IN VITRO STUDY OF BONE MARROW

I. A Method for Marrow Culture

By Claus Munk Plum, Ph.D.

FOR several years numerous investigators have devoted themselves to the culture of cells and tissue in vitro, but to date only a comparatively small number of works dealing with the culture of the bone marrow in vitro have been published. Studies of the bone marrow promise to provide a fruitful field for further investigation of fundamental hematologic problems, such as the origin of platelets and the manner of extrusion of erythrocyte nuclei.

Hematologic and cytologic theories have in general been supported by examinations of smears of blood and bone marrow supplemented by examination of embryonal tissue, smears after supravital and intravital staining, and considerations of a theoretical nature. Again and again instances have occurred in hematology in which a cell type which one investigator has apparently clearly described has been categorically declared by another investigator to be an "artefact" produced by improper treatment of the material. Now, however, it is possible to culture bone marrow in vitro, and to study changes in the relative numbers and types of the various cells as the cells of the marrow develop. At the same time, more recent experimental methods have enabled us to follow the influence exercised by changes in the surrounding media on the development of the marrow cells. These investigations, therefore, withdraw more and more from purely theoretical considerations toward an exact physical recognition of the actual physiological and morphological status of the blood corpuscles.

Two methods were employed in the original investigations of marrow culture: In the first of these, fragments of bone marrow or clots were cultured; in the second (a more recent development) suspensions of bone marrow were cultured. In 1910 Carrel and Burrows[1] using the clot culture technic studied the bone marrow of the cat and found that in vitro the marrow rapidly lost its hematopoietic cells and became overgrown with fibroblasts. Carrel and Burrows's technic was subsesequently employed by many investigators, most of whom used bone marrow from birds. More recently, investigators began to examine mammalian marrow, but human marrow has been studied only since 1937.

The first authors who investigated the cells and blood corpuscles of the bone marrow in vitro disagreed considerably as to the developmental stages which the cells underwent and as to the types of cells which developed from different anlagen. They were in agreement on only one conclusion, namely, that the fully developed polymorphonuclear granulocyte was unable in vitro to develop into any other form of cell, but, after a short life of 2–3 days (under optimal conditions), degenerated and died.

From the Biological Laboratories of Medicinalco Ltd., Copenhagen S., Denmark. The constructions of the apparatus described in this report were made possible by a grant from the Carlsberg Foundation and the work with them was supported by a grant from the King Christian X Foundation.

Two factors were responsible for the variations of results in these earlier investigations. First, explanting of the bone marrow into a clot resulted in a rapid overgrowth of the fibroblasts which prevented the full activity of the cells. Second, if it was desired to undertake a microscopic examination of the culture, it was necessary either to stain the whole of the clot at the same time and then subject it to direct microscopy, or else to fix the clot and make histologic slides in the usual way. This latter method was not suitable for demonstration of the very delicate elements of which the bone marrow is composed. Neither of these methods was particularly suitable since in either case it seems that, after staining, the primitive blood cells came to look exactly alike and all of them resembled the lymphocyte. This fact made the determination of the relationship of the individual cells an extremely difficult matter and made many authors (e.g., Maximow) regard the lymphocyte as the original form of all blood cells.

In 1936, at the time when the culture of human bone marrow was begun, Osgood[2] published a new method which rendered the examination of fluid bone marrow possible. Osgood's apparatus attempted to place the bone marrow suspension under conditions which were as nearly physiological as possible. The apparatus supplied the functions of a lung, kidney, and circulation for the marrow and, at the same time, allowed withdrawal of samples of bone marrow under sterile conditions during the course of an experiment. With the publication of his method Osgood submitted a series of proposals for studies on the culture and development of the cells under the nearly physiologic conditions afforded by his culture apparatus.

Despite these projects, however, Osgood and his collaborators after only six months gave up this method in favor of a simpler one.[3] In this the attempt to procure as nearly physiological conditions as possible was abandoned, and neither the composition of the air nor the composition of the culture medium was kept constant during the experiment.

