Special Issue

Basal medium development for serum-free culture: a historical perspective

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Received and accepted 17 June 1996

Abstract

The evolution of basal synthetic formulations to support mammalian cell culture applications has been facilitated by the contributions of many investigators. Definition of minimally-required nutrient categories by Harry Eagle in the 1950's spawned an iterative process of continuous modification and refinement of the exogenous environment to cultivate new cell types and to support emerging applications of cultured mammalian cells. Key historical elements are traced, leading to the development of high potency, basal nutrient formulations capable of sustaining serum-free proliferation and biological production. Emerging techniques for alimentation of fed batch and continuous perfusion bioreactors, using partial nutrient concentrates deduced from spent medium analysis, can enhance medium utilization and bioreactor productivity.

Introduction

Developmental progress of nutrient media, from initial reports in the late 19th century to the present day, has been characterized by evolutionary advances (Jayme and Blackman, 1985; Gruber and Jayme, 1994). The external fluids for mammalian cells and tissues evolved from bathing environments of isosmotic salt solutions to approximations of the native cellular environment through addition of buffering components, more complex salt species, and substrates and co-factors to support intermediary metabolism, such as carbohydrates, amino acids, and vitamins (Ham, 1982).

While these nutrients were adequate to maintain viability and biological function for brief studies, supplemental additives (e.g., serum, tissue extracts, other humoral fluids) were required to support proliferation and to sustain cellular activity for extended periods. Motivation to reduce these ill-defined additives derived from desire to minimize lot-to-lot variation, limited availability, cost, interference with product purification, inability to cultivate certain cell types, and concern with foreign antigens or adventitious viral contaminants (Ham, 1982; Jayme and Greenwald, 1991).

Initial efforts to develop serum-free media exclusively through addition of defined growth factors failed to account for the broader contributions of serum

(Jayme, 1991). These properties included both specific biological activities (such as cytokines, supplemental metabolites, nutrient binding and transport factors, and substratum conditioning factors) and bulk protein functions (such as pH buffering, toxin inactivation, protease activity, and protection from shear stress and vessel adsorption) (Jayme and Blackman, 1985; Gruber and Jayme, 1994).

Pioneering efforts to characterize critical serum components and identify biochemically-defined substitutes initially emphasized cell growth and biological function in clonal density cultures. Subsequent applications of cultured cells, however, focused upon biological production and maintenance or directed-differentiation of cell function. These studies evolved more complex nutrient media designed to sustain performance at elevated cell densities and to emphasize metabolic efficiency under culture conditions designed to minimize cell proliferation.

This paper will focus upon the development of basal nutrient formulations used for research and biotechnology applications, with particular emphasis upon research of the late Professor Hiroki Murakami resulting in the eRDF basal synthetic formulation. Innovative techniques currently utilized to optimize nutrient composition and delivery to mammalian cell bioreactors are also described.

Foundational contributions

Primary historical elements for this discussion begin with Eagle's 1955 publication of a basal nutrient formulation (Eagle, 1955), an 'isosmotic, pH-balanced mixture of salts, amino acids, sugars, vitamins, and other necessary nutrients'. Four years later, Eagle published a modified formulation (Eagle, 1959), termed 'Minimal Essential Medium (MEM)', which augmented the original basal amino acid composition based upon the protein composition of cultured human cells. This modified formulation, while more nutritionally complex than most previous synthetic media, still required supplemental protein in the form of plasma, serum, or tissue extracts to support cell growth. The components of Eagle's basal medium formulations provided the basis for most nutrient media prepared today (Gruber and Jayme, 1994). Table 1 compares the biochemical composition of selected basal media used for mammalian cell culture applications.

From this point, two parallel lines of investigation emerged. One track elucidated nutrients required to eliminate serum while supporting proliferation of clonal isolates of targeted cell types, particularly examining primary cultures and established lines of finite population expansion. The complementary track focused less initially upon elimination of serum, but instead pursued optimization of nutrient levels for high density culture of immortalized cell types.

Two noteworthy contributors to the development of defined culture environments were Richard Ham and Charity Waymouth. From their laboratories emerged a broad range of nutrient formulations, supporting the low density growth and maintenance of many cultured cell types (Ham, 1984; Ham, 1965; Waymouth, 1984). Of these formulations, the media most frequently cited and consumed in the greatest quantity are Ham's Nutrient Mixtures (F-12 and F-10) and several formulations of the MCDB series.

