



Common Reagents and Medium for Mammalian Cell Culture

Srirupa Mukherjee, Parth Malik, and Tapan Kumar Mukherjee

Contents

1	Introduction	139
2	Physiological Parameters Necessary for the Culture of Mammalian Cells	141
2.1	Culture Vessels with Surface Property	141
2.2	Composition of Cell Culture Medium	141
2.3	pH of the Cell Culture Medium	141
2.4	Temperature and Light	141
2.5	Humidity of the CO ₂ Incubator	141
2.6	Subculture and Feeding with Culture Medium	142
3	Cell Dissociation Agents	142
4	Cell Adhesive Agents	143
4.1	Fibronectin	143
4.2	Laminin	144
4.3	Cell Adhesion to the Extracellular Matrix	146
5	Cell Culture Medium and its Constituents	146
5.1	Composition of Cell Culture Medium	147
5.2	Nutritional Composition of the Cell Culture Medium	148
5.3	Usefulness of Serum in the Cell Culture Medium	155
5.4	Essential Nutrients Are Provided by the Serum	155
5.5	Adherence and Spreading of Cells Are Mediated by the Serum	156
5.6	Hormones and Various Growth Factors Are Provided by the Serum	156
5.7	Serum-Mediated Protection for Some Specific Cells	156
5.8	Disadvantages of Using Serum in the Cell Culture Medium	156
6	Classification of Media Based on the Presence or Absence of Serum	157
6.1	Basal Medium	157
6.2	Reduced Serum Medium	158

S. Mukherjee

Department of Immunopathology, Research Block A, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India

P. Malik

School of Chemical Sciences, Central University of Gujarat, Gandhinagar, India

T. K. Mukherjee (✉)

Biotechnology, Amity University, Noida, Uttar Pradesh, India

6.3	Serum-Free Medium	158
6.4	Advantages of Using A Serum-Free Medium	159
6.5	Cautions to be Exercised in a Serum-Free Culture	159
6.6	Regarding the Use of Antibiotics and Antimycotics in Serum-Free Medium	159
6.7	Regarding the Maintenance of Cell Density in Serum-Free Medium	160
6.8	Morphological Changes During Shifting of Cells from Serum-Containing to Serum-Free Medium	160
6.9	Clumping During Shifting of Cells from Serum-Containing to Serum-Free Medium	161
6.10	Formulation of Serum-Free Medium	161
6.11	Protein-Free and Chemically Defined Media	162
6.12	Types of Culture Media	162
7	Common Cell Culture Media	163
7.1	Minimum Essential Medium	163
7.2	Alpha Minimum Essential Medium	164
7.3	Glasgow Minimum Essential Medium	165
7.4	Dulbecco's Modified Eagle's Medium	166
7.5	Iscove's Modified Dulbecco's Medium	167
7.6	Roswell Park Memorial Institute Medium	167
7.7	M 199 Medium	168
7.8	HAM'S F-10 Medium	168
7.9	Ham's F-12 Medium	169
7.10	Kaighn Modified Ham's F-12 Medium	169
7.11	DMEM-F12 Medium	170
7.12	RPMI-1640/DMEM/F-12 Medium	170
7.13	McCoy's 5A Medium	170
7.14	Leibovitz L15 Medium	171
7.15	Waymouth's Mb752/1 Medium (Waymouth 1959)	171
7.16	Trowell's T-8 Medium	171
8	Medium Recommendations for Common Cell Lines	172
8.1	Recommended Media for Common Cell Types	172
9	Common Buffer and Solutions for Cell Culture	174
9.1	Mechanism of Action of Various Basal Salt Solutions	174
9.2	Preparation of Earle's Balanced Salt Solution	175
9.3	Preparation of Dulbecco's Phosphate Buffer Saline	177
9.4	Normal Saline	178
9.5	Alsever's Solution	179
10	pH Maintenance of the Cell Culture Medium	180
10.1	Maintenance of Osmolarity/Osmolality of the Cell Culture Medium	181
11	Antibiotics and Antimycotics	181
12	Conclusions	183
13	Cross-References	183
	References	184

Abstract

This chapter describes all common materials and reagents necessary for mammalian cell culture. Mammalian cells are normally present in tissues and organs. These cells attach to the extracellular matrix (**ECM**). So, **cell-dissociating agents** are highly essential to detach cells from the tissues/organs. Most mammalian cells are adherent in nature, except for some blood cells (e.g., B and T lymphocytes). The next important material needed for mammalian cell culture is **cell adhesive**

agents which are extremely essential for the attachment of mammalian cells with culture vessels (Petri plate/flasks). The most important material necessary for mammalian cell culture is the **medium** and its various necessary constituents. A complete cell culture medium contains all the necessary materials needed for the proliferation and growth of mammalian cells such as carbohydrates (e.g., glucose), protein/peptides (e.g., amino acids), lipids (fatty acids and glycerol), minerals, vitamins, hormones, growth factors, cytokines, and other materials. One of the most important components of the cell culture medium is a **fetal bovine serum (FBS)** or **fetal calf serum (FCS)** which contains many of the constituents of the mammalian cell culture medium. However, low serum or serum-free media are also used in particularly large-scale cell cultures for industrial purposes. The need for the above-mentioned materials and their relative quantity may vary from one type of cell to another, and therefore, **various types of cell culture** media have **been developed** with varying compositions. Finally, **various buffers** necessary for isolation, culture, and maintenance of mammalian cells are also mentioned.

Keywords

Cell dissociating agents · Cell adhesive agents · Buffers for cell culture · Cell culture medium · Fetal bovine serum (FBS) · Fetal calf serum (FCS) · Serum free medium · Antibiotics · Antimycotics

1 Introduction

Several studies including that of Carrel explained the requirements of various materials for in vitro culture of tissues (Carrel 1912). Several materials are needed for mammalian cells isolation from various tissues and organs and subsequent culturing. In a mammalian tissue or organ, various cells are joined together by extracellular matrix (ECM) proteins. Of the various proteins present in the ECM, structural protein collagen (triple helix protein) and adhesive proteins, fibronectin and laminin are highly important. To separate cells from tissues or organs, it is highly essential to dissociate or break collagen, fibronectin, and laminin that help in ECM-cell surface joining. Based on the type of tissues or organs, either mechanical disaggregation or enzymatic dissociation of cells is necessary. Cell-dissociating agents such as various proteolytic enzymes (e.g., collagenase, trypsin) and their usefulness are discussed in this chapter.

Once the cells are isolated from tissues and organs, the next important materials needed for cell culture are containers (vessels) of cell culture and cell adhesive agents. Special types of polystyrene-made sterile cell culture containers and vessels, plasticware, as well as different heat-resistant autoclavable glassware, transfer aides, and membrane filters, are extensively used in mammalian cell culture.

Except for some blood cells (e.g., leukocytes), all other cells in the mammalian body are adhesive. Since blood cells are suspended in the plasma, it is easier to

isolate them by a range of procedures including differential centrifugation. Adhesive cells grow in attached mode with the cell culture containers for their survival and growth. Various cell adhesive agents like gelatin, laminin, etc. are especially needed for the culture of all adhesive cells. For harvesting and subculturing adhesive cells from the cultured containers, trypsin-EDTA is used, which would remove the cell from the culture containers. However, for trypsin-sensitive cells, a cell scraper can be used. Nonadhesive cells such as B or T lymphocytes grow in suspension and therefore do not require any adhesive agents for their culture medium (Fig. 1).

Besides cell-dissociating and adhesive agents, another important material discussed in this chapter is the cell culture medium. Medium is the artificial environment in which mammalian cells survive and grow. A cell culture medium comprises all the nutrients including glucose, amino acids, hormones, growth factors, vitamins, inorganic salts, etc. needed for mammalian cell survival and growth. (5–10)% fetal bovine serum (**FBS**) or fetal calf serum (**FCS**) is also generally supplemented to the cell culture medium for growth and proliferation. However, serum may inhibit the proliferation of some cells. Also, the serum is one of the most expensive components of the cell culture medium. Therefore, the serum-free medium is generally utilized for some mammalian cell culture as well as industrial-scale cell culture volume, where the cost of the cell culture products is highly important. Additionally, the need for nutrition varies from one to another cell type, including some cells that require special nutrition for their growth. For example, endothelial cells grow well in the presence of vascular endothelial growth factors (**VEGFs**). Medium not only supplies nutrition but also maintains pH, osmolarity, temperature, and moisture alongside the O_2/CO_2 tension of the cells.

Finally, several buffers are utilized for various purposes in mammalian cell culture. Some **common buffers** such as phosphate buffer saline (**PBS**), Hanks Balanced Salt Solution (**HBSS**), etc. are widely used for washing mammalian cells and cell culture containers. This chapter discusses all the above-mentioned subjects/materials necessary for mammalian cell isolation and culture.

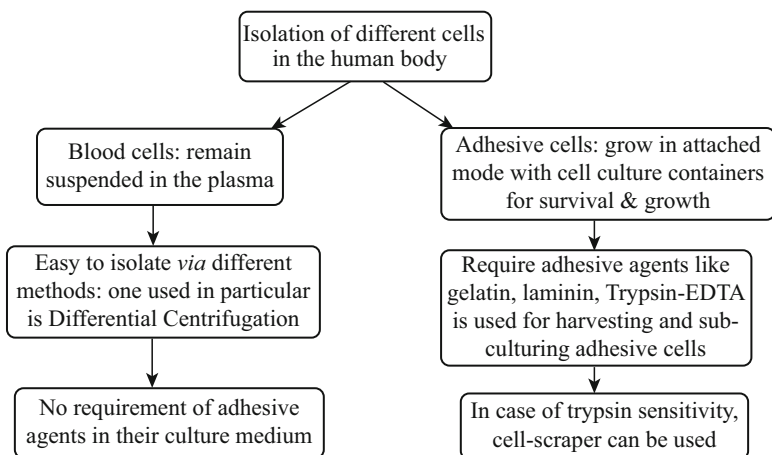


Fig. 1 Isolation distinctions of adhesive and suspended cells for their subculturing

2 Physiological Parameters Necessary for the Culture of Mammalian Cells

Besides an adequate supply of common materials for the isolation and culture of mammalian cells, ideally, some physiological parameters are also needed to be strictly maintained for mammalian cell culture.

2.1 Culture Vessels with Surface Property

Proper cell culture plates or flasks made of **polypropylene/polystyrene** are used for the growth of adherent mammalian cells. Of note, the mammalian cell membrane possesses a net negative charge in its surface. Nonadherent cells such as leukocytes (e.g., B/T cells) grow in suspension.

2.2 Composition of Cell Culture Medium

Medium with essential nutrients, minerals, vitamins, amino acids, growth factors, hormones, etc. are appropriate for culturing particular mammalian cells.

2.3 pH of the Cell Culture Medium

In general, the cell culture medium should have a physiological pH between 7.2 and 7.5 (average 7.4 which is the pH of the human blood). This pH is maintained either via HEPES buffer and/or sodium bicarbonate addition. Cells should be kept in an incubator in which the gas phase of 5% CO₂ tension and bicarbonate in the medium are kept in equilibrium (Howarth 1975).

NB: Human blood pH is 7.4 and human cell cytoplasm pH is 7.2. Human cell cytoplasm maintains a reducing environment.

2.4 Temperature and Light

All mammalian cells in the culture medium are maintained at 37 °C in a CO₂ incubator usually in a dark environment (**no light or illumination is necessary**).

2.5 Humidity of the CO₂ Incubator

The CO₂ incubator maintains nearly 95% humidity, which is needed for mammalian cell culture. Humidity is maintained at a residual **95% of sterile air** either using sterile water or via a water-jacketed CO₂ incubator.

2.6 Subculture and Feeding with Culture Medium

Depending on the cell type, when cells are divided to optima and culture medium is spent, cells are again harvested, spent medium is discarded, and fresh medium is added which is called the **subculture** of cells. If the culture vessels are full, the cells will be split into more culture vessels. For example, cells harvested from one 75 mm flask may be divided into two to three 75 mm flasks. Sometimes, subcultures are done either once or twice per week. Many times, spent medium is discarded in between and fresh medium is added gently without disturbing the cells. This procedure is also called as **feeding** of cells (Masters and Stacey 2007).

3 Cell Dissociation Agents

- Mammalian cells are isolated from their tissues and organs. Smaller tissues or organs could be cultured directly, even as **explant culture**.

NB: Explant culture is not popular and requires lots of standardization besides varying from culture to culture.

