detectable in human coccidioidomycosis, early precipitins and later complement-fixing antibodies. By using concentrated precipitin sera and dilute coccidioidal antigen, arcs of precipitation were produced which appeared to correspond to the mobility of 19S β_2 macroglobulin. Complement-fixing sera and cerebrospinal fluids reacted with coccidioidal antigen to give precipitation arcs with the mobility of 7S γ_2 globulin.

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Received September 3, 1964. P.S.E.B.M., 1965, v118.

A Premixed Powder for Preparation of Tissue Culture Media.* (29774)

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During the development of a program for certification and preservation of cells sponsored by the Cell Culture Collection Committee(1), cooperating laboratories were frequently unable to confirm results with a given cell line because of variations in batches of media prepared in different laboratories. A partial solution was to exchange prepared media along with cell cultures, a common practice between tissue culture laboratories, but of limited applicability. Another measure adopted was to coordinate orders for 10× and 100× liquid media so each laboratory received a large quantity of the same lot. However, this solution is limited by the lot size of liquid media, stability on storage, and by cold storage space possessed by each laboratory. Similar problems have frequently been encountered in laboratories attempting to grow human diploid cell strains for the first time, and investigation usually indicated that the recommended medium had not been used (2).

One approach to the solution of some of these problems has been development of entirely synthetic media. Although the use of synthetic media for growth and maintenance of several cell cultures has been successful(3, 4), the majority of animal cell cultures utilized in most laboratories still requires a medium supplemented with some type of natural product. In lieu of synthetic media, various schemes have been devised by research investigators and commercial supply houses for standardizing the basic constituents of culture media, *i.e.*, the serum and the organic and inorganic chemicals. All these procedures had some degree of success, but the basic problems still remain.

One recent advance which appears to solve most of the problems is the development of

* Supported by grant CA-4953 from USPHS.

dry powdered tissue culture media,† using a ball mill to ensure solubility and uniformity, as described by Swim and Parker(5). Great uniformity and rapid solubility are enhanced by the thorough mixing and small particle size. Stability is encouraged by sealing the desiccated powder in weighed aliquots in the final container which requires only distilled water to prepare the complete medium. Uniformity is further assured by selecting large bulk lots of organic and inorganic chemicals which meet the most rigid specifications of the National Research Council. Although Swim and Parker demonstrated the feasibility of using a premix dry powder for preparing tissue culture media, their report created little interest until Meyer, Stevenson and Morgan(6), representing the Cell Culture Collection Committee, and Hayflick(2) at the Wistar Institute, encouraged commercial production of dry ball milled tissue culture media as a possible solution to some of the evasive tissue culture media problems. The present report compares the growth of several cell lines in the new powdered media as compared to media prepared from commercial 10× concentrates which are in use in most tissue culture laboratories.

Materials and methods. 1. Chemical ingredients in the powdered media. The quality of the chemicals used in the formulations was equal to the specifications for Eagle's medium requested by the Virology Research Resources Branch, NCI, and the collaborating laboratories of the Cell Certification Collection Committee(6). Amino acid specifications were based upon NAS-NRC Publication 719, Specifications and Criteria for Biochemical Components. The purity of the amino acids was tested on large bulk lots up to 100 kg by optical rotation, elemental analysis, ash, color, solution, paper chromatography, Van Slyke, ninhydrin, manametric D-amino acid oxidase, ultraviolet adsorption and infra-red spectroscopy. The chemical analyses of each amino acid were determined by the manufacturer and by 2 additional independent laboratories. The vitamins used were USP grade. The inorganic salts in the balanced saline were Mallinkrodt analytical grade, with the exception of the NaCl which was Baker analytical grade. There were no antibiotics in the media.

2. Preparation of the powdered media. Individual ingredients are weighed and thoroughly blended. This mixture is desiccated 18 hours under vacuum to remove traces of moisture, then ball milled until an intimately mixed free-flowing fine powder is obtained. The fine powder blend is again desiccated under vacuum to remove any traces of moisture and packaged in aliquots suitable for preparation of a desired quantity of medium.

The ball mill contains porcelain "Burundum" balls, 1¼″ in diameter and 1¼″ long. They are especially chosen for their smooth surface to prevent accidental carry over of chemicals to successive batches of media processed in the ball mill. The ball mills are used only in preparation of the powdered tissue culture media.

