THE POPULATION-DEPENDENT REQUIREMENT BY CULTURED MAMMALIAN CELLS FOR METABOLITES WHICH THEY CAN SYNTHESIZE*

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Although most strains of cultured mammalian cells can synthesize serine from glucose in amounts sufficient for survival and sustained growth, if the population density is reduced to less than 100 cells/ml most of the cells die unless exogenous serine is provided, while the survivors grow only slowly (1). Similarly, although cultured human cells can synthesize cystine from methionine and glucose by way of homocystine and serine (2), they nevertheless die in a cystine- and homocystine-free medium unless the population density is maintained in excess of 200,000 to 500,000 cells/ml. Even if the cells are provided with preformed homocystine, growth is observed only if the initial population density is in excess of 10,000 to 60,000 cells/ml.

Additional instances of a population-dependent requirement for metabolites which cells can in fact synthesize are described in the present paper, involving asparagine, glutamine, serine, inositol, and pyruvate. In at least some of these cases, and perhaps all, the critical factor is the loss of a newly synthesized metabolite to the medium in amounts which exceed the biosynthetic capacity of the cell.

Materials and Methods

Cell Culture Strains.—The human cell lines here used were HeLa, KB, liver, conjunctiva, and intestine (see reference 3). The mouse strain requiring serine or pyruvate was the P388 leukemia, cultured by Herzenberg and Roosa (4). The rabbit fibroblast culture requiring serine (5) was obtained through the courtesy of Dr. H. Earle Swim.

Culture Medium.—The basal medium used in these studies embodied only the 28 demonstrably essential growth factors (6) supplemented with either dialyzed or gel (sephadex)-filtered serum (7). In the experiments involving cystine biosynthesis, the serum used in the culture medium was previously treated with dithionite in order to remove bound half-cystine residues, as previously described (2). The cells were grown in either monolayer or suspension culture, the latter in an appropriately modified medium (6). In general, monolayer cultures

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were employed unless otherwise stated, with a minimum of three replicate flasks for each determination. Cellular growth was estimated by a modified Lowry method (8) in the case of monolayer cultures, or by direct enumeration in the suspension cultures.

Amino Acid Analysis.—Samples were analyzed for their amino acid content on an automatic amino acid analyzer (9, 10), using sufficient material to give 0.1 to 1 μmole of the amino acids of interest.

When radioactivity was to be measured, a manual ion exchange procedure based on the automatic method (10) was employed. The column was operated exactly as described except that the flow rate was approximately halved, to about 15 ml/hour. Buffer from the gradient device (varigrad) was pumped to the column by a finger type pump (Sigma motor model T8) through Tygon tubing R-3603, $\frac{1}{32}$ inch inside diameter and $\frac{3}{16}$ inch outside. To prevent the formation of air bubbles in the column, the effluent was led to the collector through Tygon tubing R-3603, $\frac{1}{16}$ inch inside diameter and $\frac{5}{16}$ inch outside, partially closed with a screw clamp. With the column in operation, the clamp was gradually tightened until a gauge on the influent line to the column registered 30 to 35 lb/in^2, about 10 lb greater than that developed in the column. Sample sizes were chosen to give 1 to 5 μmoles of the amino acids of interest. 2 ml fractions were collected, diluted with 2 ml of water, and 1 ml portions were taken for ninhydrin analysis and for radioactivity measurement (11).

In these ion exchange chromatograms glutamine overlaps threonine and serine. When these amino acids were to be determined, the samples were treated with glutaminase as previously described (25), prior to chromatographic analysis.

RESULTS

Population-dependent Requirement for Serine.—Three distinct situations were encountered in which cells capable of synthesizing serine nevertheless required the exogenous amino acid for survival and growth.

1. At low population levels, less than 100 cells/ml, at which serine becomes essential for the survival of most animal cells (1), the concentration which permitted half the cells to survive was approximately 0.01 mM, and the concentration which permitted those surviving cells to grow at about half the maximal rate was 0.005 mM (Table I). These external levels were in equilibrium with the metabolically effective intracellular concentration of approximately 0.1 mM.[1] At a population density of 100 cells/ml, the volume ratio of cells:medium was approximately 1:2,500,000. It follows that in a serine- and glycine-free medium, in which these amino acids[2] must be synthesized from glucose and glutamine, each cell, in order to retain an effective intracellular level, had to secrete into the medium approximately 5000 to 25,000 times as much serine and glycine as was retained in the intracellular "pool."[1] Extremely low cell populations could not synthesize these amounts before dying of what was in effect a serine and glycine deficiency. When the initial population density in

[1] The intracellular concentration of serine was 6 to 60 times that in the medium, varying with the absolute concentration in the medium and the specific cell line (H. Eagle and K. Piez, unpublished observations; see also references 12, 13).

