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Argininosuccinate synthetase
citrulline
citrullinemia
fibroblasts
hemocitrulline
lymphoblasts
urea cycle

# Citrullinemia: Enzymatic Evidence for Genetic Heterogeneity

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## Extract

**The specific activity of argininosuccinate synthetase (micromoles of $^{14}CO_2$ per milligram of protein per hour) was 0.00104 and 0.00087 in fibroblasts derived from two patients with citrullinemia, and was undetectable in both fibroblasts and cultured lymphocytes from a third patient. In five obligate heterozygotes the specific activity in fibroblasts was 0.012–0.029 and in nine control subjects was 0.058 ± 0.014 (0.030–0.076). In both control and patient cells, the maximum activity was obtained at pH 8.5 and there was no inhibition of normal argininosuccinate synthetase by any of the mutant cells.**

**Kinetic studies were consistent with decreased binding of citrulline and/or aspartate in fibroblasts from all three patients. The $K_m$ for citrulline was $4.2 \times 10^{-3}$ and $>2.0 \times 10^{-2}$ M for the enzyme from patients 1 and 2, respectively (normal $1.1 \times 10^{-4}$ M). Corresponding $K_m$ values for aspartate were $1.8 \times 10^{-2}$ and $>6.5 \times 10^{-3}$ M, respectively (normal $3.4 \times 10^{-5}$ M). Hill constants derived from the citrulline substrate curves from patients 1 and 2 were 1.01 and 1.50 (normal 0.91). Corresponding constants obtained from aspartate binding curves were 1.06 and 1.35, respectively (normal 0.97).**

**The activity of homoargininosuccinate synthetase was undetectable in fibroblasts from all three patients and a control subject.**

## Speculation

**Heterogeneity of the primary enzyme defect will become a frequent observation in many inborn errors of metabolism. In disorders of the urea cycle, alternative and as yet unknown pathways of urea formation may be discovered.**

Citrullinemia is an inherited disorder of the urea cycle associated with deficient activity of argininosuccinate (ASA) synthetase (L-citrulline: L-aspartate ligase, EC 6.3.4.5). This enzyme occurs primarily in liver, but also in kidney, brain, and cultured skin fibroblasts. The clinical severity of the 12 reported cases of citrullinemia has varied from severe, with death in the neonatal period, to mild, with retarded or even normal mental development (2). In some patients severe hyperammonemia has been prevented by protein restriction; three such patients have been retarded, although one treated from an early age appears to be developing normally (3). The clinical severity of the disease has not appeared to correlate with the serum citrulline levels, or with the blood or urinary urea, which have been normal in some patients and low in others. Genetic heterogeneity of the primary enzyme defect has not been investigated. This report presents the range of ASA synthetase activity in citrullinemic patients, heterozygotes, and control subjects. It describes the results of a study of the kinetic properties of the mutant enzyme in cultured skin fibroblasts from three unrelated patients with citrullinemia, and attempts to correlate the findings with the clinical disease. Investigations of the proposed "outer urea cycle," based on the conversion of homocitrulline to homoarginine and then to urea, are also included.

## EXPERIMENTAL PROCEDURE

### MATERIALS

L-[*carbamoyl*-$^{14}$C]Homocitrulline was prepared from [$^{14}$C]cyanate and lysine by the method of Smith (22).

L-[*carbamoyl*-$^{14}$C]Citrulline (61 mCi/mmol) was obtained from Amersham-Searle and freed from contaminating urea by chromatography on Dowex 50-X12. The urea fraction was eluted with 0.1 N pyridine formate, pH 2.5, and citrulline with 0.1 N pyridine formate, pH 3.5. After lyophilization, the specific activity was adjusted to 0.05 mCi/mmol.

Urease type VI and pyruvate kinase type II were obtained from Sigma Chemical Company and arginase from Worthington Biochemical Corporation. Argininosuccinase was prepared from human liver by the method of Ratner (14) as far as the heat denaturation step, followed by repeated ammonium sulfate precipitations at 30% saturation until free of argininosuccinate synthetase activity.