Although Osgood's second method[3] was technically easier, I considered the physiological conditions so important that I decided to base my construction as far as possible on the principles of Osgood's first method.[2] It was found necessary to make two constructions, one for the prolonged experimentation on fairly large samples, and another embodying as far as possible the same principles, but allowing continuous microscopic observation of the individual cells under culture.

I. THE CULTIVATION OF LARGE SAMPLES

After a number of preliminary experiments with an apparatus very similar to Osgood's original construction, I adopted a somewhat modified arrangement which, while still fulfilling the primary requirements, was in many ways easier to work with. For practical reasons four identical apparatus were constructed and all of them mounted in the same thermostatic water bath, a point of considerable importance for quantitative comparative studies.

As already mentioned, normal physiological conditions were the main considerations, and it is possible to regard the apparatus as made up of sections representing respectively the supply of nourishment, the respiration, the circulation, and the excretion of waste products. These sections are to a certain extent separated

by membranes permeable to gases and crystalloids, which correspond more or less to the vascular endothelium and the kidney glomerular membrane. In Osgood's original apparatus "parlodion" was used for these membranes, but owing to the war this substance was unobtainable. The first membranes were prepared from collodion, which was difficult to obtain in uniform quality. Later, the ultrafiltration membranes described by Rehberg[4] were obtained and prepared by the Copenhagen firm Kapcello.* These were made to specifications in two sizes: viz., diameter 18 mm., length 110 mm.; and diameter 9 mm., length 50 mm. Both are practically impermeable to colloids. The following is a description of the apparatus, illustrations of which are reproduced in figures 1 and 2.†

General Remarks

The marrow solution is suspended within a membrane (*2*: 8)‡ which is fastened to a glass funnel (*2*: 9) that rests on three projections inward from an outer vessel (*1* and *2*: 10). The entire apparatus lies within a thermostatically controlled tank (*1*: 22). Inlet tubes (*1*: 1, 2, 3, 4, 5) and outlet tubes (*1* and *2*: 6, 7) allow entrance and exit of nutritive fluid respectively. Air free of carbon dioxide enters the marrow suspension via appropriate tubes (*1*: 12, 13, 14, 15, 11) and leaves via other tubes (*1*: 16, 18, 17) containing appropriate chemicals for carbon dioxide and water determinations. Waste products are removed and measured by an appropriate system (*1*: 10, 26, 27, 28, 29, 30, 31, 32, 33) and fresh solution added (*1*: 21, 23, 24, 25, 10). Marrow is introduced by way of tubes (*1*: 42, 15, 11) into the containing membrane (*2*: 8), and specimens can be withdrawn at intervals through the reservoir (*1*: 15) by way of the outlet (*1*: 40).

The Provision of Nutriment

In large scale tissue culture it is important to provide a continuous supply of food for culture. This is done in this apparatus by a constant flow of the appropriate solution through the internal membrane (*2*: 5). The nutritive solution enters through the T-tube (*1*: 2), the drip vessel (*1*: 3) (internal diameter 18 mm.), and the capillary tube (*1* and *2*: 4) (internal diameter 1.2 mm.) which opens near the bottom of the 9 mm. membrane (*2*: 5). From here the fluid rises through the membrane and the tube (*2*: 6) (internal diameter 7 mm.) to which the membrane is attached, and leaves through the side tube (*1*: 7) where it can be collected in a bottle (not shown). The rate of the flow of the fluid in my investigations was 80 to 90 ml. per hour. The nutritive substances diffuse through the membrane (*2*: 5), thereby supplying the culture in the membrane (*2*: 8). The concentration here will remain approximately constant when the supply is large compared with the amounts used up. The membrane (*2*: 8) containing the cell suspension under culture

*My thanks are due Mr. Hawlik, Civil Engineer of this firm, for his kind assistance in providing the necessary membranes.

†This macro apparatus was made by H. Struer Chemische Laboratories, Copenhagen, who are ready to supply it under my supervision. My thanks are due Mr. Rasmussen, Cand. Pharm., of this firm for his helpfulness during the construction.

‡Italic numbers refer to figures 1 and 2.

