this respect, it is similar to guinea pig semen but differs from human semen, which normally liquefies completely within 20 minutes of ejaculation. The coagulum may serve to retain spermatozoa within the vagina post coitum.

Summary. A method for electroejaculation of the macaque was successful in 8 of 11 subjects. Repeated electroejaculation at 2-3 day intervals did not cause a refractory state. Coagulation occurred almost immediately on ejaculation. Liquefaction began shortly thereafter, but was incomplete. Sperm concentration in the liquefied portion of the ejaculate ranged between 93 and 807 million/cc. Tail length was approximately 70 μ, as compared with 50 μ in human material. Electroejaculation is suggested as a practical means of obtaining monkey semen.

1. Dalziel, C. F., Phillips, C. L., *Am. J. Vet. Res.*, 1948, v9, 225.

2. Emmens, C. W., Black Shaw, A. W., *Physiol. Rev.*, 1956, v36, 277.

3. Freund, M., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 538.

4. Rowan, R. L., Howley, T. F., Nova, H. R., *J. Urol.*, 1962, v87, 726.

5. MacLeod, J., Gold, R. Z., *Fertil. and Steril.*, 1953, v4, 10.

6. van Wagenen, G., *Anat. Rec.*, 1936, 411.

Received February 18, 1963. P.S.E.B.M., 1963, v112.

Growth of L Cell Suspensions on a Warburg Apparatus.* (28243)

WILLIAM S. RUNYAN AND ROBERT P. GEYER (Introduced by F. J. Stare)
Department of Nutrition, Harvard School of Public Health, Boston, Mass.

Suspension cultures of mammalian cells are frequently agitated by devices which promote a circular motion of the suspension(1,2,3). A simple, inexpensive modification of a Warburg apparatus allowed studies of the growth of L cells when agitated by a reciprocal motion. These are reported here.

Materials and methods. The basal medium was a modification of Waymouth's MB 752/1 (4) with tris(hydroxymethyl)aminomethane (tris) in a final concentration of 14 mg/100 ml substituted for $NaHCO_3$; 0.05% methylcellulose (4000 cps) (Dow Chemical Co., Midland, Mich.), 0.1% Pluronic F68, or 0.1% Pluronic F88 (Wyandotte Chemical Corp., Wyandotte, Mich.)† (all w/v) were usually added as supplements. In serum-free suspensions, 2 of the supplements sometimes were used. Horse serum (10%, v/v), which had been inactivated at 52°C for 1 hour and passed through a sterile Seitz filter, also was added for cultures of cells previously grown in serum-containing media. All cells were sub-lines of NCTC clone 929 (Strain L). Those grown in suspensions with serum had previously been carried on glass in the basal medium with 5% (v/v) horse serum. Those propagated in serum-free suspensions had been cultivated on glass in serum-free basal medium with bicarbonate.

Suspension cultures were grown in 125 ml untreated, screw-capped Erlenmeyer flasks which were held in the Warburg bath (Precision Scientific Co., Chicago, Ill., Cat. No. 6691) by specially designed holders (Fig. 1) attached to manometer supports. The holders with flasks in place could be placed on the bench top without tipping. They may vary in size according to the type of Warburg used but should allow the flasks to be immersed within approximately an inch of the neck while the bath is at the correct water level.‡

* This study was aided in part by grants from Am. Cancer Society and from U. S. Public Health Service, Nat. Inst. Health, and by the Fund for Research and Teaching, Harvard School of Public Health, Dept. of Nutrition.

† The assistance of Dr. Trix of Wyandotte Corp. in obtaining the Pluronics is gatefully acknowledged.

‡ The holders also will fit regular Erlenmeyer flaskc and can be made in a variety of sizes and conveniently applied to many operations where shaking in a constant temperature water bath is desired.

