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Cell culture medium formulation and its implications in cancer metabolism

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Abstract

Historic cell culture media were designed to ensure continuous cancer cell proliferation *in vitro.* However, their composition does not recapitulate the tumor's nutritional environment. Recent studies show that novel media formulations alleviate the non-physiological constraints imposed by historic media, and lead to cell culture results more relevant to tumor metabolism.

Keywords

Cancer metabolism; Physiological medium; Cancer models

A tumor is a complex, dynamic and disordered structure within an organism, composed of mixed populations of normal and cancer cells. In order to understand the role of the biological units of this biological system, more than 60 years ago researchers embraced a reductionist approach and started to culture cells in isolation. Since then, the majority of the experiments in cancer research have been performed with cell lines cultured as monolayers. This implies that a substantial advance in cancer cell biology has been achieved with cultured cells, often referred to as *in vitro*. It is undeniable that culturing cancer cells is informative and has advantages that overall exceed its obvious limitations. Tumors consist of different niches depending on vascularization, genetic clonality and infiltration of immune and stromal cells. Since a cell culture dish overtly differs from the growth conditions of cells in tumors, researchers continuously attempted to refine culture conditions by modulating oxygen concentration, by allowing cells to form self-contained three dimensional structures, i.e. spheroids, or by supplying extracellular matrixes with different chemical-physical properties. Recently, more complex co-culture systems have also allowed to study *in vitro* the interaction between different cell types.

However, most of the experiments currently ongoing are still performed in historic cell culture media, some of which were formulated at least half a century ago, and whose composition clearly differs from the nutritional environment that cells withstand in tumors. For example Eagle's Minimal Essential Medium, MEM, and its Dulbecco's modified

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version, DMEM, were designed to supply cancer cells with only those nutrients essential for their continuous proliferation. These are widely used and in 2016 more than half of the published cell culture-based studies employed one or other of these media [1]. Another frequently used cell culture medium, F12, was optimized for the clonal growth of Chinese Hamster Ovary (CHO) cells under reduced serum supplementation [2]. In general, currently available commercial cell culture media were thought to allow continuous growth of specific cell types, not to recapitulate the metabolic environment of the tissue of origin [2]. As a result they often lack metabolites normally present in human fluids, while others, such as glucose, glutamine or pyruvate, are often found in media at supra-physiological concentrations (Table 1). On the contrary compounds irrelevant for human pathophysiology, such as L-alanyl-L-glutamine dipeptide (e.g. GlutaMAX™), are commonly supplemented at millimolar concentrations, with uncharacterized, yet inevitable consequences on cell metabolism.

Only in recent years, has it been shown that excessive concentrations of nutrients affect the metabolism of cultured cells and lead to discrepancies in metabolic phenotypes between cultured cells and tumors. For example, the proliferation of cancer cells has been shown to depend less on mitochondrial respiration when cultured with excessive concentrations of pyruvate, as indicated by their decreased sensitivity to metformin [3] (Figure 1). Additionally, high concentrations of cystine found in historic media enhance glutamine consumption and dependency of cancer cells in culture [4]. Undoubtedly, the effects of non-physiological levels of nutrients present in culture media, are not limited to cancer cells. BrainPhys™ is an example of a medium developed to recapitulate in culture, specific functional phenotypes observed in the brain. In 2015, Bardy et al. formulated BrainPhys™ with a reduced concentration of neuroactive ions and amino acids in comparison to DMEM/F-12 and Neurobasal™ media. This specialized medium enabled researchers to study the electrical activity of neurons derived either from primary tissue, or from induced pluripotent stem cells-, as well as *ex vivo* brain explants, in culture [5]. The logic applied in the designing of BrainPhys™ raises questions on what is currently known about the availability of nutrients and metabolites in specialized tissues, and in the tumor environment. Are cells in tumors exposed to nutrient concentrations comparable to those of plasma? Do adjacent cells directly exchange nutrients between each other or *via* extracellular interstitial fluid? Answer to these broad questions remains largely speculative, however recent evidences suggest that in poorly vascularized pancreatic adenocarcinomas, the concentrations of specific nutrients in the interstitial fluid significantly deviates from the circulating levels [6].

