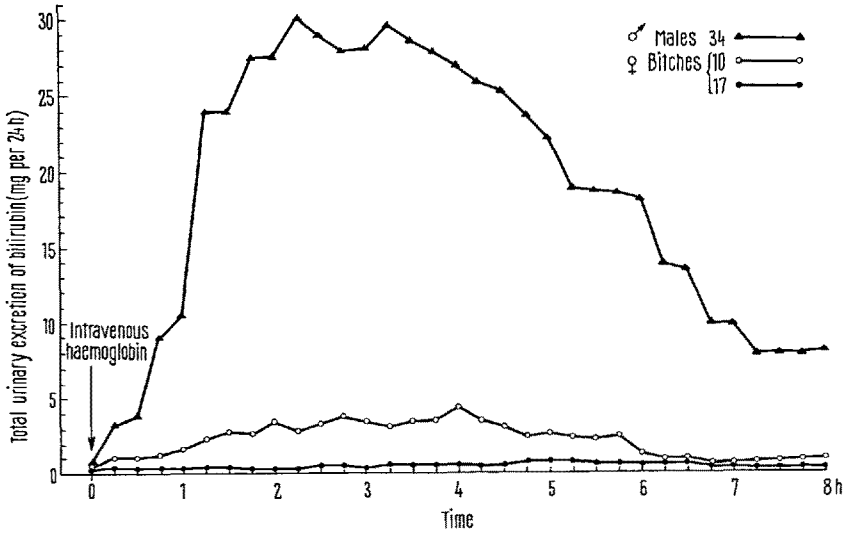
Influence of Sex on the Urinary Bilirubin Excretion at Increased Free Plasma Haemoglobin Levels in Whole Dogs and in Isolated Normothermic Perfused Dog Kidneys

In a previous paper we have communicated that the normal daily urinary excretion of bilirubin in whole male dogs (0.72 ± 0.64 mg/24 h) was higher than in bitches (0.32 ± 0.09 mg/24 h[1]. The excretion of bilirubin in the urine of whole dogs remained very constant after a single i.v. injection of a haemoglobin solution, if the haemoglobin binding capacity of the plasma did not exceed 50 mg/100 ml[2]. When the free plasma haemoglobin concentration was higher than 50 mg/100 ml, there was, in 82% of the cases, a marked increase in the total 24 h bilirubin excretion in the urine[3]. In continuing our observations, we remarked that there was a significant difference between the excretion of bilirubin in male dogs and bitches. When the free plasma haemoglobin level was higher than 50 mg/100 ml in a group of 61 dogs, all 34 male dogs had a marked increase of the urinary elimination of bilirubin, as shown in the Figure, with a highest excretion rate of an average 30.1 ± 26.2 mg/24 h bilirubin. In the bitches, 10 had a slight increase with a highest average 4.4 ± 5.4 mg/24 h bilirubin, and 17 showed no change in the excretion rate of bilirubin (Figure), in which the highest value was 0.7 ± 0.5 mg/24 h bilirubin. Since there were great variations in the total blood volume of the dogs by changes in diuresis and infusion, it was not possible to determine the difference in total amount of bilirubin in the plasma before and at the end of the experiment.

In order to prove that this increased urinary excretion of bilirubin was due to a formation of bilirubin from reabsorbed haemoglobin in the kidney, the same experiments were repeated with isolated normothermic perfused dog kidneys. The perfusion apparatus was a modification of the apparatus of CUYPERS and NIZET[4]. In these isolated kidneys we also found some indication that the normal daily excretion of bilirubin in the urine was higher in males (0.55 ± 0.61 mg/24 h) than in bitches (0.15 ± 0.14 mg/24 h), although both values are lower than in vivo (cfr. supra). Because there was a certain amount of blood in the perfusion system, we could measure the total amount of bilirubin before and at the end of 8 h of perfusion, and calculate the difference between both. The results of these determinations and calculations are listed in Table I. When the free plasma haemoglobin levels were

[1] J. DE SCHEPPER and J. VAN DER STOCK, Vlaams diergeneesk. Tijdschr. *39*, 191 (1970).
[2] J. VAN DER STOCK and J. DE SCHEPPER, Verh. K. Vlaam. Acad. Geneesk. Belg. *31*, 141 (1968).
[3] J. VAN DER STOCK and J. DE SCHEPPER, Experientia, *25*, 814 (1969).
[4] Y. CUYPERS, A. NIZET and A. BAERTEN, Archs. Int. Physiol. Biochim. *72*, 245 (1964).



The excretion of bilirubin in the urine after total saturation of the plasma haptoglobin with haemoglobin and an excess of free plasma haemoglobin >50 mg/100 ml in 61 dogs.

Table I. Changes in the total amount of bilirubin in 34 isolated perfused dog kidney systems during 8 h of perfusion

Free plasma haemoglobin (mg/100 ml)	Total amount of bilirubin (mg)					
	Male kidneys (17)			Female kidneys (17)		
	Begin	End	Increase	Begin	End	Increase
<50 mg/100 ml	(7)			(6)		
average[a] ± S.D.	0.20 ± 0.16	0.73 ± 0.48	0.53 ± 0.34	0.21 ± 0.10	0.43 ± 0.40	0.22 ± 0.21
>50 mg/100 ml	(10)			(2)		
average ± S.D.	0.29 ± 0.11	3.23 ± 1.34	2.94 ± 1.22	0.31 ± 0.12	3.31 ± 2.25	3.00 ± 2.05
>50 mg/100 ml				(9)		
average ± S.D.				0.30 ± 0.14	0.56 ± 0.39	0.26 ± 0.20

[a] Values are averages ± standard deviations. Figures between brackets are number of experiments.

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.