[2] Since all the cell glycine derives directly from serine (6), the total amount of serine synthesized by the cell is the sum of (serine + glycine + the cellular components and metabolic intermediates deriving from these two amino acids).

TABLE I

The Concentrations of Serine Permitting Cellular Growth at Low Cell Population Densities

60 mm Petri dishes were inoculated with 8 ml of a "liver" cell suspension at 12.5 cells/ml. The number of clones, and the average number of cells per clone, were determined by staining 3 replicate dishes on days 4, 6, and 8. The numbers in the body of the Table are the total number of cells. Gel-filtered serum was used to minimize contamination with protein-derived serine (7).

Serine concentration, *mM*	0	0.002	0.005	0.01	0.02	0.05	0.2
No. of clones after 8 days	8	15	15	36	64	68	74
Average No. cells/clone	2.4	2.4	6.4	9.4	21.2	34.6	51.8
Generation time in clones of surviving cells, *days*	6.3	6.3	2.97	2.47	1.81	1.56	1.40
Total No. cells	19	36	98	239	1358	2354	3833
Average generation time, based on total cell count, *days*	—	—	—	6.90	2.12	1.75	1.53

TABLE II

The Rate of Cellular Growth in a Serine-Free Medium as a Function of the Population Density, and of the Concentrations of Serine and Glycine Attained in the Medium

(Experiment with KB cells; see legend to Table I for experimental details).

Initial population density	Amino acid concentrations in medium ($mM \times 10^{-3}$) after									Degree of cellular multiplication*		"Average generation time"‡	
	4 days			8 days			12 days						
	Serine	Glycine	Total	Serine	Glycine	Total	Serine	Glycine	Total	8 days	12 days	8 days	12 days
number cells/ml													
0§	2.5	1.5	4.0	2.0	2.3	4.3	3.3	2.9	6.2	—	—	—	—
10				2.3	2.2	4.5	3.3	2.5	5.8	—	4.6	—	8.7
20				3.0	2.5	5.5	7.0	2.7	9.7	2.8	6.9	5.4	4.3
50				3.3	1.3	4.6	6.4	2.2	8.6	11.0	212	2.3	1.55
100				5.5	2.0	7.5	6.0	1.0	7.0	21.6	378	1.8	1.4
200				7.5	1.7	9.2				45	433	1.45	1.39
500	4.9	1.5	6.4	10.5	0.6	11.1				136	503	1.13	1.33
1000	7.3	1.7	9.0							217	—	1.03	—
2000	11.0	1.0	12.0							—	—		
5000	10.0	1.4	11.4							—	—		

* Referred to inoculum as 1, and based on either cell counts (at low inocula) or total cell protein (at high inocula).

‡ Assuming all of the inoculum to have survived, and assuming a uniform growth rate over the entire period of the experiment. In actual fact, with small inocula only a portion of the cells survived; further, the growth rate usually increased as serine and glycine accumulated in the medium (see 8 and 12 day results).

§ The slow accumulation of serine (and glycine) in these cell-free media reflects the breakdown of serum protein by serum proteases (14).

the cell culture was progressively increased, the levels of serine and glycine attained in the medium (and thus, within the cell) increased progressively, as did the proportion of survivors and their average rate of proliferation. In the experiment of Table II, for example, the population density which permitted the cells to grow in a serine-free medium at half the optimum rate was approxi-

TABLE III

The Growth of KB Cells in a Serine-Free Medium Containing Homocystine in Place of Cystine: The Role of Population Density and of Serine-Glycine Accumulation in the Medium

In the cultures with exogenous serine (lower portion of Table) effective concentrations were obviously present from the very beginning of experiment. In the cultures with no added serine, the extracellular concentrations increased only slowly to reach the concentrations indicated; this is reflected in the higher "average generation times."

