Enzymologic and Metabolic Studies in Two Families Affected by Argininosuccinic Aciduria

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Summary

Both the affected families studied provide another example of the autosomal recessive inheritance of argininosuccinic aciduria. The fasting plasma levels of argininosuccinic acid in the two probands did not correlate with the levels of argininosuccinic acid lyase (ASAL) in erythrocytes. There was 210 μ M argininosuccinic acid with indications of anhydride B content in the family I proband, having an enzyme activity of 13%; while the family II proband gave an argininosuccinic acidemia reading of 64.6 μ M with no activity of RBC ASAL. There was a reduced enzyme activity in all the members of affected families due to a significantly reduced $V_{\max}$ value as compared to control. Within family I, the differences were solely determined by the $V_{\max}$ of the enzymes. In family II, the two siblings had a significantly reduced K_m value as compared to the parents (0.09 and 0.10 against 0.16 and 0.18 mM).

Plasma citrulline and glutamine were generally elevated in the two affected families, particularly in the probands, whereas arginine was normal. A dietary protein-loading trial conducted on members of family II showed more urinary ammonia and α -amino nitrogen excretion as compared to a control family, while urea excretion was comparable. Only the proband showed a urinary excretion of argininosuccinic acid.

Speculation

Research work on the structural and kinetic characterization of human ASAL from various organs and tissues may throw light on the importance of RBC enzyme in the pathogenesis and diagnosis of argininosuccinic aciduria. Such studies will also help in clarifying the regulatory gene hypothesis of ASAL activity in various organs.

Argininosuccinic aciduria, a metabolic disorder of the urea cycle, caused by the deficiency of the enzyme L-argininosuccinic acid lyase (EC 4.3.2.1), is now a well known disease entity (11, 29, 32). Its incidence in newborn screening programs is variously quoted at 1 in 250,000 births (29) to 1 in 70,000 (23). In the Quebec Network for Genetic Medicine program of mass screening for amino acid disorders, two confirmed cases have been discovered; another was found to have transitory argininosuccinic aciduria, and one newborn is under investigation. This is an incidence of 1 in 80,000 newborns in the Province of Quebec in the population tested since 1973 (20).

Since first reported in 1958 (3), observations on 51 children showing argininosuccinic acid excretion and two affected fetuses have appeared in the literature, giving this disease the most extensive incidence amongst hereditary hyperammonemic disorders. The published observations include 32 cases studied in respect of ASAL deficiency in liver, kidney, brain, cultured fibroblasts, or red blood cells. However, the enzyme has been studied mostly in erythrocytes because of its easy accessibility and observed deficiency in the diseased state (4,9,19,26,31,36).

Familial studies on argininosuccinic aciduria have also generally employed ASAL activity measurements in red blood cells. It has been possible to identify the heterozygous or normal relatives of the patient on the basis of the level of active enzyme in erythrocytes (5,7,8,14-17,21,22,25,30,35).

As a part of the Quebec Network of Genetic Medicine program in 1973 we studied two families of French-Canadian origin in which, on routine neonatal screening, one child in each was discovered to excrete argininosuccinic acid. The diagnosis was confirmed by follow-up studies and erythrocyte enzyme measurement in early 1975. This paper describes the results of the familial biochemical, nutritional, and enzymologic studies undertaken recently.

SUBJECTS AND CONTROLS

Affected family I is composed of father and mother ages 35 and 31, male and female siblings ages 9 and 8, and a male proband born November 6, 1973. Family II is composed of father and mother ages 40 and 33, female and male siblings ages 10 and 7 and a female proband born September 14, 1973. No blood sample was received for the female sibling in family I.

The control for enzymologic studies was a healthy male adult. For nutritional investigation, the control family was carefully selected for comparison with family II, considering the composition and age of family members and availability of children of both sexes. Interassay variation was avoided by analysing all samples from subjects and controls in the same batch against common standards.

METHODS

ENZYMOLOGIC STUDIES

The activity of erythrocyte ASAL was measured on the basis of the method proposed by Takahara and Natelson (34). This method was considered more accurate than the method generally reported in literature (35), since it measures the direct product of the enzymatic reaction. It is sensitive to lower levels of enzyme activity and more suited to kinetic studies because no coupling enzyme is involved. The substrate contained argininosuccinic acid (Na^+ salt), buffered to pH 7 with phosphate, 0.2 M. Ethylenediaminetetraacetate (EDTA), 0.2 M, was added to inhibit arginase by complexing manganese (Mn^{++}) and other metals. Hemolyzed erythrocytes were incubated for 1 hr, proteins were precipitated with trichloroacetic acid, and arginine was measured by the application of the Sakaguchi reaction to the filtrate.

