Zusammenfassung. Isolierte Submandibularisdrüsen des Meerschweinchens wurden in physiologischer Pufferlösung mit sekretionsstimulierenden Substanzen inkubiert. Dopamin, Noradrenalin, Adrenalin und 5-HT induzieren die Sekretion von Peroxydase und Amylase, während der Serotoninantagonist BOL 148 (2-Bromo-D-Lysergsäure Dietylamid) eine beinahe vollständige Sekretionshemmung hervorruft. BOL 148 hat sich also nicht als spezifisch 5-HT-hemmend an der Meerschweinchenspeicheldrüse erwiesen.

B. Carlsöö, A. Danielsson, S. Marklund and T. Stigbrand

Departments of Histology and Physiological Chemistry, University of Umea, S-901 87 Umea (Sweden), 13 January 1975

Dissociation of Erythrocyte Catalase into Subunits and Their Re-association

Catalase (E.C. 1.11.1.6.) of various origin has been shown to consist of 4 identical subunits with a molecular weight of approximately 60,000[1-3]. The dissociation of this oligomer-enzyme into dimer and monomer particles proceeds slowly when kept at neutral pH in dilute solution[4]. However, this decomposition can be accomplished within a few minutes in concentrated urea solution or by a shift of pH (below 4 or above 11,[5,6]). Under appropriate experimental conditions, this interconversion is reversible, thus permitting a study of the decomposition of the catalase molecule as well as its re-association from subunits.

In order to get more insight into the forces acting between subunits, both reactions have been followed. This is facilitated by the fact that only the catalatic activity – but not the peroxidatic activity – of this enzyme is lost after its dissociation into subunits. Furthermore, antigenic determinants specific for the tetramer particle disappear upon dissociation. This dissociation – re-association process permits a direct follow-up of alterations in both enzymatic and antigenic properties of the particles involved. The rate of re-association can be influenced by other proteins in the solution.

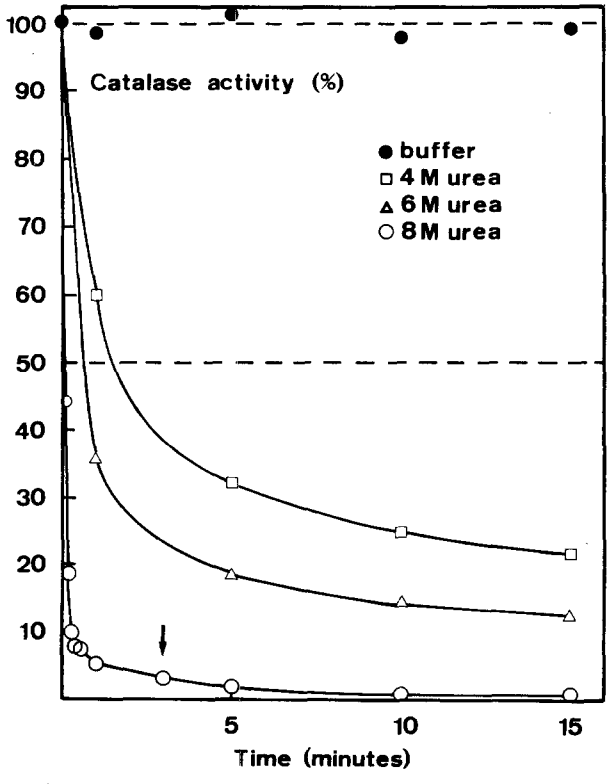

Fig. 1. Inactivation of human erythrocyte catalase in a final concentration of 4 *M* urea (□—□); 6 *M* urea (△—△); 8 *M* urea (○—○); control in 0.1 *M* phosphate buffer pH 7.2. (●—●).

Materials and methods. Human erythrocyte catalase was isolated from normal blood by the method of Mörikofer et al.[7] and Aebi et al.[4]. The effector proteins used were Bovine serum albumin, human IgM (gift from Prof. Barandun) and the IgG fractions of the anti-human erythrocyte catalase antibody[8].