(bone marrow cells suspended in Ringer's solution) is fastened to the glass funnel (*2*: 9) resting on three projections inward from the outer vessel (*1* and *2*: 10).

Aeration and Determination of the Respiratory Metabolism

The cell suspension (*2*: 8) is kept in continuous movement by a current of air or other gas mixture through the tube (*1* and *2*: 11), thus avoiding sedimentation. The rate of the air flow was 100 ml. per hour.

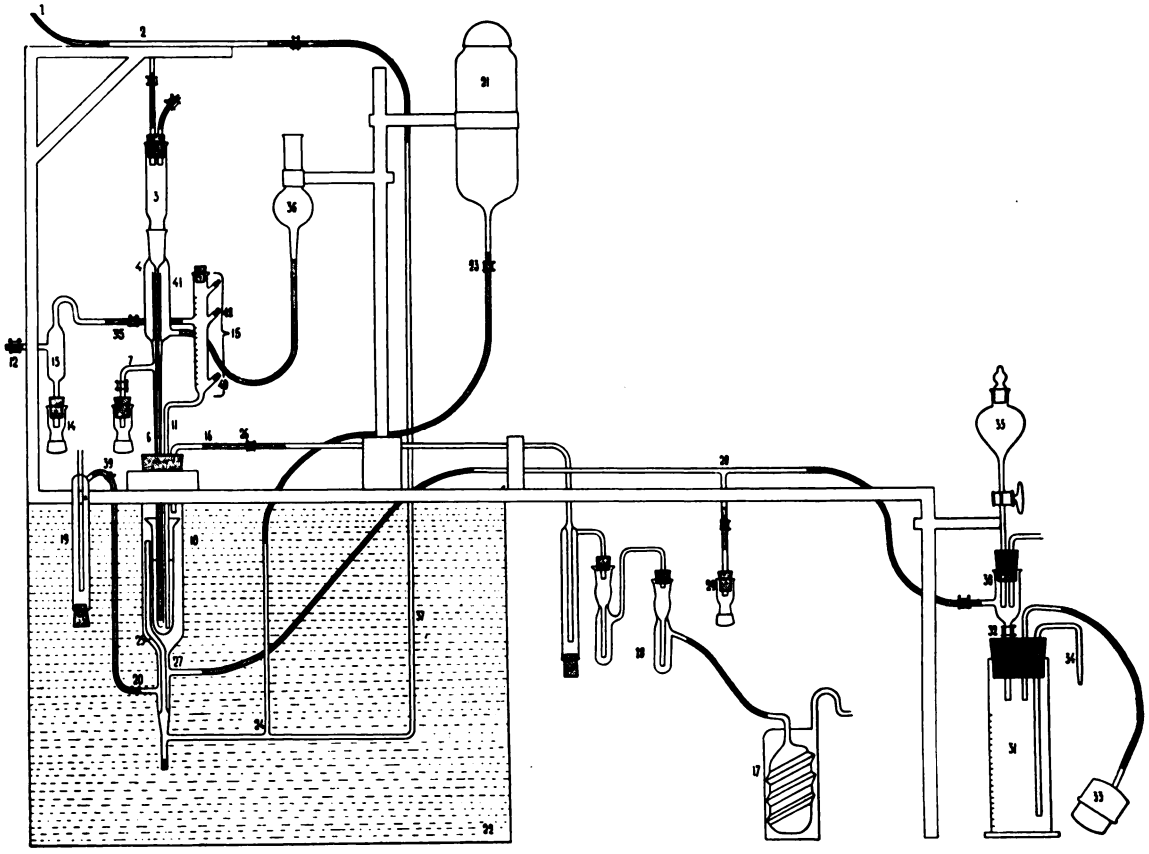

FIG. 1. DIAGRAM OF THE APPARATUS FOR CULTIVATION OF LARGE SAMPLES OF BONE MARROW IN SUSPENSION

The air, which is previously made CO_2-free, is let into the apparatus through the tube (*1*: 12) and passes through the vessel (*1*: 13) for the condensation of water vapor. Attached to this vessel is an arrangement for taking gas samples for analysis through the rubber by means of a cannula and a common air recipient (*1*: 14). Passing on to the vessel (*1*: 15) inserted for sampling of the cell solution (see later), the air is filtered through cotton wool and passed through the tube (*1* and *2*: 11) to the culture which lies within the membrane (*2*: 8), where some oxygen will be absorbed and CO_2 added. The (expired) air passes through the tube (*1*: 16), first to