Fasting samples of 5-7 ml blood were collected in heparinized Vacutainer tubes by venipuncture and immediately stored at 4° until centrifuged. Enzyme activity was measured within 24 hr of sampling, since whole blood samples have been found to retain erythrocyte ASAL activity up to 24 hr. Hemolysates of washed and packed erythrocytes were prepared in distilled water (dilution 1:5) by repeated freezing and thawing in Dry Ice-methanol

mixture. This method for achieving hemolysis was preferred to the saponin solution used in the original technique (34) due to possible interference in kinetic studies. For point activity measurement, the same final substrate concentration (0.4 mM) was used as recommended in the original method.

In kinetic study measurements, final substrate concentrations ranging from 0.031–1.56 mM were utilized, as compared to 0.1–1.6 mM deduced from the original article. Each substrate level was run in duplicate tubes. Incubation time was kept at 1 hr because of the lower enzyme activity expected in the samples received. The method has been showing good linearity up to 1 hr in earlier studies. This technique for erythrocyte ASAL measurement has consistently shown good reproducibility in our laboratories. The coefficient of variation (CV) for point activity measurement has always been less than 8%, whereas the CV for K_m and V_{max} studies was calculated to be 4.5% and 3.7%, respectively. Erythrocyte arginase was measured by the modification of the Tomlinson and Westall technique (35), proposed by Shih *et al.* (33). Hemoglobin content in packed erythrocytes was determined by the Cyanmethemoglobin method, utilizing a commercial kit (Hycel cyanmethemoglobin reagent, Hycel, Inc., Houston, TX).

Kinetic data was plotted in the form of Lineweaver-Burk plots ($1/(S):1/v$) to obtain $-1/K_m$ on the x-intercept, $1/V$ on the y intercept and K_m/V on the slope of the regression line. The data were also analyzed by the Hofstee method ($v/(S):v$) to obtain $-K_m$ on the slope and by Hanes method ($(S):(S)/v$) to get $1/V$ on the slope (12). The different methods of plotting and analysis were used to isolate various parameters on the slope for statistical comparison. The *t*-test for the comparison of slopes was taken from Dixon and Massey (13).

MEASUREMENT OF PLASMA AMINO ACIDS AND UREA

The plasma obtained after erythrocyte separation was kept frozen at -20° (Dry Ice) until sent for amino acid analysis. Quantitative amino acid determination was done on an automatic TSM amino acid analyzer (Technicon Instrument Corp., Tarrytown, NY) with ion exchange columns. Plasma urea was measured by the diacetyl monoxime-antipyrine technique as adapted by Ceriotti (10).

NUTRITIONAL AND METABOLIC STUDIES

Members of family II, along with a selected control family, were given dietary load of protein through a normal supper intake. The mothers were interviewed in advance by a trained dietician to plan the test menu and receive necessary instructions for keeping intake records on special forms. The test menu, rich in meat and milk products, was designed to provide about 1.5–3.0 g of protein/kg body wt. Intake of the meal was voluntary but was motivated towards maximum possible consumption. The family members were instructed to discard the urine voided just before supper. After the test meal, all urine samples were collected in special plastic bottles containing a small measured quantity of 0.1 N acetic acid to prevent decomposition of the NH_4^+ ion. Total collection was for about 12 hr, with the voiding just before breakfast included. A record of food intake was kept by the mother and delivered the next day along with urine samples.

Protein in the test supper was calculated on the basis of standard tables of food composition (2). Urinary urea nitrogen was measured by the modified Ceriotti technique (10). Creatinine was analyzed on a Beckman/Spinco model 150 ultramicroanalytical system by the alkaline picrate-picric acid technique of Jaffe (6). Urinary ammonia nitrogen was measured by a specific ammonia gas-sensing electrode (24) against ammonium chloride standard. These analyses were completed within 48 hr of urine collection.

Urinary amino acids were analyzed by a technique based on ion-exchange column chromatography (TSM amino acid ana-

lyzer, Technicon). Urinary orotic acid was measured by the technique of Adachi (1) as modified by Goldstein (18). α -Amino nitrogen was determined by the spectrophotometric method of Wells (38).

