onstrates a striking constancy of change. Despite variance in methods for determining cardiac output and oxygen consumption, and varying basal or nonbasal conditions of the subjects, the ratios of change are the same. For each 10% increase in oxygen consumption an average increase of 7.07% in cardiac output results. 4. Relative changes in cardiac output are the same regardless of whether one uses the ballistocardiograph, right heart catheterization, or ethyl iodide method in the determination of cardiac output.

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The Culture of Tissue Cells in Clots Formed from Purified Bovine Fibrinogen and Thrombin.*

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The plasma clot has long been accepted as the most satisfactory support for the growth of tissue cells *in vitro*. Presumably the strands of fibrin form a meshwork along which the cells migrate and by which they maintain a distribution and spacing which permits the free intercellular diffusion of nutrients and metabolites. Regardless of the precise relationship between cells and clot, their growth in the plasma coagulum is better than on any other supporting surface.[1]

As the source of coagulum, chicken plasma has been most favored. The clot formed is firm and transparent and is more resistant to the lytic effect of tissue explants, particularly those of heterologous origin, than are clots of other whole plasmas.[2-4] There are, however, several undesirable features connected with its use. There are obvious problems inherent in the collection and storage of chicken plasma. There are considerable variations in clotting properties and in the influence of the plasma on culture growth which apparently are related to the age and health of the donor.[5] Whole plasma is, moreover, of complex and, to a great extent, unknown composition. Finally, it can be the medium of transmission of infectious agents, especially viruses.[6-9] What is required, then, is the source of a clot or its equivalent more conveniently obtained than chicken plasma, free of complicating infectious agents, and acceptable to the cells as a supporting structure.

The methods for the fractionation of plasma proteins developed in the Department of Physical Chemistry of the Harvard Medical School have made available many of the proteins of both human and bovine blood plasma in states of greater purity and in larger amounts than could have been obtained

* The initial results of these studies were described at the Hershey Conference on Tissue Culture, sponsored by the Committee on Growth of the National Research Council and held at Hershey, Pa., November 10-13, 1946.

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1 Fischer, A., *Biology of Tissue Cells*, Copenhagen, Gyldendalske Boghandel Nordisk Forlag, 1946.

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readily by previous methods.[10-13] To a great extent, clots formed from 2 of these, fibrinogen and thrombin, meet the requirements of the tissue culture technic. Fibrinogen is not so complex as whole plasma and its degradation products are known approximately.[14] It is obtainable in large quantities in a stable form and so can be considered as constant for any long series of experiments. It is unlikely to carry any viruses. By the use of these purified clotting substances and by controlling simple conditions under which clotting takes place, the investigator may form clots of varied mechanical and optical properties suited to the problem at hand. These, and other considerations related to the technical use of these purified proteins are the substance of this report.

Materials and Methods. a. *Preparation of Solutions.* The fibrinogen used in these studies was that contained in Fraction I of bovine blood plasma,‡ prepared according to the methods of Cohn and his associates.[13] A 0.5% solution of the fibrinogen in Tyrode's solution§ has been found a convenient concentration to use. Sterilization, if necessary, is easily accomplished by pressure filtration through a Seitz asbestos filter. The filtrate can be stored at 0°C for several weeks without apparent loss of clotting properties.

The thrombin preparation used to bring about polymerization of the fibrinogen was that contained in Fractions II and III of bovine plasma.‖ The sterile dry powder was dissolved in Tyrode's solution to give a final concentration of 10 to 20 units/cc. This concentration is convenient because one drop will contain 0.5 to 1 unit of thrombin which is sufficient to clot 1 cc of a fibrinogen-nutrient mixture in a culture flask and to allow enough time for orientation of the tissue explants. It has been found that at 0°C thrombin in solution loses its activity quite rapidly and is not of value after 5 or 6 weeks. However, at the lower temperature of "dry ice" storage (−50°C to −70°C) the property of eliciting clot formation appears to be retained unaltered over several months. This seems to be a convenient as well as an economical method for making thrombin in solution readily available.

b. *Preparation of Clots.* The following procedure is presented as one that has been found satisfactory for the preparation of cultures with clots from purified fibrinogen. It has been used only in flask cultures and only with flasks adapted for the roller-tube rack.[17] Regular Carrel flasks could be used in the

[10] Cohn, E. J., McMeekin, T. L., Oncley, J. L., Newell, J. M., and Hughes, W. L., *J. Am. Chem. Soc.*, 1940, **62**, 3386.