### ARGININOSUCCINATE SYNTHETASE

ASA synthetase activity was measured by the radiochemical procedure of Schimke (18). The incubation mixture contained Tris buffer, pH 8.5 (100 mM), $MgCl_2$ (2.4 mM), KCl (20 mM), phosphoenolpyruvate (10 mM), ATP (2.4 mM), L-aspartate (5 mM), and [$^{14}$C]citrulline (10 mM). Arginase (158 U/ml), pyruvate kinase (6 U/ml), and argininosuccinase (1.5 U/ml) were included. The amounts of added enzymes were in excess within the range of activities measured. Samples were incubated for 1 hr and the reaction stopped by boiling for 1 min. The [$^{14}$C]urea formed was converted to $^{14}CO_2$ and trapped as described (18). Blanks containing boiled sample gave the same values as blanks containing no sample. Background values at 10 mM citrulline were around 400 dpm/assay. For control cells, the amount of protein used per assay was 0.1–0.3 mg. The reaction was linear with sample within this range. For patient samples, 0.4–1.4 mg protein was used/assay. The reaction was also linear with time up to at least 1 hr. Confidence limits for individual assays were calculated from duplicate or triplicate determinations from which the mean blank value had been subtracted.

### HOMOARGININOSUCCINATE SYNTHETASE

Activity of homoargininosuccinate synthetase was measured as described for ASA synthetase except that [$^{14}$C]homocitrulline was substituted for [$^{14}$C]citrulline in the assay.

### REFERENCE ENZYMES

Lactic dehydrogenase (LDH) activity was measured at 37° by a

modification of the method of White (29). The reaction mixture contained 0.01 M phosphate buffer, pH 7.4, 2.3 mM sodium pyruvate, and 0.13 mM DPNH in a final volume of 1 ml. The change in absorbance during the first 5 min of reaction was measured on a Gilford model 2400 recording spectrophotometer.

The activity of 6-phosphogluconate dehydrogenase (6-PGD) was determined at 37° by a modification of the method of Glock and McLean (6). The incubation mixture contained 12.7 mM Tris buffer, pH 8.0, 16.7 mM $MgCl_2$, 3.33 mM 6-phosphogluconate, and 0.25 mM NADP in a final volume of 1 ml. The change in absorbance was recorded as above.

### PROTEIN

Protein was determined by the method of Lowry (10).

### PATIENTS

A detailed clinical report of *patient 1* has been published (3). Cells from *patient 2* (13) were obtained from the American Type Cell Repository. Cells from *patient 3* and his parents were obtained from Dr. J. V. Higgins (20). The five heterozygotes comprise both parents of *patients 1* and *3* and the maternal grandmother of *patient 1*, previously diagnosed as a carrier by the elevated fasting citrulline level in her plasma (3). Control cells were obtained from a variety of normal subjects and patients with disorders unrelated to nitrogen metabolism.

### FIBROBLAST CULTURES

Skin fibroblasts were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum. Cultures were fed every 2–3 days until confluent, then daily, and were harvested at 16–21 days after confluency. Lysis of the washed cell pellet was achieved by freeze-thawing four times in water. All enzyme assays were performed on the crude homogenates on the day of harvesting.

Heterozygote and control cell lines had been passaged 3–10 times with the exception of one control which had been passaged 20 times. Cells from *patients 1*, *2*, and *3* were at passage 8–10, 18–20, and 6–14, respectively, when studied. There was no correlation between the number of passages and the activity of ASA synthetase in control cells.

Mycoplasma testing was performed by Dr. L. L. Coriell (30), Dr. L. Hayflick (31), or Dr. G. Kenny (32). Cells from the three patients, five heterozygotes, and several of the controls were tested for the presence of mycoplasma. All were negative on all occasions tested.

### CULTURED LYMPHOCYTES

Peripheral blood lymphocytes from *patient 3* and a control were established in long term culture and provided to us as frozen cell pellets by Dr. A. D. Bloom. The growth conditions of these cells and their behavior under selective conditions *in vitro* have been published by Spector and Bloom (23).