- Depending on the cell types and nature of experiments, different cell-dissociating reagents are used for both isolation of cells from tissues and organs in primary culture as well as detachment of adherent cells from the surface of culture plates or flasks (during subculture). Generally, Trypsin-EDTA is used for the detachment of cells from the cell culture containers.
- The molecules or agents that dissociate cells from the tissues or organs are called **cell-dissociating agents**. These agents are used for aseptically dissociating cells from complex mammalian tissues and organs, after dissection. Once dissociated, these cells remain suspended in a culture medium and are called **single-cell suspension**. These single-cell suspensions are allowed to grow in vitro within a CO₂ incubator.
- Either mechanical disaggregation or different types of cell-dissociating agents are used depending upon the used cells, tissues, and organs. Some dissociating agents are **proteolytic (enzymes that digest the proteins, e.g., trypsin)** and **collagenolytic (enzymes that digest the matrix triple helix protein, e.g., collagenase type 1 from *Clostridium histolyticum*)** enzymes while others are active in a nonenzymatic salt solution. The isolation of various cells from tissues and organs and the primary cultures, such as endothelial cells, smooth muscle cells, fibroblasts, and epithelial cells is discussed in chapter 08.
- Also since most mammalian cells are adherent in nature, they will attach to the polystyrene-coated cell culture containers before their growth. For harvesting or subculturing, the cells must be detached. When cells adhere to the surface of the culture flask or plates, the dissociating agents are again used to detach these cells from the surface before being pooled in suspension. This technique is called as **harvesting of cells**. The most abundantly used proteolytic dissociating agents are

0.25% or 0.01% trypsin. EDTA solution is used as a nonenzymatic cellular dissociation agent. **EDTA is used with or without trypsin to prevent metallic toxicity to the cells.** The source(s) of the metal constituents may be medium constituents or culture vessels (Allen and Snow 1970; Dalen and Todd 1971; Heng et al. 2009).

4 Cell Adhesive Agents

- Most mammalian cells are adhesive, so attachment of these cells to the cell culture containers is a prerequisite for the ideal growth.
- Thus, for culturing adherent cells, culture containers must be pre-coated with various adhesive agents or matrix adhesion proteins.
- Most commonly used adhesive agents are *gelatin*, *collagen*, *fibronectin*, *laminin*, *poly-L-Lysine*, etc.
- Gelatin is the **most common** and cheapest adhesive used in endothelial and other adhesive cell cultures.
- Matrix adhesion proteins are responsible for linking the matrix components to one another and the cell surfaces. They interact with collagen and proteoglycans to specify matrix organization and are the major binding sites for **integrins** (Curtis 1962; Curtis et al. 1983; Ramsey et al. 1984).

Here is a brief discussion of the two most important matrix adhesion proteins, that is, fibronectin and laminin.

4.1 Fibronectin

- Fibronectin is an adhesive glycoprotein that prevails present in the ECM. This protein exists in both soluble secretory form (liver) in the plasma as well as insoluble principle adhesive texture of connective tissues. It is also expressed as a cell surface protein. Initially, it is produced as a preprotein before being finally processed to form a mature protein.

Here are the brief structural and functional details of fibronectin.

4.1.1 Structure of Fibronectin Protein

- While fibronectin is produced by a single gene, several alternative splice variants of this gene are detected.
- Fibronectin is recognized as a high molecular weight (MW) glycoprotein, with the MW ranging from ~ (440–530) kDa.
- This protein exists as a **dimer of two nearly identical polypeptide chains linked by a pair of C-terminal disulfide bonds.**
- Each polypeptide chain is made up of nearly 2500 amino acids, having the MW within ~ (230–275) kDa.

- The polypeptide functional fibronectin domains could be of a structurally distinct type, namely, FN1 (I), FNII (II), and FNIII (III) repeating subunits (Fig. 2).
- All three fibronectin domains consist of antiparallel β strands with conserved residues in their hydrophobic cores.
- The binding of fibronectin to integrins (transmembrane adhesive protein with bidirectional signaling, inside out and outside in) unfolds fibronectin molecules, forming the dimers, which can function properly.

4.1.2 Functions of Fibronectin

- This protein is implicated in several functions, including the most prominent of which is **adhesion**. At present, fibronectin is recognized as a **classical cell adhesion molecule and is regarded as a master organizer of ECM**. It interacts or cross-talks with **collagen, fibrils, and GAGs**.
- **Reorganization of the cytoskeleton** is another important function of fibronectin. The binding of fibronectin with collagen and cell surface integrins reorganizes the cytoskeleton of the cells besides facilitating cell movement.
- The other well-recognized functions of fibronectin are oncogenic transformation, cell migration, phagocytosis, hemostasis, and embryonic differentiation.
- It is now well accepted that fibronectin has a role in wound healing via binding to platelets at the site of tissue injury apart from the cellular movements during wound healing.

4.2 Laminin

Laminin is a glycoprotein of ECM that interacts with other plasma membrane proteins/receptors located at the basal lamina. The major function of this protein is adhesion.

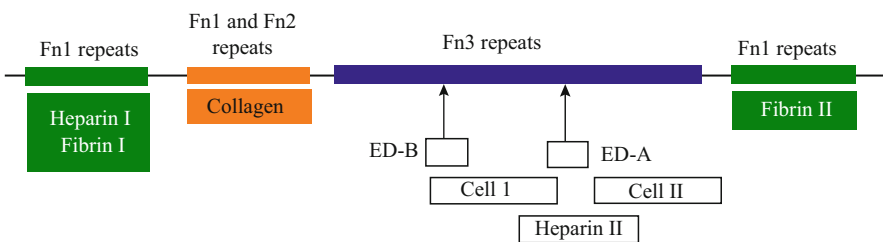


Fig. 2 Typical representation of a fibronectin polypeptide chain, comprising of multiple repeat elements (Fn1, Fn2, and Fn3) and binding sites for multiple matrix proteins and cells. Two regions can bind heparin and fibrin while the other two are involved in cell binding. ED-A and ED-B are the isoforms generated by alternative splicing which may or may not contain certain Fn3 domains. Other splice variations in the second cell-binding domain generate distinct isoforms

Here is a very brief discussion of the structure and functions of laminin.

4.2.1 Structure of Laminin

- Laminin is a very high MW (~900 kDa) glycoprotein. This complex protein is a heterotrimer of α , β , and γ subunits.
- Further, these subunits are the products of five α , four β , and three γ genes.
- The α , β , and γ chains are linked together in a cruciform structure (with globular and rod-like domains) stabilized via disulfide linkages (Fig. 3).
- So far, 15 laminin isoforms have been identified.
- Like other matrix proteins such as type IV collagen, laminins can self-assemble into mesh-like polymers.

4.2.2 Functions of Laminins

- The major functions of laminins are cell adhesion and cell-to-cell interaction.
- To fulfill these functions, laminin interacts with various proteins such as cell surface receptors integrins, type IV collagen, heparin sulfate proteoglycan (a perlecan), entactin, and other molecules, forming a cross-linked network in the basal lamina.
- Laminin may have a role in neural development and peripheral nerve repair.

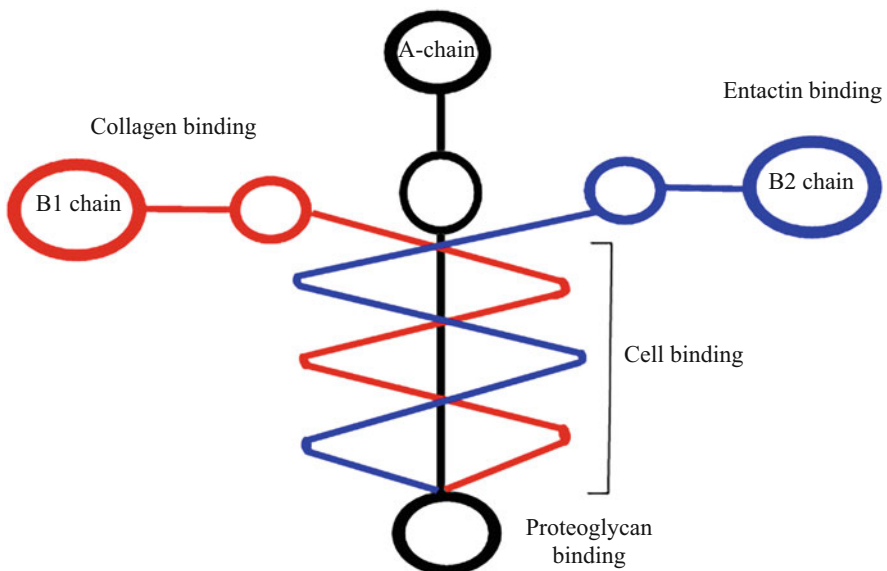


Fig. 3 Multimeric binding domains of laminins, characterized by A, B1, and B2 polypeptide chains (nowadays referred to as α , β , and γ chains), which are held together in a cruciform structure stabilized by disulfide linkages

4.3 Cell Adhesion to the Extracellular Matrix

- Many cells (plasma membrane) bind to the ECM components.

Cell adhesion can occur in the following two ways:

4.3.1 Focal Adhesions

It is a process of connecting the ECM to actin filaments of the cell.

4.3.2 Hemi-Desmosomes

These molecular structures are involved in connecting the ECM to intermediate filaments such as keratin.

- **Integrins**, a family of transmembrane proteins perform highly important roles in cell-ECM cross-talk-dependent adhesion. This specialized protein sends signals bidirectionally, from outside the cell to inside and from inside the cell to outside (commonly referred to as inside out and outside in).
- This protein consists of α and β subunits. Various types of α and β subunits joined together to form at least 24 different integrins.
- **Fibronectins and laminins** bind to ECM macromolecules and facilitate their binding to transmembrane integrins.
- *Integrins facilitate binding with several cytoskeleton proteins such as vinculin, paxillin, talin, and focal adhesion kinase (FAK), maintaining the structural integrity of the cells* (Fig. 4).

NB: Collagen, fibronectins, and laminins are natural proteins comprising the mammalian ECM.

- **Gelatin** is a derivative of mammalian collagen.
- **Poly-L-Lysine** is a positively charged synthetic protein that was originally produced from bacteria.

Gelatin and Poly-L-Lysine are also used as cell adhesive agents. They are used for coating the cell culture containers.

5 Cell Culture Medium and its Constituents

- **Cell Culture Medium** is an artificial environment that comprises essential nutrients, amino acids, vitamins, minerals, growth factors, etc. for supporting cell growth and proliferation by providing optimum nutrition.
- The cell culture medium is the artificial environment that not only supplies all the nutrients necessary for the culture and growth of mammalian cells but also maintains the pH, temperature, moisture, and O_2/CO_2 tension of the culture environment.

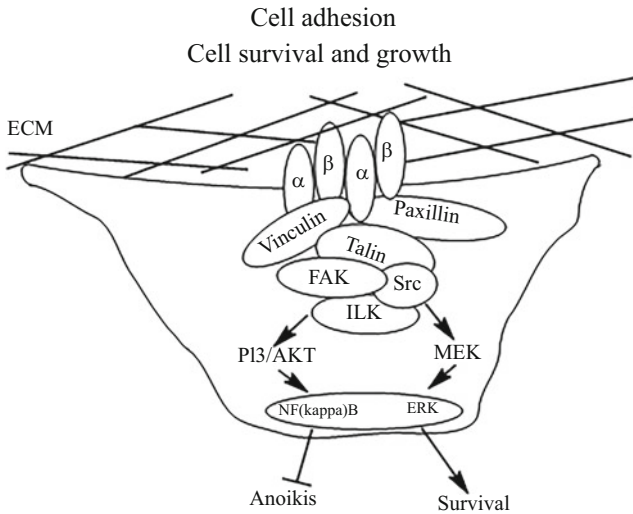


Fig. 4 Representative scenario of ECM-bound cell growth, where α and β subunits of integrins play key roles in keeping the cells glued to matrix proteins and gap junctions. The sole mandate of such a growth pattern pertains to regular nutrient intake from the matrix alongside regular communication with surrounding cells. Cells devoid of basal membrane growth support are designated as following **Anoikis** (characteristic of tumor cells)

- Every cell, including those of immortal tumor or cancer cells, needs the culture medium for its proper growth and development. However, the need for the various cell culture medium nutrients may vary from one to another cell type. Thus, cell culture medium composition varies for different cells.
- The choice of a specific medium and its supplements depends upon the grown cell type and its specific origin. For cells from special tissues needs, more complex culture medium is needed with special supplements, such as **VEGFs** are needed for endothelial cell culture.
- When ingredients of culture medium for a specific cell type are not known, different culture media with growth factors and serum are tested for optimum results by monitoring their growth microscopically.
- Cell culture medium helps to maintain the pH (7.4–7.5), temperature (37 °C), and moisture and humidity (95%) for the best growth of cultured cells (White 1946; Fischer et al. 1948; Morgan et al. 1950; Parker 1961).

5.1 Composition of Cell Culture Medium

As mentioned above, the cell culture medium maintains every factor responsible for the proper growth and maintenance of mammalian cells, including nutrition, pH, and osmolarity. Many of the contributions to designing the nutritional composition of the mammalian cell culture medium were initially established separately by *Carrel and Eagle* (Carrel and Baker 1926; Eagle 1955).

The specific composition of a cell culture medium is based on the following four important functions:

- To supply necessary nutrition to the cultured cells
- To maintain pH of medium/cultured cells
- To maintain osmolarity of the medium/cultured cells
- To provide and maintain proper temperature, moisture, and O₂/CO₂
- Tension of the mammalian cells using a CO₂ incubator

The following paragraphs provide a brief discussion on the nutritional composition, pH, and osmolarity maintenance of the cell culture media.

5.2 Nutritional Composition of the Cell Culture Medium

Cell culture medium contains the following materials.

