Defined Media and Supplements

TABLE 9.6. Selecting a Suitable Medium

Cells or cell line	Medium	Serum
3T3 cells	MEM, DMEM	CS
Chick embryo fibroblasts	Eagle's MEM	CS
Chinese hamster ovary (CHO)	Eagle's MEM, Ham's F12	CS
Chondrocytes	Ham's F12	FB
Continuous cell lines	Eagle's MEM, DMEM	CS
Endothelium	DMEM, M199, MEM	CS
Fibroblasts	Eagle's MEM	CS
Glial cells	MEM, DMEM/F12	FB
Glioma	MEM, DMEM/F12	FB
HeLa cells	Eagle's MEM	CS
Hematopoietic cells	RPMI 1640, Fischer's, αMEM	FB
Human diploid fibroblasts	Eagle's MEM	CS
Human leukemia	RPMI 1640	FB
Human tumors	L15, RPMI 1640, DMEM/F12	FB
Keratinocytes	αMEM	FB
L cells (L929, LS)	Eagle's MEM	CS
Lymphoblastoid cell lines (human)	RPMI 1640	FB
Mammary epithelium	RPMI 1640, DMEM/F12	FB
MDCK dog kidney epithelium	DMEM, DMEM/F12	FB
Melanocytes	M199	FB
Melanoma	MEM, DMEM/F12	FB
Mouse embryo fibroblasts	Eagle's MEM	CS
Mouse leukemia	Fisher's, RPMI 1640	FB, HoS
Mouse erythroleukemia	DMEM/F12, RPMI 1640	FB, HoS
Mouse myeloma	DMEM, RPMI 1640	FB
Mouse neuroblastoma	DMEM, DMEM/F12	FB
Neurons	DMEM	FB
NRK rat kidney fibroblasts	MEM, DMEM	CS
Rat minimal-deviation hepatoma (HTC, MDH)	Swim's S77, DMEM/F12	FB
Skeletal muscle	DMEM, F12	FB, HoS
Syrian hamster fibroblasts (e.g., BHK 21)	MEM, GMEM, DMEM	CS

محیط کشت

سلول ها در یک محیط مایع شیمیایی مناسب کشت می شوند.
فرمولاسیون محیط های استاندارد زیادی که برای رشد انواع سلول ها توسعه پیدا کرده اند، وجود دارد.

برای تعیین این که کدام محیط برای لاین سلولی ویژه مناسب است، توصیه می شود:
۱– به نوشته های قبلی مراجعه شود.
۲– مطالعه رشد با استفاده از ۳ یا ٤ فرمولاسیون مختلف

بعضی از محیط ها مانند DMEM غلظت بالایی از آمینواسیدها و ویتامین ها را دارند و برای رشد طولانی سلول ها مناسب هستند.

محیط هایی مانند Ham's F-12 حاوی محدوده وسیعی از ترکیبات مختلف هستند که برای برآورده کردن نیاز بعضی از لاین های سلولی مورد نیاز است.

گاهی می توان از ترکیبی از فرمولاسیون های استاندارد برای رشد سلول استفاده کرد. مثلا یک نسبت حجمی یک به یک DMEM و Ham's F12 به عنوان یک محیط بازال خوب برای فرمولاسیون های بدون سرم مناسب است.