Inactivation of catalase was achieved essentially by the method of Shpitsberg[6]. Samples containing 5.6 mg/ml catalase were brought to an 8, 6 and 4 *M* urea-concentration by using a 10 *M* urea solution adjusted to pH 7 with HCl. The catalase in urea was left at 20 °C for time periods from a few sec up to 15 min. Inactivation was stopped by diluting the samples with 0.1 *M* phosphate buffer pH 7.2 to a 3 *M* urea-concentration.

Re-activation studies were performed with samples containing 16.3 mg/ml catalase, which had been inactivated with urea for 3 min at 20 °C (final concentration 8 *M*). The samples were then diluted to a 3 *M* urea-concentration by the addition of 0.1 *M* phosphate buffer pH 7.2, or by the same buffer containing 10 mg/ml final concentration of various proteins. Re-activation was achieved by open dialysis of 1–2 ml samples in an ice-bath against 500 ml of the phosphate buffer for up to 8 h with a change of buffer after 6 h. The samples in the dialysis bags were mixed prior to the removal of aliquots for analysis at various time intervals.

Catalase activity was determined spectrophotometrically by direct measurement of the decrease of light absorption at 240 nm, caused by the decomposition of hydrogen peroxide by catalase[9,10]. Urea concentration was mea-

[1] H. Sund, K. Weber and E. Mölbert, Eur. J. Biochem. *1*, 400 (1967).
[2] T. Samejiama, W. J. McCabe and J. T. Yang, Arch. Biochem. Biophys. *127*, 354 (1968).
[3] W. A. Schroeder, J. R. Shelton, J. B. Shelton, B. Robberson and G. Apell, Arch. Biochem. Biophys. *131*, 653 (1969).
[4] H. Aebi, S. R. Wyss, B. Scherz and F. Skvaril, Eur. J. Biochem. *48*, 137 (1974).
[5] V. L. Shpitsberg, Biochemistry *30*, 687 (1965).
[6] V. L. Shpitsberg, Biofizika *11*, 766 (1966).
[7] S. Mörikofer-Zwez, M. Cantz, H. Kaufmann, J. P. von Wartburg and H. Aebi, Eur. J. Biochem. *11*, 49 (1969).
[8] Y. Ben-Yoseph and E. Shapira, J. Lab. clin. Med. *81*, 133 (1973).
[9] B. Chance and A. C. Maehly, in *Methods in Enzymology* (Academic Press Inc., New York 1955), vol. 2, p. 764.
[10] H. Aebi in *Methoden der enzymatischen Analyse*, 2dn edn. (Ed. H. U. Bergmeyer; Verlag Chemie, Weinheim 1970), vol. 1, p. 636.

