Biochemical Pharmacology, 1964, Vol. 13, pp. 1587–1592. Pergamon Press Ltd., Printed in Great Britain.

# THE INFLUENCE OF TREATMENT WITH *SALMONELLA TYPHI* ENDOTOXINS ON THE BEHAVIOUR OF SOME ACID HYDROLASES OF THE RAT LIVER*

G. PETTI, L. BOLIS, C. CARTONI and V. AMORMINO

Department of Biology, Istituto Superiore di Sanitá, Rome

(*Received* 30 *April* 1964)

**Abstract**—The behaviour of four acid lysosomal hydrolases (acid phosphatase, DNAse, β-glucuronidase, cathepsin) of rat liver after administration of *Salmonella typhi* endotoxin has been studied.

The treatment increases the unsedimentable enzymic activities of all studied hydrolases.

The authors have found also a condition of water imbibition of the liver, which may be important in the mechanism of activation of lysosomal hydrolases.

WITH regard to the physiopathologic mechanism of endotoxin action in the animal organism, it is now accepted that these substances produce a series of circulatory disturbances in different organs and particularly in the liver (alterations of caliber and of vascular permeability, ischemic and hemorrhagic phenomena comparable, according to Delaunay, to those occurring during shock) which, eventually, compromise the cellular trophism, above all the oxygen supply, determining anoxia at first and cellular degeneration later.[1,2]

The anoxic cell is obliged to use anaerobic glycolysis and, as a consequence, there is a rise of the lactic acid production and a contemporary lowering of the cellular pH (acidosis): this lactic acid rise in the liver (and in the blood) is considered as a sure index of anoxia.[3,4] A great variety of biochemical alterations has been found in the liver and in the other organs of animals treated with endotoxins:[5–8] above all, there is a lowering of the hepatic glycogen, which, evidently, is linked to the increased glycolysis.

We must remember too, among the biochemical alterations, the inhibition 'in vitro' of the liver pyruvic-oxidase observed by Kun[9] as an effect of endotoxins: this fact suggests a direct action of the toxins on the endocellular enzymic systems for the oxidation of pyruvate, quite independent from the vascular perturbations, which indirectly may compromise the cellular trophism.

A few words now about the histopathologic picture of the liver in rats killed 6 hr after the introduction of *Salmonella typhimurium* endotoxins. Cameron[10] has observed: (1) disappearing of the liver glycogen; (2) presence of wide zones of strongly

* A preliminary note has been presented to the 8 Internationaler Vitalstoff Konvent, Garmisch 24–30 Sept. 1962.

lytic necrosis, after strong doses, and of slightly lytic necrosis, after lower doses; (3) portal congestion and hemorrhage.

More recently Erede and Durst[11] have reported that mitochondrial swelling of the liver and the kidney in animals treated with *Salmonella abortus equi*, is very early and may be morphologically observed within a few minutes after the introduction of these organisms.

These observations led us to investigate the behaviour of some acid hydrolases localized in the lysosomes of the liver of rats treated with endotoxins in order to look for a correlation between the toxic action of endotoxins, and the activity and intracellular localization of these enzymes, which play an important role in the regressive and degenerative processes of the cell.[12] De Duve,[13] who has discovered a more or less abundant release of lysosomal hydrolases in the liver homogenates of animals submitted *in vivo* to hepatotoxic treatments, on the other hand, has pointed out that the principal factor for lysosomal membrane rupture is the lowering of the endocellular pH.[14]

## EXPERIMENTAL

We have studied first the activity and the intracellular distribution of four lysosomal hydrolases (cathepsin, DNAse, β-glucuronidase, acid phosphatase) in rats treated with *Salmonella typhi endotoxins*; successively we have studied the water content of liver of rats treated with the same dose of endotoxins.

*Preparation of the endotoxins.* For isolation of the colony, at the stage S, from the Salmonella typhi 0/901 stock and for the extraction, we used a method analogous to that of Boivin and Mesrobenau,[15] slightly modified by Di Nardo.[16]

*First group of experiments*

Experiments were performed on two groups of female Long Evans rats, weighing about 170 g, fed on the standard diet of this Institute. One group was used as control and the other one was treated with *Salmonella typhi* 0/901 endotoxins in the amount of 1·30 mg/100 g of body weight: this dose had revealed itself sublethal in the previous experiments.

