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INFLUENCE OF VARIOUS TREATMENTS ON THE BEHAVIOUR OF SOME ACID HYDROLASES OF RAT LIVER*

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Abstract—The behaviour of five acid lysosomal hydrolases (acid phosphatase, DNAse, β-glucuronidase, cathepsin, β-galactosidase) of the rat liver in three different experimental conditions (hypophysectomy, administration of an antagonist of B_{12}, repeated injections of serum gonadotrophin) has been studied. Hypophysectomy increases the total enzymic activity of the acid phosphatase, β-glucuronidase and DNAse, as well as the unsedimentable activity of acid phosphatase, β-glucuronidase and cathepsin. The anilide of cyanocobalamin significantly increases the total activities of β-glucuronidase, acid phosphatase and β-galactosidase. Serum gonadotrophin seems to decrease the total activity of β-glucuronidase.

AFTER many studies on the distribution of acid phosphatase and, later on, of other acid hydrolases (deoxyribonuclease, ribonuclease, cathepsin, β-glucuronidase) found in subcellular fractions obtained by differential centrifuging of rat liver homogenates, de Duve[1–13] reached the conclusion that these enzymes have a common and unique localization in a particular type of cellular granules, different from all those already identified.

In fact, the overmentioned enzymes basically show two common characteristics:

(1) a very slight enzymic activity when the homogenates are obtained with rather gentle means (Potter & Elvehjem homogenizer);

(2) they are all activated by the following treatments: homogenization in a blendor, sonic vibration, thermal shock, incubation (37°, pH 5) in media of low tonicity or salt solutions, ageing, exposure to surface-active agents such as saponin, triton X-100 or bile salts, CCl_4, enzymic treatment with lecithinase, trypsin and chymotrypsin.

A different distribution, in subcellular fractions, of acid phosphatase in respect with cytochrome-oxidase (an enzyme characteristic of the mitochondria) and with glucose-6-phosphatase (a microsomal enzyme) obtained by a modification of the classical fractionation procedures, led de Duve to the conclusion that the acid phosphatase is contained in an individual group of cytoplasmic granules, the "lysosomes", which have a sedimentation constant (250,000 *g*/min) intermediary between mitochondria (33,000 *g*/min) and microsomes (3,000,000 *g*/min).

* The present work is the synthesis of three communications[41, 42, 43] to the Società Italiana di Biologia Sperimentale: the first in collaboration with M. Galamini (hypophysectomy) and the third in collaboration with L. Bolis (gonadotrophin).

Recent studies on the histochemical localization of acid phosphatase[15] have confirmed that this enzyme is bound to granules which are different from mitochondria.

The "lysosomes" have a diameter ranging between 0·3–0·5 μ and are surrounded by a semipermeable membrane of lipoprotein nature. They are very few in proportion to mitochondria and microsomes, and they preserve their integrity at 0° and 0·25 M sucrose for some time.

Besides the five described hydrolases, other enzymes have been identified in the lysosomes: arylsulphatase, N-acetyl-β-glucosaminidase, phosphoprotein phosphatase, β-galactosidase.

The injury of the membrane, which may be caused by many treatments, is followed by the relase into the cellular medium of the enzymes, allowing them to interact with the different cellular substrates.

The lysosomes seem to have a great importance in the intracellular acid digestion of foreign substrates in relation with phagocytosis and pinocytosis phenomena[16, 17] and, above all, in physiological and pathological autolysis processes[8, 18–20]

If one wishes to know the degree of activation of the different lysosomal enzymes (that is the quantity of active enzyme released in the cellular medium by the lysosomes) without following all the phases of the differential centrifuging, one can centrifuge the initial homogenate (1:10 g/ml) at 3,000,000 g/min in order to obtain a supernatant deprived of granules. The enzymatic activity of this supernatant, expressed as the percentage of total activity of the same enzyme in the original homogenate, may be considered as representative of the grade of liberation of the enzyme inside the cell. So we can distinguish:

(1) a "*total enzymic activity*"[8] that may be determined by submitting the *initial homogenate* to a treatment with triton X-100, which completely releases the hydrolases;

(2) an "*unsedimentable activity*" which is measured in the *supernatant* in the same conditions as the total activity.

