Alternative Molecular Forms of Erythrocyte Catalase

Catalase in mammalian tissues is believed to be heterogeneous in nature. By chromatographic separation procedures multiple molecular forms of liver catalase[1-3] as well as erythrocyte catalase[4-6] have been observed. By column chromatography on DEAE cellulose catalase activity in hemolysate of normal human red cells can be separated into 3 fractions[4]. They differ from each other in respect to electrophoretic mobility and molarity of the buffer required for elution (A < B < C). THORUP et al.[4] have shown that fractions A and B are unstable, inasmuch as they tend to convert to fraction C. This is in agreement with an earlier finding that catalase activity in hemolysate shows an increase in electrophoretic mobility upon storage[7].

Such molecular conversions are not consistent with the concept of isoenzymes. Therefore it seemed of interest to investigate further this heterogeneity of erythrocyte catalase by studying its electrophoretic and chromatographic properties under various experimental conditions. Evidence has been obtained by chromatography that erythrocyte catalase is present in a single form exclusively (corresponding to THORUP's fraction A), provided that all operations from hemolysing the cells to eluting the column are carried out in an oxygen-free environment. On the other hand, any other proportion of A:B:C can be obtained according to the mode of handling the hemolysate prior to chromatographic analysis. Thus, after prolonged storage in presence of oxygen all catalase activity is eluted in form of fraction C only. So far this variability of the elution pattern has been observed in erythrocyte catalase of man and horse. Although there might be some species differences, similar results have been obtained with both preparations.

For the study of the chromatographic pattern of erythrocyte catalase on DEAE cellulose, 3 types of experiments were performed. In the standard procedure according to THORUP et al.[4] the hemolysate was prepared with distilled water, freed from stroma by filtration, dialysed against 0.003 *M* phosphate buffer pH 6.8 and applied to the column. In the case of the human enzyme (Figure 1a) catalase activity is eluted in 2 peaks, the first at 0.012 *M* consisting of fractions A and B, and the second (fraction C) at 0.05 *M* phosphate pH 6.8, respectively. In Figure 2b the pattern of catalase activity from horse erythrocytes shows 3 peaks, 2 at 0.012 *M* (A and B) and 1 at 0.05 *M* phosphate (fraction C). A comparison of the elution patterns in Figure 1a and 2b reveals that in the latter case a better separation of fractions A and B can be achieved.

In another series of experiments the hemolysates were prepared in an oxygen-free environment and in presence of EDTA in order to prevent oxidation. All buffers were saturated with nitrogen, the DEAE cellulose was washed with EDTA. Figure 1b and 2a demonstrate that both catalase of human and horse erythrocytes are eluted in 1 homogenous peak by 0.012 *M* phosphate.

For the third type of experiment the hemolysate prepared as usual was dialysed for 60 h at 4 °C against oxygen saturated starting buffer. The chromatographic pattern shown in Figure 2c also reveals one single peak, but here catalase is eluted only after the concentration of the buffer has been raised to 0.05 *M* phosphate. The overall yield in all chromatographic separation experiments was more than 80%, indicating that there were no gross changes in the specific activity.

The fractions obtained from human hemolysate by chromatography differ in electrophoretic mobility on starch gel at pH 8.0, as could be expected from the chromatographic behaviour: catalase from the 0.012 *M* elution step (A) had a slower anodic electrophoretic mobility than catalase from the 0.05 *M* step (C). An analogous difference in electrophoretic mobility was observed when freshly prepared hemolysate was compared with hemolysate stored for several days at 4 °C, catalase