RESULTS

ACTIVITY OF ERYTHROCYTE ENZYMES

Erythrocyte ASAL measurement in family I showed a residual activity of 13% in the propositus, whereas other family members had an activity ranging from 33–42% of the control value. In family II, the propositus showed no residual activity whereas parents and siblings had 36–45% of the control activity (Table 1).

The activity of erythrocyte arginase was somewhat reduced in the family I propositus, but tended to be normal in all other family members. The family II values at 1641–1812 μmol urea/g Hb/hr were also in the vicinity of control values at 1650 and our laboratory normal range of 1439–1952 μmol /g Hb/hr.

ERYTHROCYTE ASAL KINETICS

The values of K_m and V_{max} obtained by the Lineweaver-Burk plot of kinetic data are presented in Figure 1. As compared to the control value of K_m (0.16), the figures are generally equivalent or slightly reduced. In family II, there is a considerable reduction of K_m in the two siblings, being almost half that of the control. The V_{max} picture is much more clear, with less than 50% of control value in members of both Families. Family I propositus shows the least V_{max} at 0.8 as compared to 5.7

Table 1. Point activity of erythrocyte enzymes in affected families

Subject	ASAL, μmol Arg/g Hb/hr	ASAL, % of Control	Arg, μmol urea/g Hb/hr
Family I			
Father	1.83	42	1778
Mother	1.73	40	1754
Brother	1.43	33	1727
Propositus	0.57	13	1154
Family II			
Father	1.68	39	1676
Mother	1.60	37	1641
Brother	1.57	36	1771
Sister	1.94	45	1666
Propositus	Nil		1812
Control	4.36	100	1650

Fig. 1. K_m and V_{max} values of RBC ASAL from various family members, obtained by calculation of data on the basis of Lineweaver-Burk plot. The control values are from a healthy male adult.

the control. Kinetic study of the enzyme from the family II propositus was not possible because of no activity shown.

Figures 2 and 3 illustrate the differences in K_m and V_{max} in members of the two families in the form of double reciprocal plots. In Figure 2, there is a wide gap in the $1/V$ intercepts between the family I propositus and father as well as the brother, whereas the K_m values are apparently equivalent. In Figure 3, V_{max} values are very near to each other, while the $-1/K_m$ intercepts on the x-axis are wide apart for the parents and siblings.

In order to verify and characterize the apparent differences between various members of the affected families, as brought out by the kinetic parameters of the double reciprocal plot, a statistical analysis was undertaken. The aim was to compare the slopes in the regression line of the plot, for which a relatively simple method is available (13). The slope of the Lineweaver-Burk plot corresponds to the mathematical value K_m/V , which is determined by both K_m and V_{max} variations. For this reason, we isolated the K_m and V_{max} separately on the slope of the regression line, by calculating the data by two methods other than the Lineweaver-Burk plot, as outlined in *Methods*.

The calculated values of K_m and V_{max} , which are somewhat different than those shown in Figure 1 but show a similar trend, along with their statistical comparison (*t*-test) are given in Table 2. The K_m/V comparison shows a significant difference between the control and the affected family members. There is a similar significant difference on comparing the slope corresponding to

Fig. 2. Lineweaver-Burk plot of kinetic data from RBC ASAL of affected family I. All points are means of duplicate determinations.

Fig. 3. Lineweaver-Burk plot of kinetic data from RBC ASAL of parents and siblings from affected family II. All points are means of duplicate determinations. ($P < 0.01$): statistical comparison of slopes (K_m/V).

Table 2. Statistical comparison of kinetic parameters from affected family members against control

Subject	K_m/V	K_m	V_{max}
Family I			
Father	0.054 ¹	0.16	3.2 ¹
Mother	0.066 ¹	0.17	2.7 ¹
Brother	0.058 ¹	0.14	2.4 ¹
Propositus	0.183 ¹	0.16	0.7 ¹
Family II			
Father	0.066 ¹	0.18	2.6 ²
Mother	0.067 ¹	0.16	2.5 ¹
Brother	0.042 ¹	0.09	2.6 ¹
Sister	0.040 ²	0.10	2.3 ²
Control	0.028	0.13	5.8

¹ $P < 0.01$.

² $P < 0.05$.

