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## **Culture of Lymphocytes and Hemopoietic Cells in Serum-Free Medium**

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Freshly explanted lymphocytes and hemopoietic cells can grow and differentiate to terminally functioning cells in culture. Such cultures provide an opportunity to study, in cells not previously subjected to *in vitro* selection, such phenomena as induction of growth in physiologically resting cells, immune recognition, and the function of specific polypeptide factors and cell surface receptors. There are circumstances in which it would be desirable to perform these cultures in the absence of serum. One may wish to assay or study the function of added polypeptide factors that may already be present in serum in unknown concentrations. In other situations, the desired responses may be inhibited by substances in serum, or may be induced via mechanisms irrelevant to the one under study. Particularly significant in the case of immune responses are the antigens and antibodies contained in serum over which the experimenter has no control.

In eliminating serum from the cultures to avoid interpretational difficulties, it is important not to introduce new ones. This could happen if responses in serum-free conditions were inferior to those obtainable with serum through failure to fulfill nutritive requirements of the cells. When the conditions described here were developed, emphasis was placed on achieving responses in serum-free cultures that were at least as good as those in conventional conditions. As it turned out, responses in serum-free cultures significantly surpassed those normally seen in conventional serum-containing media, particularly in lymphocyte cultures [Iscove and Melchers, 1978; Iscove et al., 1980; Schreier, 1981].

The essential serum-replacing elements in lymphocyte and hemopoietic cell cultures are transferrin, lipids, albumin [Guilbert and Iscove, 1976; Iscove and Melchers, 1978; Iscove et al., 1980; Schreier, 1981] and, in certain instances, cell type-specific polypeptide factors. Transferrin is recognized by now as almost universally required for cell growth in culture [Perez-Infante and Mather, 1982]. Lipids have received less attention, apparently because most work on serum replacement has been done with established cell culture lines that show little dependence on exogenous lipids. If the experience with lymphocytes and hemopoietic cells can be extrapolated, lipids may turn out to be a universal requirement for freshly explanted cells. The need for albumin is at least partly related to lipid supply and the inhibitory potential of unbound fatty acids [Milausen, 1978]. Strong dependence on the classical endocrine hormones has not been identified with these cells. Insulin has shown some stimulating effect in B-cell and erythroid cell cultures, which is marked only in suboptimal conditions (unpublished observations).

For all its disadvantages, serum forgives many sins in tissue culture technique. Details to which the experimenter may have given little thought become important in successful serum-free culture. The transition from conventional methods will require a considerable investment in time devoted to unlearning bad habits, to optimization, and above all to trouble-shooting.

## **NUTRIENT MEDIUM**

### **General Remarks**

The nutrient formula used in our studies is a modification of Dulbecco's medium and is designated IMDM (Table 1) [Guilbert and Iscove, 1976; Iscove and Melchers, 1978; Iscove et al., 1980]. It originated as a convenient way to approximate the composition of  $\alpha$ -medium [Stanners et al., 1971] at a time when the latter was not yet widely available. In its earliest version, it simply involved addition to Dulbecco's medium of a cocktail of "nonessential" amino acids, pyruvate, biotin, and vitamin B12 at their concentrations in  $\alpha$ -medium. Later, during studies involving serum replacement, osmolarity and pH were systematically optimized with corresponding changes in salt and bicarbonate concentrations. Hepes was added as a buffer against minor changes in ambient CO<sub>2</sub>. Selenite was included because it was effective on red cell precursor growth in low-serum conditions [Guilbert and Iscove, 1976]. Other trace metals (zinc, copper, manganese, molybdenum, vanadium) were repeatedly without effect, presumably because they are normally present at sufficient levels as contaminants in the nutrient mixture and in other constituents such as methyl cellulose. Nevertheless it may ultimately prove wiser to include them in the medium. Finally, the effect of individually doubling and quadrupling the concentration of each component (except the