3. Laboratory preparation of the culture media. The components of the nutrient media tested in these experiments have been described by their developers in earlier publications. These experimental media were 1) Eagle's BME(7), and MEM with the non-essential amino acids; 2) Medium 199 of Morgan, Morton and Parker(8); 3) NCTC 109 of Evans *et al*(4); and 4) NCTC 127 of Sanford *et al*(9).

A. The control media consisting of Eagle's Basal Medium (BME) or Eagle's Minimum Essential Medium (MEM) with non-essential amino acids, were prepared from 10× and 100× liquid concentrated stock solutions purchased commercially.‡ Single strength concentrations of the media were made by diluting vitamins and amino acids in Earle's salt solution. Five percent glutamine/liter and 2.2 g/liter of $NaHCO_3$ were added to the reconstituted media. The Minimum Essential Medium differs from its precursor Basal Medium Eagle mainly in increased concentration of essential amino acids.

B. Powdered dehydrated media. 1) GBI§ Eagle's MEM without L-glutamine: Control

† General Biochemicals, Laboratory Park, Chagrin Falls, Ohio.

‡ Hyland Laboratories, Los Angeles, Calif.

§ General Biochemicals, Laboratory Park, Chagrin Falls, Ohio.

TABLE I. Plating Efficiency of 9 Cell Cultures in Media Prepared from Powdered BME.

	% of cells producing colonies*								
						Chinese hamster			
Medium	HeLa S.J.	Conjunctiva clone 22	HEp-2	Chang liver	Syrian hamster kidney	Clone G-3	Strain Don	Bovine MDBK	Mouse NCTC 2555
Powdered BME	26	37	33	21	54	35	11	0	19
Control BME	25	29	32	22	52	23	7	17	30

* Avg of 2 separate plating experiments of 5 plates for each test media.

4339C, Lot No. 64088. 9.4 g of dry powder were added to one liter of distilled water, plus 12 ml 5% L-glutamine and 2.2 g $NaHCO_3$. The final pH after filtration was 7.4.

2) GBI Eagle's BME: Control 31356, Lot No. 630658. This was prepared by adding 9.086 g dry powder to one liter of distilled water plus 2.2 g $NaHCO_3$. The final pH after filtration was 7.4.

3) GBI NCTC 109: Control 4023E, Lot No. 640805. This was prepared by adding 11.55 g of dry powder to one liter of distilled water. Monosodium glutathione has been omitted from this medium as it is now considered unnecessary. $NaHCO_3$ is present in the medium and final pH after filtration was 7.4.

4) GBI NCTC 127: Control 4868A, Lot No. 640127. Prepared by adding 11.41 g of dry powder to one liter of distilled water. The $NaHCO_3$ is present in the dry powder. Final pH was 7.4.

5) GBI Medium 199: Control 4210C, Lot No. 64033. This was prepared by adding 11.08 g of dry powder to one liter distilled water, plus 0.075 g of $NaHCO_3$. Final pH was 7.0-7.2.

The distilled water was pre-warmed to approximately 35°C and stirred for one-half hour with a magnetic stirrer after adding the powdered media. All the test media were filtered through a Millipore GS pad.

The serum source for all media was inactivated newborn calf serum obtained from Hyland Laboratories, and both control media and media prepared from dry powder received penicillin 100 units/ml and streptomycin, 100 μg/ml, at time of starting cell cultures.

C. Experimental cell lines. Growth of 5 human cell lines and 5 animal cell lines was observed for short-term growth periods in control and test media. Cells studied were: Chang liver, HEp2, Kilbourne-Wong conjunctiva clone 22, HeLa S. J., WI-38, mouse NCTC 2555, Syrian hamster kidney, Chinese hamster clone G-3, Chinese hamster Don strain, and bovine kidney, MDBK. These cells are all accepted or are candidates for inclusion in the cell registry by the Cell Culture Collection Committee. They meet all the standards established by the Committee for certified cell lines(1).

D. Evaluation of cell growth. Cells tested for growth were taken from frozen stocks stored in liquid nitrogen or from tissue cultures actively growing in milk dilution bottles, and duplicate inocula placed into test and control media simultaneously. Cell growth in the experimental media was evaluated by the plating efficiency technique, growth curves in stationary tubes, continuous passage, and by cell morphology.

