Introduction

Cells are the core of cell technology. Medium to cells is like food to humans. It is the most important factor that impacts a cell's growth, metabolism, and other biological activities. For industrial processes, it dictates the productivity and often the product quality. Over the past three decades, we have drastically expanded our understanding of the chemical environment that enables cells that have been isolated from different tissues to grow, as well as the environment that allows cells to differentiate from one state to another. Cells which have been isolated from different tissues or species, or which are at different differentiation state, have different needs in terms of growth factors and cytokines. But the basic nutritional requirements of mammalian cells in culture are strikingly similar among cells of different tissue origins and species.

Early in the industrialization of cell culture, media design efforts focused on enhancing cell proliferation and on eliminating animal serum or other animal-derived media components. The work was mostly spearheaded by bioscientists using a mechanistic understanding of cells' nutritional needs (Panel 7.1). The arrival of the antibody era in the second half of the 1990s and the sudden demand for large quantities of antibody product called for increased cell concentration and productivity and changed the face of cell culture processes. Fed-batch culture and concentrated feed medium were used more frequently. Instead of providing cells with optimal growth conditions as had been done during the previous decade, cells were increasingly cultured under conditions suboptimal for growth, or even under stressed conditions that favored

product formation. The intense emphasis on maximizing the productivity and modulating product quality demanded that medium development work take a stoichiometric and kinetic perspective. The philosophy of medium optimization changed from pampering cells with inviting growth conditions to maximizing cell density and stressing cells in order to squeeze out every last bit of productivity. Much of the success in increasing product titer by at least an order of magnitude over the course of a decade can be attributed to better media formulation. As optimizing cell growth was de-emphasized, the media that was optimized for production was also used for cell expansion in seed cultures.

Over the life cycle of a product, cells spend far more time expanding their population—from single-cell cloning and cell banking to seed train—than in the final production bioreactor. An optimized production medium maximizes productivity. A well-balanced cell expansion medium optimizes cell growth and minimizes deleterious metabolite formation, and sometimes even enhances the cells' structural and phenotypical stability. A holistic view should be taken in medium development. The industrial cell culture medium should not be monolithic; on the contrary, it should be a multitude of media that enable cells to proliferate optimally at a fast rate while maintaining cellular, structural, and genomic stability during the expansion stage, while also allowing cells to achieve conditions favorable to maximum productivity and high product quality (Panel 7.2).

The renewed emphasis on cell expansion in media design is critical for cell therapy and regenerative medicine. In those applications, cells are the product that is administered to patients. They must have high viability and functional activity. In the past decade, genomic science has profoundly changed our approach to biomedical and biochemical exploration. Modern medium design will increasingly exploit the genomic data of the cell of interest and design the medium

Panel 7.1. Historical Approaches in Industrial Cell Culture Medium Design

Classic Medium Design

- Optimized for cell growth
- Eliminates serum and animal-derived components
- Relies on a metabolic understanding of cells' nutritional requirements and meticulous experimentation

Antibody Era

- Maximizes productivity even at the expense of the growth rate
- Emphasizes stoichiometric and kinetic perspectives

Integrated Approach

- Combines mechanistic, stoichiometric, and kinetic perspectives
- Optimized for both cell expansion and production
- Employs genomic and transcriptome information on cells' biological capabilities

Panel 7.2. Industrial Cell Culture Media for Cell Expansion and Production

Industrial Cell Culture Process

- A long period of cell expansion followed by a relatively short production/differentiation period

Medium Design Objectives

- Cell expansion medium
  - Optimized for growth
  - Minimizes deleterious metabolites and reactive oxygen species (ROS)
  - Enhances cells' structural integrity and production stability
- Production medium
  - Maximizes cells' activity, productivity, or product quality

Table 7.1. Cellular Chemical Environment *in Vivo**

Approximate Concentrations in a Cellular Environment		
	Interstitial (mM)	*Intracellular (mM)*
Na^+	140	14
K^+	4.0	140
Ca^{2+}	1.2	0.01
Mg^{2+}	0.7	20
Cl^-	108	4
HCO_3^-	28.3	10
HPO_4^{3-}, $H_2PO_4^{2-}$	2	11
SO_4^{3-}	0.5	1
Amino acids	2	8
Lactate	1.2	1.5
Glucose	56	
Protein	0.2	4
Total chemical species (mmole/L)	301.8	302.2
Corrected osmolar activity (mM)	281.3	281.3

*Adapted from *Textbook of Medical Physiology*, A.C. Guyton.

Panel 7.3. Some Characteristics of Interstitial and Intracellular Fluid

- Interstitial fluid represents cells' native niche environment
- Total osmolality is ~280–300 mOsm
- Na^+ and Cl^- are the most abundant in interstitial fluid
- There is a large concentration difference between extracellular and intracellular fluid in the concentration of:
  - Na^+, Cl^-, and Ca^{2+} (high outside the cell)
  - K^+, Mg^{2+}, and PO_4^{3-} (high inside the cell)

based on the cell's intrinsic capabilities as gleaned from it genetic and epigenetic features.

A Guide for Medium Design: Body Fluids

Originally, all cells in culture were isolated from animal tissues. The composition of the chemical environment of their natural niche is a relevant reference for designing an optimal medium for their cultivation. The vast majority of cells in the body are not in direct contact with blood, and are instead surrounded by interstitial fluid. The protein and hormone content of interstitial fluid differs in various tissues, but the general chemical composition of small molecular weight solutes in interstitial vs. intracellular fluid is similar among cells of different tissues. A representative composition of interstitial fluid is shown in Table 7.1, along with that of intracellular fluid.

The low molecular weight solute composition of interstitial and intracellular fluid bears a few important characteristics. The total osmolality is around 280–300 mOsm, or about a total of 280–300 mM of dissociated solutes in both the intracellular and extracellular environments (Panel 7.3). The media for cells used in research has largely kept the total osmolality in the range of 280–300 mOsm, close to that of interstitial fluids. Bear in mind that a dissociable ionic compound contributes its total dissociated ions to the osmolality, whereas an undissociated molecule contributes an equivalent of its concentration. Thus 1 mM of $MgCl_2$ contributes a total 3 mOsm; while 1 mOsm of glucose contributes 1 mM in an ideal solution. A couple of percentage points of error notwithstanding, the osmolarity can be taken as the sum of the molarity of all dissolved species in the fluid. The largest contributor to the final osmolarity of the interstitial fluid is Na^+, followed by Cl^-. A number of other inorganic species including K^+, Mg^{2+}, and Ca^{2+} are present at low concentrations in the millimeter range.

The major anions are Cl^- and the bicarbonate ion (HCO_3^-) (10–30 mM) that serves as a pH buffer.

A number of ion species are present at very different concentrations between the interstitial space and the cytoplasm. Both Na^+ and Cl^- are present outside the cell at a 10-fold higher concentration than inside the cell, as is Ca^{2+}. In contrast, K^+, Mg^{2+}, and PO_4^{3-} concentrations are much higher on the intracellular side.

Cells' tolerance to deviations from "optimal" conditions varies with the physical and chemical conditions. The sublethal but non-lethal range of different compounds also varies considerably. For example, cells in culture can tolerate low (80 mM) or high (140 mM) sodium concentrations, or high osmolarity (up to 400 mOsm), for a period of days. Hamster cells may tolerate or even grow with a reduced growth rate in hypothermic conditions of 33 °C or a low pH of 6.8, but not at the higher temperature of 40 °C at a pH of 7.8.

Types of Media and Classes of Medium Components

A complete cell culture medium has two major categories of components: basal medium and growth supplements (Panel 7.4). The basal medium consists of the small molecular weight components such as sugar, amino acids, vitamins, nucleosides, and inorganic salts. The basal medium provides sugar as an energy source and various nutrients for making new cell mass and product. It also provides balanced salt concentrations and osmolarity to allow for cell growth. In general, the basal medium can be prepared as a mixture of chemicals in powder form and is commercially available. Upon dissolution in pure water and sterile filtration, it is ready for use. In practice, a number of components that are unstable (i.e., spontaneously degraded) in solution or are hygroscopic if present in the power mixture are excluded from the powder medium. Many amino acids and vitamins are heat labile, so the basal medium is typically sterilized by membrane filtration.

Many cell lines and primary cells explanted from tissues will not proliferate if provided with basal medium alone, as basal medium does not contain growth factors or other factors necessary for growth. Growth supplements that may be added to basal medium include growth factors, phospholipids, soy hydrolysate, and serum. These supplements may promote cell growth by providing molecules that modulate

Panel 7.4. Major Classes of Medium Components

Basal Medium
- Sugar
- Amino acids
- Fatty acids, lipids, and their precursors
- Vitamins
- Nucleosides
- Bulk salts
- Trace elements
- pH buffer

Supplements
- Serum
- Hydrolysates
- Growth factors
- Carrier proteins

specific signaling pathways, or by filling some nutritional needs (such as delivering cholesterol). Some signaling molecules, instead of stimulating cell growth, trigger a signaling pathway that directs cellular differentiation or maintains cells at a particular differentiation state.

Types of Media

Serum-free and animal component-free medium

Animal serum has been a regular component of cell culture medium, used at 5–20% (by volume) levels in addition to basal medium. Serum is highly complex in its chemical composition. It contains a large mixture of proteins, from serum albumin, immunoglobulins, clotting proteins, and lipids to small molecular weight compounds including sugar and amino acids. The outbreak of the prion disease bovine sponge form encephalopathy in the United Kingdom in the late 1980s compelled the cell culture industry and the regulatory agencies to undertake large efforts to eliminate serum from media. Since then, the use of serum-free and animal component-free media has become the industrial manufacturing norm (Panel 7.5). Removing raw materials of animal origin minimizes animal virus or prion contamination. It also eliminates the possibility of some antibodies present in the serum reacting with a cell antigen or the product (especially important for viruses produced for use as vaccines), which would reduce productivity. The use of serum-free and animal component-free media has been extended to the cell line development stage to minimize the exposure of cells to adventitious agents throughout the entire cell banking and manufacturing process. In the early days of serum-free medium, this effort was thwarted by the requirement of growth factors or other materials that were isolated from animal tissue, such as serum albumin and transferrin. Most of those supplements are now produced by recombinant DNA technology in microbial or mammalian cells. However, animal serum is still widely used in manufacturing viral vaccines. In the cultivation of human primary differentiated cells for clinical applications, inactive human serum is often used.

Panel 7.5. Serum-Free, Animal Component-Free Medium

- Serum-free media consists of a nutritionally complete basal medium supplemented with an empirically determined mixture of hormones, growth factors, cell attachment proteins, and binding proteins
- Many serum-free media contain a complex mixture of undefined components, such as soybean-meal, another plant hydrolysate, peptone, or beef hydrolysate
- Animal component-free media eliminates all materials derived from animals, such as growth factors, transferrin, and beef peptone
- The growth rate in serum-free media is often slower than in serum-containing media

Complex vs. chemically defined media

Many medium components that have been traditionally used in cell culture are a complex mixture of different components, such as animal

serum, plant hydrolysate, soybean phospholipids, and yeast extract. The inclusion of any of those materials in the medium make its composition incompletely characterized. Any medium that has an undefined chemical composition is a complex medium. In addition to a complex mixture like beef peptone, some seemingly "pure" components may readily associate with impurities that are not easily characterized; for example, serum albumin is a carrier of lipids and many metabolites that are not easily removed during purification. Those components are also regarded as having a complex composition.

A chemically defined medium contains only components whose chemical composition is known and characterized, and has all of its chemical species specified (Panel 7.6). It does not contain any mixture of components with an unknown or undefined composition. For example, "lipids" or "phospholipids" are not well-defined compounds, but are mixtures of a class of compounds that are not chemically specified. A chemically defined medium may contain purified growth factors, cytokines, and carrier proteins. A chemically defined medium is not necessarily protein-free.