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[14] Brand, E., Kassell, B., and Saidel, L. J., *J. Clin. Invest.*, 1944, **23**, 437.

‡ We are indebted to Armour and Company of Chicago, Ill., Chemical Research and Development Department for a generous supply of Fraction I (Lot C-185) of bovine plasma. Of the total protein of this lot, approximately 70% has been shown to be fibrinogen. Preparations of greater purity can be obtained, but for preliminary experiments they did not seem essential. This preparation is distributed in the form of a dry powder. In addition to the fibrinogen there are also present small quantities of the other plasma proteins and a portion of sodium citrate (about 40% of the dry weight).

§ Earle's modification of Tyrode's solution.[15]

[15] Earle, W. R., *J. Nat. Cancer Inst.*, 1943, **4**, 165.

‖ A Parke-Davis preparation of thrombin designed for topical application was used. This can be obtained commercially as a sterile preparation, distributed as a dry powder in small glass ampoules containing approximately 5000 units. The unit is defined as the amount required to clot 1 cc of a standard fibrinogen solution in 15 seconds,[16] or more precisely, the amount which clots a 1% solution of fibrinogen in a test tube 1 cm in diameter at 25°C, pH 6.3, in approximately 45 seconds.[18]

[16] Seegers, W. H., Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Biol. Chem.*, 1938, **123**, 751.

[17] Porter, K. R., Claude, A., and Fullam, E. F., *J. Exp. Med.*, 1945, **81**, 233.

same way, but for roller tube and slide cultures other procedures would, of course, be necessary.

The tissue explants are first placed in the neck of the tube, close to the flask portion. All the excess Tyrode's solution is withdrawn from around them. The fibrinogen solution (0.5% or 0.25%) and nutrient¶ are pipetted to give a total volume of one cubic centimeter. One drop of thrombin solution (containing 0.5 to 1 unit) is next added and all the ingredients are mixed by gentle shaking. The flask is quickly placed in a horizontal position and the explants are oriented. At room temperature clotting will take place in a minute or less. The resulting coagulum is rigid and fairly transparent. In a few hours at 38°C it will undergo syneresis and exude about one-half of its volume.

Recent detailed studies on the clotting mechanism have been reported by Ferry and Morrison.[18] They showed that such characteristics of experimental clots as degree of opacity, rigidity and syneresis could be varied within wide limits by the control of such conditions as hydrogen ion concentration, temperature, and concentrations of the reacting proteins. These investigators used fibrinogen and thrombin preparations of human origin. We have repeated certain of their experiments of special interest to those concerned with tissue cultures, and have used the above described bovine proteins. The results were essentially similar to those obtained with human fibrinogen and thrombin and form the basis of the following recommendations.

If one desires a transparent clot like that obtained from chicken plasma, it is important to have the solutions at room temperature (20°C). Ice-cooled nutrient and fibrinogen solutions give a rather opaque clot with inferior optical properties. If the pH of the solution is below 7.5 during clot formation, the opacity is appreciable and can be shown to increase as the hydrogen ion concentration increases. A clot of satisfactory transparency is readily obtained by having the fibrinogen-nutrient mixture at pH 7.6 to 7.8. After clotting has been completed, the pH of the nutrient may be changed without loss of transparency of the formed clot.