5.2.1 Carbohydrates

- Biochemically, carbohydrates are called **poly-hydroxy-aldehydes** and **ketones**. Carbohydrates can be divided into **monosaccharides**, **disaccharides** (two molecules of monosaccharides joined via glycosidic linkage), and **polysaccharides** (more than two molecules of monosaccharides joined together via glycosidic linkage).
- Carbohydrates are one of the most important energy sources. Disaccharides such as fructose and polysaccharide such as glycogen cannot be metabolized by mammalian cells. Therefore, they must be broken into monosaccharides before their metabolism inside the cells.
- Intestinal cell absorptive capacity varies among different monosaccharides with the highest for glucose and the least for galactose. **Glucose** is the most abundant monosaccharide in the mammalian blood, which is transported to the cells and subsequently metabolized to produce energy (ATP), and therefore is evidently the most **common sugar (used as a carbon source)** in the medium.
- Depending upon the cell type, the culture medium may have distinctive glucose contents.
- Some media may contain **dextrose** (a monosaccharide), **galactose** (a monosaccharide), **fructose** (a monosaccharide), and **maltose** (a disaccharide, consisting of two glucose molecules).
- **These monosaccharides are isomers of glucose and convert to glucose before metabolism and releasing energy (ATP/GTP)** (Lewis 1922).
- Sometimes, in addition to glucose, sodium pyruvate, a metabolic product of glycolysis (in the mammalian cell cytoplasm one glucose molecule is converted to two pyruvic acid molecules), oxaloacetate, alpha-ketoglutarate, etc. (Neuman and McCoy 1958) is freshly added to the medium to ensure an easier energy supply or even cyclic GMP (cGMP) may also be added (Seifert and Rudland 1974).

5.2.2 Amino Acids

- A protein is a polymer of amino acids joined together by a peptide bond (a type of covalent bond). In other words, amino acids are the smallest constitutional units of a protein.
- Structurally, an amino acid consists of an amino group ($-\text{NH}_2$), a carboxylic group ($-\text{COOH}$), and a side chain ($\text{R}-\text{CH}$).
- So, the basic formula of an amino acid is $\text{NH}_2\text{-RCH-COOH}$.
- The smallest and simplest amino acid is glycine with a formula of $\text{NH}_2\text{-CH}_2\text{-COOH}$, where side chain R is replaced by hydrogen.
- During peptide formation, the amino group of an amino acid joins with the carboxylic group of another amino acid to form a peptide bond (CONH), releasing one molecule of water (H_2O).
- In the cellular system, nitrogen released by an amino acid/protein is used to form nitrogenous bases such as purines and pyrimidines.
- Without purine and pyrimidine bases, DNA and RNA cannot be synthesized (replicated and transcribed, respectively). Additionally, without replication, transcription cell division or proliferation is not possible, amino acids are added as obligatory ingredients of all known cell culture media.
- Although in nature *around 300 amino acids have been discovered so far, only 20 amino acids are involved in peptide bond formation and thus protein synthesis.*
- **Glycine** is the simplest amino acid in the human body.
- While some amino acids are synthesized by the mammalian cells, others cannot be and therefore must be supplied through food, called **essential amino acids**.
- The **twelve essential amino acids** that must be supplied through foods are arginine, cysteine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, histidine, tyrosine, and valine. All these amino acids are **L-amino acids**.
- During mammalian cell culture medium preparation, special attention should be given to amino acid **glutamine**, since it is necessarily needed to synthesize nitrogenous bases for DNA and RNA.
- **The L-glutamine** concentration varies from one another culture media. For instance, in M-199 media, it is **0.68 mM** and for Dulbecco's Modified Eagle's Medium (DMEM) it is **4 mM**.
- **NB:** A proper concentration of amino acids in the mammalian cell culture medium must be maintained, failing which proper cell growth would not be possible. On the other hand, concentration higher than the optimum concentration may produce a large quantity of ammonia which is toxic for mammalian cells.
- Besides essential amino acids, several nonessential amino acids are added to the mammalian cell culture medium, since these may be depleted during cell culture.
- Supplementation of a mammalian cell culture medium with nonessential amino acids stimulates growth and prolongs cell viability (Hosios et al. 2016).

NB: The amino acid L-glutamine is easily degraded and is, therefore, generally added freshly in the culture medium.

5.2.3 Peptides and Proteins

- Besides amino acids, proteins and peptides can be directly added to the cell culture medium.
- The most commonly used proteins and peptides are **albumins**, **transferrin**, **fibronectin**, **aprotinin**, and **feutin** (Fig. 5).

Human Serum Albumin

Human serum albumin (**HSA**) is one of the most important serum proteins that not only maintains blood **osmolality** but also binds with various molecules such as steroid hormones and acts as their carrier in the mammalian body. HSA is used to eliminate the toxic substances in the cell culture medium by binding to them. It also stabilizes various small molecules (including administered drugs) by binding to them and safely delivers them to the cells.

NB: Since HSA shows antioxidant activities, it cannot be used in the mammalian cell culture medium as and when the potential anti-oxidant activities of an unknown or newly developed compound are the experimental purpose.

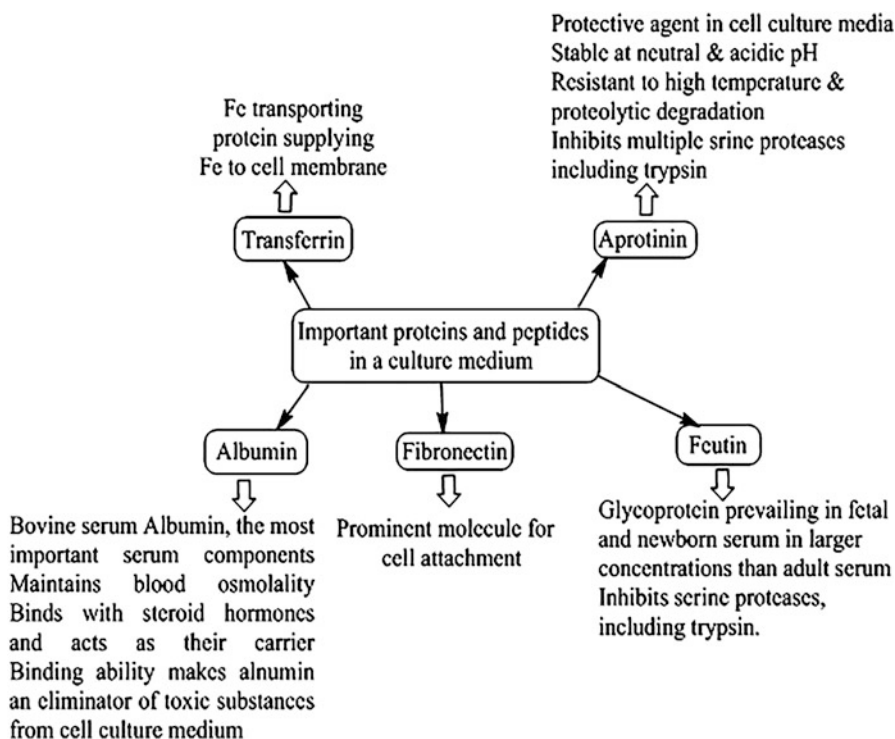


Fig. 5 Important functions of some proteins and peptides included in different culture media

Aprotinin

- It is the protein used in mammalian cell culture media to destroy the effect of various proteases that may be released by the injured or apoptotic cells.

Fetuin

- The name fetuin designates a protein released in the circulation because this glycoprotein is produced by the fetus and newborn baby. However, a detectable but very low concentration of fetuin is also released in adult human beings. Just like aprotinin, fetuin also destroys proteases, particularly serine proteases released by the injured or apoptotic cells.

Fibronectin

- As discussed in the previous sections, fibronectins are added to the mammalian cell culture medium to facilitate the attachment of adherent cells to the culture containers.

Transferrin

- Transferrin is a plasma glycoprotein that is utilized to transport iron (two molecules of ferric iron per transferrin) in the cells. This protein is almost exclusively produced by the liver.

NB: These proteins may be added to the serum-free medium. However, in the serum-containing cell culture medium, there is no need to add these proteins.

5.2.4 Fatty Acids and Lipids

- Generally, a serum-containing medium does not require fatty acids and lipids, as they are generally present in serum.
- They are particularly important in serum-free medium.
- **Linoleic, oleic, and arachidonic acids** are important fatty acids associated with albumins in nature.
- **Albumin** helps in improving the solubility of linoleic, oleic, and arachidonic acids as well as their oxidative protection.

5.2.5 Minerals and Trace Elements

- Various minerals and trace elements (micronutrients) are present in human circulation, which are necessary for various biological functions including the functioning as a cofactor for various enzymes or proteins and vitamins, pattern of glycosylation (a type of posttranslational modification which enzymatically adds carbohydrates to protein), protein folding and unfolding, and finally, growth and proliferation of mammalian cells.
- It is discovered that around **21 minerals** that are needed for various biological functions are not produced by the human body and therefore are called **essential minerals**. Therefore, deficiency of one or more of these elements leads to pathophysiological conditions.

- Out of these 21 minerals, the most important trace elements that affect the culture, growth, and bioprocessing process are **zinc, aluminum, manganese, molybdenum, and iron**. To some extent, a second category includes **copper and nickel**. It is experimentally observed that all these elements are necessary for the glycosylation of protein.
- Other minerals that are not only necessary for maintaining the osmolarity of cells/plasma, but also for various biological functions are **sodium, potassium, calcium, chlorine, phosphorus, bicarbonate, zinc, selenium, and copper**.
- The most common minerals that exhibit deficiency in humans are **calcium, iron, and iodine**.

NB: Since serum contains all the mineral and trace elements, there is no need for the addition of these materials in the mammalian cell culture medium, except in a serum-free medium. Various companies use the serum-free medium in their mammalian cell culture-based product development since the serum is a cost additive for cell culture medium (Eagle 1956; Cinatl 1969).

5.2.6 Vitamins

- Vitamins are a type of micronutrient that is not produced by the human body and are therefore called essential micronutrients. There are **13 essential vitamins**.
- While some vitamins are water-soluble (e.g., vitamin B complex and vitamin C), others are fat-soluble (e.g., vitamins A, D, E, and K).
Vitamins are supplemented through various foods.
- The common vitamin components of any culture medium are **biotin, folate, nicotinamide, pantothenic acid, pyridoxine, riboflavin, thiamine, and vitamin B12**.
- Vitamins mainly act as **coenzymes or prosthetic groups** in cell metabolism and therefore are necessary for mammalian cell growth and proliferation.

NB: In general, since mammalian serum contains all vitamins, there is no need to separately supply vitamins to the cell culture medium unless special attention is needed because of an enhanced need for certain vitamins.

5.2.7 Hormones

- Hormones are the biochemical messengers produced by the ductless **endocrine glands** of a mammalian body. Being secreted by an endocrine gland, hormones diffuse into the blood and reach various target sites through the blood.
- The major biochemical nature of the hormones are **peptides** (e.g., oxytocin/vasopressin), **proteins** (insulin/glucagon), biological **amines** (e.g., thyroxine), and **steroids** (estrogens/testosterone).
- The hormones are involved in growth, metabolism, sexual characteristics, and other functions.
- Of note, various hormones have distinct biological functions.
- Some cell culture media may require hormone addition from outside.

- Hormones like **insulin**, **cortisol**, **estrogen**, etc. may be added in different cell culture media. For example, the culture of MCF-7 breast cancer cells requires estrogen for their proliferation.

5.2.8 Growth Factors

- Growth factors are small proteins or peptide molecules that induce cell division or proliferation and are, therefore, **mitogenic**.
- Various mammalian tissues produce growth factors, many of which are secreted in the blood.
- Some important growth factors discovered to date are **vascular endothelial growth factors (VEGF)**, **fibroblast growth factors (FGF)**, **platelet-derived growth factors (PDGF)**, **neuronal growth factors (NGF)**, **insulin-like growth factors (IGF)**, etc.
- Every growth factor has **its receptors** and binding of a growth factor with its cognate receptor causes conformational changes, leading to sending growth-promoting signals to the promoter of various genes inside the nucleus.
- Biochemically, both the growth factors and their receptors are proteins, so the interaction of growth factors and their receptors is said to be **protein-protein interaction**.
- Each growth factor acts on specialized cells. For example, VEGF activates the growth of vascular endothelial cells and vascular smooth muscle cells (Hornsby et al. 1983) (see Table 1).

5.2.9 Ethylene Diamine Tetraacetic Acid

- **Ethylene diamine tetraacetic acid (EDTA)** is a polyprotic acid containing four carboxylic groups and two amine groups with a lone pair of electrons that chelate calcium and several other metal ions.