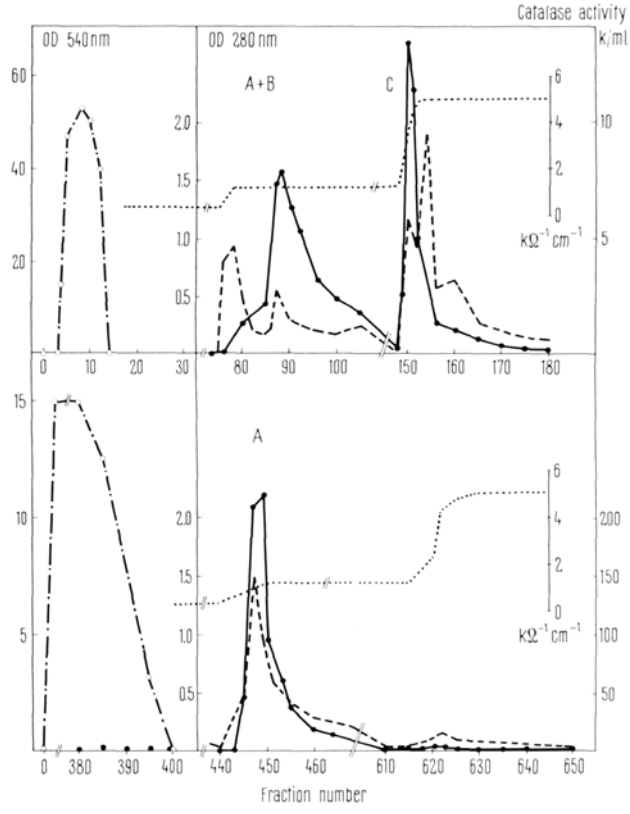

Fig. 1. Chromatography of hemolysates from human erythrocytes on DEAE cellulose columns. Elution with Na-K-phosphate buffer, pH 6.8, 0.003, 0.012 and 0.05 *M* respectively, 4 °C; ● = catalase activity (k/ml)[13]; o = hemoglobin (optical density 540 nm); --- OD 280 nm; ----- conductivity (k Ω^{-1}cm^{-1}). (1a) Standard procedure (THORUP et al.[4]), without special precautions. Dimensions of column 22 × 1.6 cm; flow rate 40 ml/h; 10 ml fractions. (1b) Preparation of hemolysate and chromatography in the absence of oxygen and free heavy metals. Addition of EDTA at a final concentration of 0.1 *M* to the hemolysate; dialysis of hemolysate against starting buffer. The DEAE cellulose was washed with 0.1 *M* EDTA and equilibrated with starting buffer. All solutions and buffers were N_2 saturated. Column 45 × 4.9 cm; flow rate 200 ml/h; 10.5 ml fractions. Note: The elution patterns were independent of the load within the range used above.

[1] V. E. PRICE and R. E. GREENFIELD, J. biol. Chem. *209*, 363 (1954).
[2] T. HIGASHI and T. PETERS, J. biol. Chem. *238*, 3945 (1963).
[3] R. S. HOLMES and C. J. MASTERS, Archs Biochem. Biophys. *109*, 196 (1965).
[4] O. A. THORUP JR., J. T. CARPENTER and P. HOWARD, Br. J. Haemat. *10*, 542 (1964).
[5] S. MATSUBARA, HEDI SUTER and H. AEBI, Experientia *22*, 428 (1966).
[6] M. OGATA and S. TAKAHARA, Medicine and Biology (Japan) *73*, 292 (1966).
[7] A. BLUMBERG, H. R. MARTI, F. JEUNET and H. AEBI, Schweiz. Med. Wschr. *92*, 1324 (1962).

in old hemolysates moving faster than the one in a fresh sample. If mixtures containing 'fast' and 'slow' moving catalase are analysed, a complete electrophoretic separation can be obtained (Figure 3). A more detailed study of this 'ageing' phenomenon has disclosed that under aerobic conditions (i.e. in equilibrium with air) electrophoretic mobility of catalase in hemolysate steadily increases within a period of about 8 days. However, if iodoacetamide is added immediately after hemolysis (20 mM, which is estimated to be a 4-fold molar excess over the thiol groups in hemolysate; pH 8.7) no alteration of electrophoretic mobility is observed within 10 days. A similar effect is obtained by adding cystamine to the hemolysate (10-fold molar excess, 50 mM, pH 8.7). Consequently, alkylation of SH-groups or formation of a mixed disulphide seem to inhibit the alterations in the molecular structure of catalase responsible for this increase in electrophoretic mobility.