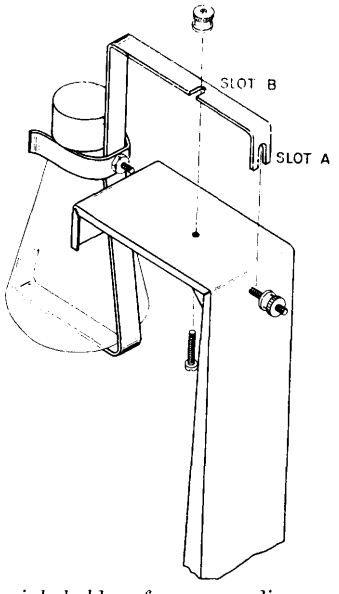

FIG. 1. Special holder for suspending culture flasks in Warburg bath shown with flask and section of manometer support. A manometer support and flask were used as guides for forming of $\frac{3}{8}'' \times \frac{1}{16}''$ brass strips. The neck clamp and body of the holder were joined with a small brass bolt. Slots A and B were so designed to facilitate attachment and removal of the holder from the manometer support.

Cultures were started by scraping cells from glass into a small volume of the desired medium, diluting to a cell count between 3 and 5×10^5/ml, and using 25 ml of the suspension per flask. Difficulty was encountered in establishing cultures with lower cell densities although subsequent cultures could be successfully started with as few as 10^5 cells/ml taken from the third generation of an established suspension culture. Incubations were done at 36°C using a shaker speed of 100 reciprocations/minute with the tops of the manometer supports travelling through a 1 and $\frac{1}{8}$ inch path.§ The gas phase was air and the pH of the cultures was adjusted when necessary by addition of sterile 10% (w/v) tris solution.

When the cell count approximately doubled, 25 ml of medium was added to the flasks. The medium was subsequently changed by centrifuging out the cells and resuspending them in fresh medium. Subcultures were established by centrifuging the desired aliquot of the parent culture and resuspending the cells in the original volume of fresh medium. Cell counts were made with a hemocytometer and metabolic activity of the cells was checked periodically by staining(5).

Results. Within 2 hours, initial cultures supplemented with serum only (Fig. 2) showed production of extracellular debris similar to that described by Kelley *et al.*(6) and some of the precautions used by the same authors were necessary when counting the cells. Such cultures showed no signs of recovery during extended incubation accompanied by periodic medium changes. The amount of debris increased with time until it was so predominant as to enmesh most of the cells. Initial cultures supplemented with methylcellulose or Pluronic, in addition to serum, did not experience such difficulty. Serum-free initial cultures produced extracellular debris in the manner described above except when the other supplements were present (Fig. 3). Sub-cultures established from the third generations of successful initial cultures required methylcellulose or Pluronic only when serum was not present (Fig. 4) and in their absence cell count diminished and extracellular debris again appeared. This finding agrees with other evidence as to the necessity of some protective agent for cells in rapidly agitated suspension(7). No significant loss of metabolic activity or visual evidence of cell damage or cell debris was detected up to a count of 10^8 cells per successful culture (2×10^6/ml). Continued growth did produce such phenomena as indicated by the erratic nature of the upper reaches of some of the curves in Fig. 2. Results of the daily cell counts of 6 replicate cultures are shown in Table I. A repeat of the experiment produced similar results.

Discussion. Growth curves of successful cultures are essentially logarithmic. Replicate cultures of the same cells agitated by the circular motion of a magnetic stirring de-

§ If a Warburg apparatus has some type of expanding pulley for shaking speed control, it may be advantageous to replace it with a solid pulley of the proper size for the desired speed since some variable pulleys do not stand up well under long-term continuous operation.

