Macromolecular Basis for Homocysteine-Induced Changes in Proteoglycan Structure in Growth and Arteriosclerosis

Kilmer S. McCully, MD

Cell culture monolayers deficient in cystathionine synthetase bound more inorganic sulfate than normal cell monolayers during growth to confluence; this was correlated with the production of granular proteoglycan by the abnormal cells and fibrillar proteoglycan by normal cells. Homocysteine was demonstrated to be an active precursor of esterified sulfate, confirming our previous finding of this sulfation pathway in liver. The cell cultures deficient in cystathionine synthetase were found to assume an abnormal cellular distribution on the surface of the culture dish, resembling the distribution assumed by neoplastic cells with loss of contact inhibition; the degree of abnormality of the cellular distribution was correlated with the amount of granular proteoglycan produced by the cells and the amount of inorganic sulfate binding by the cell monolayers. Pyridoxine was found to increase the growth rate of cell cultures from a patient with pyridoxine-responsive homocystinuria and to increase the production of fibrillar proteoglycan by the cells; no effect of pyridoxine was observed in the cell cultures from a patient who failed to respond to pyridoxine therapy. The findings suggest that the change in macromolecular conformation of cellular proteoglycans from fibrillar to granular is due to increased sulfation of the carbohydrate envelope of the molecule. The significance of the findings is related to the pathogenesis of homocystinuria, the phenomenon of contact inhibition, the action of growth hormone and initiation of arteriosclerotic plaques. (Am J Pathol 66:83–96, 1972)

HOMOCYSTEINEMIA was found to be associated with accelerated arteriosclerosis in two individuals with different enzymatic disorders of sulfur amino acid metabolism.[1] Administering homocysteine to rabbits resulted in vascular lesions that reproduced the essential features of the lesions found in the human cases.[2] Cells cultured from the skin of 2 individuals with cystathionine synthetase deficiency and homocysteinemia were found to produce an abnormal granular proteoglycan in culture, and adding homocysteine to the culture medium of normal skin cells resulted in conversion of some of the normal fibrillar proteoglycan to the granular form.[3] These findings were interpreted to indicate that

From the Department of Pathology, Harvard Medical School, and the James Homer Wright Pathology Laboratories, Massachusetts General Hospital, Boston, Mass.

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Address reprint requests to Dr. Kilmer S. McCully, Department of Pathology Harvard Medical School, James H. Wright Pathology Laboratories, Massachusetts General Hospital, Boston, Mass 02114.

homocysteinemia produces pathologic changes in arteries and other connective tissues by altering the state of aggregation and normal fibrillar structure of proteoglycan molecules produced by vascular and connective tissue cells.[3]

In this report, evidence is presented that demonstrates that cell cultures deficient in cystathionine synthetase bind more inorganic sulfate than normal cell cultures, supporting our previous suggestion that increased sulfation of proteoglycan molecules is the factor responsible for altering the macromolecular conformation from fibrillar to granular.[3] The production of highly sulfated, granular proteoglycan by the abnormal cells results in abnormal cellular orientation and alteration of contact inhibition in culture. Pyridoxine was found to stimulate the growth of cultured cells from an individual deficient in cystathionine synthetase who responds clinically to high doses of pyridoxine. We further show that homocysteine sulfur is a precursor of esterified sulfate, providing additional evidence for a sulfation pathway in which homocysteine is oxidized to homocysteic acid, which is a precursor of active sulfate, 3′ phosphoadenosine 5′ phosphosulfate (PAPS).[4] The importance of the findings in understanding the pathogenesis of homocystinuria, the phenomenon of contact inhibition, mediation of the action of growth hormone, and initiation of arteriosclerotic plaques is discussed.