Panel 7.6. Chemically Defined Medium

- Contains no complex nutrient mixtures
- All components are completely characterized in terms of chemical composition
- Its adoption was accelerated by the drive to exert control over all aspects of production and downstream processing for licensing by the FDA
- Its wide use is facilitated by
  - High-throughput screening of media components
  - The availability of growth factors produced by rDNA technology
  - Adaptation of cells to leaner growth environments
  - The development of small molecules or synthetic peptides that are biologically active in stimulating cell proliferation or directing differentiation

A large number of manufacturing processes for protein therapeutics employ chemically defined media. Many cell cultures for cell therapy or regenerative medical applications also employ chemically defined media. The application of chemically defined media in industrial manufacturing has become more widespread largely due to the drive of regulatory requirements, the availability of better-characterized raw materials, and the increasing ease in adapting cells to different culture environments (Panel 7.6).

Protein-free medium

By definition, a protein-free medium contains no protein. Most protein-free media are chemically defined. However, a protein-free medium may contain undefined lipids or fatty acids and may therefore not be chemically defined. Many cells that grow well in protein-free media are highly adapted or transformed. Most normal diploid cells, including stem cells and various differentiated cells, require some growth factors or cytokines in order to proliferate in culture.

Classes of Medium Components

Stoichiometric vs. unconsumed medium components

Medium components fall into two general categories: stoichiometric and habitable (also called "unconsumed"). Stoichiometric components are taken up by cells, metabolized, and used to make more cell mass and product (Panel 7.7). Virtually all organic components are stoichiometric components, except for some antioxidants. Some inorganic salts are also consumed to become a significant portion of cellular materials, like phosphate is a constituent of nucleic acids. A stoichiometric relationship between the biomass and the medium components that are consumed can be established for stoichiometric medium components. If one wishes to grow more cells or produce more product, these components must be supplied in larger quantities.

Medium components of the second category, mostly inorganic compounds, play the critical role of providing a chemical environment that is suitable for cell growth, but they are barely consumed by cells. They are called habitable (or habitat-enabling) components. Many of these salts are also components of the intracellular chemical environment (e.g., Na^+ and Cl^-). Even water is taken up by cells; as the total cell volume expands, water must be taken up along with other components that constitute cellular materials. However, their intracellular concentrations are low and the quantity taken up by growing cells from the medium is unappreciable. In medium design, the level of unconsumed components is to be maintained in a range that sustains the habitable environment.

Panel 7.7. Two General Types of Medium Components

Stoichiometric Components

- Medium components that are consumed in appreciable amounts
- e.g., glucose, amino acids, fatty acids, some growth factors, and some salts like phosphate
- Should be supplied in some proportion to the cell and product concentrations to be produced

Habitable (Unconsumed) Components

- Components that are hardly consumed by cells but are needed in some level to provide conditions necessary for cell proliferation
- Many such components are taken up by cells because they are constituents of the intracellular environment. However, their consumption rate is very low and almost unmeasurable.
  - e.g., Cl^- and Na^+
- Should be maintained at relatively constant levels

The classification of stoichiometric and unconsumed nutrients is therefore operational. A normally unconsumed component may be taken up in a substantial quantity at a high cell concentration. Thus, unconsumed medium components may become stoichiometric components under high-density cultivation conditions; this is illustrated with phosphate in Panel 7.8.

Macromolecules as consumed and unconsumed medium components

Cell culture medium often contains macromolecules such as insulin, fibroblast growth factor (FGF), and serum albumin. These constituents serve a variety of purposes; some are carrier proteins that ferry ligands into the cell, while others

Panel 7.8. Phosphate as a Stoichiometric Medium Component

- PO_4^{3-} concentration in medium is typically ~1 mM, while its cellular concentration is 11 mM. The intracellular ATP/ADP concentration is about 2mM, or ~6mM of phosphate. Total DNA and RNA contain another 35 mM equivalent of phosphate. A culture with cells of an average volume of 2×10^{-12} L and at a cell concentration of 10^{10} cells / L takes up about 1 mmol / L of PO_4^{3-} [(11 + 6 + 35) mmol / L x 2×10^{-12} L / cell x 10^{10} cell / L].
- Cell consumption of PO_4^{3-} will drastically reduce its concentration at such a cell concentration

are growth factors that bind to receptors on the cell's surface. Molecules that are taken up by cells may need to be replenished in order to maintain their concentration, whereas molecules that transmit signals by binding to cell surface receptors (and are therefore not internalized and degraded) may not need to be replenished.

Panel 7.9. Macromolecular Medium Components

- Some growth factors and carriers are unconsumable
  - e.g., fibroblast growth factor, transferrin
- Some are consumed or degraded
  - e.g., insulin at high levels, low-density lipoproteins (LDL)

Many macromolecules are internalized, some are degraded (consumed), and others are recycled (Panel 7.9). For example, the ferric ion carrier, transferrin, binds to a transferrin receptor and is internalized. It is then translocated to the lysosome, where it releases ferric ions in the lysosome's low pH environment. Lastly, the transferrin is recycled to the extracellular medium. As long as ferric ions are available (i.e., replenished), transferrin can continue its role as a ferric ion carrier.

Insulin, another commonly used growth factor, binds to the insulin receptor and triggers a signaling event that does not involve its own internalization. However, when present at a high concentration (a few micrograms per mL), insulin is internalized by adipocytes and degraded.

An example of a consumable macromolecule is low-density lipoprotein (LDL). LDL is the carrier for lipids and cholesterol. After an LDL particle binds to an LDL receptor on the plasma membrane, the receptor-ligand complex is internalized in a clathrin-coated pit that pinches off intracellularly to become a coated vesicle. Subsequently, the clathrin coat depolymerizes, resulting in an uncoated (smooth-surfaced) vesicle, often called an endosome. The endosome then fuses with an uncoupling vesicle that has an internal pH of about 5.0, which causes the LDL particles to dissociate from the LDL receptors. The LDL receptors are then recycled back to the plasma membrane. The vesicles containing the LDL particles fuse with lysosomes, in which the cholesterol esters are hydrolyzed to fatty acids and cholesterol. Cholesterol is then incorporated into cell membranes.

Process Medium Design: Kinetics, Stoichiometry, Omics & Optimization

Historic media, such as MEM, DMEM, and RPMI, were used in conjunction with animal serum. The development of media in those early days of cell culture largely focused on compounds that were present in high concentrations and are stoichiometrically important (e.g., sugar, amino acids and vitamins) in order to reduce the reliance on serum. Bulk salts were added to balance osmotic pressure and pH.

The drive to develop serum-free media in the 1970s created a new generation of media in which cells' nutritional needs as well as their growth/hormonal factor requirements are experimentally identified. The work in that period largely defined the general nutritional needs of mammalian cells in culture. The baseline of nutritional needs is generally quite similar among cells isolated from different tissues and different species. Some variability does occur, as a gene essential for the transport or catabolism of a particular nutrient may not be expressed in some tissues or in some species, but overall such variabilities are exceptions.

Optimal Growth Media via Clonal Growth Assay

A technique commonly used in the early period of cell culture enlightenment is clonal growth (Panel 7.10, Figure 7.1). Cells of interest were plated at the very low density of a few dozen per cm^2 in Petri dishes containing media of different nutrient compositions. At such a low density, each cell is well separated from the others. The dish was then set aside in an incubator and left undisturbed for about 10 days. During that time, some cells grew into colonies on the dishes' surface. At the end of the experiment, each dish was stained with a dye that colored the cell colonies. The extent of the color stain then gave a semi-quantitative measure of cell growth. Since the cell density used was so low, and the number of nutrients consumed through the entire period very small, the medium composition was relatively constant and the number of metabolites accumulated very small. This allowed the growth-promoting capability and the concentration of each medium component to be determined, as well as enabling simultaneous evaluation of many different chemical combinations. When this method is applied to cells grown in suspension, the cells are embedded in agar or another type of gel.

Panel 7.10. Determining Nutritional Requirements by Clonal Growth

- The test cell is plated on dishes at low density in a test medium
- Cells grow and become well-separated colonies (~10–15 days)
- The dish is stained with a dye; the degree of staining reflects the extent of cell growth
- The growth extent reflects the capability of the test nutrient at that particular level to support cell growth
- This method defined the general nutritional needs of cells in culture and paved the way for the industrialization of cell culture process

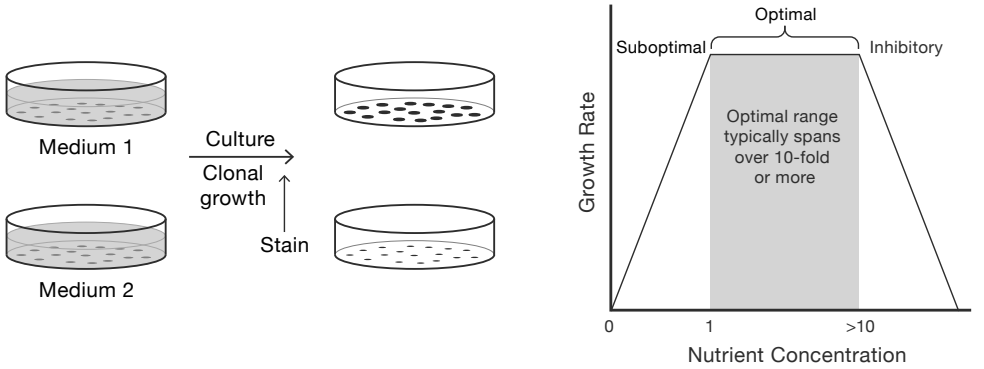

Figure 7.1. Clonal growth and optimal range of nutrients for cell growth.

By the mid-1980s, various serum-free and chemically defined media (e.g., F-12, MCDB104) had been developed, and insulin and PDGF were shown to be required for the growth of human diploid fibroblasts.[1] Clonal growth is still being performed today in small well plates to determine the growth factor/cytokine requirements of many types of cells. Since different nutrients and growth factors may have an interactive effect on cell growth, the design of experiments (DOE) approach is often taken in choosing combinations of nutrient levels to be tested (Figure 7.2).

Many cell strains or cell lines are now grown with serum-free chemically defined or semi-defined (sometimes containing serum-albumin, as will be discussed later in this chapter) media, including pluripotent and many other stem cells, and many NS0 and CHO cell lines. The discovery was made around this time that the concentration range of most nutrients that give cells their maximum growth rate (i.e., a growth-optimal concentration) spans at least 10-fold (Figure 7.1). This suggests that, in a media optimization experiment, the stepping of concentrations must not be too close to each other.

The first generation of serum-free media defined the general nutritional requirements of cells in culture. These achievements greatly facilitated media development for many cell lines, as well as for industrial processes.

