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).

(Table II), in good agreement with published data obtained at higher pH values[9,10]. This is significantly larger than the maximum value for the association of pepsin with native ovalbumin.

The temperature-dependence of K_m (Table I) yields values for the changes in normal enthalpy and entropy that are not significantly different for the 2 forms of the substrate. The large errors inherent in the method used for obtaining those values do not allow a fine interpretation, but it is significant that the association of pepsin and ovalbumin is an endothermic process with a large increase in entropy. This also occurs in the peptic hydrolysis of small synthetic substrates[11], and would indicate that hydrophobic bonding plays a preponderant role in the association of pepsin with its substrate.

Table II. Effect of temperature on the rate of acid denaturation of ovalbumin

Temperature (°C)	25.0	30.0	35.0	40.0
$k_1 \times 10^3$ (min^{-1})	7	20	55	130

The first-order rate constants (k_1) were estimated by following the decrease of solubility of $2.5 \times 10^{-4} M$ ovalbumin solutions kept at pH 0.8. The solubility was determined by periodically removing aliquots that were diluted 40-folt with $2 M$ acetate buffer (pH 4.75) containing $0.5 M$ $MgSO_4$, and reading the absorbance of the filtrate at 275 nm.

Résumé. Partant de l'effet de la température sur la cinétique de la protéolyse peptique de l'ovalbumine native ou denaturée, on a estimé l'énergie d'activation, l'enthalpie et l'entropie de l'association enzyme-substrat.

Q. S. Tahin and A. C. M. Paiva

Department of Biophysics and Physiology, Escola Paulista de Medicina, São Paulo, S.P. (Brazil), 28 January 1970.

[9] H. K. Cubin, Biochem. J. *23*, 25 (1929).
[10] R. J. Gibbs, M. Bier and F. F. Nord, Arch. Biochem. Biophys. *33*, 345 (1951).
[11] E. J. Casey and K. J. Laidler, J. Am. chem. Soc. *72*, 2159 (1950).
[12] We are indebted to the Brazilian National Research Council (CNPq) for support.

Norepinephrine-Sensitive Na^+/K^+ ATPase Activity in Brown Adipose Tissue[1]

Norepinephrine (NE) released from sympathetic neuronal terminals appears to mediate the increased thermogenesis in brown adipose tissue during cold stress[2,3]. This response, which is blocked by β-adrenergic antagonists, apparently involves activation of lipolysis via the adenyl cyclase, 3′,5′-cyclic AMP system[4,5]. However, the biochemical mechanisms underlying the NE-stimulation of respiration in brown fat are still controversial[3].

The uncoupling agent, 2,4-dinitrophenol (DNP), injected i.v. into cold-acclimated rats, enhances the thermogenic response of the brown fat during cold stress as well as during NE administration; this implies that in activated brown fat, respiration is coupled to oxidative phosphorylation[6]. We thus proposed a) that the NE-induced respiratory elevation of the brown fat initially reflects increased availability of substrate rather than of ADP, and b) that this is accompanied by an increased cellular ATP requirement[6].

The finding that NE (whether of exogenous or neuronal origin) depolarized the membranes of these cells in vivo[7], suggested that in the Na^+/K^+ distribution and perhaps also the Na^+/K^+ pump, alterations might occur during stimulation of thermogenesis by the catecholamine. Hence we examined the effect of NE on the Na^+/K^+ ATPase system associated with the membrane ion pump.

The methodology entailed removal of brown adipose tissue from decapitated male, Long-Evans rats that had been cold acclimated (exposure to 5 °C, 50% R.H., with 12 h high/low light cycle for 4–8 weeks). The brown fat was cleared of extraneous tissues and then homogenized in a mixture (9/1, volume/weight) containing 250 mM sucrose, 2 mM EDTA, and 2 mM TES (N *Tris* (hydroxymethyl) methyl-2-aminomethane sulfonic acid), pH 7.0 at 0 °C. The homogenate was centrifuged 10 min at $14{,}000 \times g$, the overlying fat removed and the supernatant decanted for assay. ATPase activity was determined in 2 media (final volume 1.5 ml): i.e., (A) in millimoles per liter: 20 TES (pH 7.2), 27 sucrose, 8.7 KCl, 70 NaCl, 2.7 EDTA, 5 $MgCl_2$, 4 NaCN, 6.7 Na_2ATP (Sigma); (B) differed from (A) only in containing 190 mM sucrose and no KCl or NaCl. Each reaction system contained 0.477 ± 0.013 mg supernatant nitrogen[8]. After incubation at 30 °C for 20 min, the reaction was terminated with 0.5 ml $1.0 N$ $HClO_4$, the tubes centrifuged at 0 °C for 10 min at $1000 \times g$ and the inorganic phosphate in the supernatant assayed[9] against appropriate controls. The Na^+/K^+ ATPase activity is here defined as the inorganic phosphate released in the presence of Na^+, K^+, and Mg^{++} (medium A) minus that in the absence of KCl and NaCl (medium B)[10].

In the presence of NE, the Na^+/K^+ ATPase was markedly stimulated. The increase was dose dependent (Figure) and maximal with 6 mM NE. The fact that this enhancement was abolished by ouabain (Table) suggests that this ATPase is part of the Na^+/K^+ pump associated with the cell membrane.

[1] Supported in part by research grants NASA No. NGR-05-004-035 and USPHS No. HD-03268. P.A.H. is a PHS predoctoral fellow (No. 1-GM-33, 505-04). A preliminary report of this work was presented at the 1969 Fall meeting of the Am. Physiol. Society; an abstract of this talk is contained in The Physiologist *12*, 257 (1969). We thank J. F. Detrick for his technical assistance.
[2] D. Hull and M. M. Segall, J. Physiol. *181*, 458 (1965).
[3] R. E. Smith and B. A. Horwitz, Physiol. Rev. *49*, 330 (1969).
[4] A. Beviz, L. Lundholm and E. Mohme-Lundholm, Br. J. Pharmac. *34*, 198P (1968).
[5] N. Reed and J. N. Fain, J. biol. Chem. *243*, 2843 (1968).
[6] B. A. Horwitz, P. A. Herd and R. E. Smith, Can. J. Physiol. Pharmac. *46*, 897 (1968).
[7] B. A. Horwitz, J. M. Horowitz jr. and R. E. Smith, Proc. natn. Acad. Sci., USA *64*, 113 (1969).
[8] T. S. Ma and G. Zuazaga, Ind. Eng. Chem. Analyt. Edn. *14*, 280 (1942).
[9] G. A. Gomori, J. Lab. clin. Med. *27*, 955 (1942).
[10] R. L. Post and A. K. Sen, in *Methods in Enzymology, Oxidation and Phosphorylation* (Eds. R. W. Estabrook and M. E. Pullman; Academic Press, New York, 1967), p. 762.