17231. Some Effects of Dilution on the Nutritive Value of Dialyzed Plasma and Embryo Juice.*

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The classic substratum for tissue cultures, developed by Harrison,[1] Burrows,[2] and Carrel,[3] consists of chick plasma clotted with chick embryo juice and with an overlay of embryo juice variously diluted. Such a plasma clot without embryo juice overlay is insufficient to support prolonged tissue growth except when frequently renewed by transplanting the cultures to fresh substratum. It supplies an ill-defined but far from negligible level of nutritional factors. In any precise study of cell nutrition, these factors must either be clearly defined or, if possible, eliminated before the substratum can be satisfactorily evaluated as a base line. Various attempts have been made to arrive at such an evaluable base line. Porter and Hawn[4] have used clots prepared from purified fibrinogen coagulated with thrombin. Both these substances are more clearly defined and more nearly inert than are plasma and embryo juice, but they are still not completely defined. Evans and Earle[5] have been successful in developing a procedure for cultivation of tissue cultures on sheets of perforated cellophane, which is nutritionally fully inert. This is a very satisfactory solution of the nutritional problem, but such cellophane is not easily available to all workers throughout the world. Lewis,[6] White,[7] and others have grown cultures directly on glass. This is likewise a fully satisfactory solution of the nutritional problem but the results obtained are somewhat unreliable.

Fischer[8] has approached the problem from quite a different angle by seeking to eliminate the nutritional factors from the classic plasma clot. He showed that when chick plasma and embryo juice, after thorough dialysis against a dextrose-Ringer's solution, were used in the preparation of clots on which to cultivate chick fibroblasts, no growth whatever would occur thereon. He concluded that such a clot was nutritionally inert and was not attacked by the tissue enzymes. This being true, it could safely be used as an inert substratum against which to study the nutritive properties of various supernatants. Dialyzed plasma and dialyzed embryo juice have been rather extensively used in such studies.[9]

Fischer states[8] that implanted tissues grew less and disintegrated more quickly on dialyzed plasma than they did in Tyrode's solution alone. If the defect is merely one of nutrient deficiency, it is difficult to see how Tyrode's solution could be the less deficient of the two. The alternative possibility exists that the defect may be due not merely to a low level of nutritive value but to some more positive injurious effect. Indeed, Fischer himself speaks of "den Eindruck einer direckten toxischen Einwirkung."[8] For this and other reasons we decided to repeat Fischer's work. Preliminary experiments were carried out by somewhat simplified procedures, using hanging drop cultures in place of Carrel flasks and chick heart fibroblasts in place of frontal bone fibroblasts.

Fischer used an undiluted dialyzed embryo

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2 Burrows, M. T., *J. Am. Med. Assn.*, 1910, **55**, 2057.