Table 1 The table describes some common growth factors, their target cells, and the specific concentration needed for cell culture

Growth factor	Common arget cell	Specific target cell	Recommended concentration
EGF	Epiblasts, mesoblastoma	Keratinocytes, fibroblasts, chondrocytes, etc.	(1–2) ng·ml ⁻¹
FGF	Mesoblastoma, neuroectoderm	Fibroblasts and vascular cells	(0.5–10) ng·ml ⁻¹
IGF	Most cells	Most cells	(1–10) ng·ml ⁻¹
VEGF	Endothelial, smooth muscle, epithelial, etc.	Endothelial cells	(1–10) ng·ml ⁻¹
PDGF	Mesenchymal cells	Fibroblasts, myocytes, neurogliocytes	(1–50) ng·ml ⁻¹
NGF	Neuronal cells	Neurocyte, neuroglycyote	(5–100) ng·ml ⁻¹

EGF = Epidermal Growth Factor, **FGF** = Fibroblast Growth Factor, **IGF**: Insulin-like Growth Factor, **VEGF** = Vascular Endothelial Growth Factor, **PDGF** = Platelet-Derived Growth Factor, **NGF** = Neuronal Growth Factor

- EDTA is described as a **metal chelator**, particularly for its strong reaction capacity with calcium and magnesium. Calcium and magnesium are necessary for cell attachment and therefore lead to cell aggregation and clumping following treatment with trypsin for cell harvesting from the culture containers. Thus, for cell harvesting from the culture medium, EDTA is always added to the culture medium.
- Figure 6 depicts the chemical structure of EDTA, wherein manifold OH groups near oxygen infer a significant role of intramolecular H-bonding. The disrupted nitrogen substituted aliphatic chains in the middle impart a distributed homogenization which helps in avoiding a sudden or random aggregation due to hydrophobic excess.
- EDTA is also used for **detaching adherent cells** from cell culture vessels either for passaging or just harvesting. The working concentration is 0.02%.
- Trypsin/EDTA treatment is a combined method for detaching cells. Of note, trypsin is a proteolytic enzyme. Generally, a mixture of 0.05% trypsin and 0.02% EDTA is directly added to the cell culture vessels for mammalian cell detachment.

5.2.10 Beta Mercaptoethanol

- **Beta mercaptoethanol (β -ME or 2-ME)** should be added afresh in some cell culture media.
- **β -ME or 2-ME is a reducing agent which breaks down disulfide bonds** and is added to the culture medium for T-cell proliferation. β -ME is supplied as 14.3 M (Fig. 7).
- The final concentration of β -ME in the culture medium is 50 μ M.

Fig. 6 Chemical structure of ethylene diamine tetraacetic acid (EDTA), with a distributive philicphobic force distribution-driven aggregation control and chelating ability

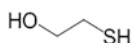
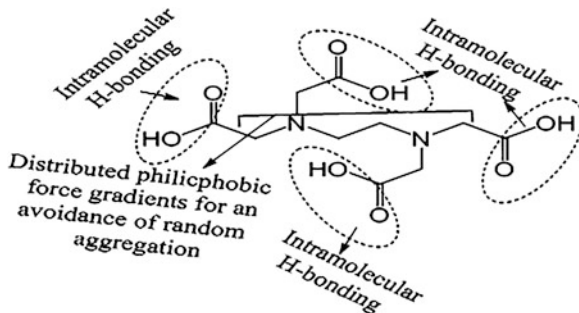


Fig. 7 Chemical structure of beta-mercaptoethanol (β -ME), with twin hydrogen donating sites, separated by an alkyl chain. Availability of H^+ confers reduction ability to this cell culture additive, which aids in T-cell proliferation

5.2.11 Serum

- *The straw color serum is the blood plasma devoid of clotting factors.*
- The cell culture medium is supplemented with (5–10)% **Fetal Bovine Serum (FBS)** or **Fetal Calf Serum (FCS)** for promoting the healthy growth of mammalian cells.
- FBS/FCS has the highest quality because the fetal bovine is not exposed to the outside environment and has the lowest antibodies and complement.
- Before use, both FBS and FCS should be freed from the **complement (destroying the serum complement proteins)** by **heating the serum at 56 °C for 30 min**, otherwise complements may lyse the cells in conjunction with antibodies.

NB: Complements are a series of around twenty heat-labile proteins secreted by the liver, and through a series of signaling cascades, enable antibodies and phagocytic cells to destroy or eliminate pathogens/antigens.

- The serum contains numerous hormones, growth factors, amino acids, peptides, and proteins (e.g., human serum albumin or HSA), glucose, fatty acids and glycerol, vitamins, minerals, enzymes, etc.
- Some specific culture conditions need specially treated serum. For example, when working on the effects of estrogens/other steroids on the in vitro cultured mammalian cells such as endothelial cells, *charcoal-dextran treated serum* is used. Charcoal-dextran **stripped all the steroids from the serum** and therefore effects of estrogens/other steroids on the endothelial cells would inevitably be prominent (von Seefried and Macmorine 1976; Hornsby et al. 1983).

5.3 Usefulness of Serum in the Cell Culture Medium

The addition of serum to the in vitro cultured mammalian cells has many beneficial effects including growth and proliferation, neutralization of trypsin and proteases that are used for the detachment of cells from the cell culture containers, neutralization of toxins that may be present in the cell culture medium, pH maintenance of the cell culture medium, and so on.

Here are some important beneficial effects of serum in cultured mammalian cells.

5.4 Essential Nutrients Are Provided by the Serum

While most mammalian cell culture medium contains 3–5% FCS/FBS, some cells need as high as 10% serum in the cell culture medium. Serum supplies all the essential nutrients including amino acids, vitamins, inorganic minerals, fats, and nucleic acid derivatives. Essentially, these materials help in cell growth and proliferation.

5.5 Adherence and Spreading of Cells Are Mediated by the Serum

As described previously, most mammalian cells are adherent in nature. So, these cells are not only cultured in polystyrene-made containers but also mandate the addition of cell-adhesive agents such as gelatin. The serum contains **fibronectin**, **laminin**, etc. which act as **adhesive agents** and therefore help in the cell adherence with the culture containers.

5.6 Hormones and Various Growth Factors Are Provided by the Serum

The serum contains various growth factors such as vascular endothelial growth factors (VEGFs), epidermal growth factors (EGFs), insulin-like growth factors (IGFs), platelet-derived growth factors (PDGFs), fibroblast growth factors (FGFs), neuronal growth factors (NGOs), and others. The serum also contains both peptide/protein (e.g., insulin, glucagon, somatostatin, etc.) and steroid ((estradiol, testosterone, progesterone, etc.) hormones.

5.7 Serum-Mediated Protection for Some Specific Cells

The anti-protease ingredients present in the serum neutralize the various proteases released by the cells such as epithelial and myeloid cells.

Serum albumin maintains the viscosity of the mammalian cell culture medium and prevents mechanical damage to the cells.

Metabolic detoxification of the *in vitro* cultured cells is mediated by the trace elements and ions, such as SeO_3 and selenium.

The proteolytic enzymes such as trypsin facilitate the detachment of cultured adherent cells from the cultured containers, and harvesting of the cells is neutralized or diluted by the serum-containing used-up medium, before centrifugation of the cell suspension. This prevents trypsin-dependent damage to the plasma membrane of the mammalian cells.

5.8 Disadvantages of Using Serum in the Cell Culture Medium

- While there are several positive effects of adding a serum to a mammalian cell culture medium dedicated for *in vitro* mammalian cell culture, it has some negative effects also.
- Following digestion and absorption of food materials, all molecules enter the circulation through the intestine, from where they are once again transported into various cells. The intracellular composition and levels of these molecules differ from their plasma concentration, and unless there is an injury or damage to a cell, there is no direct contact of serum with intracellular compartments (exceptions

herein include the endothelial cells which are in direct contact with serum/blood). Thus, by adding a serum to the cell culture medium, the cultured cells are deliberately exposed to an artificial environment compared to physiological conditions.

- Serum composition may vary from batch to batch and, therefore, there may be a variation in results. This depends upon the nutrition and physiological status of the animals being used for the collection of serum.
- In highly proliferated cells, serum constituents such as spermine and spermidine can react with the polyamine to form toxic poly-spermine.
- In general, while serum is used for the growth promotion of mammalian cells (such as endothelial cells), it may inhibit the proliferation of a few cells such as **epidermal keratinocytes**.
- Serum may contain bacteriotoxin and antibodies against various pathogens that can affect cell growth or can even lead to cell death.
- Serum increases the difficulty of maintaining cultures during downstream processing.
- The serum is the most expensive component of a culture medium.
- Serum may be responsible for chance pathogenic contamination such as from mycoplasmas and some deadly human pathogenic viruses.

6 Classification of Media Based on the Presence or Absence of Serum

There are three types of media based on the presence or absence of serum in mammalian cell culture medium (Mather 1998).

They are as follows:

1. Basal medium
2. Reduced serum medium
3. Serum-free medium

One should choose the appropriate medium depending on the specific cell type to be cultured.

Ahead is a brief discussion of the three basic types of media.

6.1 Basal Medium

A **basal medium** is a defined medium containing essential and nonessential amino acids, vitamins, inorganic salts, organic compounds, and trace elements, but no other growth supplements are necessary for cell growth, for example, RPMI 1640 and DMEM with or without L-glutamine.

- Basal media without **Fetal Bovine Serum (FBS)** or **Fetal Calf Serum (FCS)** is called an **incomplete medium**.

- For providing full growth of cells, **10% decomplexed and endotoxin-free serum** is always added to all basal media like RPMI-1640 and DMEM prior experiment.
- All culture media supplemented with 10% decomplexed FBS or FCS is referred to as **complete media** (Hornsby et al. 1983).

6.2 Reduced Serum Medium

- **Reduced Serum Medium** is the basal medium enriched with nutrient-derived factors, which reduce the needed serum content.
- Normally, basal mediums with (2–5)% **serum** are called **reduced serum medium**.

6.3 Serum-Free Medium

- **Basal medium** with lots of additional supplements in absence of any serum is called **serum-free medium**.
- Several published papers and books are available describing the process of development of serum-free medium (Hewlett 1991; Karmiol 2000).
- The major benefit of using a **serum-free medium** includes the flexibility to choose a medium in absence of serum, depending on different nutrients and growth factors for specific cell types.
- Thus, a **serum-free medium** replaces the necessity of adding animal serum with appropriate nutritional and hormonal formulations.
- In the case of many primary cultures and cell lines like Chinese Hamster Ovary (CHO), hybridoma cell lines, **VERO**, and **MDCK**, a **serum-free medium** is used.
- The **basal medium** contains **vitamins, amino acids, nucleic acids, lipids, inorganic salts**, and an **energy source**.
- Additional supplements in a **serum-free medium** include growth factors such as **insulin-like growth factors** and **epidermal growth factors**.
- **Attachment factors present in serum-free media include collagen and fibronectin, transport proteins, detoxifying agents like albumin and transferrin, trace elements (Fe, Cu, Sn, Co, Mn), and lipids like essential fatty acids and phospholipids and various hormones, etc.** (Iscove and Melchers 1978; Bottenstein et al. 1979; Barnes and Sato 1979, 1980; Brunner et al. 2010).
- **Hematopoietic cells** were cultured in a serum-free medium supplemented with selenite, transferrin, albumin, and lecithin (Guilbert and Iscove 1976).

6.4 Advantages of Using A Serum-Free Medium

- The major use of the serum-free medium is bioprocessing industries for the production of various products in a cost-savings way (cheaper).
- The serum is the source of various microbial contaminations in the cell culture medium including mycoplasma. By eliminating the use of serum in the cell culture medium, the chances of microbial contamination will be reduced.
- One of the major products of bioprocessing industries is the production of various proteins that are utilized for different purposes including the production of antibodies, vaccines, industrial utilization as enzymes, or even therapeutic use. The serum contains a large number of proteins which interferes with the purification of the protein of interest. Therefore, in this case, the absence of serum is highly preferred for easier purification and downstream processing.
- In absence of serum constituents, it will be easier to control the physiological responsiveness of the cultured cells in response to various types of drugs/toxins or other molecules.
- Following the experimental observation, the evaluation of data is very precise and consistent.
- As mentioned above, serum-free media are widely utilized for the production of various recombinant proteins by culturing various mammalian cells such as CHO cells (Keen and Rapson 1995).
- Protein-free media are also utilized to culture CHO cells (Hamilton and Ham 1977).
- Also, hybridoma cells that are cultured for monoclonal antibody production use a serum-free medium (Murakami et al. 1982; Murakami 1989).

6.5 Cautions to be Exercised in a Serum-Free Culture

Overall, cells in serum-free culture are more sensitive to extremes of pH, temperature, osmolality, mechanical forces, and enzyme treatment. Several precautionary steps must be needed not only to protect the cultured cells in a serum-free medium but also to protect the cell culture products (Merten 1999).

The following precautionary measures should be followed for serum-free culture of the mammalian cells.

6.6 Regarding the Use of Antibiotics and Antimycotics in Serum-Free Medium

Antibiotics are substances produced by one group of microorganisms and act against another group of microorganisms in a low concentration. On the other hand, antimycotics acts against fungi. In general, a serum-containing medium that is used in the research laboratories used antibiotics such as penicillin/ampicillin and

antimycotics such as amphotericin B to prevent the contamination of microorganisms during the mammalian cell handling and culture.

For mammalian cell culture in serum-free medium, either no antibiotics/antimycotics should be used or if one intends to use antibiotics and antimycotics in the cell culture medium, these must be used at 5–10 times lower concentration. This is because, in serum-containing media, various proteins present in the cell culture medium interact with antibiotics and therefore reduce the availability of free antibiotics and antimycotics. Without serum, the entire concentration of antibiotics and antimycotics remains unbound and therefore may directly act on mammalian cells and damage them.

6.7 Regarding the Maintenance of Cell Density in Serum-Free Medium

During subculturing or passaging of cells, a fixed proportion of cells always perishes due to various reasons. So, seeding at a higher density is always helpful to achieve the required cell population in a fixed time.

