AN IMPROVED CHEMICALLY DEFINED BASAL MEDIUM (CMRL-1415) FOR NEWLY EXPLANTED MOUSE EMBRYO CELLS

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ABSTRACT

An improved chemically defined medium, CMRL-1415, has been devised by testing the response of trypsinized, newly explanted mouse embryo cells in stationary cultures to various modifications of an earlier medium, CMRL-1066. The improvements are attributed to changes in amino acid levels, in the vitamin-coenzyme composition, and to an enhanced buffering capacity resulting from the use of free base amino acids, galactose and pyruvate, together with greatly reduced levels of glucose and sodium bicarbonate and the inclusion of both monobasic and dibasic sodium phosphate in the ratio 1:4. When the medium is equilibrated with 5% CO₂ in air, an initial pH of 7.2-7.4 is achieved, with excellent buffering capacity. CMRL-1415 contains 50 ingredients (9 fewer than CMRL-1066) and is prepared from six stable stock concentrates. By omitting sodium bicarbonate (to give CMRL-1415-ATM), the medium may be used in unsealed cultures in free gas exchange with air. CMRL-1415 and CMRL-1415-ATM are intended for use with and without serum protein and other supplements; and by preparing them double strength they may be combined with agar or other gelling agents to provide a semi-solid substrate.

The first defined medium reported from this laboratory, No. 199, was developed especially for freshly explanted chick embryo cells, which remained alive in the medium for an average period of 33 days (17). Because estimates of survival time became impracticable as the medium was improved, the next series of media reported by us, Nos. 612, 635, 703, 858, and CMRL-1066, were tested on replicate cultures of Earle's strain L cells from mouse, and the response of these cells to the media was estimated from hemocytometer counts of nuclei isolated from representative cultures after various periods of cultivation (9-11). The final medium of this series, CMRL-1066, has been used to advantage in many undertakings in this and other laboratories. Because it was devised especially for L cells, it was easy to adapt sublines of L cells to it, and one such line has multiplied continuously in CMRL-1066 for almost 10 yr without protein supplement of any sort at any time. Because it is now realized that most established cell lines can be adapted quite easily to unsupplemented defined media, all recent variations of CMRL-1066 have been tested on cultures of newly explanted mouse embryo cells prepared from trypsinized suspensions. This report describes an improved basal medium, CMRL-1415, that contains fewer ingredients than CMRL-1066, yields cultures of mouse embryo cells that continue to multiply for several weeks, and includes an improved buffering system. CMRL-1415 has been devised especially for use with certain protein and nonprotein supplements that are described in the following paper in this issue of the Journal.
MATERIALS AND METHODS

Development of Cell Populations

All embryos from one female mouse (CMRL Swiss strain; 14 to 16 days' gestation) were cut into fine fragments, washed twice with 40- and 30-ml quantities, respectively, of phosphate-buffered saline (PBS) (3), and both washings were discarded. The fragments were then treated twice with like amounts of 0.25% trypsin (Difco Bacto-trypsin, 1:250) in Puck's saline A (18) to disperse the cells. Cell dispersal was carried out 37°C with magnetic stirring in two stages of approximately 15 and 20 min each. The dispersed cells from each treatment were transferred through two layers of sterile gauze to a centrifuge bottle containing about 70 ml of growth medium (15% horse serum in CMRL-1066 or CMRL-1415), and centrifuged (International, No. 2) for 15 min at 1000 RPM. After the cells had been resuspended in 50 ml of growth medium, hemocytometer counts were made, and the suspension was adjusted with growth medium to 1 × 10^6 cells/ml. Primary cultures were set up by seeding 4-oz Brockway prescription bottles with 10 ml each and 32-oz Blake bottles with 80 ml. Finally, all cultures were gassed with a mixture of 8% CO₂ in air.

The cells were usually subcultured twice weekly, with one medium change between transfers. Cells were harvested with 0.25% trypsin in trisodium citrate saline (20) and an equal volume of growth rate saline (20) and an equal volume of growth medium was added to the suspension before centrifuging. Subcultures were usually made in 3 Blake bottles seeded with 0.5 × 10^6 cells/ml and 3 seeded with 0.8 × 10^6 cells/ml. Cells from primary cultures were subcultured at least once, but not more than five times before being used for experiments. Preferably, cells were used at the 3rd trypsinization (2nd subculture) 6 to 9 days after explantation; they were never used later than 30 days after explantation.