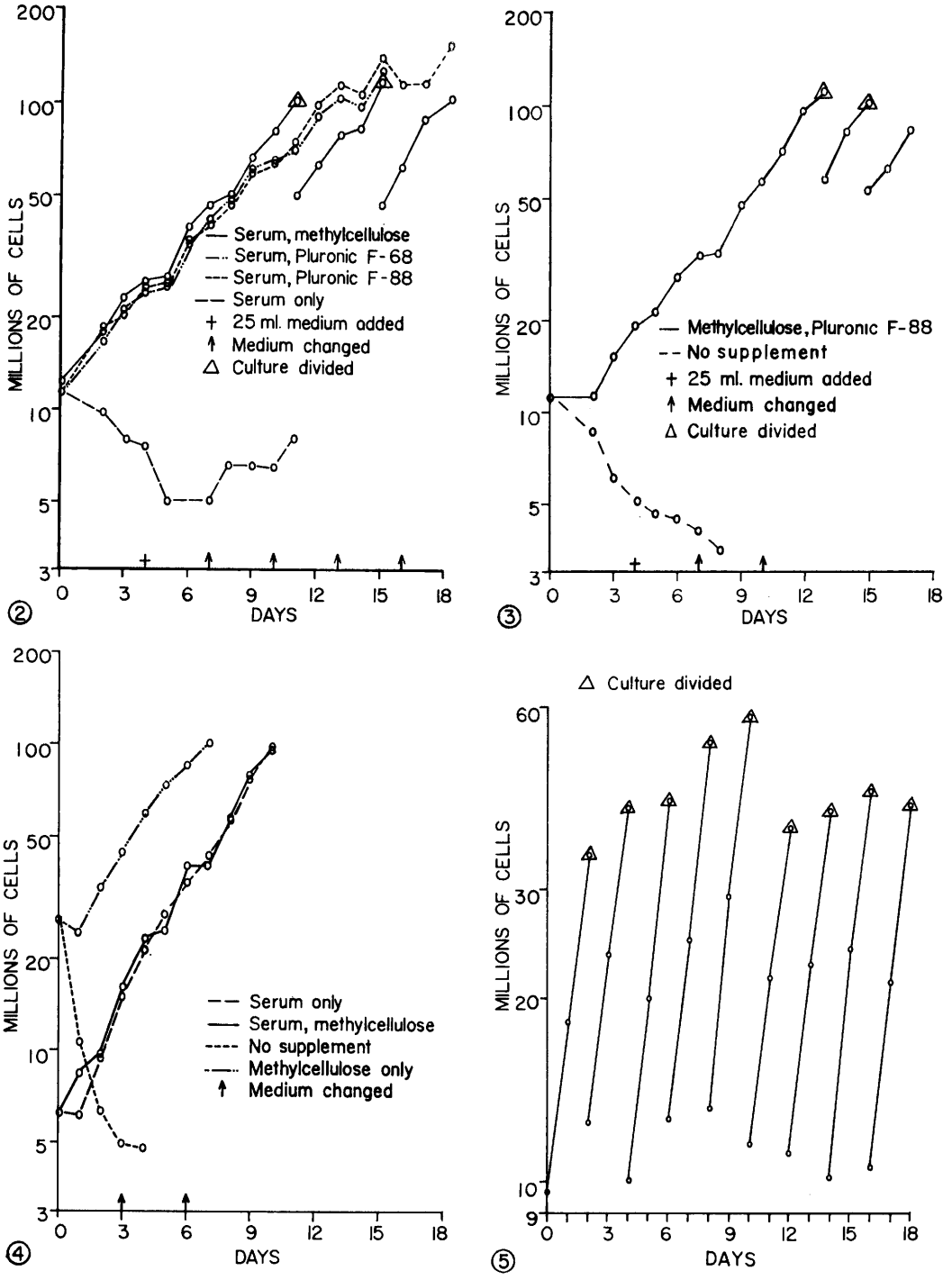

FIG. 2. Growth curves of initial serum-containing cultures with and without additional supplements.

FIG. 3. Growth curves of initial serum-free cultures with and without other supplements.

FIG. 4. Growth curves of subcultures from the third generation of successful initial cultures showing effect of removal or change of supplement.

