β -Glucuronidase in Lymphatic Tissues

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In the course of our biochemical investigations of lymphatic tissue in normal and pathological states the enzyme β -glucuronidase has been studied in conditions of atrophy and proliferation.

In the fasting animal lymphatic tissue undergoes a very marked involution (Hammar, 1905), the weight loss beginning earlier and at a higher rate than in any other tissue (Jolly, 1914; see Dougherty, 1952, for a comprehensive review). Subsequent refeeding brings about rapid reconstitution of the tissue (Frank, Kumagai & Dougherty, 1953). Adrenalectomy causes an increase in the weight of lymphatic tissue (Dougherty, 1952). Tissues undergoing such sharp variations in weight, clearly related with rates of breakdown and proliferation of the cells, offer promising material with which to test the much discussed relationship between β -glucuronidase and the process of cell growth (Levy, Kerr & Campbell, 1948; Kerr, Campbell & Levy, 1949, 1950; Mills, Paul & Smith, 1953).

The present communication is concerned with measurements of the β -glucuronidase activity in lymphatic organs made on tissue undergoing both degeneration during fast and regeneration on resumption of nutrition. The effects of adrenalectomy on the enzyme have also been studied. Enzyme activities have been assessed, both in terms of tissue weight and on the basis of cell counts.

EXPERIMENTAL

Young male albino rats of the 'Italiceo' strain, bred in this Laboratory and weighing between 78 and 118 g., were used throughout. They were kept on a standard balanced diet (Randoine & Causeret, 1947) supplemented by green vegetables. Adrenalectomy was performed by the dorsal approach under light ether anaesthesia. Animals were killed by exsanguination. Lymphatic tissues (lymph nodes, thymus and in some experiments the spleen) were immediately dissected free of connective tissue and fat and kept at ice temperature during the collection of the rest of the tissue. As representative of the lymph nodes, those superficial to the neck, a total of six, were taken. After weighing, the tissues were dispersed in distilled water (adjusted to pH 7.4) with a glass homogenizer (Potter & Elvehjem, 1936) kept in cracked ice. The final dilution of the dispersions was 1:200 (w/v). These dispersions were stored in the cold room at 2° and were always used within 12 hr. of preparation.

Determination of β -glucuronidase activity. Recrystallized phenolphthalein glucuronide prepared biosynthetically (Fishman, Springer & Brunetti, 1948) was used as sub-

strate. The composition of the incubated mixtures was: 0.1 ml. of 0.01M phenolphthalein glucuronide, 0.8 ml. of 0.20M acetate buffer, pH 5.2, and 0.1 ml. of tissue dispersion. Mixtures were incubated in a water bath at 37° for 1 hr. After addition of 1.0 ml. of glycine-NaOH-NaCl buffer (pH 12.4), followed by centrifuging, the intensity of colour was measured with a Leitz photoelectric colorimeter, using cuvettes 0.5 cm. wide, and with a filter having peak transmission at 550 m μ . Freshly prepared dilute solutions of phenolphthalein were taken as standards, and all measurements were made against substrate blanks. The determinations with enzyme were made in duplicate. Results are expressed in Glucuronidase units (G.U.), one unit being defined as the amount of enzyme which liberates 1 μ g. of phenolphthalein in 1 hr. in the conditions defined. The measurements of pH were made with a glass electrode standardized with buffer at pH 6.5.

Nitrogen determinations. The total nitrogen content of dispersions was determined by the micro-Kjeldahl method; digestions were carried out in the presence of potassium persulphate, and ammonia, distilled in the Markham (1942) apparatus, was collected in 0.0143N-H₂SO₄ for back titration, using Tashiro's indicator.

Isolation of lymphocytes. The method was based on that of Hechter & Johnson (1949). Pooled thymus glands (2-4 from normal rats, and 3-5 from fasting rats) were minced with scissors and suspended in cold 0.9% (w/v) NaCl solution. The suspension was filtered through folded sterile muslin and the lymphocytes were washed three times by centrifuging with cold saline. After being brought to the original volume with the saline a portion was diluted by addition of ten times its volume of Türk fluid in a blood white-cell pipette and counted in a Bürker camera by two observers. Smears were also made and stained according to May-Grünwald-Giemsa and Pappenheim. This showed that in both normal and fasting animals almost all the cells present were lymphocytes. These were mostly small, but some medium-size and large cells were also present. Smears from fasting animals often showed nuclear pyknosis and a cytoplasm less readily stained than in normal material.

The bulk of the preparation was again centrifuged, resuspended in the original volume of distilled water at pH 7.4 and dispersed in the homogenizer before determination of β -glucuronidase activity, which was calculated in terms of a standard count of ten million cells.

