The Influence of Age on the Activities of Some Acid Hydrolases in the Rat Liver and Kidney

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The process of senescence is generally held to be one involving degenerative changes. This process is particularly evident in the rat kidney where there is extensive atrophy of the functional elements (Kennedy, 1957). In recent years there have been several reports that in degenerating tissues there is an activation of intracellular hydrolases which have an acid optimum pH, and that the increase is due to a release of these enzymes from the lysosomes. Thus de Duve & Beaufay (1959) showed that the autolysis of the liver induced by complete ischaemia is accompanied by a rapid increase in the free activity of several acid hydrolases; a number of liver poisons produced a similar effect (Beaufay, Campenhout & de Duve, 1959). Brachet, de Croly-Briers & Hoyez (1958) found that there was a similar redistribution of typical lysosomal enzymes in certain involuting embryonic tissues. The present work deals mainly with the changes in the free and bound activities of a typical lysosomal enzyme, acid phosphatase, in the liver and kidney of the rat from infancy to advanced senility. The liver has been included since it shows relatively little degenerative change in senescence (Barrows, Falzone & Shock, 1960). Less-detailed measurements have been carried out on the arylsulphatase B and acid deoxyribonuclease of the kidney. A study has also been made of the changes in the activities of acid phosphatase and arylsulphatase B which occur during the degeneration induced by the complete ischaemia of the kidney.

METHODS

Animals. White rats of the laboratory Wistar strain of both sexes were used. Animals that had been weaned were starved for 12 hr. before the enzyme assays. Sucking-rats were allowed to remain with the mother until immediately before the experiment.

Preparation of homogenates. The animals were killed by a blow on the back of the head and allowed to bleed freely. Liver and kidneys were removed and weighed in ice-cold sucrose (0.25 M). The organs were homogenized in a Perspex homogenizer of the Potter-Elvehjem type having a clearance of 500 μ . After six strokes of the pestle at 1000 rev./min., the homogenate was made up with 0.25 M-sucrose to a volume of 10 to 20 times the weight of the tissue and filtered through muslin. In some experiments unbroken

cells, nuclei and larger debris were removed by centrifuging the filtered homogenate at 116g for 3 min.

Enzyme assays. (1) Acid phosphatase. The free activity of the enzyme was measured by incubating a 10% (tissue wt./vol.) homogenate (0.25 ml.) with 0.1 M-sodium β -glycerophosphate (0.25 ml.) in 0.1 M-sodium acetate buffer, pH 5.0, made 0.25 M with respect to sucrose, for 10 min. at 37°. The reaction was stopped by the addition of 1 ml. of 10% (w/v) trichloroacetic acid. The total activity of the enzyme was measured in the presence of Triton X-100 (supplied by Charles Lennig and Co. Ltd., 26-28 Bedford Row, London, W.C. 1) at a final concentration of 0.1% (v/v) (Wattiaux & de Duve, 1956). The precipitated protein was separated by centrifuging and the inorganic phosphate of the supernatant was determined by the method of Chen, Torribara & Warner (1956). According to these authors this method is about eight times as sensitive as that of Fiske & Subbarow (1925) and it provides more accurate determination of the inorganic phosphate at the extensive dilutions required to prevent interference by Triton X-100 (Wattiaux & de Duve, 1956).

(2) Arylsulphatase B. This was measured by the method of Roy (1958) by using dipotassium 2-hydroxy-5-nitrophenyl sulphate (Sigma Chemicals Co. Inc., St Louis, Mo., U.S.A.) as the substrate. The homogenates used in this experiment were 5% (tissue wt./vol.).

(3) Acid deoxyribonuclease. Three different methods were used for the estimation of the acid-soluble nucleotides released by the action of this enzyme. In one method, the ultra-violet absorption of the perchloric acid supernatant was measured as described by Wattiaux & de Duve (1956). In the second method, the acid-soluble nucleotides were determined by the method of Burton (1956). In the third technique ^{32}P -labelled DNA was used as substrate. It was prepared by isolating the DNA by the method of Kirby (1957) from the thymus of rats 3 hr. after injection with 100 $\mu\text{C}/100$ g. body wt. of carrier-free [^{32}P]phosphate (The Radiochemical Centre, Amersham, Bucks.). The enzyme activity was determined by measuring the radioactivity of the perchloric acid supernatant after the incubation period in a Geiger-Müller liquid counter. The conditions of incubation throughout were as described by Strauss (1957a).

