*THE SYNTHESIS OF HOMOCYSTINE, CYSTATHIONINE, AND CYSTINE BY CULTURED DIPLOID AND HETEROPLOID HUMAN CELLS**

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In a "minimal" medium, containing only those factors demonstrably essential for survival and growth, and under the usual conditions of cell culture, cyst(e)ine was required for survival and growth by a variety of cultured human cells (HeLa, KB, conjunctiva, "liver").[1] At sufficiently high population densities, however, some of these cell lines were able to survive and grow in a cystine-free medium, presumably because the concentration of newly synthesized cystine and its precursors in the medium and in the cellular pool could then be brought up to metabolically effective levels before the cells died of the cystine deficiency.[2-4] It seemed clear that the capacity to synthesize cystine *in vivo* was not limited to the liver, but was a general biosynthetic capacity of human cells.

A case of cystathioninuria reported by Frimpter *et al.*,[5] with the strong suggestion that the metabolic error was familial,[5, 6] appeared to offer an opportunity for the isolation and cultivation of a cell line with a genetically determined biochemical defect. Cultures initiated from finely minced skin explants did in fact die in a cystine-free medium, regardless of the precursors provided, down to and including cystathionine, and even at the highest population densities used (400,000 cells/ml). However, since these were diploid cells, while the strains previously studied were heteroploid, a number of normal human diploid cell lines were studied as controls. Unexpectedly, these behaved like the skin cells from the cystathioninuric patient. Regardless of the population density, they did not survive in a medium containing methionine or homocystine as the cystine precursor, and most were similarly unable to synthesize cyst(e)ine from cystathionine in amounts sufficient for survival or growth. The possible relationship of the chromosomal aberrations in the heteroploid cells to their cystine-synthesizing activity will be discussed.

Methods.—The cell lines used are listed in Table 1. All the heteroploid[6a] strains were epitheloid, three deriving from cancers and the rest from normal tissues. Of the ten diploid cultures, the primary amnion cultures were epithelial and the rest fibroblastic. Four were from fetal tissues, three from postnatal skin, two from amnion, and one from a human cancer.[9] The latter line (RPMI 2650) has recently been reported to be quasi-diploid, rather than diploid;[10] but as will appear, it behaved like the other diploid lines with respect to cystine synthesis. There was some apprehension as to whether the differences here reported between diploid and heteroploid cells might be referable to the presence in the latter of pleuropneumonia-like organisms (PPLO), and particularly since none of the eight diploid strains tested in this respect were demonstrably infected. However, three heteroploid cell cultures containing no demonstrable PPLO behaved like strains of the same cell known to be contaminated (cf. last paragraph of *Discussion*).

Cells were grown in a minimal medium containing only those growth factors essential for survival and growth,[14] and with all the amino acids in L-configuration.

L-Cystathionine was obtained from Cyclo Chemical Co. Calf serum was freed of protein-bound half-cystine residues by treatment with dithionite as previously described,[2] followed by dialysis, and was used at the 10 per cent level. Monolayer cultures were used throughout: the heteroploid cell cultures were refed every 1–2 days, and divided 20–80-fold at each weekly culture passage; the more slowly growing diploid cells were divided 2–8-fold.

The growth response to various cystine precursors was measured by protein determination on washed cultures in triplicate, using a modified Lowry procedure.[16] In experiments with S^{35}-labeled methionine or homocystine as cystine precursors, the cold 8 per cent TCA precipitate of the washed cells was hydrolyzed for 18 hr in 6 *N* HCl, and in some cases for 6 hr in 2 *N* HCl. Amino acids were determined in ultrafiltrates of the growth medium and in the cell hydrolysate by Moore-Stein ion-exchange chromatography, and their radioactivity was measured by flow scintillation counting of the column effluent prior to reaction with ninhydrin.[17]

TABLE 1

HUMAN CELL CULTURES USED IN PRESENT STUDY

Euploid	Minor chromosomal aberrations	Grossly heteroploid
Amnion[a]	RPMI 2650[f]	Amnion-FL[i] (ref. 11)
Fetal adrenal[b]	H-46[g]	Amnion-WISH[12]
Fetal conjunctiva[b]	H-53[g]	Conjunctiva[h]
Fetal bone[b]	C4[j]	HeLa[h]
Fetal lung[7]	C33[j]	HEp2[13]
Fetal thymus[b]		Intestine[h]
BAL[c]		KB[h, i]
Detroit 510[d]		Liver[h, i]
F-1[e]		

[a] Drs. H. P. Klinger and Jørgen Føgh generously supplied both freshly isolated amnion cells, and primary cultures of those cells. In early passage these cells were epithelioid; in serially propagated cultures, they were gradually replaced by diploid fibroblastic cells. It is not yet clear whether this gradual replacement was a process of selection or transformation.