Other investigators recognized that, even in the presence of serum, once cell cultures achieved relatively high density, growth would cease. Analysis of spent fluids and trial and error supplementation revealed that superior growth and sustained culture viability could be achieved by amplifying the basal levels of amino acids and vitamins contained in Eagle's original formulation. Dulbecco reported enhanced viral plaque formation in cells cultivated in a modified formula containing 4X nutrients, termed Dulbecco's modified Minimal Essential Medium, or DMEM (Dulbecco and Freeman, 1959). Further support of high density prolifer-

ation was accomplished (Iscove, 1984) by adjustment of sugar levels and by providing supplemental buffering capacity (Iscove's modified DMEM, or IMDM). High density lymphocyte proliferation was achieved in developing the RPMI media series by elevating various nutrients while maintaining relatively constant salt content and medium osmolality (Moore, Gerner and Franklin, 1967).

The art of synthesis

Future generations of cell culture researchers and biotechnologists may reflect upon the evolution of nutrient medium and recognize the enormous contribution of Gordon Sato and his co-workers (Bottenstein *et al.*, 1979). Perhaps their most frequently cited accomplishment was the merger of these two independent media development tracks into a simple composite, a 1:1 volumetric admixture of DMEM and F-12 media formulations, termed DMEM/F12 medium. By combining the favorable properties of both individual formulations, a fortified basal medium was created which supported both clonal isolation and high density culture (Barnes and Sato, 1980).

Although other combinations of classical formulations (e.g., IMDM/F12, M199/F10) have been implemented for selected applications, DMEM/F12 has emerged as the most widely utilized basal synthetic medium. Supplemented by serum, it was capable of sustaining bioreactor production applications. Augmented by a defined cocktail of peptide and steroid hormones, perhaps in combination with attachment factors or preconditioned matrices, it formed the basis for serum-free media to cultivate a broad range of cell types.

In addition to the direct achievements noted in the publications from Sato's laboratory, the fertile environment created by his leadership spawned many of the scientists who would carry the evolution of nutrient optimization to its next level. Among the great scientists trained within this environment who would contribute significantly to the development of basal media and to the progress of serum-free culture was Hiroki Murakami.

Murakami focused primarily on the nutrient requirements of various non-adherent cell types (e.g., hybridomas, lymphocytes, human tumor cells) for sustained proliferation and generation of specific monoclonal antibodies (Murakami and Yamada, 1987). Beginning with the basal DMEM/F12 formulation,