## RESULTS

The activities of ASA synthetase, LDH, and 6-PGD in tissues from three citrullinemic patients, five heterozygotes, and nine control subjects are shown in Table 1. Except for *patient 3*, all individual values of ASA synthetase were significant (above background) at the 95% confidence limits; most were significant at the 99% limits.

The activity of ASA synthetase in both normal subjects and patient's cells was measured at pH 6.5, 7.5, 8.5, and 9.5. In all cases, maximum activity was obtained at pH 8.5. Mixing experiments between normal and patient cell lysates indicated no inhibition of normal ASA synthetase activity by any of the mutant cells.

Experiments were carried out to determine the effect of increased concentrations of two substrates, L-aspartate and L-citrulline, on the activity of ASA synthetase in the patient's cells. These results are shown in Figure 1. The specific activity of ASA synthetase in *patient 3* was so low that the value was not significantly different from the blank on any of five occasions tested under standard assay conditions or at 25 mM aspartate but was significant at 50 mM citrulline.

Further kinetic studies of the normal and mutant enzymes from *patients 1* and *2* are shown in Figure 2. Hill constants derived from the citrulline and aspartate substrate curves were 1.50 and 1.35, respectively, for *patient 2*. Corresponding values for control liver were 0.97 and 0.91, control fibroblasts 0.91 and 0.97, and *patient 1* fibroblasts 1.01 and 1.06, respectively. The apparent $V_{max}$ and $K_m$ or $S_{0.5}$ values obtained from these binding curves are shown in Table 2 and compared with the values obtained from two other tissues.

Cultured blood lymphocytes from *patient 3* and a control subject were examined for ASA synthetase activity (Table 1). Under standard assay conditions, the specific activity in the cells from

Table 1. *Specific acitivity of argininosuccinate (ASA) synthetase, lactic dehydrogenase (LDH) and 6-phosphogluconate dehydrogenase (6-PGD), in tissues from three patients with citrullinemia, five known heterozygotes, and nine control subjects*[1]

| Subject | Tissue | ASA synthetase, μmol $^{14}CO_2$/mg protein/hr | LDH μmol/mg protein/min | 6-PGD μmol/mg protein/min |
|---|---|---|---|---|
| *Patients* | | | | |
| *1* | Fibroblast | 0.00104 | 7.5 | 0.027 |
| *2* | Fibroblast | 0.00087 | 5.9 | 0.031 |
| *3* | Fibroblast | N.S.[2] | 6.3 | 0.030 |
| *3* | Lymphoblast | N.S. | | |
| Heterozygotes | | | | |
| Mother of *patient 1* | Fibroblast | 0.012 | 8.5 | 0.029 |
| Father of *patient 1* | Fibroblast | 0.021 | 10.2 | 0.048 |
| Grandmother of *patient 1* | Fibroblast | 0.017 | | 0.027 |
| Mother of *patient 3* | Fibroblast | 0.017 | 7.3 | 0.034 |
| Father of *patient 3* | Fibroblast | 0.029 | 5.7 | 0.027 |
| Control subjects ($n$ = 9) | Fibroblast | 0.058 ± 0.014 (0.030–0.076) | 8.78 ± 1.51 (6.63–10.60) | 0.034 ± 0.007 (0.027–0.047) |
| | Lymphoblast | 0.059 | | |

[1] Control values are expressed as mean ± SD with the range shown in parentheses.

[2] NS.: not significantly different from blank at the 95% confidence limits.

