

DANCE ROUND



WE DANCE ROUND IN A RING AND SUPPOSE,
BUT THE SECRET SITS IN THE MIDDLE AND KNOWS.
ROBERT FROST

CHEMICALLY DEFINED CULTURE MEDIA

Rational recipes or witches' brew?

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*Scale of dragon, tooth of wolf,
Witches' mummy, maw and gulf
Of the ravin 'd salt-sea dark,
Liver of blaspheming Jew,
Gall of goat, and slips of yew
Sliver 'd in the moon's eclipse,
Nose of Turk, and Tartar's lips,
Finger of birth-strangled babe
Ditch-delivered by a drab,
Make the gruel thick and slab:*

*Add thereto a tiger's chaudron,
For the ingredients of our cauldron.*

*Double, double toil and trouble;
Fire burn and cauldron bubble.*

*Cool it with a baboon's blood,
Then the charm is firm and good.*

William Shakespeare
Macbeth, Act IV, scene 1

• A rational approach to study cells, tissues or even organs is to isolate them from the body and bring them into a controlled, and therefore reproducible, environment. *In vivo*, cells are surrounded by the extracellular matrix, and the body fluids nourish them. *In vitro*, these fluids are replaced by culture media. In the early days of tissue culture, tissue was cultured in a drop of clotted lymph. The early-day natural nutrient media have gradually become replaced by media of a more defined composition, culminating in the advent of completely defined culture media.

The elimination of serum from culture media was motivated by the wish to study the role of growth factors and hormones *in vitro* (1). The composition of serum is unknown, and it may contain factors masking the effects of these agents. In addition, the composition of serum varies greatly from batch to batch, which makes it difficult to conduct reproducible, quantitative studies. Toxic and growth-inhibiting effects of serum have also been reported (2). *In vivo*, cells are surrounded by lymph or cerebrospinal fluid, and not by serum. Therefore,

the elimination of serum may very well approximate the *in vitro* conditions to the conditions *in vivo*. For neuronal culture, the omission of serum is of special interest, since the presence of serum allows proliferation of non-neuronal cells, which might otherwise come to outnumber the neurons.

Chemically defined media (CDM) may be composed of more than 70 ingredients. This poses a methodological problem, since it is not possible to assay every possible combination of ingredients. This *Dance Round* describes the development of CDM for neural tissue in a historical perspective, emphasizing methodological considerations.

At present, many CDM are available, tuned for specific purposes. Most of these media are based on DMEM or Ham F12 and supplemented with hormones and growth factors, nutrients, binding and attachment factors. The recipes of twenty-nine CDM were compared with respect to their content of supplements, and their reported suitability. An attempt was made to trace the original introduction, and the reasoning be-

hind the introduction of each hormone and growth factor in the CDM recipes. Disappointingly, the rationale behind a novel ingredient was often not mentioned, and many hormonal additions seemed to be related to the availability of the pure substance. The various recipes were compiled in Table 1.

The group of compounds that differs most between the various media, is that of hormones and growth factors, suggesting that the requirements of these compounds are the most significant, with respect to the intended CDM specificity. We will argue that this significance reflects the developmental history of CDM, rather than specific virtues of these compounds. Finally, the question is addressed whether CDM are truly defined in the chemical sense.

HISTORY

- The history of CDM can be divided into several periods. A genealogical tree of chemically defined neural culture media was drawn to illustrate the description (Fig.1).

- **Purely natural media**

In 1907, Harrison succeeded in culturing the medullary tube of a frog embryo in a drop of clotted lymph (3). His observations of neurite outgrowth *in vitro* bear evidence to the viability of his preparation. This event marks the introduction of neural tissue culture as an experimental tool. Burrows (4), working in the same group, started using a plasma clot instead of a lymph clot. Shortly afterwards, it was discovered that embryo extracts possessed strong growth-promoting capacities (5). The use of plasma clots supplemented with embryo extracts soon became standard practice.

- **Partially synthetic media: definition of essential low molecular weight compounds**

From the early beginning, the factors in the medium necessary for growth and survival were under investigation. This work was started by Lewis and Lewis in 1911 (6). Harrison's coworkers also attempted to identify the essential ingredients

in their media (7). It was found that dialyzed culture medium, which is deficient in low molecular weight components, was completely unable to maintain the life of cells (8). In contrast, dialyzed medium supplemented with salts, amino acids and substances that are important in the Krebs cycle (vitamins, choline, creatine, C₄-acids) was able to maintain cells in culture (9). This finding provided the rationale for a systematic analysis of the low molecular weight substances.