[b] Cultured from human embryonic material by Dr. T. Ward.

[c] Cultured in this laboratory from skin of case of hyperglycinemia.[8] The biopsy material was generously provided by Drs. W. L. Nyhan and B. Childs.

[d] Cultured from skin of 5-month-old galactosemic female by Dr. C. S. Stulberg.

[e] Cultured in this laboratory from skin of normal 6-year-old male.

[f] Epithelioid quasi-diploid culture of human cancer,[9] with at least one abnormal chromosome.[10]

[g] Fibroblastic skin cultures karyotyped by Dr. H. P. Klinger: H-47 was trisomic in the E group and H-53 was from a case of Turner's syndrome, with a 45/×0 chromosome complement. The biopsy of H46 was provided by Dr. J. Linsten.

[h] Source of cell line cited in ref. 13.

[i] PPLO-free lines through courtesy of Dr. G. B. Gori. The diploid cultures here used were also PPLO-free. The courtesy of Drs. G. B. Gori, C. S. Stulberg, and T. Carski in performing these PPLO-examinations is gratefully acknowledged.

[j] Hypodiploid cancer cells isolated by Auersperg.[25]

Experimental.—(1) *Growth experiments:* Some of the heteroploid[6a] human cell cultures had previously been found to survive and grow in a cystine-free medium;[2, 3] and this was true of the additional lines here studied. The critical population density necessary for survival or growth varied with the specific precursors provided, ranging from 200,000 cells/ml and more when the cells were given only methionine and glucose (and were therefore under the necessity of synthesizing and retaining metabolically effective levels of homocyst(e)ine, serine, cystathionine, and cystine), to as few as 1–10 cells/ml when they were provided the immediate precursor of cysteine, cystathionine[3, 4] (cf. Table 2).

In contrast, the diploid, hypodiploid, and trisomic fibroblastic cells here studied were unable to utilize any cystine precursor effectively. Even with cystathionine, there was either no growth, or a trivial increase in cell protein occasionally observed at extremely high inocula, several hundred thousandfold greater than in the case of the heteroploid cells (cf. Table 3 and Fig. 1). It was therefore not surprising that these diploid cells regularly died in media containing methionine or homocystine as cystine precursors, even at the highest population densities feasible to use as inoculum.

Epithelial amnion cells in early passage, and an embryonic kidney cell culture

TABLE 2

GROWTH OF HETEROPLOID AND DIPLOID HUMAN CELLS IN A CYSTINE-FREE MEDIUM

Cystine precursor in medium[a]	Compounds which cell must synthesize and retain	Heteroploid Cells[c] Growth	Heteroploid Cells[c] Minimum inoculum[e] per cm²	Euploid Cells[c] Growth	Euploid Cells[c] Minimum inoculum[e] per cm²
Methionine glucose	Homocysteine Serine Cystathionine Cystine	0–+[d]	≷40,000	0	∞
Homocystine serine	Cystathionine Cystine	+	1–10,000	0	∞
Cystathionine	Cystine (serine)[b]	+	0.2–2	0[f]	—
Cystine	(Serine)	+	0.2	+	200±

[a] Methionine → homocysteine (+ serine) → cystathionine → cysteine + homoserine.
[b] Necessary for growth, but not in relation to cystine synthesis.[18]
[c] Cf. Table 1. Of the five cell strains with minor chromosomal aberrations, four behaved like euploid cells in their inability to grow in a cystine-free medium. The hypodiploid culture C33, however, like the heteroploid lines, could utilize cystathionine for growth.
[d] Growth or survival with KB and conjunctiva (Chang); other heteroploid cells gave inconsistent results.
[e] In culture flask with 0.2 ml medium per cm² surface.
[f] Trivial increase in cell protein with a few lines at extremely high population densities (cf. Table 3).