the glass vessels (*1*: 18) for the condensation of water vapor, and then to the CO_2 absorber (*1*: 17). In ordinary experiments the absorber contains lime water [Ca $(OH)_2$] stained with phenolphthalein to allow estimation of the CO_2 produc-

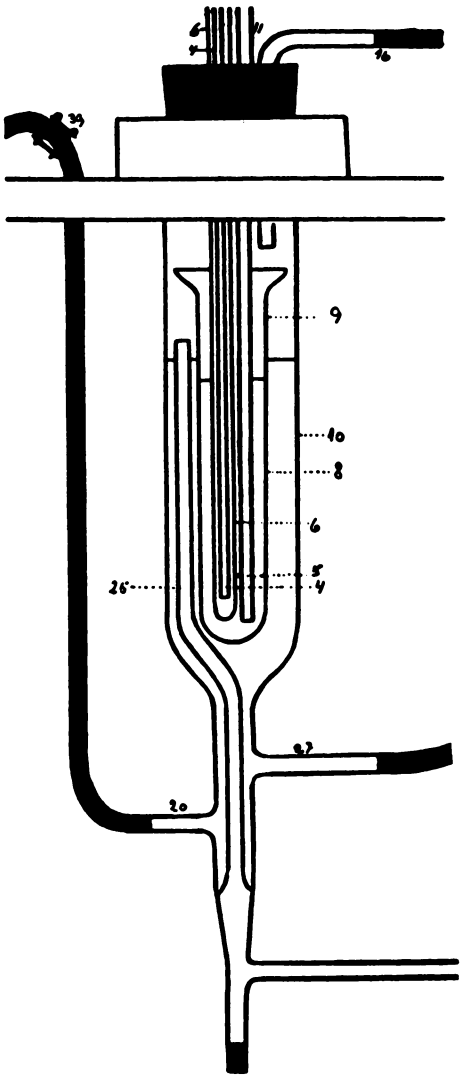

FIG. 2. A PORTION OF FIG. 1 SHOWING THE MEMBRANCES PLACED IN THE APPARATUS

tion, but in special respiratory experiments the absorber is replaced by an almost vertical tube (75° angle) of length 750 mm. and diameter 12 mm., which contains barium hydroxide [$Ba(OH)_2$], and the air is admitted from below through a capillary tube. This arrangement was found very effective for absorbing and titrating small quantities of CO_2. The Ba $(OH)_2$ was emptied into a small retort by removing

the rubber stopper, and titrated by the method of Winkler.[5] When determinations of CO_2 production were made, one of the four apparatus was used as a control and the membrane (*2*: 8) was filled with Ringer's solution not containing cells. Some O_2 will also diffuse through the "glomerular" membrane (*2*: 8) into the outer vessel (*1* and *2*: 10) and, to secure this, "inspired air" is added through (*1*: 19, 20) and allowed to bubble through the solution for removal of waste products and leave with the air from the cell suspension.

Removal of Waste Products

For technical reasons it was not found practical to have a continuous flow of solution along the outside of the membrane (*2*: 8). A large volume of solution (15 times the cell suspension) was therefore placed between the membrane (*2*: 8) and the outer vessel (*1* and *2*: 10) and changed at 30 minute intervals. The change is performed as follows: The solution in the container (*1* and *2*: 10) is removed by clamping the expired air tube (*1*: 26). This raises the pressure and drives out the fluid through (*1* and *2*: 27) (which is provided with a device for withdrawing a sample [*1*: 29]) to the funnel (*1*: 30) and the cylinder (*1*: 31) where it is collected. The funnel (*1*: 30) has a reservoir with alcoholic neutral red for determination of the pH, when desired. The cylinder can be emptied by means of the pump (*1*: 33) when the appropriate clamp (*1*: 32) has been closed.