Plating efficiencies were determined by placing 200 single, viable cells into plastic 60 mm tissue culture plates containing control or test media and 20% calf serum. Cell dilutions for plating were prepared in the individual test media so that the suspending medium of frozen ampules was diluted approximately 10,000-fold after removal from the ampule. The plates were incubated in an atmosphere of 5% CO_2-95% air for 8-10 days, then fixed with methyl alcohol and stained with Giemsa (10).

Growth curves of the cell cultures were determined by inoculating 5×10^4 cells into control and test media in replicate roller tubes incubated at 37°C in 5% CO_2-95% air. The tubes were refed with the same medium on day 4 and day 7. The cells were harvested at 48-hour intervals with 0.25% trypsin and the viable cell count ascertained with trypan blue staining in a hemocytometer. The average number of cells in four tubes, divided

TABLE II. Plating Efficiency of Cell Cultures in Media Prepared from Dry Powdered Media.

	% of cells producing colonies*					
	Experimental culture media					
Cell cultures	Control	MEM	BME	NCTC 127	Medium 199	NCTC 109
HeLa S.J.	85	84	75	71	64	—
Conjunctiva	36	41	35	38	38	—
Chang liver	38	42	36	42	39	39
HEp-2	44	49	41	44	38	—
WI-38	8	7	6	5	8	7
Mouse—NCTC 2555	55	57	52	47	48	36
Syrian hamster kidney	61	65	54	58	61	33
Chinese hamster clone G-3	57	58	48	47	46	—
" " strain Don	10	11	10	12	7	—
Bovine MDBK	3	12	5	2	2	2

* Avg of 2 separate plating experiments of 5 plates for each test media.
Control = MEM prepared from liquid stocks. — = Not tested.

by the number of cells in the inoculum gave a value referred to as "proliferation index."

Results. Nine different cell lines were plated in the powdered BME and control BME, each with 20% calf serum. The data presented in Table I indicate that 8 cell lines grew as well in GBI-BME as they did in the control medium routinely used in this laboratory. The differences were not statistically significant except with one cell culture, MDBK, a bovine kidney cell line which grows well in mass culture but poorly in plating experiments. The cell lines tested were from 5 species, 4 human, 2 Chinese hamster, 1 Syrian hamster, 1 mouse and 1 bovine.

In an extension of these studies, the same 9 mammalian cell cultures plus the human diploid cell line WI-38 were plated in the same manner. In these tests powdered BME, powdered MEM, powdered NCTC 127, powdered Medium 199 and powdered NCTC 109 were compared with control Eagle's MEM. The data are presented in Table II. In 8 of the 10 cell lines studied, colony counts in GBI-MEM were slightly higher than colony formation in either GBI-BME or the control media. Plating efficiency results in GBI-BME, NCTC 127 and Medium 199 were comparable or only slightly less than in the control medium. Somewhat lower plating efficiencies were obtained in NCTC 109 with 2 cell lines. However, confluent cell growth of all 10 cell lines was observed in this medium (NCTC 109 and 10% calf serum) in heavily seeded milk dilution bottles and roller tubes.

Colony formation of WI-38 and MDBK was minimal in most media; however, the cells of both cell lines as well as the diploid Don strain of Chinese hamster grew well in all the media in milk dilution bottles after being inoculated with large numbers of cells.

The growth rate data of HeLa, HEp2, and diploid Chinese hamster "Don strain" cells in the experimental media and in the control MEM media are presented in Table III. These data illustrate good cell growth in all the media. Cell counts in the experimental media varied above and below cell counts in control media from day to day, but the differences were not considered significant.

The 10 cell lines cultured on the 5 experimental powdered media showed no variations or changes in morphological characteristics which differed in any manner from cell cultures grown on the control media. Both epithelial-like and fibroblast-like cells retained their identifying appearance. Colony morphology in the plating efficiency studies revealed little variation in colony morphology between cell lines grown on the 5 test media and the control media. Epithelial-like cell lines formed colonies of tightly packed polygonal shaped cells in all the media, whereas colonies developing from fibroblast cells demonstrated the typical "rough, hairy, colonies" described by Puck(11).

HeLa cells cultivated in the 5 reconstituted powdered media for 10 serial passages in our laboratory have shown no change in morphology or growth rate. Cells plated after the

TABLE III. Growth Rate of Cell Cultures in Media Prepared from Dry Powder.