TABLE I. Composition of IMDM<sup>a</sup>

Component	Mr	Molarity	mg/liter
L-Alanine	89.1	2.81 x 10 <sup>-4</sup>	25
L-Arginine • HCl	210.7	3.99 x 10 <sup>-4</sup>	84
L-Asparagine • H <sub>2</sub> O	150.1	1.89 x 10 <sup>-4</sup>	28.4
L-Aspartic acid	133.1	2.25 x 10 <sup>-4</sup>	30
L-Cystine • 2HCl	313.3	2.91 x 10 <sup>-4</sup>	91.2
L-Glutamic acid	147.1	5.10 x 10 <sup>-4</sup>	75
L-Glutamine	146.1	4.00 x 10 <sup>-4</sup>	584
L-Glycine	75.1	3.99 x 10 <sup>-4</sup>	30
L-Histidine • HCl • H <sub>2</sub> O	209.7	2.00 x 10 <sup>-4</sup>	42
L-Isoleucine • HCl • H <sub>2</sub> O	131.2	8.00 x 10 <sup>-4</sup>	105
L-Leucine	131.2	8.00 x 10 <sup>-4</sup>	105
L-Lysine-HCl	182.7	7.99 x 10 <sup>-4</sup>	146
L-Methionine	149.2	2.01 x 10 <sup>-4</sup>	30
L-Phenylalanine	165.2	4.00 x 10 <sup>-4</sup>	66
L-Proline	115.1	3.48 x 10 <sup>-4</sup>	40
L-Serine	105.1	4.00 x 10 <sup>-4</sup>	42
L-Threonine	119.1	7.98 x 10 <sup>-4</sup>	95
L-Tryptophan	204.2	0.784 x 10 <sup>-4</sup>	16
L-Tyrosine • 2Na	227.2	4.58 x 10 <sup>-4</sup>	104
L-Valine	117.2	8.02 x 10 <sup>-4</sup>	94
Glucose	180.2	2.50 x 10 <sup>-2</sup>	4,500
Pyruvate • Na	110.0	1.00 x 10 <sup>-3</sup>	110
Choline • Cl	139.6	2.87 x 10 <sup>-5</sup>	4
i-Inositol	180.2	4.00 x 10 <sup>-5</sup>	7.2
Nicotinamide	122.1	3.28 x 10 <sup>-5</sup>	4
Pyridoxal • HCl	203.7	1.96 x 10 <sup>-5</sup>	4
Riboflavin	376.4	1.06 x 10 <sup>-6</sup>	0.4
Thiamine • HCl	337.3	1.19 x 10 <sup>-5</sup>	4
D-Ca-Pantothenate	238.3	1.68 x 10 <sup>-5</sup>	4
Folic acid	441.4	9.06 x 10 <sup>-6</sup>	4
Biotin	244.3	5.32 x 10 <sup>-8</sup>	0.013
Vitamin B12	1,355	9.59 x 10 <sup>-9</sup>	0.013
NaCl	58.45	7.71 x 10 <sup>-2</sup>	4.505
KCl	74.55	4.43 x 10 <sup>-3</sup>	330
CaCl <sub>2</sub>	111.0	1.49 x 10 <sup>-3</sup>	165
MgSO <sub>4</sub>	120.4	8.11 x 10 <sup>-4</sup>	97.7
NaH <sub>2</sub> PO <sub>4</sub>	120.0	1.04 x 10 <sup>-3</sup>	125
KNO <sub>3</sub>	101.1	7.52 x 10 <sup>-7</sup>	0.076
Na <sub>2</sub> SeO <sub>3</sub>	172.9	1.00 x 10 <sup>-7</sup>	0.0173
Hepes (free acid)	238.3	2.50 x 10 <sup>-2</sup>	5.958
NaHCO <sub>3</sub>	84.0	3.60 x 10 <sup>-2</sup>	3.024
Phenol red	376.4	3.99 x 10 <sup>-5</sup>	15

<sup>a</sup>Composition of complete powered medium. When the medium is prepared from Dulbecco's powdered medium, the concentrations of most of the components will be only 80% of those listed here.

salts) was examined in cultures of erythroid, granulocyte, and macrophage precursors containing 1% fetal calf serum (FCS). A positive effect was observed with increased cystine. Its concentration was finally increased 50% above that in the original Dulbecco's formulation. Other components were neither helpful nor inhibiting at the higher concentrations.

The osmolarity of Dulbecco's medium (Gibco formulation 074-2100) is about 350 mosM, at the limit of tolerance of mouse lymphocytes and hemopoietic cells. Optimum osmolarity (20% dilution, to 280-285 mosM) was determined from the effects of incremental addition of water to the formula. The inclusion of hepes imposed an additional osmotic burden. Once 280 mosM had been determined to be optimal, it was subsequently set in medium containing hepes by appropriate reduction of the amount of NaCl in the powder formula. Optimum osmolarity for human hemopoietic cells in methylcellulose cultures is somewhat lower, near 250 mosM (T. Hoang and C. Nissen, personal communications). pH balance was similarly optimized by stepwise addition of isoosmotic HCl or NaOH to the medium to produce incremental alteration in the equilibrium incubator pH with CO<sub>2</sub> held constant at 5%. The amount of added acid or base at which optimum growth occurred was noted, and the amount of NaHCO<sub>3</sub> in the formula was correspondingly altered. The final result was a medium in which growth was optimal without addition of acid or base. Cells of other vertebrate classes (fish, amphibia, birds) will have osmolarity and pH optima different from those of mammalian cells.

