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The concentrations of glutamine and ammonia in commercially available cell culture media

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The amino acid glutamine is an essential nutrient for cells in culture. In aqueous solutions such as liquid culture media, glutamine spontaneously decomposes into ammonia. In this study, we examined the toxicity of ammonia for two different cell lines. In mouse hybridoma cell cultures, viable cell counts were reduced at exogenous ammonia concentrations of 1000 μM. In the human promyelocytic cell line however, viable cell counts were shown to be reduced at exogenous ammonia concentrations of 300 μM. Next, we determined ammonia and glutamine levels in 11 commercially available media on the day of delivery. It was found that all media contained significantly less glutamine than prescribed. Ammonia was found in all media with concentrations ranging up to 1000 μM. Storage at both 4°C and 20°C caused a further degradation of glutamine and significant accumulation of ammonia in all media. The degradation curves of the various media were used to calculate the first order degradation constant k, which can be used to determine the kinetics of the spontaneous decomposition in culture media. These results suggest that precautions must be taken to avoid the deterioration of commercially available culture media, because of the decay of glutamine. Long storage times lead to a rapid decay of glutamine and an accumulation of the toxic degradation product ammonia.

Key words: In vitro; Culture medium; Glutamine; Ammonia; Proliferation

Introduction

Culture media provide the physicochemical conditions (e.g., pH, osmotic pressure and nutritional factors such as amino acids, carbohydrates and vitamins), vital for maintenance of growth and function of cultured cells. Liquid culture media contain relatively large amounts of the amino acid glutamine, which is not only an important energy substrate (Baechtel et al., 1976; Crawford et al., 1985; Hartmann et al., 1991; Reitzer et al., 1979) but also provides precursors for biosynthetic processes (Engstrom et al., 1984; Zetterberg et al., 1981). Glutamine spontaneously decomposes into ammonia and pyrrolidonecarboxylic acid, at a decomposition rate dependent on pH and temperature (Tritsch and Moore, 1962). Glutamine metabolism in cultured cells results in the formation of ammonia, glutamate, alanine and aspartate (Ardawi et al., 1983; Newsholme et al., 1989). Only ammonia has been reported to be toxic for cells in culture. Inhibition of cell multiplication in 3T3 cells (Visek et al., 1972) and of interferon synthesis by human fibroblasts (Ito et al., 1981) have been ascribed to

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the presence of ammonia. Ammonia also reduces viability and mitogenic responsiveness of mammalian lymphocytes in culture (Klucinski et al., 1984; Targowski et al., 1983). Spontaneous degradation, as well as enzymatic breakdown of glutamine can therefore have important consequences for the cellular function in culture.

In this study, we have determined the effects of ammonia on the proliferation of cultured cells and determined glutamine and ammonia levels in commercially available media. The data show that, at delivery, commercially available culture media contain amounts of ammonia which are toxic for cells in culture.

Materials and methods

Culture media

Decomposition of glutamine was determined in the commercially available liquid cell-culture media RPMI 1640, MEM, DMEM, and Ham F12, purchased from Gibco (Gibco, Paisley, Scotland), Sera Lab (JHR Biosciences, Sussex, UK) and Sigma (St. Louis, MO). 2×100 ml bottles of each media, except 2×500 ml bottles of RPMI 1640 and DMEM from Sera Lab, were purchased. Samples for the determination of glutamine and ammonia were taken under aseptic conditions on the day of delivery and stored at 4°C and at 20°C. In a separate experiment, the decay of glutamine was determined at 37°C. Culture media (10 ml) (Gibco) was incubated at 37°C in a CO_2 incubator (5% CO_2, 90% humidity). Samples (100 μl) were taken for glutamine determinations every 12 h for 4 days.

Determination of amino acids and ammonia

Amino acids were determined by HPLC as described previously (Van Eijk et al., 1989). Ammonia was assayed spectrophotometrically (Neeley et al., 1988) (Cobas Bio, Roche, Basel, Switzerland) by standard enzymatic methods using a commercial kit. All samples were stored at −70°C until further analysis.