3 Carrel, A., *J. Exp. Med.*, 1913, **17**, 14.

4 Porter, K. R., and Hawn, C. v. Z., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 309.

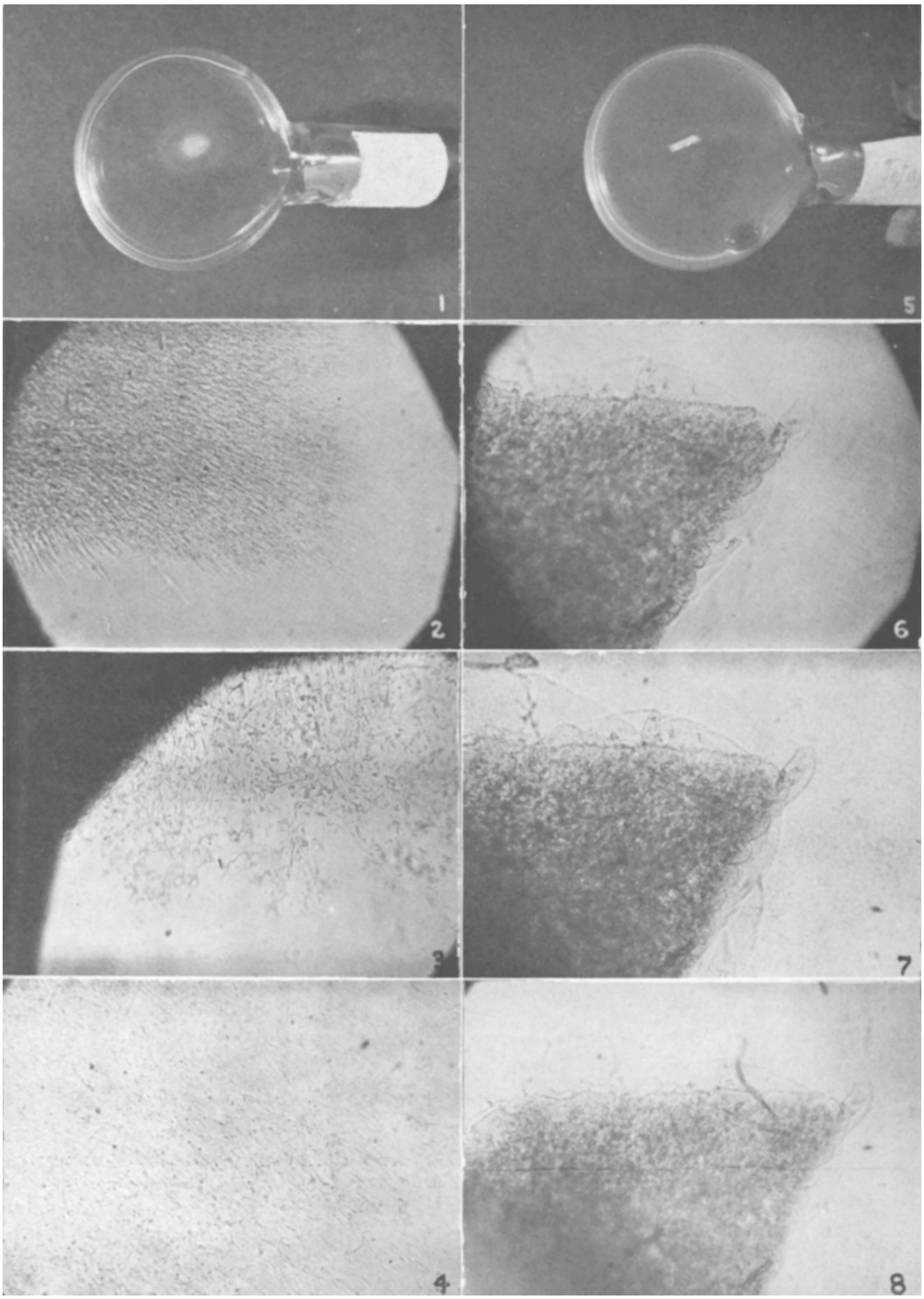
5 Evans, V. J., and Earle, W. R., *J. Nat. Cancer Inst.*, 1947, **8**, 103.

6 Lewis, Warren H., *Carnegie Inst. Wash., Contrib. to Embryology*, 1926, **18**, 1.

7 White, P. R., *Growth*, 1947, **10**, 231.

8 Fischer, A., *Acta Physiologica Scandinavica*, 1941, **2**, 143.

9 Fischer, A., Astrup, T., Ehrensvard, G., and Oelenschlager, V., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 40.



(All photographs are of living material, made with 16 mm panatomic film. Fig. 1, 5, 9, and 13 (Carrel flasks) are × 1. All others are about × 76).

FIG. 1. Twenty-four hour culture of chick frontal bone fibroblasts on the control medium consisting of chick plasma clot with overlay of chick embryo juice diluted 1:9 with Tyrode's solution. The halo of migrating cells is clearly evident.

FIG. 2. Detail from the above culture showing the compact, uniform growth of finely granular cells.

FIG. 3. Margin of the same culture at the end of 4 days. The cells contain many small fat globules and are larger than in Fig. 2, but are in excellent condition.

FIG. 4. Area from about midway between the original implant and the edge of the flask, taken at the end of 10 days. The uniform character of the growth and relatively clear cytoplasm of the cells are evident.

FIG. 5. Twenty-four hour culture of chick frontal bone fibroblasts on an undiluted dialyzed plasma-dialyzed embryo juice clot, with undiluted dialyzed embryo juice overlay. No growth has occurred.

