lower than 50 mg/100 ml, there was in all dogs a slight increase in the total amount of bilirubin after 8 h of perfusion possibly due to determination and calculation errors and to formation of bilirubin in circulating blood[5]. When the free plasma haemoglobin concentration was higher than 50 mg/100 ml, there was a marked increase in the total amount of bilirubin in all 10 male kidney perfusion systems, but only in 2 of 11 bitches systems. Calculating the difference between the increases at values below and at values above 50 mg/100 ml, there was a true average 7.8-fold increase in the amount of bilirubin in the male systems, and an average 8.8-fold in the females, which represented an average of respectively 2.2 (0.99–4.50) and 2.7 (1.81–4.80) mg bilirubin increase during 8 h of perfusion of 1 kidney. Taking into account that 1 g haemoglobin yields 34 mg of bilirubin, there was a bilirubin formation of respectively average 65 (29–132) and 79 (53–141) mg haemoglobin. This amount of haemoglobin must be broken down by the kidney. At the end of the perfusion the increased amount of bilirubin could be found, either in the plasma, or in the urine. The results are listed in Table II. In male isolated kidneys with free plasma haemoglobin levels of more than 50 mg/100 ml, the percentage excretion (63.2 ± 26.8%) of the total amount bilirubin in the urine was much higher than in all other groups, as shown in Table II, and could be compared with the much higher urinary bilirubin excretion in male whole dogs, as shown in the Figure.

Table II. Excretion of bilirubin in the urine in 34 isolated normothermic perfused dog kidneys

Free plasma* haemoglobin (mg/100 ml)	% of total amount of bilirubin excreted in the urine	
	Male kidneys (17)	Female kidneys (17)
<50	(7) 16.0 ± 8.1	(6) 9.4 ± 7.6
>50	(10) 63.2 ± 26.8	(9) 18.0 ± 16.5
		(2) 6.9 ± 0.1

*Values are averages ± standard deviations. Figures between brackets are number of experiments.

Recently BARAC[6] reported that the urinary elimination of bilirubin in dogs after injection of unconjugated bilirubin was higher in males than in bitches. We can conclude that there are really sex-linked differences in whole dog kidneys and in isolated perfused dog kidneys, concerning the formation, handling and excretion of bilirubin by the kidney, possibly due to the existence of sexual differences[7] in the renal complements of certain enzymes.

Résumé. Si le taux sanguin en hémoglobine libre dépasse les 50 mg/100 ml, l'excrétion rénale de la bilirubine est fortement augmentée chez le chien, tandis qu'elle reste faible chez la chienne. Les mêmes résultats sont obtenues par perfusion de reins isolés avec du sang hépariné ou défibriné.

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[5] T. K. WITH, *Bile Pigments. Chemical, Biological and Clinical Aspects* (Academic Press, New York and London 1968).
[6] G. BARAC, C. r. Scéanc. Soc. Biol., Paris *164*, 916 (1970).
[7] J. B. LONGLEY in *The Kidney* (Eds. C. ROUILLER and A. F. MULLER; Academic Press, New York and London 1963), p. 158.

The Effect of Heat on the Isoelectric and Size Properties of Horseradish Peroxidase

Heat treatment is still the best method for enzyme inactivation in food processing. As a result of residual enzyme activities, however, undesired organoleptic changes are frequently observed. This could at least partly be attributable to heat-resistant isoenzymes.

The inactivation of peroxidase in foodstuffs is of technological importance, due to its high resistance to heat treatment. Isoelectric focusing in gel-stabilized layers[1], and thin-layer gel filtration[2] offer an excellent opportunity for a study of both charge and size properties of this enzyme. Thin-layer isoelectric focusing revealed more than 20 distinct isoenzymes of horseradish peroxidase, a heterogeneity which had not been found previously by any other method[3]. No size differences between the isoenzymes were observed by gel filtration. A study of the effects of heat on the complex peroxidase isoenzyme system was undertaken with the aim of gaining a better understanding of the molecular processes which proceed during inactivation of enzymes in foodstuffs. Further work with isolated isoenzymes is in progress.