Cellular inoculum per ml	Serine added to medium	Time	Final medium concentration ($m\mu \times 10^{-3}$)			Degree of cellular growth*	Average generation time‡
			Serine	Glycine	Total		
	$m\mu = \times 10^{-3}$	*days*					*days*
0§	0	4	3	2	5§	—	—
2,000	0	4	3.9	3.1	7	0	∞
5,000	0	4	4.7	4.5	9	0	∞
10,000	0	4	17	16	33	2.4	3.15
20,000	0	4	30.5	32	62	3.1	2.44
30,000	0	4	30	59	89	4.1	1.96
60,000	0	3	29	71	100	4.6	1.81
5,000	0	4	4.7	4.5	9	0	∞
5,000	20	4	22.6	12.9	35.5	2.3	3.32
5,000	50	4	42.5	19.5	62	10.3	1.19
5,000	200	4	114	34	148	22.3	0.89

* Referred to inoculum as 1, and based on protein analysis.

‡ Assuming the survival of all cells in inoculum, and a uniform rate of growth during the 4 days of incubation.

§ The accumulation of serine and glycine in the medium in the absence of added cells reflects the breakdown of the serum protein by serum proteases (14).

mately 50 cells/ml. At this population density, the extracellular concentrations of serine attained 0.0033 mM after 8 days, approximately the same as the concentrations which had to be added to lower cell populations in order to permit growth at a comparable rate.

The serine requirement at low cell populations has recently been discussed along similar lines by Moser (15), who has further found one cell line to develop a requirement for pyruvate under cloning conditions. The qualitative similarity to the serine or pyruvate requirement of the P388 mouse leukemia (see page 34) is of particular interest.

2. In a medium containing homocystine instead of cystine, and in which serine must be used for cystine synthesis, populations of less than approximately 10,000 cells/ml did not survive unless exogenous serine was provided (2). The concentration which permitted growth at approximately half the maximum rate was then approximately 0.04 mM (see bottom section of Table III). In that experiment, the exogenous serine became unnecessary with inocula of 10,000 cells/ml or more. At this initial population density, the extracellular levels of serine and glycine attained a total of 0.033 mM within 4 days, the same as the concentration of serine which had to be added to lower population densities in order to achieve comparable growth.

TABLE IV

The Concentrations of Serine Necessary for the Growth of Presumably Serine-Requiring Strains

Cell strain	Inoculum	Time		Concentrations of serine added to medium, mM						
				0	0.002	0.005	0.01	0.02	0.05	0.2
	No. cells/ml	*days*								
RT 6 (Rabbit fibroblast)	10,000*	14	Degree of cellular multiplication...	0	3 ×	7 ×	22 ×	29 ×	46 ×	27 ×
			Generation time, days.....	∞	8.8	5.0	3.1	2.9	2.6	2.9
P-388 (Mouse leukemia)	50,000‡	11	Degree of cellular multiplication...	0	10.7 ×		139 ×		1020 ×	3160 ×
			Generation time, days.....	∞	3.1		3.1		1.1	0.95

* Monolayer culture, totally re-fed with fresh medium every 2 days.

‡ Suspension culture, diluted daily with fresh medium to bring cell count back to 50,000 cells/ml.

When cells were grown in a medium containing effective concentrations of homocystine and serine, as much as 80 per cent of the glycine, serine, and cystine residues of the cell protein then derived from the glucose of the medium, rather than from the preformed serine (2). It therefore seems probable that the primary function of the exogenous serine at low population densities was to condition the medium, *i.e.*, to limit the continuing elution of the newly synthesized amino acid from the cell.

3. Several serially propagated cell lines have been isolated which require exogenous serine for growth under normal culture conditions, including strains deriving from rabbit testicle (5) and the P388 mouse leukemia (4). In both cases, the serine concentration permitting growth at half the maximum rate was approximately 0.005 mM (see Table IV). With both strains also, the requirement proved to be population-dependent, disappearing at initial cell counts in excess of 50,000 and 150,000 cells/ml, respectively, the precise levels varying with the conditions of the experiment (Table V). In both instances

the critical population densities permitted the accumulation of serine and glycine to approximately the same level as that which had to be added to smaller populations to ensure survival and growth. These two cell lines therefore differed from other serially propagated strains only in a quantitative sense, in that higher cell population densities were necessary to build up external levels of serine (and glycine) in equilibrium with a metabolically effective intracellular pool.