in second passage containing both epithelial and fibroblastic elements, behaved like the fibroblastic cells. Two of three epithelial cancer cells with minor chromosomal aberrations [C4 (hypodiploid) and RPMI 2650 (quasidiploid)] similarly could not utilize cystathionine for growth (cf. Table 3). However, a third hypodiploid cancer line (C33)[25] did grow in the cystathionine medium. Unlike the four other culture strains with minor chromosomal aberrations, and like the six grossly heteroploid lines, this hypodiploid cancer cell presumably had sufficiently enhanced cystathionase activity to permit the effective utilization of cystathionine as a cystine precursor.

(2) *The synthesis of homocystine and cystathionine by cultured human cells:* Most of the cell lines here tested, diploid and heteroploid alike, formed S^{35}-homocystine from S^{35}-methionine (Table 4). The diploid cells were therefore not blocked in the synthesis of homocyst(e)ine from methionine, although their average activity was somewhat less than that of the heteroploid cells. Similarly, although two diploid lines did not demonstrably synthesize cystathionine from preformed S^{35}-homocystine and serine, most were as active as the heteroploid strains in this respect. (There is no present explanation for the wide variations sometimes found in repeat experiments with the same strain.)

Discussion.—In confirmation of previous findings,[2–4] a number of grossly heteroploid human cell lines were found to carry out all three of the sequential reactions involved in the synthesis of cystine from methionine:[21] all could synthesize homocystine from methionine, and cystathionine from homocystine, and all could utilize cystathionine effectively for growth in a cystine-free medium. The ten diploid cultures here studied could similarly form homocysteine from methionine, and eight of the ten could condense homocysteine and serine to form cystathionine (cf. Table 4). All the diploid strains, fibroblastic and epithelial alike, were blocked, however, in the conversion of cystathionine to cystine, as evidenced by their inability to grow in a cystine-free medium containing L-cystathionine. At extremely high population densities (200,000 cells/ml and 40,000/cm²), exceeding the maximum levels spontaneously achieved in a complete growth medium, a few diploid lines may have effected a minor utilization of cystathionine, evidenced by an initial slight in-

TABLE 3

GROWTH RESPONSE OF DIPLOID AND HETEROPLOID HUMAN CELLS TO L-CYSTATHIONINE IN A CYSTINE-FREE MEDIUM

Karyotype	Cell Strain		Growth Response[a] after 5–8 Days with Inocula (per ml)[b] of: 400,000	200,000	100,000	50,000	20,000	Plating Efficiency (%)[c] with Inocula (per ml) of: 20	5	2
Euploid	Fetal	Adrenal	1.3	2.4	0.54	0.3		0	0	
			1.2	0.1	0.11	0.13				
		Bone	1.1	1.1	1.6	0.96		0	0	
			1.1	1.5	1.71	0.96				
		Conj.	0.93	1.2	0.4	<0.1		0	6	
				1.2	1.2	0.31				
		Lung	0.8	0.6	0.8	0.9		0	0	
		Thymus	1.1	1.7	2.0	1.4	<	0	0	
		Thymus		1.3	0.3	<0.1				
	Skin	BAL	0.9	1.1	0.6	<0.1		0	0	
				1.3	0.3	<0.1				
		F1	0.8	0.6	0.5	0.2		0	0	
			1.0	0.5	0.5	0.3				
		Det. 510	0.7	0.4	0.2			0	0	
	Amnion (epithelial)	168-2	1.6	2.0	0.9	0.2		0	0	
			1.0	0.8	0.02					
	Amnion (fibroblastic)	168-12	1.0	0.8	0.1			0	0	
	Cancer	RPMI 2650[d]	1.6	1.0	0.7	0.8		0	0	
			0.5	0.7	0.7	—				
Minor chromosomal aberrations	H-46	(Trisomic in E group)		0.84	1.17	0.11				
	H-53	(45 Chromosomes)	0.85	0.6	0.3	0.2				
	C4	Hypodiploid cancer								
	C33	Hypodiploid cancer	2.7	4.6	4.7	3.0				
				10.8	5.3	1.9	0			
Heteroploid	"Transformed" amnion	FL			1.5	3.0	5.8		80	
					1.8	3.8	13.3			
		WISH			2.9	3.2				
					1.9	2.1	3.2		40	
	HEp2				6.1	15.3				
	Conjunctiva (Chang)				3.9	5.3	−9.1			
	KB				4.3	6.6	8.1	46	57	60
	HeLa					16.4	28.1	63	85	102
					6.0	7.3	5.3			