Materials and Methods

Binding of $^{35}SO_4$ and ^{35}S-Homocysteine Thiolactone to Human Skin Cells

The cell lines (21 HC and 49 HC, cystathionine synthetase–deficient; 26 JL, 120 JL and Dunn-4, normal), methods of cultivation and examination by light microscopy were described previously.[3] To determine ^{35}S binding, equal numbers of cells were passaged into Eagle's minimal essential medium (MEM) with 10% fetal calf serum, containing $^{35}SO_4$ (New England Nuclear), 1 μCi ml and 0.8 mM, or ^{35}S-homocysteine thiolactone (Amersham), 1 μCi ml and 1.0 mM, and refed twice weekly. The sulfate concentration of some media was doubled by adding 0.2 g liter of $MgSO_4 \cdot 7H_2O$. The two concentrations of sulfate used, 0.8 and 1.6 mM, are both within the normal physiologic range found in plasma. After the cultures reached confluence (2–8 weeks), the medium was removed, and the monolayer was rinsed twice with cold phosphate-buffered saline (PBS), pH 7.0, and scraped from the dish into fresh PBS. The mixture was homogenized thoroughly in a glass homogenizer, and aliquots were taken for counting by liquid scintillation, using Bray's solution, and for protein determination, using the Lowry method.[5] Aliquots of some homogenates were mixed with cold 5% trichloroacetic acid (TCA) and filtered through Millipore filters; the filters and filtrates were counted in Bray's solution. Aliquots of some homogenates were exhaustively dialyzed for 24 hours against cold calcium- and magnesium-free buffer [6] containing ethylenediaminetetraacetate (EDTA), 0.2 g liter, and K_2SO_4, 0.28 g liter, and the radioactivity remaining within the dialysis bag was determined.

Homogenates of cells cultured in ^{35}S-homocysteine thiolactone were dialyzed against buffer containing EDTA and K_2SO_4, hydrolyzed with 6 N HCl at 110 C for 22 hours under N_2 and chromatographed on sulfonated polystyrene resin.[7] The radioactivity of the fractions was determined using Bray's solution, and the positions of the peaks were compared to those of authentic compounds.

Results

Cells from the individuals with cystathionine synthetase deficiency, 21 HC and 49 HC, grown to confluence in medium containing $^{35}SO_4$, bound more sulfate per milligram of protein than cells from normal individuals, 26 JL and 120 JL (Table 1). When the concentration of unlabeled sulfate was increased in the medium, all the cell lines bound more $^{35}SO_4$. Adding unlabeled homocysteine thiolactone to the medium had no consistent effect upon binding of $^{35}SO_4$. In similar experiments using ^{35}S-homocysteine thiolactone as a precursor (Table 1), the normal cell lines bound more ^{35}S from ^{35}S-homocysteine than from $^{35}SO_4$. The amounts of bound ^{35}S from ^{35}S-homocysteine and from $^{35}SO_4$ were comparable in the cystathionine synthetase–deficient cell lines. Increased concentrations of unlabeled sulfate in the medium had no effect on ^{35}S binding from ^{35}S-homocysteine in three of the lines, but an increase was observed in the normal 120 JL line.

The nature of the binding of $^{35}SO_4$ and ^{35}S-homocysteine to cell culture

Table 1—Binding of $^{35}SO_4$ and ^{35}S-Homocysteine Thiolactone to Cultured Human Skin Cells*

Cells	$SO_4^=$ (mM)	HCT (mM)	$^{35}SO_4$ (nmole/mg protein)	^{35}S-HCT (nmole/mg protein)
26 JL	0.8	—	14, 16	44, 69, 49
26 JL	1.6	—	20, 19	41, 51, 54
26 JL	0.8	1.0	9.3, 6.3, 9.3	—
120 JL	0.8	—	7.8, 8.1, 9.1, 9.4	55, 69
120 JL	1.6	—	18, 20, 30, 26	104, 94, 54, 85
120 JL	0.8	1.0	7.0, 7.2, 8.6, 8.2	—
49 HC	0.8	—	29, 26, 33	31, 51, 50
49 HC	1.6	—	58, 62, 64	54
49 HC	1.6	1.0	88, 98, 112	—
21 HC	0.8	—	79, 67	42, 24, 38
21 HC	0.8	1.0	84, 62, 85	—
21 HC	1.6	1.0	—	40