Medium for Industrial Cell Culture

Before the arrival of therapeutic proteins, cell culture media were developed for biomedical research and sustained only relatively low levels of cell density. For industrial processes, which require higher cell and product concentrations, the medium's nutrient content must be increased (Panel 7.11, Figure 7.2). The general approach is to provide a more complete nutritional content at a higher concentration while minimizing the increase of osmolality, or simply by using a mixture of DMEM (which has higher nutrient levels and supports higher cell concentration) and

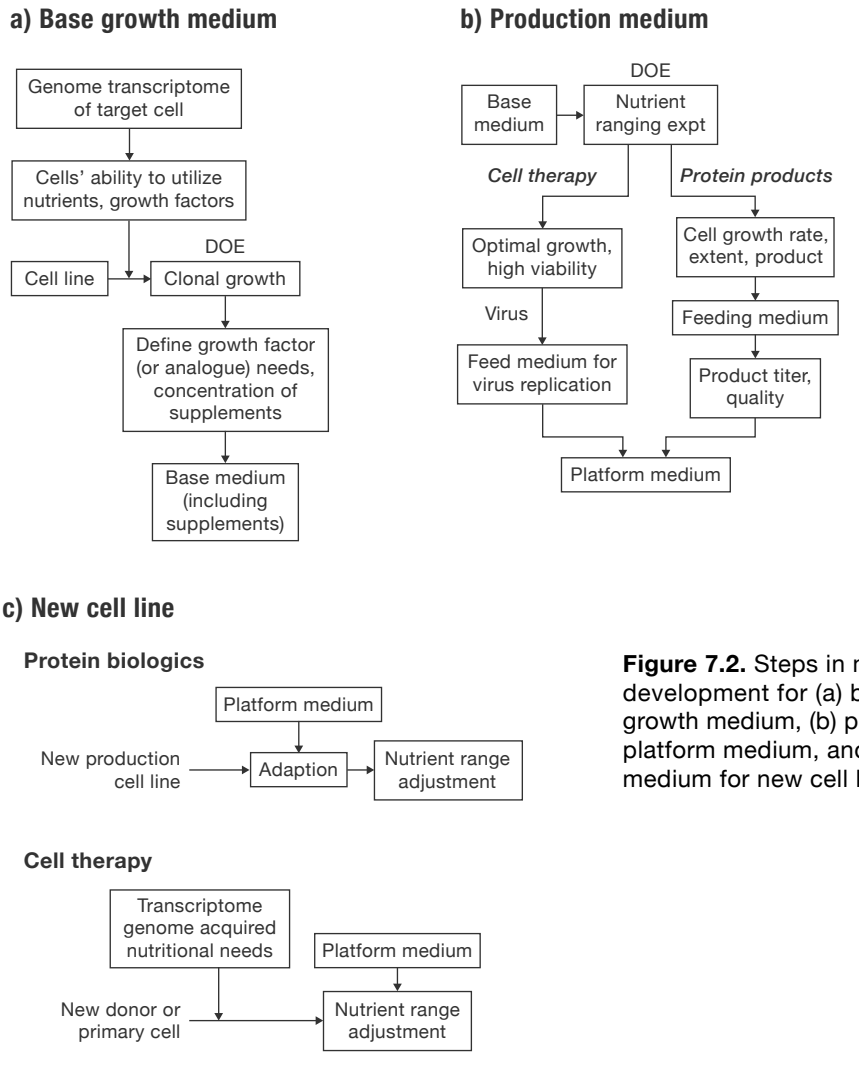

Figure 7.2. Steps in medium development for (a) base growth medium, (b) production platform medium, and (c) medium for new cell lines.

F-12 (which has a more complete nutritional content). Nowadays, most industrial media have higher levels of nutritional components, including amino acids, other organics, and a small number of salts (such as phosphate and magnesium, which will be discussed later), in order to meet the stoichiometric needs of generating more biomass. The medium composition, including the feed medium for fed-batch cultures, is typically determined empirically. In industrial settings, this is often performed using high-throughput instrumentation with pH monitoring and control and automatic sampling. The fortified nutrient levels can give many-fold higher cell and product concentrations. Inevitably, metabolites accumulate as cell concentration increases and cell viability decreases in the late stage of the process.

The increased nutrient level and osmolality takes a toll on cells' growth rate. Furthermore, for cost reasons growth factors are often eliminated when possible or used at suboptimal levels for growth in industrial manufacturing. Hence, the cell growth rate in industrial production is often lower than in a medium optimized for growth. Nevertheless, the high cell density and product titer achieved through the employment of these industrial media contributed to the rapid growth of antibody therapeutics at the turn of the millennium. In many cases, the same medium is used for both cell expansion and production.

Panel 7.11. Developments of Industrial Cell Culture Media

- Basal media include more complete nutrients (e.g., include all nutrients in Ham's F-12) at higher levels (like in DMEM or higher)
- Adapts cells to proliferate in leaner media with low or no growth factor supplement
- Adjusts the levels of key stoichiometric medium components to enable cells to grow to a high concentration
- Small flask cultures or high-throughput automated instrumentation are often used to conduct DOE-based experimentation to determine the nutrient range
- May result in higher osmolality, higher metabolite accumulation, and slower growth rate, but higher cell and product concentration
- Establishes a platform medium that is used as the basis for rapid development of new media for cell lines producing new products

Platform Medium and Extension to Additional Cell Lines

To streamline the process development of a new product, the medium that gives rise to high productivity for a given product is adopted as a platform medium for future media development for cell lines (Panel 7.11, Figure 7.2). The platform medium provides the basis for the fine-tuning of medium composition for a new cell line. Some cell lines have a high variability at the genome and transcriptome levels, causing different sublines to have different metabolic behavior. One may first adapt the new cell line to grow in the platform medium. Subsequent adjustment of nutrient concentrations can then be undertaken to optimize process performance.

Integrating Physiological Insight and Genomic Data into Medium Design

Work in the past few decades has established the nutritional needs of cells in culture. Medium design efforts today often concern culturing a new cell type, or a new cell isolate of a particular differentiation lineage. Different tissues in mammals have different specialized metabolic functions, and different tissue cells respond differently to growth regulators. Cells derived from a particular tissue generally inherit the characteristics of the tissue from which they are derived. However, upon cell isolation, gene expression changes may occur to the isolated cells; this is partly caused by changes in cell-tissue interactions and local chemical cues. Importantly, the acquisition of the capability to grow in culture is accompanied by major alterations in cellular growth regulatory architecture.

Hence, even primary cells in culture often bear significant differences in gene expression from the tissue cells of their origin. Such differences in gene expression may affect the cells' nutritional needs. Furthermore, even though all mammals share highly similar nutritional needs, there are many variabilities among different species.

Further contributing to the variability of nutritional needs are the genetic and epigenetic changes that occur in the process of deriving cell clones from a progenitor cell line. This is especially true in the case that clones are isolated under selective pressure following genetic manipulation. Through genomic, transcriptomic, proteomic, and even metabolomic measurement, or by gleaning omics data available in the public domain, much insight on cells' metabolic capability in utilizing specific components in the medium can be gained to facilitate medium design (Panel 7.12).

Modern medium design should thus return to a mechanism-based approach while integrating omics information. Figure 7.3 illustrates the supply and utilization of typical nutrients in a medium. Glucose is always the most abundant organic nutrient. Sometimes, alternative sugars such as fructose, galactose, and mannose are included with the aim of modulating energy metabolism or to influence the glycosylation profile of the product. Additional components include amino acids and other organics. Every nutrient must have a route (a transporter or transport system), to enter the cell. Many transporters have multiple isoforms with different transport kinetics that are expressed differently in different tissues and cell lines.

Panel 7.12. Physiological Considerations in Medium Design

- A cell's ability to use a medium component is dictated by its expression of the relevant genes
- Depending on the tissue source, species, and epigenetic events, cells have varying metabolic capabilities
- The effective utilization of a nutrient requires the expression of a transporter, utilization genes, and degradation/catabolic enzymes
- "Alternative" substrates (such as fructose, mannose, or galactose in the place of glucose) also require an efficient route of entering the main pathway
- Reactive nutrients that may otherwise cause damage to cells or other nutrients additionally require "sequesters" or carriers both extracellularly and intracellularly
- To respond to a growth regulator and signaling molecule, cells must have the corresponding receptor
- Transcriptome and proteome data can reveal a cell's nutrient utilization capability and can be used to guide medium development

Upon entering the cell, some nutrients (e.g., glucose and most amino acids) enter their metabolic pathways (e.g., glycolysis and tRNA loading) readily. Some other nutrients, such as galactose and mannose, may need to be converted to another intermediate in order to enter the main metabolic artery. For example, galactose and fructose are first converted to UDP-galactose and fructose 1-phosphate, respectively, to enter glycolysis. The proteins for the entry must be expressed at a sufficiently high level to sustain a flux that is high enough for the desired biochemical objective.

Some nutrients are highly damaging to cells at high levels. *In vivo*, these nutrients

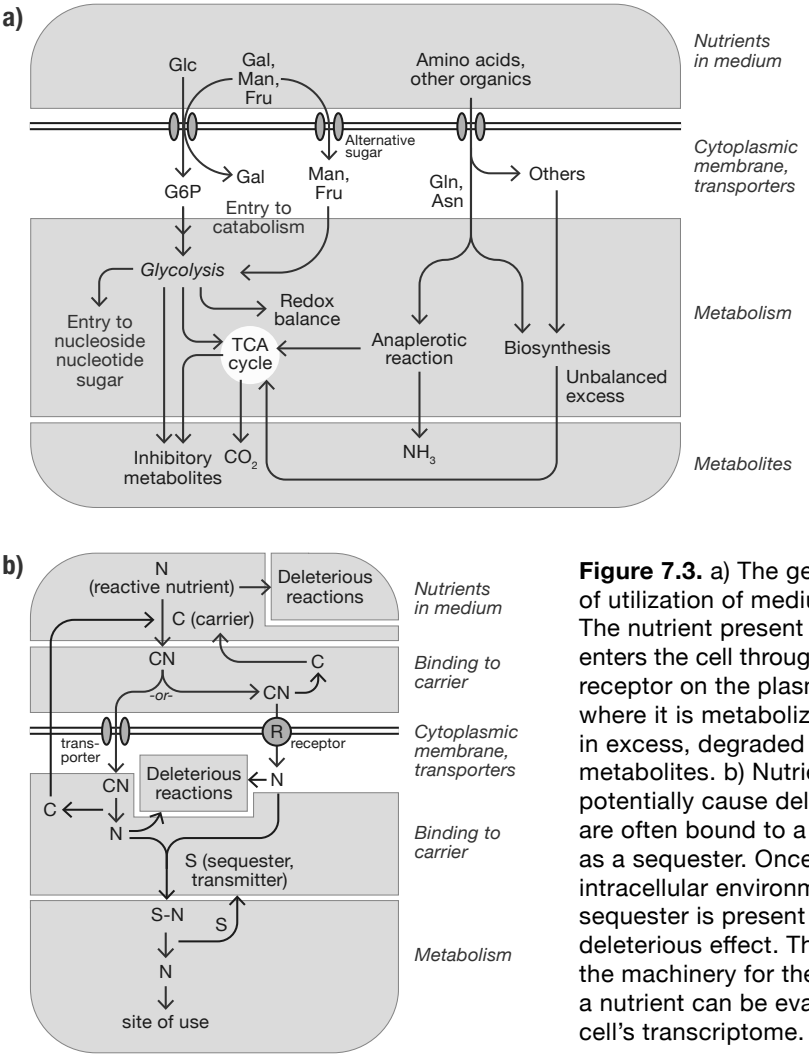

Figure 7.3. a) The general scheme of utilization of medium components. The nutrient present in the medium enters the cell through the transporter/receptor on the plasma membrane where it is metabolized and, if in excess, degraded to generate metabolites. b) Nutrients that can potentially cause deleterious effects are often bound to a carrier that acts as a sequester. Once entering the intracellular environment, another sequester is present to minimize its deleterious effect. The presence of the machinery for the utilization of a nutrient can be evaluated using a cell's transcriptome.

are sequestered outside and inside the cell; in the latter case, they conjugate to a binding protein. For example, both copper and ferric ions are highly oxidative. Ferric is bound to transferrin in blood circulation to prevent it causing damage to cells. Carrier or sequester compounds may need to be included in culture medium to safeguard the extracellular environment. For highly reactive species, such as ferric ion, carrier or sequester proteins are present in the cell to mitigate their deleterious effect on cellular constituents.

Nutrients that are taken up by cells in excess of what is needed for cell growth and other functional activities will enter pathways for their degradation and excretion. Nutrient levels in industrial media typically far exceed the optimal for growth. However, the somewhat reduced growth rate results in a high product titer and productivity. At a high concentration, some nutrients are taken up by cells far in excess and are

shunted to degradation pathways. Some metabolites from the degradation are growth inhibitory or even toxic at high levels. The expression level of enzymes involved in the degradation pathways of compounds leading to inhibitory metabolites will therefore affect the performance of the culture. Hence, medium design may need to take the presence or absence of degradation pathways into consideration.

Components of Basal Medium

Water

Mammalian cells are exceedingly sensitive to the quality of water used for media preparation. City water, the usual source of water for medium preparation, contains particulates, including bacteria that can introduce endotoxins, trace organics, and various inorganic ions such as potentially harmful heavy metals (Panel 7.13). Those contaminants must be removed before water is used in cell culture. Typical water preparation processes include deionization through ion exchange, microfiltration to remove particulates and bacteria, and finally reverse osmosis to reduce conductivity (or increase resistance) to >20 MΩcm.