FIG. 6, 7, 8. Details of the above culture after 24 hours, 4 days, and 10 days. There has been no migration or change other than disintegration of the cells within the implanted bit of clot.

juice in the preparation of both his substratum and his experimental supernatant.[8] We have obtained our most satisfactory control results (undialyzed media) with nutrients in which the embryo juice* was diluted about 1 to 9 with Tyrode's solution. In order that our experimental cultures might be more nearly comparable with the controls, we therefore carried out cultures not only with undiluted embryo juice but with dilutions approximating those of our controls. Our experiments with undiluted embryo juice fully confirm Fischer's findings, but, to our surprise, cultures prepared with diluted dialyzed embryo juice grew quite well over the 48-hour periods which can be followed in hanging drops. This suggested that the undiluted material remaining after dialysis was perhaps not *deficient,* that is, lacking in essential nutrient factors, but merely *defective* due to unsatisfactory concentrations or proportions of its constituents. These initial experiments were, however, not duplicates of those reported by Fischer. We therefore carried out another series of cultures, repeating as nearly as possible Fischer's second procedure[8] and extending the study to the use of diluted media.

One of us (E.L.) spent a month in the summer of 1946 in Fischer's laboratory in Copenhagen and was therefore familiar with his methods. In the preparation of plasma and in dialysis of both plasma and embryo juice we followed Fischer's procedures exactly. Twenty-five ml of plasma, from blood drawn by heart puncture from a young cockerel into a heparinized syringe, were transferred to a sterile (autoclaved) cellophane dialysis tube under sterile precautions and were dialyzed for 8 days in the refrigerator at about 4°C against 1.5 liters of Ringer's solution containing 0.1% dextrose. The solution was not renewed.[8] Freshly prepared juice from 9 day chick embryos was similarly treated. All dilutions were made with Tyrode's solution without dextrose. It was found that satisfactory clots could not be obtained with dilutions higher than one part of embryo juice in two parts of Tyrode's solution. A series was set up with 16 Carrel flasks, 4 flasks containing each of the following solutions: 1) control, undiluted plasma (0.5 ml) coagulated with 0.5 ml of embryo juice diluted 1:2, supplied after clotting with a supernatant of embryo juice diluted 1:9; 2) Fischer's cultures, consisting of undiluted dialyzed plasma (0.5 ml) clotted with 0.5 ml of undiluted dialyzed embryo juice, and with a supernatant of undiluted dialyzed embryo juice; 3) similar to the latter, but with 0.5 ml of undiluted dialyzed plasma clotted with dialyzed embryo juice diluted with an equal part of Tyrode's solution (0.5 ml), and a supernatant of dialyzed embryo juice likewise diluted with an equal part of Tyrode's solution; and 4) undiluted dialyzed plasma (0.5 ml) clotted with dialyzed embryo juice diluted with 2 parts of Tyrode's solution (0.5 ml), and a supernatant of dialyzed embryo juice similarly diluted. In each flask was placed a single fragment of a rapidly growing homogeneous culture of fibroblasts derived from the frontal bone of a 14-day chick em-

* Embryo juice was prepared by finely triturating with sharp scissors without addition of fluid, allowing to stand for one-half hour, and then centrifuging.