* The cell lines were all passaged two to six times before the isotope-binding studies were started. Equal numbers of normal (26 JL, 120 JL) and cystathionine synthetase–deficient cells (49 HC, 21 HC) were allowed to grow to confluence in the presence of either $^{35}SO_4$, 1 μCi/ml, or ^{35}S 1-homocysteine thiolactone, 1 μCi/ml and 1.0 mM, in the culture medium, Eagle's MEM with 10% fetal calf serum. After the cells reached confluence, the medium was removed, the monolayer was rinsed with PBS and homogenized. Aliquots were taken for protein determination[5] and counting in Bray's solution.

monolayers was studied by dialyzing aliquots of the homogenates against buffered saline containing EDTA and unlabeled sulfate. More of the bound $^{35}SO_4$ was nondialyzable from homogenates of the cystathionine synthetase–deficient lines than for the normal line (Table 2). The amount of nondialyzable ^{35}S from ^{35}S-homocysteine thiolactone was approximately the same for all three lines. In all the cell lines studied, a larger percentage of ^{35}S was nondialyzable or precipitable by TCA if ^{35}S-homocysteine thiolactone rather than $^{35}SO_4$ was used as a precursor.

The chemical form of the nondialyzable ^{35}S from homocysteine thiolactone was determined by chromatography of the acid-hydrolyzed nondialyzable fraction (Table 3). Most of the radioactivity was recovered in fractions corresponding to the elution of sulfate, and a small fraction of the radioactivity was recovered in fractions corresponding to methionine sulfoxide. A slightly greater proportion of the nondialyzable ^{35}S was recovered as $^{35}SO_4$ from cystathionine synthetase–deficient cells, compared to normal cells (Table 3). The fraction recovered as $^{35}SO_4$ represents ^{35}S-homocysteine thiolactone that was oxidized and converted to esterified ^{35}S sulfate in the cultured cell monolayer. The fraction recovered as ^{35}S-methionine sulfoxide represents peptide-bound ^{35}S-methionine, arising from cellular methylation of ^{35}S-homocysteine, cellular protein synthesis and oxidation of ^{35}S-methionine to ^{35}S-methi-

Table 2—Solubility and Dialysis of Bound $^{35}SO_4$ and ^{35}S-Homocysteine Thiolactone in Cultured Human Skin Cells*

Cells	Precursor	Nondialyzable or TCA Insoluble	
		Percent	nmole ^{35}S/mg protein
		Nondialyzable	
120 JL	$^{35}SO_4$	8	1.2
120 JL	^{35}S-HCT	28	15.1
21 HC	$^{35}SO_4$	17	12.4
21 HC	^{35}S-HCT	33	11.4
49 HC	$^{35}SO_4$	12	3.5
		TCA Insoluble	
49 HC	$^{35}SO_4$	7.5	2.2
49 HC	^{35}S-HCT	35	15.4

* The cell lines were allowed to grow to confluence in medium containing $^{35}SO_4$, 1.0 μCi/ml and 0.8 mM, or ^{35}S-homocysteine thiolactone, 1.0 μCi/ml and 1.0 mM. The monolayers were homogenized and aliquots were either precipitated with 5% cold TCA and collected on Millipore filters or dialyzed against buffer containing EDTA and sulfate.[6] The radioactivity of the precipitate or the contents of the dialysis bag was related to the radioactivity and protein content of the homogenate.

Table 3—Recovery of ^{35}S Compounds from Hydrolysis of Bound ^{35}S-Homocysteine Thiolactone in Cultured Skin Cells*

Compound	Recovered ^{35}S (%)	
	Dunn-4	49 HC
$^{35}SO_4$	73	88
^{35}S-Methionine sulfixide	12	9.8
Methionine	<0.1	<0.1
Homocysteine	<0.1	<0.1
Homocystine	<0.1	<0.1
Homocysteine thiolactone	<0.1	<0.1
Unidentified	15	2.4