Table 1. Comparative biochemical composition of basal nutrient formulations^a

Component	MEM	F-12	DMEM	DMEM/F12	RPMI 1640	RDF	eRDF
Inorganic salts							
CaCl ₂ (anhyd)	200.00	33.22	200.00	116.60			
CaCl ₂ ·2H ₂ O						77.90	108.77
Ca(NO ₃) ₃ ·4H ₂ O					100.00	49.58	
CuSO ₄ ·5H ₂ O		0.0024		0.0013		0.00062	0.00075
FeSO ₄ ·7H ₂ O		0.83		0.417		0.208	0.222
Fe(NO ₃) ₃ ·9H ₂ O		0.10		0.05		0.025	
KCl	400.00	223.60	400.00	311.80	400.00	358.00	373.00
MgSO ₄ (anhyd)	97.67	57.22	97.67	48.84	48.84	49.35	66.20
MgCl ₂ (anhyd)				28.64			
MgCl ₂ ·6H ₂ O						30.48	
NaCl	6800.00	7599.00	6400.00	6995.50	6000.00	6505.00	6435.00
NaHCO ₃	2200.00	1176.00	3700.00	2438.00	2000.00	1050.00	1050.00
NaH ₂ PO ₄ ·H ₂ O	140.00		125.00	62.50		31.20	
Na ₂ HPO ₄ (anhyd)		142.00		71.02	800.00		
Na ₂ HPO ₄ ·12H ₂ O						1100.00	1220.00
ZnSO ₄ ·7H ₂ O		0.86		0.43		0.22	0.23
Sub-total	9837.67	9232.83	10922.67	10073.80	9348.84	9251.96	9253.42
Amino Acids							
L-Alanine		8.90		4.45		2.23	6.68
L-Arginine·HCl	126.00	211.00	84.00	147.50	200.00	194.00	582.00
L-Aspartic acid		13.30		6.65	20.00	13.30	39.90
L-Asparagine·H ₂ O		15.01		7.50	50.00	31.50	94.50
L-Cysteine·HCl·H ₂ O		35.12		17.56		8.80	105.40
L-Cystine						36.00	
L-Cystine·2HCl	31.00		63.00	31.29	65.00		
L-Glutamic acid		14.70		7.35	20.00	13.20	39.70
L-Glutamine	292.00	146.00	584.00	365.00	300.00	333.00	1000.00
Glycine		7.50	30.00	18.75	10.00	14.30	42.80
L-Histidine·HCl·H ₂ O	42.00	21.00	42.00	31.48	15.00	25.00	75.00
L-Hydroxyproline					20.00	10.50	31.50
L-Isoleucine	52.00	4.00	105.00	54.47	50.00	52.50	157.50
L-Leucine	52.00	13.10	105.00	59.05	50.00	55.10	165.30
L-Lysine·HCl	73.00	36.50	146.00	91.25	40.00	65.80	197.30
L-Methionine	15.00	4.50	30.00	17.24	15.00	16.40	49.20
L-Phenylalanine	32.00	5.00	66.00	35.48	15.00	24.80	74.30
L-Proline		34.50		17.25	20.00	18.40	55.30
L-Serine		10.50	42.00	26.25	30.00	28.40	85.10
L-Threonine	48.00	11.90	95.00	53.45	20.00	36.90	110.80
L-Tryptophan	10.00	2.00	16.00	9.02	5.00	6.10	18.40
L-Tyrosine						29.00	87.00
L-Tyrosine·2Na·2H ₂ O	52.00	7.81	104.00	55.79	29.00		
L-Valine	46.00	11.70	94.00	52.85	20.00	36.30	108.90
Sub-total	871.00	614.04	1606.00	1110.02	994.00	1051.53	3126.58

Table 1. Continued.

Component	MEM	F-12	DMEM	DMEM/F12	RPMI 1640	RDF	eRDF
Vitamins							
p-Aminobenzoic acid					1.00	0.51	0.51
Biotin		0.0073		0.0035	0.20	0.10	0.10
D-Ca Pantothenate	1.00	0.50	4.00	2.24	0.25	0.67	0.67
Folic acid	1.00	1.30	4.00	2.65	1.00	1.81	1.81
Niacinamide	1.00	0.036	4.00	2.02	1.00	1.50	1.50
Pyridoxal HCl	1.00		4.00	2.00		1.00	1.00
Pyridoxine HCl		0.06		0.03	1.00	0.50	0.50
Riboflavin	0.10	0.037	0.40	0.22	0.20	0.21	0.21
Thiamine HCl	1.00	0.30	4.00	2.17	1.00	1.60	1.60
Vitamin B12		1.40		0.68	0.005	0.34	0.34
Sub-total	5.10	3.64	20.40	12.02	5.66	8.24	8.24
Miscellaneous							
Choline chloride	1.00	14.00	4.00	8.98	3.00	6.14	12.29
D-Glucose	1000.00	1802.00	4500.00	3151.00	2000.00	1700.00	3423.00
Glutathione (reduced)					1.00	0.50	0.50
HEPES						1190.00	1190.00
Hypoxanthine (Na salt)		4.77		2.39		1.00	1.00
i-Inositol	2.00	18.00	7.20	12.60	35.00	23.40	46.80
Linoleic acid		0.084		0.042		0.021	0.021
Lipoic acid		0.21		0.105		0.052	0.052
Phenol red	10.00	1.20	15.00	8.10	5.00	6.56	5.00
Putrescine·2HCl		0.161		0.081		0.04	0.04
Pyruvate (Na salt)		110.00		55.00		55.00	110.00
Thymidine		0.70		0.365		0.18	0.18
Sub-total	1013.00	1951.13	4526.20	3238.67	2044.00	2982.89	4788.88
Total Formulation	11726.77	11801.64	17075.27	14434.51	12392.50	13294.62	17177.12