TABLE V

The Growth of "Serine-Requiring" Strains in a Serine-Free Medium at Sufficiently High Population Densities

Inoculum	Rabbit fibroblast strain RT6			Mouse leukemia strain P388*		
	Time	Cellular multiplication‡, given supplement of		Time	Cellular multiplications,‡ given supplement of	
		0	0.2 mM serine		0	0.2 mM serine
No. cells/ml	*days*			*days*		
1,000	4§	1.0	11.3	10	0	48
2,000	4	10.0	16.3			
5,000	4	21.0	24			
50,000	6‖	2.1	36	9	0	1133
100,000	6	3.2	62	9	0	
150,000				9	453	
200,000	6	8.2	27	9	214	476
250,000				9	308	
400,000	6	12.6	17			

* Suspension culture, diluted daily with fresh medium to bring cell count back to indicated initial level.

‡ Referred to inoculum as 1.

§ Experiment in which cultures were not re-fed during 4 days of incubation.

‖ Experiment in which cultures were re-fed on days 2, 4, and 5.

As in the cases previously discussed, the function of the exogenous serine at low populations appeared to be primarily to limit the loss of the amino acid from the cell to the medium. When P388 cells were grown in a medium containing uniformly C^{14}-labeled glucose, and serine at 0.02 mM, the specific activity of serine and glycine residues in the cell protein were, respectively, 86 and 89 per cent of the precursor glucose. Clearly, the cells continued to synthesize serine from glucose, and to use that newly synthesized amino acid for protein synthesis.

Inositol Requirement.—Meso-inositol has been shown to be required for the survival and growth of all but one of 22 cell lines tested (3). The one inositol-

independent strain synthesized its inositol from glucose, and actually released enough into the medium to permit the parabiotic growth of inositol-dependent cells (16). Even inositol-dependent strains, however, growing in the necessary presence of preformed inositol, were found to be synthesizing 25 per cent of their inositol residues *de novo*, from glucose. These observations suggested that the difference between the inositol-dependent and -independent strains might

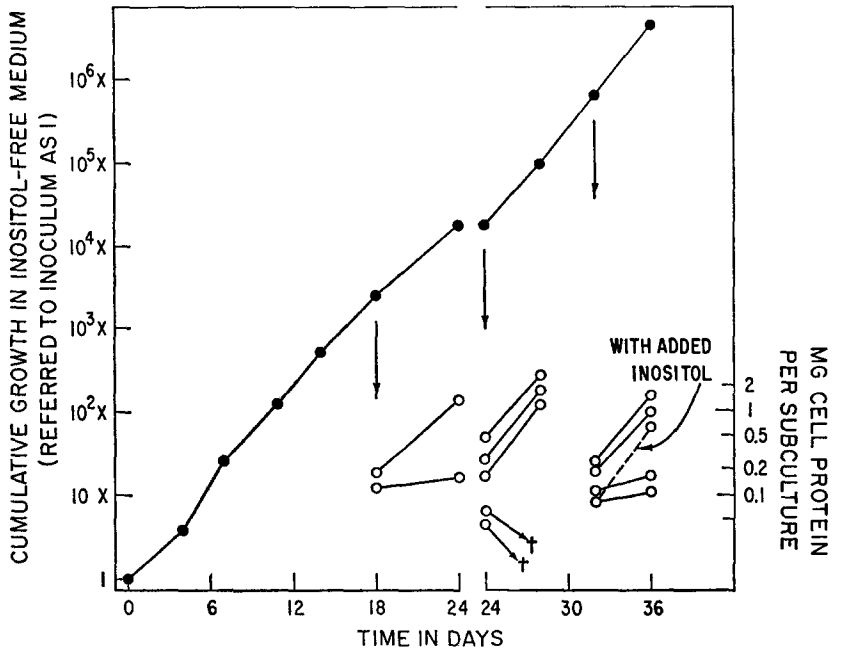

FIG. 1. The sustained growth of HeLa cells in an inositol-free medium, if the cell population density was maintained in excess of 240,000 cells/ml (●—●). When the culture was subdivided to less than 50,000 cells/ml (approximately 0.1 mg cell protein in 3 ml of culture medium) the cells died (○—○).

again be merely one of degree, and that the dependent cells failed to grow in an inositol-free medium only because prohibitive amounts of the newly synthesized inositol were lost from the cellular pool in the course of its equilibration with the medium. In at least one instance this proved to be the case. When HeLa cells were grown with care to keep the population density in excess of 240,000 cells/ml, there was in some experiments sustained growth in an inositol-free medium (see Fig. 1). (For reasons which are not yet clear, this experiment could not be duplicated with other cell lines, and was not regularly successful even with the HeLa strain.) Even after prolonged growth under these condi-

tions, however, when the population density was reduced to less than approximately 50,000 cells/ml, the cells died unless exogenous inositol was added.

