doubtful or negative results. Its selective depigmenting action was first observed within a few weeks of starting experiments on its carcinogenicity in which applications (0.3% w/v in acetone, twice weekly) were made to the clipped dorsal skin of C57BL × IF F_1 hybrid mice from about 8 weeks of age.

Subsequent comparative tests showed that depigmentation occurred more readily in pure line C57BL mice than in the hybrids, but less readily in IF mice. Male mice were always much less affected than females of the same strain.

Female C57BL mice have therefore been used in most experiments. In these mice, 0.1% 8-HQ in acetone applied twice weekly caused just noticeable depigmentation. At 0.5%, however, new hair growth consisted to a major extent of non-pigmented hair, giving rise to a variety of coat patterns as illustrated in Figures 1 and 2. Further increase to 1.0%, or increased frequency of application, made little apparent difference and complete depigmentation has not been observed.

The lesser effect of 0.5% 8-HQ on female IF mice is shown in Figure 3. Several strains of brown mice (C3H, CBA, DBA, MP) given similar treatment also developed patterns of depigmentation (Figure 4).

Applications at 0.5% in acidified aqueous solution or in liquid paraffin similarly induced selective depigmentation in C57BL females. Administration in the drinking water at strengths up to 0.5% had no effect, but this is probably not surprising as 8-HQ is inactivated by intact red blood cells[3] and is unlikely to reach the follicles in an active form.

In contrast to the striking effect of 8-HQ on young adult female mice, applications to C57BL or C57BL × IF mice from 4 days of age had no effect on pigmentation of the first coat of hair. Maximum variegation appears to occur in growth cycles G3 and G4 at approximately 3 to 9 months of age, after which treated mice develop less distinct patterns of depigmentation apparently correlated with the more patchy growth of hair which occurs in older animals.

No report has been found of 8-HQ as an inhibitor of melanogenesis in vivo, though it has been reported to inhibit potato tyrosinase in vitro[4]. Many other substances have long been known to interfere with some stage of melanogenesis, which involves the copper enzyme tyrosinase, but applications of various other copper reagents (2-mercaptopyridine, 8-mercaptoquinoline hydrochloride, cupron, 2,2′-biquinolyl, 2,9-dimethyl-1,10-phenanthroline and zinc dibenzyldithiocarbamate) all failed to inhibit pigmentation in female C57BL mice.

This suggests that the depigmenting action of 8-HQ, like its antibacterial and fungistatic action[3], may depend on the toxic action of a metal complex rather than to direct combination with tyrosinase copper. Although it is suggested that the selective action against follicles in the treated area is probably related to the hair growth cycles, the mode of action of this selectivity is not known.

Zusammenfassung. Die Melanogenese wird bei Mäusen durch 8-Hydroxychinolin selektiv gehemmt. Besonders bei behandelten weiblichen Tieren des Stammes C57BL entwickelt sich ein aussergewöhnliches Haarmuster mit abwechselnden schwarzen und weissen Streifen oder Kreisen. Diese reversible Wirkung wurde bei neugeborenen Mäusen oder nach Behandlung mit Cu-reaktiven Substanzen nicht beobachtet.

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1 K. Borum, Acta path. microbiol. scand. *34*, 521 (1954).
2 A. Haddow, L. A. Elson, E. M. F. Roe, K. M. Rudall and G. M. Timmis, Nature *155*, 379 (1945).
3 A. Albert, *Selective Toxicity*, 4th Edn. (Methuen, London 1968), Chapt. 9.
4 C. Tsukamoto and T. Taniguchi, J. pharm. Soc. Japan *77*, 462 (1957). – Chem. Abstr. *51*, 16604 (1957).
5 I wish to thank Professor D. G. Harnden and Dr. Mary Lyon for their interest and advice, and Mr. C. R. Richardson for technical assistance. The work was supported by the Birmingham Branch of the British Empire Cancer Campaign for Research.

Heterogeneity of Erythrocyte Catalase: Variability of the Isoelectric Point

Human as well as horse erythrocyte catalase can be separated into three enzymatically active fractions designated as A, B and C, according to the molarity of the buffer required for elution from DEAE-cellulose at pH 6.8 (A < B < C)[1–3]. In the absence of oxygen and heavy metal catalysts, fraction A is found exclusively[2–4]. In order to elucidate whether purified fraction A corresponds to the native form of catalase, the isoelectric point (IEP) of this fraction was compared with the one of catalase in a fresh hemolysate.

