷⁷ The fact that Parker warns of the danger of cell contamination lends credence to the suggestion that such contamination actually occurred. When Dr. Helen Morton joined R. C. Parker in Toronto in 1947, they were following exactly the methods learned by Parker from Carrel and she has described these in detail. In the early 1950s Dr. Morton attempted to assess the adequacy of the technique outlined above, and found living cells in two out of five preparations. Although these cells were presumably damaged because they did not grow, Dr. Morton thinks it probable that contamination with growing cells did occur at various times during the life of the "immortal" strain.⁷⁸ Dr. Charity Waymouth believes it very likely that the preparative techniques used were inadequate to kill cells present in the embryo extract. She recalls that Fischer as late as 1946 was not freezing and thawing extract prepared in his Copenhagen laboratory.⁷⁹ However, other laboratories that were unable to grow cells

⁷² Carrel, *op. cit.*, note 21 above, p. 17.

⁷³ Ebeling, *op. cit.*, note 27 above, p. 533.

⁷⁴ R. C. Parker, *Methods of tissue culture*, New York, Paul B. Hoeber, 1938, p. 70.

⁷⁵ K. R. Porter, letter to the author, 29 May 1979.

⁷⁶ Parker, *op. cit.*, note 74 above.

⁷⁷ A. H. Drew, 'Three lectures on the cultivation of tissues and tumours in vitro', *Lancet*, 1924, i: 785-787, p. 785.

⁷⁸ H. Morton, letter to the author, 6 May 1979.

⁷⁹ C. Waymouth, letter to the author, 3 August 1979.

Dr. Carrel's immortal cells

indefinitely were using exactly the same preparative methods as Carrel's laboratory and it would have been expected that at least one or two of these would have been as successful as Carrel at contaminating their cultures. I believe that although some cell contamination probably occurred, it is unlikely that it could have done so consistently over a thirty-four-year period, and particularly in the last ten years of the immortal strain's life when Carrel's laboratory was well aware of the problem and presumably took great care to prevent cell contamination of the extract.

(c) *The "re-stocking" theory*

In a paper published in 1914, Carrel said that "if we exclude accidents, connective tissue cells . . . may proliferate indefinitely."⁸⁰ Is it possible that the cells of the "immortal" strain did die, but because these deaths must have been (according to Carrel's dogma) "accidental", the cultures were begun afresh after each accident? Dr. Margaret Murray tells me that such a rumour was current in New York tissue culture circles in the 1930s,⁸¹ but there is now anecdotal evidence in its support.

Carrel spent some thirty years at the Rockefeller Institute but was notorious for his reserve and isolation from the life of the Institute. G. W. Corner in his *History of the Rockefeller Institute* described Carrel's relationship with other workers in the Institute: ". . . [he] held himself aloof from the general life of the Institute. Working largely by himself he rarely consulted colleagues . . . People from other laboratories, fearing to carry infectious germs into the laboratory, did not drop in for casual visits, and never learned about what was going on, except when specially invited."⁸²

Occasionally, however, visitors from other institutes were able to visit Carrel's laboratory, although such visits were not welcome and required some determination and stubbornness to succeed.⁸³ Dr. Ralph Buchsbaum⁸⁴ made such a visit to the Rockefeller Institute in the summer of 1930 and his account of this visit is fascinating for the picture it gives of life in Carrel's laboratory. Of particular interest is that his account suggested a third possibility for Carrel's success in maintaining the "immortal" strain of fibroblasts for thirty-four years. Dr. Buchsbaum has very kindly given me permission to quote his story and I cannot do better than reproduce it in full, together with his conclusion:

As a graduate student at the University of Chicago, I worked jointly in the Zoology Department under the guidance of Dr. W. C. Allee and in the Anatomy Department under the guidance of Dr. William Bloom,

⁸⁰ Carrel, *op. cit.*, note 26 above, p. 2.