Panel 7.13. Water for Cell Culture Media

- Types of contaminants in city water
  - Inorganics: heavy metals, iron, calcium, chlorine
  - Organics: byproducts of plant decay, detergents
  - Bacteria: endotoxins, pyrogens
  - Particles: colloids, particles
- A typical water preparation process for cell culture involves deionization, microfiltration, and reverse osmosis. An ultrafiltration step is sometimes included.
- WFI, which is prepared by water vapor compression or by multiple-effect distillation, is also used in cell culture manufacturing

In some applications, especially cell therapy, the product is subjected to minimal purification before final filling as a drug product; to minimize the entry of any pyrogenic contaminants, water for injection (WFI) is used in preparing cell culture medium. WFI is prepared by low evaporation rate distillation, which minimizes the chance of a water droplet in the evaporating steam carrying a solute or particle from the water.

Hexoses

Glucose and glutamine are the primary nutrients that supply a cell's energy needs in culture (Panel 7.14). The physiological concentration of glucose in blood is ~0.8 g/L (4.4 mM). In culture, glucose is typically present from 1–5 g/L (5.5–27.5 mM). In the production reactor, sometimes a high level of glucose, as much as 15 g/L (82.5 mM), is used. In this case, glucose is a large contributor to the osmolarity of the medium, and adjustments to the medium's composition must be made (by reducing sodium and chloride concentrations) to maintain osmolarity in a growth-permissible range.

Other sugars, especially galactose, mannose, and fructose, may also be used as alternative sugars. All cultured cells express the GLUT1 transporter at a significant level, and take up glucose readily under a normal glucose level in medium. Galactose is also transported by GLUT1, and can thus be used as an alternative sugar to glucose. The K_M for galactose uptake is higher than for glucose. In the concentration range commonly used for glucose, galactose is taken up by cells at a lower rate, resulting in lower lactic acid production in the culture. Fructose is transported by the GLUT5 transporter. The K_M for fructose transport by GLUT5 is also high. Thus, similar to galactose, the uptake rate for fructose is lower than for glucose unless a high concentration of fructose is used. However, not all cells can utilize fructose as some do not express GLUT5.

Panel 7.14. Six-Carbon Sugars

- Glucose is typically used in 1-5 g/L
- Physiological level is ~0.8 g/L
- Fructose and galactose may also be used
- Galactose and glucose can both be transported by the GLUT1 transporter, which is present in most cells
- Fructose is transported by a different transporter (GLUT5); unless the transporter is expressed, the cell may not be able to use fructose efficiently
- An alternative sugar is often taken up by cells at a slower rate, which may reduce lactate production and help cell maintenance
- Pyruvate and ribose are sometimes supplied in small quantities that are insufficient to supply a cell's energy needs

Galactose and fructose do not get phosphorylated at their C6 position and enter the glycolysis pathway directly. They can serve as the main hexose source for cells through alternative entry points to glycolysis. Galactose is phosphorylated at C1 to become galactose 1-phosphate, and then through a transferase and an epimerase catalyzed reaction it becomes glucose 1-phosphate, which is converted to glucose 6-phosphate. Fructose, once in the cytosol, is converted to fructose 1-phosphate and split into dihydroxyacetone 3-phosphate and glyceraldehyde by aldolase. Both are then converted to glyceraldehyde 3-phosphate and enters glycolysis (Figure 7.4).

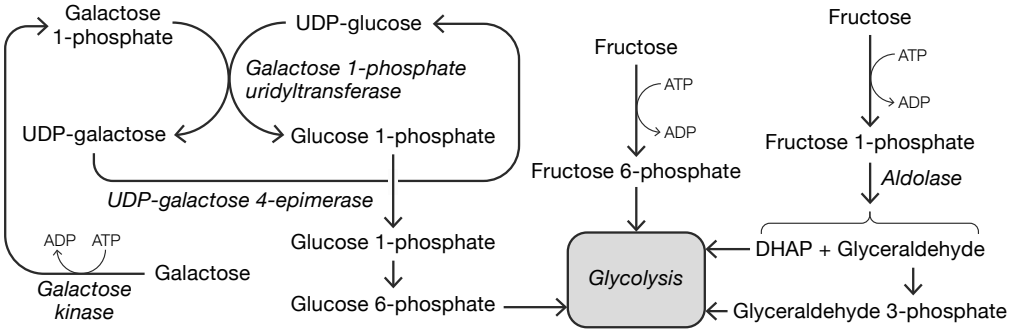

Figure 7.4. Entry of galactose and fructose to glycolysis.

With their slower uptake rates, galactose and fructose give a low flux type of metabolism when used as the main hexose. The alternative sugar can also be used in conjunction with glucose. As glucose concentration decreases to a low level, the consumption rate of galactose or fructose declines, facilitating the switch of the glycolysis to a low flux state. Galactose and mannose have also been used to increase galactosylation of N-glycans. However, when used together with glucose, a high concentration may be necessary in order to compete with glucose for transport through GLUT1. It is important to keep in mind that when using galactose, fructose, or other sugars as the sole course of sugar, the carbon flux distribution and the intracellular nucleotide sugar concentrations are different than if glucose had been used. Since nucleotide sugars are the precursors to glycan, the effects of alternative sugar on the glycan profile must be examined if glycosylation is a product quality attribute.

Amino Acids

Glutamine is an essential amino acid for cells in culture. Most tissues express glutamine synthase and make glutamine from glutamic acid. Glutamine is thus not an essential amino acid (Panel 7.15). However, glutamine becomes an essential nutrient in culture. Among all the amino acids, glutamine is consumed in the highest quantity and is present in the medium at the highest concentration. It is the second most abundant organic component in the medium after glucose and is consumed at roughly 1/3 to 1/10 of the molar consumption rate of glucose. The exceptions are cell lines that have been engineered to express glutamine synthetase, which enables cells to synthesize glutamine. Glutamine supplies the amino group for incorporation into proteins and for nucleotide biosynthesis. It also supplies the carbon backbone of TCA cycle intermediates by converting to α-ketoglutarate in anaplerotic reactions (see Chapter 3). Glutamine spontaneously degrades in aqueous solutions and releases ammonium. Consequently, in order to avoid degradation glutamine is typically added to culture medium immediately prior to use.

Panel 7.15. Glutamine as a Nutrient

- Glutamine is an essential amino acid for cultured cells, but not for mammals
- Its main roles as a nutrient:
  - Used in protein and nucleotide synthesis
  - Enters the TCA cycle after deamination for anaplerotic metabolism
  - Consumed at a high level, second only to glucose, at about 1/3 to 1/10 of glucose
- Spontaneously degrades in aqueous solutions

Amino acids are classified as essential or nonessential based on nutritional studies using animals or tissue culture cells (Table 7.2). Mammals lack the synthetic pathway of aspartate family amino acids that produce lysine, isoleucine, methionine, and threonine. The synthetic pathways for the aromatic amino acid family (phenylalanine and tryptophan), and for leucine, valine, and histidine are also incomplete. Mammals thus acquire these amino acids

through food to meet growth needs. Some amino acids are essential only for cells in culture, but not for animals (indicated by a * in Table 7.2). In animals, different tissues may cross feed each other, such that amino acids synthesized in one tissue (especially liver) may be transported to cells in other tissues. The expression levels of enzymes involved in some amino acid biosyntheses may decrease in cultured cells to make the amino acid become essential. Glutamine is nonessential for animals; it is synthesized from glutamic acid through glutamine synthase. Proline becomes an essential amino acid for some cell lines.

Cell culture media developed in the 1960s and 1970s contained at least the 14 essential amino acids. Those media were designed to be used with serum supplementation, which supplies some additional amino acids. Media designed for serum-free culture typically include all amino acids.

Some nonessential amino acids may be secreted by cells and accumulate in culture medium, including alanine, asparagine, glutamic acid, and proline. In principle, nonessential amino acids can be synthesized by cells, and thus the depletion of a nonessential amino acid is not detrimental. However, it has been reported that the depletion of asparagine in medium resulted in some degree of its substitution by serine in the therapeutic protein produced.[2] Caution should be taken in medium development to monitor possible side effects of nutrient depletion.

Table 7.2. Essential and Nonessential Amino Acids

Essential	*Nonessential*
L-arginine*	L-alanine
L-cysteine*	L-asparagine
L-histidine	L-aspartic acid
L-isoleucine	L-glutamic acid
L-leucine	L-glycine
L-lysine	L-proline
L-methionine	L-serine
L-phenylalanine	
L-threonine	
L-tryptophan	
L-tyrosine*	
L-valine	
L-glutamine*	

*Essential for cells in culture, but not for animals.

Vitamins

Many vitamins serve critical biochemical roles in metabolism. Their nutritional deficiency in humans is the root cause of numerous diseases and led to their discovery in the early twentieth century. Even though they are grouped as a common class of nutrients, their chemical natures and biological roles are diverse. They are all essential for the vitality of humans, but are needed only in minute quantities compared to glucose and amino acids. Some vitamins are cofactors of enzyme reactions, while others are a prosthetic group (i.e., bound to the protein) of an enzyme. These vitamins are essential for the catalytic activity of those enzymes.

Not all vitamins are needed by cells because of species or tissue variability. For example, vitamin C is essential for humans but not for mice. Vitamin K is nonessential only for some differentiated cells (or genetically engineered cells) that synthesize proteins that require glutamic acid γ-carboxylation. The nutritional deficiency of these vitamins leads to various diseases in humans, and their deficiency in cell culture can impair

energy metabolism or biosynthetic functions (for review see reference 3). The vitamins that are commonly included in cell culture media are listed in Table 7.3.

Biotin (vitamin B7) is a prosthetic group tightly bound to many enzymes involved in carboxylation reactions. It functions as a CO_2 carrier in enzymes such as pyruvate carboxylase that are involved in anaplerotic reactions, and acetyl-CoA carboxylase in fatty acid synthesis.

Thiamine (vitamin B1) participates in biochemical reactions as thiamine pyrophosphate. It forms a transient C−C bond with the α-carbon of α-keto acid in α-keto acid dehydrogenase reactions to facilitate the departure of CO_2 in the decarboxylation reaction. It is also a coenzyme of transketolase in the pentose phosphate pathway.

Pantothenate (vitamin B5) is a precursor of CoA and a critical participant in a large number of reactions in energy metabolism, lipid catabolism, and biosynthesis. Plants, yeast, and many bacteria can synthesize CoA *de novo* from aspartate and ketovalerate through pantothenate. Mammals lack the capacity to synthesize pantothenate and must take it up from a food source.

Table 7.3. Vitamins

Compound	***Vitamin***	***Biological Roles***
Participate in vital biochemical reactions required by all mammalian cells		
Biotin	Vitamin H	A prosthetic group as a CO_2 carrier in carboxylase reactions
Ca-pantothenate	Vitamin B5	Precursor of CoA
Cyanocobalamin	Vitamin B12	Catalyzes reactions involving the exchange of an alkyl group with hydrogen
Folic acid	Vitamin B9	C1 (methyl, methylene, formyl) carrier
Niacinamide niacin, nicotinic acid	Vitamin B3	A precursor of NAD, NADP
Pyridoxine HCl	Vitamin B6	A prosthetic group in amino group transfer reactions
Riboflavin	Vitamin B20	Cofactor in many redox reactions (as FMN, FAD)
Thiamine HCl	Vitamin B1	Thiamine pyrophosphate participates in decarboxylation reactions
Antioxidants		
Ascorbic acid	Vitamin C	Electron donor, reduces free radicals
D-alpha-tocopheryl acetate	Vitamin E	Antioxidant, protects polyunsaturated fatty acids
Required only in cells carrying out certain reactions or undergoing specific differentiation		
Phylloquinone, menadione, menaquinone, sodium bisulfite	Vitamin K	Cofactor of vitamin K-dependent γ-carboxylation
Cholecalciferol	Vitamin D	Involved in calcium homeostasis, supplemented to culture media of some differentiated cells

Folic acid plays the role of a C1 (methyl, methylene, formyl) carrier. It is involved in glycine synthesis through the use of serine as a methylene donor. Its derivative, dihydrofolate, participates in the nucleotide salvage pathway. Dihydrofolate reductase is a target enzyme used in the DHFR gene amplification system.

