increase in RNA synthesis might therefore be expected in cells which synthesize large amounts of this enzyme (*10*). The functional significance of vitamin A–induced alkaline phosphatase synthesis is uncertain, but some of the effects of vitamin A in bone formation (*11*) and in lipid deposition and transport (*12*) might be mediated through this enzyme, since it is required for phosphate transfer in both types of metabolism (*13*). Alkaline phosphatase is also increased by vitamin A in certain protein-secreting cells having membrane-bound ribosomes (*14*) where it may be more directly concerned with membrane phospholipid metabolism.

Alkaline phosphatase is increased by hypervitaminosis A in tissues other than epidermis, as in the epiphyseal osteoblasts of rats (*15*) and in human blood plasma (*16*), which suggests that in general it may be vitamin A–dependent. In the body it occurs as a variety of genetically independently determined molecular forms (isoenzymes) which are often tissue specific although having similar enzymic activity (*17*). This variation might have evolved fortuitously, but there could be a functional significance if, as seems possible, alkaline phosphatase is in general vitamin A–dependent and if these isoenzymes have different threshold requirements for synthesis. This would explain why, for example, neurones and the blood vessel endothelium are rich in alkaline phosphatase in the presence of relatively low vitamin A concentrations (~ 100 μg/100 ml) (*18*), whereas the epidermal prickle cells require ~ 50,000 μg/100 ml for comparable synthesis to occur. In view of this, it is interesting to note that Fell and Mellanby (*19*) considered that the various tissues probably have different threshold requirements for vitamin A.

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Cystathionine Synthase in Tissue Culture Derived from Human Skin: Enzyme Defect in Homocystinuria

Abstract. *Fibroblasts derived from normal human skin and from cells in amniotic fluid and grown in tissue culture have cystathionine synthase activity. Skin from homocystinuric patients gives rise to fibroblast lines with normal activities of methionine-activating enzyme, but with very low or undetectable cystathionine synthase activity. Thus, the enzyme lesion in homocystinuria is demonstrable in readily available human cells. Neither cystathionine synthase nor methionine-activating enzyme could be detected in intact normal skin.*

Homocystinuria is an inherited metabolic aberration (*1*) characterized by mental retardation, dislocation of the crystalline lens, arachnodactyly, and thromboembolic episodes. Homocystinuric patients have a specific deficiency of cystathionine synthase activity in liver (*2*, *3*) and in brain (*4*). The affected enzyme is in the methionine-to-cysteine pathway. It catalyzes the condensation of homocysteine with serine to form cystathionine.

We have studied this transsulfuration pathway in a variety of mammalian cells grown in tissue culture and have tried to find a readily available source of human cells exhibiting cystathionine synthase activity. Studies of this enzyme in humans have been limited by the need to work with either liver biopsy specimens or material obtained postmortem.

We have determined the cystathionine synthase activity and methionine-activating enzyme [adenosine triphosphate: L-methionine adenosyltransferase, E.C. 2.5.1.6. (*5*)] activity in fibroblasts cultured from small skin biopsies of homocystinuric patients and control subjects. We used cells after 2 to 27 serial subcultivations in monolayer culture.

The results are summarized in Table 1. For controls, cells derived from 21 normal volunteers or patients with diseases other than homocystinuria were studied. The specific activities of cystathionine synthase are expressed relative to the number of cells extracted and to the protein content of the extract. These two measurements correlated well with one another ($r = .93$, $P < .001$). Evaluation of the data did not suggest that a correlation exists between enzyme activities and such variables as the length of time the cells had been stored after harvest, the age or sex of the donor, the presence or absence of disease other than homocystinuria, the number of subcultivations, or the age or density of the cell sheet at the time of harvest. There appeared to be no trend toward either increasing or decreasing enzyme activities during the 20-month course of the study. The specific activities of methionine-activating enzyme and cystathionine synthase appeared to vary independently.

Cell lines were derived from skin biopsies of six patients, each of whom excretes excessive amounts of homocystine in his urine, has dislocated lenses and, in some cases, other of the clinical stigmata of homocystinuria. In their characteristics in culture, the cell lines derived from these homocystinuric patients were indistinguishable from the control lines. The fibroblasts of four of these lines had no detectable cystathionine synthase as measured by a method (*6*) which is about ten times more sensitive than the one used for the control cells. The modified assay permitted chromatographic identification of

the cystathionine formed during incubation with enzyme. By this means it was demonstrated that the extracts of the cells from homocystinuric patients D.A. and J.I. did have low levels of cystathionine synthase activity; that is, they converted serine to cystathionine at a low rate. As further confirmation, it was shown that the activity in the extract from J.I. was dependent on addition of homocystine (as was the activity in extracts of control cells). An extract from homocystinuric cells did not inhibit cystathionine synthase activity when incubated with an extract from control cells. None of the values for methionine-activating enzyme in the cells from homocystinurics fell significantly outside of the control range, illustrating the specificity of the enzyme defect in these cells.