A sulfhydryl compound, either  $\beta$ -mercaptoethanol ( $5 \times 10^{-5}$  M) or  $\alpha$ -thioglycerol ( $7.5 \times 10^{-5}$  M) is routinely added to the medium at the time of preparation. These compounds have a marked beneficial effect on growth of lymphocytes [Broome and Jeng, 1973] and on hemopoietic cells particularly of the erythroid lineage [Iscove and Sieber, 1975]. Their activity is independent of whether they are added in the reduced or oxidized state [Broome and Jeng, 1973; Iscove and Sieber, 1975]. They are not known to have adverse effects on any type of cell at these concentrations. Both compounds are similarly effective in practice.  $\alpha$ -thioglycerol might be preferable because it is less volatile, having the advantages of being more stable in concentration and less strongly smelling.

It is preferable to make up the medium from powder rather than to obtain it from the commercial supplier in liquid form, where the user relinquishes control over the quality of the water and the conditions of storage.

### Preparation

**From Dulbecco's medium.** The following stock solutions are prepared:

1. Double-strength Dulbecco's medium (Gibco 074-2100)

Dissolve 1 package of powder (sufficient for 1 liter of single-strength medium) in 500 ml H<sub>2</sub>O. Filter-sterilize (0.2 μm) and store at 20° C in the dark.

2. NaHCO<sub>3</sub>, 25 g  
H<sub>2</sub>O to 250 ml  
Dissolve at 37° C. Filter-sterilize and store at room temperature.
3. Vitamin B12, 1 mg  
Biotin, 1 mg  
HCl 1 mM, 4 ml  
Dissolve and store at 20° C.
4. Amino acids  
L-alanine, 250 mg  
L-asparagine, 250 mg  
L-aspartic acid, 300 mg  
L-glutamic acid, 750 mg  
L-proline, 400 mg  
Na pyruvate, 1100 mg  
H<sub>2</sub>O 96 ml  
Dissolve at 40° C with agitation. Add B<sub>12</sub>/biotin (stock 3), 0.64 ml.  
Filter-sterilize and store in aliquots at 20° C.
5. Cystine, 280 mg  
H<sub>2</sub>O, 30 mg  
HCl 1M, 10ml  
Dissolve, filter-sterilize, and store in aliquots at 20° C.
6. Sodium selenite, 1.73 mg  
H<sub>2</sub>O, 10ml  
Dissolve, filter-sterilize, and store at 4° C.

IMDM is prepared from the stock solutions:

	<u>Stock</u>	<u>Volume</u>
Double-strength Dulbecco's	1	410 ml
NaHCO <sub>3</sub>	2	25
Hepes 1 M, pH 7.3 (Gibco or KG Biologicals)		25
Amino acids	4	8
Cystine	5	4
Sodium selenite	6	0.1
β-Mercaptoethanol 1% vol./vol.		0.35
or		
α-Thioglycerol 1% vol./vol.		0.63
Antibiotics		
H <sub>2</sub> O		to 1,000 ml

**From powdered IMDM.** Dissolve the contents of 1 package of powder (17.7 g, sufficient for 1 liter of single-strength medium) together with 3.024 g NaHCO<sub>3</sub> in about 900 ml water. Add the appropriate amounts of antibiotics and either  $\beta$ -mercaptoethanol or  $\alpha$ -thioglycerol. Bring the total volume to 1,000 ml with H<sub>2</sub>O.

Filter-sterilize (0.2  $\mu$ , ignoring any residue that has not dissolved after 15 min of stirring. Store at 4° C in the dark for periods up to 2-3 weeks, preferably under 5% CO<sub>2</sub>/air. Storage for longer periods should be at -20° C. Medium containing albumin, transferrin, and lipids can also be stored for 2-3 weeks at 4° C, but the liposomes will not tolerate freezing.

It is illogical to adjust the pH of medium with acid or base at the time it is prepared. Its equilibrium pH is governed primarily by ambient CO<sub>2</sub> and by the formula concentrations of bicarbonate and hepes. The contribution of the amino acids and the phosphate is quantitatively minor. The final pH under 5% CO<sub>2</sub> is not significantly affected by substitution of a few individual amino acids by their corresponding hydrochlorides or sodium salts.

### Photosensitivity

Culture media that contain riboflavin deteriorate on exposure to light in the visible blue and near ultraviolet regions of the spectrum (absorption maxima of riboflavin at 475, 444, 370, 270, and 225 nm). Hydrogen peroxide and possibly superoxide radicals are produced and accumulate in the medium on such exposure [Wang and Nixon, 1978]. It is therefore counterproductive to store medium in an illuminated cold room. Exposure to fluorescent light at the work place should also be minimized. "Gold" fluorescent tubes that have no emission at wavelengths shorter than 500 nm are available from General Electric.