The activity of lysosomal enzymes and the integrity of the lysosomes themselves may be influenced, *in vivo* and *in vitro*, by hormonal[8, 20–30] and nutritional[8, 21, 31] factors. We therefore studied the behaviour of five lysosomal hydrolases of rat liver (acid phosphatase, β-glucuronidase, cathepsin, deoxyribonuclease, β-galactosidase) under different experimental conditions:

(1) hypophysectomized rats,

(2) rats treated with a vitamin B_{12} analogue,

(3) rats which were submitted to repeated doses of serum gonadotrophin.

In the case of hypophysectomy, Stevens and Reid[30] have found a slight increase of the deoxyribonuclease activity of the cytoplasmic fraction of rat liver and particularly of the activity bound to the mitochondrial fraction while the ribonuclease activity increased principally in the supernatant. These studies led to the conclusion that, under such conditions, there is an increase in the fragility or in the permeability of the lysosomes.

On the other hand, de Duve[8] has pointed out the effect of malnutrition on the liver lysosomes: a necrogenic diet determines a slight rise of acid phosphatase, deoxyribonuclease, ribonuclease, and a significant release of all the lysosomal hydrolases. This release was also observed after a prolonged fast.

We therefore decided to study the effect of the anilide of the monocarboxyilic acid of vitamin B_{12}[32] in rats already deprived of vitamin B_{12}, since, this anilide (a substance that antagonizes many effects of B_{12}[33, 34]) inhibits partially, *in vitro*, and proportionally to the dose, protein synthesis in the liver cytoplasmic fractions extracted at pH 5 according to Wagle.[35]

Finally, we have submitted rats to a repeated treatment with serum gonadotrophin for two reasons (Bolis[36, 37]): (a) strong doses of this hormone cause in liver an injury that, at the beginning, appears as a serous hepatitis of Roessle type. It is followed by parenchymal degeneration, histologically revealed by the disappearing of cytoplasmic and nuclear boundaries of the hepatocytes; (b) massive doses depress the rat liver β-glucuronidase activity.

EXPERIMENTAL

1. *Hypophysectomy*

The experiments were performed on female rats, weighing about 90 g. The animals were divided into two groups: one was hypophysectomized and the other was used as a control. All the animals were fed *ad libitum*, on the standard diet of this Institute.

About 20 days after the hypophysectomy, both the operated and the normal rats, were weighed and killed. The autopsy revealed the success of the operation.

2. *Treatment with anilide of the monocarboxylic acid of vitamin* B_{12}

The experiments were performed on two groups of thirteen male rats of an initial average weight of 45 g. The first group (which had an initial average weight of 44·5 g), was fed on a soya-lactose diet (Wagle[38]) and received a supplement of 50μg of vitamin B_{12}/Kg of basal diet. The second group (which had an initial average weight of 43·3 g) was fed on the basal diet only.

Thirty-one weeks later, the average weight of the first group was 243 g and the average weight of the second group was 212·5 g.

The growth difference between the two groups (29·1 g) is slightly significant ($t = 2{\cdot}21 - P < 0{\cdot}05$).

The anilide of B_{12} was supplied to the animals fed on the basal diet only, in order to aggravate their deficient vitaminic level: five rats received a total dose of 100 μg distributed in five injections and six rats a dose of 500 μg in nine injections.

The animals belonging to the three groups (diet + B_{12}, diet + 100 μg, diet + 500 μg of anilide of B_{12}) were alternatively killed.

3. *Treatment with serum gonadotrophin*

The experiments were performed on male rats, weighing from 100 to 130 g. The animals were divided into two groups. A first group was treated for 18 days with 10 U.I., daily, of "standard" serum gonadotrophin, the other one remaining as a control.

The period of treatment was inferior to that necessary for the formation of substances with an antigonadotrophin action[39]; one treatment was less intense and more prolonged than the one performed by Bolis[36].

The animals were killed about 18 days after the beginning of the experiments.

In these three series of experiments, the animals were killed by decapitation after 15 hours of fast. Their liver was cooled and weighed in ice-cold 0·25 M sucrose.