Table 1. Cystathionine synthase and methionine-activating enzyme activities in cultured fibroblasts. After cells had attained confluency, they were harvested for enzyme assay from 32-ounce prescription bottles with 0.05 percent trypsin and 0.005 percent ethylene diaminetetraacetate. A sample of washed cells was removed for enumeration in the Coulter counter. The sedimented cells (600*g*) were stored at −55°C until assayed. The cells were suspended in potassium phosphate buffer (*p*H 6.9, 0.03 mole/liter) and disrupted in the microattachment of the Sorvall homogenizer or by the Branson Sonifier with the microtip. Samples of the supernatant fluids (5000*g*) were used for assays of cystathionine synthase and protein content according to previously described methods (*13*) and for assay of methionine-activating enzyme by a modification (*14*) of a published method (*13*). In all assays for cystathionine synthase, pyridoxal phosphate was added at a final concentration of 1 mmole/liter. A unit (U) of cystathionine synthase or methionine-activating enzyme is herein defined as the amount of enzyme catalyzing the formation of 1 nmole of cystathionine in 135 minutes or 1 nmole of *S*-adenosylmethionine in 30 minutes under the standard conditions (*13*, *14*). At least two batches of cells of each homocystinuric patient have been tested. The values presented here are based on use of the assay modified for maximum sensitivity. Values for two batches of cells from J.I. are shown.

Donor	Enzyme activity		
	MAE* (U/mg protein)	CS † (U/mg protein)	CS † (U/10^6 cells)
	Homocystinuric patients		
C.T.	2.2	< 0.2	< 0.04
J.H.	1.6	< 0.4	< 0.06
D.R.	2.8	< 0.2	< 0.02
M.R.	2.1	< 0.2	< 0.04
J.I.	1.6	3.3	0.3
		2.0	0.2
D.A.	1.8	0.5	0.08
	Control subjects		
Mean ± SE	2.5 ± 0.22	32.4 ± 3.7	5.0 ± 0.6
No.	14	21	19
Range	1.7 – 4.5	3.7 – 55.3	0.3 – 9.2

* MAE, methionine-activating enzyme. † CS, cystathionine synthase.

We have also cultured a number of cell lines from the fetal cells present in amniotic fluids (obtained after 12 to 20 weeks of gestation by transabdominal amniocentesis or at therapeutic abortion). Cystathionine synthase was assayed in four of these lines. The activities ranged from 55.1 to 162 units per milligram of protein or 8.2 to 12.5 units per 10^6 cells. Thus, cultures derived from amniotic fluid may make possible the detection of cystathionine synthase deficiency in the developing fetus before the end of the second trimester.

Our results demonstrate (i) that cystathionine synthase is present in fibroblasts derived from normal human skin and (ii) that in fibroblast lines derived from the skin of patients with homocystinuria, cystathionine synthase is not detectable or is present at very low specific activity. The ranges of specific activities of control subjects and homocystinuric patients nearly overlap due to the very low value found in the cells of one control subject and the high values (compared to other homocystinuric patients) for J.I. The hepatic enzyme activity of neither patient is known. Homocystine was not found in the urine of the control. How accurately the enzyme activities in these two fibroblast lines reflect the donors' hepatic enzyme activities is not resolvable in the case of the control since he is deceased. Thus, it cannot now be unequivocally stated that the high homocystine excretion of J.I. is due to cystathionine synthase deficiency. There appears to be little uncertainty about the other homocystinuric patients whose cell lines have enzyme activities far below the control range. Indeed, the hepatic enzyme activities of C.T. and J.H. have been reported to be extremely low (*2*, *4*).

It has been reported that cystathionine synthase is not detectable in stratum corneum from human finger tips (*2*). We have now shown that, even with the most sensitive assay, no cystathionine synthase was detected in full thickness human skin, dermis or epidermis, extracted in a variety of ways. Extracts from skin did not inhibit cystathionine synthase in extracts of fibroblasts grown in vitro. Because much of the protein in a specimen of skin is extracellular, its DNA content rather than its protein content seemed to be a better indication of the number of cells present. The activities of cystathionine synthase in dermis and in cultured fibroblasts were thus compared on the basis of both DNA content and protein content of the preparations (*7*). The comparison showed that cystathionine synthase activity of dermis was less than 0.4 percent of the mean value for cultured fibroblasts based on protein content and less than 0.6 percent based on DNA content.

Our data do not rule out the possibility that 1 percent, or less, of the cells in dermis contain a concentration of cystathionine synthase equal to that found in cultured fibroblasts, and that these are the cells which proliferate in culture. Selective growth in vitro because of cystathionine synthase possession is unlikely, however, since the mutant cells lacking this enzyme have normal growth rates. Another possibility is that the presence of cystathionine synthase in the cultured fibroblasts but not in skin is a result of differing cellular responses to the environments in culture and in vivo. Further interpretation is difficult because of uncertainty about which of the cell types in dermis gives rise to the fibroblasts of tissue-culture lines (*8*, *9*).