⁸¹ M. R. Murray, letter to the author, 15 June 1979.

⁸² Corner, *op. cit.*, note 37 above, p. 153.

⁸³ A close friend of Carrel had been turned away on two consecutive days by Carrel's secretary who was acting on Carrel's general instructions. The friend gained access only on the third day when he lost his temper and insisted on admission. (W. S. Edwards and P. D. Edwards, *Alexis Carrel, visionary surgeon*, Springfield, Illinois, Charles C Thomas, 1974, p. 54.)

⁸⁴ Dr. Ralph Buchsbaum (b. 1907) studied at the University of Chicago where he also obtained his Ph.D in zoology. He became an instructor at the University of Chicago and entered the United States Air Force as a captain at the outbreak of the Second World War. There he continued his scientific interests, being involved with the Arctic, Desert and Tropic Information Center. Dr. Buchsbaum was appointed professor of Zoology at the University of Pittsburgh in 1950, and in addition he worked with UNESCO on the teaching of biology in Africa. Among his publications are a book on tissue culture (*Methods of tissue culture*, Chicago, University of Chicago Press, 1936) and a very popular textbook of invertebrate zoology, *Animals without backbones*, (Chicago, University of Chicago Press; Harmondsworth, Penguin Books, 1951). Dr. Buchsbaum retired in 1972 and now publishes books (The Boxwood Press) in California.

J. A. Witkowski

on a problem in tissue culture. I was eager to visit Alexis Carrel's laboratory to see the great man and his work, especially the "immortal" strain. Accordingly, in the summer of 1930 I drove to New York (in those days and in my 5-year-old 4-cylinder Chevrolet – quite a feat).

I phoned Carrel's laboratory, and as I expected Dr. Carrel to be away in Spain on vacation, I talked to Carrel's chief assistant, Dr. R. C. Parker. He said he had a "big experiment" on that day and told me to come back tomorrow. I could not wait, so I phoned Dr. A. H. Ebeling, Parker's associate, who cordially invited me to come to the laboratory right away. He showed me around the whole series of laboratories but when I asked to see the famous strain of cells he told me it was too delicate to risk being shown to visitors. I did see some other cell strains and they looked fine. As he bid me goodbye at the elevator, Dr. Parker was also about to depart, clad in golf clothes and with a golf bag – this explained the "big experiment" planned for the morning.

I just could not bear to return immediately to Chicago without seeing the famous immortal strain, so I returned to the floor where I had met a young woman technician. I pleaded with her to let me see the cultures. She said that Dr. Carrel and Dr. Parker would have a fit if they knew, but "what harm could it do for me to see them?" When I looked at them and said that they were full of fat globules and obviously on the way out, she said slyly, "Well, Dr. Carrel would be so upset if we lost the strain, we just add a few embryo cells now and then . . . We make new strains for new experiments. Dr. Parker says he will retire the strain soon, it costs too much to keep it going."

For me this was a great relief. I was meticulous with my own cultures, yet I could not keep any strain going under the then standard methods (chicken plasma and chick embryo extract in Tyrode's solution, on coverslips, dividing the cultures in half every other day) for more than perhaps a year, mostly less. I don't have the actual records in front of me at this moment, but I recall well my disappointment (and criticism from Bloom, who thought I should do better – after all, Carrel showed the way) when the cultures slowed their growth and failed to increase and finally died.

I told this story, of my visit to Carrel's laboratory, to various people. Dr. Bloom refused to believe it. Others chuckled gleefully. Dr. Carrel was to blame only in that he did not keep on top of what was really going on in the laboratory (mostly, he wrote the papers). Dr. Parker and Dr. Ebeling probably suspected something, hence the "retirement". In the interest of truth and science, the incident should have been thoroughly investigated. If it had been, some heads might have rolled, sacrificed to devotion to a wrong hypothesis – immortality of cell strains.