The treated animals were killed 7 hr after the injection. The animals of two groups were killed by decapitation after 16 hr fasting. For details of the techniques of homogenization and determination of the enzymic activities, we recall our preceding work.[17]

The liver was homogenized in ice-cold 0·25 M sucrose by three up-and-down runs of the pestle, rotating at 780 rev/min, of a Potter-Elvehjem homogenizer. A part of the homogenate was centrifuged in a Spinco model L, n·40 rotor, at 40,000 rev/min for 30 min and the supernatant was taken to determine the unsedimentable activities of enzymes.

The enzymic activities studied are: deoxyribonuclease, cathepsin, β-glucuronidase and acid phosphatase, and were determined according to de Duve.[18]

We must distinguish, according to de Duve,[13] *a total enzymic activity*, that may be determined by submitting the homogenate to triton X–100 (which is able to completely release hydrolases) and *an unsedimentable activity*, which may be measured by centrifuging the initial homogenate (untreated with a triton) at 40,000 rev/min for 30 min (in order to obtain a supernatant deprived of subcellular granules, in particular

lysosomes). By comparison between these two activities, we can calculate the quantity of released enzyme.

Total enzymic activities are referred to the total nitrogen content of the liver (Table 1).

The total nitrogen of the liver was measured by the microkjedahl method.

TABLE 1. THE EFFECTS OF ENDOTOXIN ON THE BEHAVIOUR OF TOTAL AND UNSEDIMENTABLE ACTIVITIES OF ACID PHOSPHATASE, CATHEPSIN, DEOXYRIBONUCLEASE AND β-GLUCURONIDASE OF RAT LIVER

| Group and no. of animals | Normal no. 5 | Treated no. 5 |
|---|---|---|
| Weight of animals (g) | 167·8 (± 19·2)* | 171·4 (± 7·0) |
| Liver: | | |
| weight | 4·71 (± 0·79) | 5·61 (± 0·60)† |
| weight/100 g of animal | 2·81 (± 0·29) | 3·28 (± 0·21)† |
| Nitrogen (mg/g of liver) | 32·3 (± 2·00) | 28·4 (± 2·3)† |
| Nitrogen (mg/100 g of animal) | 90·7 (± 11·6) | 92·8 (± 8·4) |
| Total enzymic activities (Units/g of N): | | |
| Acid phosphatase | 164·7 (± 35·79) | 151·7 (± 19·49) |
| Cathepsin | 30·3 (± 6·79) | 28·3 (± 4·81) |
| Deoxyribonuclease | 30·3 (± 2.91) (3) | 31·7 (± 2·79) (4) |
| β-glucuronidase | 29·7 (± 4·76) | 28·2 (± 5·08) |
| Unsedimentable activities (% of total): | | |
| Acid phosphatase | 8·32 (± 1·46) | 17·00 (± 0·49)‡ |
| Cathepsin | 4·38 (± 2·24) | 14·80 (± 1·55)‡ |
| Deoxyribonuclease | 1·73 (± 0·26) (3) | 7·40 (± 0·70)‡ (4) |
| β-glucuronidase | 5·12 (± 1·12) | 14·80 (± 3·43)‡ |

* = Standard Deviation
† = $P < 0.05$
‡ = $P < 0.001$

TABLE 2. THE EFFCTS OF ENDOTOXIN ON THE FRESH AND DRY WEIGHT OF RAT LIVER

| | Normal no. 3 | Treated no. 3 | Difference | % |
|---|---|---|---|---|
| Body weight of animals (g) | 158·0 (±7·7) | 160·0 (± 7·4) | | |
| Liver: | | | | |
| weight (g) | 4·86 (± 0·55) | 5·88 (± 0·33)* | + 0·60 | 19·0 |
| weight/100 g of animal (g) | 3·06 (± 0·21) | 3·66 (± 0·26)* | | |
| Dry weight (g) | 1.49 (± 0·61) | 1·49 (± 0·18) | 0 | 0 |
| Ratio: fresh weight/dry weight | 3·27 (± 0·12) | 3·95 (± 0·09)† | 0·68 | 21 |
| Water content: fresh weight/dry weight | 3·37 (± 0·37) | 4·39 (± 0·28) | + 1·02 | |

* = $P < 0.05$
† = $P < 0.01$

*Second group of experiments*

We have determined the ratio between fresh weight and dry residue of liver in rats treated or not with endotoxins. The dry residue was determined at 104°C (Table 2).