Thrombin concentration, varied over a wide range, does not greatly influence the transparency of the clot. In these studies it has been reduced to as low a concentration as 0.1 unit per cc of fibrinogen-nutrient mixture and yet a clot was obtained.** This was slightly opaque, but not enough to make it unsuitable, and it supported good cell growth. Clotting was, of course, greatly delayed, 10 minutes being required, but for some types of culturing a large portion of this time might be needed. With such a wide range of concentrations effective in providing transparent clots, the concentration recommended is, therefore, that which gives the operator sufficient time to arrange the tissue explants.

c. *Cell Growth in Clots from Purified Fibrinogen.* The ability of the clot from purified fibrinogen to support the growth and migration of cells is, of course, the real measure of its usefulness. In these studies this has been examined with rat fibroblasts, rat myoblasts, Jensen rat sarcoma cells†† and chick fibroblasts. In general, the rate of growth observed, whenever the clot has not undergone lysis, has been similar to that obtained in chick plasma (Fig. 1). Minor differences in cell structure and colony archi-

¶ This is composed of Tyrode's solution (5 parts), placental cord serum (3 parts), and embryo extract (2 parts). Using 2 parts of nutrient to one of fibrinogen solution further dilutes the latter so that the final concentrations of fibrinogen are approximately 0.08 to 0.16%.

[18] Ferry, J. D., and Morrison, P. R., *J. Am. Chem. Soc.*, 1947, **69**, 388.

** Calculations based on the dry weight of the original material show that by way of the solution about 0.025 mg is ordinarily added to a culture and that 0.005 mg is more than enough. When it is considered that only a small fraction of this is thrombin some impression is gained of the extreme dilution at which thrombin is active.

†† This tumor was kindly provided by Dr. R. L. Ladd, Northwestern University.

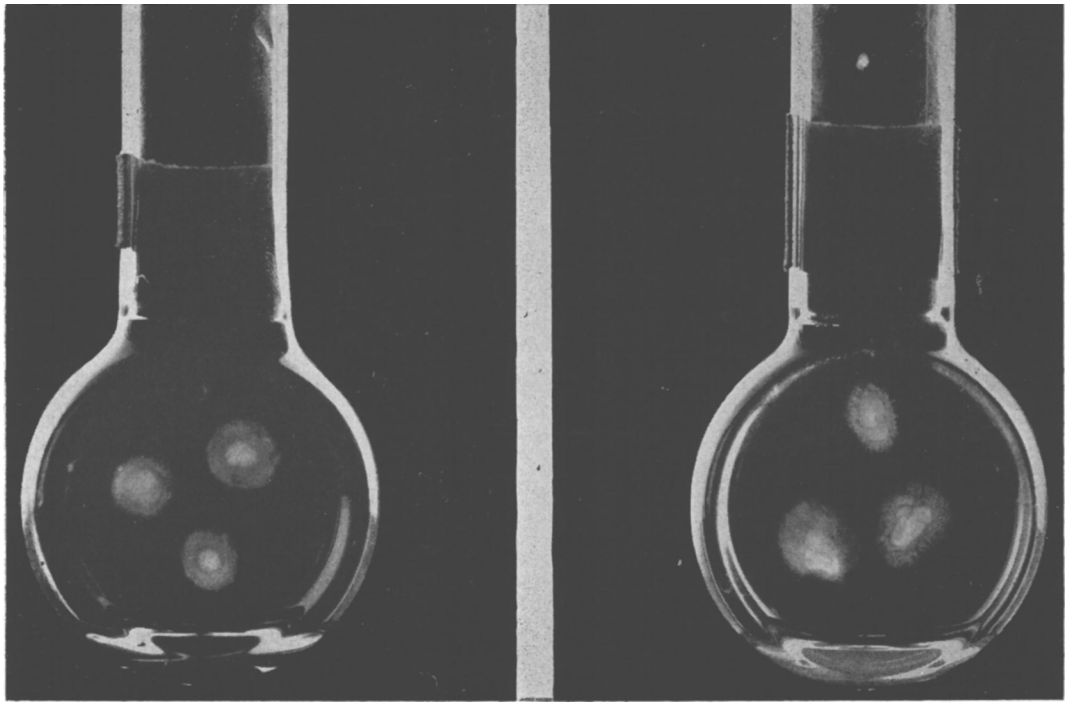

FIG. 1a. FIG. 1b.

FIG. 1a. Colonies of cells from skeletal muscle of chick embryo growing in clot from chick plasma.

FIG. 1b. Similar colonies of the same age growing in clot from bovine fibrinogen.

tecture have been noted, but these are not important to this report. It seems fairly certain that as the material is better understood and its possibilities and behaviors have been further examined, it will be accepted as a useful substitute for the clot from whole plasma.