Fischer's supplement consisted of 47 components (9), far too many to allow the investigation of all possible combinations. Instead, he demonstrated the importance of amino acids by showing that cells died quickly when cultured with the supplement mixture lacking amino acids. He subsequently omitted single amino acids from the mixture, in order to investigate their importance. Although the omission of several amino acids greatly decreased growth, no single component was demonstrated to be absolutely essential. It was obvious that the composition of the final minimal medium greatly depended on the arbitrary choice of amino acids in the initial mixture.

Several researchers continued Fischer's work and developed synthetic media (10-12), but a major breakthrough was achieved by Eagle (13). Using the same approach as Fischer, he was able to establish the essential amino acid requirements for a mammalian cell line (strain L). Likewise, he defined the minimum vitamin requirements (14). Supplementation of the minimal essential medium (MEM) (15) with 0.25 to 2.0 % dialyzed serum was still necessary. At this point, it could not be excluded that the dialyzed serum supplied protein-bound trace elements, vitamins, or even amino acids. The amino acids and vitamins shown to be essential were obviously not present in the serum fraction. If they were present in concentrations sufficient for survival and growth, the omission of these ingredients would not lead to cell death.

Growth of mammalian cells in a completely synthetic medium was first reported in 1954 by Healey and colleagues (16). Ham (17) was the first to describe colony formation and growth of a clonal cell line in a completely synthetic medium. Chinese hamster ovary cell lines demonstrated clonal growth in the

Abbreviations: Age: E - embryonic, F -fetal, N - neonatal, A - adult. Animal: R - rat, C - chick, M - mouse, X - Xenopus laevis, Q - quail. Culture type: CL - cell line, RDIS - reaggregating dissociated cells, DIS - dissociated cells, EXP - explant, PC - primary culture, ON - optic nerve. LMWF - low molecular weight fraction.

To several media extra putrescin (already present in Ham's F12) was added. The medium of Wilson et al (52) further contained ceruloplasmin, uridin, and collagen. Creatine and fumarase (not listed in this table) were used by Annis et al (33). Likewise several media contain extra linoleic acid or lipoic acid, which are also components of Ham's F12. Zinc is already present in Ham's F12, but nevertheless further zinc was added in some recipes (32, 49, 52, 99). Glutathione cooperates with selenium and Vitamins A and E to protect the cell against damage from oxygen-derived free-radicals. Vit B12 and H are constituents of Ham's F12, but were supplemented to several media.

Table 1. A compilation of the ingredients of 29 chemically defined media, listed in chronological order. Pre-existent media were supplemented with various mixtures of ingredients and then tested for their efficacy in a specific culture situation. Often several ingredients were tested, and finally not included in the medium; these instances are indicated by a "T".

Refs	Medium name	Cell source	Age	Cell type	Culture type	LMWF	Growth factors	Hormones	Protein metabolism	Lipid metabolism	Carbohydrate metabolism	Trace elements	Vitamins	Reference nr.
21		R	GHE		CL		Insulin		Transferrin				Vit A	21
35		R	HeLa		CL	Waymouth's medium	Nerve growth factor		Prostaglandin				Glutathion	35
22	N2	R	Neuroblastoma		CL	HAMF12	Fibroblast growth factor		Testosterone				Vit H	22
23		R	Brain		RDIS	DMEM	Epidermal growth factor		Oestradiol				Vit E-acetate	23
24		C	F Brain		DIS				Progesteron				Vit E	24
37	N1	C	E DRG		DIS				alpha-Melanocyte stimulating hormone				Vit C	37
38		C	E DRG		DIS				Luteinizing hormone				Vit B12	38
39		R	N Astrocyte		CL				Cortisol				Vit A-acetate	39
40		R	N Astrocyte		CL				Thyroxin				Vit A	40
41		R	N Cerebellum		DIS				Triiodothyronine					41
42	G2	M	F Hypothalamus		DIS				Parathyroid hormone					42
42	G3	H/R	A Glioma		CL				Thyroxin releasing hormone					42
42		R	A Schwannoma		CL				Somatomedin					42
36		M	N Astrocyte		DIS				Insulin					36
43		R	N Glial progenitor		DIS ON				Nerve growth factor					43
30	R12	R	F Neocortex		RDIS				Fibroblast growth factor					30
44	SF1	R	F Oligodendrocyte		DIS				Epidermal growth factor					44
44	SF2	R	N Oligodendrocyte		DIS									44
45		R	N Astroglial		DIS									45
45	G4	R	N Astrocyte		DIS									45
46	G5	R	N Astrocyte		DIS									46
47		H	Oligodendrocyte		DIS									47
48		R	F Brain		DIS									48
49		Q	E Neural crest		PC									49
50		R	N Oligodendrocyte		PC									50
51	O1	R	N Oligodendrocyte		CL									51
52		X	E Neural crest		EXP									52
52	R16	R	N Neocortex		EXP									52
53		R	N Oligodendrocyte		PC									53
33	EOL1	R	N Brain		EXP									33

absence of serum or serum-derived components. Moreover, Ham's F12 medium contained substances that did not appear to be essential in limited experiments.