## RESULTS

The data given in Table 1 show that, 7 hr after the i.p. injection of *Salmonella typhi* endotoxins, there is a great increase of unsedimentable enzymic activities of the four studied hydrolases, while there are no very important variations of the total enzymic activities (on the whole, a slight decrease).

The results suggest an injury of the lysosomal lipoprotein membrane, which allows the release of the enzymes and thereafter their reactions with the various cellular substrates.

It is evident that these experiments do not allow to conclude that the endotoxins have a direct action on the lysosomal membrane, though this cannot be excluded *a priori*.

On the whole, except for DNAse, the hydrolases behaviour we observed is analogous to that found by de Duve[14] after a few hours, in livers of rats made ischemic as a consequence of vessels ligature. This treatment caused, evidently, cellular anoxia as in our experiment and anoxia itself might be a very important factor in order to explain the toxic action of the endotoxins.

It is known (de Duve) that anoxia alone cannot be responsible for membrane injury in isolated lysosomes: in fact, a lysosomal suspension is not injured by staying in a medium deprived of oxygen. On the contrary, there is an evident release of lysosomal enzymes in liver slices incubated anaerobically: this fact suggests that lysosomal membranes are above all sensitive to environmental modifications inside the anoxic cell. The lowering of the endocelullar pH, which might activate the cathepsin already present inside the membrane, may be especially important.

De Duve has found, in ischemic livers, [14]a constant lowering of the nitrogen content which increases progressively during the first 8–12 hr after the ligature, and does not show any more change up to 24 hr; this phenomenon is presumably caused by water imbibition from the peritoneal cavity.

In order to evaluate variations in liver weight, we studied the 'liver organic weight , that is, the percentage ratio between liver and body weight of the rat.

Considering nitrogen, we studied not only the nitrogen content per/g of liver but also, multiplying the hepatic nitrogen by the organic weight, the variations of hepatic nitrogen (thus of hepatic proteins, enzymes etc.) with respect to the total organism could be calculated.

As shown in Table 1, liver organic weight rises about 20 per cent in the treated animals, but the nitrogen content falls in a parallel way, so that the product of hepatic nitrogen per organic weight does not undergo significative variations. Thus hepatic nitrogen, that is, the hepatic nitrogen quota at the body disposal, does not show any important change.

This rapid and conspicuous rise of the organ weight led us to consider whether this variation can be related to a rise of the water content, or to a congestive state, or to both.

In order to make sure that the rise in liver weight is caused by an increased water content, we performed the second group of experiments, in which, as shown in Table 2, the rise in "liver organic weight" in animals treated with endotoxins is about 19 per cent; the ratio fresh weight/dry residue rises about 21 per cent, while the dry residue does not show any change.

Differences of liver weight in treated and normal animals (that is 1·02 g) did not induce any variation of the dry residue, as would have been expected if the fluid, which is responsible for this increase was greatly different from water.

Our hypothesis that the increase in liver weight in treated animals is due to an oedema of the organ rather than to a hyperemic-hemorrhagic condition is supported by the values obtained for the total nitrogen of the blood in normal rats. Total nitrogen/g of blood was found to be, in normal rats of the same stock and weight, about 31·9 mg/g, this is the same value as for normal liver. Thus, simple hyperemia could not induce variations of the ratio N/g of the organ.

The foregoing results suggest that there is a condition of water imbibition in the liver in treated animals. We think that this condition may be important, together with anoxia, in order to explain the toxic effects of endotoxins at the cellular and subcellular levels. It could be assumed that the aqueous fluid exudated from the vessels (or water absorbed from the peritoneal cavity, as de Duve has suggested in his studies of rats liver ischemia produced by blood vessels ligature) can penetrate in the cells and imbibe them, causing an alteration of the osmotic equilibrium; a hypotonia of the medium might cause a swelling of the subcellular particles, mitochondria or lysosomes. Quite independently, a defective oxygen supply could induce both a lag in mitochondria oxidative processes[19] and a release of lysosomal enzymes into the cell. These enzymes could induce the known phenomena of cellular injury; a turbid degeneration, at first, and a total lysis of the cell later. It is evident, on the other hand, that anoxia, in blocking oxidative processes and stimulating glycolysis and lactic acid production, makes easier the action of lysosomal enzymes, by creating a favourable pH.