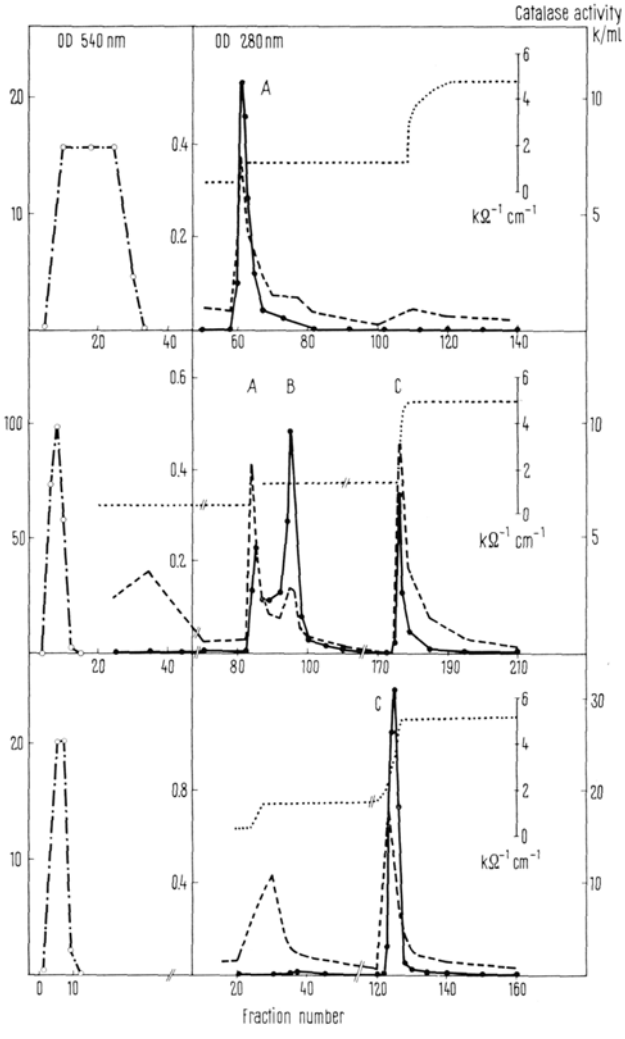

Fig. 2. Chromatography of hemolysates from horse erythrocytes on DEAE cellulose. The same buffers and symbols are used as listed in legend to Figure 1. (2a) Chromatography in the absence of oxygen and free heavy metals. Same conditions as described for Figure 1b. Dimensions of column 21.5 × 2.0 cm; flow rate 60 ml/h; 10 ml fractions. (2b) Standard procedure. Dimensions of column 23 × 1.6 cm; flow rate 60 ml/h; fraction volume 10 ml. (2c) Standard procedure with the exception that the hemolysate was dialysed for 60 h against O_2 saturated starting buffer prior to analysis. Column 16 × 1.6 cm; flow rate 120 ml/h; fraction volume 10 ml.

On the other hand, it is possible to reconvert fraction C to fraction A catalase in vitro. This has been accomplished by incubating a sample of purified fraction C with mercaptoethanol at alkaline pH: catalase in a concentration of $2.85 \times 10^{-6} M$ was treated with $5 \times 10^{-4} M$ mercaptoethanol in 0.05M borate buffer, pH 8.2 (containing 0.05M EDTA) for 75 min at 37 °C. After desalting by passage through a Sephadex G-25 column (equilibrated with 0.003M phosphate buffer pH 6.8, 0.001M EDTA and $10^{-4} M$ mercaptoethanol) the effluent was rechromatographed on DEAE cellulose at pH 6.8, the phosphate buffers containing $10^{-5} M$ mercaptoethanol. The elution pattern revealed that – under these conditions – about 85% of catalase activity have been transformed to a species of catalase which is eluted by 0.012M phosphate buffer and therefore has the same chromatographic properties as fraction A.