• **Serum-free media: definition of specific high molecular weight compounds**

Thus far, growth in CDM had only been achieved for a number of clonal cell lines. These cases may very well be examples of selection of a subpopulation of cells capable of growing in the mixture of factors. Another conceivable event is adaptation of cells to defined culture media. For the maintenance of normal function of differentiated neurons, the addition of poorly defined biological extracts was still required. Because it was realized that these supplements contained critical factors for normal function *in vitro*, many attempts have been made to isolate and characterize them. Purification of growth stimulating factors from serum proved to be extremely difficult, if not impossible. First, many liters of starting material are needed. Second, factors are sometimes bound to carrier proteins from which they gradually dissociate. Third, many serum proteins are very similar regarding charge and size which complicates purification by conventional biochemical techniques. In addition to these technical difficulties, serum factors often act synergistically, further confusing separate purification. For these reasons, Sato (18) attacked the problem in a synthetic rather than an analytic way. He hypothesized that the primary role of serum was to provide hormones. Consequently, different cell lines required different serum-replacing hormone cocktails. Experiments showed that serum depleted of certain hormones no longer supported growth of cells, unless the medium was supplemented with the hormones that were removed (19, 20). This notion was not complete, since serum also contained nutrients, as well as binding factors for nutrients and hormones, and attachment factors. Binding proteins, such as albumin and transferrin, act like buffers, keeping the concentrations of their ligands at physiological level under various conditions. Attachment factors like fibronectin and globulin replace the supporting function of stroma in culture. Sato and coworkers (20-22) used the following strategy for the development of a serum-free nutrient medium: (1) initially, cultures were maintained with a defined medium (DMEM, Ham F12) supplemented with serum; when good growth of the cells was obtained, (2) the serum concentration was decreased in a stepwise manner, and hormones were added to the medium to compensate for the decreased growth; in the end the serum could be completely omitted from the medium,

and (iii) by omission of single additives, the essentiality of every additive could be established.

The first report (21) of serum substitution by hormones described the growth of an established rat pituitary cell line (GH₁) without altering the characteristics of the individual cells or the overall population. Triiodo-L-thyronine (T₃), thyrotropin-releasing hormone, transferrin, parathyroid hormone, and a partially purified somatomedin (5000-fold purification from serum) were necessary and sufficient to compensate for complete serum elimination. T₃ became one of the most frequently used hormones in neural tissue culture (Table 1).

After this report, serum-free culturing evolved rapidly and soon many cell types could be cultured under serum-free conditions. In 1979, three groups almost simultaneously reported successful long-term culture of various types of nerve cells (22-24). In the following years, these three CDM were adapted for specific purposes. Most of these custom-made neural CDM were derived from N2 medium (22) for neuroblastoma cells (Fig.1). The hormonal requirements of cells *in vitro* are likely to be similar to those *in vivo*, so that during the design process many of the specific requirements of different cells were revealed. Research into the regulation of neural ontogeny thus profited from the refinement of the various defined media (25-28).

Interesting from a methodological viewpoint is an extensively documented elaboration of a serum-free CDM for long-term culturing of reaggregated rat cerebrocortical tissue (29-32). These studies used the CDM mentioned above (22), and subsequently improved it stepwise, and in adherence to a cumulative improvement strategy. The effects of various single components or cocktails (Bottenstein and Sato-mix) were consecutively tested, and every time the optimal concentration was used in following tests.

The condition of the reaggregated cells was evaluated on the basis of morphological and sometimes electrophysiological parameters. The mean mammalian cerebrospinal fluid level (or, if unavailable, the blood serum level) could provide a guideline for the final concentration of a component. Other considerations were its biochemical properties and possible functions in the development and maintenance of brain cells, as well as the concentration profile of all other compounds in the medium.

Most adapted CDM recipes were designed for culturing established cell lines or dissociated cells of various origins. It was

Figure 1. Genealogical tree of publications pertaining to the elaboration of chemically defined media for nervous tissue. The reference numbers specific to the present paper are indicated. Relations between the various papers are indicated by the respective arrows, and explained in the text.