Table 3. Statistical comparison of kinetic parameters within affected families (*t* values)

Comparison	$b = K_m/V$	$b = -K_m$	$b = 1/V$
Family I			
Father and mother	1.39	0.31	3.08 ¹
Father and brother	1.58	0.68	3.90 ¹
Father and propositus	6.22 ¹	0.17	33.37 ¹
Mother and brother	3.02 ¹	1.12	0.55
Mother and propositus	5.16 ¹	0.37	29.74 ¹
Brother and propositus	8.92 ¹	0.03	32.16 ¹
Family II			
Father and mother	5.15 ¹	0.99	1.66
Father and brother	12.30 ¹	5.70 ¹	0.82
Father and sister	2.90 ¹	3.39 ¹	0.47
Mother and brother	11.33 ¹	3.43 ¹	1.91 ²
Mother and sister	5.26 ¹	2.08 ²	0.50
Brother and sister	0.54	0.28	0.03

¹ $t_{.99} = 2.896$.

² $t_{.95} = 1.860$.

V_{max} value alone. However, the K_m slope comparison does not show any significant differences.

A statistical comparison within the affected family members was also undertaken. Table 3 shows the *t* values obtained on the comparison of slopes bearing K_m/V , $1/V$, or $-K_m$. In family I, there was a significant difference in K_m/V between propositus and other members of the family and between the mother and brother. The K_m value had no significant difference. However, all except one comparison of V_{max} value showed a significant difference. This indicates that within family I, all significant differences were determined by variations in V_{max} alone.

In family II, there was a significant difference ($P < 0.01$) in all comparisons of K_m/V , except between brother and sister. The V_{max} comparison showed only one significant difference ($P < 0.05$) between mother and brother. However, the K_m comparison shows an interesting picture. There is no significant difference between two parents and within two siblings, whereas each parent compared to each sibling shows a significantly different K_m . This indicates that the family II differences are generally dictated by K_m variation and that both the siblings have a different K_m than both parents. This is in contrast to the situation in family I.

PLASMA AMINO ACIDS IN AFFECTED FAMILIES

Plasma aminograms of two families are represented in Tables 4 and 5. There was an increased total plasma amino acid level in members of both families, particularly the propositi. The propositi of family I and II have argininosuccinic acidemia of the level of 210 and 64.6 μM , respectively. The plasma from

Table 4. Plasma amino acid levels in family I (micromolar concentration)

Amino acid	Father	Mother	Brother	Propositus	Normal 2-12 years	Normal adults
Taurine	245	197	167	209	60-110	50-80
Aspartic acid	38.3	42.9	37.8	43.5	20-40	10-40
Threonine	103	161	100	135	40-100	90-140
Serine	155	150	141	214	80-110	80-110
Asparagine	29.6	42.8	30.9	49.3	20-70	
Glutamic acid	320	139	175	143	20-250	50-200
Glutamine	437	764	371	1373	150-720	
Proline	126	194	90.9	148	70-150	150-250
Glycine	195	363	236	526	120-220	150-240
Alanine	493	467	417	691	140-300	350-370
Citrulline	49.6	47.4	65.8	240		tr-30
α -NH ₂ -butyric acid	35.2	11.0	9.2	14.8		10-30
Cystine	1.3	2.6		1.3		0
Valine	403	191	208	150	130-280	50-80
Methionine	26.1	29.5	18.4	31.1	10-20	10-40
Isoleucine	109	48.8	73.2	52.4	30-80	50-80
Leucine	242	130	124	94.6	60-180	100-140
Tyrosine	81.3	58.9	80	66.4	30-70	40-70
Phenylalanine	82.5	60.7	54.8	46.5	30-60	40-70
Ornithine	145	109	87.8	39.5	30-90	580-900
Lysine	266	148	141	120	70-150	160-180
Histidine	94.6	67.2	72.5	90.9	20-80	60-70
Carnosine	7.7			7.7		
Arginine	94.6	63.6	84.6	50.3	20-90	30-60
Argininosuccinic acid				210		
Anhydride B				Present (qualitative)		

Table 5. Plasma-aminoacid levels in family II (micromolar concentration)