Under a reasonable assumption that the circulating levels of metabolites constitute a relevant source of nutrients for most normal and neoplastic tissues, in 2015 we formulated a medium with glucose, pyruvate and amino acids concentrations similar to human blood (Serum-like Modified Eagle's Medium, SMEM [7, 8]). SMEM has lower concentrations of the amino acids found in DMEM, it has additional proteinogenic (e.g. alanine, glutamate) and non-proteinogenic amino acids (e.g. ornithine, citrulline), but still lacks many polar metabolites normally found in human plasma. In 2017 Cantor et al. described the effects on cancer cells of a more complex medium formulation with amino acid derivatives, ketone bodies, end products of organismal catabolism (e.g. urate) and other components at concentrations found in human plasma (HPLM, Table 1 [9]). Urate is the end product of purine catabolism, and

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Cantor et al. reported that it can regulate the biosynthesis of the pyrimidine nucleotides by inhibiting uracil monophosphate synthetase (Figure1). This enzyme is also responsible for the activation of the drug 5-flurouracil, therefore cancer cells cultured in HPLM have been shown to be less sensitive to this anticancer drug. These observations suggest that the formulation of the cell culture medium might have profound implications in the target identification and drug development processes, in particular when these focus on cell metabolism [10].

Plasmax™ is a more complex iteration of the afore mentioned SMEM and, similarly to HPLM, it aims to recapitulate more closely the nutrient composition of human plasma [11]. Plasmax™ formulation contains 66 organic components. Amongst these, arginine and pyruvate are ~10 fold less abundant in this medium than in historic ones, such as DMEM. In triple negative breast cancer (TNBC) cells, pyruvate stabilizes the hypoxia-inducible factor 1α (HIF1α) in a dose dependent manner, and at the concentrations supplemented in historic media (0.5-1mM), it induces a pseudo-hypoxic response even under atmospheric oxygen availability. Concomitantly, in cells cultured in media such as DMEM or RPMI, the high concentrations of arginine reverse the direction of the reaction catalyzed by the urea cycle enzyme, argininosuccinate lyase. This metabolic feature was not observed in cancer cells grown in Plasmax™, nor in mammary orthotopic xenografts. Furthermore, in only four days of culture in Plasmax™ the metabolic profile of TNBC spheroids resembled the metabolic landscape of orthotopic xenografts more closely than that obtained with historic media. This indicates that the metabolism of established cancer cell lines, isolated and cultured for many passages under the non-physiological selective pressure of historic media, can be rectified towards a more tumor-like state. In addition, these observations suggest that *in vitro* models could be further refined by culturing cells freshly isolated from patient-derived material directly in a more physiological medium.

In commonly employed media, essential components (e.g growth factors, non-polar nutrients, and trace elements) are largely provided by the serum. Hence, their concentration varies between different batches, thereby impairing the reproducibility of results between laboratories. In order to achieve a chemically defined medium which allows cells to be cultured without serum supplementation, essential components must be included in the formulation. Plasmax™, as well as some advanced commercial media, contain trace elements such as Fe, Se, Zn, Cu in the form of salts. Human metabolism largely depends on circulating levels of these elements bound to organic small molecules and proteins such as transferrin, selenoproteins, ceruloplasmin and albumin. Therefore, the physiological availability of these important metabolic catalysts can be achieved by supplementing relevant concentrations of the trace elements, coupled with appropriate carrier molecules. Essential components are not limited to trace elements. Vitamins, hormones, lipids and growth factors, normally contributed by serum, should also be considered in the attempt to achieve tumor-relevant media formulations.

Finally, it is common cell culture practice to incubate the cells with a fixed amount of medium for extended periods. This can lead to the exhaustion of heavily consumed nutrients (e.g. glucose) and an accumulation of metabolic products (e.g. lactate) far beyond the physiological ranges reported in humans. This consideration applies in particular to

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physiological media where the concentration of nutrients has not been artificially increased to overcome this problem. An option to prevent both nutrient exhaustion in spent medium and excessive concentrations of nutrients in fresh one, is offered by a bioreactor, called chemostat [12], which provides a constant flow of fresh and exhausted media. While this approach is widely employed in microbiology and biotechnology, it is far less practical for multiplex experiments used in cancer research. A gross approximation of a steady state level of nutrients and metabolic end-products in the cell supernatant, can be achieved by adjusting the ratio between cell number, volume of medium and time between medium renewals. This can be applied with only minor modifications of current cell culture practice, for example by increasing the frequency of medium renewal, or by adjusting the volume of medium proportionally to the number of cells.