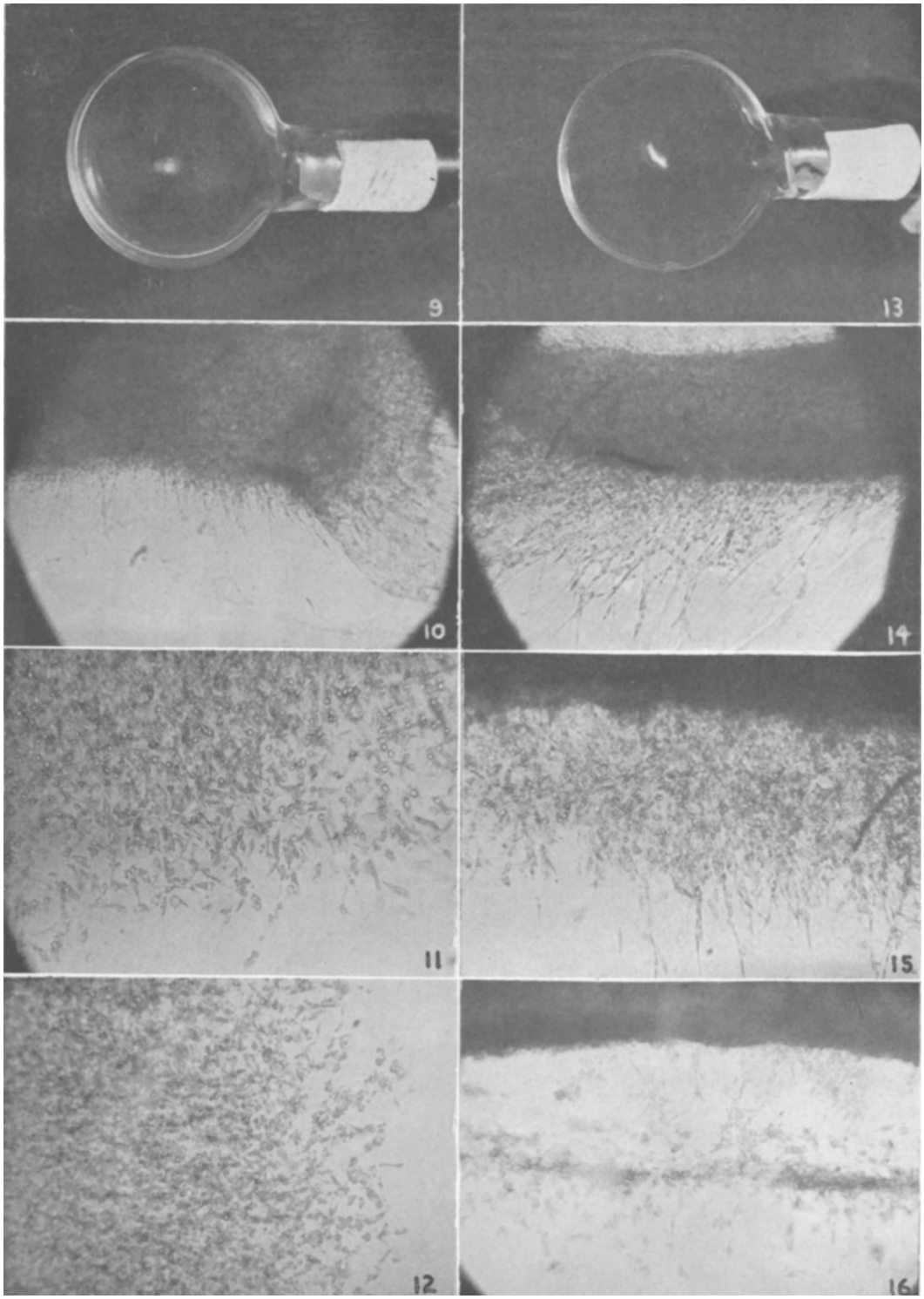


FIG. 9. Twenty-four hour culture of chick frontal bone fibroblasts on a clot prepared with dialyzed embryo juice diluted 1:1 with a dextrose-free Tyrode's solution, with overlay of this

same dilution. The halo of migrating cells is much smaller than in Fig. 1, but is still quite evident.

FIG. 10. Detail from the above culture showing the sparse but evident migration of fibroblasts.

FIG. 11. The same culture as Fig. 10 at the end of 4 days (compare with Fig. 3). The zone of migrating cells is fairly wide and quite uniform. The cells themselves are large, larger than in Fig. 3, but are packed with fat globules, many of which are very large.

FIG. 12. The same culture after 10 days. Outlines of single cells are no longer visible, disintegration having taken place.

FIG. 13. Twenty-four hour culture of chick frontal bone fibroblasts on a clot prepared with dialyzed embryo juice diluted 1:2 with dextrose-free Tyrode's solution, with overlay of the same dilution. The considerable halo of migrating fibroblasts is evident.

FIG. 14. Detail from the above culture. The area of growth is both qualitatively and quantitatively better than at a 1:1 dilution (Fig. 10).

FIG. 15. The same culture after 4 days. Growth is somewhat more compact than at a 1:1 dilution (Fig. 11) but otherwise not very different.

FIG. 16. The same after 10 days. Cells in the original migrating zone (transverse band) are fatty but in the gap left between this and the original implant by lysis and contraction of the clot, subsequently patched, the new cells have clear-cut outlines and are not excessively filled with fat. This culture is still very much alive and growing, though very slowly.

bryo and maintained on the standard chick plasma-dilute embryo juice medium through four successive passages. Cultures were examined and photographed at the end of 48 hours, 4 days, and 10 days. Nutrient was replaced in each of the flasks after 2, 4, 6 and 8 days.