Amino acid	Father	Mother	Brother	Sister	Propositus	Normal, 2-12 years	Normal adults
Taurine	410	293	399	322	306	60-110	50-80
Aspartic acid	26.3	21.7	76.7	37.3	46.5	20-40	10-40
Threonine	180	137	161	150	93.2	40-100	90-140
Serine	189	161	215	244	167	80-110	80-110
Asparagine	48.6	20.1	39.4	57.5	12.8	20-70	
Glutamic acid	212	117	189	128	183	20-250	50-200
Glutamine	725	526	588	911	548	150-720	
Proline	150	94.9	131	162	189	70-150	150-250
Glycine	304	262	260	277	367	120-220	150-240
Alanine	533	369	385	445	363	140-300	350-370
Citrulline	89.3	68.6	83.7	133	173		tr-30
α -NH ₂ -butyric acid	3.8	3.2	14.8	19.3	2.4		10-30
Cystine							0
Valine	306	190	344	323	205	130-280	50-80
Methionine	30.7	157	27.6	38.9	69.5	10-20	10-40
Isoleucine	89.7	49.5	111	100	47.2	30-80	50-80
Leucine	185	118	195	222	121	60-180	100-140
Tyrosine	81.9	62.1	96.2	125	66.7	30-70	40-70
Phenylalanine	58.6	51.1	75.1	93.9	76.0	30-60	40-70
Ornithine	116	113	133	132	229	30-90	580-900
Lysine	185	184	195	228	138	70-150	160-180
Histidine	89.5	61.4	77.2	90.8	93.7	20-80	60-70
Carnosine	4.0	6.6					
Arginine	66.8	83.0	82.7	117	102	20-90	30-60
Argininosuccinic acid					64.6		

family I propositus also showed a peak of argininosuccinic acid anhydride B, which could not be quantitated.

Citrulline, the urea cycle intermediate anterior to argininosuccinic acid, is generally elevated in the two families. There is a particularly higher level in the two propoiti. Family I propositus

shows 240 μ M citrulline, as compared to 47.4-65.8 μ M in other family members. In family II, the propositus has a citrulline level of 173 against 68.6-133 in parents and siblings. The plasma citrulline and argininosuccinic acid levels in the propoiti show a similar trend.

Glutamine, another amino acid of particular importance in ammonia detoxication, is generally elevated. In family I it has the highest level in the propositus at 1373 μM as against 371–437 μM in the parents and sibling. The family II figures range from 526 to 911 μM , with the sister of the propositus showing the highest glutamine level. These values indicate a generalized disturbance in ammonia metabolism. The plasma arginine level in members of both families is normal probably due to normal enzyme levels in some of the organs.

Plasma urea nitrogen, ranged between 7.1 to 13.3 mg/dl in family I and 7.7–11.7 mg/dl in family II. The propoiti had 9.6 and 11.1 mg/dl, respectively, which is within normal fasting range. Despite the enzyme block in the urea cycle, the patients with argininosuccinic aciduria have shown normal urea nitrogen concentrations in the plasma (11). Measurement of plasma NH_3 was not attempted due to considerable time lapse between sampling and analysis.

NUTRITIONAL AND METABOLIC STUDIES

As family I was not readily accessible, the dietary protein loading trial was conducted on members of family II, along with a carefully selected control family. Table 6 shows the results of this trial, which was designed to study the urinary nitrogen excretion pattern due to disturbed ammonia metabolism in the affected family. The urinary urea excretion in the affected family ranged between 6.9 and 15.9 mg/mg creatinine, as compared to 9.4–15.1 in the control family. The urinary ammonia excretion at 0.410–2.089 mg/mg creatinine in the affected family was much higher than 0.137–0.222 mg excreted by the control family.

Since we have evidence of a good linear correlation ($r = 0.79$) between the protein intake (1.0–3.0 g/kg body wt) and urinary urea and ammonia excretion (28), we equilibrated the varied protein intake of all family members by calculating the figures on a per gram protein-intake basis. The data presented in Table 6 show that, although the urea excretion pattern was apparently similar, 3–5 times more ammonia was excreted by the affected family members as compared to controls. The propositus excreted 15–20 times more ammonia than the control family and 3–5 times more than the members of the affected family.

The excretion of orotic acid in the affected family members ranged from 0.98 to 4.2 $\mu\text{g}/\text{mg}$ creatinine as compared to control Family figures of 0–2.48, all within the normal level of 6.8 ± 5.54 $\mu\text{g}/\text{mg}$ creatinine (18).