Nitrogen determinations. These were carried out by a micro-Kjeldahl procedure.

Ligation of kidney. The operation was carried out under light ether anaesthesia and the pedicle of the left kidney was ligated with cotton thread. After closing the incision the animals were transferred to a dry cage and maintained on 10% sucrose for the required period of time. The operation was tolerated well by the animals and they were soon moving about normally after the anaesthesia had worn off.

RESULTS

Acid phosphatase in the cortex and medulla of the kidney. Preliminary experiments were carried out in order to determine whether Triton X-100 could activate acid phosphatase in both medullary and cortical tissue of the kidney. The cortices and medullae from the kidneys of four young-adult female rats were homogenized separately, and the free and total activities assayed. The results are given in Table 1. Although both free and total activities were higher in cortex, the percentage activation of the free activity by Triton X-100 was about the same in the two renal segments. In all subsequent experiments therefore the whole organ was homogenized, thus simplifying the preparation of the homogenates from very young animals.

Effect of complete ischaemia on renal acid phosphatase and arylsulphatase B. This experiment was carried out on young adult female rats and the animals were killed at 0, 4, 6, 12 and 24 hr. after ligation of the renal pedicle. The changes in the free and total activities are given in Table 2. The free activity of the acid phosphatases rose rapidly after ligation, whereas the total activity remained relatively unchanged for 12 hr. Thereafter both free and total activities fell, though the fall was relatively greater in the total activity so that nearly all of the acid phosphatase was free 24 hr. after ligation. The free activity of the arylsulphatase rose rapidly after the operation but reached a constant level at 4 hr. and was essentially unchanged at 24 hr. The total activity of the sulphatase was apparently unaffected by complete ischaemia in the kidney for the duration of the experiment.

Effect of age on the activity of the acid phosphatase of the liver and kidney. The free and total activities were assayed in rats of the following age groups: group 1, infantile (10–15 days); group 2, immature (6–8 weeks); group 3, young adult (6–7 months); group 4, middle-aged (12–14 months); group 5, senile (23–25 months). Equal numbers of male and female rats were used and since there were no obvious sex differences the results were pooled. The results (Table 3) show that, in group 1, the percentage activation of the enzyme was high in both liver and kidney and the total activities were also high. There was a sharp decline in these values in the animals of group 2 and the figures for the percentage activation then remained essentially the same in young-adult and middle-aged animals in both tissues. In the senile animals there was a further fall in the percentage activation by Triton X-100 in the kidney, which was different from the figure for group 4 at the 5% level of significance and different from the group 3 figure at the 1% level. The percentage activation of the acid phos-

phatase of the liver was not significantly different in the senile group from the figures obtained in groups 4 and 3.

Free and total acid phosphatase of the liver showed a further fall in the young-adult animals compared with the figures for group 2 but showed no further significant changes in groups 4 and 5. The total acid phosphatase of the kidney, on the other hand, showed a progressive decline throughout the life of the animal, the fall in the senile group being especially marked. There was also a decline in the free activity of the kidney, although this was much smaller than the fall in the total activity.

Effect of age on the activity of arylsulphatase B in the kidney. The results of this experiment (Table 4) show that there was no difference between the

Table 1. Activation of acid phosphatase by Triton X-100 in renal medullary and cortical tissue homogenates

	Activity of acid phosphatase (μ g. of P/mg. of N/ 10 min.)		Activation by Triton X-100 (%)
	Free	Total	
Medulla	41.7	67.6	62.1
Cortex	52.1	86.8	66.5

Table 2. Effect of complete ischaemia on the free and total activities of acid phosphatase and arylsulphatase B of the rat kidney

Homogenates were prepared in 0.25M-sucrose, 10% (w/v) for acid phosphatase and 5% (w/v) for arylsulphatase B. Acid phosphatase was assayed at pH 5.0 and arylsulphatase B at pH 5.7. Values are the mean of results obtained from two animals in each case.