Niacin, or nicotinamide (vitamin B3), is a precursor of NAD and NADP. Riboflavin (vitamin B2) or flavin mononucleotide (FMN) and flavin adenosine dinucleotide (FAD) are important cofactors in many biochemical reactions.

Ascorbate (vitamin C) is a water-soluble reducing agent and electron donor that is involved in many antioxidant reactions. It is a cofactor in the proline hydroxylation reaction in the biosynthesis of collagen I and III. It also participates in dioxygenase catalyzed reactions and histone demethylation. Plants and most mammals can synthesize ascorbate, but the enzyme for the last step of its biosynthesis, gulonolactone oxidase, is not functional in humans, other primates, and some other species. Hence, ascorbate is a vitamin necessary for humans. In the dioxygenase reaction, ferrous ion is oxidized to ferric. Ascorbate reduces the ferric ion back to ferrous and allows dioxygenase to return to its original state (Figure 7.5).[4] The oxidized ascorbate (dehydroascorbate) is regenerated back to ascorbate enzymatically by consuming NADH or NADPH. It can also revert to ascorbate by oxidizing Fe^{2+} to Fe^{3+} while generating free radicals, which can further react with oxygen to form hydrogen peroxide, especially at a high concentration of free Fe^{3+}. *In vivo*, most iron is sequestered in transferrin in plasma or is bound to ferrin inside the cell; thus, the prooxidant effect of ascorbate is not prominent. Under cell culture conditions of high ascorbate and free Fe^{3+} concentrations, the possible role of ascorbate as a prooxidant,

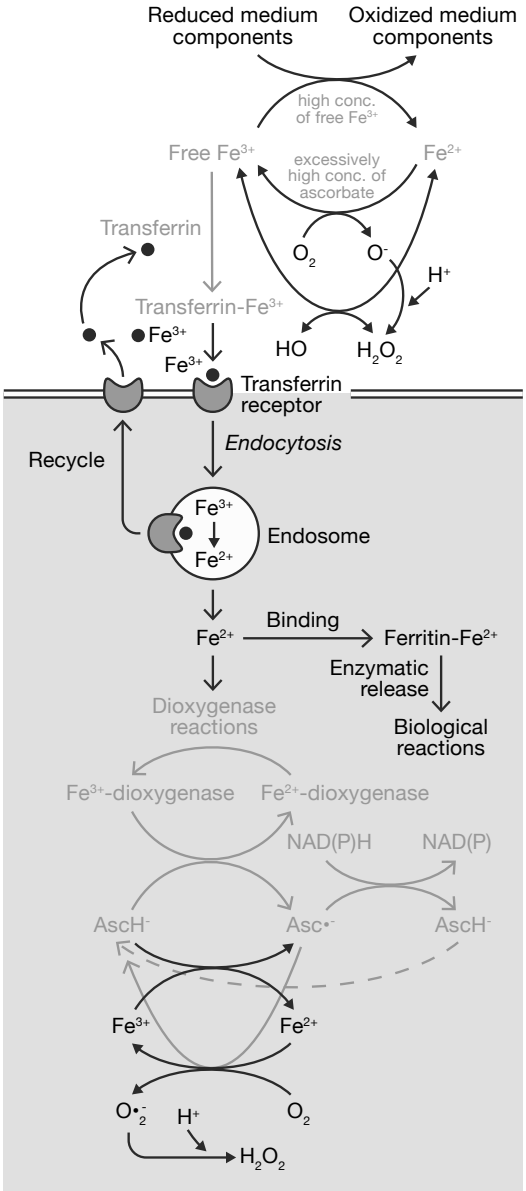

Figure 7.5. The interactions of iron, transferrin, ferritin, ascorbate, and the iron metabolism.

instead of an antioxidant, should not be ignored. To minimize the possible prooxidant effect of ascorbate, ascorbate 2-phosphate is sometimes used. Ascorbate 2-phosphate is not oxidized as rapidly as ascorbate in solution, and thus has a low prooxidant effect. It is taken up by cells at a slower rate, giving it a longer availability in cell culture media.

The vitamins discussed above are all highly soluble in water except for biotin, which has a somewhat lower solubility. A few vitamins (vitamins A, D, E, and K) have a low solubility in water and are fat-soluble.

Vitamin E (tocopherol) is an antioxidant that prevents polyunsaturated fatty acids from being oxidized and forming peroxides. Its presence thus reduces the production of free radicals and reactive oxygen species. Most of the vitamin E associated with cells resides in membranes, especially the Golgi membrane. The oxidation of polyunsaturated fatty acids generates C-centered free radicals that can propagate and generate more oxidized polyunsaturated fatty acids in membranes. The presence of vitamin E in the membrane stops such chain reactions. Vitamin E and ascorbic acid are thus complementary to each other; vitamin E has an antioxidant effect on unsaturated fatty acids in cellular membranes, while ascorbic acid prevents the formation of reactive oxygen species in the intracellular soluble environment. Both vitamin E and ascorbic acid are regenerated by glutathione.

Vitamin K is a cofactor in the γ-carboxylation of glutamate that occurs in the synthesis of many blood coagulation proteins, including prothrombin, factors VII, IX, and X, protein C, and non-coagulation proteins such as osteocalcin and transthyretin. Vitamin K is supplemented to cultures of cells carrying out γ-carboxylation reactions.

Vitamin D is a group of related compounds naturally synthesized in humans and other mammals from cholesterol (called cholecalciferol). It becomes deficient under conditions of low exposure to sunlight in the UV spectra range. The photoactivation reaction completes the last step of vitamin D3 synthesis. Individuals with low UV exposure must obtain it from dietary sources. Vitamin D3 is a classical hormone that regulates the transcription of a variety of genes in a tissue-specific manner. At the whole-body level, vitamin D regulates the homeostasis of calcium and phosphorus through its effects on their tissue uptake. It is supplemented to cultures for the purpose of modulating the differentiation process or cells' differentiated properties. At high doses, vitamin D inhibits cell growth.

A group of compounds generally referred to as retinoids possess vitamin A activities. Their forms are retinol, retinal, retinoic acids, and ester. Their most important role is the vision function, with retinal serving as the photosensitive chromophore through a covalent link to the photoreceptor protein rhodopsin. In addition to operating as a vitamin, the retinoic acid derived from retinol plays a role in the regulation of transcriptional regulation in embryo development and in stem cell

differentiation. Vitamin B is used to guide the differentiation process of specific lineages.

Nucleosides

Nucleosides and nucleic acid make up a very significant portion of a cell's content, as they constitute the genome. Most cultured cells are diploid, although many industrial cell lines are multiploid. Nucleosides constitute all RNAs whose cellular content is higher than DNA. Additionally, free ribonucleotides, like ATP, ADP, and AMP, are present in relatively high abundance in the cell. In fast-growing cells, deoxyribonucleotides (deoxyadenosine, deoxyguanidine, deoxythymidine, deoxycytidine) and ribonucleotides (adenosine, guanidine, uridine, cytidine) must be synthesized sufficiently quickly or otherwise be supplied exogenously to sustain growth. Mammals have *de novo* synthesis pathways for making purines (adenosine and guanidine) and pyrimidines (uracil, cytidine, thymidine), although the major site of such synthesis is in the liver (Panel 7.16). Both bases (purine and pyrimidine) and nucleosides (base + ribose or deoxyribose, without phosphate) can be transported into cells from tissue fluid or culture medium. Cultured cells, notably cancer cells, can develop the capability to synthesize purines and pyrimidines. Nucleic acids, especially mRNAs, are subjected to degradation due to gene expression regulation and turned over rapidly in the cell. These degradation products, mostly nucleosides or nucleoside monophosphates, are recycled to nucleoside triphosphates through salvage pathways for incorporation into nucleic acids again.

Panel 7.16. Nucleosides and Precursors

- DNA, various RNAs, and nucleotide monomers constitute a major portion of cellular materials
- *De novo* synthetic pathways exist, but most synthesis occurs in the liver in mammals
- They are subject to degradation and turnover; salvage pathways recycle (and interconvert) the nucleosides, but do not provide what is needed for growth
- Bases and nucleosides can be transported into the cell
- Hypoxanthine and thymidine are typically added to the medium, and their stoichiometric quantity can be calculated

Thymidine and hypoxanthine are commonly used as pyrimidine and purine sources in contemporary media. They are readily linked to a ribose 5-phosphate in the cell to become inosine monophosphate (IMP) and TMP. IMP enters the synthetic pathway to make AMP and GMP, which are further phosphorylated into ATP and GTP. TMP is converted to UMP and CMP. If hypoxanthine is not available in the medium, *de novo* synthesis is the primary source and must be sufficiently active to sustain cell growth since the salvage pathway only recycles the base "salvaged" from nucleic acid degradation, but does not generate the nucleosides needed for proliferation. In *de novo* synthesis, the four nitrogen atoms in each purine ring are derived from glutamine, aspartate, and glycine, while obtaining carbons requires CO_2, formate, glycine, and folic acid. The *de novo* synthesis of pyrimidine similarly requires glutamine, aspartic

acid, and CO_2. One can see that without a sufficient supply of hypoxanthine, many precursors will be needed for nucleoside biosynthesis.

As discussed in Chapter 6, dihydrofolate reductase mutants of Chinese hamster ovary cells are frequently used for co-amplification of the DHFR gene and transgene. Hypoxanthine and thymidine are supplemented for the culture of those mutants but are removed to facilitate the process of gene amplification.

Fatty Acids and Lipids

Lipids constitute a significant portion of animal biomass (Panel 7.17). Phospholipids, cholesterol, and sphingolipids make up the cell membrane. The composition of lipids in a membrane affects its fluidity and dynamic behavior. The lipid bilayer membrane forms vesicles which play key roles in protein secretion and envelop virus replication. Mammals synthesize different lipids in different tissues. Unsaturated fatty acids of different carbon lengths constitute about half of the fatty acids in phospholipids. However, mammals cannot add double bonds to w-3 and w-6 carbons. Linoleic acid (18:2 cis-$\Delta^{9,12}$, a w-6 fatty acid with an 18-carbon total and 2 double bonds at positions 9 and 12 from the carboxylic carbon) and linolenic acid (18:3 cis-$\Delta^{9,12,15}$, a w-3 fatty acid) are thus essential fatty acids for mammals. They acquire these w-3 fatty acids from diet. From linoleic acid and linolenic acid, other long-chain fatty acids with double bonds in w-3 and w-6 carbons can be synthesized. Hence, a complete medium for serum-free culture usually contains linoleic acid and sometimes also arachidonic acid (24:4 cis-$\Delta^{5,8,11,14}$) and oleic acid (18:1 cis-Δ^{9}).

Panel 7.17. Lipids and Fatty Acids

- Lipids constitute a few percent of biomass in most cells
- Phospholipids and cholesterol form lipid bilayer membranes; about half of the fatty acids in the lipid bilayer membrane are unsaturated
- Linoleic acid (18:2 cis-$\Delta^{9,12}$) and linolenic acid (18:3 cis-$\Delta^{9,12,15}$) are essential fatty acids
- Lipids have a low solubility in media, but carrier proteins (e.g., serum albumin) or synthetic carriers may increase their bioavailability
- Phospholipid precursors (ethanolamine, choline) are often present
- Most cells can synthesize cholesterol, but for some it must be provided

Although linoleic and linolenic acids are often referred to as essential fatty acids, their effects on the growth of cultured cells is subtle. The depletion of essential amino acids has an almost immediate retarding effect on cell growth, but the failure to supplement cells with essential fatty acids is not as profound and does not cause immediate cessation of growth.

The vast majority of fatty acids supplemented to media are incorporated into phospholipids and reside in cell membranes. Phospholipid supplementation to culture medium is a logical approach in medium design. Supplementing tissue extracts rich in phospholipids, cholesterol, and lipoproteins (such as Ex-Cyte derived from bovine or lecithins isolated from various sources) promotes cell growth. However, the demands to

employ media components that are non-xeno and the drive to use chemically defined medium has thwarted the use of those extracts except in special applications.