In summary, for decades cell biologists have used media disconnected from physiology. In the last decade, the growing focus on cancer cell metabolism has contributed to the exacerbation of some of the artifacts observed by culturing cells in historic media. The implications of using nutritionally skewed media in cancer research will become more evident with the use of refined and more physiologically relevant culture media by a broader research community. In parallel, more efforts to understand the nutritional environment of tumors will provide us with more defined templates for designing better cellular models of cancer.

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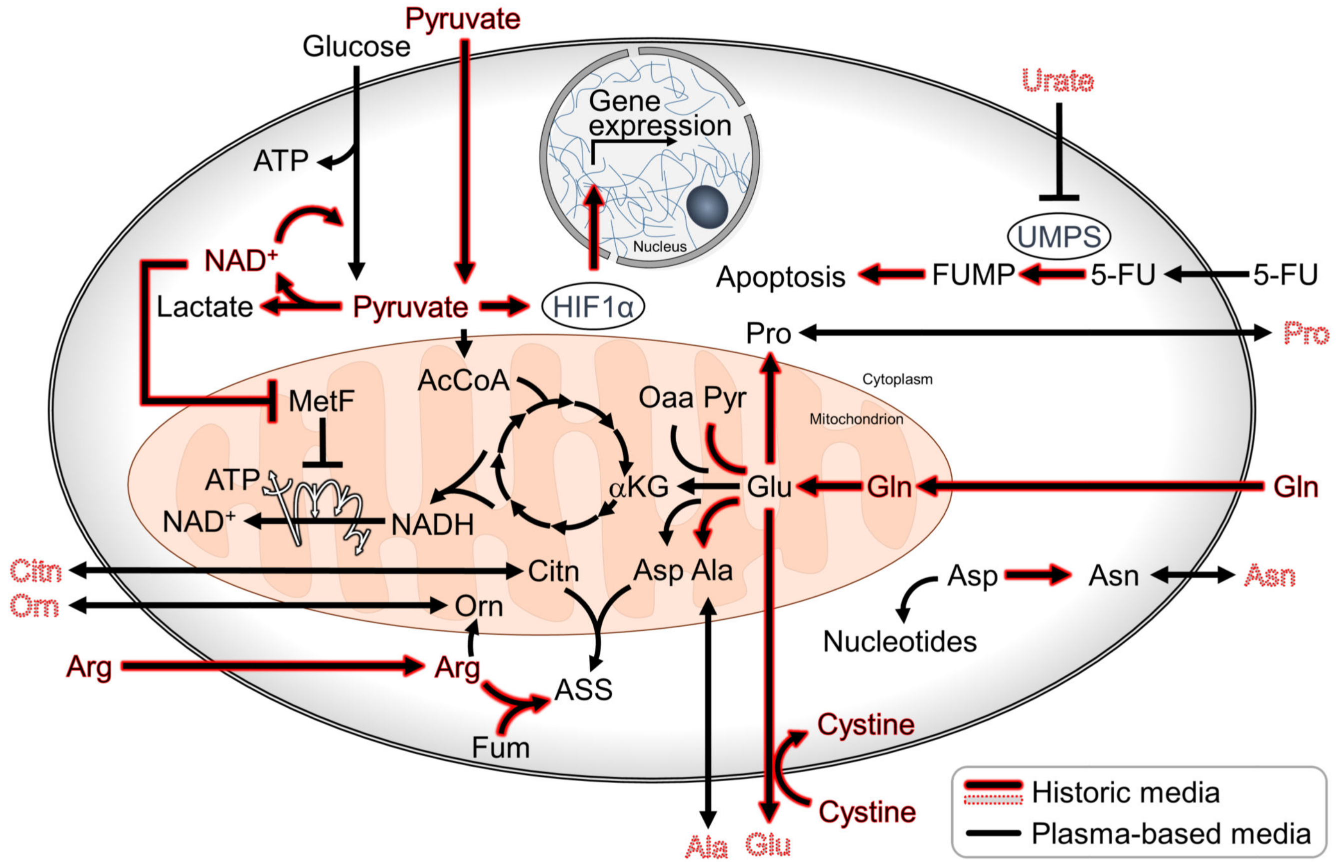

Figure 1. Metabolic reactions observed in cancer cells cultured in historic and physiological media.