EXCRETION OF URINARY AMINO ACIDS IN FAMILY II

A part of the urine samples obtained from affected family II was analyzed for aminoaciduria. The results of α -amino nitrogen measurement showed a level of 1.15 mg/mg creatinine in the propositus as compared to 0.23–0.51 in the parents and siblings.

Calculated on the basis of per g protein intake, the figures showed approximately 4–5 times (0.76) more α -amino nitrogen excretion in the propositus as compared to parents and siblings (0.14–0.20).

Only the propositus showed a urinary excretion of argininosuccinic acid of the level of 126 mg/dl urine. Anhydrides B and C were also seen in the chromatogram, but could not be quantitated. However, anhydride C had 7.06 times more surface area than anhydride B. Nothing remarkable was seen in the excretion pattern of other amino acids.

DISCUSSION

Both the affected families studied in this paper provide another example of the autosomal recessive inheritance of argininosuccinic aciduria. The parents in both families are obligate heterozygotes, showing 37–42% of erythrocyte ASAL activity. The propoiti are of different sexes, with male and female heterozygous siblings. However, we did not find any normal sibling. This situation is not beyond the limits of probability. The sibling number has to be considerably larger to achieve the theoretic incidence of 2:1:1 for heterozygotes, normal homozygotes and homozygous abnormal in the autosomal recessive pattern of gene transmission.

No residual enzyme activity was seen in red blood cells of the family II propositus, which corresponds to most of the published observations in studied cases. However, there was 13% of erythrocyte ASAL activity in the propositus of family I. To our knowledge, very few cases with residual RBC enzyme have been reported. Glick *et al.* (17) found 17% of control activity in his patient who died of neonatal argininosuccinic aciduria. Shih *et al.* (31) have reported 0–5% and 0–10% of activity in two of the six children, who were being followed up for this disease after newborn screening in the state of Massachusetts. This points towards the heterogeneity of mutant or residual enzymes in the children studied so far. However, the difference in methodology used by Glick *et al.* (17) and ourselves would also be a contributing factor toward this variation.

The fasting plasma levels of argininosuccinic acid in the two propoiti did not correlate with the levels of ASAL in erythrocytes. There was 210 μM argininosuccinic acid with indications of anhydride B content in the family I propositus, with an enzyme activity of 13%; while the family II propositus gave an argininosuccinic acidemia reading of 64.6 μM with no activity of RBC ASAL. Plasma glutamine and citrulline levels were also much more elevated in the family I propositus. This situation is understandable since the absence of RBC ASAL has not always been associated with an acute form of argininosuccinic aciduria syndrome. The clinical disease seems to be the result of an adverse balance of ASAL activity in various organs and tissues, with liver enzyme being the deciding factor. Recent report of an acute case of argininosuccinic aciduria (17) would support this

Table 6. Pattern of urinary urea and ammonia excretion after protein loading in affected family II as compared to control family

Family members	Age (years)	Body wt, kg	Protein intake, g/kg body wt	Urinary urea excretion, mg/mg creatinine	Urinary NH_3 excretion, mg/mg creatinine	Urinary urea, mg/mg creatinine/g protein intake	Urinary NH_3 , mg/mg creatinine/g protein intake
Affected family							
Father	40	60.9	1.6	6.90	0.584	4.31	0.365
Mother	33	58.2	1.5	9.38	0.410	6.25	0.270
Male sibling	10	22.7	3.1	15.88	0.882	5.12	0.285
Female sibling	7	26.8	2.6	12.60	0.787	4.85	0.303
Propositus	3	13.2	1.5	10.76	2.089	7.18	1.393
Control family							
Father	41	75.0	1.2	9.35	0.141	7.79	0.118
Mother	37	52.3	1.6	9.52	0.137	5.95	0.086
Male child	12	36.8	2.5	13.25	0.222	5.30	0.089
Female child	11	30.9	2.8	12.30	0.193	4.39	0.069
Male child	6	20.9	2.7	15.05	0.204	5.58	0.076

contention. In this child, who died at 6 days of age, there was a 17% residual activity of RBC enzyme; the brain and kidney enzymes were nearly normal whereas the liver enzyme was almost absent.