Instead of tissue extracts, precursors of phospholipids (choline, ethanolamine, inositol) are supplied. Cholesterol is supplied to its auxotrophic mutants, such as NS0 cells. The frequent addition of chemically defined phospholipids, phosphatidyl -choline, -ethanolamine, -inositol, and dipalmitoyl phosphatidic acid to culture medium had been shown to have a growth-promoting effect on human diploid fibroblasts.[1]

Fatty acids, cholesterol, and phospholipids all have low solubility in aqueous environments. In mammals or in culture media containing animal serum, lipids are bound in serum albumin. *In vivo*, cholesterol is carried in high-density and low-density lipoproteins in circulation. When lipids are used in disposable plastic bioreactors, the adsorption to the plastic surface and possibly reduced bioavailability should be determined. Ways to increase the solubility have been explored. Cholesterol can be derivatized with a dicarboxylic acid (e.g., succinic acid) to form an ester and increase its solubility, or be supplied as a cyclodextrin conjugated complex. Phospholipids may be prepared as liposomes[1] or dissolved in DMSO for delivery to laboratory cell culture; however, its industrial applications may not be imminent.

Bulk Salts

Many mineral elements, in the form of ions (e.g., Na^+ or Cl^-) or polyatomic ions (e.g., PO_4^{3-}), are essential constituents of cell mass and must be provided in the medium. A list of the ions contributed by bulk salts and their concentrations in several media are shown in Table 7.4. Among all ions, Na^+ is present in the highest concentration, followed by Cl^- by a rather large margin because Na^+ is also contributed by other anionic species, especially HCO_3^-, which is added as $NaHCO_3$ for buffering

Table 7.4. Concentrations of Bulk Ions in Basal Medium (mM)

Ion	*DMEM*	*F-12*	*RPMI*	*William's E*	*E8*
Na^+	155.29	145.02	138.52	144.44	103.53
K^+	5.33	2.98	5.33	5.33	4.16
Mg^{2+}	0.81	0.60	0.41	0.81	0.60
Ca^{2+}	1.80	0.30	0.42	1.80	1.05
Cl^-	119.28	135.80	108.78	126.18	181.24
PO_4^{3-}	0.91	1.04	5.63	1.01	0.95
HCO_3^-	44.04	14.00	23.81	26.19	40.52
SO_4^{2-}	0.81	0.07	0.41	0.81	0.41
NO_3^-	0.00	0.00	0.85	0.00	0.00

pH. Potassium concentration in the medium is typically only 1/50 to 1/25 that of Na^+. Its intracellular level is 10–20 times higher than Na^+ (Table 7.1).

Phosphate makes up part of the nucleic acids and nucleotides of cell biomass. It is also present as various phosphorylated metabolic intermediates and as inorganic phosphate. A high level of phosphate in the medium is necessary for carrying out the stoichiometric need to synthesize new biomass. Magnesium plays key metabolic roles in energy metabolism and is conjugated to many metabolites. The intracellular level of Mg^{2+} is high, but free Mg^{2+} is only at about ~.25–1 mM, as the majority is complexed to ATP and other organic acids. Phosphate, K^+, and Mg^{2+} are thus three inorganic components that appear at higher concentrations intracellularly than in the medium. Calcium is essential for signaling in some differentiated cells, and is present in high concentrations in the endoplasmic reticulum (ER) and the sarcoplasmic reticulum in muscle cells. The concentrations of the most abundant bulk ions (Na^+, Cl^-, and K^+) in commonly used media spans a small range. In contrast, HCO_3^{2+} spans a much wider range.

An important role of bulk ions is to balance the osmolarity to be in, or at least near, the physiological range of 270–300 mOsm. The most important contributors to osmolarity are Na^+, Cl^-, and HCO_3^{2+}. While the composition of bulk ions may vary in different medium formulations, the total osmolarity of most industrial media is maintained in the range of 270–330 mOsm. When bicarbonate is not used in culture medium, or when glucose is added at a very high concentration (such as 50 mM, contributing nearly 50 mOsm of osmolality), the osmolarity of the culture medium needs to be rebalanced by adding or removing bulk salts. A common practice is to add a mixture of NaCl and KCl to maintain the molar ratio at about 30.

Trace Elements

Five transition metals—iron, copper, manganese, zinc, and cobalt—play key roles in the biological functions of all mammalian cells (for review, see reference 5). They are naturally present in serum, but must be included in serum-free media (Table 7.5). Additionally, some other heavy metal ions, including molybdenum (Mo), vanadium (V), strontium (Sr), and selenium (Se), also appear to participate in biological reactions. Among these metals, iron and zinc are present in cells and human bodies at much higher levels than other trace elements (Panel 7.18).

The zinc ion has only one 2+ valence state (Zn^{2+}), is not redox active, and is less toxic to cells. All the other metals have multiple oxidation states; for example, iron has Fe^{2+} and Fe^{3+}, copper has Cu^+ and Cu^{2+}, and manganese has II, III, and IV states. These ions incur cytotoxicity when present at high concentrations, by oxidizing components of cells or the medium, or by generating reactive oxygen species. This is especially

true for copper, which has a redox potential of +0.2 to +0.8 V, and iron, which has a redox potential of 0.77 to ~1.0 V. In media and in free forms in a typical solution (not oxygen-depleted), they exist as Cu^{2+} and Fe^{3+} and are highly oxidative. In circulation, Fe^{3+} is bound to the ~200 binding sites on transferrin (Figure 7.5). At a bound state in transferrin, Fe^{3+} is not available for reactions involving Fe^{3+}/Fe^{2+} transition. Intracellularly, Fe^{3+} is bound to protein ferritin and is delivered to its site of usage through carrier proteins. The binding to carrier proteins sequesters the oxidizing effect of free Fe^{3+}. Although it is beyond the scope of this chapter to discuss this matter in detail, the other trace metals that exhibit cytotoxicity at high levels likely have similar mechanisms: extracellular and intracellular carriers that sequester the cytotoxic effect, a transportation system that allows for intake across the cytoplasmic membrane, and a protected means of delivery to reach the target site.

Table 7.5. Trace Elements in MCDB 104 (a serum-free medium for human diploid cells)

Element	*Compound*	*Concentration (μM)*
Fe	$FeSO_4 \cdot 7\ H_2O$	5.0
Cu	$CuSO_4 \cdot 5\ H_2O$	1.0×10^{-3}
Zn	$ZnSO_4 \cdot 7\ H_2O$	5.0×10^{-1}
Mn	$MnSO_4 \cdot 5\ H_2O$	1.0×10^{-3}
Mo	$(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$	1.0×10^{-3}
Ni	$NiCl_2 \cdot 6\ H_2O$	5.0×10^{-4}
Se	SeO_2	3.0×10^{-2}
V	NH_4VO_3	5.0×10^{-3}
Si	$Na_2SiO_3 \cdot 9\ H_2O$	5.0×10^{-1}
Sn	$SnCl_2 \cdot 2\ H_2O$	5.0×10^{-3}

These trace metals play key biological roles. Their multiple valence states make them easy participants in electron transfer reactions. They can form a prosthetic group, function as a cofactor of an enzyme, or be part of a complex that is integrated into a protein. Iron is responsible for the key activity of heme in hemoglobulin, which allows red blood cells, myoglobulin in muscle cells, and cytochromes in virtually every cell to carry oxygen molecules. Iron also plays a key role in electron transfer complexes in the mitochondria. Furthermore, iron is a metal ion component of many important proteins expressed at high levels in the cell. Among the trace elements, iron's cellular demand is the highest and is therefore provided in the medium at a relatively high concentration.

In comparison, the copper ion, which has an even higher oxidative potential than Fe^{3+}, is present only in small numbers of cellular proteins, many of which are extracellular or are involved in prevention of copper toxicity.[5] Copper is provided at low levels in most traditional media, about three orders of magnitude lower than iron. Nevertheless, it plays a critical role as the cofactor in cytochrome C oxidase that catalyzes the last step of the electron transfer chain by reducing oxygen to water and pumping four protons across the mitochondrial inner membrane. Increasing copper levels in traditional media has been shown to facilitate the transition of glucose metabolism from a high flux state to low flux state in the late exponential growth phase. Since copper is highly oxidative, caution should be taken when raising copper to very high levels.

Panel 7.18. Trace Elements in Serum-Free Media

- Trace elements play a variety of biological roles and must be supplied in a chemically defined medium
  - Fe^{3+} is the part of heme that carries oxygen
  - Fe^{3+} and Cu^{2+} are both involved in electron transfer in the mitochondria
  - Mn^{2+} is a component of many enzymes, including DNA/RNA polymerases and glycosyl transferases
- Fe, Zn, and Si are present in higher concentrations than all others
- Except for Zn, these elements have multiple oxidation states, are redox active, and are harmful at high concentrations
- Fe and Cu are especially reactive and can generate ROS. Their concentration is very low in body fluids.
- Fe^{3+} binds to transferrin in blood and is sequestered in cells by binding to ferritin

Zinc is present at high concentrations in the pancreas and is conjugated with insulin. Its distribution in tissues is somewhat uneven. Zinc does not participate in redox reactions, but is a cofactor of a large number of enzymes. Many metalloproteases are zinc proteins, including carboxypeptidases and matrix metalloproteases. Other zinc proteins include many zinc finger proteins that bind to DNA and play a regulatory role, as well as carbonic anhydrase, which is localized on the mitochondria membrane and facilitates the solubilization of carbon dioxide to bicarbonate.

Manganese is involved in photosynthesis in the oxidation of H_2O to O_2 in plants. It is a component of many enzymes including DNA/RNA polymerases and glycosyl transferases (involved in glycosylation). Manganese is also associated with enzymes involved in reversing oxidative stress such as catalases and peroxidases. It is an essential element, although it is required at a low level.

Cobalt is a component of vitamin B12. Selenite serves as an antioxidant. It was not realized as an essential nutrient in the early days of serum-free medium development because it was supplied as a minute contaminant in another media component. The serum-free medium F-12 developed in the 1970s had only the trace elements described above. In later development of MCDB, the list was expanded to that shown in Table 7.5.

Non-Nutritional Medium Components

Some medium components are added primarily for operational purposes instead of nutritional ones. They can be omitted without harmful effects. For example, sodium bicarbonate is a pH buffer and Pluronic F-68 is used as a protective agent from mechanical stress. Sodium bicarbonate can be replaced by another pH buffer such as HEPES. Pluronic F-68 may be removed when cells are grown in flasks without mechanical agitation.

Sodium bicarbonate buffer

Cellular respiration generates a large quantity of CO_2 that becomes bicarbonate after dissolving in water. Bicarbonate provides a natural pH buffer in a human's body fluid. In the early days of cell culture media development, sodium bicarbonate was adopted as a pH buffering reagent.

Consider that CO_2 is present in a system consisting of a gas phase and an aqueous phase. Over a period of time, CO_2 distributes between the gas phase and the liquid phase and reaches equilibrium (Eq. 7-1, Panel 7.19). At equilibrium, the concentration of CO_2 between the two phases is described by Henry's law (Eq. 7-2). Upon association with water, the dissolved $CO_{2(aq)}$ is dissociated into bicarbonate (HCO_3^-) and H^+ (Eq. 7-3). At equilibrium, the relationship between the $CO_{2(g)}$ in the gas phase and the bicarbonate and H^+ in the liquid phase is described by Eqs. 7-4 and

Panel 7.19. CO_2 as a pH Buffer

- CO_2 is the pH buffer in the human body's fluid
- Media that contains bicarbonate becomes alkaline very rapidly due to the loss of CO_2 when removed from the incubator
- The pK_a of bicarbonate is low (6.3)
- The $NaHCO_3$ buffer requires appropriate CO_2 concentrations in the gas phase. The reactions are:

CO_2 dissolves in an aqueous solution to reach equilibrium:

$$CO_{2(g)} \rightleftharpoons CO_{2(aq)} \qquad \textbf{(Eq. 7-1)}$$

The CO_2 concentration in liquid is described by Henry's Law:

$$P_{CO_2} = H\left[CO_{2(aq)}\right] \qquad \textbf{(Eq. 7-2)}$$

CO_2 in an aqueous solution forms a bicarbonate ion:

$$CO_{2(aq)} + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \qquad \textbf{(Eq. 7-3)}$$

The equilibrium is described as:

$$K_{eq} = \frac{\left[HCO_3^-\right]\left[H^+\right]}{\left[CO_{2(aq)}\right]} \qquad \textbf{(Eq. 7-4)}$$

Applying Eq. 7-2 to the equilibrium relationship:

$$K_{eq} = \frac{\left[HCO_3^-\right]\left[H^+\right]}{P_{CO_2} / H} \qquad \textbf{(Eq. 7-5)}$$

$pH = -\log_{10}[H^+]$ and $pK_a = -\log(K_{eq})$, so:

$$pH = pK_a + \log\frac{\left[HCO_3^-\right]}{P_{CO_2} / H} \qquad \textbf{(Eq. 7-6)}$$

The pH of the solution is affected by P_{CO_2} and HCO_3^-.