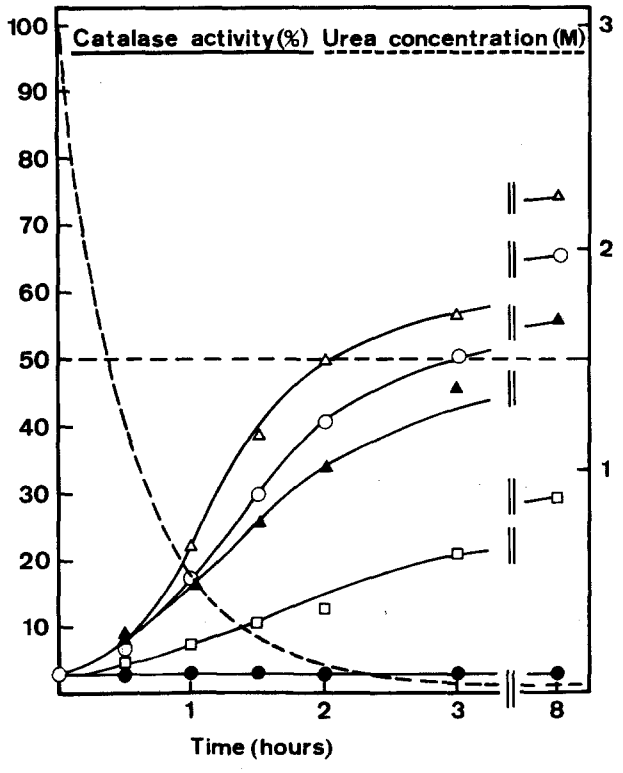

Fig. 2. Reactivation of human erythrocyte catalase after 3 min inactivation in a final concentration of 8 *M* urea, followed by dilution to 3 *M* urea with 0.1 *M* phosphate buffer pH 7.2 only or with buffer containing various proteins in a final concentration of 10 mg/ml. Open dialysis of the inactivated samples was performed for 8 h against 500 ml of the same buffer, with a change of buffer after 6 h. Dilution with: buffer only (○—○); buffer containing BSA (△—△); buffer containing anti-catalse IgG (▲—▲); buffer containing human IgM (□—□); control sample, dilution with buffer only, but no dialysis (●—●); – –, urea concentration.

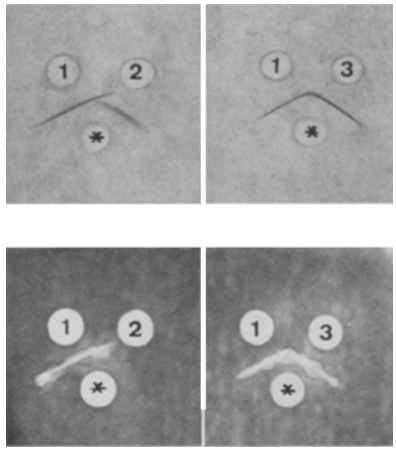

Fig. 3. Double immunodiffusion tests: Antigenic properties of inactivated and reactivated erythrocytes catalase. Upper: peroxidase activity stain; lower: catalase activity stain. 1, Human erythrocyte catalase (HEC); 2, inactivated HEC (= Dimer); 3, reactivated enzyme (= HEC); *, anti-catalase IgG.

sured with an auto-analyzer[11]. Double immunodiffusion tests were performed according to Ouchterlony[12]. Detection of peroxidase active precipitin lines was achieved by applying a substrate mixture on the plates, consisting of 5 ml 0.5% *o*-dianisidine in methanol and 95 ml 0.003% hydrogen peroxide. Catalase active precipitin lines were visualized by the method of THORUP et al.[13], after impregnation of the well washed and dried agar plates with a 2% starch solution for 2 h at 45 °C.

Results. Catalase, when brought in concentrated urea solution rapidly loses its H_2O_2-decomposing activity. As shown in Figure 1, inactivation is completed in 8 *M* urea after a few min, whereas it proceeds more slowly at lower urea-concentrations (4–6 *M*). In the standard procedure – incubation in 8 *M* urea for 3 min – activity is reduced to about 3% of the initial value (↓ in Figure 1). If such a sample of inactivated catalase is dialyzed against buffer-solution in order to remove the urea, catalase activity gradually reappears. After 2–3 h, when urea-concentration has fallen to an insignificant level, about half the original activity can be detected. As shown in Figure 2, the presence of bovine serum albumin has a slightly accelerating, IgG and IgM an inhibitory effect. After 8 h of dialysis catalase activity (per ml) has reached 60–80% of the original level. The inhibitory effect of IgG is relatively small under the conditions described above, i.e. 16.3 mg/ml catalase (Figure 2). However, the effect becomes the more pronounced the lower the catalase concentration. If the same type of experiment is performed using 5 mg/ml catalase, the reactivation after 8 h amounts to 40–50% of the original level, but reaches only 6–7% in presence of IgG.

A comparative analysis of 1. the original sample, 2. the inactivated and 3. the reactivated enzyme preparation gave the following results: Ultracentrifugation of the inactivated sample revealed a peak with a sedimentation coefficient corresponding to a molecular weight of 120,000, which is consistent with catalase dimer. Chromatography on Sephadex G-150 of the reactivated sample showed the same elution volume as the original enzyme, corresponding to a molecular weight of 240,000. The double immunodiffusion tests (Figure 3, right) demonstrate complete antigenic identity of (1) the native and (3) the reconstituted catalase. The line of identity exerts catalase and peroxidase activity. On the other hand, the precipitin line formed by the inactivated sample (2) exerts peroxidatic, but – if any – only a trace of catalatic activity. The spur formation seen in the upper left picture confirms a difference in antigenic properties between the tetramer and the dimer particle[4].