[a] Referred to inoculum as 1. [b] Three ml planted in 15-cm² flask. [c] Percentage of individual cells inoculated which grew out in the cystathionine medium to form visible clones containing at least 50 cells. [d] Diploid, but aneuploid (cf. Table 1).

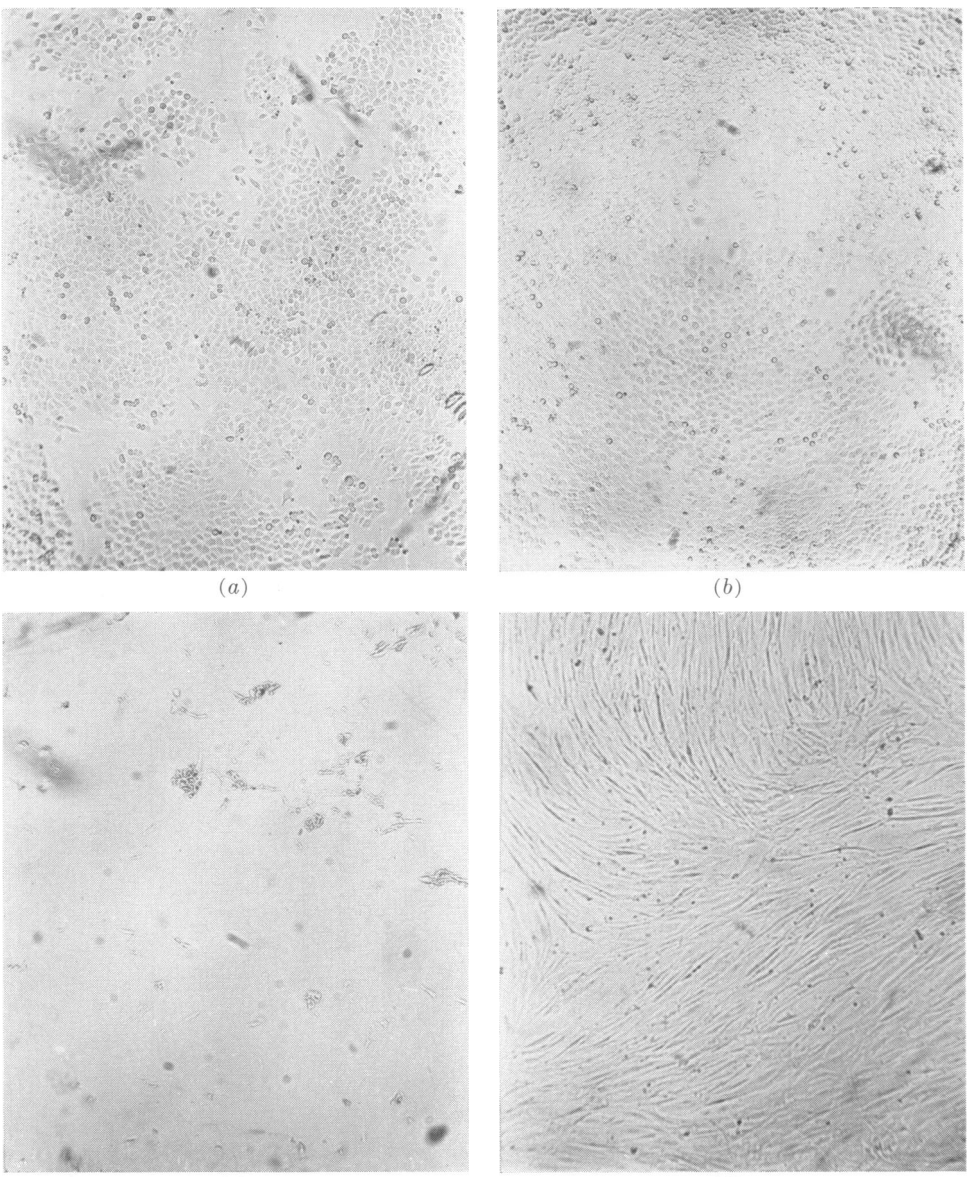

FIG. 1.—The inability of human diploid fibroblasts to utilize cystathionine as a precursor for cystine. Growth response to cystathionine: (*a*) heteroploid (liver-Chang), 3 days; (*c*) diploid (BAL), 6 days. Growth response to cystine: (*b*) heteroploid (liver-Chang), 3 days; (*d*) diploid (BAL), 6 days.

crease in cellular protein and prolonged survival, indicative of decreased rather than absent cystathionase activity in such cells. In the same medium, the heteroploid cells regularly grew even with inocula of 1–10,000 cells per ml. Experiments are in progress to determine the cystathionase activity of diploid and heteroploid human cells, and the correlation with their markedly different growth response to cystathionine as here reported.

TABLE 4

SYNTHESIS OF HOMOCYSTINE AND CYSTATHIONINE BY CULTURED HUMAN CELLS

Cell Type		Millimicromoles Synthesized per Day per Mg Cell Protein*	
		Homocystine from methionine	Cystathionine from homocystine
Euploid	Amnion (epithelial)	3.2	0
	Amnion (fibroblastic)	2.7, 4.3, 7.8	30, 32, 28
	Fetal adrenal	5.7, 6.5	5.5, 13
	Fetal bone	0, 5.4, 8.5, 6.1	30, 30
	Fetal conjunctiva	0, 5.7, 5.4	0, 21,13
	Fetal lung	6.7, 7.0	28, 19
	Fetal thymus	5.8	5.9
	BAL	11, 9.3	0, 0
	Detroit	6.5, 9.2	13, 20, 9.8
	F-1	5.2, 5.5	7.1, 0
Minor chromosomal aberrations	RPMI 2650	9.5, 11	5.0±, 5.7
	C33	9.8	8.4
Heteroploid (all epithelial)	Amnion (FL)	13, 12	47, 23