These observations suggest that in the red cell neither catalase isoenzymes can be observed, nor is there a significant equilibrium between several labile molecular forms. If secondary oxidation is avoided, fraction A is found exclusively. This native form of catalase is converted to fraction C in presence of oxygen. Since this conversion can be inhibited by alkylation of SH-groups or formation of mixed disulphides, it seems that oxidation of thiol-groups to S-S-bridges is involved in this structural change. This interpretation is also supported by the fact that this transformation can be reversed by mercaptoethanol. It is possible that the alternative forms A, B, C

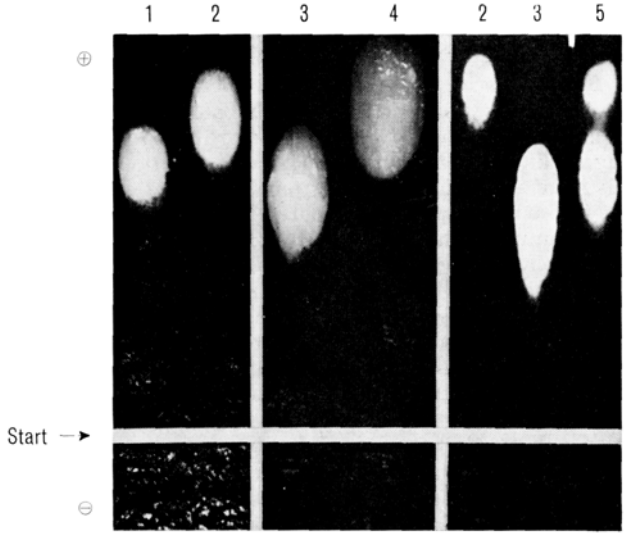

Fig. 3. Starch gel electrophoresis (SMITHIES[8]) of catalase fractions from DEAE cellulose chromatography and of fresh and stored human hemolysates. Visualization of catalase by means of THORUP's technique[9]. Vessel buffer: Na-K-phosphate pH 8.0, 0.067M; gel buffer: vessel buffer diluted 1:10. Electrophoresis at 4 °C for 16–18 h at 120 V and 12 mA for a gel of 21.5 × 6 × 0.8 cm. Samples of 20μl containing a total catalase activity of 0.15 k were applied to the gel. 1, fraction A from hemolysate; 2, fraction C from hemolysate; 3, total hemolysate immediately after hemolysis with distilled water; 4, hemolysate stored for 7 days at 4 °C; 5, mixture of equal parts of samples 2 and 3.

[8] O. SMITHIES, Biochem. J. *61*, 629 (1955).
[9] O. A. THORUP, W. B. STROLE and B. S. LEAVELL, J. Lab. Clin. Med. *58*, 122 (1961).

of erythrocyte catalase represent different conformations (conformers) of the same molecule[10].

By applying the standard separation procedure, it has been demonstrated that reticulocytes show a higher A/C ratio than old red cells[4,11]. In view of the results presented here, it seems probable that the considerably higher metabolic activity of the reticulocyte (e.g. regeneration of GSH) may cause a higher degree of reductive protection during the fractionation procedure. Secondary alterations of similar nature have been observed with other red cell enzymes such as G-6-PD and GOT[12], stressing the importance of a reducing environment for maintenance of the structure of red cell enzymes[14,15].

Zusammenfassung. Katalase aus Erythrocyten vom Menschen (und Pferd) lässt sich säulenchromatographisch und elektrophoretisch in drei Fraktionen A, B und C auftrennen, wobei die Fraktionen A und B die Tendenz haben, in die Fraktion C überzugehen. Durch Chromatographie unter Ausschluss von Luftsauerstoff konnte gezeigt werden, dass die Katalase in den Erythrozyten in der Form A vorliegt. Setzt man das Hämolysat dagegen einige Zeit Luftsauerstoff aus, wird die Katalase bei der Chromatographie in Form C eluiert. SH-blockierende Reagentien verhindern die Umwandlung von A in C, während C mit Mercaptoäthanol zu A reduziert werden kann. Es wird angenommen, dass dem Übergang von Fraktion A in B und C eine Bildung von Disulfidbrücken zugrunde liegt und dass es sich bei den beobachteten alternativen Formen möglicherweise um Katalase-Konformere handelt.

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Medizinisch-Chemisches Institut der Universität Bern (Switzerland), 31 October 1967.

[10] N. O. Kaplan, 7th Int. Congr. Biochem. Tokyo 1967 Symposium III, 3, 6, Abstracts p. 179.
[11] H. Aebi, Int. Symp. on hereditary disorders of erythrocyte metabolism, Duarte (February 1967).
[12] H. Walter, F. W. Selby and J. R. Francisco, Nature *208*, 76 (1965).
[13] B. Chance and A. C. Maehly, in *Methods in Enzymology* (Eds S. P. Colowick and N. O. Kaplan; Academic Press, New York 1955), vol. 2, p. 764.
[14] Acknowledgment: This investigation is part of project No. 3785 subsidized by the Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung.
[15] A preliminary report has been presented on 15 December 1967 at the annual meeting of the Swiss Biochemical Society in Basle.