- Another reason for maintaining the seeding density is maintaining the cell-to-cell cross-talk or communication via secreting various materials. Cells may not grow properly due to inadequate cell-to-cell communication. For example, it has been observed that experimentally, endothelial cells fail to grow if the seeding density is less than 1×10^5 in a 100 mm Petri plate.
- It is recommended that mid-log phase cells having confluency of ~60–80% should be passed. In general, 100% confluent cells (already subject to contact inhibition) take a longer time to adapt and grow when passaged into new cell culture containers.
- Adaptation to a serum-free medium should be slow, gradual, and progressive. Sudden changes from the serum-containing to the serum-free medium may generate shock, resulting in either the cell's failure to respond to the culture medium for growth or even death.

6.8 Morphological Changes During Shifting of Cells from Serum-Containing to Serum-Free Medium

During the transfer of cells from serum-containing to serum-free medium, there may be a slight change in the morphology of the cultured cells. In general, such morphological changes do not exhibit effects on the overall properties of the cultured cells. However, if the doubling time and viability of the cells alter, caution should be taken and the situation demands a change in the culture protocol.

6.9 Clumping During Shifting of Cells from Serum-Containing to Serum-Free Medium

As one gradually adapts cells from serum-containing to serum-free medium, there may be clumping of the cells. In this condition, it is recommended to gently or slowly triturate the clumps to break them before passaging of cells.

6.10 Formulation of Serum-Free Medium

To prepare a serum-free medium, several supplements are added to the basic cell culture medium.

The following are the list of supplements.

6.10.1 Adherence Substances

Previous sections of this chapter discussed in detail the adherent cells and the adherent substances such as laminin, fibronectin needed for the mammalian cell culture.

6.10.2 Growth Factors

Growth factors are needed for the optimum growth of various cells. For example, VEGF is necessary for the culture of endothelial cells. This point is discussed in the earlier section of this chapter.

6.10.3 Peptide or Steroid Hormones

Depending upon the type of cells, either peptide or steroid hormones may need to be added to the culture medium.

6.10.4 Enzyme Inhibitors

Adherent cells require trypsinization to passage. Trypsin is a proteolytic enzyme and therefore can damage or injure the plasma membrane if it is not neutralized following detachment of adherent cells from the culture containers. In such an eventuality, a **soybean trypsin inhibitor** may be used to neutralize trypsin.

6.10.5 Binding Protein(S) and Translocator

In the earlier section of this chapter, it has been mentioned that several translocators and binding proteins are required for the mammalian cell culture. The specific type and amount depend on the cells. For example, bovine serum albumin (**BSA**) and **transferrin** are required by some cells.

6.10.6 Trace Elements

In the earlier section of this chapter, it has been mentioned that several trace elements are required for the mammalian cell culture. For example, **selenium** is required by some cultured cells.

6.10.7 Process of Using Serum-Free Medium

In general, cells are grown in a serum-containing medium after which, the serum is gradually removed from the culture medium so that cells should not witness a sudden nutritional crunch.

The process of gradual adaptation is as follows:

- Grow cells in a 10% serum-containing medium.
- Subculture the cells in a 5% serum-containing medium.
- Subculture the cells in a 3% serum-containing medium.
- Subculture the cells in a 1% serum-containing medium.
- After 70–90% confluency, remove serum from the culture containers and allow the cells to adapt to a serum-free medium.

6.11 Protein-Free and Chemically Defined Media

- A chemically defined medium (**CDM**) is a medium in which the chemical nature and extents of all constituents are known.
- Serum and protein-free media may be highly useful to produce recombinant proteins using mammalian cell culture.
- In the bioprocessing industry, Chinese Hamster Ovary (**CHO**) cells are highly used for the production of various recombinant proteins. The CHO cells are adapted to grow in a protein-free medium.
- At present, several companies use the protein-free medium for the culture of mammalian cells to produce various products.

6.12 Types of Culture Media

Mammalian cell culture media can be classified as follows (Darling and Morgan 1994; Macy and Shannon 1977).

Natural Medium

Synthetic Medium

The following paragraphs describe the basics of these media configurations.

6.12.1 Natural Medium

- **Animal body fluid** (e.g., plasma, serum, lymph) or **tissue extraction** (chicken embryos leaching solution) is recognized as a natural medium.
- While natural media are rich in every type of nutrition required for the culture of mammalian cells, there are batch-to-batch variations in the composition of this type of medium.
- Therefore, these types of media are replaced by synthetic medium with a standardized/fixed concentration of various nutrients.

6.12.2 Synthetic Medium

Commercial culture media were developed as early as the 1950s. The first-ever developed medium was **Eagle's Basal Medium** by Harry Eagle during normal and malignant cell type studies. **Eagle's Basal Medium** was later modified to generate many popular media (Jacoby and Darke 1948; Ham 1965; Ham and McKeehan 1979).

Here is a brief description of some common mammalian cell culture media.

7 Common Cell Culture Media

Minimum Essential Medium (**MEM**)

Alpha Minimum Essential Medium (**α -MEM**)

Glasgow Minimum Essential Medium (**GMEM**)

Dulbecco's Modified Eagle's Medium (**DMEM**)

Iscove's Modified Dulbecco's Medium (**IMDM**)

Roswell Park Memorial Institute Medium (**RPMI**)

199medium (**M199 MEDIUM**)

Ham's F10 Medium

Ham's F12 Medium

Kaighn Modified Ham's F-12 Medium (**Ham's F-12 k**)

Dmem-F12 Medium

RPMI 1640/DMEM/F-12 (**RDF**) Medium

McCoy's 5A Medium

Leibovitz L15 Medium

Waymouth's MB752/1 Medium

Trowell's T8 Medium

Each medium has its composition and supplements (Freshney 2010).

Ahead is a brief description of the above cell culture media configurations.

7.1 Minimum Essential Medium

- Minimum Essential Medium (**MEM**), also called **Eagle's Minimal Essential Medium (EMEM)**, is the cell culture medium developed by *Harry Eagle*.
- Eagle first developed Basal Medium Eagle (**BME**), and then gradually increased the content of essential amino acids to produce **MEM** (Table 2).
- However, it only contains 12 kinds of nonessential amino acids, glutamine, eight vitamins, and some basic inorganic salts.
- Many continuous mammalian cell lines such as MCF-7 and MDA-MB breast cancer cells and adherent cells are maintained in this simple medium.

Table 2 Basic differences between the Basal Medium Eagle (**BME**) and Minimal Essential Medium (**MEM**) compositions

Medium	BME	MEM
Inorganic salts		
Calcium chloride (CaCl ₂) (anhydrous)	200	200
Magnesium sulfate (MgSO ₄) (anhydrous)	97.67	97.67
Potassium chloride (KCl)	400	400
Sodium bicarbonate (NaHCO ₃)	2200	2200
Sodium chloride (NaCl)	6800	6800
Sodium phosphate monobasic (NaH ₂ PO ₄ -H ₂ O)	140	140
Vitamins	1	
Biotin		
Choline chloride	1	1
D-calcium pantothenate	1	1
Folic acid	1	1
Niacinamide	1	1
Pyridoxal hydrochloride	1	1
Riboflavin	0.1	0.1
Thiamine hydrochloride	1	1
i-inositol	2	2
Amino acids		
L-arginine hydrochloride	21	126
L-Cystine 2HCl	16	31
L-glutamine	292	292
L-histidine	8	
L-histidine hydrochloride-H ₂ O		42
L-isoleucine	26	52
L-leucine	26	52
L-lysine hydrochloride	36.47	73
L-methionine	7.5	15
L-phenylalanine	16.5	32
L-threonine	24	48
L-tryptophan	4	10
L-tyrosine disodium salt dehydrates	26	52
L-valine	23.5	46
Other materials		
D-glucose (dextrose)	1000	1000
Phenol red	10	10

7.2 Alpha Minimum Essential Medium

- Minimum Essential Medium Eagle-alpha modification is a medium based on MEM, first published in 1971 by *Clifford P. Stanners and colleagues*.

- It contains significant nonessential amino acids, sodium pyruvate, and vitamins (ascorbic acid (vitamin C), biotin, and cyanocobalamin) compared with MEM (Table 3).
- It is also available with lipoic acid and nucleosides.

NB: Some researchers may prefer to add nucleosides as well as fresh L-glutamine, in the culture media.

7.3 Glasgow Minimum Essential Medium

Ian MacPherson and Michael Stoker modified Eagle's medium and named it **Glasgow Modified Essential Medium (GMEM)**.

The modification includes the addition of 10% tryptose phosphate and twice the normal concentration of amino acids and vitamins.

Table 3 Composition of Alpha-Minimum Essential Medium

Components	Concentration (mg/L)
Inorganic salts	
CaCl ₂ (anhydrous)	200.00
KCl	400.00
MgSO ₄ (anhydrous)	97.67
NaCl	6800.00
NaHCO ₃	2200.00
NaH ₂ PO ₄ ·H ₂ O	140.00
Other components	
D-glucose	1000.00
Lipoic acid	0.20
Phenol red	10.00
Sodium pyruvate	110.00
Amino acids	
L-alanine	25.00
L-arginine	105.00
L-asparagine-H ₂ O	50.00
L-aspartic acid	30.00
L-Cystine	24.00
L-cysteine-HCl-H ₂ O	100.00
L-glutamic acid	75.00
L-glutamine	292.00
Glycine	50.00
L-histidine	31.00
L-isoleucine	52.50
L-leucine	52.40
L-lysine	58.00

(continued)

Table 3 (continued)

Components	Concentration (mg/L)
L-methionine	15.00
L-phenylalanine	32.00
L-Proline	40.00
L-serine	25.00
L-threonine	48.00
L-tryptophan	10.00
L-tyrosine	36.00
L-valine	46.00
Vitamins	
L-ascorbic acid	50.00
Biotin	0.10
D-ca Pantothenate	1.00
Choline chloride	1.00
Folic acid	1.00
i-inositol	2.00
Niacinamide	1.00
Pyridoxal HCl	1.00
Riboflavin	0.10
Thiamine HCl	1.00
Vitamin B12	1.36

- This medium is specially designed to grow certain cells.
- Particularly, this medium was initially designed to grow the Baby Hamster Kidney cells (**BHK-21**) to examine the genetic factors impacting cell competence.

NB: The medium composition/formula is available from the websites of various companies.

7.4 Dulbecco's Modified Eagle's Medium

- *Dulbecco/Vogt* modified Eagle's Minimal Essential Medium (**MEM**) and renamed it **Dulbecco's Modified Eagle's Medium (DMEM)**.
- The modification in DMEM includes the addition of ferric nitrate (**iron**), phenol red (**to check the pH alteration**), fourfold vitamins, and twofold amino acids over the MEM.
- In addition, based on the cell's need (e.g., some tumor cells need a high concentration of glucose) of carbon source, either a **low (1000 g•L⁻¹)** or a **high (4500 g•L⁻¹)** glucose is used. So, DMEM is divided into low and high glucose regimes.

- Since the medium is very rich in vitamins, amino acids, glucose, etc., most of the cells grow easily in this type of medium.
- However, cells requiring special medium composition should grow in that specific medium composition.
- For example, to show the effects of estrogens (a female hormone) on the endothelial cells nitric oxide (NO), growth factors, hormones, phenol red, etc. must be removed from the cells.

NB: The medium composition/formula is available on the websites of various companies.

7.5 Iscove's Modified Dulbecco's Medium

- Iscove added 12 ingredients including amino acids and vitamins, sodium pyruvate, selenium, potassium nitrate, and HEPES in DMEM and renamed it ***Iscove's Modified Dulbecco's Medium (IMDM)***.
- Iron (ferric nitrate) is removed from the medium.
- Iscove claimed that this modified medium may be suitable for the growth of transformed cells after DNA transfection, low-density cell cultures, and difficult cell cultures.
- IMDM is also used as growth support for hematopoietic precursor cells and various lymphocytes.

NB: The medium composition/formula is available from the websites of various companies.

7.6 Roswell Park Memorial Institute Medium

- In 1966, ***Moore and his group*** at Roswell Park Memorial Institute, USA, developed a new medium, which was named Roswell Park Memorial Institute Medium (**RPMI**).
- The medium was further modified and renamed **RPMI-1640**. It is one of the most commonly used media, particularly useful for suspension cells, mainly **lymphoid cells**.
- However, many other types of cells including primary cells can easily grow in this medium.
- One liter of RPMI-1640 contains the following: glucose (2 g) pH indicator (phenol red, 5 mg), salts (6 g sodium chloride, 2 g sodium bicarbonate, 1.512 g disodium phosphate, 400 mg potassium chloride, 100 mg magnesium sulfate and 100 mg calcium nitrate) (Table 4).

NB: The medium composition/formula is available from the websites of various companies.