انواع شکل های محیط کشت

۱- به صورت تجاری و آماده

۲- تغلیظ شده به حالت مایع

۳- تغلیظ شده به حالت پودر

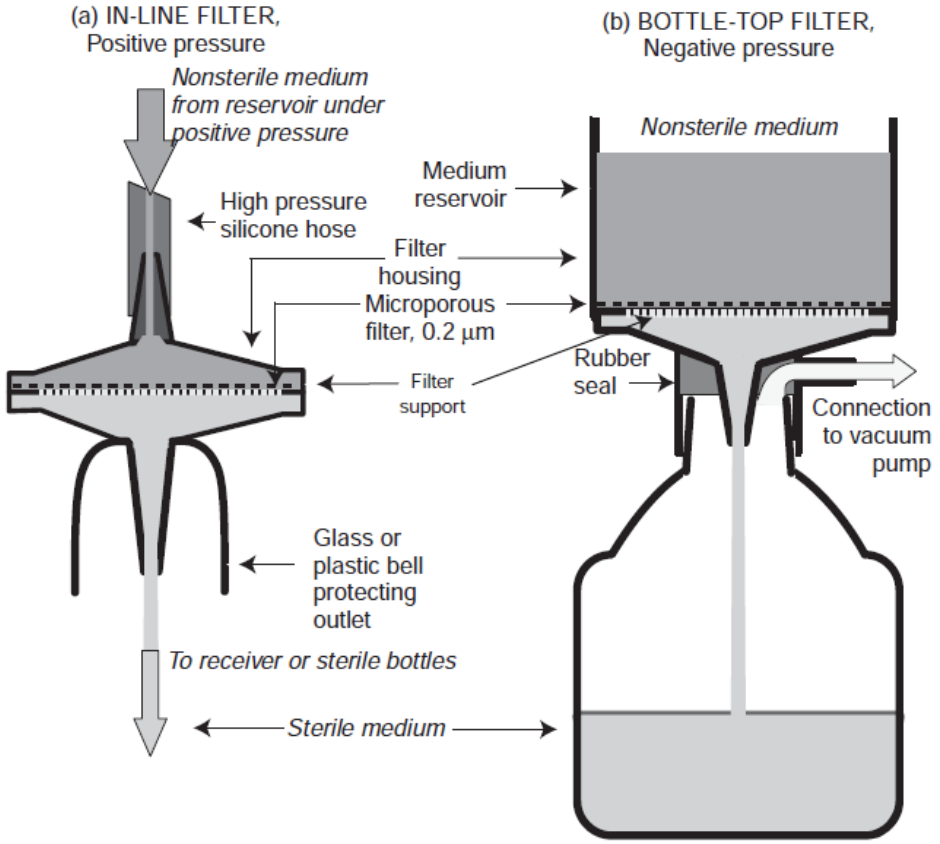

Fig. 11.11. Sterile Filtration. (a) In-line filter. Nonsterile medium from pump or pressure vessel. (b) Bottle-top filter or filter flask (designs are similar). Medium added to upper chamber and collected in lower. Lower chamber can be used for storage.