The reservoir (*1*: 21) contains fresh solution (usually suitably buffered Ringer's solution) at body temperature. When the clamp (*1*: 23) is opened, the solution flows through the tubes (*1*: 24, and *1* and *2*: 25) and fills up the chamber (*1* and *2*: 10) which has just been emptied.

Sterilization

It is of course necessary to work under sterile conditions. Since the complicated apparatus would be difficult to sterilize completely, antisepsis was accompanied by the addition of "solbrol" (methyli-para-oxybenzoate) in a concentration of 0.1 per cent to all solutions. This proved harmless to the cells studied. In the experiments carried out infections have occurred very rarely. In case of infection the experiment was discarded.

Before use the membranes were kept in water to which solbrol had been added, and the Ringer's solution used for the cell suspension and waste elimination was sterilized by boiling and solbrol added.

Technic of an Experiment

An experiment is started as follows: The membranes are mounted in the apparatus and tested for tightness by means of Ringer's solution, especially at the places where they are connected to the various glass tubes (by means of broad rubber bands, 5 mm. long and 1 mm. thick). The whole apparatus is assembled and air passed through for 15 minutes, during which time the solution in the outer chamber (*1* and *2*: 10) is changed three or four times. Next, the flow of air is stopped by closing the clamps (*1*: 38, 39, 26), and the Ringer's solution in the membrane (*1* and *2*: 8) is aspirated into the tube (*1*: 15) by means of a cannula introduced through

the vacuum rubber tube (*1*: 42) and a syringe of capacity 30 ml. Then another cannula is introduced through the vacuum rubber (*1*:40) and the Ringer's solution is withdrawn. Bone marrow is aspirated from the experimental animal or patient through a sterile cannula into a sterile 20 ml. syringe by the technic of Plum.[6] After this 0.05 ml. of the cells is suspended in 25 ml. of Ringer's solution. The cannula in the rubber tube (*1*: 40) is removed and the sterile bone marrow suspension is injected into the tube (*1*: 15) through the cannula in the rubber (*1*: 42) and the cannula is removed. The volume of the bone marrow is then read by means of the graduation of the tube (*1*: 15) which is of diameter 8 mm. and graduated in 0.5 ml. Then the clamps (*1*: 26 and *1*: 38) are opened, and the air flow will press down the bone marrow suspension into the membrane (*2*: 8). When the suspension has been pressed down, the clip (*1*: 39) is opened.

When samples of the suspension are to be taken during the experiment they are aspirated into the tube (*1*: 15), the volume is measured, and the necessary quantity is drawn out by means of a 2 ml. syringe by way of the outlet (*1*: 40).

In cases in which a flow of nutritive solution is not desired, the membrane (*2*: 5) may become compressed. This may be avoided by introducing water from the reservoir (*1*: 36) into the chamber (*1*: 41) which opens into the tube (*1*: 6) and thus raises the pressure.

In some cases it may be desired also to arrange a *flow* of the nutritive solutions through the compartment (*1* and *2*: 10). To effect this, a connection is established with the tube (*1*: 37) and also with (*1* and *2*: 10) and the solution in the chamber (*1* and *2*: 10) is changed as described.

The apparatus as here described may at first sight appear more complicated than Osgood's first model.[2] However, it was found capable of maintaining the normal structure of the blood cells and therefore also, presumably, their normal function. Evidence for this is suggested by the fact that in our first experiments, in an apparatus copied directly from Osgood, destructive changes were often observed in the erythrocytes whereas even crenation was only rarely seen in the apparatus described here.

The apparatus as described will render possible study of a number of hematological problems including metabolism determinations on the bone marrow as a whole or on isolated groups of cells. Some of the possibilities thus opened up include determinations of the optimal temperature, pH, oxygen and CO_2 tensions for cell culture; determinations of the respiratory metabolism; studies of the effect of bactericidal substances on bacterial cultures; etc. We have conducted our investigations only for a 24 hour period, but Osgood reports that he has made investigations on cultures 72 hours old. The technic as here described is to be considered as preliminary. It can be suitably modified for any purpose and also improved so as to approach more closely the normal physiologic conditions within the organism.