Discussion. Catalase – like hemoglobin – can readily be converted to its dimer form. The almost complete reversibility of the equilibrium tetramer – dimer in the system described is a prerequisite for the preparation of hybrid catalase molecules in vitro. The findings presented here support the assumption that – at least to some extent – a dynamic equilibrium of this type may also exist in vivo, e.g. in the erythrocyte. This possibility is of genetic interest, since it has been observed that individuals heterozygous for an unstable catalase mutant (Acatalasemia) possess an unusual catalase species. This enzyme, found in erythrocytes as well as leukocytes of such heterozygotes, exerts electrophoretic and heat stability properties intermediate to those of normal catalase and of the enzyme variant present in the homozygotes[14]. A possible explanation of these unexpected findings is the assumption that normal and variant catalase-monomers are produced simultaneously and a random assembly of either monomer species is taking place, thereby forming hybrid molecules.

[11] L. T. SKEGGS, Amer. J. clin. Path. *28*, 311 (1957).
[12] O. OUCHTERLONY, Progr. Allergy *5*, 1 (1958).
[13] O. A. THORUP, W. B. STROLE and B. S. LEAVELL, J. Lab. clin. Med. *58*, 122 (1961).
[14] S. R. WYSS and H. AEBI, Enzyme, in press (1975).

Zusammenfassung. Zwischen der Tetramer- und der Dimer-Form der Erythrocyten-Katalase besteht ein Gleichgewicht. Dieses lässt sich in vitro durch Variation der Harnstoffkonzentration beliebig verschieben. Das dabei entstehende Dimer zeigt Peroxidase-, nicht aber Katalase-Aktivität. Bei der Reassoziation, deren Geschwindigkeit sich durch andere Proteine beeinflussen lässt, entsteht ein Produkt, das vom nativen Enzym nicht unterscheidbar ist.

[15] Acknowledgments. This study is part of project No. 3.8460.72 subsidized by the Swiss National Science Foundation. One of the authors (Y. B-Y., present address: Dept. of Immunology, The Weizmann Institute, Rehovot) is grateful to the Roche Studienstiftung for a fellowship.

H. Aebi, B. Scherz, Y. Ben-Yoseph and S. R. Wyss[15]

Medizinisch-chemisches Institut der Universität, CH-3000 Bern 9 (Switzerland), 17 January 1975.

Effect of Nucleoside Di- and Triphosphates and $MgCl_2$ on the Activity of 5′-Nucleotidase from Bull Seminal Plasma[1]

In recent years considerable attention has been given to the control of the activity of 5′-nucleotidase (EC 3. 1. 3. 5.). The enzyme, partially purified from mammalian, avian and bacterial sources, has been found to be inhibited by nucleoside di- and triphosphates[2-9]. Such inhibition may constitute a control of the 5′-nucleotidase activity to prevent unregulated catabolism of 5′-mononucleotides required for nucleic acid and coenzyme synthesis[10]. Furthermore, a role of Mg^{++} ions in overcoming this inhibition has been suggested by Sullivan and Alpers[6] and by Magni et al.[11] for the rat heart muscle and guinea-pig skeletal muscle 5′-nucleotidase respectively.

The occurrence of a 5′-nucleotidase in bull seminal plasma has long been known[12], and the fundamental kinetic parameters have been reported by Heppel and Hilmoe[13] and Levin and Bodansky[14]. However, the regulation of this enzymic activity had never been reported.

The present comunication describes the inhibition exerted by nucleoside di- and triphosphates on bull seminal plasma 5′-nucleotidase and the role of magnesium ions in overcoming the inhibition imposed by ATP and ADP. The experiments have been conducted at pH 7.2, where the activity does not show any magnesium dependence.