Aspiration Pump

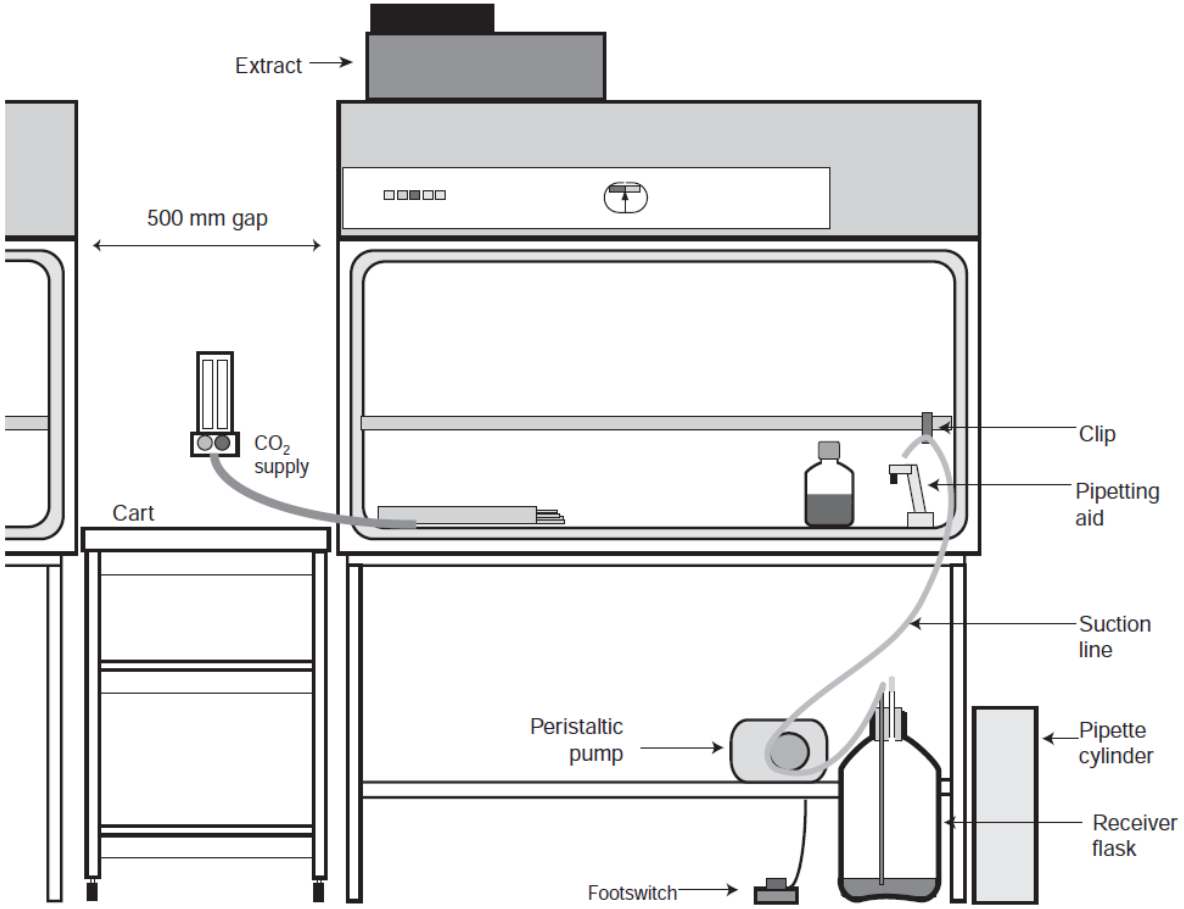

Fig. 5.1. Laminar-Flow Hood. A peristaltic pump, connected to a receiver vessel, is shown on the right side below the hood, with a foot switch to activate the pump. The suction line from the pump leads to the work area, and a delivery tube from a gas mixer provides a supply of CO_2 mixed in air.

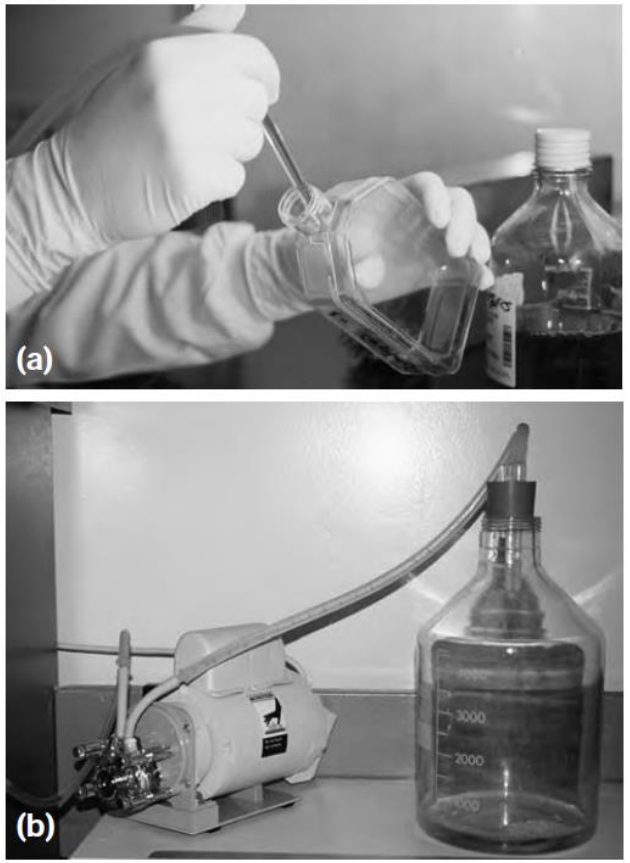

Fig. 5.2. Withdrawing Medium by Suction. (a) Pipette connected via tube to a peristaltic pump being used to remove medium from a flask. (b) Peristaltic pump on the suction line from the hood leading to a waste receiver.