* The cystathionine synthetase–deficient cell line, 49 HC and a normal skin cell line, Dunn-4, were grown to confluence in medium containing ^{35}S-homocysteine thiolactone, 1.0 μCi/ml and 1.0 mM. The media were removed, and the monolayers were homogenized and dialyzed exhaustively against buffer containing EDTA and sulfate.[6] The nondialyzable fractions were hydrolyzed with 6 N HCl and chromatographed to separate the resulting amino acids.[7] The positions of the peaks were compared to those of authentic compounds.

onine sulfoxide during acid hydrolysis. No ^{35}S-labeled methionine, homocysteine, homocystine or homocysteine thiolactone were detected in the hydrolysate of the nondialyzable fraction of the monolayer homogenates.

The intercellular orientation and distribution of the cystathionine synthetase–deficient cells over the surface of the culture dish was abnormal, since areas of sparse growth alternated with areas of growth in which cells formed multiple layers containing mitotic figures (Fig 1). The abnormal distribution of the cells was more marked in the 21 HC line than in the 49 HC line, and the amount and degree of abnormality of granular proteoglycan was somewhat more prominent in the 21 HC line than the 49 HC line (Fig 5 and 6). The 21 HC line bound more $^{35}SO_4$ than the 49 HC (Table 1), which was correlated with both the amount of granular proteoglycan and with the degree of abnormal cellular distribution on the surface of the culture dish.

When pyridoxine was added to parallel cultures of the 21 HC cell line, the growth rate of the cells and production of protein was greatly increased (Fig 2), but there was no effect of added pyridoxine on the 49 HC or the 120 JL line. The effect of pyridoxine correlates with the clinical response of the two cystathionine synthetase–deficient individuals from whom the cell lines were obtained, since pyridoxine therapy decreased the excretion of homocystine and the plasma homocystine concentrations in the individual from whom the 21 HC line was obtained but did not affect the donor of the 49 HC line. When added pyridoxine was

present in the culture medium of the 21 HC line, more fibrillar proteoglycan was produced by the cells, but the granular form was also produced. The growth rate of the 21 HC line was greatly reduced by added homocysteine thiolactone, 1.0 mM, in the medium (Fig 3), and added pyridoxine in the medium completely reversed the growth inhibition effect of added homocysteine (Fig 4).

Discussion and Conclusions

The finding that cultured cell monolayers deficient in cystathionine synthetase bind more inorganic sulfate than normal monolayers (Table 1) supports our previous suggestion[3] that the conversion of fibrillar to granular proteoglycan produced by the abnormal cells is due to an increase in anionic charge on the carbohydrate envelope surrounding the polypeptide core of the macromolecule. The increased charge may result either from increased numbers of sulfate groups on the carbohydrate component of the proteoglycan or from increased numbers of both sulfate and carbohydrate groups per mole of polypeptide of the proteoglycan. Separation, purification and chemical characterization of the sulfated polysaccharide components of the proteoglycans from normal and cystathionine synthetase–deficient cells are needed to distinguish between these possibilities. Since very little is known concerning the protein component of cellular proteoglycans, a detailed understanding at a molecular level concerning the influence of increased numbers of sulfate groups on the fibrillar and granular forms of proteoglycans must await further investigation of their composition, structure and macromolecular conformation.

Several lines of evidence suggest that the change in macromolecular conformation of the proteoglycan that results from excessive binding of sulfate is accompanied by a decreased solubility of the substance in a physiologic milieu. First, flocculent precipitated material was found in the cystathionine synthetase–deficient cell monolayer.[3] Second, the solubility in buffered saline of protein-bound hexuronic acid of cystathionine synthetase–deficient monolayers was decreased compared to that of normal monolayers.[8] Finally, the solubility of the glycoprotein and proteoglycan fraction was found to be decreased in the aorta of a patient with homocystinuria.[9] Further studies are needed to substantiate and explain these findings, since a change in the solubility of arterial wall sulfated proteoglycans may explain why metachromatic substance accumulates in early arteriosclerotic plaques[10] and in arterial plaques produced by homocysteinemia.[2,11]