### Stability of the Powder Formulation

When IMDM is to be used in cultures containing serum, problems with stability of the IMDM powder may not be apparent. On the other hand, the powdered formulation has proved unreliable for use in serum-free cultures. When tested in cultures of erythroid precursors and spleen T cells, fresh powder supplied in sealed packages sufficient for preparation of 10 liters of liquid medium performed well on receipt. However, performance declined after storage of unopened packages at 37° in a dry atmosphere for 7 weeks. This period of time would correspond to approximately 6 months of storage at 4° C. In contrast, no such decline has been observed with the powdered Dulbecco's formulation (Gibco No. 074-2100). For the purpose of serum-free culture, IMDM prepared from powdered Dulbecco's medium has accordingly proved the most reliable. The cause of instability of the complete formulation is not clear.

### Hepes

Commercially supplied hepes can be toxic in culture, presumably because of heavy-metal contamination. The liquid 1 M solution (supplied by Gibco or KC Biologicals already titrated to pH 7.3) has usually been satisfactory. One can only hope that hepes of a similar grade of purity is used for preparation of the complete IMDM powder, a step over which the user has no control. Passage of an inhibitory hepes solution over a column of Chelex 100 (Bio-Rad) can be helpful. Chelex resin preferentially complexes transition metals.

### WATER AND GLASSWARE

Our experience has been limited to the use of water purified sequentially through deionizing resin, activated charcoal, and three stages of glass distillation. The water is collected fresh from distillation into glassware prerinsed with the same water.

Glassware fresh from manufacture should not be used for preparation or storage of medium or medium components. Such glass contains metal impurities that can slowly enter solution at the glass surface. The vessels should be soaked in water with daily changes for several days before use. The use of fresh glassware may account for the consistent unsuitability of commercially bottled medium for serum-free culture in our experience.

### TRANSFERRIN

#### General Remarks

Transferrin is the major iron transport protein in the plasma [MacGillibray et al., 1982]. It is required by most cells for growth in serum-free medium, and there is evidence to suggest that its role involves iron delivery [Perez-Infante and Mather, 1982]. Each transferrin molecule (Mr 80,000) has two combining sites for ferric iron ( $\text{Fe}^{3+}$ ). The affinity for iron is very high but is reversibly lost at pH below 7. These sites can also hold other transition metals (more weakly than  $\text{Fe}^{3+}$ ), and a role for transferrin in supplying zinc to cells has been suggested [Phillips and Azari, 1974]. A suitably purified preparation of human apotransferrin is available from Hoechst-Behringwerke, Marburg, Federal Republic of Germany. Unusable batches have been infrequent and the major potential problem to be anticipated would be contamination with buffers or salts used in its purification.

#### Preparation

Ferric chloride solution is prepared by rapidly weighing a large lump of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  (hygroscopic!) and dissolving it in 1 mM HCl. A 10 mM stock

solution is then made by dilution into 1 mM HCl and stored in aliquots at  $-20^{\circ}$  C.

Purified human transferrin (Hoechst-Behringwerke), 120 mg, is dissolved in 4 ml of either bicarbonate-free Dulbecco's medium containing 10 mM hepes, or bicarbonate-free IMDM containing 25 mM hepes. In either case, the pH of the vehicle is brought to 7.4 with 1 M NaOH before addition of the transferrin. When the transferrin has dissolved, 270  $\mu$ l of 10 mM ferric chloride is added for 90% saturation of the iron sites. Mix immediately. Binding is practically complete within seconds. Iron in excess of saturation of these sites may be inhibitory. Filter-sterilize and store indefinitely at  $4^{\circ}$  C.

Although problems with this method of iron addition have not been encountered in practice, the reactions of ferric iron in neutral solution are not straightforward [Bates and Schlabach, 1973].

### Use

Typical concentration optima are 30  $\mu$ g/ml in liquid cultures and 300  $\mu$ g/ml in semisolid cultures. Serum contains about 3 mg/ml, so these are the amounts supplied by 1% and 10% serum, respectively.

## ALBUMIN

### General Remarks

Albumin is the major plasma protein. Most of the unesterified fatty acid in plasma is albumin-bound [Specter, 1975]. Some freshly explanted cells (erythroid precursors, for example) are absolutely dependent on exogenous unsaturated fatty acid for growth. However, unbound fatty acids are generally toxic to cells in culture except at concentrations too low to satisfy the requirement of growing cells for any length of time [Nilausen, 1978]. Albumin provides a convenient means of adding sufficient fatty acid while buffering it to a very low free concentration. Although this is probably the major function of albumin in these cultures, it is also able to bind other hydrophobic substances (including some amino acids) as well as metal ions (including  $\text{Ca}^{2+}$ ) [Allen et al., 1977] and might play a role in reducing their free concentrations to tolerable levels.