Complete Media

The term ***complete medium*** implies a medium that has had all its constituents and supplements added and is sufficient for the use specified.
It is usually made up of a **defined medium component**, some of the constituents of which, such as **glutamine**, may be added just before use, and various supplements, such as serum, growth factors, or hormones.

Defined media range in complexity from the relatively **simple Eagle's MEM**, which contains essential amino acids, vitamins, and salts, to complex media such as medium **199 (M199), CMRL 1066, MB 752/1, RPMI 1640,** and **F12** and a wide range of serum-free formulations.
The complex media contain a larger number of different amino acids, including nonessential amino acids and additional vitamins, and are often supplemented with extra metabolites (e.g., nucleosides, tricarboxylic acid cycle intermediates, and lipids) and minerals.

Amino Acids

- The essential amino acids (i.e., those that are not synthesized in the body) are required by cultured cells, plus cysteine and/or cysteine, arginine, glutamine, and tyrosine.
- Although individual requirements for amino acids will vary from one cell type to another.
- Other nonessential amino acids are often added as well, to compensate either for a **particular cell type's incapacity to make them** or because they are made, but **lost by leakage into the medium**.
- The concentration of amino acids usually **limits the maximum cell concentration attainable**, and the balance may influence **cell survival and growth rate**.
- Glutamine is required by most cells, although some cell lines will utilize **glutamate**; evidence suggests that glutamine is also used by cultured cells as a source of **energy and carbon**.
- **Glutamax** (Invitrogen) is a alanyl-glutamine dipeptide which is more stable than glutamine.

Vitamins

- Eagle's MEM contains only the water-soluble vitamins (the B group, plus choline, folic acid, inositol, and nicotinamide)
- other requirements presumably are derived from the serum.

- Biotin is present in most of the more complex media and *p*-aminobenzoic acid (PABA) is present in M199, CMRL 1066 (which was derived from M199), and RPMI 1640.

- All the fat-soluble vitamins (A, D, E, and K) are present only in **M199**, whereas vitamin A is present in **LHC-9** and vitamin E in **MCDB 110**.

- Some vitamins (e.g., choline and nicotinamide) have increased concentrations in serum-free media.

- Vitamin limitation—for example, by precipitation of folate from concentrated stock solutions—is usually expressed in terms of **reduced cell survival and growth rates** rather than maximum cell density.

- Like those of the amino acids, vitamin requirements have been derived empirically and often relate to the cell line originally used in their development; e.g., **Fischer's medium** has a high folate concentration because of the folate dependence of L5178Y, which was used in the development of the medium.

Salts

- The salts are chiefly those of **Na^+**, **K^+**, **Mg^{2+}**, **Ca^{2+}**, **Cl^-**, **$SO4^{2-}$**, **$PO4^{3-}$**, and **$HCO3^-$** and are the major components contributing to the **osmolality of the medium**.
- Most media derived their salt concentrations originally from Earle's (high bicarbonate; gas phase, 5% CO2) or Hanks's (low bicarbonate; gas phase, air) BSS.
- Divalent cations, particularly **Ca^{2+}**, are required by some cell adhesion molecules, such as the **cadherins**.
- **Ca^{2+}** also acts as an intermediary in signal transduction,
- and the **concentration of Ca^{2+}** in the medium can influence whether cells will proliferate or differentiate.
- **Na^+**, **K^+**, and **Cl^-** regulate membrane potential, whereas **$SO4^{2-}$**, **$PO4^{3-}$**, and **$HCO3^-$** have roles as anions required by the matrix and nutritional precursors for macromolecules, as well as regulators of intracellular charge.
- Calcium is reduced in suspension cultures in order to minimize cell aggregation and attachment.
- The **sodium bicarbonate** concentration is determined by the concentration of CO2 in the gas phase and has a significant nutritional role in addition to its buffering capability.