	Amnion (WISH)	20, 14	11, 5, 19
	Conjunctiva (Chang)	12, 10	10, 8.4
	HeLa	11, 16	17, 17
	KB	8.0, 10	9.2, 21, 21
	Liver (Chang)	17, 10, 23	6.7, 40, 20, 13

Cells were overlaid for 24–48 hr with a medium containing (*a*) S^{35}-methionine and 0.1–0.2 mM homocystine, or (*b*) S^{35}-homocystine and 0.05–0.1 mM cystathionine. The exogenous homocystine or cystathionine enhanced rather than inhibited its own synthesis.[19] Serine (0.2 mM), cystine (0.1 mM), and methionine (0.1 mM) were present throughout to assure the survival of the cells during the period of the experiment. The values in the table for the newly synthesized homocystine and cystathionine in the medium do not take into account the relatively minor amounts bound to serum protein in the medium[20] or used to make cystine.

* Based on average of cell protein at beginning and end of experiment. The individual numbers in the table represent different experiments, carried out at different times.

The absent or weak cystathionase activity of amnion cells in primary culture suggests that this may be an inherent property of most human diploid cells, and not caused by the special conditions of culture *in vitro* (unless one assumes the amnion cells had lost cystathionase activity in their first culture passage). This is indicated also by the tissue enzyme assays recently reported by Mudd *et al.*[21] The fact that cystine is not an essential amino acid *in vivo* may therefore reflect the biosynthetic activity of a few organs, such as the liver, pancreas, and kidney.[21] This would be analogous to the situation with arginine and tyrosine, similarly essential in culture but not in the whole animal.[14]