^a All biochemical quantities are expressed in milligrams per liter. Sub-totals for each category are rounded to two decimal places. Selected media represent the most frequently-used, glutamine-containing derivative of the referenced formulation. MEM is based upon reference #8; Nutrient Mixture F-12 upon reference #9; DMEM upon reference #11; DMEM/F12 upon a 1:1 mixture of the parent formulations, as defined in reference #14; RPMI 1640 upon reference #13; RDF and eRDF formulations are based upon concentrations defined in reference #18.

he and other colleagues from Dr. Sato's laboratory reported a common requirement for supplementation of the basal formulation with three additional nutrients, insulin, transferrin and selenium. The metabolic requirements for a cell cycle progression factor, iron-binding activity, and various trace elements (including selenium) persist as universal constituents of serum-free media.

Murakami's careful studies identified yet another nutrient commonly required as an ingredient for serum-free cultivation of hybridomas and other rapidly-proliferating cell types, ethanolamine (Murakami *et*

al., 1982). This cocktail of four additives (insulin, transferrin, selenium, ethanolamine) (ITES) has been commercialized by several suppliers, both as a serum extender to permit superior culture performance with reduced serum supplementation and as an additive to DMEM/F12 and similar basal media to permit serum-free cultivation of many cell types.

This augmented formulation, termed DF-ITES, remains as a principal fortified basal medium for many investigators engaged in serum-free culture applications. However, in the spirit of continuous improvement, Murakami still pursued additional refinements.

He noted sub-optimal levels of critical constituents in the DMEM/F12 formulation which had been refined by Poste and co-workers in developing RPMI formulations for lymphocyte culture. Following the lead of his mentor, Murakami experimented with mixtures of these formulations, eventually reporting superior performance with a 1:1 mixture of RPMI 1640 and DMEM/F12 formulations, which he termed RDF medium (Murakami *et al.*, 1984).

Further enrichments in the basal RDF formulation to sustain high density growth evolved from the efforts of Murakami and co-workers (Murakami and Yamada, 1987). For some applications, he found lactoferrin to be a superior substitute for transferrin. Slight (5%) increases in medium osmolality achieved by adjustment of electrolyte levels resulted in enhanced monoclonal antibody productivity. Further, he recognized that exhaustion of critical nutrients became both growth and production limiting in hybridoma cultures, so he proposed increasing the levels of amino acids and glucose by two-fold over the original RDF formulation (Murakami and Yamada, 1987; Murakami *et al.*, 1985; Murakami, 1989). Part of Hiroki Murakami's legacy is the recognition that this high potency, basal formulation, termed eRDF (for enriched RDF medium) is widely used, either by name or by composition, throughout much of the global biotechnology community.

Iterative improvements

Early nutrient formulation development efforts were based largely upon the ability of cultured cells to thrive with addition or omission of a particular constituent. With the assay endpoint being cell number and inability to quantitate utilization of individual nutrients within a complex mixture, sub-optimal nutrient formulation was inevitable. Additionally, nutrient formulations were developed for less fastidious cell types, cultured in the presence of serum or other additives at relatively low densities (Gruber and Jayme, 1994; Jayme and Gruber, 1994).

Advanced analytical procedures have demonstrated that nutrient utilization by cells cultured in production bioreactors at high density in the absence of serum is both qualitatively and quantitatively different. The negative impacts of lactate and ammonia accumulation on culture viability and biological production have been observed both in batch and sustained bioreactor environments (Jayme, 1991).

As culture alimentation regimens progress from batch to fed-batch and semi-continuous feeding schedules, there is growing evidence that initiating bioreactors in nutrient medium containing *all* of the nutrients that the culture will eventually consume may *reduce* utilization efficiency and biological productivity. Various investigators (Jayme and Gruber, 1994; Fike *et al.*, 1993) have demonstrated that significant lowering of the initial hexose, amino acid and lipid levels, combined with periodic replenishment of critical nutrients by addition of a concentrated supplement, can profoundly affect bioreactor performance.

Elevated initial hexose can lead to medium acidification and lactate accumulation, particularly in the absence of adequate oxygenation through culture sparging. Critical limitation of hexose can also result in glycosylation heterogeneity in the target product and loss of culture viability. By contrast, initiating cultures at relatively low, sustainable hexose concentrations and matching utilization and supplementation rates can minimize these culture artifacts (Jayme, 1991; Jayme and Gruber, 1994).