Time after ligation (hr.)	Activity of enzyme		Activation by Triton X-100 (%)
	Free	Total	
Acid phosphatase (μ g. of P/mg. of N/10 min.)			
0	43.5	72.5	66.7
4	57.8	77.6	34.3
6	59.1	66.0	11.6
12	68.4	79.0	15.5
24	38.4	39.5	2.9
Arylsulphatase B (μ g. of nitrocatechol/mg. of N/15 min.)			
0	313	519	65.6
4	382	459	20.1
6	388	458	18.0
12	396	471	19.8
24	460	531	15.6

Table 3. *Effect of age on the activities of acid phosphatase and of kidney and liver*

Experimental conditions were as in the text. Figures in parentheses indicate the number of animals used; for the 10-15-day-old animals the organs from four rats were pooled and assayed together making four groups in all. Values given are mean $\pm$ S.E.M.

Age	Acid phosphatase (μ g. of P/mg. of N/10 min.)		Significance of difference	Activation by Triton X-100 (%)	Significance of difference
	Free	Total			
Kidney					
10-15 days (16)	36.80 $\pm$ 3.36	86.50 $\pm$ 4.84	$P < 0.001$	135.10 $\pm$ 13.45	$0.01 > P > 0.001$ $0.05 > P > 0.01$
6-8 weeks (6)	45.00 $\pm$ 3.21	75.20 $\pm$ 3.65		67.10 $\pm$ 4.10	
6-7 months (11)	44.50 $\pm$ 1.86	73.10 $\pm$ 2.52		64.20 $\pm$ 2.90	
12-14 months (12)	43.00 $\pm$ 1.68	70.20 $\pm$ 2.07		63.20 $\pm$ 5.48	
23-25 months (12)	42.00 $\pm$ 1.14	62.90 $\pm$ 1.30		49.80 $\pm$ 4.09	
Liver					
10-15 days (16)	30.00 $\pm$ 2.02	75.30 $\pm$ 6.55	$P > 0.1$	151.00 $\pm$ 11.50	$P > 0.1$
6-8 weeks (6)	29.10 $\pm$ 1.65	59.00 $\pm$ 2.11		102.90 $\pm$ 5.39	
6-7 months (11)	25.50 $\pm$ 1.48	51.90 $\pm$ 2.07		103.60 $\pm$ 5.33	
12-14 months (12)	25.80 $\pm$ 0.98	54.40 $\pm$ 1.87		111.00 $\pm$ 7.26	
23-25 months (12)	25.40 $\pm$ 0.86	52.20 $\pm$ 1.69		105.10 $\pm$ 8.50	

Table 4. *Comparison of the activation of arylsulphatase B by Triton X-100 in homogenates of kidney from young-adult and senile female rats*

Assay was carried out with 5% (w/v) homogenates in 0.25M-sucrose with 6 mM-nitrocatechol sulphate (dipotassium salt) in acetate buffer, pH 5.7, at 37°. Figures in parentheses indicate the number of animals used. Values given are mean $\pm$ S.E.M.

Age (months)	Activity of arylsulphatase B (μ g. of nitrocatechol/mg. of N/15 min.)		Activation by Triton X-100 (%)
	Free	Total	
6-7 (6)	283 $\pm$ 8	491 $\pm$ 14	73.4 $\pm$ 3.6
23-25 (6)	266 $\pm$ 10	467 $\pm$ 11	73.6 $\pm$ 6.8

percentage activation by Triton X-100 of this enzyme in the kidneys of young-adult and senile female rats. There was an indication of somewhat lower free and total activities in the older group.

Effect of age on the activity of acid deoxyribonuclease in the kidney. Table 5 indicates the results obtained by the three different techniques used. The reason for the lower value for activation given by the labelled-DNA method is not clear, but it may have been due to contamination of the DNA with 32 P-labelled phosphoprotein and the release of phosphate from this by a soluble phosphoprotein phosphatase. However, with each method used, a decline in the stimulation of enzyme activity by Triton X-100 in the senile group was demonstrated in these preliminary experiments.