The organ was cut in small pieces and homogenized by three up-and-down runs of the pestle, rotating at 780 rev/min, of a Potter-Elvehjem homogenizer, in 15 ml of 0·25 M sucrose.

The homogenate was subsequently brought to a volume corresponding to ten times the weight of the original tissue, by adding 0·25 M sucrose.

A part of the homogenate was centrifuged in a Spinco model L, n.40 rotor, at 40,000 rev/min for 30 min (3,000,000 *g*/min).

In all these experiments, the experimental conditions were kept constant, and the temperature remained at 0°.

The total enzymic activity and the unsedimentable enzymic activity of all the studied enzymes were determined. The activities of the five acid hydrolases were determined according to de Duve *et al.*[2,5,13].

The incubations were performed, for all the enzymes, at 37° in acetate buffer pH 5 except for cathepsin that has an optimum of activity at pH 3·6.

As enzymic unit for acid phosphatase, β-glucuronidase and β-glactosidase, we considered the quantity of enzyme that decomposes a micromole of substrate/min under the conditions of the assay. For cathepsin, the molarity of the products of the enzymatic activity was conventionally expressed in terms of tyrosine equivalents of the colour developed with the Folin and Ciocalteu reagent, while the products of the breakdown of DNA were expressed in terms of liberated mononucleotides, assuming an average extinction coefficient at 260mμ: $8{\cdot}5 \times 10^6$ cm^2 mole^{-1} (Stimson and Reuter 1945).

The total enzymic activities are reported, in the first experiments, to the weight of the fresh organ and, in the other two experiments, to the content of nitrogen.

The unsedimentable enzymic activities are always expressed as per cent of the total activities.

The total nitrogen of liver, in the two last experiments, was measured by a micro-Kjeldahl method.

RESULTS

The results are summarized in tables 1, 2 and 3.

1. *Hypophysectomy* (*Table* 1)

(a) A slight, but significant, increase of the total enzymic activities of the acid phosphatase, β-glucuronidase and deoxyribonuclease was observed in hypophysectomized animals;

(b) the unsedimentable enzymic activities, expressed as per cent of the total homogenate activities, are nearly twice for cathepsin, acid phosphatase and β-glucuronidase. The unsedimentable activities of deoxyribonuclease and β-galactosidase, are slightly increased, but this increase has no statistical meaning.

2. *Anilide* (*Table* 2)

We observed—as shown by Table 2—a slight increase of the total enzymic activities of all the hydrolases of the animals that received 500 μg of anilide. This increase has a statistical meaning only for β-glucuronidase, acid posphatase and β-galactosidase. The per cent relationship between the unsedimentable enzymic activities and the total activities remained unchanged in all the groups.

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

Group and no. of animals	Normal no. 10	Hypophysectomized no. 6
Weight of animals (g)	120·1 (±14·75)*	98·5 (±7·84)‡
Liver: weight/100 g of animal (g)	3·9 (± 0·37)	2·8 (±0·14)§
Total enzymic activities (Units/g of liver):		
Acid phosphatase	5·26 (± 0·96)	6·56 (±1·02)†
β-glucuronidase	1·03 (± 0·37)	1·52 (±0·40)†
β-galactosidase	0·73 (± 0·17) (7)	1·06 (±0·35)
Cathepsin	1·21 (± 0·26)	1·27 (±0·15)
Acid deoxyribonuclease	0·85 (± 0·28)	1·23 (±0·18)†
Unsedimentable activities (% of total):		
Acid phosphatase	8·14 (± 1·81)	16·00 (±2·76)§ (5)
β-glucuronidase	8·63 (± 4·66)	14·85 (±3·54)† (4)
β-galactosidase	14·71 (± 5·55) (7)	16·40 (±9·57) (4)
Cathepsin	9·16 (± 2·65)	21·00 (±8·66)‡ (5)
Acid deoxyribonuclease	5·85 (± 1·00)	7·90 (±3·41) (5)

* = Standard deviation (σ).
† = $0{\cdot}05 > P > 0{\cdot}01$.
‡ = $0{\cdot}01 > P > 0{\cdot}001$.
§ = $P < 0{\cdot}001$.
( ) = No. of animals.