Albumin is difficult to purify exhaustively on a large scale. The Cohn fraction V material is only a crude serum product, enriched in albumin but containing significant quantities of other proteins. Cleaner preparations are commercially obtainable, but none, including "crystallized" preparations, would exceed 98% purity. In addition to contaminating proteins, albumin preparations can be presumed to contain other hydrophobic substances including fatty acids and steroid hormones, and traces of salts and buffers used in the purification along with their contaminating transition metal ions.

The procedures given here are designed to remove most of the ionic and smaller hydrophobic passengers, but not contaminating proteins, from commercially obtained albumin. In situations where the required concentration of albumin is relatively low, e.g., 500  $\mu\text{g}/\text{ml}$  in liquid lymphocyte cultures, it may be possible to dispense with delipidation or deionization depending on the particular albumin batch. At the higher albumin concentrations used in methyl cellulose cultures, deionization has always been helpful, and delipidation has occasionally salvaged an otherwise unusable batch.

Our only experience has been with the purified albumin supplied by Hoechst-Behringwerke. Batches are usually but not always suitable for use, and it may be necessary to test samples of different lots. Such selection has been most important for the culture of erythroid precursors. The basis of the variation is not known.

### Preparation

Dissolve 200 mg of Dextran T40 (Pharmacia) in 200 ml  $\text{H}_2\text{O}$  in a 500-ml beaker. Add 2 g activated charcoal (Norit SX-1 or Norit A) and suspend with occasional agitation at room temperature for 30 min. Add 10 g BSA powder (Hoechst-Behringwerke) to the top of the fluid and allow it to dissolve completely at room temperature without stirring (1-2 h). Then slowly add 1 M HCl dropwise with rapid stirring to bring the pH down to 3.0. Heat in a water bath to 56° C for 30 min with frequent agitation. Centrifuge for 30 min at 2,700g to remove the charcoal. Adjust to pH 5.5 with 1 M NaOH with rapid stirring. Remove the remaining charcoal by successive passage through 47-mm-diameter 1.2- $\mu\text{m}$  and 0.45- $\mu\text{m}$  Millipore filters. Deionize overnight at 4° C in a 500-ml to 1,000-ml Erlenmeyer flask over about 20  $\text{cm}^3$  of Amberlite mixed bed ion exchange resin MB-2 (Serva). Diffusion distances are minimized if the solution overlying the resin is shallow. Remove the resin by filtration through prewashed cotton gauze and concentrate by ultrafiltration over an Amicon Diaflo UM-10 membrane to 50 ml. Adjust the pH to 7.0 with 1 M HCl or NaOH added dropwise with rapid stirring. Filter-sterilize and store aliquots indefinitely at 4° C. The precise albumin concentration can be determined by measurement of optical density at 280 nm.

The pH often changes during deionization. This may reflect the presence of buffer salts (e.g., ammonium bicarbonate) used in preparation of the albumin and incompletely removed by the supplier.

See Materials and Methods in Iscove et al. [1980] for additional comments and references on these procedures.

### Use

Typical concentration optima are 500  $\mu\text{g}/\text{ml}$  in liquid cultures and 10 mg/ml in semisolid cultures. Serum contains up to 40 mg/ml, and these amounts could be supplied by 1% and 25% serum, respectively.

## LIPIDS

### General Remarks

Serum-free growth of freshly explanted lymphocytes and hemopoietic cells depends partially or entirely on added lipid. The lipid requirement for colony formation by CFU-E is satisfied by the combination of unsaturated fatty acid (oleic acid), dipalmitoyl phosphatidyl choline ("lecithin"), and a high concentration of cholesterol [Iscove et al., 1980]. The same mixture also supports the growth of granulocyte and macrophage colonies but has not been optimized for them. The requirements of T and B lymphocytes remain to be defined. At present they grow with a crude lipid composite from soybean. This lipid extract contains about 50% plant lecithin (phosphatidyl choline) along with other phosphatides (phosphatidyl ethanolamine, phosphatidyl inositol), sterols including cholesterol and sterol glycosides, triglycerides, tocopherols (vitamin E), and possibly free fatty acids. T and B lymphocytes depend increasingly on exogenous lipid with decreasing seeding densities. At a relatively low initial density of  $2 \times 10^5$  spleen cells/per 1 ml of serum-free medium, lipid enhances the B-cell mitogenic response to LPS fourfold, and the T-cell mitogenic response to Con A by 50% [Iscove and Melchers, 1978; and unpublished observations].

Different cells vary in their requirement for cholesterol. Terminal erythroid maturation depends on a high concentration of cholesterol in the medium ( $8 \mu\text{g/ml}$ ), probably related to the high concentration of cholesterol in the erythrocyte cell membrane. On the other hand, high levels of cholesterol can be toxic to B and T lymphocytes with inhibition becoming increasingly apparent at cell concentrations below  $5 \times 10^5$  per 1 ml.