Preparation of Test Cultures

Cells to be used for a test experiment were trypsinized and then centrifuged as before in growth medium (to stop the action of trypsin). If all the solutions to be tested in a given experiment were made up in the same basal medium (e.g., CMRL-1066 or modifications of it), the cells were washed and centrifuged a second time in the basal medium. If the solutions to be tested were made up in different types of media, the cells were washed and centrifuged a second time in PBS. The cells were then resuspended in a second, smaller volume of basal medium or PBS, passed through nylon cloth (400-mesh; Tabler, Ernst and Traber, Inc., New York) placed over the metal screen of a Swinny hypodermic adapter (Millipore Filter Corp., Bedford, Massachusetts), counted in a hemocytometer or Coulter cell counter, and adjusted to 2 × 10^6 cells/ml. Usually, two replicate cultures were prepared for each test solution by mixing 9.8 ml of the test solution with 0.1 ml of penicillin and 1.1 ml of the cell suspension, and 5 ml of this mixture were placed in each of two 30-ml Falcon plastic flasks (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles) fitted with silicone stoppers. Care was taken to adjust the pH of the cultures with gas mixtures, if necessary, before placing them for incubation at 37°C. Complete fluid changes were made twice a week.

Estimation of Results

Because of many variables encountered in attempting to make comparable experiments from newly explanted mouse embryo cells cultivated in the absence of whole serum, it was felt that photographic records of replicate cultures after various time intervals would be more informative than cell numbers, for numerical data from cultures prepared from separate batches of newly explanted cells cultivated without serum cannot be compared, even when the cells are derived from the same group of embryos. Also, newly explanted cells cultivated without serum are exceedingly sensitive to minor variations in media that do not affect cells cultivated in serum or cells from established lines, which adapt much more readily than newly isolated cells to adverse conditions.

The most revealing experiments were always those in which cells in the defined basal media serving as controls would begin to degenerate long before those in media containing useful modifications. Often, however, the effects of useful modifications in the medium were revealed more clearly by improved cellular morphology and mode of growth than by length of survival or measurable differences in population density.

In a few selected experiments, the population-doubling time was estimated by making hemocytometer counts of the entire population of individual replicate cultures prepared with the same medium. Such counts were made at intervals of 2, 3, or 4 days, during the logarithmic phase of growth. Because cells in defined media are as difficult to dislodge from Falcon flasks as they are from glass, treatment with trypsin (0.25%) was prolonged to 30 to 35 min and its action was interrupted by including an equal amount of soybean trypsin inhibitor (Pen- tex, Inc., Kankakee, Illinois) in the diluting fluid used in preparing the cells for counting.

Fixation and Staining for Photography

For photography, the cultures were fixed in Ringer-formol, stained with Weigert's iron hematoxylin, and cleared by filling the plastic flasks with 50% glycerin.
EXPERIMENTS AND RESULTS

Development of Basal Medium CMRL-1415

(Table I)

1. AMINO ACIDS: Medium CMRL-1415 contains 20 natural amino acids that are present in the free form in mammalian blood plasma and in hydrolysates of nutritionally adequate proteins. The levels of certain amino acids in CMRL-1415 are based on values previously established in this laboratory (9, 10) and upon values established by Eagle et al. (7) for the "essential" amino acids. The levels of certain other amino acids were readjusted so as to be more consistent with analytical values for human plasma as reported in the literature (22, 12). Also, the hydrochlorides of l-arginine, l-cystine, l-histidine, and l-lysine were replaced by their free bases and the level of l-arginine was greatly increased, to improve the buffering capacity of the medium (25, 13).

2. BUFFERS: For many years the pH of cultures has been regulated by means of a buffer system modeled after the naturally occurring CO₂-bicarbonate system present in blood plasma. Various attempts to replace bicarbonate as the principal buffer in defined media with Tris(hydroxymethyl)aminomethane or with glycylglycine (24, 15) resulted in excellent pH control but the growth response was variable. Swim (23) extended these studies of nonbicarbonate buffered media and has recently clarified the essential nature of bicarbonate. If an insufficient amount of CO₂ is produced and retained in the culture system, some bicarbonate and/or CO₂ must be supplied. It was decided, therefore, to retain bicarbonate as part of the buffer system, at a reduced level (1.0 g/liter), and to supplement it with mono- and dibasic sodium phosphate. In an effort to achieve a final pH of 7.4 with this lesser amount of bicarbonate (25) and a constant ratio of mono- and dibasic sodium phosphate (1:4), the proportion of phosphates was varied in the presence of 5% CO₂ in a closed system. As a result, it was found that adequate buffering capacity of the medium, consistent with good growth and ease of handling the cultures, was achieved by a combination of 1.0 g/liter NaHCO₃, 0.05 g/liter NaH₂PO₄, and 0.2 g/liter Na₂HPO₄; and, as already mentioned, the level of l-arginine was increased to augment still further the buffering capacity of the medium. Because arginine in solution dissolves