Table 4 Composition of the RPMI medium

Roswell Park memorial institute (RPMI) media	RPMI 1640 (Moore and Gerner 1967)	This medium is originally formulated based on the 5A Medium which was developed by McCoy et al. in 1959. The purpose to develop this medium was the long-term culture of peripheral blood lymphocytes and hybridoma cells characterized by low levels of calcium and magnesium and high levels of phosphate. Later on, the medium composition was modified several times and the media were renamed (e.g. RPMI 1629, 1630, and 1634).
--	-----------------------------------	---

7.7 M 199 Medium

- In 1950, *Morgan, Morton, and Parker* for the first time developed a nutritionally defined medium for cell culture. Thus, it is one of the earliest culture media. The medium was named “medium 199 or M-199.”
- Composition analysis of M-199 shows more than 60 distinct components including almost all amino acids, vitamins, growth hormones, nucleic acid derivatives, etc., to varying extents.
- HBSS or ESS was used as a basic solvent to prepare this medium.
- AT094A is M199 with Earle’s salts and 25 mM HEPES buffer. It does not contain L-Glutamine.
- Originally, this medium was developed to culture fibroblasts from chick embryos.
- It was observed that explanted tissues could survive in Medium 199 without serum but long-term cultivation of cells required supplementation with serum.
- A large number of cells including various endothelial and epithelial cells, transformed cells for vaccine and virus production, and primary explants culture can be grown in M199 with serum supplementation.

NB: The medium composition/formula can be obtained from the websites of various companies.

7.8 HAM’S F-10 Medium

- Developed in 1962–1963 by *Ham*.
- This medium was originally designed in 1962, to support the growth of mouse and human diploid cells.
- Originally, no serum was added to the medium, but subsequently, albumin and fetuin were added as serum constituents.
- Trace elements, copper, and zinc were added for the first time in this culture medium.

- It was observed that CHO cells grew well in this medium without serum supplementation. This was a major boon for biopharmaceutical and bioprocessing industries to produce various cell-based compounds.
- However, culturing other cells in this medium may require serum supplementation.

NB: *The medium composition/formula is available from various company websites.*

7.9 Ham's F-12 Medium

- In 1965, *Ham* developed this medium.
- This medium is claimed to be the world's first chemically defined medium.
- The basic distinctions between Ham's F10 and Ham's F12 media comprise the serum albumin and fetuin replacements with linoleic acid and putrescence, respectively.
- Most of the amino acids are present to a greater extent in Ham's F12 medium than in Ham's F10 medium while those of the vitamins (except choline and inositol) and potassium phosphate are lower in Ham's F12 medium.
- The concentration of zinc is also reduced for a protein-free culture of mammalian cells such as the CHO cells.
- This medium is also available with a 25 mM HEPES buffer, yielding a more effective buffering within the 7.2–7.4 optimum pH range.
- MCDB301 was later developed with 20 trace elements for a protein-free culture of CHO cells in Ham's F-12 medium.
- Some cell types cultured in this medium are primary rat hepatocytes, rat prostate epithelial cells, and CHO cells, without any serum requirement.

NB: *The medium composition/formula is available on the websites of various companies.*

7.10 Kaighn Modified Ham's F-12 Medium

- In 1974, *Kaighn* modified Ham's F12 medium, thereby renaming it Ham's F-12 K culture medium.
- This modification was aimed at the primary culture of mammalian cells.
- Concerning Ham's F-12, the concentrations of the amino acids, pyruvate, biotin, calcium, magnesium, putrescence, and phenol red were higher in this culture medium.

NB: *The medium composition/formula is available on the websites of various companies.*

7.11 DMEM-F12 Medium

- This medium is a **1:1** mixture of Ham's **F-12** and **DMEM**, combining the richness of F12 with DMEM nutritive potency.
- It is widely used for cell culture in serum-free conditions.
- In 1979, *Barnes and Sato* formulated a 50:50 mixture of nutrient-rich DMEM medium and Ham's F12 medium. This medium mixture was subsequently recommended as the basal medium for the serum-free culture of several mammalian cells.

NB: The medium composition/formula is available on the websites of various companies.

7.12 RPMI-1640/DMEM/F-12 Medium

- In 1984, a new cell culture medium was developed by *Murakami* for the serum-free culture of hybridomas.
- It is typically used as a serum-free mode or after being supplemented with insulin, transferrin, ethanolamine, and selenite.
- This medium was developed in a 2:1:1 mixture of RPMI 1640, DMEM, and Ham's F-12, resulting in the terminology of **RDF medium**.

NB: The medium composition/formula is available on the websites of various companies.

7.13 McCoy's 5A Medium

- In 1959, *McCoy* and his colleagues reported the amino acid requirements for in vitro cultivation of *Novikoff Hepatoma Cells*.
- These studies were performed using Basal Medium 5A, resulting in subsequent modification and creation of a new medium known as McCoy's 5A Medium.
- At present, McCoy's 5 M medium is used for the culture of several primary as well as established cell lines.
- The primary cultures derived from adrenal glands, bone marrow (normal), gingiva, lung, mouse kidney, skin, spleen, and other tissues use this culture medium.

NB: The medium composition/formula is available on the websites of various companies.

7.14 Leibovitz L15 Medium

- In 1963, *Leibovitz* developed the L15 medium.
- The buffering capacities of this medium are attributed to phosphates and free basic amino acids instead of sodium bicarbonate so that the culture's pH is maintained in ambient air without a CO₂ incubator.
- Amino acids were added to this medium at their highest concentration.
- Instead of glucose, pyruvate (and galactose) is added at a high concentration to control pH decline due to lactic acid production.
- At present this medium is rarely used.

NB: The medium composition/formula is available on the websites of various companies.

7.15 Waymouth's Mb752/1 Medium (Waymouth 1959)

- In 1959, *Waymouth* developed this medium.
- This medium was developed with a simple composition as feasible, so that **mouse L929 cells** could be cultured without adding a serum and other proteins.
- This medium is composed of a total of 40 components, including glucose, inorganic salts, amino acids, vitamins, purine bases, and hypoxanthine.
- It is characterized by high concentrations of glucose, histidine, lysine, glutamine, choline, and thiamine.

NB: The medium composition/formula is available from the websites of various companies.

7.16 Trowell's T-8 Medium

- In 1959, *Trowell* developed this medium.
- This medium is suited for the long-term culture of **adult rat liver epithelial cells**.
- With its reasonably simple composition, this medium is devoid of any nonessential amino acids as well as vitamins but rather has high glucose and insulin contents.
- It is used for short-term organ culture.

7.16.1 General Observations Regarding Mammalian Cell Culture Media

- The above-stated media are well-commercialized with each having different physical or packaged forms, such as powder or liquid, large or pouch pack.
- The liquid form is subdivided into 10x concentrated solution, 2x concentrated solution, and working solution (1x).
- Ca⁺² and Mg⁺² are missing in some culture media.

- Phenol red (pH indicator of cell culture medium) is absent in some culture media since it may interfere with a certain cell culture treatment.
- Glucose may be added to the media in low, medium, or high concentrations.
- The laboratory personnel/users can choose the product according to experimental requirement(s), particularly the type(s) of cells used.

8 Medium Recommendations for Common Cell Lines

- In the research laboratories or the research and development section in a pharmaceutical company/bioprocessing industry, many continuous mammalian cell lines can be maintained on a relatively simple medium such as **MEM** supplemented with serum.
- However, as mentioned in the previous section, large-scale culture in the bioprocessing industry requires serum-free and protein-free media, for cost-saving and other reasons.
- For desired specialized cells, a specialized medium must be used for optimum growth.
- Since the information for selecting the appropriate medium for a given cell type is usually available in published literature, the responsible person must read and survey the literature. For example, cells available from American Type Culture Collection (**ATCC**) carry all the details of a cell culture medium and its composition.
- If there is no information available about a specific medium for a specific cell type, one can choose the growth medium and serum empirically or test using several different media for best results.
- It is generally recommended that a good way to start mammalian cell culture is by using *MEM for adherent cells and RPMI-1640 for suspension cells*.
- In general, for large-scale culture of mammalian cells in a bioreactor or similar containers for the production of specific cell culture products, a serum-free medium is preferable.
- Serum-free medium is not only cost-effective but also decreases the contamination of deadly microbes or viruses through serum contamination (Eisenblatter et al. 2002).

Table 5 describes some recommended media for the culture of common mammalian cells.

8.1 Recommended Media for Common Cell Types

The following table describes various cell lines isolated from the *Homo sapiens* (humans).

Table 5 Recommended medium for the culture of common mammalian cells

Cell line	Cell type	Species	Tissue	Medium
HUVECS	Endothelial (primary cell)	Human	Umbilical cord	F-12 K and 10% FBS plus 100 µg/ml heparin or M199 with 5% serum VEGF and heparin
HPAECs	Endothelial (primary cell)	Human	Human pulmonary artery	M199 with 5% serum, VEGF, heparin
HMEC-1	Endothelial Cell line	Human	Dermal microvessels	M199 with 5% serum, VEGF, heparin
MCF-7	Epithelial cell line	Human	Breast adenocarcinoma	MEM and 5–10% FBS plus NEAA and 10 µg.ml ⁻¹ insulin DMEM can also be used instead of MEM
MDAMB-468	Epithelial Cell line	Human	Metastatic breast adenocarcinoma	ATCC-formulated Leibovitz's L-15 medium, with 10%FBS, cells also grow well in MEM/DMEM and (5–10)% FBS plus NEAA and 10 µg/ml insulin.
A549	Epithelial cell line	Human	Lung carcinoma	Cells grow well in F-12 K medium (with 10% FBS and penicillin (100 U.ml ⁻¹), streptomycin 100 µg.ml ⁻¹). Cells also grow well in DMEM with high glucose (4.5 to 5 gm glucose/liter of medium).
BEAS-2B	Epithelial cell line	Human	Bronchus of lung	BEGM, also known as LHC-9 with modification or BEBM bullet kit (available from Clonetics) is claimed to be the best culture medium.
HeLa	Epithelial Cell line	Human	Cervical carcinoma	MEM and 10% FBS plus NEAA (in suspension, S-MEM)
LNCaP	Epithelial cells	Human	Male prostate adenocarcinoma	RPMI-1640 with 10%FBS, 100 U/ml penicillin, 100 µM streptomycin sulfate
PC3	Epithelial cell	Human	Male prostate neuroendocrine carcinoma	F-12 K with 10% FBS, 100 U/ml penicillin, 100 µM streptomycin sulfate
HT-1080	Epithelial Cell line	Human	Fibrosarcoma	MEM and 10% HI FBS plus NEAA
Caco-2	Epithelial Cell line	Human	Colon adenocarcinoma	MEM and 20% FBS plus NEAA
VERO	Epithelial cell line	African green monkey	Kidney cancer	EMEM (ATCC formulated) with 10%FBS, or DMEM with high glucose.
HEK-293	Fibroblast Cell line	Humans	Embryonic kidney	MEM and 10% FBS

(continued)

Table 5 (continued)

Cell line	Cell type	Species	Tissue	Medium
COS-7	Fibroblast Cell line	Monkey	Kidney	DMEM and 10% FBS
RAW	Macrophage cell line	Abelson mouse	Blood cell (macrophage) Abelson murine leukemia virus-induced tumor, ascites	DMEM with high glucose, 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate.
Jurkat	Lymphoblast cell line	Human	Lymphoma (T cell)	RPMI-1640 and 10% FBS

HUVECs: Human Umbilical Vein Endothelial Cells; **HPAECs:** Human Pulmonary Artery Endothelial Cells; **HMEC-1:** Human Microvascular Endothelial Cells-1; **MCF-7:** Michigan Cancer Foundation-7; **MDAMB-468:** MDA stands for “M.D. Anderson, MB stands for Metastatic Breast Cancer” and 468 is an abrupt number; **HeLa:** **Henrietta Lacks;** **HEK-293:** Human Embryonic Kidney 293; **Caco-2:** Cancer coli-2; **COS-7:** CV-1 (Simian) in Origin, and carrying the SV40 genetic material; **MEM:** Minimum Essential Medium; **DMEM:** Dulbecco’s Modified Eagle Medium; **NEAA:** Nonessential Amino Acids Solution, **F12K:** Kaighn’s modification of Ham’s K12 medium; **FBS:** Fetal Bovine Serum

9 Common Buffer and Solutions for Cell Culture

In the absence of a culture medium, **Balanced Salt Solution (BSS)** can serve as an in vitro microenvironment for cells for a short period that helps to maintain the structural and physiological integrity of the cells. They also maintain **pH**, osmotic pressure (**osmolality**), and **membrane potential** (Na^+ , K^+ , Ca^{+2}) besides providing **ions** for **attachment** and binding of **enzyme cofactors** (Eagle 1956; Eagle 1971).

9.1 Mechanism of Action of Various Basal Salt Solutions

- Na^+ and K^+ maintain an isotonic solution, resulting in optimum osmolality, and permeability of mammalian cells.
- Ca^{+2} and Mg^{+2} maintain the internal structure of the cells as well as the integrity of the plasma membrane.
- Phosphate and bicarbonate are the buffering agents. They maintain the H^+ concentration of the solutions.
- Glucose or pyruvate acts as a possible energy source.

Some regularly used BSS for keeping cells at room temperature are as follows:

1. Earle’s balanced salt solution
2. Hanks balanced salt solution
3. Phosphate buffer saline

4. Dulbecco's phosphate buffer saline with or without Ca^{++} and Mg^{++}
5. Normal saline
6. Alsever's solution

Here is the description of the process of preparation of the above buffers/solutions.