Exogenous cholesterol and some of its oxidation products can interfere with cholesterol synthesis in cells [Kandutsch et al., 1978; Cornell et al., 1977]. The effect occurs via inhibition of hydroxymethylglutarate-CoA reductase, blocking production of mevalonate, a cholesterol precursor. Addition of mevalonic acid to cultures bypasses this block but does not alleviate the inhibitory effect of cholesterol on growth of B and T lymphocytes (unpublished observations). It is therefore unlikely that the inhibitory effect of excess cholesterol on lymphocyte growth is related to the presence of oxidized impurities.

When soybean lipid is titrated in B- and T-cell cultures, the optimum effect occurs in a narrow dose range ( $\pm 30\%$ ). The inhibitory effect of excess concentrations is due at least in part to the ability of phospholipid vesicles to extract cell membrane cholesterol [Cooper et al., 1975]. Low concentrations of cholesterol (e.g.,  $1 \mu\text{g/ml}$  medium, 2.5% wt./wt. of the soybean lipid) can be beneficial in broadening the usable lipid dose range. However, there is a variable amount of cholesterol already present in the commercial soybean lipid mixture. The optimal amount of cholesterol to add (if any) must therefore be determined empirically for each soybean lipid batch.

The addition of lipids to the medium is complicated because of their insolubility in water. One method is to add them in the form of a solution in a water-miscible organic solvent such as ethanol. Another is to prepare them as phospholipid liposomes in stable aqueous suspension. This approach avoids the use of toxic solvents and also permits the introduction of the lipid into the medium in a controlled physical state. The latter method will be described in detail. The suspending medium for the lipids contains albumin. Suspensions were found to be less stable without albumin, precipitating on storage at 4° C. Its role is likely to involve binding of free fatty acid. (Indeed, small amounts of fatty acid, e.g., 4 µl linoleic acid in 5 ml 1 % bovine serum albumin [BSA], can be readily transferred to albumin by a minute or so of sonication. The result is a stable clear solution without need to resort to the use of organic solvents.)

### Preparation of Lipid Suspensions

*Suspending medium* is prepared each time lipids are to be sonicated.

Acid Dulbecco's (see below), 47.5 ml

BSA (20% stock, charcoal extracted and deionized), 2.5 ml

Adjust pH to 6.8 with 1 M NaOH.

(Preparation of vesicles small enough to pass through a sterilizing filter requires less energy at pH 6.8 than at the pH of 5.1 reported earlier) [Iscoe and Melchers, 1978; Iscoe et al., 1980].

Acid Dulbecco's is prepared by dissolving powdered Dulbecco's medium (Gibco 074-2100), sufficient for 1 liter of medium, in 1 liter of water without bicarbonate or other additives. Filter-sterilize and store at 20° C.

*Lipids* are weighed out onto the bottom of a beaker. When mixtures of lipid stocks are prepared (e.g., soybean lipid plus added cholesterol), the lipids are uniformly mixed by being dissolved completely in a few drops of chloroform. The solution is then dried onto the bottom of the beaker under a gentle stream of nitrogen. Drying is complete a few minutes after the chloroform odor can no longer be detected.

**For lymphocytes and granulocyte/macrophage colonies.** Soybean lipid "Astec FLPV," is obtained from Associated Concentrates, 32 61st Street, Woodside, Long Island, New York 11377. It is a highly viscous fluid. Store at -20° C and protect from moisture. Weigh out into a 100 ml beaker.

Soybean lipid, 200 mg

Cholesterol (optional), 4 mg

If cholesterol is included, dissolve with the soybean lipid in a few drops of chloroform and dry. Add 50 ml suspending medium and sonicate (see below).

**For erythroid colonies.** Dipalmitoyl phosphatidyl choline (=dipalmitoyl lecithin, or dipalmitoyl PC), cholesterol and oleic acid are stored at 4° C in the dark in air-tight containers. Oleic acid is solid at 4° C, liquid at room temperature. Weigh out into a 20 ml beaker.

Dipalmitoyl phosphatidyl choline, 20 mg  
Cholesterol, 21 mg  
Oleic acid, 15.4 mg (=17.2  $\mu$ l)  
Add 5 ml of suspending medium and sonicate.

*Sonication* disperses the lipid in the form of vesicles ("liposomes," reviewed in Poste et al. [1976]). The object is to produce vesicles sufficiently small to pass through the pores of a sterilizing filter (0.45  $\mu$ m). The following considerations are only approximate but provide guidelines for the length of sonication time required. The final limiting size of the liposomes is a function of the intensity of the sonic field and the time the liposomes spend within it. The sonic field is most intense in the cylindrical volume projecting downward from the flat face of the sonic probe. Its intensity there is inversely proportional to the area of the face of the probe. Sonication efficiently circulates the fluid, and average time of a particle within the high-energy field is therefore inversely proportional to the total volume of fluid, and directly proportional to the area of the probe face.