Glutamine degradation according to Tritsch

Glutamine degradation in the media used in our study was compared with the calculations of Tritsch and Moore (1962) for glutamine degradation in aqueous solutions. These authors calculated a first order rate constant k (4°C $\log k = -1.9$, 20°C $\log k = -1.4$, 37°C $\log k = -0.95$). The degradation of glutamine after t days equals (with $[GLN_0]$: glutamine at day 0; F: first order constant k; and t: time in days.

$$\log[GLN_t] = \log[GLN_0] \cdot 10^{F} \times (t/2.303)$$

Cell lines and culture conditions

HL-60 (ATCC CCL 240), a human promyelocytic leukemic cell line and 2F1, a mouse hybridoma, product of a fusion with SP_20 cells (ATCC CRL 1581, mouse myeloma) were used. Cells were cultured in RPMI 1640 medium (Gibco, glutamine-deficient), 10% heat-inactivated bovine calf serum (Hyclone, UT), 2 mM L-glutamine (Flow LAB, Irvine, Scotland), penicillin (5000 U/ml) and streptomycin (5000 μg/ml) (Flow LAB) and grown in 25 cm^2 culture flasks (Costar, Cambridge, MA), kept at 37°C in a CO_2 incubator (5% CO_2, 90% humidity).

To study the effects of ammonia, cell cultures (10^5/ml) were incubated with NH_4Cl (filter sterilized (0.22 μm)) in RPMI 1640. After an initial culture period of 4 h, NH_4Cl was added ($t = 0$ h) to reach final ammonia concentrations of 0, 300, 1000 and 3000 μM (three separate cell cultures for each concentration). The pH of the media did not change after addition of NH_4Cl (not shown). Cell viability was determined by trypan blue dye exclusion. Cell counts were determined 48 h after addition of the NH_4Cl.

Pilot studies demonstrated that during a culture period of 48 h glutamine and several other amino acids (lysine, tryptophan and valine as illustrated for 2F1 in Table I) were either depleted or reduced to minimal amounts. Therefore, the medium was renewed after 24 h, so that cell growth was not limited by a reduction in any of the components of the medium.

Statistical analyses

Results are presented as mean ± SEM for ammonia. Amino acids were assayed once only since the coefficient of variance (CV) for the HPLC method was low (3%) and glutamine was consid-

ered to act as an internal control for accumulation of ammonia. The level of significance was set at $P < 0.05$. ANOVA was used for statistical analyses.

Results

To examine the toxic effects of ammonia on cell cultures, the mouse myeloma cell line 2F1 and the human promyelocytic cell line HL-60, were cultured for 48 h with increasing concentrations of exogenous ammonia. A significant difference in viable cell numbers was observed after the addition of increasing ammonia concentrations to cultures of mouse hybridoma 2F1 ($p < 0.001$) and human promyelocyte HL-60 ($p < 0.001$) (Fig. 1). In HL-60 cultures, a significant reduction in viable cell counts was seen with ammonia concentrations as low as 300 μM ($p < 0.01$). In contrast, the growth inhibition in 2F1-cultures became evident after the addition of 1000 μM ammonia ($p < 0.01$). However, it should be noted that ammonia concentrations represent

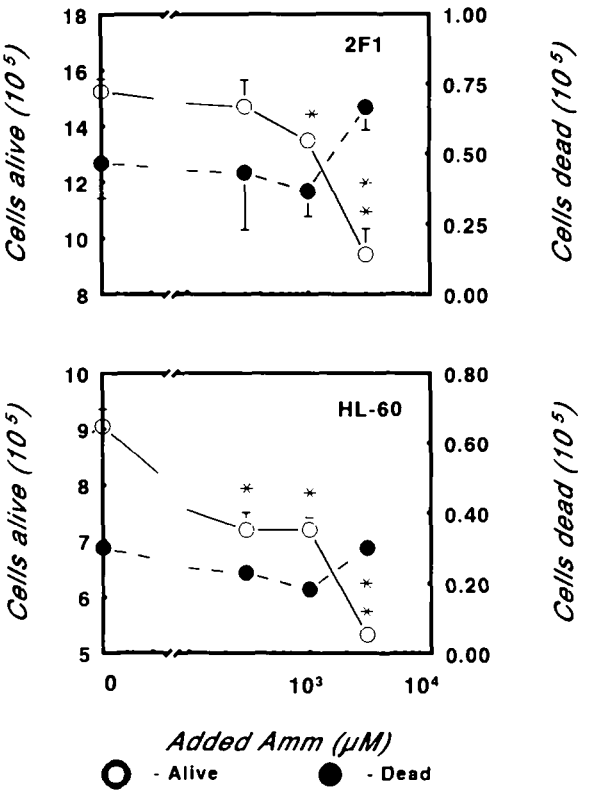

Fig. 1. Viable (left axis) and dead cell counts (right axis) of 2F1 and HL-60 cells after 48 h culture in the presence of 0, 300, 1000 and 3000 μM exogenously added ammonia (log axis). * $p < 0.01$, ** $p < 0.001$. Data are means ± SEM.