| TABLE I: Chemically Defined Medium CMRL-1415* |
|-------------|-----------|-----------|
| Mg per Level in 1000 ml CMRL-1066:~ |
| l-Alanine   | 30        | 25        |
| l-Arginine  | 500       | 70        |
| L-Aspartic acid | 10    | 30        |
| L-Cysteine  | 179§      | 260       |
| L-Cystine   | 24        | 20        |
| L-Glutamic acid | 10    | 75        |
| L-Glutamine | 292       | 100       |
| Glycine     | 17        | 50        |
| l-Histidine | 31§       | 20        |
| Hydroxy-l-proline | 10  | 10        |
| l-Isoleucine| 52        | 20        |
| l-Leucine   | 52        | 60        |
| L-Lysine    | 47§       | 70        |
| L-Methionine| 13        | 15        |
| L-Phenylalanine | 32   | 25        |
| L-Proline   | 30        | 40        |
| L-Serine    | 12        | 25        |
| L-Threonine | 48        | 30        |
| L-Tryptophan| 10        | 10        |
| L-Tyrosine  | 36        | 40        |
| L-Valine    | 46        | 25        |
| Ascorbic acid | 50     | 50        |
| d-Biotin (USP) | 1     | 0.01      |
| Calcium pantothenate | 0.5  | 0.01      |
| Choline chloride | 1     | 0.50      |
| Folic acid (USP) | 1     | 0.01      |
| Inositol (NF) | 2      | 0.05      |
| Cocarboxylase | 1      | 1         |
| Codecarboxylase | 1      | 1         |
| Diphosphoryridine nucleotide | 7     | 7         |
| Flavin adenine dinucleotide | 1     | 1         |
| Glutathione | 10        | 10        |
| Triphosphoryridine nucleotide | 1     | 1         |
| Uridine triphosphate | 1     | 1         |
| Deoxyadenosine | 10     | 10        |
| Deoxycytidine | 10     | 10        |
| Deoxyguanosine | 10     | 10        |
| 5-Methyldeoxycytidine | 0.1   | 0.1       |
| Thymidine   | 10        | 10        |
| d-Galactose | 500       | 500       |
| d-Glucose   | 500       | 1000      |
| Phenol red  | 20        | 20        |
| Sodium pyruvate | 225   | 225       |
| NaCl        | 6800      | 6800      |
| KCl         | 400       | 400       |
| CaCl₂       | 140       | 200       |
| MgSO₄·7H₂O  | 240       | 200       |
| NaH₂PO₄·H₂O | 50        | 140       |
| Na₂HPO₄     | 200       | 200       |
| NaHCO₃      | 1000      | 2200      |

* Reg'd trademark.
† 13 ingredients of CMRL-1066 are not included in CMRL-1415.
§ Free base.
CO₂, a high level of arginine in the medium facilitates equilibrium between CO₂ and bicarbonate.

3. CARBOHYDRATES AND PYRUVATE: The importance of glucose in tissue culture media has long been recognized (14, 27, 1, 21, 8, 2, 6, 16). It is now known that other sugars can substitute for glucose (6), but when certain sugars (e.g., mannose and glucose) are metabolized approximately half of the carbohydrate used appears as lactic acid, which imposes a great burden on the buffering capacity of the medium. Chang and Geyer (2) observed that galactose in the presence of pyruvate could also substitute for glucose for the growth of certain human cell lines. Eagle et al. (6) confirmed this for certain other cell lines and observed that galactose was used more efficiently than other sugars, for as little as 3% of the galactose metabolized appeared as lactic acid. In 1959, Eagle (5) reported pyruvate to be required by embryonic mouse cells. Later, Leibovitz (13) devised a medium (L-15) containing 900 mg/liter of galactose as the only carbohydrate, and 550 mg/liter of sodium pyruvate, for the propagation of certain established cell lines in free gas exchange with the atmosphere. In view of the glucose-sparing action of galactose, especially with added pyruvate, and the corresponding decrease in lactic acid production, half of the glucose of CMRL-1066 was replaced with galactose and 225 mg/liter of sodium pyruvate were added. This combination of sugars and pyruvate gave good attachment and growth of mouse embryo cells; and excellent buffering action was obtained when the improved medium also contained monobasic and dibasic phosphates, four free-base amino acids, and a reduced level of sodium bicarbonate (Figs. 1 to 3).