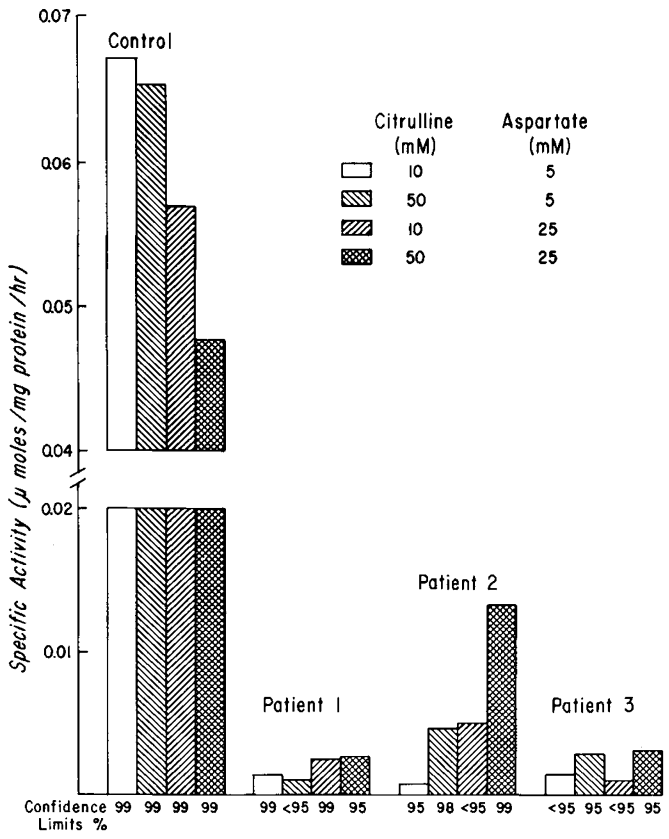


*Fig. 1.* Specific activities of argininosuccinate synthetase from normal and mutant fibroblasts under standard assay conditions (5 mM aspartate and 10 mM citrulline) and at increased concentrations of substrate. The confidence limits for each individual assay were calculated from duplicate or triplicate determinations from which the mean blank value had been subtracted.

*patient 3* was virtually undetectable. No measurable activity was obtained when the concentration of aspartate was increased to 25 mM or when the ATP was increased to 12 mM. At 50 mM citrulline, a low activity was discerned; however, it was not significantly different from the blank at the 95% confidence limits.

Fibroblast lysates were examined for activity of homoargininosuccinate synthetase at 10 mM homocitrulline in samples from one control subject and *patients 2* and *3*, and at 50 mM homocitrulline in samples from the same control subject and from *patients 1* and *3*. In all cases, the specific activity was $<0.005$ μmol/mg protein/hr and was not statistically significant.

## DISCUSSION

The very low levels of ASA synthetase activity in the citrullinemic fibroblasts and the intermediate levels of activity in cells from the heterozygotes are shown in Table 1.

Investigation of the effects of raised substrate concentrations on the activity of ASA synthetase in the patients' cells (Fig. 1) was consistent with altered binding of aspartate to the enzyme from both *patients 1* and *2* and of citrulline to the enzyme from *patients 2* and *3*. As shown in Figure 2, the enzyme from *patient 2* exhibited sigmoidal characteristics with respect to both citrulline and aspartate binding in comparison with both the normal subject and *patient 1*. Cooperativity of binding was further illustrated by the elevated Hill constants derived from the curves for *patient 2*. The sigmoidal shape of the citrulline binding curve confirms the findings of Tedesco and Mellman (26) with these cells; aspartate binding had not been studied previously.

Comparison of the $V_{max}$ and $K_m$ or $S_{0.5}$ values obtained from the binding curves (Table 2) showed that the $K_m$ for citrulline varied somewhat depending on the tissue examined. The value obtained for liver was approximately twice that found by Rochovansky and Ratner (15) using a purified preparation from bovine liver, whereas the value for fibroblasts was approximately one-fifth that previously reported (26). Alteration of the aspartate concentration from 5 mM to 25 mM had little effect on the $K_m$ for