9.2 Preparation of Earle's Balanced Salt Solution

- **Earle's Balanced Salt Solution (EBSS)** is an isotonic buffer solution containing inorganic salts such as sodium chloride (NaCl), potassium chloride (KCl), and carbohydrate as an energy source, bicarbonate and phosphate for buffering agents (Table 6).
- EBSS is manufactured with and without phenol red and Ca^{+2} and Mg^{+2} .
- This buffer is designed for the short-term maintenance of cells in a CO_2 environment.
- EBSS is commonly used for different cell culture applications such as washing, transporting, and diluting the cells.

NB: *Added phenol red content maybe 10 mg per liter.*

9.2.1 Procedure for Making Earle's Balanced Salt Solution (1 Liter)

1. Take 800 ml distilled water in a suitable container.
2. Add 200 mg CaCl_2 (anhydrous) to the solution.
3. Add 200 mg MgSO_4 (heptahydrate) to the solution.
4. Add 400 mg KCl to the solution.
5. Add 2.2 gm NaHCO_3 to the solution.
6. Add 6.8 gm NaCl to the solution.
7. Add 140 mg Na_2HPO_4 (monobasic) to the solution.
8. Add 1gm glucose or dextrose to the solution.
9. Add 10 mg phenol red to the solution.
10. Adjust solution to final desired pH using HCl or NaOH.
11. Add distilled water until the final volume is adjusted to 1 liter.

Table 6 Composition of Earle's Balanced Salt Solution (EBSS)

Name	Concentration (M)	The amount for 1 liter (mg)
CaCl_2	0.001	200
MgCl_2	0.0008	200
KCl	0.005	400
NaHCO_3	0.026	2200
NaCl	0.117	6800
Na_2HPO_4	0.001	140
D glucose	0.006	1000

9.2.2 Preparation of Hank's Balanced Salt Solution

- **Hanks' Balanced Salt Solution (HBSS)** consists of inorganic salts including **phosphate** apart from being supplemented with glucose and used to maintain a physiological pH and osmotic pressure of the cultured mammalian cells (Table 7).
- It is also used to wash cells besides maintaining them in a viable state.
- Additionally, HBSS provides water and essential inorganic ions to cells.
- HBSS is available in multiple formats including Ca and Mg-free versions for washing cells before trypsinization.
- Phenol red can also be added to HBSS.

9.2.3 Procedure for Making Hanks Balanced Salt Solution

1. Take 800 ml distilled water in a suitable container.
2. Add 8 gm NaCl to 800 ml distilled water.
3. Add 400 mg KCl to the solution.
4. Add 140 mg CaCl₂ to the solution.
5. Add 100 mg MgSO₄·7H₂O to the solution.
6. Add 100 mg MgCl₂·6H₂O to the solution.
7. Add 60 mg Na₂HPO₄·2H₂O to the solution.
8. Add 60 mg of KH₂PO₄ to the solution.
9. Add 1 gm glucose to the solution.
10. Add 350 mg NaHCO₃ to the solution.
11. Add distilled water until the final volume is 1 liter.

9.2.4 Preparation of Phosphate Buffer Saline

- **Phosphate-buffered saline (PBS)** is a buffer solution commonly used in biological research.
- It is a water-based salt solution containing Na₂HPO₄ **disodium hydrogen phosphate**, NaCl, and in some formulations, KCl and KH₂PO₄ (Table 8).
- It closely mimics the pH, osmolarity, and ion concentrations of the human body.
- The buffer helps to maintain a constant pH.
- PBS has many uses because it is isotonic and nontoxic to cells.

Table 7 Composition of Hanks' Balanced Salt Solution (HBSS)

Name	Concentration (M)	Amount per 1 liter (mg)
NaCl	0.14	8000
KCl	0.005	400
CaCl ₂	0.001	140
MgSO ₄	0.0004	100
MgCl ₂	0.0005	100
Na ₂ HPO ₄	0.0003	60
KH ₂ PO ₄	0.0004	60
Glucose	0.006	1000
NaHCO ₃	0.004	350

- The major use of sterile PBS buffer in a mammalian cell culture laboratory is for the aseptic washing of cells/tissue, washing of cell culture containers, following adhesive molecule treatment, washing of the cells following trypsinization, dilution of medium constituents and water-soluble drugs, toxins, etc.

9.2.5 Procedure for Making Phosphate Buffer Saline

Take 800 ml distilled water in a suitable container.

Add NaCl, KCl, KH_2PO_4 , and $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$.

The final pH is adjusted between (7.2–7.4) using NaOH or HCl.

Adjust final volume to 1 liter using double-distilled H_2O , autoclave, and then store.

NB: PBS is the cheapest, most widely used buffer during cell culture including isolation of cells and posttreatment (with the drug of interest) washing.

9.3 Preparation of Dulbecco's Phosphate Buffer Saline

- *Dulbecco* is the name of a scientist who modified the composition of the PBS that suits some mammalian cells washing in a PBS. It is named after the discoverer.
- **Dulbecco's Phosphate Buffered Saline (DPBS)** is intended to provide a **buffer** system for maintaining cell culture medium in the physiological **pH** range of 7.2–7.6.
- It provides cells with water and certain bulk inorganic ions essential for normal cell metabolism. Table 9 contains the Ca^{+2} and Mg^{+2} devoid of DPBS constituents.

NB: The presence of Ca^{+2} and Mg^{+2} promote adhesions. So, the removal of Ca^{++} and Mg^{++} helps in cell detachment.

Table 8 Composition of phosphate-buffered saline (PBS)

Name	Concentration	Amount (in g) per liter
NaCl	0.137 M	8
KCl	0.0027 M	0.2
Na_2HPO_4	0.01 M	1.44
KH_2PO_4	0.0018 M	0.240

Table 9 Composition of Dulbecco's Phosphate Buffered Saline (DPBS) without calcium and magnesium

Name	Concentration (mM)	Amount (in gm) for 1 liter
NaCl	136.9	8
KCl	2.67	0.2
KH_2PO_4	1.47	0.2
Na_2HPO_4 , anhydrous	8.10	1.15

9.3.1 Procedure for Making Dulbecco's Phosphate Buffer Saline Without Ca^{+2} and Mg^{+2}

Take 800 ml distilled water in a suitable container.

Add NaCl, KCl, KH_2PO_4 , and $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$.

Adjust final pH within (7.2–7.4) using NaOH or HCl.

Adjust final volume to 1 liter using double-distilled H_2O , autoclave, and then store.

9.3.2 Procedure for Making Dulbecco's Phosphate Buffer Saline with Ca^{+2} and Mg^{+2} (1 Liter)

Here is the composition of DPBS with Ca^{+2} and Mg^{+2} (Table 10).

Add all components to 800 ml distilled water and mix well.

Adjust final pH within (7.2–7.4) using NaOH or HCl.

Adjust final volume to 1 liter, using double-distilled H_2O , autoclave, and then store.

NB: Generally, Ca^{+2} and Mg^{+2} free PBS/DPBS is used to wash the adhesive cell culture containers before treatment with trypsin-EDTA because Ca^{+2} or Mg^{+2} may react with trypsin. However, since EDTA is already present with trypsin, it helps to chelate Ca^{+2} and Mg^{+2} .

9.4 Normal Saline

- **Normal saline** is the name for **0.89% sodium chloride (NaCl) solution** in double-distilled water.
- This solution is **called normal saline** because its osmolarity ($308 \text{ mOsmol} \cdot \text{L}^{-1}$ (calc)) is nearly the same as that of blood. It contains $154 \text{ mEq} \cdot \text{L}^{-1}$ Na and $154 \text{ mEq} \cdot \text{L}^{-1}$ Cl.

To prepare normal saline the following requirements need to be met.

NaCl: 0.89 gm.

The final volume of double distilled water is 1000 ml.

The pH of normal saline is 4.5 to 7.0.

Table 10 Composition of Dulbecco's Phosphate Buffered Saline (DPBS) with calcium and magnesium

Name	Concentration (mM)	Amount (in g) for 1 liter
NaCl	136.9	8
KCl	2.67	0.2
KH_2PO_4	1.47	0.2
Na_2HPO_4 (anhydrous)	8.10	1.15
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.90	0.10
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	0.50	0.1

9.5 Alsever's Solution

- **Alsever's solution** was first used in 1941 by the American hematologist *John Bellows Alsever*.
- Alsever's solution is a saline liquid used as an anticoagulant of blood (Table 11).
- An equal volume of Alsever's solution is gently and thoroughly mixed with the blood to prevent coagulation.

9.5.1 Role of Alsever's Solution

- The solution is frequently used as a blood cell preservative as it permits the storage of blood cells at refrigerator temperatures for nearly 10 weeks.
- The benefits of using Alsever's solution are its ability to support the antigenic life of red blood cells (RBCs), enabling its use in RBC screening, identification panels, as well as RBCs, drawn for serological investigation.
- Alsever's solution use is not restricted to human RBCs; it is also used to preserve RBCs from animals.
- Sheep and poultry are the most common animals that have their RBCs preserved in Alsever's solution.

9.5.2 Precautions and Limitations of Alsever's Solution

Like all solutions, Alsever's solution may have the following limitations:

- The solution should only be used for in vitro diagnostics purposes.
- The solution is light-sensitive, so should be stored in a dark place.
- Turbidity of the Alsever's solution generally indicates contamination which means the solution should not be used.
- Blood specimens showing signs of hemolysis or contamination should not be suspended in Alsever's solution as it may severely reduce the shelf life of the suspension.
- Bottles or vials of Alsever's solution which are leaking should be discarded.
- For the Alsever's solution to remain stable until the end of the shelf life, it should be stored in a fridge between (2–8) °C.

Table 11 Composition of Alsever's solution

Name	Amount (in g) per liter
D-glucose/ dextrose	2.05
Sodium citrate	0.8
Citric acid	0.055
NaCl	0.42

NB: During autoclaving, all solutions containing D-glucose can be charred at high temperatures. Therefore, D-glucose solution is prepared in minimum water and filter sterilized with a Millipore membrane filter. The remaining ingredients are added to water having a final volume of 1 liter minus the D-glucose solution volume. The filter-sterilized D-glucose solution is later added to the autoclaved solutions to get the final volume of reconstituted 1 liter BSS. HEPES and NaHCO_3 are common buffers used to balance the pH of the culture medium.

10 pH Maintenance of the Cell Culture Medium

pH is the negative logarithm of H^+ concentration. pH ranges from 0 to 14. At 25 °C, pH 7 is neutral. At 25 °C, solutions with a pH less than 7 are acidic and solutions with a pH > 7 are basic. pH maintenance of a cell culture medium is highly essential.

- All cell culture media are checked for pH before adding the supplements.
- The pH of all culture media is checked with pH paper, but not with a pH meter as a medium can interfere with an electrode of a pH meter.
- The pH of the medium is adjusted with NaOH and HCl.
- All mammalian cell culture medium pH is maintained in a lower alkaline range like blood that is, **between 7.2 and 7.4 (while human cell cytoplasm pH is 7.2, blood pH is 7.4)**.
- This is called the **physiological pH range** as this is the optimum pH for mammalian cells and helps the cells to maintain proper ionic balance and keep the optimum activity of cellular enzymes, hormones, and growth factors in cell cultures.
- **In a CO_2 incubator, the regulation of mammalian cell culture medium pH uses the buffering system as a bicarbonate – 5% CO_2 system.**
- To detect the pH of the culture medium visually, **phenol red, a chemical pH indicator** is added to most commercially available culture media. Phenol red indicates medium pH changes by cell metabolites by readily changing its color.
- **In presence of phenol red, the alkaline pH of the medium is detected by light pink or orangish color whereas acidic pH is determined by its yellow color.**
- An acidic pH below 6.8 (yellowish color) indicates either bacterial contamination or retarded cell growth in the spent medium. So, when the pH of the medium falls below 7.0, the medium needs to be changed as early as possible (Howorth 1975).

NB: Phenol red has structural similarity with estrogens and estrogen-like phenolic/polyphenolic compounds. Therefore, phenol red must be removed while experimentally working with estrogens/other steroids/phenolic compounds.

10.1 Maintenance of Osmolarity/Osmolality of the Cell Culture Medium

- Mammalian cells need an isotonic environment. So, the cell culture medium must maintain isotonic conditions.
- The *ideal osmotic pressure of the cell culture medium intended for culturing the human cells is 290 mOsm·kg⁻¹*.
- This osmotic pressure is needed as it is the **osmotic pressure of human plasma**.
- However, in most mammalian cells, osmotic pressure ranges between (260–320) mOsm·kg⁻¹.
- The osmotic pressure of a mouse plasma is about **320 mOsm·kg⁻¹**.
- Mainly various salts, glucose, and amino acids maintain the osmotic pressure of mammalian cell culture medium to an isotonic level (**~300 mOsm**).
- The **osmolality** of the **culture medium** should be similar to the **osmolality** of the physiological environment.
- While osmolality can affect both cell growth and function, the medium hypermolality can shrink the cells and hypomolality can make them swell.
- Thus, the culture medium is always kept isotonic and all other buffers and solutions are maintained at this level only (Waymouth 1970).