Prompt Effect of Insulin in vitro on the Esterolytic Activity of Tissue Homogenates not Modified by Actinomycin D

In the course of our study of the mechanism of insulin action in the lipolytic system, we centred our attention on the role of group-specific esterases. It is possible that these enzymes might, besides lipases, take part in lipolysis. Esterases, in contradistinction to lipases[1,2], act predominantly on aqueous solutions of the substrates. In spite of the fact that most tissues contain complex mixtures of esterases with high total activities[3,4], their physiological function has so far remained unexplained[5].

In the experiments which were partly reported elsewhere[6,7], we have found that the esterolytic system of adipose tissue from starved rats is sensitive in vitro to insulin. These results were obtained after preincubation of samples of the whole tissue with insulin in the presence of NaCl. In this paper we have studied the influence of insulin in vitro on the esterolytic activity of tissue homogenates taken from fed as well as from starved rats in the presence of KCl or NaCl in the medium. Moreover, we have examined whether the tissue esterolytic activity changes during fasting and whether insulin also acts in vivo as an inducer or suppressor of the synthesis of esterases.

Materials and methods. Albino Wistar rats (inbred strain) weighing 150–200 g were used. They were fed on the usual Larsen diet[8] and, before the experiment, were fed or fasted for 48 and 96 h respectively.

One group of rats was made diabetic by using alloxan-monohydrate[9]. Diabetes produced by alloxan was stable as indicated by persistant glycosuria and hyperglycemia. Glucose was estimated by glucose oxidase test[10]. Insulin (Insulin Spofa pro injectione) and actinomycin D were applied to alloxan-diabetic rats during 24 h[9].

Epididymal fat pad or myocardium, femoral muscles, lungs, liver, and kidney were weighed immediately after killing the animals and homogenated in distilled water by using a glass homogenizer of Potter-Elvehjem type as well as sea sand (under standard conditions). For estimation were used samples of 0.1 ml of supernatant fluid obtained after centrifugation at *2000 g*. Each sample represented 2 mg of tissue.

For the determination of esterolytic activity was used 2-naphtolacetate dissolved in 0.2 *M* veronal buffer pH 7 (adipose tissue and skeletal muscle) or 7.5 (myocardium and lungs). The liberated 2-naphtol was determined colorimetrically[11] or fluorimetrically[12]. In the samples of tissue homogenates proteins were determined similarly by

[1] P. Desnuelle and P. Savary, J. Lipid Res. *4*, 369 (1963).
[2] L. Sarda and P. Desnuelle, Biochim. biophys. Acta *30*, 513 (1958).
[3] C. I. Markert and R. L. Hunter, J. Histochem. Cytochem. *7*, 42 (1959).
[4] J. G. Huggins and S. H. Moulton, J. exp. Med. *88*, 169 (1948).
[5] D. K. Myers, in *The Enzymes*, 2nd edn (Eds P. D. Boyer, H. Lardy and K. Myrback; Acad. Press Inc., New York 1960), vol. 4, p. 475.
[6] M. Chmelař and M. Chmelařová, Plzen. lék. Sb. Suppl. *16*, 145 (1966).
[7] M. Chmelařová and M. Chmelař, Abstracts of the 3rd Fedn Europ. Biochem. Socs Meeting, Warsaw, 1966, p. 329.
[8] P. Fábry, Čsl. Fysiol. *8*, 529 (1959).
[9] A. Gellhorn and W. Benjamin, Science *146*, 1166 (1964).
[10] A. St. G. Hugget and D. A. Nixon, Biochem. J. *66*, 12P (1957).
[11] M. M. Nachlas and A. M. Seligman, J. biol. Chem. *181*, 343 (1949).
[12] M. Chmelařová, M. Chmelař and B. Večerek, Colln Czech. chem. Commun. Engl. Edn *31*, 1886 (1966).