TABLE I

REDUCTION OR AUGMENTATION OF AMINO ACIDS IN 2F1 CULTURES AFTER 48 h

Values are expressed as a percentage of $t = 0$ h. 2F1 cultures (2×10^5/ml) were cultured for 48 h, with *no* renewal of the culture medium at $t = 24$ h.

	Percentage present after 48 h culture
Glutamate (Glu)	135
Asparagine (Asn)	75
Serine (Ser)	41
Glutamine (Gln)	0
Glycine (Gly)	149
Threonine (Thr)	27
Histidine (His)	49
Alanine (Ala)	1418
Arginine (Arg)	77
Tyrosine (Tyr)	36
Valine (Val)	8
Methionine (Met)	19
Isoleucine (Ile)	29
Phenylalanine (Phe)	11
Tryptophan (Trp)	0
Leucine (Leu)	23
Lysine (Lys)	0

added amounts of ammonia. Table II gives ammonia levels detected in the 2F1 culture after 48 h. This suggest that substantial amounts of endogenous ammonia accumulate both in control cultures and in cultures with exogenously added ammonia. The effects of ammonia on the cells is also illustrated by the reduction in endogenous ammonia production (Table II).

The concentration of glutamine and of its degradation product ammonia were measured in various commercially available media. Table III shows that glutamine concentrations in RPMI and DMEM differ considerably from the concentrations given by the manufacturer. Amino acid concentrations in MEM and HAM F12 show a similar pattern (data not shown). These data are not claimed to be representative but are illustrative since they are based on the analysis of single samples. In some media (e.g., RPMI/Sigma), the

TABLE II

ADDED AND MEASURED AMMONIA (μM) IN 2F1 CULTURES AFTER 48 h

Values are mean ± SEM. 2F1 cell cultures (10^5/ml) were incubated with NH_4Cl for 48 h. After an initial culture period of 4 h, NH_4Cl was added (t = 0 h) to the medium to final ammonia concentrations of 0, 300, 1000 and 3000 μM (three separate cell cultures for each concentration). Media was renewed at t = 24 h. Data represents the ammonia accumulation during the final 24 h of cell culture (after renewal of culture medium). δ ammonia represents endogenous ammonia production during cell culture.

Added ammonia	Measured ammonia	δ ammonia
0	1372 + 12	1372
300	1573 ± 23	1270
1000	2159 ± 71	1159
3000	3791 ± 66	791

reduction in glutamine concentration was more than 40%, e.g., in RPMI 1640 (from Gibco), only 1447 μM glutamine was found whereas 2055 μM was expected. The accumulated amount of ammonia appears to indicative clearly the extent of glutamine decomposition, since the ratio between glutamine degradation and ammonia accumulation was approximately 1 : 1 for most media. Moreover, the concentrations of other amino acids, particularly lysine, tyrosine and methionine differed considerably from the values given by the manufacturer (Table III).

Table I shows the consumption pattern of 2F1. Glutamine and several other amino acids (valine, phenylalanine, tryptophan and lysine) were either depleted or reduced to minimal amounts. It seems that the concentrations of these amino acids in the media were insufficient to maintain the growth and function of cells in the culture for 48 h.

The breakdown of glutamine in the media was investigated both at 4°C and at 20°C. Table IV gives the degradation data for RPMI 1640, which is illustrative for all other media studied (data not shown). Storage at 20°C resulted in much higher degradation rates, than storage at 4°C.

The degradation rate of glutamine in the media studied did not fit with the data of Tritsch and Moore (1962). We therefore recalculated the first order rate constant k in the formula of Tritsch, using our data of glutamine decomposition and we found that the first order constants were as follows: $\log k = -2.55 \pm 0.09$ (4°C) and $\log k = -1.86 \pm 0.03$ (20°C). The glutamine degradation curve, based on these constants (Fig. 2), accurately represented the actual glutamine

TABLE III

AMINO ACIDS AND AMMONIA IN RPMI 1640 AND DMEM FROM VARIOUS MANUFACTURERS

Data in μM. Exp. = expected: medium composition according to the manufacturers' catalogue. Found: medium composition on day of delivery. +/− >10%, ++/−− >20%, +++/−−− >30% deviation from expected value. Only amino acids with >10% difference are shown.