Arrows and names highlighted in red indicate reactions or metabolite levels enhanced in historic media, such as DMEM. Nutrients and metabolites with a dashed outline are absent in DMEM. 5-FU: 5-fluorouracil, AcCoA: acetyl-Coenzym A, ASS: argininosuccinate, ATP: adenosine triphosphate, Citn: citrulline, Fum: fumarate, FUMP: 5-fluorouracil monophosphate, HIF1α: hypoxia-inducible factor 1α, αKG: α-ketogluterate, MetF: metformin, NAD: nicotinamide adenine dinucleotide, Oaa: oxaloacetate, Orn: Ornithine, Pyr: pyruvate, UMPS: uridine monophosphate synthetase.

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Table 1

Comparison between the formulations of physiological and historic media.

Formulations of Plasmax™, Human Plasma-Like Medium (HPLM), Minimal Essential Medium (MEM), Iscove's Modified Dulbecco's Medium (IMDM), Dulbecco's modified Eagles Medium (DMEM, high glucose), DMEM/F-12 nutrient mix (1:1), Ham’s F-12 Nutrient Mix (F-12), and Roswell Park Memorial Institute 1640 Medium (RPMI 1640, low glucose). The range of normal concentration values for human plasma are reported in the first column. All the concentrations are reported in μM. NA: not available. The colors represent the relative abundance of each component across the different media. White represents the level in Plasmax™, and blue and red represent lower and higher concentrations, respectively. An eightfold cutoff on color scale was applied. Grey and yellow cells are not part of the color scale.

	Human plasma	Plasmax™	HPLM	MEM	IMDM	DMEM	DMEM/F-12	F-12	RPMI 1640
Proteinogenic Amino Acids									
L-Alanine	230 - 510 [13]	510	430	NA	281	NA	50	100	NA
L-Arginine	13 - 64 [13]	64	110	597	399	398	699	1000	1149
L-Asparagine	45-130 [13]	41	50	NA	189	NA	50	100	379
L-Aspartic acid	0 - 6 [13]	6	20	NA	226	NA	50	100	150
L-Cysteine	23.2 - 43.8 [14]	33	40	NA	NA	NA	100	200	NA
L-Glutamate	32-140 [13]	98	80	NA	510	NA	50	100	136
L-Glutamine	420-720 [13]	650	550	2000	4000	4000	2500	1000	2055
Glycine	170 - 330 [13]	330	300	NA	400	400	250	100	133
L-Histidine	26 - 120 [13]	120	110	200	200	200	150	100	97
L-Isoleucine	42 - 100 [13]	140	70	397	802	802	416	31	382
L-Leucine	66 - 170 [13]	170	160	397	802	802	451	100	382
L-Lysine	150 - 220 [13]	220	200	399	798	798	499	199	219
L-Methionine	16 - 30 [13]	30	30	101	201	201	116	30	101
L-Phenylalanine	41 - 68 [13]	68	80	194	400	400	215	30	91
L-Proline	110-360 [13]	360	200	NA	348	NA	150	300	174
L-Serine	56 - 140 [13]	140	150	NA	400	400	250	100	286
L-Threonine	92 - 240 [13]	240	140	403	798	798	449	100	168
L-Tryptophan	44.8 - 64.2 [14]	78	60	49	78	78	44	10	25
L-Tyrosine	45 - 74 [13]	74	80	199	462	399	214	30	111
L-Valine	150 - 310 [13]	230	220	393	803	803	452	100	171
Non-proteinogenic Amino Acids									
α-Aminobutyrate	15 - 41 [13]	41	20	NA	NA	NA	NA	NA	NA
L-Citrulline	16 - 55 [13]	55	40	NA	NA	NA	NA	NA	NA
L-Cystine	30 - 65 [13]	65	100	99	292	201.3	100	NA	207.7
L-Homocysteine	6.1 - 12.1 [15]	9	NA	NA	NA	NA	NA	NA	NA
4-Hydroxy-L-proline	3 - 23 [16]	13	20	NA	NA	NA	NA	NA	152.7
L-Ornitine	27 - 80 [13]	80	70	NA	NA	NA	NA	NA	NA
L-Pyroglutamate	12.2 - 15.3 [17]	20	NA	NA	NA	NA	NA	NA	NA