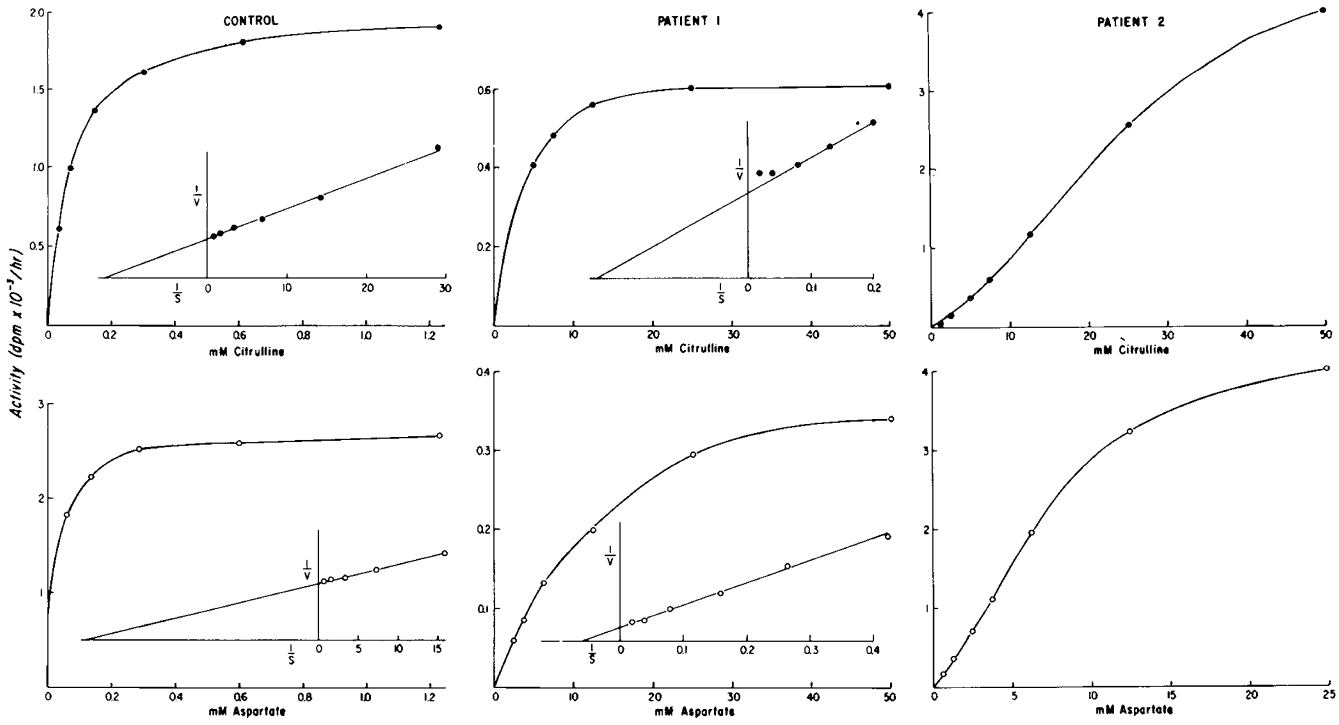


*Fig. 2.* The effect of citrulline (●——●) and aspartate (○——○) concentrations on argininosuccinate synthetase activity in normal and mutant fibroblasts. Citrulline binding curves were obtained at 5 mM aspartate for normal and 25 mM aspartate for mutant cells. Aspartate binding curves were obtained at 10, 20, and 50 mM citrulline for the enzyme from the control subject, *patient 1*, and *patient 2*, respectively. With control cells, a correction (7) was made for the amount of substrate used in the reaction (maximum 25% for citrulline binding and 40% for aspartate binding). With mutant cells, the amount of substrate used was less than 1%.