It is very difficult to determine the motives behind such an admission or to assess its accuracy. Dr. Margaret Murray recalls that one of Carrel's technicians of that time was passionately anti-fascist and detested Carrel's political and social ideas. Dr. Murray believes that this technician would willingly have discredited Carrel scientifically if possible.⁸⁵

Nevertheless the "immortal" cell strain was of considerable importance to Carrel and his colleagues and it is quite probable that the cultures could have been replenished in the way described by Dr. Buchsbaum.

The culture was begun in an attempt to silence his early critics, and Corner has described it as a "consummate piece of scientific enterprise and showmanship."⁸⁶ It was clearly essential for Carrel to maintain these cultures, particularly as he had declared that the cells could be grown "indefinitely" as early as 1913.⁸⁷ Having committed himself to this opinion, the cultures assumed a new significance and Carrel's laboratory was committed to maintaining them indefinitely. Furthermore, the culture was the perfect example of the "pure" cell line, a concept repeatedly emphasized by Carrel. He drew an analogy between the microbiologist studying cultures of a single type of bacterium and the cell culturist studying cultures of a single cell type. "Pure" cell cultures were essential before useful experiments could be carried out with cell cultures: "The isolation and maintenance of pure strains of various types of tissues was the first and most indispensable step in the adaptation of the method of

⁸⁵ Murray, *op. cit.*, note 81 above.

⁸⁶ Corner, *op. cit.*, note 37 above, p. 127.

⁸⁷ Carrel, *op. cit.*, note 24 above, pp. 297-298.

Dr. Carrel's immortal cells

tissue culture to physiological research.”⁸⁸

The cells of the immortal strain were the standard cells on which so much of the work of his department was based for twenty-seven years. Finally, the demonstration that cells were immortal once removed from the influences of the body was an essential part of Carrel's understanding of physiology and of his mystical ideas on the nature of life.⁸⁹ Corner described the importance of the “immortal” cultures to Carrel: “This experiment, surely one of the most extraordinary in the history of science, with its demonstration of unending life force released from the mortal body, gave Carrel a vivid sense of closeness to Nature's secrets.”⁹⁰

The importance attached to these cells can hardly have failed to impress those concerned with the maintenance of the cultures. It is certainly conceivable that Carrel's staff might have “helped” the cultures along whenever they were declining in growth.⁹¹ This is not the first occasion on which such a suggestion has been made. P. B. and J. S. Medawar have written that “an alternative and less creditable possibility [to accidental cell contamination] is that the cultures *did* die out, and were simply started anew from fresh tissues on the grounds that their death could only have been due to lack of attention, to the use of a toxic medium or to some other accident.”⁹²

I have attempted to contact colleagues of Carrel who were working with the immortal strain of cells between 1930 and 1939 for their comments on Dr. Buchsbaum's story. Unfortunately I have had no success and it is unlikely that any confirmation or denial of the “re-stocking” theory by people who actually worked with the cells will be obtained.

6. CONCLUSION

Of all Carrel's work, the “immortal strain” was the most remarkable for the public interest it aroused and for its influence on theories of ageing. The effect produced by the belief that cells *in vitro* were immortal was to lead research away from all consideration of possible cellular changes during senescence. Instead it was emphasized that ageing was the result of a breakdown of the interaction and co-operation of tissues in the body and a necessary consequence of metazoan organization. Fischer in the historical introduction to his book on tissue culture wrote

⁸⁸ A. Carrel, ‘The method of tissue culture and its bearing on pathological problems’, *Br. med. J.*, 1924, ii: 140-145, p. 140.

⁸⁹ A. Carrel, *Man the unknown*, London, Hamish Hamilton, 1935, chapter V: ‘Inward time’. It is difficult now to appreciate the immense popular interest aroused by Carrel's book; it is a curious mixture of scientific, mystical, and political thought that I find almost unreadable. Nevertheless, the series of lectures given by Carrel in 1935 were so popular that police had to be called out to control the crowds. ‘Science and Death’, *New York Herald Tribune*, 14 December 1935.