FIG. 5. Growth curve of a fast growing suspension culture after having been changed from a magnetic stirring device to the Warburg apparatus.

TABLE I. Reproducibility of Cell Counts of 6 Replicate Suspension Cultures when Agitated by the Warburg Apparatus in Basal Medium with Horse Serum and Methylcellulose.

Day	1	2	3	4	5	6	7	8	9
Mean cell count $\times 10^5$	1.61	2.07	2.54	3.29	3.93	4.50	5.39	6.74	8.82
Standard deviation $\times 10^5$	2.73	7.29	6.81	10.18	14.96	15.00	17.02	20.33	52.79

vice in this laboratory produced growth curves superimposable on those shown here. After completion of the above studies, rapidly growing L cells from a spinner culture were obtained from Littlefield and propagated as described by him(2) using the Warburg apparatus in place of the spinner. Growth curves (Fig. 5) comparable to those shown by the cells in spinner culture were obtained with no intermittent lag period. Thus the smooth reciprocal motion of the Warburg apparatus can be used successfully for cultivation of suspension cultures.

A Warburg apparatus is a common item in many biological laboratories and suspension cultures can be grown by this method without additional elaborate or expensive equipment. The water bath offers greater precision of temperature control than is available with air incubators and all cultures are agitated at precisely the same speed and in the same manner, thus promoting good reproducibility of growth curves which is advantageous in many types of growth studies. Cells from the cultures can be subjected to Warburg manometric studies without greatly altering their environment. A refrigerated Warburg, such as was used in these studies, conveniently allows work with suspension cultures at low temperatures. The method could be combined easily with the continuous CO_2 detection method of Karler and Woodbury(8), which involves use of a Warburg apparatus, to measure the CO_2 output of suspension cultures at various stages of the growth curves.

Summary. A study was made of the growth of suspension cultures of L cells when agitated by a Warburg apparatus through use of specially designed holders for the culture flasks. The basal medium was supplemented with certain compounds as protectants for the cells. Logarithmic growth curves were obtained with serum-free and serum-containing cultures. Good reproducibility of growth curves of replicate cultures was obtained. Certain advantages for growing suspension cultures were realized.

1. Earle, W. R., *Fed. Proc.*, 1958, v17, 967.
2. Littlefield, F. W., *Exp. Cell Res.*, 1962, v26, 318.
3. Cherry, W. R., Hull, R. N., *J. Biochem. Microbiol. Tech. and Eng.*, 1960, v2, 267.
4. Geyer, R. P., Bennett, A., Rohr, A., *J. Lipid Res.*, 1962, v3, 80.
5. Phillips, H. J., Terryberry, J. E., *Exp. Cell Res.*, 1957, v13, 341.
6. Kelley, G. G., Adamson, D. J., Oliver, K. L., *Am. J. Hyg.*, 1960, v71, 9.
7. Bryant, J. C., Schilling, E. L., Earle, W. R., Evans, V. J., *J. Nat. Cancer Inst.*, 1960, v24, 859.
8. Karler, R., Woodbury, D. M., *J. Appl. Physiol.*, 1962, v17, 365.

Received January 16, 1963. P.S.E.B.M., 1963, v112.

Plaque Production with Infectious Bovine Rhinotracheitis (IBR) Virus.* (28244)

H. ROUHANDEH AND A. A. WERDER (Introduced by Perry Morgan)
Department of Pediatrics and Hixon Memorial Laboratory and Department of Microbiology, University of Kansas School of Medicine, Kansas City, Kan.

Infectious bovine rhinotracheitis (IBR) virus was isolated by Madin *et al.*(1) and shown to produce a cytopathic effect on various primary bovine embryo tissue culture cells. To our knowledge literature does not reveal plaque formation by IBR virus. This report

* Supported in part by grants from Lederle Laboratories and the National Foundation.