Fraction B and C of erythrocyte catalase are formed out of fraction A by irreversible oxidation of sulfhydryl groups in the presence of oxygen and heavy metal catalysts[4]. Evidence for the formation of acidic groups during this transition was obtained by comparing the IEP of these fractions.

Materials and methods. Purified fractions A and C of human and horse erythrocyte catalase, as well as hemolysates, were prepared as described previously[4]. Catalase activity was determined spectrophotometrically (Beckman DB) at 240 nm and 25 °C[5]. The number of sulfhydryl groups, disulphide bridges and irreversibly oxidized sulfhydryl groups present in the fractions used for isoelectric focusing was determined by titration with *p*-Chloromercuribenzoate (pCMB)[4,6]. The results are summarized in the Table.

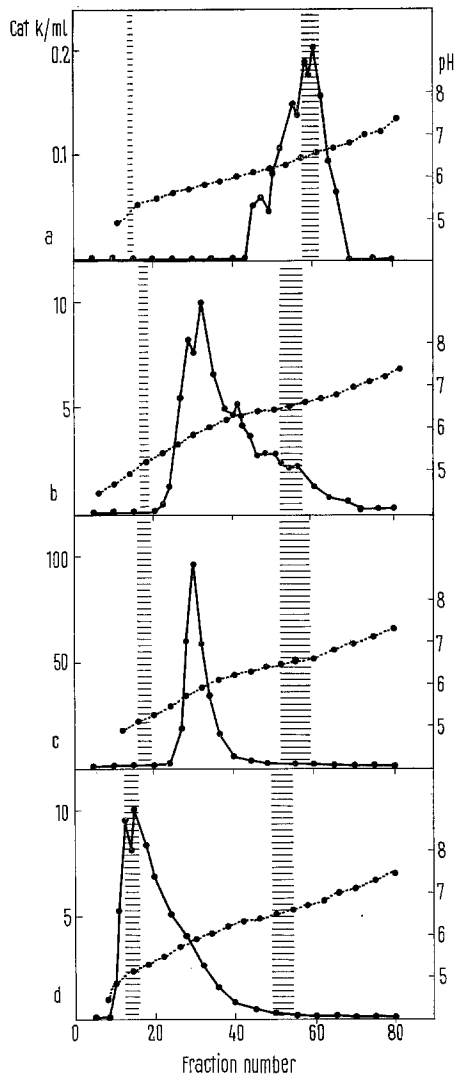
The isoelectric focusing experiments were performed in a Uniphor apparatus (LKB-Produkter AB, Stockholm-Bromma, Sweden) according to the method of Vester-

1 O. A. Thorup, J. T. Carpenter and P. Howard, Br. J. Haematol. *10*, 542 (1964).
2 M. Cantz, S. Mörikofer-Zwez, E. Bossi, H. Kaufmann, J. P. von Wartburg and H. Aebi, Experientia *24*, 119 (1968).
3 H. Aebi, in *Exposés annuels de Biochimie Médicale* (Masson & Cie., Paris 1969), 29ième série, p. 139.
4 S. Mörikofer-Zwez, M. Cantz, H. Kaufmann, J. P. von Wartburg and H. Aebi, Europ. J. Biochem. *11*, 49 (1969).
5 H. Aebi and H. Suter, in *Biochemical Methods in Red Cell Genetics* (Ed. J. J. Yunis; Academic Press, New York, 1968), p. 272.
6 P. D. Boyer, J. Am. chem. Soc. *76*, 4331 (1954).

Sulfhydryl group content and isoelectric point of different molecular forms of erythrocyte catalase

Species	Molecular form	IEP (pH)	No. of groups per molecule (mol wt 225,000) Sulf-hydryl	Disul-phide	Irreversibly oxidized sulfhydryl
Human	Native enzyme	6.5	–	–	–
Human	Fraction A	5.95	13.8	1	0
Human	Intermediate fraction[a]	5.8	11.8	1	~ 2
Human	Fraction C	5.2	3.5	1	~10
Horse	Native enzyme	7.3	–	–	–
Horse	Fraction A	5.9–6.3	15.8	0	0
Horse	Fraction C	5.7	5.6	0	~10

[a] For explanation see text.



Isoelectric focusing patterns of different molecular forms of human erythrocyte catalase. a) Fresh hemolysate (polarity of the electrodes inversed); b) fraction A (14 sulfhydryl groups); c) fraction C (2 sulfhydryl groups oxidized irreversibly); d) fraction C (10 sulfhydryl groups oxidized irreversibly). For experimental details see text. ●—●, catalase activity (k/ml); ●···●, pH (measured at 0 °C). ▒, pH 6.45–6.55; ▒, pH 5.10–5.20.