Similarly, increases in amino acid levels, particularly glutamine, can be harmful to productivity, as spontaneous and enzyme-catalyzed deamidation can rapidly generate cytotoxic accumulation of ammonia. Initiating cultures at relatively lower, sustaining levels of glutamine and other critically-limiting amino acids, combined with a concentrated feed-stream which matches utilization with replenishment, can minimize ammonia accumulation and deliver augmented biological product yields (Jayme, 1991; Jayme and Gruber, 1994).

Although successful results have been obtained with a simple fold-amplification of amino acids (and other nutrients) within the basal formulation (Bibila *et al.*, 1994; Mahadevan *et al.*, 1994), superior results may be achieved by preparing a supplemental nutrient cocktail which matches the nutrient utilization pattern for the desired cell type, bioreactor system and target product (Fike *et al.*, 1993). As an example, the amino acid profile from a typical high density production bioreactor reveals that some analytes are exhausted rapidly, others remain relatively constant over the production campaign, and still others accumulate due to transamination and other intermediary pathways. Analysis of spent fluids during the initial consumption phase permits approximation of a pseudo first-order nutrient utilization rate.

From these data, a customized nutrient concentrate may be designed which delivers a stoichiometrically-balanced cocktail of growth or production limiting

nutrients upon demand. Once this balanced cocktail is developed, direct on-line monitoring of a single limiting nutrient (e.g., glucose) effectively approximates the utilization of all critical nutrients. Linking automated delivery upon demand of the supplemental nutrient cocktail when the level of a target analyte falls below a setpoint maintains *all* critical nutrients at adequate levels while reducing accumulation of cytotoxic catabolites (Fike *et al.*, 1993).

Concentrated nutrient supplements may be effectively introduced either in fed-batch or continuous perfusion mode. Such nutrient cocktails reduce the overall media cost and minimize the volume of waste medium by introducing only required nutrients to a buffered, isosmotic extracellular environment (Roth *et al.*, 1995). This feeding technique also avoids exposing cells to the variable nutrient deprivation or inhibitory levels of metabolic by-products characteristic of many medium recirculation systems.

Additional nutrient medium optimization may be achieved by recognizing that cellular proliferation and biological production are often independent or even competing activities. Hence, the medium formulation which supports maximal proliferation may be a poorer nutrient mixture for biological production. The qualitative differences may be cell cycle associated, may relate to allocation of metabolic energy to support proliferative vs. synthetic events, or may result from different nutritional building blocks required for the two cellular processes (Jayme, 1991; Jayme and Gruber, 1994).

Successful results have been obtained by segregating, where possible, the two events by providing a growth medium and a production medium. In some cases, the production medium is almost identical to the growth medium, but may lack serum or other additives which stimulate cell growth but inhibit product synthesis or downstream purification. Alternatively, the production medium may contain additives which induce or stimulate biological production. In other situations, there can exist significant differences both in physical and nutritional properties of the two media (Jayme, Kubiak and Fike, 1995).

Segmented bioreactors, such as hollow fiber cartridges, can exploit yet another form of medium optimization. Depending upon the exclusion porosity of the fiber matrix and the permeability of the desired product, nutrient media may be designed for the extracapillary space to optimize viability at high cell density, while a less costly blend of rapidly-consumed nutrients can be perfused through the luminal space to replen-

ish the extracellular bathing fluids by osmosis. This process has been demonstrated to sustain viable hollow fiber bioreactors at high cell density and improve production efficiency while minimizing the cost of perfusion medium (Jayme, Kubiak and Fike, 1995).

Summary

Scientific understanding of the nutritional requirements of cultured mammalian cells has evolved with development of serum-free media, implementation of high density bioreactors for biological production applications, validation of quantitative analytical methods, and qualification of fed-batch and continuous perfusion feeding regimens. Stepwise improvements in basal, fortified medium formulations, established by thoughtful, deductive reasoning and careful experimentation as exemplified by the contributions of Hiroki Murakami, which are shared with the scientific community through publication and open dialog, will continue to accelerate the technical and commercial reality of cell-based biotechnology.

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