RESULTS

Variations in enzyme activity due to various factors

In preliminary experiments the optimum conditions for the assay of β -glucuronidase in lymphatic tissue were established. For this purpose homogenates from pooled neck lymph nodes were used exclusively.

Variations in activity with pH. The pH-activity curve (Fig. 1) showed two well-marked optima at pH 4.2 and 5.2, the latter being the point of highest activity. This result is substantially similar to those of Kerr *et al.* (1949) for liver and kidney. The pH chosen finally for assays was therefore 5.2.

Variation in activity with concentration of substrate. There was optimal glucuronidase activity when the final concentration of phenolphthalein

glucuronide was 0.001M, as in the assay method of Talalay, Fishman & Huggins (1946). This concentration was chosen for use in the present investigation.

Effect of enzyme concentration. In the range 0–1.25 mg. of tissue/ml. of final mixture, the activity was directly proportional to the weight of tissue (Fig. 2). Subsequent assays were set up with 0.5 mg. tissue/ml., the tissue being taken as suspensions, 0.5% (w/v), in water.

Fig. 1. Effect of pH on the liberation of phenolphthalein from 0.001M phenolphthalein glucuronide by lymph nodes dispersions. Acetate buffers, 0.16M. Other conditions of the assay as described in the text.

Fig. 2. Relationship between hydrolysis of phenolphthalein glucuronide and concentration of the enzyme. Standard conditions of assay as described in the text.

Table 1. Wet weights and β -glucuronidase activities of neck lymph nodes, thymus and spleen of normal rats, of rats fasted for 96 hr. and of adrenalectomized rats (means $\pm$ S.E.M.)

Condition	No. of rats	Wet wt. (mg./100 g. initial body wt.)	Wet wt. as % of wet wt. of normals	β -Glucuronidase			Total amount of enzyme per organ or pooled organs (g.u.)
				g.u./g. wet wt.*	g.u./mg. N	g.u./mg. N in % of the values of the normals	
Neck lymph nodes							
Normals	15	153 $\pm$ 14	100	14 400 $\pm$ 800	523 $\pm$ 34†	100	2 203 $\pm$ 221
Fasted for 96 hr.	9	71 $\pm$ 5†	46	22 040 $\pm$ 1600†	711 $\pm$ 50†	135	1 565 $\pm$ 136†
Adrenalectomized for 96 hr.	7	155 $\pm$ 8	101	16 280 $\pm$ 1480	569 $\pm$ 76	108	2 523 $\pm$ 209
Thymus							
Normals	14	195 $\pm$ 14	100	5 920 $\pm$ 340	195 $\pm$ 4†	100	1 155 $\pm$ 136
Fasted for 96 hr.	8	56 $\pm$ 8†	29	16 400 $\pm$ 1800†	449 $\pm$ 47††	270	918 $\pm$ 103
Adrenalectomized for 96 hr.	7	263 $\pm$ 19†	135	5 800 $\pm$ 720	178 $\pm$ 14	91	1 526 $\pm$ 83
Spleen							
Normals	7	857 $\pm$ 178	100	15 200 $\pm$ 860	—	—	12 986 $\pm$ 1596
Fasted for 96 hr.	10	255 $\pm$ 44†	30	42 800 $\pm$ 1140†	—	—	10 914 $\pm$ 1418
Adrenalectomized for 72 hr.	5	756 $\pm$ 145	88	20 200 $\pm$ 900†	—	—	15 271 $\pm$ 605

* For definition of G.U. see text.

† Seven rats only.

‡ Difference with normals statistically significant ($P < 0.05$; Student's t test).

Effect of incubation period. Hydrolysis proceeded linearly with time over a period of at least 2 hr. The standard incubation period decided upon was of 1 hr.

β -Glucuronidase activity in lymphatic tissue after 96 hr. fasting and after bilateral adrenalectomy

In a preliminary survey, levels of β -glucuronidase were determined in two experimental conditions such that the weight of lymphatic tissue had undergone variations in opposite directions. In the first place a condition of fasting was enforced. This

ectomy, a significant increase in β -glucuronidase activity was apparent only in the spleen, corresponding with a slight loss in weight. In the lymph nodes and thymus there were slight and not significant variations in the enzyme, although the weights of the thymus had increased markedly in comparison with the normal.

Fig. 3. Effect of fasting and refeeding of rats on the weight and glucuronidase activity of neck lymph nodes. Wet weight (mg./100 g. body weight at the beginning of the experiment): upper curve; β -glucuronidase activity (G.U./g. wet weight of tissue): lower curve. The vertical segments correspond to $2 \times \text{s.e.m.}$ Each experimental point corresponds to the mean of from five to eleven individual results.