	RPMI 1640						DMEM					
Company:	Gibco		Sigma		Sera Lab		Gibco		Sigma		Sera Lab	
Cat. no.:	041-02400H		R8758		51-536		041-02320		D8788		51-436	
Lot no.:	10G5012		90H-0637		1G3428		11G5012		21H-4600		1E3380	
	Exp.	Found	Exp.	Found	Exp.	Found	Exp.	Found	Exp.	Found	Exp.	Found
Amm	0	659 +++	0	832 +++	0	343 +++	0	1043 +++	0	1115 +++	0	722 +++
Glu	136	118 −	136	112 −	136	123	0	43 +++	0	8 +++	0	4 +++
His	97	98	97	88	97	93	271	192 −−	271	182 −	271	191 −−
Arg	1378	1471	1148	1085	1148	1165	482	447	482	396 −	482	404 −
Ser	285	293	285	271	285	256 −	400	396	400	379	400	363
Gln	2055	1447 −−	2055	1241 −−−	2055	1757 −	3973	3210 −	4000	2973 −−	4000	3439 −−−
Tyr	110	106	159	100 −−−	159	106 −−−	397	398	573	368 −−	573	385 −−−
Met	101	84 −	101	77 −−	101	86 −	201	186	201	179 −	201	174 −
Ile	381	494 ++	381	480 ++	381	376	800	854	800	831	799	789
Trp	24	23	24	19 −−	24	20 −	78	80	78	75	78	74
Lys	274	189 −−−	274	188 −−−	274	189 −−	999	785 −−	999	765 −−	1000	740 −−

TABLE IV

DEGRADATION OF GLUTAMINE AND ACCUMULATION OF AMMONIA IN RPMI 1640 FROM THE VARIOUS MANUFACTURERS

RPMI 1640 as a illustrative example of glutamine degradation and ammonia accumulation in the various media. Data are in μM. Ammonia values are mean ± SEM. Statistical analyses: analyses of variance effects in time per media [a] $p < 0.001$ [b] $p < 0.05$. Media were stored at 4°C for 8 weeks and at 20°C for 4 weeks.

	Time (days)	Glutamine		Ammonia	
		4°C	20°C	4°C	20°C
Gibco	0	1447		659 ± 2	
	14	–	1165	–	901 ± 13
	28	1357	905	720 ± 9	1167 ± 10 [a]
	56	1339	–	823 ± 5 [a]	–
Sigma	0	1241		832 ± 15	
	14	–	1088	–	980 ± 14
	28	994	896	1077 ± 10	1131 ± 3 [b]
	56	685	–	1397 ± 12 [a]	–
Sera Lab	0	1757		343 ± 2	
	14	–	1467	–	592 ± 4
	28	1732	1345	429 ± 3	791 ± 12 [a]
	56	1589	–	498 ± 5 [a]	–

degradation in the media studied. All individual degradation curves of the media used fitted the degradation curve within a range of 95%. Incubation of culture media at 37°C resulted in a considerable decay of glutamine (Fig. 2). At this temperature, the degradation of glutamine corresponded with the data previously calculated by Tritsch and Moore.

Discussion

Glutamine is an important nutrient for cells in vitro (Baechtel et al., 1976; Hartmann et al., 1991; Reitzer et al., 1979; Zielke et al., 1984). The main degradation product of glutamine metabolism, ammonia is known to be toxic to cultured cells (Hassell et al., 1991; Klucinski et al., 1984; Targowski et al., 1983; Visek et al., 1972). Ammonia is also released following spontaneous degradation of glutamine (Tritsch and Moore, 1962). In the present studies, it was obvious that ammonia concentrations present in liquid media were inhibitory for human HL-60 and mouse 2F1, although the two cell lines clearly differed in their sensitivity to increasing concentrations of ammonia. The inhibitory effect of am-