The studies of binding of ^{35}S-homocysteine to the cell culture mono-

layers show that homocysteine sulfur is a more active precursor of nondialyzable sulfur than inorganic sulfate (Table 2) and that most of the nondialyzable homocysteine-derived ^{35}S is in the form of sulfate ester (Table 3). This finding is independent confirmation of our discovery of a pathway for active sulfate formation in guinea pig liver, in which homocysteine is oxidized to homocysteic acid, and then the homocysteic acid and ATP react to form 3′ phosphoadenosine 5′ phosphosulfate (PAPS),[4] the precursor of esterified sulfate.[12] The finding that an increased concentration of inorganic sulfate in the culture medium did not reduce the formation of esterified sulfate from ^{35}S-homocysteine (Table 1) demonstrates that ^{35}S from homocysteine does not equilibrate with extracellular inorganic sulfate during formation of PAP-^{35}S and esterified $^{35}SO_4$. In addition, the increased binding of $^{35}SO_4$ to cystathionine synthetase–deficient cell monolayers (Table 1), part of which is in the form of nondialyzable esterified sulfate (Table 2), suggests that homocysteic acid, which is presumably present in elevated concentration in the enzymatically deficient cells, may increase the rate of reaction between ATP and inorganic sulfate to form PAPS, resulting in increased formation of esterified sulfate. The observation that cystathionine synthetase–deficient cell monolayers bind ^{35}S from homocysteine in an amount approximately equal to or lower than that of normal cell monolayers (Table 1) may be explained by assuming that cellular synthesis of unlabeled homocysteine and homocysteic acid derived from methionine [4,13,14] was increased because of inability of the cystathionine synthetase–deficient cells to convert excess homocysteine to cystathionine. The increased amount of unlabeled homocysteic acid derived from methionine in the abnormal cells would be expected to dilute the activity of the ^{35}S-esterified sulfate derived from the ^{35}S-homocysteic acid formed from ^{35}S-homocysteine.

The phenomenon of increased binding of $^{35}SO_4$ to cystathionine synthetase–deficient cell monolayers compared to normal monolayers (Table 1) is similar to the *in vitro* effect of growth hormone–dependent serum sulfation factor, which increases the binding of $^{35}SO_4$ to cultured cartilage fragments.[15] The increased binding of $^{35}SO_4$ by the cystathionine synthetase–deficient cells is presumably related to increased cellular synthesis of homocysteic acid from homocysteine and methionine. Homocysteic acid possesses other properties of sulfation factor, including heat stability, dialyzability, and co-chromatography with the amino acids on gel filtration.[16] Furthermore, homocysteic acid increases the growth rate of normal young guinea pigs.[4] Most of the individuals with cystathionine synthetase deficiency are unusually tall,[17] their increased growth rate is

presumably secondary to increased homocysteic acid synthesis, resulting from their inability to convert homocysteine to cystathionine.

The amount of increased binding of $^{35}SO_4$ in the 21 HC and 49 HC cell monolayers (Table 1) correlates with the amount of granular proteoglycan material produced in the respective cultures and the degree of abnormality of distribution of the cellular growth within the culture dish (Fig 5 and 6). The appearance of the cellular areas of growth in which multiple layers of proliferating cells cover one another (Fig 2 and 4) is similar to the appearance of cultured malignant or transformed cells with altered contact inhibition.[18,19] These findings suggest that increased sulfation of the proteoglycans synthesized by cells in culture is an important factor in loss of normal intercellular orientation and contact inhibition of growth and movement. However, a detailed interpretation of these findings requires further investigation, since the phenomenon of contact inhibition is poorly understood at a molecular level.