Glucose

- Glucose is included in most media as a source of energy.
- It is metabolized principally by **glycolysis** to form pyruvate, which may be converted to lactate or acetoacetate and may enter the citric acid cycle and is oxidized to form CO2 and water.
- The accumulation of **lactic acid** in the medium, particularly evident in **embryonic and transformed cells**, implies that the citric acid cycle may not function entirely as it does *in vivo*, and recent data have shown that much of its carbon is derived from glutamine rather than glucose.
- This finding may explain the exceptionally high requirement of some cultured cells for **glutamine** or **glutamate**.

Organic Supplements

A variety of other compounds, including **proteins**, **peptides**, **nucleosides**, **citric acid cycle intermediates**, **pyruvate**, and **lipids**, appear in complex media.

Again, these constituents have been found to be necessary when the serum concentration is reduced, and they may help in cloning and in maintaining certain specialized cells, even in the presence of serum.

Hormones and Growth Factors

Hormones and growth factors are not specified in the formulas of most regular media, although they are frequently added to **serum-free media**.

Antibiotics

Antibiotics were originally introduced into culture media to reduce the frequency of contamination.
However, the use of laminar-flow hoods, coupled with strict aseptic technique, makes antibiotics unnecessary.
Indeed, antibiotics have a number of significant disadvantages:

(1) They encourage the development of **antibiotic resistant organisms**.
(2) They hide the presence of low-level, **cryptic contaminants** that can become fully operative if the antibiotics are removed, the culture conditions change, or resistant strains develop.
(3) They may hide **mycoplasma infections**.
(4) They have **antimetabolic effects** that can cross-react with mammalian cells.
(5) They encourage **poor aseptic technique**.

- Hence it is strongly recommended that routine culture be performed in the absence of antibiotics
- and that their use be restricted to **primary culture** or **large-scale labor-intensive experiments** with a high cost of consumables.
- If conditions demand the use of antibiotics, then they should be removed as soon as possible, or, if they are used over the long term, parallel cultures should be maintained free of antibiotics.

- A number of antibiotics used in tissue culture are **moderately effective** in controlling bacterial infections.
- However, a significant number of bacterial strains are resistant to antibiotics, either **naturally or by selection**, so the control that they provide is **never absolute**.
- **Fungal and yeast contaminations** are particularly hard to control with antibiotics; they may be held in check, but are seldom eliminated.

Serum

- Serum contains **growth factors**, which promote cell proliferation, and **adhesion factors** and **antitrypsin activity**, which promote cell attachment.
- Serum is also a source of **minerals**, **lipids**, and **hormones**, many of which may be bound to protein.
- The sera used most in tissue culture are **bovine calf**, **fetal bovine**, **adult horse**, and **human serum**.
- Calf (CS) and fetal bovine (FBS) serum are the **most widely** used, the latter particularly for more demanding cell lines and for cloning.
- Human serum is sometimes used in conjunction with some **human cell lines**, but it needs to be screened for viruses, such as HIV and hepatitis B.
- **Horse serum** is preferred to calf serum by some workers, as it can be obtained from a closed donor herd and is often more consistent from batch to batch.
- Horse serum may also **be less likely to metabolize polyamines**, due to lower levels of **polyamine oxidase**; polyamines are mitogenic for some cells.

1. Protein

- Although proteins are a major component of serum, the functions of many proteins *in vitro* remain obscure.
- It may be that relatively few proteins are required other than as carriers for minerals, fatty acids, and hormones.
- Those proteins for which requirements have been found are **albumin**, which may be important as a carrier of lipids, minerals;
- and **globulins**, **fibronectin** (cold-insoluble globulin), which promotes cell attachment, although probably not **as effectively as cell-derived fibronectin**;
- and ***α*2-macroglobulin**, which inhibits trypsin.