BERG and SVENSSON[7]. The preparation of the saccharose-gradient was adapted to the volume of the Uniphor-column (2.5 × 34 cm, 165 ml). Carrier ampholytes 'Ampholine' (LKB-Produkter AB) covering a pH-range from 5 to 7 were used throughout. In all electrofocusing experiments, a tension of 600 volts was applied for 44–63 h. The temperature was kept constant at 9 °C by cooling with tap water. Unless otherwise stated, the anode was positioned at the bottom of the column. After each focusing experiment, 1.6 ml fractions were collected from the bottom of the column.

Results. The isoelectric point (IEP) of human and horse erythrocyte catalase was determined by isoelectric focusing of freshly prepared hemolysates (Figure a and Table). The resulting values were pH 6.5 for the human and pH 7.3 for the horse enzyme. The enzyme present in these hemolysates was considered to represent the native form of catalase. In both types of experiments catalase was completely separated from hemoglobin. The slight asymmetry of the activity curve possibly indicates the formation of some other forms of catalase during the isoelectric focusing.

Fraction A of human erythrocyte catalase is chromatographically homogenous[4]. However, the isoelectric focusing experiment shown in Figure b suggests the presence of several molecular forms of catalase within this fraction. The main part of the activity is focused at pH 5.95. Only a small amount of native catalase as found in hemolysates (IEP = 6.5) seems to be present. Similar results were obtained with fraction A of the horse enzyme.

In the presence of oxygen, but in the absence of heavy metal catalysts, fraction A of the human enzyme is converted to a relatively stable intermediate, containing 2 irreversibly oxidized sulfhydryl-groups (Table). This intermediate fraction is eluted from DEAE-cellulose as fraction C. It has an IEP of 5.8 and apparently consists of only one molecular species (Figure c). In a second oxidation step, taking place in presence of oxygen and heavy metal catalysts, more than 2 sulfhydryl groups may be irreversibly oxidized. This results in a further lowering of the IEP of catalase fraction C without affecting its activity or its chromatographic properties. Figure d shows the isoelectric focusing of a fraction C which contained approximately 10 irreversibly oxidized cystein residues (Table). The main part of the active material is focused at pH 5.20.

In horse erythrocyte catalase, the transition from fraction A to fraction C takes place only in presence of oxygen and heavy metal catalysts[4]. A stable intermediate, as detected in human erythrocyte catalase, was not found. Fraction C, containing approximately 10 irreversibly oxidized cystein residues, has an IEP of 5.70 (Table).

Discussion. The transition of fraction A of human as well as horse erythrocyte catalase to fraction C is characterized by an irreversible oxidation of sulfhydryl groups[4] and is paralleled by a considerable shift of the IEP. The results indicate that the IEP is related to the number of irreversibly oxidized sulfhydryl groups (Figure b, c and d). This observation is compatible with the assumption that, during the transition of fraction A to C, acid derivatives of cystein are formed by oxidation of sulfhydryl groups present in fraction A.

The difference in IEP between the enzyme present in a fresh hemolysate and the oxidized fraction C is larger

[7] O. VESTERBERG and H. SVENSSON, Acta chem. scand. *20*, 820 (1966).

than the one between fractions A and C, and amounts to 1.3 pH-units in the human enzyme (pH 6.5–5.2) and 1.6 in horse erythrocyte catalase (pH 7.3–5.7). Thus, additional factors other than SH-oxidation affect the IEP of erythrocyte catalase. Fraction A, although isolated under mild, non-oxidizing conditions, has a lower IEP than catalase in a fresh hemolysate and therefore does not correspond to the native enzyme. Since fraction A contains no irreversibly oxidized sulfhydryl groups[4], the difference cannot be attributed to the formation of acid derivatives of cystein. During the isoelectric focusing of hemolysates, hemoglobin is present in a large excess and might affect the result by protein-protein interaction. This possibility can however be ruled out, since catalase is always completely separated from hemoglobin. Furthermore, the addition of hemoglobin does not significantly influence the IEP of purified, hemoglobin-free fraction A. The observed difference is possibly due to conformational changes occurring during the purification of the enzyme.

The data on the IEP of different catalase preparations reported in the literature[8] correspond to the value obtained for fraction C. It is concluded that those catalase preparations must have contained a high percentage of irreversibly oxidized sulfhydryl groups[9].