The finding that pyridoxine stimulates the growth of the 21 HC cell line (Fig 2 and 4) but not that of the 49 HC cell line correlates with the clinical response of the patients to pyridoxine therapy.[20] Since pyridoxine is a cofactor for cystathionine synthetase, the increased amount of fibrillar proteoglycan synthesized by the 21 HC cells in the presence of pyridoxine (Fig 2) provides additional evidence that the abnormality of the proteoglycan produced by the cells[3] is secondary to the metabolic effects of the enzyme deficiency. Pyridoxine also prevents the marked inhibition of growth of the 21 HC cells produced by added homocysteine in the culture medium (Fig 4), presumably by enabling the cells to convert excess intracellular homocysteine to cystathionine.

The metabolic and macromolecular abnormalities demonstrated in the cystathionine synthetase–deficient cell cultures have aided interpretation of the initiation of arteriosclerotic plaques,[2] led to the discovery of a new pathway for synthesis of sulfate esters,[4] increased our understanding of the pathogenesis and treatment of homocystinuria,[3] provided a clue for further investigation of the phenomenon of contact inhibition and contributed to an increased understanding of the action of growth hormone. The molecular formulation of the role of sulfur metabolism in arteriosclerosis has suggested a new approach to prevention of that disease [2,3] and further work in this area may suggest new methods of therapy for arteriosclerosis and abnormalities of growth.

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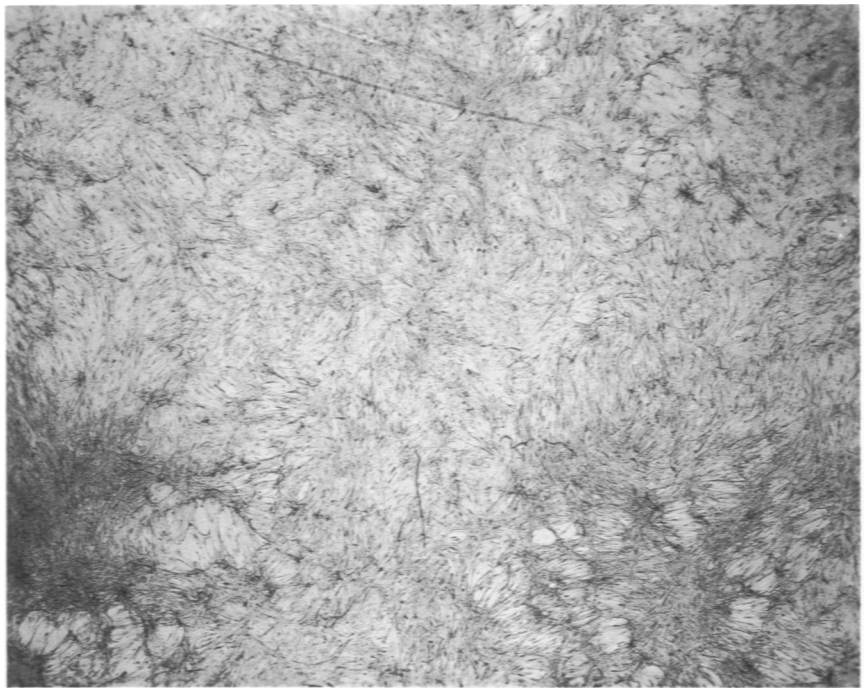
[*Illustrations follow*]