⁹⁰ Corner, *op. cit.*, note 37 above p. 128.

⁹¹ Corner describes Carrel as winning “almost fanatical devotion from some of his immediate helpers” (*ibid.*, p. 153). It is perhaps significant that two of Carrel's technicians, Mrs E. Hull and Miss D. Olmstead, accompanied the cultures when they went with Ebeling to American Cyanamid in 1939. Ebeling, *op. cit.*, note 34, above, p. 24.

⁹² P. B. Medawar and J. S. Medawar, *The life science*, London, Wildwood House, 1977, pp. 125-126. There is also the remarkable instance of Mendel's work on inheritance. Sir R. A. Fisher demonstrated that the values obtained by Mendel were far closer to the expected theoretical values than could be reasonably expected on probability theory and Fisher suggested that Mendel may have been deceived by his gardeners who gave him the figures that they knew he wanted! R. A. Fisher, ‘Has Mendel's work been rediscovered?’ *Ann. Sci.*, 1936, 1: 115-137.

that as a result of Carrel's studies: ". . . it seems as if the factors of senescence are not to be found in the cells themselves but are far more profound phenomena correlated to the entire function of all the cells in the organism and their 'milieu interieur'."93

This point was made more forcefully by Pearl in his wide-ranging and careful review of ageing. He regarded senescence as: ". . . an attribute of the multicellular body as a whole . . . If this conception of the phenomenon of senescence is correct in its main features . . . it shows the essential futility of attempting to investigate its causes by purely cytological methods."94

On the contrary, since 1961 when Hayflick and Moorhead demonstrated the limited *in vitro* lifespan of cells,95 a considerable amount of research has been carried out using cell ageing *in vitro* as a model system.96 It is not clear what contribution the changes observed in cells senescing *in vitro* make to ageing *in vivo*, but there is no doubt that cells in culture do undergo a variety of changes with time and exciting research is being carried on the biochemical basis of these changes. For whatever reason it appears that Carrel's results were spurious and that they diverted attention away from an important phenomenon.

Unfortunately, as Strehler has put it, ". . . the ultimate effects of the ageing process have made it impossible for Carrel to respond in his own defense . . ."97 The cultures outlived Carrel. He returned to France at the beginning of the Second World War and died in disgrace in Paris on 5 November 1944. The "immortal" strain was eventually discarded on 26 April 1946 and its demise was recorded by the *Herald Tribune* on 2 October 1946.98

SUMMARY

A strain of embryonic chick heart fibroblasts established by Alexis Carrel on 17 January 1912 was grown for thirty-four years. Recent investigations on the ageing of cells have shown that cells in culture have a limited lifespan; embryonic chick fibroblasts undergo about thirty cell doublings over a period of four months before dying. Two explanations have been advanced to account for the longevity of Carrel's cells. (1) The cells underwent a spontaneous transformation with the result that they were able to grow indefinitely. This is unlikely because none of the other changes associated with spontaneous transformation was described, and no other chick cells have been known to undergo spontaneous transformation. (2) The cultures were accidentally replenished by cells present in the chick embryo extract included in the culture medium. It is possible that cells might have survived the procedures used for preparing the embryo extract, but it is very unlikely that such contamination could have occurred consistently over so many years and in only one laboratory. A third explanation has now been proposed; the cultures were deliberately renewed by the addition of fresh tissue. There is reputable anecdotal evidence for this and the explanation is credible in view of the importance of these cultures for Carrel's theories.

93 Fischer, *op. cit.*, note 8 above, p. 24.

94 Pearl, *op. cit.*, note 40 above, p. 335.

95 Hayflick and Moorhead, *op. cit.*, note 15 above.

96 Hayflick, *op. cit.*, note 42 above.

97 Strehler, *op. cit.*, note 14 above, p. 41.

98 Corner, *op. cit.*, note 37 above, p. 533.