*ARGININOSUCCINATE SYNTHETASE ACTIVITY AND CITRULLINE METABOLISM IN CELLS CULTURED FROM A CITRULLINEMIC SUBJECT**

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Citrullinemia is one of several human diseases caused by a defect in the Krebs-Henseleit urea cycle. In this disorder a deficiency of liver argininosuccinate synthetase has been reported.[1] Morrow and his colleagues have recently described an infant with this condition[2] and a fibroblast-like cell line has been derived from a skin biopsy of this child.

In studying minimal media requirements for heteroploid human cell cultures, Eagle observed that citrulline, but not ornithine, could substitute for arginine as an essential nutrient.[3] This evidence for the existence of the citrulline to arginine portion of the Krebs-Henseleit cycle had also been noted in cultured chick embryo heart fibroblasts.[4] We have found that extracts of diploid fibroblast-like cells cultured from human skin normally can convert citrulline to arginine, but not ornithine to citrulline (ornithine transcarbamylase) or arginine to urea (arginase).[5]

We have compared the growth requirements and the properties of argininosuccinate synthetase in normal human cells and cells cultured from the skin of an infant with citrullinemia. Our findings provide evidence for the genetic nature of this human disorder.

Methods and Material.—Procedure for cell culture: Fibroblast-like cell lines were established from skin biopsies of human subjects and were subcultured in monolayers according to the procedure of Hayflick and Moorhead.[6] Control lines were from three different human subjects and the citrullinemia cell line was derived from the skin of a 4-month-old female with citrullinemia ("citrullinemia cells"). The complete medium included 0.2 mM arginine and was made up of 90% (v/v) double-strength Eagle's basal medium in Earle's balanced salt solution and 10% (v/v) fetal calf serum. For arginine-free medium a special preparation of Eagle's amino acid concentrate without arginine was used (Flow Laboratories, Rockville, Md.). Because the arginine-free medium contains 10% fetal calf serum, the final medium described as "arginine-free" does contain some arginine (approximately 2 μM). Penicillin (100 units/ml), streptomycin (100 μg/ml), and aureomycin (50 μg/ml) were added to the medium. The aureomycin was effective in preventing contamination with mycoplasma as described by Hayflick.[6]

Cells were harvested for enzyme studies several days after they became confluent and there was no microscopic evidence of mitotic activity.[7] The experimental cells on subcultivation had the growth characteristics of human diploid cells in Phase II of their culture life as defined by Hayflick.[8]

Determination of rates of cell growth in various media: The control and citrullinemia cell lines were each subcultured into 12 bottles (1 liter, Blake type): 4 bottles contained complete medium with 0.2 mM arginine, 4 contained arginine-free medium, and 4 contained arginine-free medium supplemented with 0.2 mM citrulline.

At the time of harvesting, the cells from each set of 4 bottles were combined and the total number of cells were estimated by direct chamber counts.

Incorporation of C^{14}-citrulline into trichloroacetic acid (TCA)-precipitable cell material: Control and citrullinemia cells, grown in a complete medium until they became a confluent sheet, were washed twice with Earle's balanced salt solution. Arginine-free medium without serum was added

and the cultures were maintained at 37° for 16–20 hr. The medium was poured off and replaced with fresh arginine-free medium containing 0.02 mM ureido-C^{14}-citrulline (New England Nuclear, Inc., Boston; 1.15 μc/μmole). After 6 hr incubation the cells were washed twice with balanced salt solution and then harvested. The cell suspensions were counted and an aliquot was removed for DNA determination.[7] The cells were centrifuged at 1000 *g* for 10 min, resuspended in 1 ml of water, and frozen and thawed five times. A milliliter of 8% (w/v) TCA was added to the lysate and the mixture was centrifuged for 15 min at 1000 *g*. The supernatant solution was counted and is referred to in Table 2 as the "TCA-soluble fraction." The precipitate was dissolved in 1.5 *N* NH_4OH and counted as the "TCA-precipitable fraction" (expt. 1, Table 2).

To test the possibility of nonspecifically bound radioactivity in the TCA precipitate, a suspension of cells from a control cell line was treated as follows: The cell suspension was divided into two equal portions and both were precipitated with TCA as in experiment 1. The precipitate from one portion of the suspension was solubilized with 1.5 *N* NH_4OH containing unlabeled citrulline and reprecipitated two times with TCA. The supernatant solutions from the two reprecipitations were combined and counted. The final precipitate was solubilized and counted (expt. 2, Table 2).