Table 2. *Kinetic properties of argininosuccinate (ASA) synthetase in tissues from control and citrullinemic patients*[1]

| Subject | Tissue | $V_{max}$, μmol/mg protein/hr | $K_m$ (citrulline), M | $K_m$ (aspartate), M |
|---|---|---|---|---|
| Control | Liver | 0.83 | $1.0 \times 10^{-4}$ (0.005) | $4.2 \times 10^{-5}$ (0.010) |
| Control | Lymphoblast | 0.086 | $1.5 \times 10^{-4}$ (0.005) | $3.5 \times 10^{-5}$ (0.010) |
| Control | Fibroblast | 0.065 | $7.9 \times 10^{-5}$ (0.005) | $3.4 \times 10^{-5}$ (0.010) |
| Control | Fibroblast | | $1.1 \times 10^{-4}$ (0.025) | |
| *Patient 1* | Fibroblast | 0.0086 | $4.2 \times 10^{-3}$ (0.025) | $1.8 \times 10^{-2}$ (0.020) |
| *Patient 2* | Fibroblast | >0.025 | $>2.0 \times 10^{-2}$ (0.025)[2] | $>6.5 \times 10^{-3}$ (0.050)[2] |

[1] $K_m$ values for the control and *patient 1* enzyme were determined from plots of 1/V against 1/S. The $S_{0.5}$ (the concentration of substrate giving one-half the maximum observed rate) for the enzyme from *patient 2* was estimated from curves of V plotted against S. Concentrations of fixed substrate are shown in parentheses. With control cells a correction (7) was made for the amount of substrate used in the reaction (maximum 25% for citrulline binding and 40% for aspartate binding). With mutant cells, the amount of substrate used was less than 1%. $V_{max}$ was estimated from the same curves with aspartate as the variable substrate in all cases.

[2] Values depict $S_{0.5}$ rather than $K_m$.

citrulline in control fibroblasts (Table 1). Both mutant cell lines examined here showed a decreased affinity for both citrulline and aspartate. The $K_m$ for citrulline in *patient 1* was approximately 40 times normal whereas the $K_m$ for aspartate was 500 times normal. The $S_{0.5}$ for citrulline in *patient 2* was over 200 times normal and agrees well with the value previously reported (26). The $S_{0.5}$ for aspartate in these fibroblasts was also over 200 times normal. It can be seen that the $V_{max}$ obtained with these cells approaches the normal range.

The development of an "outer urea cycle," based on the conversion of lysine via homocitrulline and homoargininosuccinic acid to homoarginine and thence to urea, has been proposed to explain the increased excretion of homocitrulline and homoarginine as well as the normal production of urea in *patient 3* (19, 20). The evidence for such a cycle includes the following: lysine can be converted to homocitrulline in rat liver (16, 21); homocitrulline is a substrate for argininosuccinate synthetase from bovine liver although the activity is about 10% of that obtained with citrulline (15); small amounts of homoargininosuccinic acid have been synthesized from homoarginine and fumaric acid using an enzyme prepared from pig kidney, suggesting that the reverse reaction might also take place (24); finally, homoarginine can be converted to urea and lysine in rat liver (16).

We attempted to examine the possible conversion of homocitrulline to urea by substituting [*ureido*-$^{14}$C]homocitrulline for citrulline in our assay system. Using this modification, the production of $^{14}CO_2$ would depend upon the conversion of any homoargininosuccinic acid formed to homoarginine and then to urea. No significant activity could be discerned in either control or citrullinemic cells. These findings, taken in conjunction with the very low activities of the proposed steps of the "outer urea cycle" support the conclusion of Levin *et al.* (9) that this is not likely to be a significant pathway for the formation of urea. Since lysine can be converted to homocitrulline and homoarginine in adult humans, the latter by a transamidination reaction in kidney (17), the increased excretion of homoarginine and homocitrulline may simply be secondary to the elevated levels of lysine in *patient 3*. Whether this elevation is caused by inhibition of normal lysine catabolism by citrulline (9), or to the fact that lysine is a normal substrate for ornithine transcarbamylase (4) is of little consequence to the present problem. The question that remains to be answered is how *patient 3* is able to maintain normal blood ammonia levels and produce normal amounts of urea.