Cell line	Proliferation index* Day 2	Day 3	Day 4	Day 7	Day 9	Day 11
HeLa S.J.						
Control	2.0	3	—	8	6	15
Powdered MEM	1.9	2.5	—	8	9	15
" BME	1.9	2.8	—	7.8	9.5	12
" NCTC 127	2.0	2.5	—	8.0	11	12
" Medium 199	1.8	2.5	—	6.2	10	10
Chinese hamster Don strain						
Control	1	—	2.4	3	3.4	3.5
Powdered BME	1	—	2.6	6.8	3.9	3.5
" NCTC 127	1	—	1.6	5.2	3.1	2.2
" Medium 199	1.2	—	1.9	4.4	5.4	3.5
" NCTC 109	1	—	1.6	3.8	6.6	4.8
HEp-2						
Control	<1	—	<1	4	2.5	3.3
Powdered MEM	<1	—	<1	2.5	2.2	2.6
" BME	<1	—	<1	3.1	3.5	3.7
" NCTC 127	<1	—	<1	1.6	1.6	2.5
" Medium 199	<1	—	<1	3.0	2.2	2.8
" NCTC 109	<1	—	<1	1.8	2.6	2.1

* Proliferation index = No. of viable cells at day of harvest/No. of viable cells inoculated at 0 day. Each reading is avg of 4 replicate tubes. Original inoculum of each tube was 5×10^4 viable cells.

Control = MEM prepared from liquid stocks.

sixth serial passage gave results comparable to cells carried for a similar time in control media (Table IV). This suggests that the powdered media contain all essential nutrients for growth and do not depend on trace elements carried over from previous control media. This is further established by Hayflick(2) who has grown the diploid cell strain WI-38 for at least 40 passages in reconstituted powdered Eagle's BME.

Discussion. Ten cell cultures grown in 5 different tissue culture media prepared from ball milled dry powder grew as well or better than cells grown in Eagle's MEM media prepared from concentrated liquid stock solutions. The basis for comparing media was colony formation from single cell inocula, because this technique has been shown to be more sensitive to nutritional deficiencies than growth of heavily seeded culture(7,10). The number of colonies formed from single cells of the different animal cell cultures grown in the reconstituted powdered media was equivalent to, or greater than the number of colonies produced in the control media. In those cell lines where the plating efficiencies were low (WI-38, Don strain and MDBK) excellent cell growth was observed in roller tubes seeded with large cell inocula. These data were reinforced by controlled growth curves of HeLa S.J., HEp-2 and diploid Chinese hamster cells. NCTC 109 and 127 were developed by Evans *et al*(4) and Sanford *et al*(9) as chemically defined, protein-free medium, but they were tested in these experiments in the presence of serum, because the cell cultures being tested were not adapted to growth in serum-free media.

TABLE IV. Plating Efficiency of HeLa Cells Cultured for 6 Continuous Passages in Media Prepared from Dry Powder.

Cell culture	% of cells producing colonies Control	Experimental culture media MEM	BME	NCTC 127	Medium 199	NCTC 109
HeLa S.J.	80	87	94	90	71	56

Avg of 5 plates for each test media. Control = MEM prepared from liquid stocks.

The advantages of dry powdered tissue culture media are numerous. They can be prepared in large batches, up to 100 kg of dry powder, and dispensed in various sized containers for preparation of large or small batches of medium. Long-term stability of the dry powder permits tests to be carried out with the same lot of media, over a period of many months or in different laboratories.

Another benefit accruing from the use of the dry powdered media is the increased solubility of the finely powdered chemicals as a result of the ball milling technique. No chemical manipulations are required to dissolve certain amino acids which are usually difficult to get into solution. The need for solubilizers and stabilizers that could be toxic to certain cells is eliminated. Many of the components added to synthetic media are rather unstable and the only way to store them effectively for long periods is in the dry state, preferably in the cold(12). Studies with the powdered media have indicated that they are stable for at least one year in the dry state, which would allow the use of the same bulk lots for preparing successive batches of media(2). The requirement of extensive refrigerator or deep freezing facilities for large amounts of liquid media is also eliminated by the dry media, which requires only minimal storage space. Investigators can conduct extensive sterility tests on a large batch of media and have greater confidence in the results than is possible when smaller batches of liquid stock solutions are tested. Collaborative programs requiring the transfer of new cultures and media between laboratories can be more conveniently implemented since the powdered media can be shipped great distances over long periods, with decreased shipping charges and greater stability of the culture media. A great additional advantage of the powdered media of prime importance to all investigators is its low cost. It can be made available at approximately one-tenth the cost per liter now charged for media prepared from liquid concentrates.