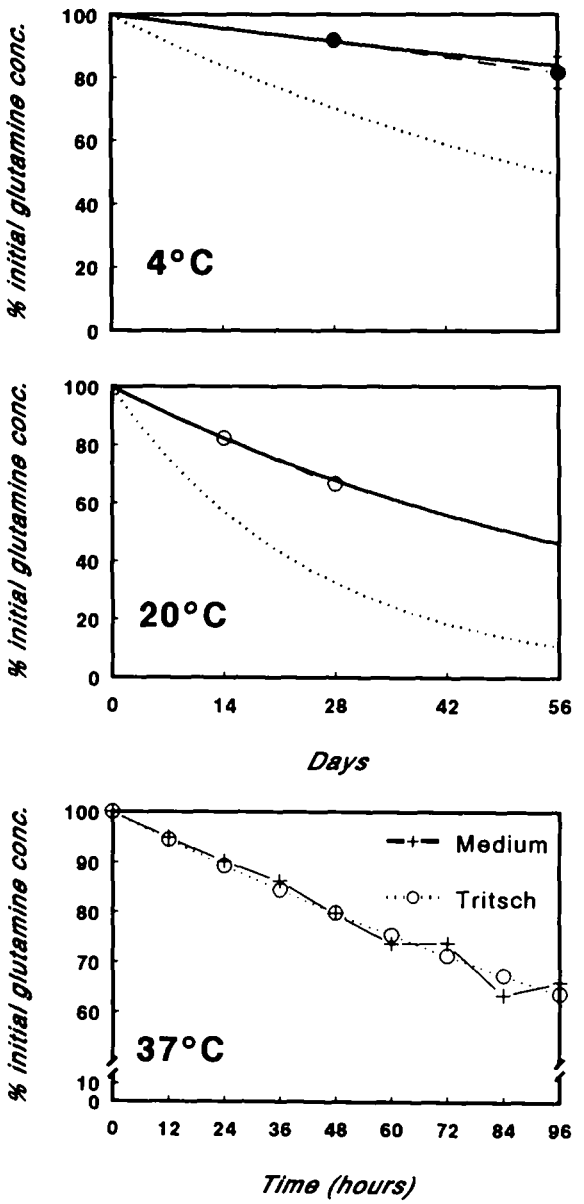

Fig. 2. Decomposition of glutamine as a percentage of the initial glutamine concentration. Glutamine degradation rate is plotted at 4°C (top panel), at 20°C (middle panel) and at 37°C (bottom panel). Top and middle panel: open and closed circles represent mean degradation in all analyzed media. Dashed lines represent theoretical glutamine degradation as calculated according to Tritsch and Moore (1962), solid lines represent recalculated degradation.

monia on cell culture growth was also reported for mammalian lymphocytes (Klucinski et al., 1984) and mouse fibroblasts (Visek et al., 1972). Our data indicate that ammonia concentrations present in commercially prepared media can inhibit the growth of cells.

Storage of glutamine containing media leads in time to glutamine degradation and ammonia accumulation. The rate of spontaneous decomposition is dependent on temperature, pH and the ionic milieu, as previously reported by Tritsch and Moore (1962). These authors examined several cell culture media and phosphate buffers and calculated a daily decay of 10% at 37°C, while at 4°C this would take 9 days. As shown in Fig. 2, the theoretical rate of glutamine degradation calculated by Tritsch and Moore exceeded the observed degradation in the media investigated. The recalculated k appears to approximate the spontaneous glutamine degradation in the investigated media more accurately. The consequence of this spontaneous glutamine decomposition is obvious; not only is less glutamine available for cells in culture, the resulting ammonia is toxic for those cells.

Culture media provide a mixture of nutrients. For cell culture, one can choose between different media, of similar composition. The measured concentrations in the commercially available media of several amino acids (e.g., lysine, tyrosine, tryptophan and methionine) were substantially lower than those given. As shown in Table I, 2F1 cultures rapidly consumed these amino acids during a culture period of only 48 h (during which the culture medium was not renewed). Insufficient availability of these essential amino acids leads to an inhibition of cell growth. Reduced concentrations of any essential amino acid, especially those which are consumed most rapidly, diminishes directly the usefulness of the medium. Furthermore, if the composition of the media were better adapted to the consumption pattern of the cells, a more physiological and economic use of the nutrients in the media could be achieved.

In conclusion, we recommend the use of fresh glutamine-deficient media, in which glutamine is added just before culture. An alternative is the use of media containing glutamine-dipeptides (e.g., glycyl-L-glutamine) which are stable in solution and are consumed by cells efficiently as glutamine (Brand et al., 1989).

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