All of the fractions described above were counted by spotting measured aliquots of each on 1-in. squares of filter paper and placing the papers directly into scintillation fluid. Radioactive counting was done in a Packard scintillation counter (model 314EX) using 15 ml of 0.4% 2,5-*bis*-2(5-*tert*-butylbenzoxazolyl)-thiophene (Packard reagent 6008012) in toluene as the scintillation fluid.

Procedures for enzyme assays in cell extracts: Cell lysates contained 20–30 $\times$ 10^6 cells per ml of 0.01 *M* Tris buffer, pH 8.5.[7] Citrulline conversion to arginine was measured by the determination of urea production in the presence of excess arginase. Urea was detected colorimetrically by the method of Archibald as modified by Ratner.[9] The combined argininosuccinate synthetase and argininosuccinase system was measured in lysates of cells grown on complete medium, arginine-free medium, and arginine-free medium supplemented with citrulline. Urea production was found to be proportional to both time (1 and 2 hr) and enzyme concentration (0.1 and 0.2 ml lysate).

The Michaelis constant (K_m) for citrulline of the argininosuccinate synthetase enzyme was determined with ureido-C^{14}-citrulline (spec. act. 1.15 μc/μmole) by measuring C^{14}-urea production at citrulline concentrations of 0.125–10 mM. Enzyme activities were determined by incubating 0.1 ml of cell lysate in a final volume of 0.2 ml at 37° for 1 hr. The final reaction mixture based on the assay procedure described by Schimke[10] contained 80 mM Tris buffer, pH 8.5, 5 mM aspartic acid, 2.4 mM $MgCl_2$–6 H_2O, 2.4 mM adenosine triphosphatase (ATP), 10 mM phosphoenolpyruvic acid, 20 mM KCl, 0.1 mg/ml pyruvatekinase (Sigma, 1 mg converts 3.8 μmoles phosphoenolpyruvate/min at pH 7.6, 37°), 100,000 units/ml arginase (Sigma, 1 mg converts 20 μmoles arginine/min at pH 9.5, 25°) and 400 units/ml argininosuccinase. The argininosuccinase was prepared from hog kidney and was generously supplied by Dr. Robert Schimke. An identical incubation system that contained boiled lysate was used as a control for each concentration of citrulline tested. At the lowest concentration of substrates used in these experiments the maximum substrate utilization did not exceed 10%.

After incubation a protein-free supernatant solution was obtained by heating the incubation mixtures at 100° for 5 min and then centrifuging them. The silica gel on an 8 $\times$ 8 in. thin-layer sheet (Chromagram, Rochester, N.Y., Eastman type K-301-R-2) was scribed into 12 channels so that there were gel-free spaces between each sample. Aliquots (20 μl) of the supernatant solutions from the incubations were spotted in each channel and carrier urea (10 μg) was added to each spot. Authentic urea and citrulline were spotted on the outside channels of each sheet with a 20-μl portion of protein-free supernatant solution.

The chromatograms were developed in isopropanol-ammonia-water (20:1:2, v/v/v) and usually required 2.5–3 hr for a solvent migration of 15 cm. The channels containing standards were cut from the edges of the sheet and sprayed with Ehrlich's reagent. Citrulline had an R_f of 0–0.06; the R_f of urea varied from 0.46 to 0.62 but was uniform in all channels on the same sheet. The channels with the various supernatant solutions were cut into individual strips, taped to filter-papers, and scanned (Nuclear-Chicago strip scanner) to locate the radioactive peak corresponding to urea. This area was then cut from the strips and placed directly into 15 ml of scintillation fluid and counted.

TABLE 1

CELL GROWTH IN VARIOUS MEDIA

Cell line	Expt. date	Cell Yields × 10^6 — Medium: Complete	Arginine-free	Arginine-free + citrulline
Control	1-13-66	27	14 (52%)	26 (96%)
	2-3-66	25	9 (36%)	23 (92%)
Citrullinemia	1-7-66	97	35 (36%)	18 (19%)
	1-26-66	60	21 (35%)	24 (40%)

In each experiment a cell line was subcultured into 12 bottles, 4 contained complete medium which included 0.2 mM arginine, 4 arginine-free medium, and 4 arginine-free medium supplemented with 0.2 mM citrulline. All 12 subcultures were harvested simultaneously. The cells from 4 bottles subcultured in a particular medium were pooled and counted. Percentages represent the fraction of growth observed on complete medium.

Results.—Rates of cell growth: Growth as estimated by cell counts was poorer in the cultures grown on arginine-free media (35–52% of growth on complete media) for both control and citrullinemia cell lines. When the arginine-free medium was supplemented with citrulline, the control cells grew as well as on the complete medium, while the citrullinemia cells did not improve their growth (Table 1).