The demonstrated ability of a wide variety of grossly heteroploid human cultures to effect the cleavage of cystathionine in amounts sufficient for growth in a cystine-free medium may reflect derepression of the cistron controlling cystathionase synthesis, e.g., due to gene dosage effects, deletion or suppression of a regulator gene, or may instead reflect enzyme activation or stabilization. The generally enhanced capacity of such cells to synthesize homocystine falls into the same pattern. The possibility may in fact be considered that it is the many tetraploid cells in the liver[26] which are responsible for the cystine-synthesizing capacity of that organ, by virtue of an enhanced cystathionase activity analogous to that here described for heteroploid cells. Relatively minor chromosomal abnormalities did not usually increase cystathionase activity, at least to the point of cellular growth on cystathionine. Thus, the epithelial (cancer) RPMI 2650 strain, recently shown to have an abnormal chromosome in the D group,[10] and two fibroblastic skin cultures supplied by Dr. H. P. Klinger and derived from patients with chromosome aberrations [one from a Turner's syndrome, with a 45/×0 chromosome complement, and one

with a 47th chromosome in the E group (cf. Table 1)], all behaved like euploid cultures in their inability to utilize cystathionine for survival and growth in a cystine-free medium, regardless of the initial population density.

In cloned variants of a single heteroploid human cell strain, DeCarli *et al.*[22] have found a positive correlation between the number of acrocentric chromosomes and cellular alkaline phosphatase activity. A similar comparison of the cystathionase activity of individual clones deriving from the heteroploid lines here studied would be of interest.

The ability to synthesize cystine is only one of many phenotypic differences between cultured diploid and heteroploid human cells, including the escape of the latter from contact inhibition of locomotion and growth, their "immortality" as contrasted with the finite life expectancy of diploid strains, their ability to grow in suspension, and their high plating efficiency.[23, 24] Some of these heteroploid cells derived from cancers (cf. Table 1), and may have been aneuploid originally. Others derived from normal tissues, and the profound chromosomal aberrations now present presumably developed in the course of their serial cultivation; but in these cases the parent diploid strains are no longer available for comparison. Human amnion cells, however, can be cultured afresh at will; and the many phenotypic differences between such euploid amnion cultures and the heteroploid, spontaneously "transformed" FL and WISH amnion lines, including the enhanced capacity of the latter to synthesize cystine, may well be a consequence of those chromosomal aberrations.

The possible relevance of these findings to the problem of cancer is self-evident. Since most human cancer cells show varying degrees of chromosomal aberration, the possibility may be considered that the chromosomal changes in these cells are associated with changes in biosynthetic activity analogous to the derepression of cystine synthesis here described, but which permit the cell to escape from the unknown control mechanisms which limit the growth of normal cells *in vivo*. This does not mean that the postulated metabolic changes responsible for the malignant character are necessarily the same in all cancer cells; nor does it specify whether those changes involve nonstructural products of cellular metabolism, or alterations in their surface composition, for example.

However, at least one alternative possiblity must be considered which would vitiate most of the entire preceding discussion. Although 3 "PPLO-free" heteroploid lines (FL, KB, and Chang liver) did not differ in the biosynthetic activities here studied from strains of the same cell known to be PPLO-contaminated, other widespread but unrecognized microcontaminants in the heteroploid cells, all of which have been carried in culture for years, could conceivably be responsible for the enhanced ability of the latter to synthesize homocystine and to cleave cystathionine.

Summary.—Cystine as such was required for growth by all ten diploid human cell strains here tested. Although all could form homocysteine from methionine, and eight could condense homocysteine with serine to form cystathionine, none could utilize cystathionine in lieu of cystine, indicative of absent or deficient cystathionase activity. In contrast, a number of grossly heteroploid human cell lines could carry out all three reactions involved in the synthesis of cystine from methionine. In consequence, these cells could grow in a cystine-free medium if given homocystine or cystathionine; and at high population density a few lines could grow with

methionine alone. They behaved as if the chromosomal aberrations resulted in enhanced cystathionase activity.

Four aneuploid cell strains with relatively minor chromosomal aberrations behaved like euploid cells in that they could not utilize cystathionine for growth and did not survive in the absence of preformed cystine. A fifth such strain, a hypodiploid cancer cell, did grow under these conditions.

Possible implications of these findings with respect to the nature of the malignant transformation are discussed.

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[6a] The term "heteroploid" will be used to describe those cell lines in which the chromosome number, and usually chromosome structure as well, departed greatly from the euploid complement, and varied markedly within the same culture.

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