Summary. Five different tissue culture media prepared as a dry powder by blending the ingredients in a ball mill followed by desiccation were reconstituted and evaluated for their ability to support cell growth of ten different tissue culture cell lines. Eagle's BME, Eagle's MEM, Medium 199, NCTC 127 and NCTC 109 supported good cell multiplication as compared to the control Eagle's MEM prepared from 10× or 100× concentrated liquid stock solutions. Plating efficiencies of 8 of 10 cell lines were higher in media prepared from the powdered Eagle's MEM than in the control media. Plating efficiency data were confirmed by growth curves in roller tubes and by growth of massive inocula in milk dilution bottles. Additional important advantages of using dry powdered culture media include: low cost of powdered media as compared to the present commercial liquid concentrates; greater stability; greater uniformity of the chemical ingredients because of the ball milling process and the rigid selection of large bulk lots of basic ingredients; low shipping charges; and ease of storage for long term experiments.

The authors wish to thank Drs. Richard C. Meyer and Robert E. Stevenson, Nat. Inst. Health, and Herbert R. Morgan, Univ. of Rochester Med. School, N. Y., whose efforts as members of the Subcommittee on Media and Substrates of the Cell Culture Collection Committee initiated this study. Thanks are also due Dr. Leonard Hayflick, Wistar Inst., for helpful advice, and Mr. Michael Peluse and Mrs. Elizabeth Kauderer for technical assistance.

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Received September 18, 1964. P.S.E.B.M., 1965, v118.

Liberation of Bactericidal Histone from Inactive Deoxyribonucleohistone Complex by DNase.* (29775)

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In 1887, Ribbert(1) observed the extracellular destruction of microorganisms surrounded but not ingested by leucocytes. This extracellular antibacterial activity has been substantiated more recently by Dubos and MacLeod(2) and Cromartie *et al*(3). Skarnes and Watson in 1956(4) isolated Leukin, a bactericidal factor from peritoneal leucocytes, under conditions simulating the acidic environment existing in an inflammatory area. They suggested that this cationic protein was derived from the nucleus upon cell destruction and was then able to exert its extracellular bactericidal activity.

The present studies demonstrate a plausible means by which the cationic histones might be liberated from the deoxyribonucleohistone complex (DNP) within the nucleus by means of deoxyribonuclease (DNase).

Materials and methods. DNase I was obtained from Worthington Biochemical Corp., Freehold, N. J. The fast green FCF and safranin counterstain (Gram) were obtained from Hartman-Leddon Co., Philadelphia, Pa.

Group A streptococci, T-18, were isolated at NMRU-4, Great Lakes, Ill. The organisms were grown out of a lyophilized state, transferred at 12 hours and the subsequent 12-hour culture was used in the bactericidal assays.

The modified Stock's dialyzable medium (5) was employed because it was capable of supporting the growth of the streptococci and yet it did not combine nonspecifically with the cationic histones.

Calf thymus obtained from local meat packing plants was the source of nuclei. The method employed was that of Allfrey(6) in which the calf thymus is mildly homogenized in 0.25 M sucrose solution with a Waring blendor followed by centrifugation to obtain the nuclei. The nuclei were then resuspended in the dialyzable medium at a concentration of 50% T at a wave length of 600 mμ.

The nucleohistones were extracted from calf thymus with solutions of low ionic strength according to the method of Crampton *et al*(7). This procedure also provides a method for preparation of histone and DNA based upon the observations that high salt concentrations (2.6 M) tend to dissociate the nucleohistone complex. Addition of alcohol to this high salt solution precipitates out the DNA. All 3 substances, nucleohistone, DNA and histone were lyophilized after their isolation.

The antibacterial effects were determined in two ways: The first method was performed by adding the substances to be tested for bactericidal activity to the solvent (water or medium) and incubating them for 1 hour at 37°C. These solutions were then inoculated with a "standard loop" of a 12-hour culture and reincubated for 30 minutes at 37°C. The assay was designed so that a subsequent loop inoculation from the above solutions into tubes of trypticase soy agar at 45°C contained approximately 50-100 organisms. Bactericidal activity was expressed as a decrease in the colony counts on the poured plates after 24 hours incubation at

* This investigation was supported by USPHS Grants HTS 5360 and 5 T1 AI 90-05 from Nat. Inst. Health.

† Supported by USPHS research career program award, AI-K6-11651.