The Population-Dependent Requirement for Pyruvate.—The P388 leukemia cell had been shown by Herzenberg and Roosa (4) to require either serine or

TABLE VI

The Ability of Cultured Human Cells to Grow in a Cystine-Free Medium as a Function of the Initial Population Density, and of the Specific Precursors Provided

Illustrative experiments with (*a*) HeLa, (*b*) KB, (*c*) Conjunctiva, and (*d*) KB cells in monolayer culture.

Cystine precursors in medium	(a) Methionine + glucose		(b) Homocystine + glucose		(c) Homocystine + serine		(d) Cystine + serine	
Cystine intermediates which must be synthesized and retained	Homocystine Serine Cystathionine Cystine		Serine Cystathionine Cystine		Cystathionine Cystine			
Inoculum	Incubation time	Degree of cellular growth	Incubation time	Degree of cellular growth	Incubation time	Degree of cellular growth	Incubation time	Degree of cellular growth
cell no./ml.	*days*		*days*		*days*		*days*	
500,000	1	1.5 ×						
250,000	3	1.88 ×	7	7.8				
100,000	4	0.4 ×	7	16.6				
50,000	11	<0.2 ×	9	45.6				
20,000			9	<0.5				
250					8	10.7*		
125					8	3.5*		
60					8	1.8*		
25					8	0.53*	8	76*
Minimum cell population required for survival and growth‡	200,000 to 500,000		10,000 to 60,000		50 to 500		1	

* Based on cell counts in stained clones; all other values for cellular growth are based on determination of cell protein.

‡ In a number of similar experiments with HeLa, KB, conjunctiva, and liver cells.

pyruvate for growth in serial culture. As discussed in a previous section, these requirements disappeared at population levels in excess of approximately 50,000 cells/ml. No explanation can yet be offered for the mutual replaceability of serine and pyruvate at lower population levels. In the case of the Detroit 98C6b strain also, Moser (15) has shown a need for pyruvate under cloning conditions, with inocula of less than 100 cells/ml, a requirement which was not evident under ordinary culture conditions.

The Population-Dependent Repuirement for Cystine and Homocystine.—In a cystine-free medium, when cells were given only methionine and glucose as cystine precursors, they had to make, and retain, metabolically effective levels of homocystine, serine, cystathionine, and cystine. Under these circumstances, the cells died unless provided with traces of exogenous cystine or homocystine, or unless the population density was maintained in excess of 200,000 to 500,000

TABLE VII

Illustrating the Population-Dependent Requirement for Glutamine and Asparagine by Glutamic Acid—"Adapted" HeLa Cells

Monolayer cultures were "adapted" for several weeks to growth in 20 mM glutamic acid in lieu of glutamine, and then maintained in suspension culture in that glutamic acid medium. The dispersed cells were planted at the indicated population densities in replicate monolayer flasks, and re-fed with fresh medium on days 3, 6, 9, and 12.

Cellular inoculum	Glutamic acid concn. in medium	Degree of cellular multiplication* in 13 days, given					
		0	Glutamine at			Asparagine at	
			0.01 mM	0.1 mM	1 mM	0.05 mM	0.1 mM
no./ml.	*mM*						
5,000	2	0	0	0			
10,000		0		2.0			
20,000		0		11.0			
50,000		0		9.7			
100,000		0					
1,000	20	0		3.1	85		
2,000		0		63	111	0	
5,000		0	0	72		0	0
10,000		0	5.0			9.9	2.4
20,000		0					10.7
50,000		15					
100,000							

* Referred to inoculum as 1, and based on protein determinations in the washed flasks.

cells/ml (2). If preformed homocystine was provided, the critical population density, permitting growth in a cystine-free medium was reduced to 10,000 to 60,000 cells/ml (Table VI). The metabolite then limiting growth was serine; and, as in the experiment of Table II, the critical population density was that at which metabolically effective levels of serine (and glycine) were attained in the medium, and thus, in the intracellular pool. Given both homocystine and serine as cystine precursors, the critical population density was reduced to 50 to 500 cells/ml. Finally, given preformed cystine and serine, a single cell would grow even in 10 ml of culture medium, a volume ratio of medium:cells of 2 billion:1 (Table VI).