3. *Gonadotrophin* (*Table* 3)

The activities and the intracellular distribution of the five acid hydrolases of the liver is not influenced by the serum gonadotrophin.

If we compare—as shown in Table 3—the total enzymic activity of β-glucuronidase to the content of hepatic nitrogen, the diminution of this activity, in the treated animals, has no statistical meaning ($t = 1{\cdot}93 - 0{\cdot}1 > P > 0{\cdot}05$).

On the other hand, if we compare the activity of β-glucuronidase to the weight of the fresh organ, we find that the difference of activity between control and treated animals is significant: the β-glucuronidase activity of control animals = 1·16 (± 0·2) units/g of liver, while that of the treated animals = 0·97 (± 0·2) ($t = 2{\cdot}53 — P < 0{\cdot}05$).

DISCUSSION

The enzymic pattern of hypophysectomized rats liver, regarding deoxyribonuclease, agrees with the work of Stevens and Reid [30]: it is essentially the same as that found by de Duve[8] for animals submitted to hepatotoxic treatments. An increase of the unsedimentable activities is observed 20 days after hypophysectomy: this increase does not seem to be due to the post-operative shock, which might only produce a release of lysosomal enzymes a few hours after the operation.

A slight increase (about 20 per cent) of the content of acid hydrolases, which is significant for phosphatase, β-glucuronidase and β-galactosidase, was observed in animals to which 500 μg of anilide of vitamin B_{12} were supplied. An increase of the cathepsin and deoxyribonuclease activities was also observed, but it does not reach a

TABLE 2. THE EFFECTS OF CYANOCOBALAMIN ANILIDE ON THE BEHAVIOUR OF TOTAL AND UNSEDIMENTABLE ACTIVITIES OF ACID PHOSPHATASE, β-GLUCURONIDASE, β-GALACTOSIDASE, CATHEPSIN AND ACID DEOXYRIBONUCLEASE OF RAT LIVER.

Group and no. of animals	Normal no. 9	100 μg of anilide no. 5	500 μg of anilide no. 6	Var. %
Weight of animals (g)	233·4 (±42·3)*	206·0 (±23·8)	205·0 (±48·6)	
Liver: weight/100 g of animal (g)	2·98 (± 0·28)	3·22 (± 0·29)	3·26 (± 0·45)	
mg N/g of liver	31·21 (± 4·70)	31·77 (± 4·95)	32·00 (± 5·33)	
mg N/100 g of animal	92·71 (± 7·35)	102·02 (± 7·92)	104·73 (±17·6)	
Total enzymic activities (Units/g of N):				
Acid phosphatase	214·4 (±22·31) (8)	192·1 (± 31·38)	255·4 (±34·38)†	(+19·1)
β-glucuronidase	33·7 (± 4·05)	38·6 (± 5·82)	40·6 (± 4·08)‡	(+20·5)
β-galactosidase	15·6 (± 3·84)	16·5 (± 3·79)	21·3 (± 3·50)‡	(+36·5)
Acid deoxyribonuclease	19·9 (± 2·99) (8)	21·2 (± 3·86)	23·1 (± 3·04)	(+16·1)
Cathepsin	35·7 (± 5·70)	35·3 (± 7·20)	41·9 (± 7·19)	(+17·5)
Unsedimentable activities (% of total):				
Acid phosphatase	11·32 (± 8·18)	8·85 (± 1·54)	9·50 (± 2·41)	
β-glucuronidase	7·23 (± 3·58)	6·90 (± 2·56)	9·72 (± 4·13)	
β-glactosidase	13·40 (± 8·90)	12·40 (± 4·38)	17·40 (± 6·00)	
Acid deoxyribonuclease	4·10 (± 2·50) (7)	3·77 (± 1·11)	7·21 (± 3·28)	
Cathepsin	12·30 (± 2·99)	9·60 (± 3·19)	14·30 (± 3·38)	

* = Standard deviation (σ).
† = 0·01 > P > 0·05.
‡ = 0·001 > P > 0·01
( ) = No. of animals.

statistical value. This is in agreement with de Duve[8], who points out that the content of these enzymes increases in liver under unfavourable conditions.