4. VITAMINS AND COENZYMES: Medium CMRL-1415 contains six coenzymes that are as effective in mammalian cell cultures as their parent vitamins (9, 10). The nutritional value of the coenzymes that are substituted for their parent vitamins has been confirmed by Eagle (4) who also observed that p-aminobenzoic acid (PABA) was not required in the presence of folic acid. The following B-vitamins that were present in medium CMRL-1066, and are constituents of corresponding coenzymes, have been omitted from CMRL-1415: niacin and niacinamide (in DPN and TPN), pyridoxine (in codecarboxylase), thiamine.HCl (in cocarboxylase), and riboflavin (in FAD). PABA and coenzyme A have also been omitted from CMRL-1415 without adverse effects, and the level of calcium pantothenate has been increased 50-fold (Figs. 4 and 5). Two lipid sources (cholesterol and Tween 80) and ethanol, that were used in our earlier media as adjuncts to the lipid-soluble vitamins (17), have likewise been omitted from CMRL-1415.

5. CELL MULTIPLICATION IN MEDIA CMRL-1066 AND CMRL-1415: In a typical experiment, the population-doubling time of newly explanted mouse embryo cells in replicate cultures prepared with media CMRL-1066 and CMRL-1415 was approximately 12 and 5 days, respectively.

Preparation of Basal Medium CMRL-1415 for Use in Sealed Culture Containers

The ingredients of medium CMRL-1415 are obtained commercially and employed without further purification. Tenfold concentrated stock solutions are prepared with water passed through a Barnstead still and then through a Barnstead Bantam Demineralizer (Barnstead Still and Sterilizer Co., Boston, Massachusetts). All stock solutions are stored in the frozen state in Pyrex bottles, without filtration.

**SOLUTION 1:** To nearly 1 liter of water, heated to about 80°C and stirred continuously, are added the following: L-alanine, 0.3 g; L-arginine (free base), 5 g; L-aspartic acid, 0.1 g; L-cysteine (free base), 1.79 g; L-glutamic acid, 0.1 g; glycine, 0.17 g; L-histidine (free base), 0.31 g; hydroxy-L-proline, 0.1 g; L-isoleucine, 0.52 g; L-leucine, 0.52 g; L-lysine (free base), 0.47 g; L-methionine, 0.15 g; L-phenylalanine, 0.32 g; L-proline, 0.3 g; L-serine, 0.12 g; L-threonine, 0.48 g; L-tryptophan, 0.1 g; and L-valine, 0.46 g. After the solution has cooled to room temperature, 2.92 g of L-glutamine are added and the final volume is adjusted to 1 liter, with water.

**SOLUTION 2:** To nearly 100 ml of 0.3 N HCl heated to about 100°C and stirred continuously, are added: L-cystine, 0.24 g, and L-tyrosine, 0.36 g. After the solution has cooled to room temperature, the volume is adjusted to 100 ml, with water.

**SOLUTION 3:** To nearly 1 liter of water at room temperature, stirred continuously, are added the following: Ascorbic acid, 500 mg; d-biotin, 10 mg; calcium pantothenate, 5 mg; choline chloride, 10 mg; folic acid, 10 mg; mesoinositol, 20 mg; cocarboxylase, 10 mg; codecarboxylase, 10 mg; 1 Registered trademark.
Figure 1 Thirty-day-old culture (34306-17) of mouse embryo cells in medium CMRL-1066; MsE 206:Ts(7). (Embryos from mouse No. 206; culture is one of a series prepared after 3rd trypsinization of cells, 7 days after tissues were explanted from embryos). × 65.

Figure 2 Thirty-day-old culture (34306-1) in medium CMRL-1415; MsE 206:Ts(7). × 65.