Therefore:

For a given probe and energy level, sonicate twice as long for a volume twice as large.

For a given volume and energy level, a 1-cm diameter probe can finally produce smaller vesicles than a 2-cm probe but may take longer to finish the job.

We use a 1-cm-diameter probe on a 60-W Measuring and Scientific Equipment, Ltd. sonicator, and allow about 1 min sonication time per 1 ml of fluid at maximum energy. Sonication is performed under air. Heat is dissipated by surrounding the beaker containing the sonicate with an abundant quantity of ice (in water!) and renewing the ice supply as necessary during the course of sonication. Particularly when using soybean lipid, it may be helpful to begin by suspending the material coating the bottom of the beaker by manually moving the beaker so as to expose all portions of the bottom to the high-energy sonic field.

At the end of the sonication period, a few small aggregates of soybean lipid may have escaped dispersion. These are ignored. The suspension is passed once through a membrane of 1.2  $\mu$ m pore size (47 mm diameter for 50 ml) after which it usually passes without difficulty through a sterilizing 0.45  $\mu$ m filter. If this filter blocks rapidly, it may be helpful to pass the suspension once more through a 1.2  $\mu$ m membrane. The grey residue on the filter is particulate metal shedded from the sonicator probe. Filtration is easiest immediately after sonication and should not be delayed.

The resulting suspensions range from moderately opalescent in the case of the synthetic mixture to almost opaque in the case of the soybean lipid preparations. Cholesterol and triglyceride increase opacity. The lipid suspensions are stored at 4° C. They may become somewhat more opaque in the

first day or two of storage. This change probably reflects some coalescence of the liposomes to larger average size but has no influence on their effectiveness in culture. Freezing will probably destroy the liposomes. The synthetic mixture for erythroid colonies is stable for at least several months. The soybean lipid preparations remain effective for at least a few weeks.

### Use

Because of quantitative variation in the completeness of dispersion and retention on the sterilizing filters, each individual lipid preparation should be titrated in culture to determine its optimal concentration. Guidelines for appropriate concentrations in erythroid cell cultures can be found in Iscove et al. [1980]. In liquid cultures of  $2 \times 10^5$  unseparated mouse spleen cells per 1 ml, optimal soybean lipid concentrations are approximately  $50 \mu\text{g/ml}$  for the B-cell mitogenic response to bacterial lipopolysaccharide and  $12.5 \mu\text{g/ml}$  for the T-cell mitogenic response to concanavalin A.

Filter-sterilization of medium which has already been supplemented with lipid should be avoided, since there is a risk of retaining an indeterminate amount of lipid on the filter.

## SEMISOLID CULTURES

### General Remarks

The choice of "gelling" agent ordinarily lies between agar and methyl cellulose. The usual "bacto agar" used for this purpose is a rather crude product containing biologically active substances (discussed in Hoang et al. [1981]). A more purified agent might be preferred for work in defined conditions. Agarose might be one choice. Our experience has been with methyl cellulose (Methocel A4M Premium, Dow Chemical; the premium grade has been more thoroughly washed of salts and metals). Methyl cellulose has the advantage in some applications that medium containing it is viscous rather than semisolid at room temperature. This property permits cells and colonies to be observed in a single plane at the bottom of the culture vessel and also facilitates their physical manipulation or resuspension if desired.

The gelling properties of methyl cellulose are inverse to those of agar: Methyl cellulose solutions of the A series gel at temperatures above  $45^\circ \text{C}$ . Although the solutions are fluid at lower temperatures, the thixotropic properties of A series methyl cellulose result in a gel-like state at incubator temperature ( $37^\circ \text{C}$ ) if the fluid is not disturbed. This property appears to be important for preservation of colony morphology. Hydroxypropylmethyl celluloses of the E and F series, with gelling points above  $50^\circ \text{C}$ , fail to preserve colony morphology at  $37^\circ \text{C}$  despite having viscosities identical to that of A series methyl cellulose.

The solubility of methyl cellulose is a function of the extent of methyl substitution achieved in manufacture. This varies from batch to batch and determines the concentration of undissolved fibers visible in the cultures. A batch of methyl cellulose A4M with a low "fiber rating" can be specifically requested from Dow. Hydroxypropylmethyl celluloses are more soluble but, as already mentioned, are unsuitable for semisolid culture.

Details on the preparation and use of methyl cellulose are given elsewhere [Iscove and Schreier, 1979].

The optimum concentrations of both albumin and transferrin are 10 times higher in methyl cellulose than in liquid cultures. It is not clear whether this higher requirement arises because of decreased convection or because of the need to buffer inhibitory substances present in the methyl cellulose.