- **Fetuin** in **fetal** serum enhances cell attachment, and **transferrin** binds iron, making it less toxic and bioavailable.

- Other proteins, as yet uncharacterized, may be essential for cell attachment and growth.

- Protein also increases the **viscosity of the medium**, reducing **shear stress** during pipetting and stirring.

2. Growth Factors

- **Natural clot serum** stimulates cell proliferation more than serum from which the cells have been removed physically (e.g., by centrifugation).
- This increased stimulation appears to be due to the release of **platelet-derived growth factor (PDGF)** from the platelets during clotting.

- PDGF is one of a family of polypeptides with **mitogenic activity** and is probably the **major growth factor in serum**.
- PDGF stimulates growth in **fibroblasts** and **glia**,
- but other platelet-derived factors, such as **TGF-*β***, may inhibit growth or promote **differentiation in epithelial cells**.

- Other growth factors, such as **fibroblast growth factors (FGFs)**, **epidermal growth factor (EGF),** endothelial cell growth factors such as **vascular endothelial growth factor (VEGF)** and **angiogenin**, and **insulin-like growth factors IGF-I and IGF-II**, which have been isolated from whole tissue or released into the medium by cells in culture, have varying degrees of specificity and are probably present in serum in small amounts.

- Many of these growth factors are available commercially as **recombinant proteins**, some of which also are available in long-form analogs (Sigma) with increased mitogenic activity and stability.

3. Hormones

- **Insulin** promotes the uptake of glucose and amino acids and may owe **its mitogenic effect** to this property or to **activity via the IGF-I receptor**.
- IGF-I and IGF-II bind to the insulin receptor, but also have their own specific receptors, to which insulin may bind with lower affinity.

- **IGF-II** also stimulates glucose uptake.

- **Growth hormone** may be present in serum—particularly fetal serum—and, in conjunction with the somatomedins (IGFs), may have a **mitogenic effect**.

- **Hydrocortisone** is also present in serum—particularly fetal bovine serum—in varying amounts and it can promote cell attachment and cell proliferation,
- but under certain conditions (e.g., at **high cell density**) may be cytostatic and can induce cell differentiation.

4. Nutrients and Metabolites

- Serum may also contain **amino acids**, **glucose**, **oxo (keto) acids**, **nucleosides**, and a number of other nutrients and **intermediary metabolites**.
- These may be important in simple media but less so in complex media, particularly those with higher amino acid concentrations and other defined supplements.

5. Lipids

Linoleic acid, **oleic acid**, **ethanolamine**, and **phosphoethanolamine** are present in serum in small amounts, usually bound to proteins such as albumin.

6. Minerals

Serum replacement experiments have also suggested that **trace elements** and **iron**, **copper**, and **zinc** may be bound to serum protein. McKeen et al. (1976) demonstrated a requirement for **selenium**, which probably helps to detoxify free radicals as a cofactor for GSH synthetase.

7. Inhibitors

- Serum may contain substances that inhibit cell proliferation.
- Some of these may be artifacts of preparation (e.g., bacterial toxins from contamination before filtration, or antibodies, contained in the γ-globulin fraction, that cross react with surface epitopes on the cultured cells),
- but others may be **physiological negative growth regulators, such as TGF-β.**

- **Heat inactivation** removes complement from the serum and reduces the cytotoxic action of immunoglobulins without damaging polypeptide growth factors,
- but it may also remove some more labile constituents and is not always as satisfactory as untreated serum.

Testing Serum

The quality of a given serum is assured by the supplier, but the firm's quality control is usually performed with one of a number of continuous cell lines. If your requirements are more demanding, then you will need to do your own testing.
There are four main parameters for testing serum:

1.Plating efficiency. During cloning, the cells are at a low density and hence are at their most sensitive, making this a **very stringent test**.
Plate the cells out at 10 to 100 cells/mL, and look for colonies after 10 days to two weeks.
Stain and count the colonies, and look for differences in **plating efficiency (survival)** and **colony size (cell proliferation).**
Each serum should be tested at a range of concentrations from 2% to 20%.
This approach will reveal whether one serum is equally effective at a lower concentration, thereby saving money and prolonging the life of the batch, and will show up any toxicity at a high serum concentration.