Of the several conditions which doubtless favor or discourage the growth and migration of cells in these pure fibrin clots, the concentration of fibrinogen is of first importance. In our experience, if the final concentration is much greater than 0.16%, the clot is so dense that the cells have difficulty in making their way through it. Under such conditions the greatest migration is on the surface of the clot. Some cells push through but their pseudopodia show very fine arborizations very much as though they are trying to extend through extremely small spaces. Thus, the concentrations of fibrinogen supporting the most typical cell colonies have been 0.16% and 0.08%. Lower concentrations have not been tested.

Experiment 1. Cultures of rat fibroblasts from explants of one-day-old rat skeletal muscle were set up in clots containing 2 parts of nutrient (5:3:2) and 1 part of fibrinogen solutions. The latter was used in 3 different concentrations, 1.0%, 0.5% and 0.25% in Tyrode's, to give final concentrations in the nutrient-fibrinogen mixtures of 0.33%, 0.16% and 0.08% of clotting fibrin. Four flask cultures, each with 3 explants, were prepared with each concentration of fibrinogen. To bring about clotting, one drop of Tyrode's solution of thrombin containing 20 units per cc was added to each culture. Control cultures were simultaneously prepared with 2 parts of nutrient and 1 part whole chick plasma. The cultures were each fed twice weekly for 3 weeks with 1 cc of the 5:3:2 nutrient mixture and during this time comparisons were made of cell growth and migration and colony structure.

In the 0.33% fibrin clot, migration of the

TABLE I.
Measurement of Growth by Increase in Width of Colony Margin.

Age of culture	Avg for controls mm	Avg for cultures in 0.16% fibrin, mm	Avg for cultures in 0.08% fibrin, mm
4	1.5	1.3	1.2
6	2.0	1.8	2.0
8	3.1	3.1	3.2
10	3.5	4.0	4.2
12	4.0	5.0	5.6
14	4.0	5.2	6.2

cells was always restrained relative to behavior in control cultures. It appeared that the cells were not able to find a way through the clot. Their pseudopodia developed fine arborizations, as though probing the small interstices. After one week, long cords of cells having the appearance of hyphae of mold mycelia could be seen, but the best migration was only on the upper and lower surfaces of the coagulum.

Growth and migration of cells in the 0.16% fibrin clot approached more nearly their extent in the control cultures. Hyphae-like filaments of cells appeared but were more numerous and more branched. After 3 weeks the growth in these cultures was almost as extensive as in the controls.

The cultures having clots with a concentration of 0.08% fibrin developed at essentially the same rate as the controls and the cells showed no striking abnormalities. After 2 weeks, the clots of these cultures began to undergo lysis but the process never became extensive or interfered with the continued growth of the cultures.

Another experiment, similar to the above, was carried out with cells of the Jensen rat sarcoma. In this the fibrin content of the clot was varied from 0.33% to 1.0%. Migration of cells was possible only through the clot made from the lower concentration (0.33%) and even in this the cells extended from the explant as columns of more or less cuboidal units.

It has been noted that, as might be expected, growth starts up more promptly after making the culture if the nutrient is incorporated in the clot. In some cultures, for example, explants were set up in clots developed solely from fibrinogen in Tyrode's solution. The nutrient mixture was then added as a liquid phase above the clot. The initial growth here was poor‡‡ but after a week or 10 days it had overtaken the other cultures started in the usual manner.

If the concentration of fibrin is suitable and other nutritional conditions are the same, the migration and growth of cells in clots of fibrinogen origin seem to be essentially equal to that in chick plasma (Fig. 1). An experiment made with fibroblasts from skeletal muscle of an 11-day-old chick embryo illustrates this.

Experiment 2. Cultures of chick embryo skeletal muscle were set up as follows:

4 flasks—3 explants per flask with 1 part chick plasma, 2 parts nutrient (5:3:2).
4 flasks—3 explants per flask, 1 part fibrinogen 0.5% and 2 parts nutrient (5:3:2).
4 flasks—3 explants per flask, 1 part fibrinogen 0.25% and 2 parts nutrient (5:3:2).

