Citrullinemia: Elevated Serum Citrulline Levels in Healthy Siblings

Citrullinemia is a rare disorder of the ornithine urea cycle. Only 2 cases have been studied and reported up to now[1,2]. Our own observation refers to a new-born baby who was admitted in spring 1969 to the hospital and died after a short clinical course with signs of severe intoxication. The amino acid analysis of the patient's serum and urine revealed high amounts of citrulline which led to the diagnosis. Subsequently the serum citrulline levels of the family members have been determined.

Material and methods. Blood samples: Blood samples in family members and controls have been taken by venipuncture after overnight fasting. Blood was allowed to clot, centrifuged, the serum removed and immediately deproteinized with picric acid, then centrifuged again and the supernatant stored at 2 °C (up to 24 h) until further processing could be done.

Methods. 1. High Voltage Electrophoresis (HVE): HVE-apparatus with water-cooled tank (Camag, Muttenz, Switzerland). Acetic-acid/formic-acid buffer: pH 1.9 (120/26 ad 1000). Running time: 14 min, Potential-difference 100 V/cm Filter-paper: Schleicher-Schüll 2043 B (Camag, Muttenz, Switzerland).

Dipping reagents: a) Ninhydrin in acetone (2 g/l). b) Ehrlich's reagent 2 g dimethylbenzaldehyde/100 ml HCl 20% + 300 ml acetone (freshly prepared). Ascending paper-chromatography with butanol/acetic/water (12:3:5) may follow in the second dimension. Urine samples corresponding to 15 µg creatinine for one-dimensional HVE or 7.5 µg for two-dimensional HVE and chromatography are used (half quantities in new-born babies). Serum is deproteinized with acetone (1:1) and 40 µl of the supernatant is used.

2. Column chromatography according to the method of Stein and Moore[3]. Amino acid analyzer BC 200 (Biocal Instrument, GmbH, Munich, Germany). Double column method: Acidic and neutral amino acids with sodium citrate buffer pH 3.25 and 4.25 respectively (temperature 30 °C and 55 °C). Basic amino acids with sodium citrate buffer pH 4.26 and 5.28 respectively. Staining with ninhydrin. Serum is deproteinized with picric acid followed by absorption of the remaining precipitant. The eluate is dried by evaporation in vacuum.

Diagnosis. An urine sample of the 5-day-old patient has been examined by HVE. As a suspicious pattern of amino acids was detected, the HVE was followed by paper-chromatography in the second dimension. Unusually high amounts of the following groups of amino acids were detected: Lysine/ornithine, alanine, glutamine/citrulline. Presence of citrulline was suspected because staining with Ehrlich's reagent over the staining with ninhydrin produced an orange colour. Citrulline could infact be identified by direct staining with Ehrlich's reagent which gave at the expected position the yellow colour characteristic for citrulline. The serum of the patient was examined on the following day and a generalized hyperaminoacidemia was detected. The amino acid pattern was very similar to that found in the urine: alanine and glutamine/citrulline were found to be present in excessive amounts and the concentrations of lysine/ornithine were elevated as well. The corresponding stainings with Ehrlich's reagent led to the same results as observed in the urine. In this way on the 6th day of life the diagnosis of citrullinemia seemed very probable. Unfortunately our patient died on the same day. On the day of death he had received no proteins at all, the day before only 0.3 g/kg body weight. The diagnosis of citrullinemia has been verified by column chromatography on the patient's serum. The amount of citrulline was determined to be 144 mg/l.

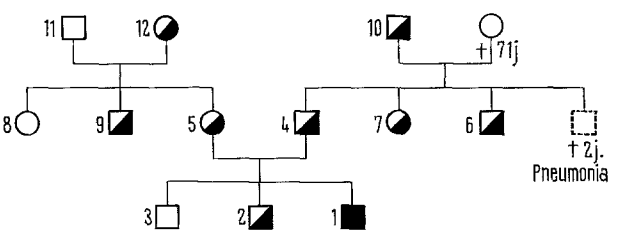
Inheritance. The family tree (Figure) is compatible with an autosomal heterocygote inheritance. The two families of the grand-parents come from different villages of the Black Forest. The two preceding generations do not show any consanguinity and further back a consanguinity is very improbable too. In the numerous kinship there is neither a high neonatal mortality nor an increased number of abortions.