Our results thus agree on the whole with those of de Duve concerning the effects of anoxia, but we think it is important to insist on the importance of tissue imbibition in activating the acid hydrolases, as already pointed out by Novelli and Michelazzi, according to Erede and Durst.[11]

Finally, it is a well known fact that cortisone protects the animals from the toxic phenomena caused by the endotoxins of *Salmonella typhi*; according to Lurie,[20] it is likely that this phenomenon reduces the permeability of the cellular membrane to the toxic factors. Remembering the favourable effect of the cortisone discovered by de Duve[13, 21] on the lysosomal membrane integrity, it may be suggested that cortisone acts on the subcellular membranes too.

This is suggested also by the very recent studies of Weissman *et al.*[22] though there are some differences between the authors' results and ours probably depending upon different experimental conditions. These authors have also found that the lysosomal membrane is injured as a consequence of endotoxins introduction.[22]

## REFERENCES

1. L. Thomas, *Annu. Rev. Physiol.* **16**, 467 (1954).
2. A. Delaunay, P. Boquet, J. Lebrun, Y. Lehoult and M. Belaunay, *J. Physiologie (Paris)* **40**, 89–110 (1948).
3. E. Kun and C. P. Miller, *Proc. Soc. exp. Biol. N.Y.* **67**, 221 (1948).
4. E. Kun and C. P. Miller, *Proc. Soc. exp. Biol. N.Y.* **68**, 496 (1948).
5. A. De Barbieri and M. E. Scevola, *Boll. Istituto Sieroter Milanese* **35**, 550 (1956).
6. M. Hamosh and B. Shapiro, *Brit. J. exp. Path.* **41**, 372 (1960).
7. M. H. Weil and B. S. Miller, *J. Lab. clin. Med.* **57**, 5 (1961).

8. M. W. WOODS, M. LANDY, S. L. WHITBY and D. BURK *Bact. Rev.* **25**, 447 (1961).
9. E. KUN and L. G. ABOOD, *Proc. Soc. Exp. Med. N.Y.* **71**, 362 (1949).
10. G. R. CAMERON, M. E. DELAFIELD and J. WILSON, *J. Path, Bact.* **32**, 223 (1940).
11. P. EREDE and F. DURST cited by L. MICHELAZZI and A. NOVELLI in *Riv. di Istochimica* **5**, 429 (1959).
12. C. DE DUVE, *Exposé Annuales Bioch. Méd.* **6**, 197 (1956).
13. H. BEAUFAY, E. VAN CAMPENHAUT and C. DE DUVE, *Biochem. J.* **73**, 617 (1959).
14. C. DE DUVE and H. BEAUFAY, *Bioch. J.* **73**, 610 (1964).
15. A. BOIVIN and L. MESROBENAU, *Rev. Immunol.* **1**, 553 (1935).
16. A. DI NARDO *Boll. Istituto Sierot. Milanese* **35**, 20 (1956).
17. G. PETTI, C. CARTONI and D. RUGGIU, *Biochem. Pharmacol.* **12**, 1297 (1963).
18. C. DE DUVE, B. C. PRESSMAN, R. GIANNETTO, R. WATTIAUX and F. APPELMANS, *Biochem. J.* **60**, 604 (1955).
19. M. U. DIANZANI—cited by L. MICHELAZZI and A. NOVELLI in *Riv. di Istochimica* **5**, 429 (1959).
20. M. B. LURIE—cited by L. CHEDID, F. BOYER and M. SAVIARD in *Ann. Inst. Pasteur* **86**, 347 (1954).
21. C. DE DUVE, *Biochem. Pharmacol.* **8**, 99 (1961).
22. G. WEISSMANN and L. THOMAS, *J. Exp. Med.*, **116**, 433 (1962).