2.Growth curve. A growth curve should be plotted for cell growth in each serum, so that the **lag period**, **doubling time**, and **saturation density** (cell density at "**plateau**") can be determined.
A **long lag** implies that the culture has to adapt to the serum;
short doubling times are preferable if you want a lot of cells quickly;
and a **high saturation density** will provide more cells for a given amount of serum and will be more economical.

***3.Preservation of cell culture characteristics*.** Clearly, the cells must do what you require of them in the new serum, whether they are acting as host to a given virus, producing a certain cell product, differentiating, or expressing a characteristic sensitivity to a given drug.

***4.Sterility*.** Serum from a reputable supplier will have been tested and shown to be free of microorganisms. However, in the unlikely event that a sample of serum is contaminated but has escaped quality control, the fact that it is contaminated should show up in mycoplasma screening.

Heat Inactivation

Serum is heat inactivated by incubating it for 30 min at 56°C. It may then be dispensed into aliquots and stored at −20°C.

Originally, **heating was used to inactivate complement for immunoassays**, but it may achieve other effects not yet documented.

Often, heat-inactivated serum is used because of the adoption of a previous protocol, without any concrete evidence that it is beneficial.

Claims that heat inactivation removes mycoplasma are probably unfounded, although heat treatment may reduce the titer for some mycoplasma.

OTHER SUPPLEMENTS

In addition to serum, **tissue extracts and digests** have traditionally been used as supplements to tissue culture media.

1. Amino Acid Hydrolysates

- Many such supplements are derived from microbiological culture techniques and autoclavable broths.

- **Bactopeptone**, **tryptose**, and **lactalbumin hydrolysate** (Difco—B-D Biosciences) are proteolytic digests of beef heart or lactalbumin and contain mainly **amino acids and small peptides**.

- Bactopeptone and tryptose may also contain **nucleosides** and other heat-stable tissue constituents, such as **fatty acids** and **carbohydrates**.

2. Embryo Extract

- Embryo extract is a crude homogenate of 10-day-old chick embryo that is clarified by centrifugation.

- The crude extract was fractionated by Cahn et al. [1966] to give fractions of either **high or low molecular weight**.

- The low-molecular-weight fraction promoted cell proliferation, whereas the high-molecular-weight fraction promoted pigment and cartilage cell differentiation.

- Although Cahn did not fully characterize these fractions, more recent evidence would suggest that the low-molecular-weight
- fraction probably contains peptide **growth factors** and the high-molecular-weight fraction proteoglycans and other matrix constituents.

3. Conditioned Medium

- Puck and Marcus [1955] found that the survival of low density cultures could be improved by growing the cells in the presence of **feeder layers**.

- This effect is probably due to a combination of effects including **conditioning of the substrate and conditioning of the medium by the release into it of small molecular metabolites and growth factors.**

- Hauschka and Konigsberg [1966] showed that the conditioning of culture medium that was necessary for the **growth and differentiation of myoblasts** was due to collagen released by the feeder cells.

- **Using feeder layers and conditioning the medium with embryonic fibroblasts or other cell lines remains a valuable method of culturing difficult cells.**

- Attempts have been made to isolate active fractions from conditioned medium, and the original supposition is still probably close to the correct interpretation.

- Conditioned medium contains both **substrate-modifying matrix constituents**, like collagen, fibronectin, and proteoglycans, and growth factors, such as those of the heparin-binding group (FGF, etc.), insulin-like growth factors (IGF-I and-II), PDGF, and several others, in addition to the intermediary metabolites.

- However, conditioned medium adds undefined components to medium and should be eliminated after the active constituents are determined.