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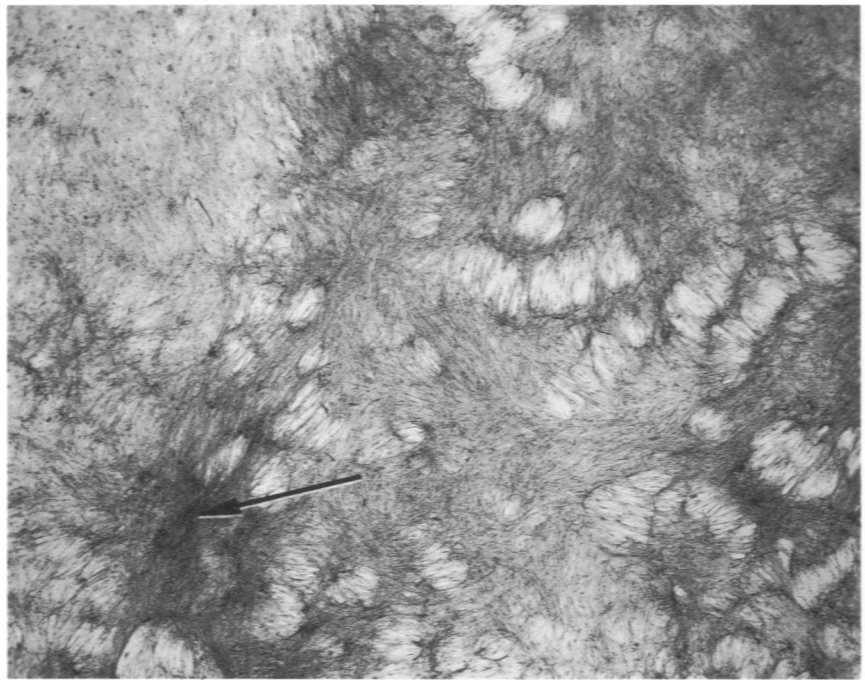
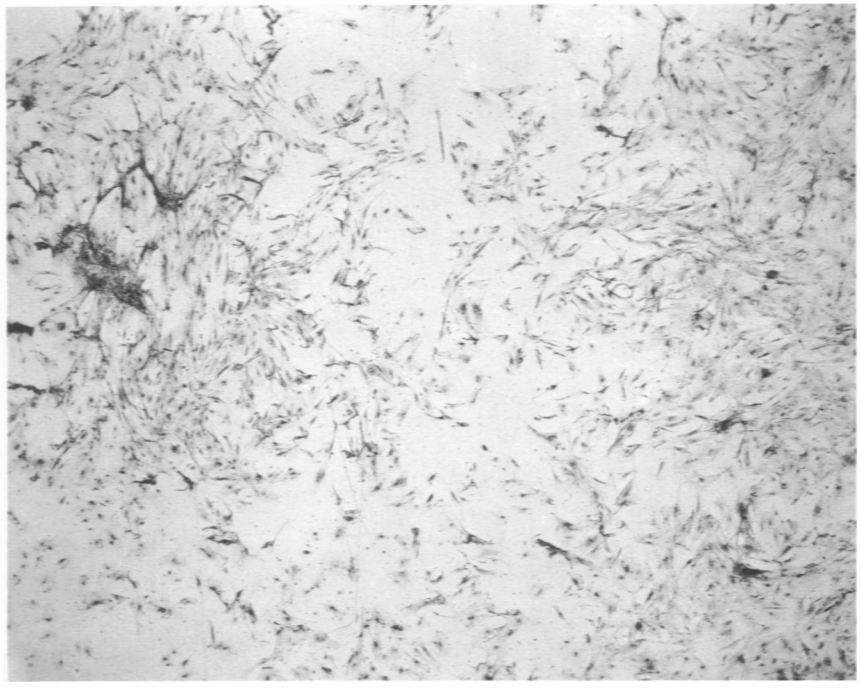


2

Fig 1—Cultured cystathionine synthetase–deficient skin cells, 21 HC, form abnormal intercellular orientation and distribution (Giemsa, × 11). **Fig 2**—Parallel culture of 21 HC cells with added pyridoxine, 0.5 μg/ml of medium, shows increased growth of cells, abnormal intercellular orientation and areas with multiple layers of cell growth (*arrow*) (Giemsa, × 11).