7-5. By taking a logarithmic transformation and applying the definition of pH ($\log_{10}[H^+]$) and pK_a ($\log_{10}K_{eq}$), one obtains the relationship between the pH and the gas phase level of CO_2 and HCO_3^-.

In a cell culture medium, HCO_3^- is supplied as sodium bicarbonate ($NaHCO_3$). In order to reach the target pH, some amount of CO_2 must be provided in the gas phase depending on the sodium bicarbonate concentration in the medium. The buffering capacity in the medium is dependent on the concentration of the bicarbonate in the medium, and the buffering action of bicarbonate requires the presence of CO_2. At a given concentration of bicarbonate, the pH of the medium is dependent on the CO_2 level in the gas phase. Similarly, at a constant CO_2 level the pH at equilibrium is affected by the bicarbonate concentration. The relationship can be plotted from Eq. 7-6 as shown in Figure 7.6.

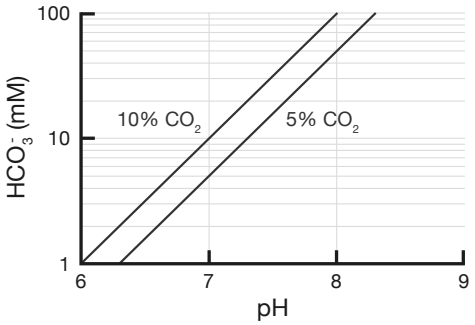

Figure 7.6. The relationship between the sodium bicarbonate concentration, pH, and atmospheric carbon dioxide level.

When selecting a buffer, one normally chooses a compound whose pK_a is near the target pH. The optimal pH for most cells is in the neutral range, or even 7.2–7.4, except in bioprocess, where the pH is often controlled at the low side, around 6.9–7.0, to reduce lactate production. The pK_a of bicarbonate is about 6.3, making it not an ideal buffer for neutral pH. A typical cell culture medium also contains sodium phosphate and HEPES, which has a pK_a in the neutral range, to increase the pH buffer capacity in neutral pH.

Buffering effect of bicarbonate, lactate, and CO_2

Typical cell culture medium contains 14–44 mM $NaHCO_3$. In a cell culture incubator with 10% CO_2 in ambient air, the pH in a DME medium will be initially ~7.4. As cells grow, lactate and CO_2 are produced. Each mole of lactate excreted by cells is accompanied by a mole of H^+. The pK_a of lactic acid is 3.7. Hence, all lactic acid produced from cell metabolism is dissociated to lactate and H^+ under cell culture conditions. Similarly, $CO_{2(aq)}$ produced from metabolism associates with water to generate a bicarbonate and a H^+. Because the pK_a of bicarbonate is pH 6.3, when in neutral pH the ~40 mM of bicarbonate in a DME medium has only ~20 mM of buffer capacity.

The bicarbonate level in a human's blood is about 25 mM, while the normal lactate level is about 2 mM. The buffer capacity of bicarbonate is sufficient to hold blood pH neutral. However, the buffer system provided by CO_2 in our blood is a dynamic system. CO_2 is continuously produced by cells and ventilated in the lungs, creating a balance of production and ventilation that maintains the CO_2 level in blood at ~25

mM. The ventilation is necessary to maintain the blood's pH. The other metabolite produced in very large quantities that influences blood pH is lactate. Lactate, once it is generated through muscle exercise or other activities, is consumed by some tissues like muscle and liver (Figure 7.7).

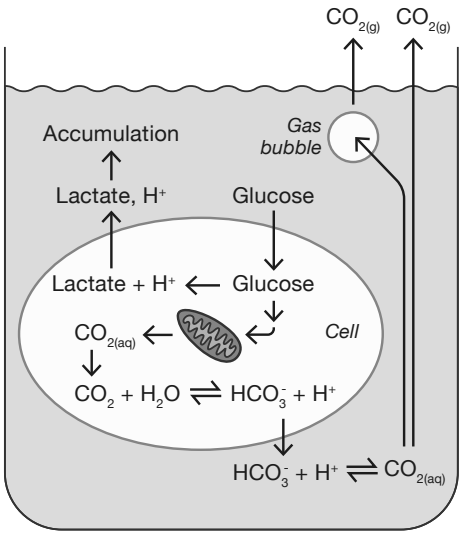

Figure 7.7. Stripping of CO_2 from the bioreactor.

The buffering of pH in a cell culture process is not only provided by the sodium bicarbonate initially present in the medium, but also by the CO_2 produced by cells. Like the dynamic system present in the blood, there should be a balance between production and removal through aeration. At the beginning of a culture process, CO_2 is present at a level that is appropriate for the sodium bicarbonate concentration in the medium and for the maintenance of the pH. As cells grow and lactate accumulates, the CO_2 level in the gas phase should be reduced to strip $CO_{2(aq)}$ from the medium. This neutralizes H^+ stoichiometrically (Eq. 7-3 and Figure 7.7) and requires that the CO_2 stripping capacity in the reactor is sufficient. However, the CO_2 stripping capacity of most industrial bioreactors is insufficient, as will be discussed in Chapter 11.

Alternative buffers

Under some conditions, such as when a CO_2 incubator is not available, sodium bicarbonate is unsuitable for buffering pH. A number of alternative pH buffers are listed in Table 7.6. Most cells have a relatively high tolerance to HCO_3^- concentration (over 50 mM), but the tolerance to alternative buffers is lower. At excessively high concentrations, these pH buffers can exert a negative effect on cell growth (Panel 7.20). Among these buffers, HEPES is the most commonly used, as its pK_a is in the neutral range and most cells have a moderate tolerance to its presence.

It should be noted that CO_2 is also an essential nutrient for cell growth. It is a substrate in many biochemical carboxylation reactions. Normally, the demand for CO_2 is met by its production in cell metabolism. If cells are cultured in a bicarbonate-free medium and the ambient air is aerated vigorously through the medium to strip off the CO_2 produced by the metabolism, one may see cell growth inhibition until the aeration is stopped, thus allowing the CO_2 produced in the metabolism to accumulate. When a sodium

Table 7.6. Non-Bicarbonate Buffers Used in Cell Culture

	pK_a value at 37 °C	*Anhydrous mol. wt.*	*Working concentration (mM)*
HEPES	7.31	238.3	10–28
TRICINE	7.80	179.2	<50
MOPS	7.01	209.3	10–20
Glycylglycine	7.95	132.1	10–20

Panel 7.20. Alternative Buffers

- Sodium β-glycerophosphate (20 mM) functions as a detoxifier of ferric chloride hydroxo compounds (i.e., Fe^{3+} chelator)
- Zwitterionic buffers: HEPES (N-2-hydroxyethylpiperazine-N-2-ethane) is commonly used between 10–30 mM
- Alternative buffers can be growth inhibitory at high concentrations
- Requires balance of osmolality by adjusting bulk salt levels by the addition of NaCl/KCl

bicarbonate-free medium is used to grow cells, it is common practice to maintain the CO_2 in the gas phase at 0.2–0.5%. Many media have low concentrations of sodium bicarbonate and HEPES to provide a neutral pH at the beginning of a cell culture process. The low level of bicarbonate provides the CO_2 required for cell growth.

Antioxidants

During cell metabolism, reactive oxygen species (ROS), free radicals, and various superoxide radicals arise and accumulate both inside and outside the cell. Additionally, some medium components such as Fe^{3+} and Cu^{+} are oxidative. As well as vitamins E and C for species that do not synthesize vitamin C, physiologically relevant compounds such as glutathione, enzymes that neutralize oxidized and potentially damaging compounds, and even chemicals that are not naturally occurring in the cell (e.g., mercaptoethanol) are sometimes included in the medium (Panel 7.21). Among them, glutathione and mercaptoethanol are the most commonly seen.

Panel 7.21. Physiologically Relevant Antioxidants

- Glutathione (reduced)
- Vitamin E
- Vitamin C
- Taurine
- Selenium
- Catalase
- Superoxide Dismutase
- β-mercaptoethanol

Glutathione is a key player that cells use to reduce ROS. Glutathione is a tripeptide of glutamic acid, cysteine, and glycine. In mammals, it is synthesized primarily in the liver and transported through blood circulation to other tissue cells, where it is further transported across the cytoplasmic membrane into the cytoplasm and certain organelles. Glutathione is often included in cell culture media, especially when cultivating differentiated tissue cells.

In addition to physiological antioxidants like vitamins C and E and glutathione, non-physiological antioxidant compounds are often included in culture media to minimize oxidation of labile medium components in culture fluid. β-mercaptoethanol is routinely used in the cultivation of stem cells and differentiated cells. Caution should be taken when using reducing agents in a medium if secreted proteins containing disulfide bonds are being produced. Mercaptoethanol can reduce the disulfide bridge to free thiols, which may then become reoxidized and form incorrectly paired disulfide bonds.

Agents modulating cells' physical interactions with the environment

Some medium components are added with the intention of modulating the physiochemical interactions between cells and neighboring

cells or cells and their environment. The viscosity of the medium was speculated to be important for sustaining cell growth in culture in the early days of serum-free culture medium development. Some compounds, such as polyethylene glycol and carboxymethylcellulose, were tested in the early development of serum-free media, but are seldom used now (Panel 7.22).

Panel 7.22. Media Components that Modulate Physical Interactions

- Heparan sulfate, heparin, and dextran sulfate can be used to facilitate the adaptation of cells to suspension growth
- Pluronic F-68 is used to protect cells from mechanical damage caused by agitation and aeration

Many cells used in bioprocessing are adherent cells. To adapt them to grow in suspension, sometimes heparan sulfate or dextran sulfate is added to the medium to reduce cell aggregation and adhesion to surfaces and facilitate the adaptation to suspension growth. The effectiveness of the compound is affected by the charge and the molecular weight.

Most cells are derived from tissue cells that are not normally exposed to high fluid shear conditions like those found in a bioreactor. When adherent cells are grown on microcarriers or suspension-adapted cells are grown under agitated conditions, cell damage caused by aeration and mechanical agitation is frequently seen. To protect cells grown in stirred tank bioreactors from damages caused by extensive fluid mixing and aeration, a protective agent, Pluronic F-68, was used in the cultivation of BHK cells in 1960s. Many Pluronic surfactants are available; all are block copolymers of polyoxyethylene (POE) and polyoxypropylene (POP). The larger the POE group, the more hydrophilic the molecule is and the greater its detergent-like activity and cell cushioning effects. The larger the POP group, the greater the toxicity and the anti-foaming ability. Pluronic F-68, in a concentration range of 0.01–0.2%, provides a protective effect under agitation and aeration conditions. The degree of foaming is high. Therefore, it is desirable to determine if a suitable replacement is available. Pluronic F-88 and F-77 may also provide suitable protective effects. As a surface-active agent, Pluronic F-68 alters the interfacial properties of the cell–fluid and gas bubble–fluid interfaces. Despite this, the mechanism of its protective effect is not well understood.