II. THE CULTIVATION OF MICROSCOPIC SAMPLES

In the apparatus as described above, the changes taking place can be studied only on representative samples secured at intervals. It is often important to be able to follow the changes in a particular cell, and to enable one to do this, a culture apparatus was made which could be placed directly under an examining micro-

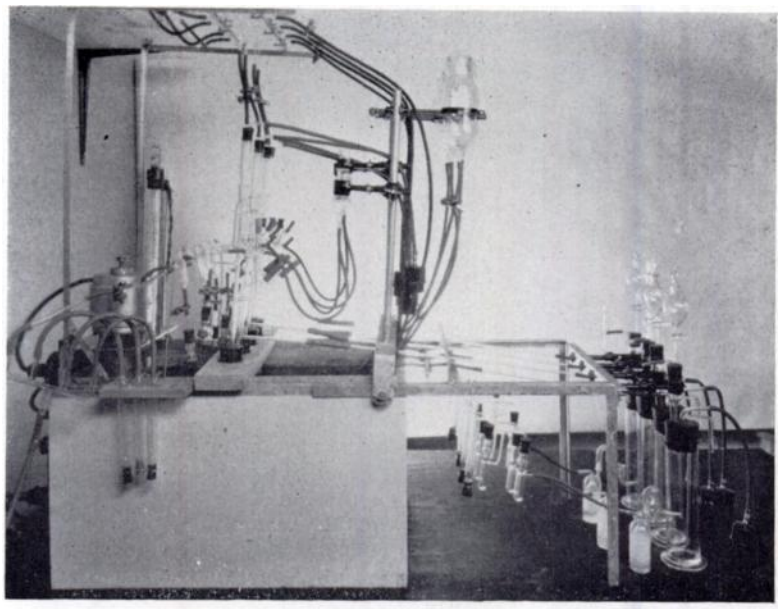

FIG. 3. THE APPARATUS FOR CULTIVATION OF LARGE SAMPLES OF BONE MARROW (IN QUADRUPLICATE)