Results. The results were clear cut, consistent both among themselves and with the results of previous experiments using hanging drop cultures. Growth on the control nutrient was normal and very rapid, so that at the end of 10 days the entire *bottoms* of the flasks were covered. The clot had undergone lysis around the implant and was patched on the 4th day; new growth had covered the patch by the 10th day (Fig. 1-4). On the undiluted dialyzed plasma dialyzed embryo juice medium, in complete agreement with Fischer's results, there had been absolutely no growth and the cells had undergone histolysis (Fig. 5-8). On dialyzed plasma containing dialyzed embryo juice diluted 1:1, however, there had been very considerable growth (migration). At 24 hours this reached a maximum width of about 0.1 mm. At 4 days the band was about 0.8 mm wide, the cells were *larger* than the controls and were packed with fat globules. At 10 days they had grown no further and were mostly dead and broken down (Fig. 9-12). Dialyzed plasma clotted with embryo juice diluted 1:2 did not give firm uniform clots, although they were sufficiently coherent to attach the cultures satisfactorily and to provide a satisfactory substratum on which growth could take place. At 24 hours the growth zone was about 0.5 mm wide, 5 times as great as at 1:1 dilution. At 4 days this had about doubled, that is, was about the same width as in the 1:1 dilution. The growth was, however, denser and the cells somewhat less fatty. At 10 days, some lysis had occurred (patched at 8 days), the band was about 2 mm wide, cells had grown out into the patched areas, and the cells were still alive (Fig. 13-16).

Discussion. The results agree with those of Fischer[8] in showing that an undiluted dialyzed plasma-dialyzed embryo juice clot with undiluted dialyzed embryo juice overlay does not support growth of a homogeneous strain of frontal bone fibroblasts and brings about rapid breakdown of implanted cells. Yet dilution of the overlay with Tyrode's solution lacking dextrose, which is a nonnutritive and purely protective solution, restores the capacity to support at least a residual degree of growth, and the degree of restoration is, within the limits set by the clotting capacity of the medium, proportionate to the degree of dilution. It is evident that the failure of the undiluted dialyzed material to support growth was not due solely to a deficiency, since any deficiency would only have been accentuated by dilution. It must, therefore, have been due to some positive defect. Such positive defects may be of two sorts. There may be present a nondialyzable growth inhibitor, either a high molecular weight substance or a prosthetic

group attached to such a substance, whose effects are minimized by dilution. One point in Fischer's data might lend support to the idea of such a toxic factor. Fischer reports that either egg albumin or insulin, when added to dialyzed plasma, will protect cells placed thereon from histolytic disintegration. He attributes this effect to the nutritive properties of the labile S-S and S-H bonds in these materials, but both egg albumin and insulin are known to be highly active adsorbents capable of detoxifying various media. This appears to be the effect of serum albumin when added to media for the cultivation of bacteria[10] and may have been a factor in Spratt's experiments[11] with explanted early chick blastoderms (not tissue cultures). It seems quite possible that the beneficial effect of egg albumin or insulin when added to dialyzed plasma may be of similar origin. On the other hand, the defect might be due to an imbalance of nutritive materials. The plasma and embryo juice were dialyzed against a Ringer's solution containing 0.1% dextrose but lacking phosphate. The diluent used in preparing media (Tyrode's solution) contained no dextrose but did contain phosphate. The diluent was thus *not* in equilibrium with the dialyzed materials, and would both extract dextrose therefrom and add phosphate thereto. If 0.1% dextrose was too high a concentration for fibroblast growth, the defect would be corrected by dilution. Since phosphate is required for growth, the defect might be a simple deficiency as postulated by Fischer, but not necessarily a deficiency of organic materials. However, Fischer's evidence, that a suitable amino-acid mixture prepared with physiological salt solution rather than Tyrode's solution does, in fact, partially correct the defect of dialyzed plasma-embryo juice, shows that phosphate was not the critical factor. It shows also that the low molecular weight materials removed by dialysis, such as the amino acids, *are* of great and at this level critical importance.

All of this evidence, however, still leaves unresolved the question of the nature of the postulated positive toxic defect in the dialyzed materials. In any case, our observations, though limited, suggest that it is unsafe to assume that dialyzed plasma or embryo juice is truly inert. Until further experiments have been carried out to clarify the effects of dilution on dialyzed extract, we feel that these materials should not be used in the preparation of substrata on which to test the nutritive value of supernatants. We believe that bare glass or cellophane are still the only really safe bases for such studies.

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17232. Effects of 4-Amino-Pteroylglutamic Acid in Dogs with Special Reference to Megaloblastosis.*

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Experimental production of macrocytic anemia and megaloblastosis of bone marrow has been achieved by administration of diets deficient in folic acid (PGA). Miller and Rhoads[1] observed in dogs fed the Goldberger

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[1] Miller, D. K., and Rhoads, C. P., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 540.