Materials and methods. Horseradish peroxidase with an absorbance ratio $A_{403\ nm}/A_{275\ nm}$ of 0.6 was purchased from Boehringer (Mannheim, West Germany). A monomeric material was obtained by column gel filtration on Sephadex G-200, by which the absorbance ratio increased to about 1.5 without changes in the isoelectric pattern. The peroxidase was heated in sealed ampoules flushed with argon as an approximately 1% protein solution in a 0.01 *M* phosphate buffer (pH 7.2). The samples were heated in a temperature-controlled (± 0.1 °C) waterbath for the desired time (5, 10, 20 and 40 min) at 90 °C. Immediately after heating, the ampoules were immersed in ice-water. Thin-layer gel filtration and isoelectric focusing were performed as described previously[1–3].

Results. In agreement with determinations of total peroxidase activity in the heated samples (guaiacol assay[4]), an overall decrease of enzyme staining was noted by thin-layer isoelectric focusing in pH 3–10 ampholytes, when equal volumes of the samples were applied (Figure 1). With equal amounts of enzyme activity applied, distinct qualitative and quantitative changes in the isoelectric patterns were demonstrated (Figure 2), which were less evident in the experiment with equal volumes of the heat-treated enzyme solution. The most basic isoenzymes (Group IV) became preferentially inactivated, and after 40 min at 90 °C nearly all isoenzymes with a pI higher than 8 disappeared. Within the Group III isoenzymes, which comprises most of the activity of the untreated enzyme, quantitative changes resulting in an increase of isoenzymes with lower isoelectric points were observed

[1] B. J. RADOLA, J. Chromat. *38*, 61 (1968).
[2] B. J. RADOLA, Biochim. biophys. Acta *194*, 335 (1969).
[3] H. DELINCÉE and B. J. RADOLA, Biochim. biophys. Acta *200*, 404 (1970).
[4] B. CHANCE and A. C. MACHLY, in *Methods in Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN; Academic Press, New York 1955), vol. 2, p. 770.

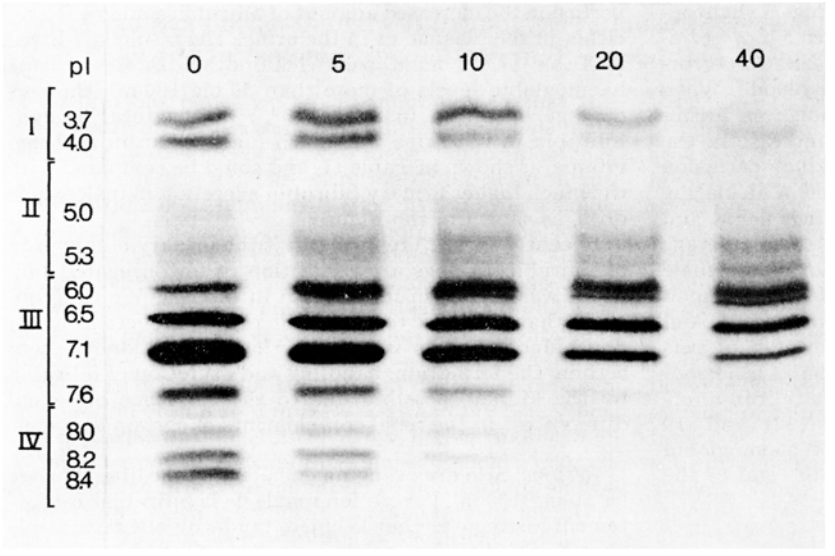

Fig. 1. Thin-layer isoelectric focusing in pH 3–10 ampholytes of heat-treated horseradish peroxidase. Heating time at 90 °C/min above patterns. Plate, 20 × 20 cm, coated with a 0.75 mm layer of a Sephadex G-75 Superfine suspension containing 1% of LKB carrier ampholytes. Focusing at 10 V/cm for 7 h at 4–6 °C. For enzyme detection a print was taken with a buffered (0.3 *M* citrate – 0.6 *M* phosphate buffer with pH 5.0) paper (Schleicher and Schüll 2043 b Mgl or 2040 b), impregnated with 1% urea-peroxide and *o*-toluidine in methanol. Equal sample volumes were applied.