Romijn who first succeeded in culturing neocortex explants instead of separate cells in a serum-free medium (32). Only recently, a CDM for organotypic slice culture in a roller tube was developed (33), which indicates the youthful stage of neural tissue culture in CDM.

Summarizing, early this century neural tissue was cultured in a drop of clotted lymph, a "natural" medium. The low molecular weight part of natural medium was then replaced by a mixture of amino acids, vitamins and salts. Upon application of the "artificial" low molecular weight fraction of the natural medium, it was discovered that the remnant fraction of the natural medium could now be replaced by a tissue-specific mixture of high molecular weight molecules, such as hormones and transport proteins. The advent of these chemically defined high molecular weight supplements in return allowed further refinement of the composition of the low molecular weight fraction. This process is strikingly similar to Baron von Munchhausen escaping the swamp by pulling himself up by his own hairs.

By first establishing the low molecular weight requirements of tissue, using these for defining the high molecular weight compounds, and subsequently readjusting the low molecular weight portion, the reliability of the resulting media critically depends on the initial choice of compounds in the low molecular weight fraction. Statements concerning the *in vivo* requirements based on the composition of these media should be distrusted, since the composition of these media was based in the first instance on *a priori* suppositions about these requirements (another von Munchhausen's manoeuvre).

Fortunately, we may, at least partially, escape from the swamp. In the first place, *in vitro* observations may be corroborated by *in vivo* observations. The fact that cells *in vitro* generally looked like their *in vivo* counterparts morphologically and electrophysiologically, and expressed plasma membrane specific proteins, convincingly supports statements about the *in vivo* requirements. Here von Munchhausen pops up again, since for the generation of the antibodies that recognize these membrane proteins, cell specific antigens purified from cultured cells were used. In the second place is it not entirely correct to state that the suppositions about low molecular weight necessities were completely *a priori*. Biochemical research had, by the time Fischer performed his experiments (9), already established many of the basal metabolic requirements (34).

Focussing on the development of serum-free media from serum-supplemented media, it should first be mentioned that from the majority of the reports reviewed here, very little information about the methodology of the specific medium elaboration could be extracted.

By omission of single additives, the essentiality of every addi-

tive could be established. In several experiments the result of the addition of single components was compared with the results obtained with the unsupplemented medium and the medium containing all supplements. Was the effect of single supplements negligible, the effect of complete supplementation optimal and the effect of single omission catastrophic, synergy of supplements had been demonstrated (22). In most reports (21, 35) only the results of single omission of the ingredients were mentioned. However, in some of these the single-addition experiments probably had been performed, since the Materials and Methods mentioned ingredients that were not a part of the unabridged supplement mixture (23, 24).

The "single omission strategy" has at least one drawback. If several compounds can be omitted individually without any effect, this does not necessarily mean that they can be omitted as group. Several compounds of the medium could be left out without influencing the quality of the cultures, but nevertheless continued their addition to the medium (24). It might be suggested that they tried the medium without these compounds with little success, and decided not to continue with a "single addition strategy" with the medium without these compounds as a basis.

For later adjustments to existing serum-free media for specific purposes not only experimental, but also theoretical arguments were considered. This approach could very well lead to an overestimation of the necessities, and therefore to unnecessarily expensive media. Glutamine was not omitted from R12 medium, despite the fact that its omission did not visibly affect the cultures, because "it certainly could not be excluded that glutamine supplementation had a beneficial effect" (30).

Not only the ingredients contribute to the contents of the medium, but also the products of the cultured cells. In a study on oligodendrocytes, for example, astrocytes may perturb the experiments by secreting factors that influence oligodendrocytes. To obtain pure cultures, selective culture media were designed, that select for a specific cell type (36). Another variable is often the number of cultured cells, which also influences the medium composition.

CONCLUSION

- The development of "chemically defined" media was a rational process, resulting in media of increasingly, but never completely, defined composition. The process was rational in that the methodology (single omission, single addition) was optimal, given the impossibility of trying every possible combination of putative ingredients. A disadvantage of the methodology is that the final composition of the media is critically dependent on which ingredients were tested, and on the order in which they were tested. Since the choice of ingredients to

be tested depended on previous reports, especially those describing the development of similar media, the ingredients of the first CDM for neural tissue culture were crucial. The term "completely defined medium" is misleading and incorrect, since all media contain undefined substances. The amount of undefined components, however, has been minimized. Given the variability of experimental conditions, complete definition of the media might not be necessary. Thus, chemically defined culture media can be viewed as *rationally designed witches' brews*.

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