Antibiotics and selective agents

Penicillin and streptomycin have been frequently used in cell maintenance in research laboratories. The practice was relatively routine in the earlier days, when medium preparation equipment and practice were not as robust. For manufacturing processes, antibiotics are rarely used, although in vaccine production neomycin, polymyxin B, streptomycin, and gentamicin are sometimes used. For the cultivation of primary cells, which are not subjected to a long duration of quality control prior to processing, the use of gentamicin reduces the rate of contamination significantly. Some antibiotics are only moderately more inhibitory to bacteria

than to cultured cells. Toxicity testing is necessary before use. Many cell lines have been subjected to selection using antibiotics or metabolic inhibitors, such as methotrexate, as discussed in Chapter 6. In some cases, a low level of selective agent is present in the cell maintenance medium to reduce the risk of gene copy loss and the subsequent reduction of productivity.

High Molecular Weight and Complex Supplements

Some cells are capable of rapid growth using only a basal medium without any further supplements. However, such an extraordinary capability is the exception rather than the norm of cells in culture. Most cells in culture require supplementation of a number of growth factors and carrier proteins. Some industrial cell lines can be grown with only a basal medium supplemented with a few non-protein compounds (thus a protein-free medium), but at a substantially lower growth rate. When an enriched medium is used, cells grow at a faster rate. Many stem cells and other normal diploid cell lines require high concentrations of serum to grow. Serum is still commonly used in the isolation of primary cells, at least in the early stage of cell cultivation. In this section we will discuss the role of a few key high molecular weight supplements in cell culture.

Serum or Biological Fluids

Serum is the fluid left behind after blood coagulates, free of blood cells and most coagulation proteins. It is a complex mixture containing nutrient substances, metabolites, hormones, substances released from damaged cells (e.g., hemoglobin and growth factors from platelets), and various plasma proteins. Fetal bovine serum (FBS) is the most widely used serum in animal cell culture because it contains high concentrations of growth stimulatory factors and low concentrations of growth inhibitory factors. Other common sources of sera are human, bovine, newborn bovine, donor bovine, and donor horse.

Serum serves many different and important roles in cell culture (Panel 7.23). In addition to providing nutrients not sufficiently present in basal media (e.g., cholesterol), serum provides factors for the attachment of adherent cells (e.g., vitronectin, fibronectin) and modulates colloid osmolarity. Serum contains protease inhibitors and neutralizes trypsin used in cell detachment and other enzymes released by dead cells. Serum contains the carrier proteins transferrin and serum albumin. Carrier proteins chaperone components that are in very low concentrations, are poorly soluble (e.g., fatty acids carried by albumin), or are unstable (e.g., ferric ions carried by transferrin).

Serum is rich in "bulk" proteins (e.g., serum albumin) that can prevent nonspecific adsorption of critical factors to culture vessels. Serum also plays an important role as a scavenger. In cell cultivation, various contaminants may arise from numerous sources. For example, minute chemical components may leach out from parts of the reactor or the filter used in medium preparation. Some of these chemicals may be detrimental to cell growth or have other negative effects on cells. Due to the presence of serum in the medium, those compounds may be sequestered by adsorption to serum proteins before they can act on cells, thus minimizing potential damage.

Animal serum, however, has numerous disadvantages in addition to cost and the difficulty of maintaining consistent quality (Panel 7.24). The most serious concern is the possibility of contamination with animal viruses or prions that are infectious to cells used in the production. The presence of serum in culture medium makes downstream processing more complicated. It also makes the task of final product characterization more complex, as the number of heterologous proteins from the serum must be lowered to beneath an acceptable threshold. Serum also carries antibodies against various antigens to which the animals have been exposed. One has to check each lot of serum to ensure no reaction to the product protein or virus. For virus production processes, if serum antibodies cross-react with the product virus the production will be drastically affected.

Panel 7.23. Roles of Serum in Cell Culture Media

- Provides hormones and growth factors
- Protease inhibitors (e.g., α-2-macroglobulin) neutralize proteases used in trypsinization or produced by dead cells
- Provides carrier proteins
  - For low molecular weight substances (e.g., transferrin)
  - For nutrients which dissolve poorly (e.g., fatty acids, cholesterol, apolipoprotein)
- Binds nonspecifically and releases slowly the medium components that are inhibitory when present in excess
- Binds and/or neutralizes toxic substances (e.g., detergents)

Panel 7.24. Disadvantages of Serum in Cell Culture Media

- Potentially introduces animal viruses and other undesirable contaminants into cell culture (e.g., adventitious agents, antibiotics, proteases)
- Variability in quality
- High running costs and unnecessary capital outlay
- Normally purchased in large lot sizes, resulting in costly storage
- Serum lot testing can be tedious and costly
- Increases the complexity of downstream processing and final product characterization

Insulin and Insulin-Like Growth Factor (IGF-1)

Insulin plays a key role in regulating glucose uptake for many cell types. In addition to modulating glucose metabolism, insulin also exhibits mitogenic effects and stimulates cell growth through an overlapping pathway with IGF-1 (Panel 7.25). IGF-1 has an acute effect on protein and carbohydrate anabolism by increasing cellular uptake of amino acids and glucose, and by stimulating glycogen and protein synthesis. IGF-1 also affects cell proliferation, differentiation, and apoptosis. It is

Panel 7.25. Insulin and Insulin-Like Growth Factor

- Insulin stimulates glucose uptake by adipocytes and other cells, and also has a mitogenic effect at high concentrations
- Insulin is used in culture at the 1–10 μg/mL range. The blood insulin level is 4μU/mL or 1.3 μg/mL (1 μU = 0.33 μg); the IGF-1 level is 100–200 ng/mL.
- Insulin and IGF-1 have an overlapping signaling pathway through IR and 1GF1R
- IGF-1 can replace insulin in cell culture at a lower concentration

a potent mitogen that increases DNA synthesis and stimulates the expression of cyclin D in a wide variety of cells.

Both insulin and IGF-1 bind to the insulin receptor (IR) and IGF receptor (IGF1R), but with different affinities. After insulin binding, IR or IGF1R is phosphorylated, leading to activation of an insulin receptor substrate (IRS). There are multiple isoforms of IRS that are distributed differently in cells of different tissues. The signal is then relayed to downstream signaling pathways.

The response of the cell to insulin and IGF is dependent on the abundance level of the different IRS isoforms. Most cells, including CHO cells, express both IR and IGFR. However, NS0 cells express only IGFR. Differential binding to IR and IGF1R, as well as differential activation of various IRS isoforms, leads to different responses to insulin and IGF-1.

Insulin is used in cell culture at concentrations that are nearly 100-fold higher than that found in blood. At such a high concentration, insulin can trigger a mitogenic response. IGF has a much stronger affinity for IGF1R and is used at a much lower concentration than insulin.

Industrial NS0 cells or other myeloma lines are grown without insulin supplementation, and many CHO cells have been adapted to grow without insulin. Some differentiated cells derived from cancer, such as PC12 cells derived from pheochromocytoma, can also grow without insulin supplements. The changes in the signal transduction pathway that enable those cells to rapidly proliferate without insulin or IGF-1 stimulation is not yet clear. IGF-1 has a short half-life and is comparatively costlier than insulin. Also available is a protein-engineered IGF-1 called Long R3-IGF-1, which has a slower degradation rate and longer bioavailability in culture. Insulin, as one of the first recombinant proteins to be made available for widespread use in the treatment of diabetes, is a commodity chemical. For the manufacturing of recombinant therapeutics, insulin is commonly used instead of IGF-1.

Transferrin

Transferrin is the iron carrier glycoprotein in blood circulation in mammals. As we discussed in the trace metal section, ferric ions are highly oxidative. In the human body, it exists primarily in bound form to heme and other proteins with iron centers, or in bound form to transferrin in blood circulation (Figure 7.5) (Panel 7.26). Transferrin has a large number of binding sites with very high binding constant for ferric ion. When bound to transferrin, Fe^{3+} is unavailable for oxidizing other chemical

species. Transferrin binds to the transferrin receptor on a cell's surface to form a complex which is internalized into an endosome. Upon internalization, Fe^{3+} is released from the transferrin into the low pH environment of the endosome, where it is reduced to Fe^{2+} by ferrireductase and transported into the cytoplasm. Transferrin and its receptor are recycled to the extracellular environment and cytoplasmic membrane, respectively. Once in the cytoplasm, Fe^{2+} is stored in ferritin or is transported into sites of use, such as the mitochondria or target proteins.

Panel 7.26. Transferrin

- Typically used at 1–30 µg/mL (MW 80kDalton, 10 µg/mL = 0.1 µM)
- Binds to iron very strongly
- May be replaced by an iron-chelating agent, such as citrate

Recombinant human transferrin is commercially available. In general, transferrin will bind to receptors from many different species, although the binding affinity may be affected by the species origin of the transferrin–transferrin receptor pair. The concentrations required for cells of different species may differ and must be empirically determined. Furthermore, the cost of recombinant transferrin is a factor in its widespread application.

In addition to transferrin-mediated iron uptake, another route, non-transferrin-bound iron uptake, is used by cells.[6] A significant portion of Fe^{3+} in blood is bound to citrate instead of transferrin. In lieu of transferrin, Fe^{3+} can be chelated to citrate in a freshly prepared stock solution for addition to cell culture medium. Depending on the chelating agent used, the resulting iron-chelator complex may sequester iron availability to cells if a membrane transport mechanism is unavailable, or may make the complex available if an uptake system exists. Evidence suggests that the iron-citrate complex is taken up by cells.

Serum Albumin and Other Carrier Proteins

Serum albumin is the most abundant protein in human plasma. It is a nonglycosylated, negatively charged protein of 585 amino acids and serves as a carrier for many compounds that may have low solubility in aqueous solutions (Panel 7.27). Most notably, serum albumin is a carrier for fatty acids, bilirubin, heavy metal ions, and other agents that may harm cells. Serum albumin is probably the most important protein that mediates scavenger functions of serum in cell culture medium.

Panel 7.27. Serum Albumin

- Most abundant protein in blood (~3.5–5 g/L), MW 66.5 Kda, used in medium at 0.1–5 g/L
- Carrier protein for many different molecules, fatty acids, lipids, Fe^{3+}, and inhibitory metabolites like bilirubin
- Highly versatile molecule, but difficult to completely chemically characterize the compounds bound to it

Recombinant forms of human albumin are available. Most recombinant proteins are easily characterized in a chemically defined medium with respect to their purity and minute contaminating species. In comparison, the diverse binding capacity of serum albumin to a wide variety of species makes determining its complete

chemical properties difficult. Serum albumin from different preparations may be bound to different amounts and varieties of fatty acids or other compounds. Hence, serum albumin may not be used in a chemically defined medium.

Protein Hydrolysates

Hydrolysates, or extracts from yeast, animal, or plant tissues, have been commonly used in cell culture processes to reduce the need for animal serum. Beef extract, such as the commercial Ex-Cyte, served as a ready-to-use source of phospholipids and other nutrients until the use of animal extracts was mostly discontinued in industrial cell culture. Some processes employ hydrolysates derived from soy, rice, and other plants by enzymatic or acid hydrolysis. Plant hydrolysate is a complex mixture consisting of various oligopeptides of different compositions and lengths, oligosaccharides, other organics, and even various metal ions including heavy metals. The composition varies with the method and conditions of hydrolysis, the source of the raw materials, and the season of harvest. Variation between lots is inevitable and difficult to quantify. The roles of hydrolysates are not completely understood. They may provide some nutrients and minerals, and may also act as scavengers through undefined molecular interactions with possible contaminants.

Concluding Remarks

Our understanding of the nutritional requirements of cells in culture has improved drastically over the years, aided by the advances in chemical and biochemical analytical tools. The aim of the development of industrial cell culture media is to harness these understandings and incorporate stoichiometric and kinetic considerations. This chapter gives succinct descriptions of the roles of key medium components in supporting cell growth. While general nutritional needs are common to all cells, certain needs arise that are specific to particular cell lines or cell types. In the coming years, industrial medium development will take a genomics-assisted approach, employing epigenomic or global gene expression profiles of the target cell to design the medium based on the cell's capability to utilize certain nutrients or its need for specific growth factors. Industrial medium development will also seek to optimize cell growth during the cell expansion stage and maximize productivity in the production bioreactor. This chapter has not discussed the potential effects of medium design on cell stability, since little is known and few systematic studies have been done. A number of medium components affect the generation of cell-damaging compounds such as ROS and free radicals. In conclusion, the use of contemporary methods of medium