Effect of various media on the bound acid-phosphatase activity of homogenates of kidney from young-adult and senile female rats. It was important to determine whether there was any difference in the stimulation of the activity of acid phosphatase

brought about by pretreatment with hypo-osmotic sucrose solutions in preparations of kidney from young and old animals since this would be a measure of any difference in lysosomal fragility between the two groups (Strauss, 1957a). Cell-free preparations of kidney from female animals of age groups 3 and 5 were prepared in the media indicated in Table 6 and kept at 0° for 15 min. The sucrose concentration was then brought back to 0.25M and the free and total activities of acid phosphatase were assayed. The results (Table 6) are expressed as the percentage depression in bound activity, i.e. in total activity minus free activity. There was no appreciable difference in the response of the homogenate from the two age groups to this type of treatment.

DISCUSSION

Strauss (1956) demonstrated that the 'droplets' in the cells of the proximal convoluted tubules of the kidney have a number of features in common with the hepatic lysosomes. He found that the droplets, or phagosomes, contained a number of acid hydrolases, which were released by treatment with water and hypo-osmotic sucrose solutions in the same way that lysosomal enzymes were. In the present investigation it has been shown that acid phosphatase may be activated by Triton X-100 in medullary tissue as well as in the cortex, an indication that the enzyme may be partially confined to lysosome-like particles throughout the kidney. Strauss (1957a, b) also showed that the renal lysosomes may be involved in the reabsorption of protein from the tubular lumen: the rapid rise in the free activity of acid phosphatase which followed the ligation of the kidney in the present work indicates that the lysosome-like particles of

Table 5. Comparison of the activation of acid deoxyribonuclease by Triton X-100 in homogenates of kidneys from young-adult and senile female rats

Three different methods were used for the estimation of the acid-soluble nucleotides released by the enzyme: ultraviolet absorption of nucleotides, diphenylamine estimation of nucleotides (Burton, 1956), and estimation of nucleotides by the use of ³²P-labelled DNA. For details see text.

Age (months)	Ultraviolet method (μmoles of nucleotide released/mg. of N)			Diphenylamine method (μmoles of nucleotide released/mg. of N)			Labelled-DNA method (counts/min./mg. of N)		
	Activation		Activation (%)	Activation		Activation (%)	Activation		Activation (%)
	Free	Total		Free	Total		Free	Total	
5-6 (4)	1.02	1.86	82.0	1.06	1.92	81.0	22.6	37.7	66.8
23-25 (4)	0.93	1.38	47.5	0.99	1.45	46.5	18.2	22.5	23.8

Table 6. Effect of various media on the bound acid-phosphatase activity of cell-free preparations of kidney from young-adult and senile female rats

Cell-free preparations of kidney were prepared in the above media and were maintained at 0° for 15 min. and were then brought back into 0.25 M-sucrose where necessary. Assay of free and total activity was then carried out in the usual manner. Bound activity = total activity - free activity.

Medium	Reduction in bound acid-phosphatase activity (%)	
	7-Month-old rats	24-Month-old rats
0.25 M-Sucrose	0	0
0.125 M-Sucrose	60.5	61.5
0.0625 M-Sucrose	84.5	88.5
0.25 M-Sucrose + 0.1 % Triton X-100	100.0	100.0

the kidney may have a role to play in the autolysis induced by ischaemia. In view of the rather different behaviour of arylsulphatase B in the ischaemic kidney it is interesting to note that Roy (1958) and Ugazio (1960) were unable to detect any change in the free or total activity of this enzyme in liver necrosis induced by carbon tetrachloride or phosphorus, whereas Beaufay *et al.* (1959) and Dianzani (1954) found that such treatment led to a significant rise of the free and a depression of the total activity of acid phosphatase. These findings together with those of the present investigation reinforce the suggestion made by Ugazio (1960) that arylsulphatase B may be in a different type of particle from that which contains acid phosphatase or in a less accessible part of the same particle.

Distinct changes with age in the activities of both liver and kidney acid phosphatase have been demonstrated. The very marked activation of the enzyme by Triton X-100 in homogenates of both liver and kidney of infant rats, when taken in conjunction with similar findings for arylsulphatases A and B (Ugazio, 1960), suggests that a high ratio of total to free activity of acid hydrolases may be a characteristic of rapidly growing tissues. This is reinforced by the observation that the decline of

the rapid growth phase was accompanied by a reduction in the activating effect of Triton X-100 on the acid-phosphatase activity of both tissues. The reason for this change is not clear but it may be concerned with the control of metabolic processes within the cell as suggested by Conchie, Hay & Levy (1961).