11 Antibiotics and Antimycotics

- By definition, **antibiotics are substances produced by a group of microorganism (e.g., *Penicillium notatum*, a yeast that produces antibiotic penicillin) and acts against another group of microorganism (e.g., Gram-positive bacteria) at low concentration**.
- Similarly, antimycotics are substances produced by the microorganisms such as yeast and prevent the growth of fungus or molds.
- Antibiotics and antimycotics are slowly degraded at 37 °C or room temperature and perhaps in refrigerators too.
- So, since there is a life span of antibiotics and antimycotics, they are required to be added fresh in every mammalian cell culture medium to prevent the growth of the contaminating microorganisms (bacteria and fungus, respectively).
- **Penicillin, streptomycin, and gentamicin** are common **broad-spectrum antibiotics** working against bacteria.
- **Nystatin** and **amphotericin B** are used as **antimycotic agents** against yeast and fungi.
- Three classes of antibiotics kill **mycoplasma** when used at relatively low concentrations: **tetracycline, macrolides, and quinolones**.
- Table 12 describes some antibiotics and antimycotics, their storage temperature, the final concentration needed for the cell culture medium, and stability at 37 °C.

Table 12 Description of antibiotics and antimycotics, their final concentrations, storage temperature, and stability in a CO₂ incubator

Name	Storage temperature	Concentration (final)	Stability in CO ₂ incubator (37 °C)	Antibiotic/antimycotic
Penicillin G Benzylpenicillin	-20 °C	100 U·ml ⁻¹	3 days	Antibiotic
Ampicillin sodium salt	4 °C	100 µg·ml ⁻¹	3 days	Antibiotic
Ciprofloxacin hydrochloride	-20 °C	10 µg·ml ⁻¹	Not known	Antibiotic
Tetracycline hydrochloride	-20 °C	10 µg	4 days	Antibiotic
Gentamycin sulfate	4 °C	50 µg·ml ⁻¹	5 days	Antibiotic
Neomycin sulfate	-20 °C	50 µg·ml ⁻¹	5 days	Antibiotic
Polymyxin B	4 °C	100 U·ml ⁻¹	5 days	Antibiotic
Hygromycin B	4 °C	500 µg·ml ⁻¹	Not known	Antibiotic
Kanamycin sulfate	-20 °C	100 µg·ml ⁻¹	5 days	Antibiotic
Streptomycin sulfate	-20 °C	100 µg·ml ⁻¹	3 days	Antibiotic
Chloramphenicol	-20 °C	5 µg·ml ⁻¹	5 days	Antibiotic
Puromycin dihydrochloride	-20 °C	20 µg·ml ⁻¹	Not known	Antibiotic
Nystatin	-20 °C	100 µg·ml ⁻¹	3 days	Antimycotic
Amphotericin B	-20 °C	2.5100 µg·ml ⁻¹	3 days	Antimycotic

The following are the cautions regarding the use of antibiotics/antimycotics in a mammalian cell culture medium:

- Antibiotics/antimycotics target specific molecules in the microorganisms. For instance, penicillin attacks penicillin-binding proteins (**PBPs**), a transpeptidase enzyme prevailing in the peptidoglycan layer of the bacteria. PBPs help in the cross-linking of the peptidoglycan layer.
- The PBPs are not produced by mammalian cells. However, a very high penicillin concentration may nonspecifically affect the cultured mammalian cells and express toxic effects. Therefore, always use a low but sufficient concentration of antibiotics/antimycotics that will affect only the microorganisms and not the cultured mammalian cells.
- The addition of serum neutralized many of the exerted toxic effects on the cultured mammalian cells.
- *As discussed in the earlier paragraphs, it is best either not to use antibiotics in a serum-free culture medium or to use them at a 5–10 times lower extent as compared to serum-containing medium.*
- Drug (antibiotic/ antimycotic)-resistant microorganisms could pose significant difficulty in mammalian cell culture.

- Since antibiotics/antimycotics are temperature-sensitive, so care must be taken during repeated freeze-thawing of antibiotics or proper replacement of cell culture medium after stipulated days of incubation at 37 °C in a CO₂ incubator.

NB: Stock antibiotics and antimycotics should be stored at – 20 °C in several aliquots.

- Antibiotics/antimycotics are not useful against virus contamination in cell culture. While contaminations with various viruses are rare, those by mycoplasma are a serious obstacle in mammalian cell culture since cell culture serum is often infected with mycoplasma. While various interferons will regulate virus growth, ***mycoplasmin*** can regulate mycoplasma growth. Peptidoglycan synthesis inhibitors such as penicillin cannot be useful against mycoplasmas because mycoplasmas do not have any cell wall (Perlman 1979).

12 Conclusions

Mammalian cells need various nutrients and other materials for culture, growth, and maintenance. The materials commonly needed for the mammalian cell culture are cell-dissociating agents, cell adhesive agents, various cell culture media, and various types of buffers. The cell-dissociating agents are necessary for dissociation and the isolation of mammalian cells from the tissues and organs. Once the cells are dissociated, the next step is to seed the isolated mammalian cells into the cell culture vessels containing the cell culture medium. Since most mammalian cells are adhesive in nature, cell adhesive agents may be added to the mammalian cell culture containers which are made up of polystyrene. After the treatment of the polystyrene-containing culture vessels with the adhesive agents, cells suspended in a cell culture medium are added to the culture vessels. The cell culture medium contains all the necessary materials necessary for the culture and growth of the cells. However, since different cells may need different amounts or types of various nutrients, different types of cell culture media are formulated accordingly. Finally, various buffers are necessary for the isolation, culture, and maintenance of mammalian cells. This chapter described all these materials necessary for the isolation, culture, and maintenance of mammalian cells.

13 Cross-References

- ▶ [Culture of Continuous Cell Lines](#)
- ▶ [Culture of Neuron and Glia Cells](#)
- ▶ [Isolation and Primary Culture of Various Mammalian Cells](#)
- ▶ [Primary Culture of Immunological Cells](#)
- ▶ [Stem Cell Culture and Its Applications](#)

References

- Allen A, Snow C. The effect of trypsin and ethylene-diamine-tetra acetate on the surface of cells in tissue culture. *Biochem J.* 1970;117:32.
- Barnes D, Sato G. Growth of a human mammary tumor cell line in a serum-free medium. *Nature.* 1979;281:388–9.
- Barnes D, Sato G. Serum-free cell culture: a unifying approach. *Cell.* 1980;22:649–55.
- Bottenstein J, Hayashi I, Hutchings SH, Masui H, Mather J, McClure DB, et al. The growth of cells in serum free hormone supplemented media. *Methods Enzymol.* 1979;58:94–109.
- Brunner D, Frank J, Appl H, Schoffl H, Pfaller W, Gstraunthaler G. Serum-free cell culture: the serum-free media interactive online database. *ALTEX.* 2010;27:53–62.
- Carrel A. On the permanent life of tissues outside of the organism. *J Exp Med.* 1912;15:516–28.
- Carrel A, Baker LE. The chemical nature of substances required for cell multiplication. *J Exp Med.* 1926;44:503–21.
- Cinatl J. Inorganic-organic multimolecular complexes of salt solutions, culture media and biological fluids and their possible significance for the origin of life. *J Theor Biol.* 1969;23:1–10.
- Curtis ASG. Cell contact and adhesion. *Biol Rev.* 1962;37:82.
- Curtis ASG, Forrester JV, McInnes C, Lawrie F. Adhesion of cells to polystyrene surfaces. *J Cell Biol.* 1983;97:1500–6.
- Dalen H, Todd PW. Surface morphology of trypsinized human cells *in vitro*. *Exp Cell Res.* 1971;1971(66):353.
- Darling DC, Morgan SJ. *Animal cells: culture and media.* New York: Wiley; 1994.
- Eagle H. Nutrition needs of mammalian cells in tissue culture. *Science.* 1955;122:501–14.
- Eagle H. The salt requirements of mammalian cells in tissue culture. *Arch Biochem Biophys.* 1956;2:356–66.
- Eagle H. Buffer combinations for mammalian cell culture. *Science.* 1971;174:500–3.
- Eisenblatter T, Psathaki K, Nitz T, Galla H, Wegener J. Cell culture media: selection and standardization. In: Lehr CM, editor. *Cell culture models of biological barriers in-vitro test systems for drug absorption and delivery.* London: Taylor & Francis; 2002. p. 20–40.
- Fischer A, Astrup T, Ehrensvar G, Oehlschlager V. Growth of animal tissue cells in artificial media. *Proc Soc Exp Biol Med.* 1948;67:40–6.
- Freshney RI. Defined media and supplements. In: Freshney RI, editor. *Culture of animal cells.* Hoboken: Wiley; 2010. p. 99–114.
- Guilbert LJ, Iscove NN. Partial replacement of serum by selenite, transferrin, albumin and lecithin in haemopoietic cell cultures. *Nature.* 1976;263:594–5.
- Ham RG. Clonal growth of mammalian cells in a chemically defined, synthetic medium. *Proc Natl Acad Sci U S A.* 1965;53:288–93.
- Ham RG, McKeenan WL. Media and growth requirements. *Methods Enzymol.* 1979;58:44–93.
- Hamilton WG, Ham RG. Clonal growth of Chinese hamster cell lines in protein-free media. *In Vitro Cell Dev Biol.* 1977;13:537–47.
- Heng BC, Cowan CM, Basu S. Comparison of enzymatic and non-enzymatic means of dissociating adherent monolayers of mesenchymal stem cells. *Biol Proc Online.* 2009;11:161–9.
- Hewlett G. Strategies for optimising serum-free media. *Cytotechnology.* 1991;5:3–14.
- Hornsby P, Sturek M, Harris S, Simonian M. Serum and growth factor requirements for proliferation of human adrenocortical cells in culture: comparison with bovine adrenocortical cells. *In Vitro.* 1983;19:863–9.
- Hosios AM, Hecht VC, Danai LV, Johnson MO, Rathmell JC, Steinhauser ML, Manalis SR, Vander Heiden MG. Amino acids rather than glucose account for the majority of cell mass in proliferating mammalian cells. *Dev Cell.* 2016;36(5):540–9.
- Howorth P. The physiological assessment of acid-base balance. *Br J Dis Chest.* 1975;69:75–102.
- Iscove NN, Melchers F. Complete replacement of serum by albumin, transferrin, and soybean lipid in cultures of lipopolysaccharide-reactive B-lymphocytes. *J Exp Med.* 1978;147:923–33.
- Jacoby F, Darke SJ. Animal tissue culture with a synthetic medium. *Nat.* 1948;161:768–9.

- Karmioli S. Development of serum free media. In: Master JRW, editor. *Animal cell culture*. 3rd ed. Oxford: Oxford University Press; 2000.
- Keen MJ, Rapson NT. Development of a serum-free culture medium for the large scale production of recombinant protein from a Chinese hamster ovary cell line. *Cytotechnology*. 1995;17:153–63.
- Lewis MR. The importance of dextrose in the medium of tissue cultures. *J Exp Med*. 1922;35:317–22.
- Macy ML, Shannon JE. Preparation of medium ATCC-CRCM30. *TCA Manual*. 1977;3:617–22.
- Masters J, Stacey G. Changing medium and passaging cell lines. *Nat Protoc*. 2007;2:2276–84.
- Mather JP. Making informed choices: medium, serum, and serum-free medium. *Methods Cell Biol*. 1998;57:19–30.
- Merten OW. Safety issues of animal products used in serum-free media. *Dev Biol Stand*. 1999;99:167–80.
- Moore GE, Gerner RE, Franklin HA. Culture of normal human leukocytes. *JAMA*. 1967;199(8):519–524.
- Morgan JF, Morton HJ, Parker RC. Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium. *Exp Biol Med*. 1950;73:1–8.
- Murakami H. Serum-free media used for cultivation of hybridomas. *Adv Biotechnol Processes*. 1989;11:107–41.
- Murakami H, Masui H, Sato GH, Sueoka N, Chow TP, Kano-Sueoka T. Growth of hybridoma cells in serum-free medium: ethanalamine is an essential component. *Proc Natl Acad Sci U S A*. 1982;79:1158–62.
- Neuman RE, McCoy TA. Growth-promoting properties of pyruvate, oxaloacetate, and α -ketoglutarate for isolated Walker carcinosarcoma 256 cells. *Exp Biol Med*. 1958;98:303–6.
- Parker RC. Chemically defined media. In: Parker RC, editor. *Methods of tissue culture*. New York: Harper & Row; 1961. p. 62–80.
- Perlman D. Use of antibiotics in cell culture media. *Methods Enzymol*. 1979;58:110–6.
- Ramsey WS, Hertl W, Nowlan ED, Binkowski NJ. Surface treatments and cell attachment. *In Vitro*. 1984;20:802–8.
- Seifert WE, Rudland PS. Possible involvement of cyclic GMP in growth control of cultured mouse cells. *Nat*. 1974;248:138.
- von Seefried A, Macmorine H. The use of foetal, calf and adult bovine sera for the growth of serially subcultivated diploid cells. *Dev Biol Stand*. 1976;37:83–9.
- Waymouth C. Rapid proliferation of sublines of NCTC clone 929 (strain L) mouse cells in a simple chemically defined medium (MB752/1). *J Nat Cancer Inst*. 1959;22:1003–1017.
- Waymouth C. Osmolality of mammalian blood and of media for culture of mammalian cells. *In Vitro*. 1970;6:109–27.
- White PR. Cultivation of animal tissues in vitro in nutrients of precisely known constitution. *Growth*. 1946;10:231–89.