(Figure 2). Already after 5 min heating, the amount of the pI 6.0 isoenzyme increased. After 20 min heating the amounts of the pI 7.1, 6.5 and 6.0 isoenzymes were nearly equal, whereas after 40 min the amount of the pI 6.0 isoenzyme exceeded that of the pI 7.1 isoenzyme, the predominant isoenzyme in the control. For the acidic pI 3.7 and 4.0 isoenzymes (Group I) also a quantitative change in the distribution was observed; the amount of the former increased, whereas that of the latter decreased. In addition, a series of enzyme zones appeared in the pH 5–6 range with very similar isoelectric points.

By thin-layer gel filtration of the heat-treated peroxidase only small amounts of enzymatically active aggregates were found in addition to the remaining monomers, when equal activities were applied (Figure 3). The R_M value[1] of these aggregates corresponded to the exclusion limit of the Sephadex G-200 gel. An increase of the amount of active aggregates was noted with increasing heating time. When the print was stained for protein with Amido black 10 B, rather than for peroxidase activity, large amounts of high molecular aggregates were observed (Figure 4). With prolonged heating the ratio of aggregates to the residual monomeric protein increased. After 10 min the amount of monomer roughly equalled that of the aggregates.

When the heat-treated peroxidase was separated into monomers and aggregates by preparative gel filtration in gel-stabilized layers, subsequent thin-layer isoelectric focusing of the monomers revealed distinct changes in the patterns of the heat-treated monomers, basically of the same type as those observed for the unfractionated samples. In contrast to the patterns of the unfractionated heated samples, the monomers contained, instead of the series of enzyme zones with very close isoelectric points in the pH 5–6 range, only 2 distinct isoenzymes with isoelectric points in this range. The enzymatically active aggregates were focused only in the pH 5–6 range with a diffuse distribution of activity. After storage for a few days, part of the aggregates precipitated, and further focusing revealed activity only on the starting line. No or only traces of precipitate were observed in the unfractionated samples.

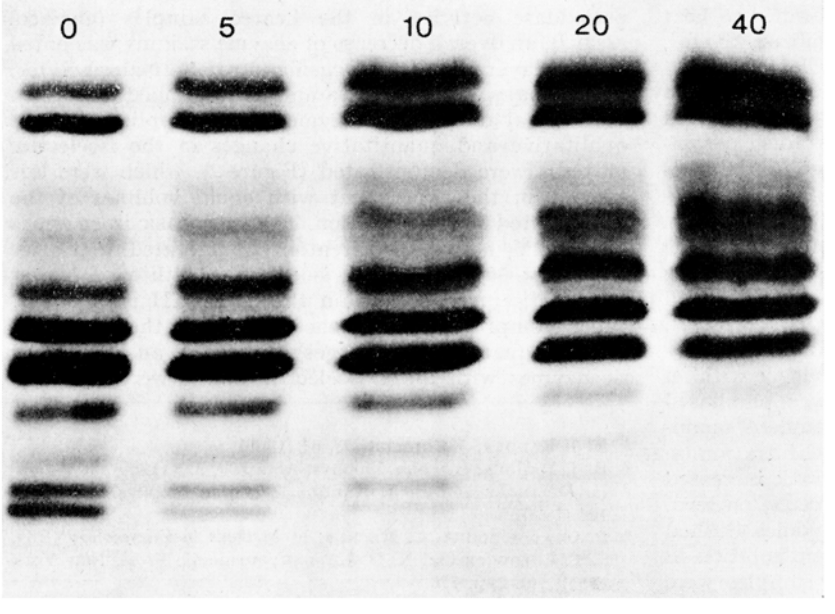

Fig. 2. Thin-layer isoelectric focusing in pH 3–10 ampholytes of heat-treated horseradish peroxidase. Equal amounts of peroxidase activity were applied.