Figure 3 Same culture (34306-1) shown in Fig. 2. Note giant cells that may have served as feeders. × 120.
Figure 4 Forty-eight-day-old culture (34355-7) in medium 1411-2 (modified CMRL-1066 with co-enzyme A replaced by 0.05 mg % calcium pantothenate); MsE 30S-T3(7). Note giant cells that may have served as feeders. × 190.

Figure 5 Another area of culture (34355-7) shown in Fig. 4. × 190.
diphosphopyridine nucleotide, 70 mg; flavin adenine dinucleotide, 10 mg; glutathione, 100 mg; triphosphopyridine nucleotide, 10 mg; and uridine triphosphate, 10 mg. The final volume is adjusted to 1 liter, with water.

**Solution 4:** To nearly 1 liter of 0.1 N NaOH at room temperature, stirred continuously, are added the following: deoxyadenosine, 100 mg; deoxycytidine, 100 mg; deoxyguanosine, 100 mg; 5-methyldeoxycytidine, 10 mg; and thymidine, 100 mg. The final volume is adjusted to 1 liter, with water.

**Solution 5:** To nearly 1 liter of water at room temperature, stirred continuously, are added the following: sodium chloride, 68.0 g; potassium chloride, 4 g; sodium dihydrogen phosphate monohydrate, 0.5 g; disodium hydrogen phosphate, 2 g; sodium bicarbonate, 10 g; and phenol red, 0.2 g. The final volume is adjusted to 1 liter, with water.

**Solution 6:** To nearly 1 liter of water at room temperature, stirred continuously, are added the following: calcium chloride, 1.4 g; magnesium sulfate heptahydrate, 2.4 g; d-glucose, 5 g; d-galactose, 5 g; and sodium pyruvate, 2.25 g. The final volume is adjusted to 1 liter, with water.

To prepare 1 liter of medium CMRL-1415, the stock solutions are completely thawed with agitation under running tap water, then allowed to warm to room temperature. To a 1-liter volumetric flask containing 400 ml of water are added the following, with thorough mixing between additions: solutions 1, 3, 4, 5, and 6, 100 ml each; solution 2, 10 ml. The final volume is adjusted to 1 liter, with ion-exchange water. The pH should be 7.4 ± 0.2 (glass electrode) and may be adjusted with 0.3 N HCl or NaOH. Finally, the completed medium is checked for isotonicity on a Fiske osmometer. The osmolarity should be 300 ± 10 milliosmols, and usually falls within this range. If the completed medium is found to be hypotonic, the addition of 30 mg NaCl per liter of medium will increase the tonicity 1 milliosmol. The completed medium is sterilized by filtration and stored at 4°C.

**Preparation of Basal Medium CMRL-1415-ATM for Use in Unsealed Culture Containers**

There are many cell and tissue culture undertakings, e.g., chromosome studies, embryological studies, and estimation of plating efficiencies, in which it is often desirable to incubate unsealed culture containers such as Petri dishes in free gas exchange with water-saturated atmospheric air, i.e., in simple moist chambers. Medium CMRL-1415-ATM, which has been especially designed for this purpose, is identical with CMRL-1415 except that the sodium bicarbonate has been omitted.

CMRL-1415-ATM, with or without serum or other supplements, may also be prepared at double strength and combined aseptically with an equal volume of nonnutrient agar (28) to provide a solid or semi-solid substrate for the cultivation of tissue fragments and developing organ structures.

**Supplementation of CMRL-1415 with Whole Serum**

With CMRL-1415, a supplement of 15% horse serum (to give CMRL-1415-SER) was necessary in order to provide the abundant cell multiplication required for subculturing the cell populations twice weekly. Lesser amounts of serum are adequate for the continuous propagation of cells multiplying at slower rates.

**Discussion**

In testing materials of nutritional interest on cells in tissue culture, it must be remembered (1) that many established cell lines (e.g., 929 L and HeLa cells) are much more readily adaptable than newly explanted cells to adverse conditions of many sorts including nutritional deficiencies in the medium, and (2) that a culture of newly explanted cells in chemically defined medium may remain healthy because the medium is favorable to all the cells or because cells unable to multiply may become giants that serve as feeders (19) for others. There is evidence in Figs. 3 to 5 that giant cells may have operated in this manner in the experiments reported here.

Newly explanted mouse embryo cells proved to be exceedingly sensitive to many of the variations that were made in the basal medium in the present study. Also, as shown in the accompanying paper, these cells were equally responsive to various protein and nonprotein supplements that have been tested in an effort to discover those things that are still lacking in defined, basal media of this sort.

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