3



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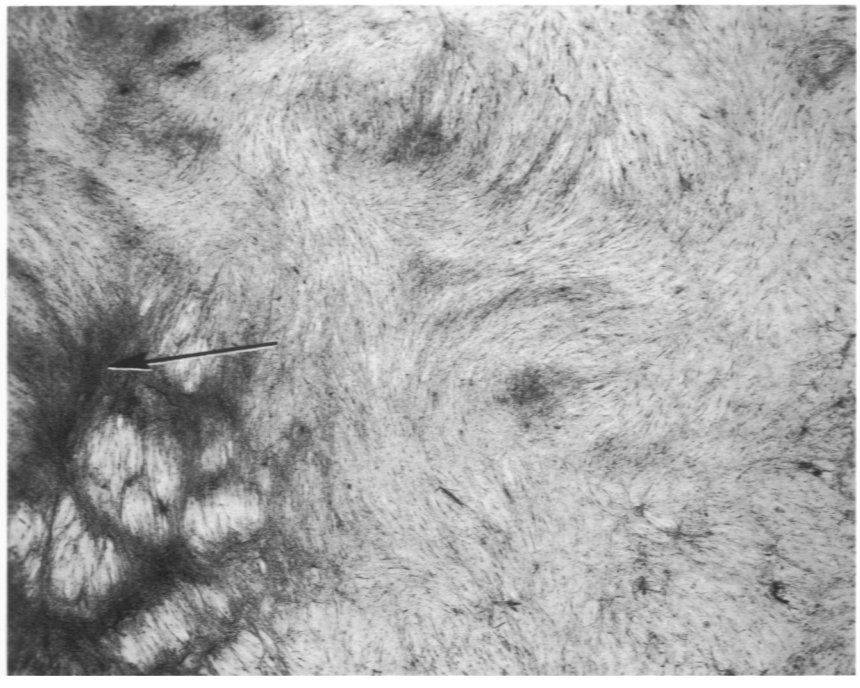

Fig 3—Parallel culture of 21 HC cells with added homocysteine thiolactone, 1.0 μmole/ml of medium, shows decreased growth of cells (Giemsa, × 11). **Fig 4**—Parallel culture of 21 HC cells with added homocysteine thiolactone, 1.0 μmole/ml, and pyridoxine, 0.5 μg/ml of medium, shows increased growth of cells and areas with multiple layers of cell growth (*arrow*) (Giemsa, × 11).

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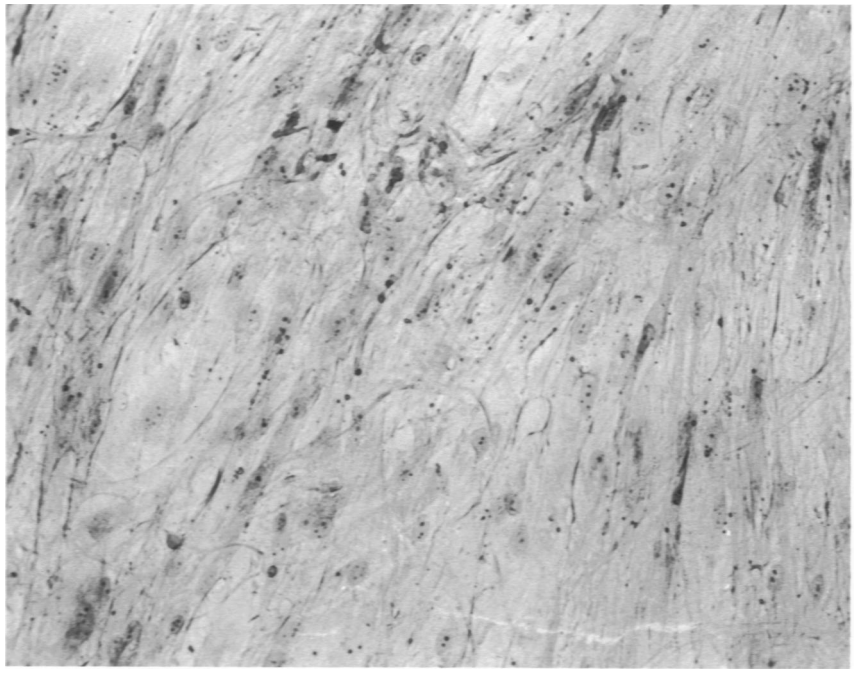

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Fig 5—Culture of cystathionine synthetase–deficient skin cells, 49 HC, forms granular substance within cells and between cells (Giemsa, × 140). **Fig 6**—Culture of cystathionine synthetase–deficient skin cells, 21 HC, forms abundant granular substance within and between cells (Giemsa, × 140).

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