The Population-Dependent Requirement for Glutamine or Asparagine by Cells Adapted to Growth in Glutamic Acid.—Although most serially cultured mammalian cells require glutamine for survival and growth, extremely high concentrations of glutamic acid (20 mM) usually substitute (17). At these high levels, the cells develop increased glutamine synthetase activity (18), and such glutamic acid–adapted cells can then grow at relatively low concentrations of glutamic acid. However, the ability of these cells to dispense with preformed glutamine proved to be population-dependent. At low population densities, even glutamic acid–adapted HeLa, conjunctiva, and KB cells regularly failed to survive without exogenous glutamine. The critical population density varied widely between individual cell strains, and for any one strain varied inversely with the glutamic acid concentration in the medium (Table VII). On the basis of the foregoing discussion, it is a reasonable presumption that the exogenous glutamine was supplementing that produced by the cells, and that the critical population density was that at which an intially glutamine-free medium could be conditioned with respect to its glutamine content before the cells died.

As was to have been anticipated from the fact that asparagine derives from glutamine in these cell cultures (19), the addition of asparagine occasionally exercised a glutamine-sparing effect, and permitted the cells adapted to glutamic acid to grow at population densities at which they would otherwise not have survived (Table VII). In this range, there was thus a population-dependent requirement for asparagine, similar to that shown for glutamine.

DISCUSSION

A number of examples have been here described of nutritional requirements (serine, cystine, homocystine, glutamine, asparagine, pyruvate, inositol) which disappeared at sufficiently high population densities (Table VIII). In all the situations so far examined, these were metabolites which the cells could synthesize in amounts sufficient for sustained growth, but which at low population densities were lost from the cell to the environment in amounts which exceeded the biosynthetic capacity of the cell. The critical population density was that which was able to "condition" the medium, *i.e.*, to build up a concentration in equilibrium with the minimum effective intracellular level, before the cells died of the specific deficiency.

In an analogous situation, it had been shown that in the synthesis of poliovirus by HeLa cells, exogenous amino acids became necessary for viral synthesis only when the population density was reduced to approximately 50,000 cells/ml (20). In that case also, the amino acid requirement reflected the depletion of the cellular pool. Similarly, it is a reasonable surmise that the previously described HeLa variant (21) which was capable of synthesizing tyrosine from phenylalanine, but which nevertheless died in the absence of exogenous tyrosine, would have been tyrosine-independent at a sufficiently high population density. The possibility also may be considered that the requirement for exogenous

TABLE VIII

Summary of Population-Dependent Requirements by Cultured Mammalian Cells for Metabolites Which They Can in Fact Synthesize

Metabolite required		Cell line	Concn. permitting growth at low population densities	Population density in excess of which exogenous metabolite becomes unnecessary*
			mM	
Serine	In medium containing cystine	Human‡	0.005 to 0.02	50 to 200
		Mouse (P388)	0.005	150,000
		Rabbit (RT6)	0.005	50,000
	In medium containing homocystine as a cystine precursor	Human‡	0.04	50 to 500
Cystine	In medium with no homocystine	Human‡	0.002 to 0.005	200,000 to 500,000
	In medium containing homocystine	Human‡	0.002 to 0.005	10,000 to 60,000
Homocystine	In medium lacking cystine	Human‡	0.01	200,000 to 500,000
Glutamine		Human (HeLa and conjunctiva cells adapted to growth in glutamic acid)	0.01 to 0.05 (varies with cell population density and glutamic acid concentration)	>50,000 (varies with glutamic acid concentration)
Pyruvate		Mouse (P388)	0.1 to 1	50,000
		Detroit 98 C6B (15)	0.1 to 1	100‡
Inositol		Human (HeLa)	0.001	200,000§

* Permitting growth at half of maximal rate, based on average generation time over period of 8 days.

‡ HeLa, Conjunctiva, KB, "Liver."

§ See text, page 35.

CO_2 by certain cell strains (22) reflects its loss to the environment from cells which produce it at a relatively slow rate. Under these circumstances, the CO_2 requirement might also be population-dependent.