Discussion. It seems that heterocygotes of citrullinemia can be detected by a simple determination of citrulline in fasting blood. The citrulline values of the parents of the second patient in the literature[4] also showed a higher

Serum citrulline levels. Table I summarizes the serum citrulline levels which was measured in the patient and his family compared with the mean and standard deviation (S.D.) of 22 healthy adults. Serum citrulline levels of the patient and his family compared with normal values

		Normal values ± S.D.[b]	Citrulline (mg/l) 5.35 + 0.99	Relationship
1.	W.N.	6 days	144	patient
2.	W.T.	2 years	18.4[a]	brother
3.	W.S.	3 years	6.4	brother
4.	W.R.	34 years	25.9[a]	father
5.	W.G.	31 years	8.01[a]	mother
6.	W.A.	28 years	8.80[a]	father's brother
7.	M.M.	40 years	16.0[a]	father's sister
8.	V.J.	32 years	5.6	mother's sister
9.	F.A.	18 years	10.4[a]	mother's brother
10.	W.A.	72 years	12.0[a]	father's father
11.	F.A.	61 years	5.15	mother's father
12.	F.E.	60 years	9.73[a]	mother's mother

[a] These healthy family members show values which distinctly surpass the normal values of our laboratory, i.e. they are higher than the mean plus 2 S.D. [b] 22 healthy adults, hospital staff.



Persons with citrulline levels exceeding 7.33 mg/l (i.e. Mean + 2 S.D., see Table) are represented by ◪ or ◪.

[1] W. C. McMurray and F. Mohyuddin, Lancet *2*, 352 (1962).
[2] G. Morrow, Am. J. Dis. Child. *113*, 157 (1967)
[3] D. H. Spackman, W. H. Stein and S. Moore, Analyt. Chem. *30*, 1190 (1958).
[4] G. Morrow, L. A. Berness and M. L. Efron, Pediatriya *40*, 565 (1967).

than normal value, which supports our view. The course of the first 2 cases was rather different as they were detected at the age of 4 years and 21 months. The diagnosis of citrullinemia is however clearly established in our patient, and death early in the neonatal period could be the rule rather than the exception. Thus citrullinemia would be diagnosed much more rarely than would correspond to its real occurrence[5].

Zusammenfassung. Die Familienuntersuchung eines 3. Falles von Citrullinaemie spricht für eine autosomal heterozygote Vererbung. Die Merkmalsträger können durch eine einfache Nüchternserum-Bestimmung des Citrullins von den nicht betroffenen Familienmitgliedern abgegrenzt werden.

H. WICK, T. BRECHBÜHLER and J. GIRARD

Children's Hospital of the University of Basel, CH-4000 Basel 5 (Switzerland), 9 February 1970.

[5] We gratefully acknowledge the very valuable help of the family doctor of our patient, Dr. K. GÉRARD, and we thank our technician, Mrs. BÉDOUCHA, for her continuous effort in pursuing promptly any suspicion of a metabolic disorder.

The Effect of Temperature on the Peptic Proteolysis of Ovalbumin

The temperature-dependence of peptic hydrolysis of a protein was studied by BULL and CURRIE[1], who employed ovalbumin as substrate. We have re-examined this problem in view of the finding that the Michaelis constant for that reaction does not depend on pH[2], and in view of the evidence that K_m for the peptic hydrolysis of serum albumin[3] and of ovalbumin[2] is the equilibrium constant for the dissociation of the enzyme-substrate complex into enzyme and substrate.

The kinetics of the action of pepsin (Nutritional Biochemicals Corp., twice recrystallized) on ovalbumin (twice recrystallized[4]), both native or acid-denatured[2], was followed by determining the trichloroacetic acid-soluble material absorbing at 275 nm, in aliquots removed periodically from the incubation media[2]. When native ovalbumin was used as substrate, care was taken to measure the initial velocity of proteolysis without interference of substrate denaturation[2,5].