### TROUBLESHOOTING

Most of the potential problems have already been mentioned. Items in the following list require continual vigilance (on the basis of bitter experience).

1. Purity of water (should only be used *fresh* from distillation)
2. Cleanliness of glassware (traces of detergent; new glassware)
3. Atmospheric contaminants (organic solvents, fixatives etc., e.g., formaldehyde; volatile additives in the incubator humidifying water (e.g., azide); volatile organics or polymerizers in plasticware, plastic air lines; volatile solvents in adhesive tape inside the incubator; ozone generated from sterilizing UV lamps especially where a transparent air supply line is exposed; ozone generated from a faulty motor inside the incubator CO<sub>2</sub> supply unit)
4. Exposure of medium to light (blue and near-UV)
5. Individual batches of plasticware (require pretesting)
6. UV irradiation of plastic tissue culture ware
7. Exposure of plastic tissue culture ware to organic vapors
8. Individual batches of albumin, transferrin, soybean lipid, hepes, powdered medium (age)
9. State of health of experimental animals

Because of the large number of variables, finding the cause when things do not work can be difficult and time-consuming. There are two cardinal rules that simplify things if followed: 1. Keep records adequate for tracing batch-related difficulties. All batches (components, mixtures, materials) should be uniquely labeled and specified in all experimental protocols. 2. Reserve samples of all components and materials (including plasticware) known to perform satisfactorily, and use them only for troubleshooting.

When serum-free culture is first attempted, stocks of known effectiveness will normally not be on hand. In this instance it may be useful to proceed by stepwise reduction of serum. A simple model system should be used for these tests, e.g., erythropoietin-dependent colony formation by mouse CFLJ-E, or the mitogenic response of unseparated spleen cells ( $3 \times 10^6$  per ml) to LPS or concanavalin A. Begin by confirming that IMDM works with the conventional concentration of serum. Then reduce the amount of serum to the point where the response falls to, say, 30% of optimum. Confirm that the combination of albumin, transferrin, and lipids is effective and then determine the optimum concentration of each addition (including the mitogen) in the presence of the others. Again reduce (or eliminate) the serum in cultures containing albumin, transferrin, and lipid, and determine once more the optimum concentration of each additive. In this way tests are performed in the presence of some nonzero response, allowing, for example, quantitative comparison of different batches of materials. When a more effective batch of a given constituent is obtained and incorporated in the mixture, optimal concentrations of the other additives should be determined again.

### CONCLUDING REMARKS

The medium described here was designed for serum-free culture of freshly explanted lymphocytes and hemopoietic precursors. The requirements of established cell lines, long selected by serial passage, are less stringent than those of freshly explanted cells. Most T- and B-cell lines and hybridomas can be grown in the serum-free conditions described here [Fazekas de St. Groth, 1983; Muzik et al., 1982; F. Melchers, M. Schreier, C. Paige, and S. Fazekas de St. Groth, personal communications]. Although full supplementation with albumin, transferrin, and lipids is the most effective with the greatest number of lines, some lines may not require exogenous lipid.

The responses of murine T and B lymphocytes, and of red cell precursors (CFU-E) meet or exceed those obtained in earlier serum-containing media. For example, the mitogenic response of  $2 \times 10^5$  spleen cells per 1 ml to bacterial lipopolysaccharide or concanavalin A is two to five times higher in optimum serum-free conditions than in RPMI 1640 supplemented with 8% fetal calf serum,  $\beta$ -mercaptoethanol, and glutamine [Iscove and Melchers, 1978; and unpublished observations]. The cloning efficiency of bone marrow CFU-E in serum-free methyl cellulose cultures [Iscove et al., 1980] is typically twice that obtained earlier in medium containing 30% fetal calf serum before the IMDM formula was optimized [Iscove and Sieber, 1975]. However, much remains to be done. There may be some benefit from a component-by-component optimization of the nutrient formula for particular cell types at very low seeding densities [Ham and McKeehan, 1978]. The lipid require-

ments of other hemopoietic precursors and of the various lymphocyte populations need to be more precisely defined. In the present conditions, multilineal colonies from pluripotential precursors and erythroid/megakaryocyte colonies can grow in methyl cellulose in the absence of serum, but the colonies are smaller and deficient in numbers of terminally differentiated red cells and granulocytes. Macrophage colonies grow well, but granulocytic maturation is not prominent. It remains to be seen whether the problems reflect only "nutritive" lack or imbalance, whether they reflect the need for "housekeeping" functions provided, for example, by serum protease inhibitors, catalase, superoxide dismutase, etc., or whether they reflect the absence of more specific factors normally provided by serum. Finally, conditions for serum-free culture of human cells as good as those for mouse cells also have yet to be worked out.

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