We could not find a clear increase of hydrolases in the supernatant; this might be due to the fact that the degree of B_{12} depletion, notwithstanding the preventive diet and the antimetabolite, was not great enough to cause important injuries.

TABLE 3. THE EFFECTS OF SERUM GONADOTROPHIN ON THE BEHAVIOUR OF TOTAL AND UNSEDIMENTABLE ACTIVITIES OF ACID PHOSPHATASE, β-GLUCURONIDASE, β-GALACTOSIDASE, CATHEPSIN AND ACID DEOXYRIBONUCLEASE OF RAT LIVER.

Group and no. of animals	Normal no. 8	Treated no. 10	Var. %
Weight of animals (g)	132·0 (±23·0)*	141·2 (±21·5)	
Liver: weight/100 g of animal (g)	3·7 (± 0·47)	3·3 (± 0·20)†	−10·8
Liver: N mg/g of tissue	32·9 (± 1·10)	32·8 (± 2·60)	
Total enzymic activities (Units/g of N):			
Acid phosphatase	188·1 (±26·7)	176·1 (±15·5)	
β-glucuronidase	34·9 (± 7·8)	28·16 (± 7·0)	−19·3
β-galactosidase	11·0 (± 2·0)	10·5 (± 0·9) (7)	
Acid deoxyribonuclease	21·3 (± 4·6)	19·6 (± 5·2) (7)	
Cathepsin	38·8 (± 2·7)	38·0 (± 6·8) (7)	
Unsedimentable activities (% of total):			
Acid phosphatase	4·8 (± 1·2)	4·4 (± 0·7) (7)	
β-glucuronidase	6·3 (± 1·1)	7·4 (± 4·4) (7)	
β-galactosidase	10·7 (± 2·2)	9·7 (± 2·5) (7)	
Acid deoxyribonuclease	2·3 (± 1·4) (7)	2·2 (± 0·9) (5)	
Cathepsin	7·2 (± 1·8)	4·9 (± 1·2) (7)	

* = Standard deviation (σ).
† = $0{\cdot}05 > P > 0{\cdot}01$.
( ) No. of animals.

At any rate, there is a conspicuous increase of the unsedimentable activity of deoxyribonuclease (78%), even if it is not highly significant.

Treatment with serum gonadotrophin has not modified, on the whole, the total activities or the unsedimentable activities of the five hydrolases we studied. The unsedimentable activity of cathepsin shows a diminution, however, but the interpretation of this result is difficult*.

As mentioned before, the diminution of β-glucuronidase, if the activity is expressed in grams of fresh tissue, rather than to the nitrogen content of the liver, reaches a certain level of significance ($P < 0{\cdot}05$); this is in agreement with what Bolis already found under slightly different conditions.

Our observations, in which the level of significance depends on the chosen parameter (hepatic nitrogen or fresh organ weight (g)), are rather similar to these of Knobil[28]: he noticed that the increase of β-glucuronidase activity in the suprarenal gland which, in rat, follows hypophysectomy, is significant at 5 per cent if reported to the contents in nitrogen, while it is significative at 1 per cent if it is related to the weight of the fresh organ.

* Similar findings have been made for the deoxyribonuclease unsedimentable activity in cortisone treated rats by de Duve[5].

It should be pointed out that, while the other acid hydrolases have a localization that is exclusively lysosomal, the β-glucuronidase activity of liver, can attributed (according to de Duve[5, 13]) to at least two different enzymes which have a different localization in the cellular particulate: one of them has a lysosomal origin and the other has a microsomal one.

We do not know whether serum gonadotrophin has a direct or an indirect action on hepatic tissue. The action of gonadotrophin on the liver does not seem to bear a relationship with the gonads activity; in fact, Bolis has obtained the same results on castrated rats (unpublished), and supply of testosterone to castrated and normal rats had not effect on the level of β-glucuronidase activity of the liver[29]. On the other hand, Gersh and Catchpole [40] have proposed the hypothesis of an ubiquitarious action of the hormone on the mesenchyma, which would express itself through the depolymerization of the connectival glycoprotides.

On the whole, our results indicate different behaviours of the hepatic lysosomal enzymes under conditions which change the normal physiological equilibrium.

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