The effect of temperature on the initial velocity of peptic hydrolysis of native and denatured ovalbumin was studied at the respective pH optima, and the results are shown in Table 1. Although we have observed that v_{max} values were consistently lower with denatured than with native ovalbumin, the temperature-dependence of v_{max} was not significantly different for the 2 kinds of substrate, yielding activation energies (E_A) of 13.7 ($\pm$ 1.0) kcal/mole and 12.7 ($\pm$ 1.0) kcal/mole for the native and denatured substrated, respectively. This would seem to indicate a difference in activation entropy (ΔS^*) for the breakdown of the enzyme-substrate complex into products. Unfortunately this difference between the 2 kinds of substrate could not be detected by estimates of ΔS^* from E_A and rate constant values because of the large errors involved. We have calculated from our data that ΔS^* for native and denatured substrates, at 25 °C, was -8 and -13 cal/°mole, respectively, but the estimated standard deviation for these values was 3.5 cal/°mole.

From the K_m values at 38 °C we obtain an estimate of -5.8 kcal/mol for the free energy of association between pepsin and ovalbumin, both native and denatured. Although our system does not allow measurement of the activation energy for the association of enzyme with substrate, the maximum value for this parameter should be 7.9 ($\pm$ 1.1) kcal/mole for the native and 6.9 ($\pm$ 1.4) kcal/mole for the denatured substrate. The absence of a significant difference between these 2 values contradicts the hypothesis[5,7] that a rate-limiting acid denaturation, as the first step in peptic proteolysis would cause the lower pH-optima observed with native, as compared with denatured, protein substrates. If this hypothesis were valid, the activation energy for the association of enzyme with native substrate would include that of the acid denaturation[8]. We have found that acid denaturation of ovalbumin, in the conditions prevailing in our enzyme reactions, has an activation energy of 33.9 ($\pm$ 3.6) kcal/mole

Table I. Effect of temperature on K_m and v_{max} for peptic proteolysis of native and denatured ovalbumin

Temperature (°C)	$K_m \times 10^4$ (M)	v_{max} (ΔA/min)
Native ovalbumin		
20.0	2.00 ($\pm$ 0.19)	0.340 ($\pm$ 0.026)
25.0	1.43 ($\pm$ 0.25)	0.530 ($\pm$ 0.035)
30.0	1.00 ($\pm$ 0.11)	0.730 ($\pm$ 0.028)
35.0	0.88 ($\pm$ 0.23)	1.130 ($\pm$ 0.015)
38.0	0.80 ($\pm$ 0.12)	1.180 ($\pm$ 0.020)
Denatured ovalbumin		
19.0	1.47 ($\pm$ 0.13)	0.173 ($\pm$ 0.007)
29.0	0.97 ($\pm$ 0.09)	0.416 ($\pm$ 0.018)
33.3	0.83 ($\pm$ 0.08)	0.476 ($\pm$ 0.006)
38.0	0.77 ($\pm$ 0.08)	0.640 ($\pm$ 0.010)
40.0	0.74 ($\pm$ 0.05)	0.675 ($\pm$ 0.006)

Initial velocities at several substrate concentrations, ranging from 5×10^{-5} to $3 \times 10^{-4} M$, were measured at pH 0.8 with the native and pH 1.65 with the denatured ovalbumin. K_m and v_{max} values were estimated from the least-square equation for the 1/v versus 1/[S] plots. v_{max} is expressed by the increase of absorbance of the trichloracetic acid filtrate at 275 nm/min. Pepsin concentration was $2.8 \times 10^{-6} M$ in all the experiments. Figures in parentheses indicate the standard deviations calculated from the error of the estimates of the intercepts and slopes of the least-square lines.

[1] H. B. BULL and B. T. CURRIE, J. Am. chem. Soc. *71*, 2758 (1949).
[2] Q. S. TAHIN and A. C. M. PAIVA, Enzymologia *37*, 153 (1969).
[3] J. R. CANN and J. A. KLAPPER JR., J. biol. Chem. *236*, 2446 (1961).
[4] R. A. KEKWICK and R. K. CANNAN, Biochem. J. *30*, 227 (1936).
[5] L. K. CHRISTENSEN, Arch. Biochem. Biophys. *57*, 163 (1955).
[6] K. J. LAIDLER, *The Chemical Kinetics of Enzyme Action* (Oxford University Press, Oxford 1958), p. 201.
[7] M. SCHLAMOWITZ and L. U. PETERSON, J. biol. Chem. *234*, 3137 (1959).
[8] K. LINDERSTROM-LANG, R. D. HOTCHKISS and G. JOHANSEN, Nature *142*, 996 (1938).