Our present study on the kinetics of the RBC ASAL in affected families has yielded interesting data. To our knowledge, this is the first study of its kind and may lend itself well to present and future interpretations of the nature of the enzyme defect and other important questions involved. The details of different plots of kinetic data and the statistical methodology used has already been discussed under *Results*. The figures of statistical comparison in Table 2 confirm the presence of a reduced enzyme activity in all the members of affected families due to a significantly reduced $V_{\max}$ value, as compared to control. There is no significant difference between the control and affected subjects in respect of K_m values. This may mean that there are no remarkable structural differences between the normal control enzyme and the enzyme from affected family members, but the quantity of available enzyme molecules differs.

The statistical comparison within family members, shown in Table 3, brings up a contrast between the two affected families. Within family I, the differences are solely determined by the $V_{\max}$ value of the enzyme. There is a significantly reduced velocity in the propositus as compared to all other family members and in mother and brother as compared to father. Since structural differences do not seem to be important, this could be interpreted to mean that the level of induced normal enzyme due to dietary or other factors was much different than each other in these heterozygotes at the time of hematopoiesis. The point activity measurement, 42% in the father against 40% and 33% in mother and sibling, would be in line with this argument.

Within family II, almost all the differences are determined by a variation of K_m values. There are no significant differences within the parents and within the two siblings, indicating a structural similarity in each case. However, there is a significant difference seen (generally $P < 0.01$) when each parent is compared against each child. The two siblings have a significantly reduced K_m value as compared to the parents (0.09 and 0.10 against 0.16 and 0.18 mM). This heterogeneity of enzyme structure or expression, between the obligate heterozygotes and heterozygote siblings, is hard to understand. It cannot be explained on the basis of different structural enzyme entities inherited from father and mother since there is no statistical difference between the parents. Yet the similarity of K_m values in the two siblings lends strong support to the probability that the enzyme has been inherited from the two parents. Unfortunately, it was not possible to study the kinetics of the enzyme in the propositus of this family, due to no activity shown. Any such study might have thrown some light on the issue. Under the circumstances we can only venture to postulate that the enzyme structure has somehow been adapted to the metabolic needs of quick growth in the face of reduced enzyme content in the tissues involved.

Glick *et al* (17), in their recent article, have presented evidence to demonstrate that more than one gene either codes for the structure of ASAL or regulates its activity in different tissues. Although some evidence is available (11) on the difference of ASAL structure in human kidney and liver, we will have to wait for studies made on purified enzymes from human organs to know whether there are similarities in the structure of enzyme from various tissues. Such a similarity would support the hypothesis of Glick *et al* (17), who postulate that different regulatory genes may be involved in controlling the level of ASAL in various organs. The statistical data on RBC ASAL kinetics presented by us is generally in line with the regulatory gene hypothesis. It demonstrates that the preponderance of maximal velocity differences in the enzyme taken from deficient erythrocytes results from the presence of residual normal molecules rather than structurally altered enzyme.

Increased levels of citrulline in the plasma of affected families, particularly in the two propositi, indicate the presence of a feedback inhibition by argininosuccinic acid of argininosuccinate synthetase, the enzyme anterior to ASAL in the urea cycle. The citrulline level also suggests that the mitochondrial enzymes of the urea cycle are intact. This is supported by a near normal level of urinary orotic acid in family II. Plasma glutamine levels are the result of a disturbed ammonia detoxication in members of both families. Recent research has indicated that even in normal acid-base conditions the amount of glutamine available to the kidney can alter the rate of ammonium excretion in urine which is proportional to the level of plasma glutamine (37).

The presence of increased plasma argininosuccinic acid and its anhydrides only in the propositi of the two affected families would mean that the residual ASAL activity in the tissues of the heterozygotes is sufficient to metabolize the available argininosuccinic acid. A protein load of moderate proportions was also not able to induce argininosuccinic aciduria in the heterozygotes of family II, although it brought about a high excretion of argininosuccinic acid in the propositus.

The present study has given an interesting picture of the kinetics of RBC ASAL in two families affected by argininosuccinic aciduria but has not provided answers to many important questions. One of these is: just what is the importance, individual or interdependent, of the RBC ASAL in the whole scheme of things? This question has a bearing on current and future research on argininosuccinic aciduria, since erythrocyte enzyme, because of its easy accessibility, would be used increasingly to confirm the diagnosis of inherited disease. A comprehensive answer to this question would involve painstaking work on the structural and kinetic characterization of human enzyme from various organs and tissues in its normal and abnormal forms, and an integrated study at the biochemical, metabolic, and clinical levels.

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39. Informed consent was obtained from parents of both affected families.
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