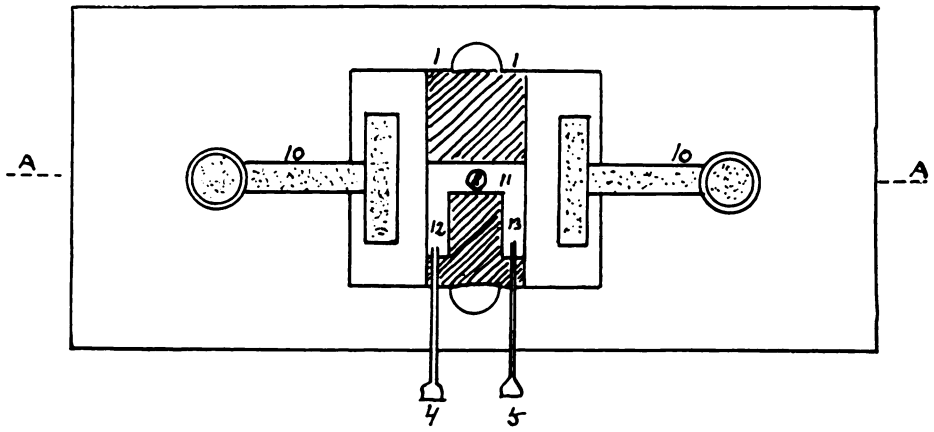

FIG. 4. DIAGRAM OF THE APPARATUS FOR CULTIVATION OF ISOLATED CELLS OF THE BONE MARROW. VIEWED FROM ABOVE

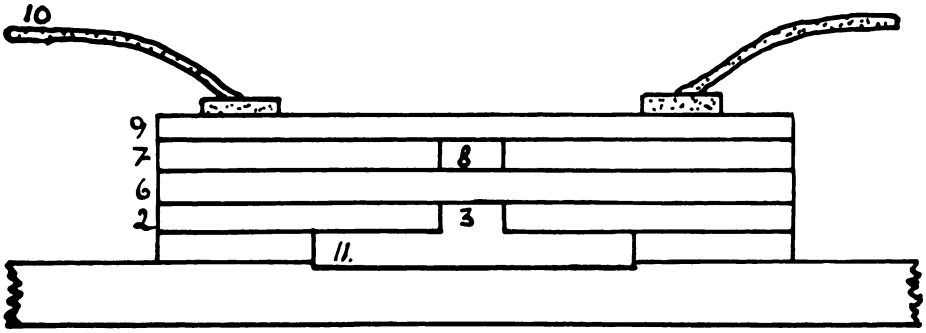

FIG. 5. AN ENLARGEMENT OF THE CENTRAL PART OF THE APPARATUS SHOWN IN FIG. 4. SIDE VIEW

scope.* As a basis for this a Bürker-Türck counting chamber was employed. As shown in figures 4 and 5, the two side trenches (*4*: 1) were filled up with asphalt and a small amount of the same substance was used to level the counting chamber. On the top of the chamber a cover slip (*5*: 2)—the usual one used for blood corpuscle counting provided with a 2 mm. central hole—was now sealed on after making a hole (*5*: 3) of 2 mm. diameter in its center. Then two cannulas were embedded (*4*: 4, 5) in the two last side trenches to be used for feeding nutritive solution and carrying off the solution respectively. In successive layers on top of the cover slip (*5*: 2) were placed a thin plane collodion membrane (*5*: 6), another thin (0.14–0.17 mm.) cover slip with a 2 mm. hole in the center to take the cell suspension (*5*: 7, 8), and finally an ordinary cover slip (*5*: 9) (thickness 0.14–0.17 mm.) held down by two clips (*4* and *5*: 10). The whole apparatus was placed on a heating table under the microscope. Nutritive solution suitably heated and saturated with oxygen was made to flow from a Dewar vessel through the apparatus at a rate of 80 ml. per hour.

This apparatus may be constructed at a slightly lower cost if, instead of a Bürker-Türck counting chamber, a 3 mm. thick object slide is used as base and then, by means of heated Canada balsam, glass pieces are mounted to form the lower drain made by the trenches of the counting chamber.

The observation of the cells is rather difficult since they must remain unstained and it is only possible to observe differences in size and shape. Usually, however, the nucleus appears a shade lighter than the rest of the cell content. The use of Zeiss "Phasenkontrast-Einrichtung"† somewhat facilitated the observations.

Observations on the production of individual red cells have been made by this method and will be described in a subsequent report.

SUMMARY

Two types of apparatus are described for the culture of bone marrow in vitro. The first, a macro method, is suited to the study of large amounts of bone marrow. The second, a micro method, allows observations of the development of individual cells.

Studies of erythropoiesis by these technics has allowed insight into production of erythrocytes. These data will be reported in a subsequent article.

REFERENCES

[1] Carrel, A., and Burrows, M. T.: Culture de moelle osseuse et de rate. C. R. Soc. Biol. *69:* 299–301, 1910.

[2] Osgood, E. E., and Muscovitz, A. N.: Culture of human bone marrow. Preliminary report. J. A. M. A. *106:* 1888–90, 1936.

[3] —— and Brownlee, I. S.: Culture of human bone marrow. A simple method for multiple cultures. J. A. M. A. *107:* 123, 1936.

[4] Rehberg, P. Brandt: Acta physiol. Scandinav. *5:* 305, 1943.

[5] Winkler, Kolthoff: Die Massanalyse II. Berlin, 1932.

[6] Plum, C. M.: The composition of the bone marrow in normal adults. I. The cell of the bone marrow. II. Histological examinations. Acta med. Scandinav. *107:* 11–52, 1941.

[7] ——: In vitro study of bone marrow. II. Studies of erythropoiesis. Blood (supp.) *1:* 42, 1947.

*This apparatus was made by the firm of Brock and Michelsen, Copenhagen.

†Kindly lent by Messrs. Brock and Michelsen.