Twenty-four hours after preparation each culture was given 1 cc of nutrient and this was changed every 3 or 4 days thereafter during the course of the experiment. The cultures were examined every 2 days and measurements were made of the width of the margin of cell migration and growth (Table I).

This type of measurement, while only an approximation, was considered adequate for the purpose of this experiment. Its significance was further limited by the observation that the population of cells in the growth margin of the control colonies seemed more dense than in the colonies in the fibrinogen clots. There was a limited lysis of plasma

‡‡ This could be related to absence of nutrients or to the presence of considerable sodium citrate.

clot in one case, but no lysis of any of the fibrinogen clots.

Besides demonstrating the suitable nature of fibrinogen clots for culturing cells of the kind studied, these data probably have little significance. The greater width of the growth margin in the fibrinogen clots may be related to a less dense population of cells and the thinner nature of the fibrinogen clot which seems to flatten more than the plasma clot during syneresis. Possibly also of influence in this respect is the absence of, or the delayed appearance of any change in the character of the purified fibrinogen clot. As is well known, it is not uncommon for the clot from whole plasma to become very opaque around the margin after 2 or 3 weeks in a flask culture. It has the appearance of denatured fibrin. This same transformation, which may restrict cell migration and colony expansion has not, in our experience, appeared at all in the clots from bovine fibrinogen.

In the transferring of tissue cultures the properties of the fibrinogen coagulum are of some importance and so have been examined. Up to the time of this report, flask cultures of rat fibroblasts and also of chick fibroblasts in fibrinogen clots have been transferred for 3 generations with as much ease and success as their equivalents in chick plasma coagula. They are not more friable on transfer; if anything, less so.

From these limited observations it appears that the purified fibrinogen clot does not resist lysis as completely as the chick plasma clot. In this respect, however, it seems similar to coagula derived from many mammalian sources. Lysis was observed to be most pronounced in the presence of rat myoblasts (or explants of young rat hearts) and less so with rat sarcoma (Jensen) cells. Growth of rat fibroblasts from explants of skeletal muscle was supported for 2 weeks without any liquefaction and it appeared then only in clots derived from the more dilute preparations of fibrinogen. Finally, the combination of explants of the skeletal muscle of chick embryos and bovine fibrinogen gave excellent cultures which showed no lysis at all. These observations, together with recorded notes on clot lysis in tissue cultures,[4] suggest that the process might be controlled to some extent by proper selection of the source of the purified fibrinogen. For example, fibrinogen prepared from chick plasma might generally prove more resistant to the lytic activity of mammalian cells than does fibrinogen from bovine sources. There may, in addition, be other methods of control, either through the selection of appropriate sera for the nutrient or by suitably treating the fibrin during or after its polymerization. Studies on this problem are being continued.

It may be shown subsequently that the size of the fibrin strands in a clot, as well as the size of the interstices, are influential in determining cell migration and even multiplication. Possibly the finer strands and smaller spaces inhibit these cell functions. If this is found to be true, it may be necessary to accept a clot of partial opacity in order to obtain larger fiber size; for it has been shown that the degree of opacity is determined by fiber size.[18,§§]

Summary. Procedures are described for the successful culture of tissue cells in clots formed from purified bovine fibrinogen and thrombin. The influence on clot characteristics of pH and temperature, effective during clot formation, and the influence of fibrinogen concentration on the subsequent cell migration in the clot are indicated. Cultures grown in these clots can be transferred with the same ease as those grown in clots derived from chick plasma. The problem of fibrinolysis is briefly mentioned.

§§ An electron microscope study of the structure of the fibrin clot is being made by the authors. There is a marked increase in fiber size in opaque clots formed at pH 6.5 as compared with fiber size in transparent clots formed at pH 8.5. Preliminary measurements show the fibers in the clot formed at the more acid pH to range roughly from 50 to 350 μ, at the more alkaline pH they vary from 15 to 90 μ. Also of interest is the presence of striations across the fibrin strands which show a surprisingly constant periodicity of 240 Å at pH 6.5.