Fig. 3. Densitograms (reflectance) of thin-layer gel filtration on Sephadex G-200 Superfine of heat-treated horseradish peroxidase, stained for enzyme detection. The peak to the right corresponds to the starting line. Equal amounts of activity were applied.

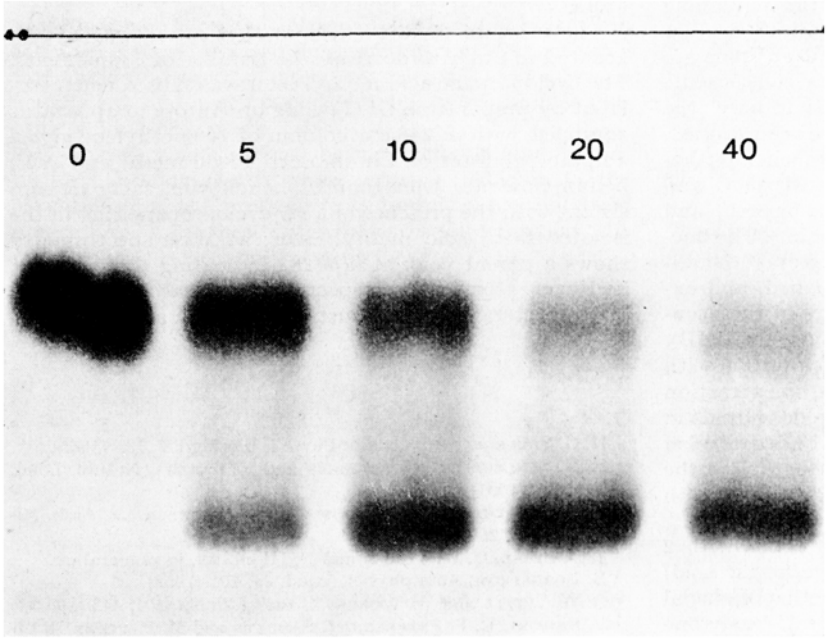

Fig. 4. Thin-layer gel filtration on Sephadex G-200 Superfine of heat-treated horseradish peroxidase, stained for protein with Amidoblack 10B. Equal sample volumes were applied.

Discussion. The results demonstrate that heat inactivation of peroxidase proceeds through 2 different mechanisms: 1. Molecular aggregation resulting in large aggregates with only traces of enzyme activity, and 2. modification of the monomeric enzyme. Already at low degrees of enzyme inactivation (short heating time) the aggregation proceeds rapidly to very large aggregates. This is in contrast to the radiation inactivation of peroxidase[5], where aggregation apparently proceeds through a dimer, trimer, etc. to the larger aggregates. The modification of the monomers after heating suggests a stepwise degradation, by which the content of basic isoenzymes is reduced. Splitting of labile amide groups or deamination could be an explanation for these effects. As opposed to radiolytic degradation of the monomeric enzyme, the multiplicity of radiation-induced enzyme zones, mainly in the pH 4–6 range, was not observed for the heat-degraded monomer. Also, the most acidic isoenzymes did not disappear in the isoelectric pattern of the heat-treated enzyme, only a distribution to more acidity was observed. Evidently, the molecular processes leading to the inactivation of peroxidase by heat or by irradiation are different[6].

Zusammenfassung. Unter Einwirkung von Hitze (90 °C) auf die Meerrettich-Peroxidase wurden die basischen Isoenzyme bevorzugt inaktiviert. Eine quantitative Verlagerung der Isoenzymverteilung mit einer Umwandlung zu Isoenzymen mit niedrigeren isoelektrischen Punkten wurde beobachtet. Es wurden nur geringe Mengen neuer enzymatisch aktiver Komponenten mit Hilfe der dünnschicht-isoelektrischen Fokussierung gefunden. Mittels der Dünnschicht-Gelfiltration wurde eine Aggregatbildung gefunden, wobei die Aggregate ein sehr hohes Molekulargewicht (>500000) aufwiesen und nur Spuren von Aktivität aufzeigten.

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1 April 1971.

[5] H. Delincée, B. J. Radola and F. Drawert, Int. J. Radiat. Biol. *19*, 93 (1971).

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