Incorporation of C^{14}-citrulline into TCA-precipitable cell material: Significant incorporation of C^{14} into cell protein (TCA-precipitable fraction) was observed after control cells were incubated with medium containing C^{14}-citrulline (28 cpm/μg DNA), while the same fraction from the citrullinemia cells had little radioactivity (0.5 cpm/μg DNA). (Table 2, expt. 1.) Solubilizing the TCA precipitate of control cells in the presence of unlabeled citrulline and reprecipitating twice did not significantly decrease the radioactivity of the TCA-precipitable fraction (Table 2, expt. 2).

Enzyme activities in extracts of cultured cells: The enzymatic conversion of citrulline to arginine was similar in the control and citrullinemia cell lines at citrulline concentrations of 10 mM, based on the colorimetric determination of urea formation in the presence of excess arginase. The range of activity of the cell preparations was 0.01–0.03 μmoles urea formed/hour/10^6 cells (Table 3).

Arginosuccinate synthetase activity at various citrulline concentrations, as measured by radioassay, revealed distinct differences in the relationship between activity and substrate concentration of the control and citrullinemia cells (Fig. 1).

TABLE 2

C^{14}-CITRULLINE UPTAKE INTO CELLS AND INCORPORATION INTO CELL PROTEIN

Experiment 1

Fraction	Control (Total cpm)	Control (Cpm/μg DNA)	Citrullinemia (Total cpm)	Citrullinemia (Cpm/μg DNA)
TCA-soluble	1072	7.85	302	1.28
TCA-precipitable	3827	28.00	126	0.50

Experiment 2

Fraction	Control A (Total cpm)	Control A (Cpm/μg DNA)	Control B (Total cpm)	Control B (Cpm/μg DNA)
TCA-soluble	102	1.24	72	1.16
TCA-precipitable	618	7.54	352	5.68
Combined supernatants after reprecipitations	—	—	30	0.48

Control and citrullinemia cells were incubated with C^{14}-citrulline and the radioactivity measured in the TCA-soluble and TCA-precipitable fractions of the cells. Expt. 1 compares control and citrullinemia cells. Expt. 2 tests the possibility of nonspecific binding of C^{14}-citrulline to the TCA precipitate. A suspension of control cells was divided into two equal fractions (*A* and *B*). Fraction A was treated as in expt. 1. The TCA precipitate of fraction B was solubilized and reprecipitated twice in the presence of unlabeled citrulline.

TABLE 3

CONVERSION OF CITRULLINE TO ARGININE BY CELL EXTRACTS FROM CELLS GROWN ON VARIOUS MEDIA*

Cell line	Medium	Urea Production (μmoles/hr/10^6 cells)	
Control	Complete	—	0.018
	Arginine-free	0.028	0.026
	Arginine-free with citrulline	0.019	0.010
Citrullinemia	Complete	0.023	0.026
	Arginine-free	0.017	0.021
	Arginine-free with citrulline	0.019	0.019

The conversion of citrulline to arginine was estimated by the colorimetric determination of urea in the presence of excess arginase. The reaction system contained 10 mM citrulline, 5 mM aspartic acid, 2.4 mM $MgCl_2$ $6H_2O$, 2.4 mM ATP, 10 mM phosphoenolpyruvic acid, 20 mM KCl, 0.1 mg/ml pyruvate kinase, 100,000 units/ml arginase and 0.2 ml cell lysate. Final volume 0.4 ml. Temperature 37°. Incubation time 1 hr. Comparable amounts of urea were formed when argininosuccinic acid was used instead of citrulline in the reaction system.

Blanks consisted of a tube in which the cell lysate had been boiled for 5 min before adding it to the incubation mixture, and a tube in which citrulline was deleted from the incubation medium.

* Same cells as those used for experiments presented in Table 1.

The Michaelis constants, as derived from the data by the Lineweaver-Burk method, showed marked differences between enzyme in control and citrullinemia cells. The K_m for the control cell enzyme preparations was 4×10^{-4} M and for the citrullinemia cells was between 10 and 100×10^{-3} M (Fig. 2).

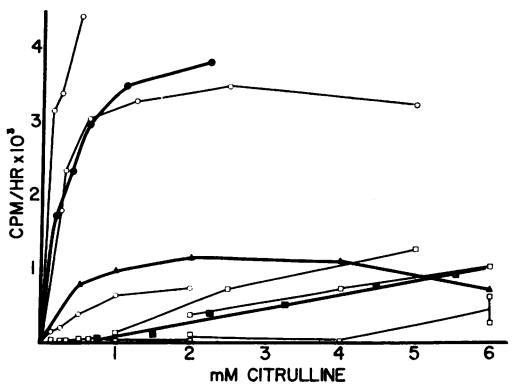

FIG. 1.—The effect of citrulline concentration on arginino succinate synthetase activity. Two control cell lines (●,▲) and the citrullinemia cell line (■) were used in these experiments. Open symbols and their connecting lines represent individual experiments. Solid symbols and bold connecting lines are the average of the individual experiments. Enzyme activity expressed as urea (cpm) produced per hour per 10^6 cells at 37°.