The requirement for metabolites which can in fact be synthesized is not

limited to mammalian cells, but was observed by Bonner, Yanofsky, and Partridge (23) in the case of "leaky" tryptophan and niacin-less mutants of *Neurospora*. These were anomalous in two respects. They synthesized significant amounts of niacin and tryptophan despite the fact that they were genetically homozygous for the recessive character, and required the metabolite despite their demonstrated capacity to produce it. In the light of the data here reported it is possible that in those situations also, the limited amounts of niacin and tryptophan synthesized were being lost to the medium, and that if the loss per cell were limited by an appropriate increase in the population density, the nutritional requirement might then disappear. The term "leaky" mutants applied to these organisms by Bonner *et al.* may therefore apply not only to their anomalous genetic character, but also to the physiological process responsible for the unexpected nutritional requirement.

The present observations may have a bearing also on the problem as to why serially cultured cells usually fail to carry out the metabolic activities characteristic of the organ from which they had been derived. A number of explanations may be suggested for this phenomenon (24). The interaction between different cell types in the same organ, or between different organs, has been regularly stressed in discussions of differentiation and function. The data here reported suggest that, over and above such heterotypic interactions, the metabolic activity of a given cell type may be greatly influenced by its own population density; and in this respect, tissues and cell cultures differ profoundly. In animal tissues, the cell number is on the order of 10^8/ml, while cultures are usually inoculated at 10^4 to 10^5 cells/ml, and even a heavy culture usually contains no more than 5×10^5 cells/ml. In tissue, the equilibration of the cellular pool with the immediate environment obviously leads to only a minor loss of metabolites, and a significant loss would result only from the following, and probably much slower, equilibration of the extracellular fluids with the circulating lymph and plasma. In contrast, the equilibration of dispersed cultured cells with the surrounding medium causes an extremely rapid and major depletion, since the cells then constitute only 0.1 to 1 per cent of the culture volume. This equilibration depletion may obviously apply to any product of cellular metabolism, and is a pertinent consideration no matter what the absolute equilibrium ratio between the interior of the cell and the environment. To the degree that the cellular metabolites so lost are essential for specialized functions, such depletion could contribute to the usual failure of dispersed cell cultures to carry out the functions of the organ from which they were derived.

In embryonic differentiation also, a number of experimental situations have been described in which the mass of differentiating tissue is a critical factor (26). The present experiments suggest that in those cases also, one may be dealing with a prohibitive loss to the environment of metabolites essential to the process of differentiation. The important consideration could then be the

ratio of cell mass to the immediate environment with which it equilibrates, rather than the absolute amount of differentiating tissue.

SUMMARY

At least seven compounds synthesized by cultured cells in amounts which should suffice for sustained growth have nevertheless proved under certain conditions necessary for survival (asparagine, cystine, glutamine, homocystine inositol, pyruvate, serine). In every instance so far examined, that requirement has been population-dependent, disappearing at cell densities sufficiently large to bring the concentration in the medium and in the cellular pool to metabolically effective levels before the cells died of the specific deficiency.

At population densities of less than 100 cells per ml, serine was required by all cultured cells so far studied. With more exigent strains, such as the RT6 strain of rabbit fibroblast and the P388 mouse leukemia, the serine requirement disappeared only at populations in excess of 50,000 and 150,000 cells per ml, respectively. The requirement for pyruvate by the latter cell as an alternative to serine also disappeared at that population density.

In a cystine-free medium there were population-dependent requirements for cystine, homocystine, or serine, depending on the experimental conditions. With methionine and glucose as cystine precursors, the critical population density permitting cellular survival and growth was in excess of 200,000 cells/ml. The provision of homocystine as an intermediate reduced the critical population density to 10,000 to 60,000 cells/ml; with the further provision of serine, the critical cell concentration permitting growth was reduced to 50 to 500 cells/ml.

Cells adapted to glutamic acid, and capable of utilizing it as a substitute for glutamine, nevertheless required exogeneous glutamine at cellular densities of less than 50,000 cells per ml. In some experiments, the provision of asparagine reduced the critical population density to 10,000 cells/ml, presumably because of its glutamine-sparing action.

Inositol is required by most cell lines, despite their demonstrated capacity to synthesize it from glucose. With at least one cell line (HeLa), sustained growth was occasionally achieved in an inositol-free medium if the population density was maintained in excess of 240,000 cells/ml.

The possible implications of these findings with respect to the loss of specific organ functions in dispersed cell culture are discussed.

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