Several conclusions can be drawn from the results presented here. First, the defect in each patient appears to be the result of a structural gene mutation and not a regulator gene mutation. Apart from the original study on fibroblasts from *patient 2* (26), there are few documented examples of a human disease caused by a mutant enzyme with altered kinetic properties. Cases where such findings have been demonstrated include the Lesch-Nyhan syndrome (12), pyruvate kinase deficiency (25), glucose 6-phosphate dehydrogenase deficiency (11), and ornithine transcarbamylase deficiency (1, 5, 8, 27). Second, we can conclude from kinetic evidence that the primary defect in all three patients is genetically distinct. The very low levels of enzyme activity attest to the severity of the disease in *patients 1* and *2*. Increased levels of activity at high substrate concentrations are not likely to be realized *in vivo*. However, therapy designed to increase the intracellular levels of aspartate might be beneficial to these patients.

The data for *patient 3* remain inconclusive, in part because of the difficulty of growing large numbers of fibroblasts for study. These cells always grew slowly and never achieved the cell densities reached by normal cells or those from *patients 1* and *2*, which were grown under identical conditions. The low activity of argininosuccinate synthetase in lymphocytes grown in long term culture supports the findings in fibroblasts and, moreover, the inability of these lymphocytes to grow in medium in which arginine is replaced by citrulline (23) supports the findings of low enzyme activity in these cells. The apparent increase in activity at 50 mM citrulline, in these cells as well as in fibroblasts, probably reflects a structural alteration of this mutant enzyme. The very low activity does not explain the relatively mild disease in this patient or account for the near normal production of urea. We have not been able to provide support for the concept of an "outer urea cycle," and the possibility of other, as yet unknown, mechanisms of urea synthesis must still be considered. Our conclusions to date have been based on the assumption that the diminished levels of enzyme activity in cultured skin fibroblasts reflect the levels *in vivo* in liver and other tissues. There is one report of a patient with citrullinemia in whom activity of ASA synthetase was undetectable in liver, but active in kidney (28). The occurrence of such isozymes could provide an alternative explanation for the clinical heterogenity seen in citrullinemia and account for the findings in *patient 3*.

## SUMMARY

Argininosuccinate synthetase is greatly reduced in fibroblasts from three citrullinemic patients and in cultured lymphocytes from one patient. Kinetic studies are consistent with decreased binding of citrulline and/or aspartate in cells from all three patients and sigmoidal binding of both substrates in one patient. No support could be obtained for the concept of an "outer urea cycle" based on the conversion of homocitrulline to homoarginine and then to urea. The kinetic results suggest that the enzyme defect in each patient is genetically distinct.

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36. Accepted for publication March 11, 1975.



*Corrigendum*

## Excessive Thyrotropin Response to Thyrotropin-releasing Hormone in Pseudohypoparathyroidism

By Werder *et al.*

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p. 13—Footnote 4 to Table 1 should have appeared as "Transient hypoparathyroidism."
p. 13—The transposed paragraphs should have appeared as follows:

### PSEUDOPSEUDOHYPOPARATHYROIDISM

These three probands were adult relatives (two mothers, one maternal uncle) of patients with pseudohypoparathyroidism whose clinical findings (brachymetacarpia, ectopic calcifications) suggested the same disorder. Because of their definite, although intermediate, cAMP response to PTE, they were classified as pseudopseudohypoparathyroid cases (4). In one case (*SM*) with moderate hypocalcemia, plasma PTH slightly elevated.

### IDIOPATHIC HYPOPARATHYROIDISM

Eight patients, including one pair of siblings (*BK*, *BA*), were tested. The diagnosis was based on documented hypocalcemia and hyperphosphatemia in all cases and on undetectable (5 cases) or low PTH (3 cases). The latter 3 patients showed normal urinary cAMP response to PTE infusion.

### CONTROL SUBJECTS

TSH response to TRH was analyzed in 28 euthyroid children. In four patients aged 5.8–17.8 years with minor brachymetacarpia, cAMP response to PTE infusion was studied. The results of the decrease in tubular reabsorption of phosphate (ΔTRP) after intravenous PTE were compared with those obtained from 13 control patients of a previous study (14). The other control values appearing on Table 1 represent the experience of our laboratories. (Informed consent was obtained on all subjects tested.)