Zusammenfassung. Mittels isoelektrischer Fokussierung wurde der IEP von nativer Erythrocytenkatalase bestimmt (Mensch pH 6,5; Pferd pH 7,3). Die im Verlauf der Reinigung auftretende Konformationsänderung ist von einer Verschiebung des IEP nach der sauren Seite begleitet (Mensch ΔpH $-1{,}3$; Pferd ΔpH $-1{,}6$).

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[8] P. Nicholls and G. R. Schonbaum, in *The Enzymes* (Eds. P. D. Boyer, H. Lardy and K. Myrbäck; Academic Press, New York 1963), vol. 8, p. 159.

[9] Acknowledgement. The skillful technical assistance of Miss V. Bissegger is gratefully acknowledged. This investigation was supported by the Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung, project No. 5021.3.

The Role of Neuraminic Acid in the Stability and Enzymic Activity of Acid Phosphatase of the Human Prostate Gland

Recently it was reported that acid phosphatase of the human prostate (EC 3.2.3.1) occurs in several fractions, which can be separated by chromatography on DEAE or CM-cellulose[1,2] and by isoelectric focusing[3]. The heterogeneity is a result of the presence of N-acetylneuraminic acid (NANA) in the phosphatase molecule, which can be split off by means of neuraminidase without loss of enzymic activity[2]. It was therefore of interest to compare some of the properties of native phosphatase, such as thermostability, optimum pH, K_m and K_i with neuraminidase-treated enzyme.

Acid phosphatase activity was assayed under standard conditions[4] with *p*-nitrophenylphosphate, disodium salt (*p*-NPP), product of Sigma (Biochem. Corp., St. Louis, Mo.) as substrate in 0.1 *M* citrate buffer, pH 5.5. Liberated *p*-nitrophenol was assayed spectrophotometrically (Uvispec, Hilger a. Watts, London) at 420 nm. Activity of the enzyme toward adenilic acid (pA, Koch-Light, Colnbrook, England) and deoxyguanosinediphosphate (d-pGp, kindly provided by Dr. M. Laskowski sr.) was also studied, in this case on the basis of Pi liberated, assayed by a modification of the Fiske-Subbarow method[5].

Acid phosphatase I was used, which represents about 70% of total activity of crude human hypertrophic prostate extract, from which it was obtained and purified by the method previously described[4]. The preparation was homogenous in ultracentrifuge and in disc electrophoresis on polyacrylamide gel. To about 1 mg of enzyme I in 1 ml of 0.05 *M* acetate buffer of pH 5.5 containing 1% NaCl and 0.1% $CaCl_2$, 30 U of neuraminidase from Vibrio cholerae (25,000 U/mg protein, Koch-Light) was added, and the mixture was incubated 20 h at 25 °C. The solution was dialyzed overnight against distilled water and stored in frozen state for further study. Phosphatase I treated with neuraminidase under these conditions, when examined by isoelectric focusing according to Svensson[6], exhibited a single symmetrical peak with isoelectric point at about pH 5.8, whereas the native enzyme gives 3 well-separated peaks at pH 4.82, 4.92 and 5.10 (Figure 1).

Treatment of phosphatase with neuraminidase causes a rise of optimum pH by ca. 0.4 U for pA and *p*-NPP as substrates. Thermostability of both enzymes was studied at 55 °C and different pH. Splitting off NANA had no effect on the sensitivity of phosphatase to heating. Figure 2 shows that both enzymes were inactivated at the same rate in the pH range 4.6–6.75.

The reaction velocities and K_m constants for various substrates of native phosphatase and phosphatase treated with neuraminidase indicate that the enzyme deprived of NANA has greater affinity for all substrates studied (Table). The values of V_{max} and K_m, determined according to the Lineweaver and Burk[7] and by the method of least squares, are always lower for the modified enzyme, indicating greater affinity for the substrate, presumably as a result of diminished surface charge of the protein molecule. Strongly polar substrate molecules possess greater ease of contact with molecules of the modified

[1] W. Ostrowski and J. Rybarska, Biochim. biophys. Acta *105*, 196 (1965).
[2] J. K. Smith and L. G. Whitby, Biochim. biophys. Acta *151*, 607 (1968).
[3] W. Ostrowski, M. Weber, Z. Wasyl, M. Gumińska and E. Luchter, in press (1970).
[4] W. Ostrowski, Acta biochim. pol. *15*, 213 (1968).
[5] M. H. Martland and R. Robinson, Biochem. J. *20*, 847 (1926).
[6] H. Svensson, Arch. Biochem. Biophys., Suppl. *1*, 132 (1962).
[7] H. Lineweaver and D. Burk, J. Am. chem. Soc. *56*, 658 (1934).