Methods. The 5′-nucleotidase reaction was carried out spectrophotometrically at 25 °C as previously described by Ipata[15], with 5′-AMP as substrate, in the presence of adenosine deaminase excess. The standard reaction mixture, in a final volume of 2.0 ml, contained 75 m*M* *Tris*-HCl pH 7.2, 0.1 unit of commercial adenosine deaminase (Boehringer) and 40 μM 5′-AMP. Linearity of the reaction rate was maintained up to at least 50 µg of protein per reaction mixture. One enzyme unit equals an activity equivalent to a decrease of 0.001 absorbance unit per min. The protein concentration was determined by the biuret method of Gornall et al.[16].

The 5′-nucleotidase was partially purified from 11 ml batches of bull seminal plasma according to Levin and Bodansky[14] through precipitation with protamine sulfate, precipitation with ammonium sulfate between 40 and 60% saturation and heat treatment. Further purification was achieved as follows: the heat treated fraction was centrifuged in the cold to remove any precipitated material and the supernatant fluid was dialyzed overnight against 0.05 *M* *Tris*-HCl buffer pH 8.2. The dialyzed material (10 ml containing 28 mg protein/ml) was adsorbed on a DEAE cellulose (Whatman DE 32) column (1.5 × 10 cm). After washing the column with *Tris*-HCl 0.05 *M*, pH 8.2, the elution was carried out with a linear gradient *Tris*-HCl 0.05 *M*, pH 8.2 (250 ml) and 0.5 *M* NaCl in the same buffer (250 ml). The enzyme activity was eluted around a concentration of 0.2 *M* NaCl. The active fractions were pooled and brought to 80% saturation with ammonium sulfate. The precipitate, dissolved in a minimal amount of water, was gel-filtered at 4 °C through a 1.8 × 60 cm Sephadex G-100 column, equilibrated with *Tris*-HCl buffer 0.05 *M*, pH 7.2. The 5′-nucleotidase was recoverde between the 30th and the 50th ml of the eluate as a sharp

Nucleotide concentrations required for 50% inhibition of bull seminal plasma 5′-nucleotidase and K_i values of inhibitory nucleotides

Nucleotide	Concentration[a] (μM)	K_i (μM)
ITP	50	25.5
CTP	15	5.1
GTP	8.0	1.73
ATP	2.5	0.6
UTP	2.3	0.45
IDP	11	4.55
CDP	3.5	1.55
GDP	2.8	0.32
ADP	2.2	0.30
UDP	0.9	0.29

[a] The values were obtained from inhibition curves, all showing hyperbolic shapes. The final 5′-AMP concentration was 40 μM.

[1] This work was supported by Italian C.N.R.

[2] P. L. Ipata, Biochem. biophys. Res. Commun. *27*, 337 (1967).
[3] P. L. Ipata, Biochemistry *7*, 507 (1968).
[4] H. P. Baer, G. L. Drummond and L. Duncan, Molec. Pharmac. *2*, 67 (1966).
[5] M. J. Edwards and H. M. Maguire, Molec. Pharmac. *6*, 641 (1970).
[6] J. M. Sullivan and J. B. Alpers, J. biol. Chem. *246*, 3057 (1971).
[7] W. B. Gibson and G. I. Drummond, Biochemistry *11*, 223 (1972).
[8] H. B. Bosman and G. Z. Pike, Biochim. biophys. Acta *227*, 402 (1971).
[9] R. A. Felicioli, S. Senesi, F. Marmocchi, G. Falcone and P. L. Ipata, Biochemistry *12*, 547 (1973).
[10] A. W. Murray and B. Friedrichs, Biochem. J. *111*, 83 (1969).
[11] G. Magni, E. Fioretti, F. Marmocchi and P. L. Ipata, Life Sci. *13*, 663 (1973).
[12] T. Mann, Biochem. J. *39*, 451 (1945).
[13] L. A. Heppel and R. J. Hilmoe, J. biol. Chem. *188*, 665 (1951).
[14] S. J. Levin and O. Bodansky, J. biol. Chem. *241*, 51 (1966).
[15] P. L. Ipata, Analyt. Biochem. *20*, 30 (1967).
[16] A. G. Gornall, C. S. Bardawill and M. M. David, J. biol. Chem. *177*, 751 (1949).