Fig. 4. Effect of fasting and refeeding of rats on the weight and glucuronidase activity of thymus glands. Wet weight (mg./100 g. body weight at the beginning of the experiment): upper curve; β -glucuronidase activity (G.U./g. wet weight of tissue): lower curve. The vertical segments correspond to $2 \times \text{s.e.m.}$ Each experimental point corresponds to the mean of from five to eleven individual results.

caused a rapid loss in weight, the maximum effect being observed in the thymus, which diminished to about 25 % of its normal weight after 96 hr. In the second case adrenalectomy was performed; this resulted in an increase in weight, also particularly evident in the thymus gland, which went up by 35 % in 4 days from operation.

Corresponding with decreased weight of tissues, a clear increase in β -glucuronidase activity was observed. The effect was greatest in thymus and spleen, where the relative loss of weight was greatest. On the other hand, 4 days after adrenal-

These results seemed to establish a link between decrease in weight of lymphatic tissue and increase in the activity of β -glucuronidase, whereas increase in weight seemed not to be accompanied by any clear variation in the activity of the enzyme. It was therefore deemed necessary to follow closely the behaviour of glucuronidase in correlation with the variations in weight, both positive and negative, of the tissues. Such conditions could be easily obtained by fasting the animals for 4 days, so that

lymphatic tissue decreased sharply in weight, and then refeeding them to bring about reconstitution of the tissue. Figs. 3 and 4 show the variations in weight and in enzyme activity under these conditions for lymph nodes and thymus glands. It is apparent that β -glucuronidase activity increased in both thymus and lymph nodes during the period in which these organs were undergoing loss in weight, and that it began to fall rapidly on refeeding.

Fig. 5. Wet weight (upper curve) and β -glucuronidase activity (lower curve) of neck lymph nodes after bilateral adrenalectomy. Each experimental point corresponds to the mean of from six to eleven experimental results. The vertical segments correspond to $2 \times \text{S.E.M.}$

Further data on the relationship between the weight of lymphatic tissue and glucuronidase activity were obtained in adrenalectomized rats (Figs. 5, 6). Whereas in the lymph nodes glucuronidase activity was slightly increased in correlation with a marked increase in weight, in thymus a very pronounced increase in weight was accompanied by only small changes in the activity of the enzyme.

β -Glucuronidase content of isolated lymphocytes

Since the specific activity of atrophic lymphatic tissues was found to be elevated, while the total amount of enzyme in the organs was if anything diminished (Table 1), it could be supposed that

Fig. 6. Wet weight (upper curve) and β -glucuronidase activity (lower curve) of thymus after bilateral adrenalectomy. Each experimental point corresponds to the mean of from six to eleven experimental results. The vertical segments correspond to $2 \times \text{S.E.M.}$

Table 2. β -Glucuronidase activity of lymphocytes isolated from the thymus (G.U./10 million cells)

	Expt. no. 1	Expt. no. 2	Expt. no. 3
Normals	8 640	24 000	30 800
Fasted for 96 hr.	18 200	39 200	38 800

fasting resulted in the preferential mobilization of enzymically inactive material. This hypothesis was tested by determinations of glucuronidase activities of lymphocytes isolated from thymus glands of normal and of fasted animals. The results (Table 2) show that during the fast an actual increase in the activity of glucuronidase per cell takes place and,

further, demonstrate that lymphocytes contribute to the increases found in the tissues. Without further evidence it is not possible to assess the part played by other elements such as reticular cells and stroma, which are relatively increased as a result of fasting.

DISCUSSION

The main result arising from the experiments reported here is that in lymphatic tissues the activity of glucuronidase increases considerably under conditions which bring about sharp decrease in weight of the tissue; under conditions in which the weight increases, β -glucuronidase levels, if previously elevated as a result of experimental intervention, return to normal values, and if previously normal show no significant change. This pattern of variation follows the same course whether activity is expressed as an enzyme:wet weight ratio, or as activity/unit weight of nitrogen (Table 1). In view of the elevated enzymic activity per cell found during fasting, it was thought that this could be due to a decrease in the content of some inhibitor normally present in the tissues (which was actually found in various organs of the rat, including the spleen, by Walker & Levvy, 1953). Experiments set up to test this hypothesis (Table 3) were not convincing, as even when four parts of the normal material were used for each part of the starved tissue the inhibition found ranged only from 14 to 21 %.