The tendency of mammalian cells to lose specialized function in culture, including some enzyme activities (*10*), has been emphasized (*11*). Yet, we now find that cystathionine synthase, an enzyme present in high activities in but a few organs (*13*) and not found in leukocytes (2), is also not demonstrable in skin but is easily detectable in fibroblasts cultivated from skin. The presence of an enzyme in nearly all of the cells of the body or in leukocytes (or in both) has been taken as a criterion for predicting the presence of this enzyme in cultured cells (*8*, *12*). Our findings suggest that the number of metabolic activities and thus the heritable metabolic diseases that can be studied in tissue culture may be less restricted than this criterion would indicate.

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of approximately ten times the usual specific radioactivity and of an additional paper chromatographic purification (*13*).

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3-Acetylpyridine: Effects in vitro Related to Teratogenic Activity in Chicken Embryos

Abstract. *Production of skeletal muscle hypoplasia by 3-acetylpyridine and its complete reversal by nicotinamide in developing chicken embryos have been confirmed. Cultures of developing embryonic chicken muscle show degenerative effects produced by 3-acetylpyridine; these effects are reversed by nicotinamide. Cartilage production in cultured chondrogenic cells is potentiated by 3-acetylpyridine; this potentiation is completely reversed by nicotinamide. It is suggested that nicotinamide- or pyridine-nucleotide–dependent reactions influence normal differentiation of limb mesoderm cells by inhibiting chondrogenic-cell and potentiating muscle-cell expression or proliferation.*

Landauer (*1*) demonstrated that 3-acetylpyridine is teratogenic when introduced into the yolk sac of 96-hour-old chicken embryos. The most striking aberration produced under this condition was skeletal muscle hypoplasia such that little or no leg musculature was apparent at hatching. Bone, skin derivatives (*1*), heart, and eye were normal (*2*). Adequate supplements of nicotinamide given at the 96-hour stage lowered the toxicity of co-injected 3-acetylpyridine and completely protected against its teratogenicity. Landauer suggested that 3-acetylpyridine interfered with synthesis or utilization of nicotinamide by the early chicken embryo, or both.

In order to perform a detailed biochemical analysis of the effects of nicotinamide-antagonized teratogens, it seems necessary to isolate certain developmental events from the complex biochemistry of the whole chicken embryo. Systems in vitro are available for this purpose, and we now report establishment of a correlation between the action of 3-acetylpyridine in vivo and in vitro. Limb cartilage-producing and muscle-producing cell cultures were employed for two reasons. (i) Since both of these cell types originate from the single-cell type of the mesoderm in limb development (*3*), a study of the action of nicotinamide-antagonized teratogens might reveal details of the control system that dictates into which of the two possible cell types mesodermal cells eventually develop, and (ii) nicotinamide-antagonized teratogens seem to affect either muscle or bone development, rarely both (*4*). Thus, the contrast between muscle- and cartilage-producing cells may serve in the evaluation of the site of action of these teratogens.

Suspensions of leg muscle myoblasts were plated at two density ranges and cultured in Konigsberg medium (*5*). Most experiments were performed with 0.5 to 2 × 10^6 cells in 3 ml of medium per 5-cm plastic petri dish (*6*). In the few cases where confluent cultures in which rapid formation of multinuclear cells were desired, 10 to 20 × 10^6 cells were plated. These single-cell suspensions consist, for the most part, of myoblasts with some contaminating fibroblasts. The myoblasts fuse to form myofibril-producing multinuclear cells. The culture medium was removed and replaced with fresh medium every 24 hours. From 0.045 to 4.5 mg of 3-acetylpyridine per 3 ml of culture medium was added either at the time of plating, or 24, 48, 72, or 96 hours after plating. The 3-acetylpyridine was renewed at each 24-hour medium change in one series (that is, continuous application) and was eliminated from the medium of other series after 24, 48, 72, 96, or 120 hours of exposure. Control myoblast cultures were compared to those supplemented with nicotinamide or 3-acetylpyridine or both. Two amounts of 3-acetylpyridine (0.23 or 2.3 mg per 3 ml of culture medium) and two amounts of nicotinamide (0.30 and 3.0 mg) were added to the culture medium either separately or in combination.

Cartilage-producing cultures of limb-bud mesoderm cells were grown in the presence of (i) 0.45 mg or 4.5 mg of 3-acetylpyridine or (ii) 0.60 or 6.0 mg of nicotinamide, or a combination of (i) and (ii). Suspensions of single cells were obtained from the mesoderm of stage 24 (*3*) limb buds (*7*). From 15 to 25 × 10^6 cells were plated on plastic petri dishes (5 cm) in Eagle's medium (*8*) with 7 percent horse serum (*9*), 3 percent fetal calf serum (*10*), and 5 percent chicken embryo extract. Cells at this density form multilayered cultures in which cartilage nodules may be seen in the living state under phase optics, as highly refractile arrays of cells or by their metachromatic staining with toluidine blue in fixed cultures (*11*).

Both 3-acetylpyridine and nicotinamide were made up in calcium- and magnesium-free Tyrode's solution and then passed through an HA Millipore filter. Portions (0.1 ml) were delivered to each experimental culture. The various controls to which 0.1 ml of calcium- and magnesium-free Tyrode's solution was added showed none of the effects observed when 3-acetylpyridine or nicotinamide was present. Cultures were observed daily with phase-contrast optics, and were fixed and stained at the end of