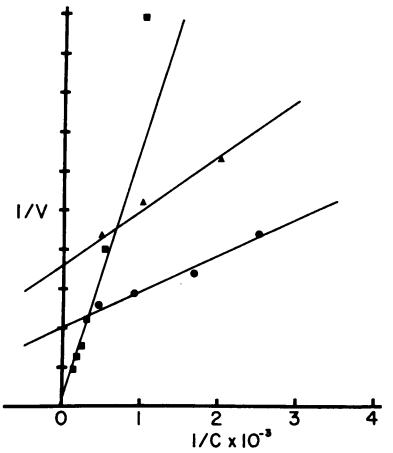

FIG. 2.—The effect of citrulline concentration ($1/C$) on argininosuccinate activity ($1/V$) in extracts of control and citrullinemia cells (data taken from averages presented in Fig. 1). Control cells, ●, ▲; citrullinemic cells, ■.

Discussion.—Experiments with a cell line derived from the skin of a child with citrullinemia have revealed that in these cells citrulline cannot substitute for arginine in the medium when the indicator of citrulline utilization is the rate of cell growth. In contrast, equimolar amounts of citrulline can substitute for arginine in control cell cultures. When radioactive citrulline is incubated with a monolayer of citrullinemia cells, incorporation of label into the acid-precipitable cell fraction was less than 10 per cent of that found with comparably prepared control cells. Schimke has shown that the label from ureido-C^{14}-citrulline incorporated in this

manner is primarily in the cell protein as labeled arginine.[10] This is further evidence that the citrullinemia cells do not utilize citrulline normally.

In contrast to Schimke's experience with several mammalian cell lines,[10] an increase in the activity of the citrulline to arginine pathway (Table 3) was not observed in our cells when deprived of arginine even though the growth of these cells was slowed by lack of arginine (Table 1).

Enzyme assays of citrullinemia cell extracts reveal that synthesis of arginine from citrulline is normal when high citrulline substrate concentrations are present in the incubation system, but the activity is markedly reduced when the substrate concentration is lowered. The K_m obtained for citrulline in the argininosuccinate synthetase reaction for control cell extracts is similar to that reported by Ratner for rat liver enzyme,[11] while the K_m of the citrullinemia cell enzyme is between 25 and 250 times that of the control cells. The degree of difference in K_m as measured in the cell extracts is clearly of biological significance, as reflected in the metabolic disturbance of the affected infant[2] and the inability of citrulline to support the growth of cells from this infant in culture. That citrulline might be utilized in these experiments by pathways other than ASA synthetase was considered. Such utilization appears unlikely because there was no detectable ornithine transcarbamylase[10] activity in either the control or mutant cells under the conditions of these experiments.

This apparent inborn error of metabolism thus appears to be the result of decreased substrate affinity rather than an absence of enzyme protein. That this is a genetic defect is demonstrated by the persistence of the metabolic disturbance in cells under long-term cultivation from an individual who demonstrates a similar biochemical disorder *in vivo*. Definition of the precise molecular defect in this disease requires purification of the enzyme. This cell line could provide an adequate source of enzyme protein for this purpose. In addition this line of human diploid cells contains a potentially useful genetic marker in that it can be placed at a growth disadvantage with selective media.

Summary.—A fibroblast cell line from the skin of an infant with citrullinemia has been used to study the biochemical defect of this inborn error of metabolism.

The growth of cell cultures was slowed when arginine was removed from the medium of both control and citrullinemia cell lines. This growth inhibition was prevented in control cells but not in citrullinemia cells by the addition of citrulline to the culture medium. Control cells incorporated C^{14} from citrulline into TCA-precipitable cell fractions, while citrullinemia cells showed essentially no incorporation.

Enzyme preparations from citrullinemia and control cells demonstrated similar capacities for converting citrulline to urea (argininosuccinate synthetase activity) when citrulline was present in high concentrations. There was no change in argininosuccinate synthetase activity when cells were grown in arginine-free medium or when citrulline was substituted for arginine in the medium. The citrullinemia cells had no argininosuccinate synthetase activity at citrulline concentrations in the range of the K_m for control cells. The citrulline K_m for the citrullinemia cells was at least 25 times greater than that for the normal cells

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