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	Human plasma	Plasmax™	HPLM	MEM	IMDM	DMEM	DMEM/F-12	F-12	RPMI 1640
Amino Acids Derivatives									
L-Acetyl glycine	69.7 [14]	70	90	NA	NA	NA	NA	NA	NA
L-Carnosine	5.5 - 7.5 [18]	6	NA	NA	NA	NA	NA	NA	NA
Glutathione (reduced)	32.2 - 41.8 [19]	37	25	NA	NA	NA	NA	NA	3.3
Putrescine	0.1 - 0.3 [20]	NA	NA	NA	NA	NA	0.5	1.0	NA
Taurine	45 - 130 [13]	130	90	NA	NA	NA	NA	NA	NA
N-Trimethylglycine (betaine)	49.6 - 94.4 [14]	72	70	NA	NA	NA	NA	NA	NA
Other Components									
Acetate	26.8 - 57 [14]	42	60	NA	NA	NA	NA	NA	NA
Acetone	24.8 - 84 [14]	55	60	NA	NA	NA	NA	NA	NA
Acetyl carnitine	2.5 - 8.6 [21]	5	NA	NA	NA	NA	NA	NA	NA
Citrate	87.2 - 141.2 [14]	114	130	NA	NA	NA	NA	NA	NA
Carnitine	34.1 - 57.3 [14]	46	40	NA	NA	NA	NA	NA	NA
Creatine	8.4 - 65 [14]	37	40	NA	NA	NA	NA	NA	NA
Creatinine	60.5 - 87.7 [14]	74	75	NA	NA	NA	NA	NA	NA
Formate	19.5 - 46.1 [14]	33	50	NA	NA	NA	NA	NA	NA
Fructose	28.0 - 34.0 [22]	NA	40	NA	NA	NA	NA	NA	NA
Galactose	53.6 - 123 [22]	NA	60	NA	NA	NA	NA	NA	NA
D-Glucose	4598.5 - 5344.1 [14]	5560	5000	5556	25000	25000	17506	10011	11111
Glycerol	331.2 - 532 [14]	82	120	NA	NA	NA	NA	NA	NA
2-Hydroxybutyrate	23.5 - 39.1 [14]	31	50	NA	NA	NA	NA	NA	NA
3-Hydroxybutyrate	10.6 - 143.2 [14]	77	50	NA	NA	NA	NA	NA	NA
3-Hydroxyisobutyrate	19.0 - 23.0 [23]	20	NA	NA	NA	NA	NA	NA	NA
Hypoxanthine	4.5 - 5.3 [24]	5	10	NA	NA	NA	15	30	NA
Lactate	1118.2 - 1860.6 [14]	500	1600	NA	NA	NA	NA	NA	NA
Linoleic Acid	45.8 - 121.8 [14]	NA	NA	NA	NA	NA	0.15	0.30	NA
Lipoic Acid	0.060 - 0.094 [22]	NA	NA	NA	NA	NA	0.5	1.0	NA
Malonate	12.3 - 14.7 [14]	NA	10	NA	NA	NA	NA	NA	NA
Methyl acetoacetate	for acetoacetate 4.1 - 77.1 [14]	41	NA	NA	NA	NA	NA	NA	NA
Phenol Red	NA	25.0	14.0	26.6	39.9	39.9	21.5	3.2	13.3
Pyruvate	9.3 - 59.7 [14]	100	50	NA	1000	1000	500	1000	NA
Succinate	23.5 [14]	23	20	NA	NA	NA	NA	NA	NA
Thymidine	0.1 - 0.3 [19]	NA	NA	NA	NA	NA	1.5	2.9	NA
Uracil	1.1 - 3.1 [19]	2	NA	NA	NA	NA	NA	NA	NA
Urate	228.9 - 315.1 [19]	270	250	NA	NA	NA	NA	NA	NA