The senile degeneration of the rat kidney is characterized by a marked loss of nephrons (Arataki, 1926) and extensive atrophy of the tubular cells (Kennedy, 1957). In view of these findings, perhaps the marked fall in the total activity of acid phosphatase in the senile rat kidney might be attributed to the degeneration of the nephrons since these structures contain the major part of renal acid phosphatase (Wachstein, 1955). Further, the constancy of the free activity on a nitrogen basis implies that there would be a raised free activity in the remaining tubular cells, and it has been shown that a raised free activity relative to the total may be a characteristic of degenerating tissues.

This raised ratio of free to total activity may be interpreted in several ways. It may have been due to a rupture *in vivo* of the lysosomes of the degenerating cells although an increased particle fragility could lead to extensive breakdown during homogenization. However, the results from the experiment on the effects of hypo-osmotic sucrose solutions on homogenates from the kidneys of young and senile rats probably exclude the latter possibility. An increased particle permeability could give rise to a higher ratio of free to total activity since Conchie *et al.* (1961) have suggested that the free activity of homogenates prepared in 0.25 M-sucrose is due to penetration of the particles by the substrate. Appelmans & de Duve (1955), on the other hand, have argued cogently against this idea. This uncertainty as to the exact nature of the free activity does give support to the experimental procedure of measuring free activity in whole homogenates rather than in high-speed supernatants as advocated by de Duve (1959), since the former method should give a more reliable indication of the activity of lysosomal enzymes *in vivo*.

The preliminary results indicating that there was a decreased stimulation of acid deoxyribonuclease by Triton X-100 in the senile kidney, i.e. an increased ratio of free to total activity, supports the impression that there is a group of similar lysosomal particles containing acid phosphatase and acid deoxyribonuclease and which behave similarly in degenerating tissues. The unchanged ratio of free to total arylsulphatase B in the senile kidney, on the other hand, adds to the evidence that this enzyme may be contained in a different class of particle or in a different part of the same particle which is more resistant to the changes occurring in a degenerating cell.

The results obtained with liver preparations demonstrated a consistently higher activating effect of Triton X-100 on acid phosphatase than in the kidney in all age groups. Once again this could have been due to a lower permeability of the liver particles, or to a greater resistance of the liver particles to the stresses in homogenization, or finally it could have been a true indication of a difference *in vivo* between the intracellular distribution of acid phosphatase in the two tissues. The unchanged ratio of, and absolute values for, free and total acid phosphatase in the liver of senile rats support the impression of other workers that senile changes are not prominent in this organ (Barrows *et al.* 1960).

The cause of the change in the lysosomal particle in the kidney of the senile animal is not known. Ischaemia of the senile organ is not likely to be of importance in the rat for arterio- and arteriolo-sclerosis are not very marked in the senile rat kidney (Kennedy, 1957). The proteinuria of the senile rat (Saxton & Kimball, 1941) could play a part in causing changes in the lysosomal particle, since Strauss (1957*a*) has shown that an induced proteinuria can bring about some release of enzymes from the kidney lysosomes.

SUMMARY

1. Activation of acid phosphatase by Triton X-100 has been demonstrated in both the cortical and medullary tissue of the rat kidney.

2. Complete ischaemia of the rat kidney produced a rapid rise in the free activity of acid phosphatase: after 12 hr. there was a fall in free activity and an even greater fall in total activity so that at 24 hr. most of the acid phosphatase was free. There was also an initial rise in the free activity of arylsulphatase B but a plateau was reached after 4 hr. The total activity of this enzyme remained unaffected for the period of this experiment.

3. The low ratio of free to total acid-phosphatase activity of liver and kidney in the infant rat increased in the first 2 months of life to a value that was essentially unchanged in rats of 6-7 and 12-14 months of age. In senile 23-25-month-old animals there was a further significant increase in the ratio in the kidney, whereas the ratio in the liver remained unchanged. Preliminary results indicated a similar increase in the ratio of free to total acid-deoxyribonuclease activity in the senile kidney.

4. Arylsulphatase B showed no change in the ratio when 6-7-month-old rats were compared with 23-25-month-old animals, although both free and total activities were a little lower in the senile group.

5. The findings are discussed from the view point of the distribution of these enzymes within the cell and changes in their activity in degenerating tissues.

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