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	Human plasma	Plasmax™	HPLM	MEM	IMDM	DMEM	DMEM/F-12	F-12	RPMI 1640
Urea	3920.4 - 8228.8 [14]	3000	5000	NA	NA	NA	NA	NA	NA
Uridine	1.8 - 4.4 [19]	3	NA	NA	NA	NA	NA	NA	NA
Inorganic Salts									
Ammonium Chloride	for NH_4^+ [25] for Cl^- [1]	50	40	NA	NA	NA	NA	NA	NA
Calcium Chloride	for Ca^{2+} and Cl^- [1]	1800	2350	1802	1487	1802	1050	299	NA
Calcium Nitrate	for Ca^{2+} [1] for NO_3^- [22]	NA	40	NA	NA	NA	NA	NA	424
Magnesium Chloride	for Mg^{2+} and Cl^- [1]	NA	480	NA	NA	NA	302	602	NA
Magnesium Sulfate	for Mg^{2+} and SO_4^{2-} [1]	813	250	814	814	814	407	NA	407
Potassium Chloride	for K^+ and Cl^- [1]	5330	4100	5333	4400	5333	4157	2981	5333
Potassium Nitrate	for K^+ [1] for NO_3^- [22]	NA	NA	NA	1	NA	NA	NA	NA
Sodium Bicarbonate	for Na^+ and HCO_3^- [1]	26191	24000	26191	36000	44048	29024	14000	23810
Sodium Chloride	for Na^+ and Cl^- [1]	118706	105000	117241	77672	110345	120612	131017	103448
Sodium Phosphate monobasic	for Na^+ and PO_4^{3-} [1]	1010	NA	1014	906	906	453	NA	NA
Sodium Phosphate dibasic	for Na^+ and PO_4^{3-} [1]	NA	870	NA	NA	NA	500	1000	5634
Trace Elements									
Ammonium Metavanadate	for NH_4^+ [25] for V [26]	0.0026	NA	NA	NA	NA	NA	NA	NA
Cupric Sulfate	for Cu [27] for SO_4^{2-} [1]	0.0052	NA	NA	NA	NA	0.0052	0.0100	NA
Ferric Chloride	for Fe [28] for Cl^- [1]	NA	NA	NA	2	NA	NA	NA	NA
Ferric Nitrate	for Fe [28] for NO_3^- [22]	0.1238	NA	NA	NA	0.2475	0.1238	NA	NA
Ferric Sulfate	for Fe [28] for SO_4^{2-} [1]	1.0428	NA	NA	NA	NA	1.5000	3.0000	NA
Manganous Chloride	for Mn [29] for Cl^- [1]	0.0002	NA	NA	NA	NA	NA	NA	NA
Sodium Selenite	for Na^+ [1] for Se [29]	0.0289	NA	NA	0.0980	NA	NA	NA	NA
Zinc Sulfate	for Zn [29] for SO_4^{2-} [1]	1.50	NA	NA	0.49	NA	1.50	2.90	NA
Vitamins									
p-Aminobenzoate	5.0 - 32.0 [24]	NA	7.3	NA	NA	NA	NA	NA	7.3
Ascorbate	57.9 - 67.3 [19]	62	NA	NA	NA	NA	NA	NA	NA

	Human plasma	Plasmax™	HPLM	MEM	IMDM	DMEM	DMEM/F-12	F-12	RPMI 1640
D-Biotin	0.0006 - 0.0019 [30]	4.100	0.800	NA	0.530	NA	0.014	0.030	0.820
Choline	9.2 - 19.8 [14]	7.1	21.5	7.1	28.6	28.6	64.1	100	21.4
Folate	0.017 - 0.025 [31]	2.30	2.30	2.30	9.10	9.10	6.00	2.90	2.27
myo-Inositol	17.1 [14]	11.1	194.3	11.1	40.0	40.0	70.0	100.0	194.4
Niacinamide	0.435 - 0.445 [32]	8.2	8.2	8.2	32.8	32.8	16.6	0.3	8.2
D-Calcium pantothenate	4.5 - 5.3 [22]	2.10	0.52	2.10	8.40	8.40	4.70	1.05	0.52
Pyridoxine	0.007 - 0.060 [33]	4.90	4.90	4.90	19.60	19.40	9.80	0.29	4.90
Riboflavin	0.0054 - 0.028 [34]	0.30	0.50	0.27	1.10	1.10	0.58	0.01	0.53
Thiamine	0.078 - 0.114 [35]	3.0	3.0	3.0	11.9	11.2	6.4	0.9	3.0
Vitamin B12	0.00017 - 0.00033 [36]	0.0050	0.0037	NA	0.0096	NA	0.5000	1.0000	0.0037

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