Undritz (1924), Schillaci (1928) and Frank *et al.* (1953). They show that the increase of glucuronidase activity in lymphatic tissue takes place during the period of reduced cellular proliferation, i.e. when the cells undergo atrophy and reduction in number. When, on the other hand, under the influence of refeeding or adrenalectomy, a marked increase in the lymphocyte count takes place, the level of enzyme activity does not vary unless an increase has been caused previously; in such a case a rapid fall in activity is observed.

The present findings are not compatible with the hypothesis that increase in β -glucuronidase is closely related to the proliferation of cells (Levy *et al.* 1948; Kerr *et al.* 1949, 1950). This concept is based mainly on the increase in enzyme activity of liver and kidney found after the administration of various toxic substances. The results of these authors could also be interpreted as indicating an increase in β -glucuronidase coinciding with cellular damage (Mills *et al.* 1953). Other reports in the literature indicate a lack of correlation between variations in β -glucuronidase levels and cell proliferation. Some of these are concerned with the influence of hormones on the genital tract (Harris & Cohen, 1951; Knobil, 1952; Beyler & Szego, 1954), others with the behaviour of peripheral nerve in Wallerian degeneration (Hollinger & Rossiter, 1952), and yet others with the enzyme activity in rat liver at various stages of growth (Mills *et al.* 1953). Further examination of

Table 3. *Influence of mixtures in various proportions of lymph nodes dispersions from normal and fasted rats on the hydrolysis of phenolphthalein glucuronide*

Mixtures of tissue dispersions 0.2 ml./ml. of incubated mixture. Acetate buffer 0.7 ml. For other conditions of assay see text.

Parts of dispersions		β -Glucuronidase activity (g.u./g. of tissue)		Inhibition (%)
From normals	From fasted	Found	Theoretical*	
Expt. no. 1				
2.5	2.5	18 000	23 600	24
4	1	14 700	18 600	21
1	4	21 900	23 500	7
Expt. no. 2				
2.5	2.5	16 700	19 200	13
4	1	14 300	16 700	14
1	4	18 800	19 800	5

* Sum of the activities found when each portion of enzyme was incubated separately.

Histological examination of lymphatic tissue revealed that during the period when the weight is declining as a result of fasting there was loss of lymphocytes, leaving a relative predominance of reticulo-endothelial cells. Mitoses, while not disappearing, were much less numerous than in normal conditions. These observations are in agreement with those reported by Dustin (1923),

the literature discloses some cases in which increases in the activity of the enzyme show positive correlation with atrophy or involution of the tissue. Knobil (1953) observed a significant increase of glucuronidase activity in adrenal glands during cortical atrophy following hypophysectomy. Fishman & Farmelant (1953) found an increased activity in the seminal vesicles of castrated mice;

which was reversed by treatment with testosterone. Greenbaum & Greenwood (1954) observed an increase in glucuronidase activity during the period of involution of the mammary gland after lactation.

The results of Fishman & Anlyan (1947), Fishman, Anlyan & Gordon (1947) and of Odell & Burt (1949) on the elevated level of glucuronidase activity in tumours (see, however, Mills & Smith, 1951, who did not find an increase in experimental hepatoma) would not conflict with the hypothesis correlating the increase in enzyme activity with regression processes in tissues, since zones of proliferation and of regression and necrosis are intimately interwoven in malignant tumours. On the other hand, the data of Fishman & Fishman (1944) and Fishman (1947) on the increase of β -glucuronidase activity in the uterus under the influence of oestradiol, and those of Rietton & Fishman (1953) and of Fishman & Farmelant (1953) on changes in kidney brought about by testosterone, cannot be reconciled with such an hypothesis. In the present study an attempt was made to determine whether injections of corticosteroids would produce any changes in the enzyme levels in lymphatic tissue. However, the result was negative; a single injection of cortisone acetate (Cortone, Merck), 6.2 or 12.5 mg./rat, did not induce any appreciable modification in the weights of the organs or of the activity of the enzyme in them.

Little can be said at present on the mechanism by which β -glucuronidase activity increases during atrophy. Further work aimed at clarifying this point is in progress.

SUMMARY

1. Variations in the levels of β -glucuronidase activity of rat lymphatic tissue have been followed, with phenolphthalein glucuronide as substrate, during periods of (i) loss in weight of the tissues caused by fasting, (ii) reconstitution by resumed feeding, and (iii) increase in weight which takes place after adrenalectomy.

2. β -Glucuronidase activity increased, while the weight of the tissue decreased. When the tissue increased in weight the enzyme level, if previously raised, fell to a normal value, or if already normal showed no significant variation.

3. Significant increases in the levels of enzyme, expressed as activity per cell, take place when mitoses are inhibited, and lymphocytes undergo atrophy and disappear.

4. The results are not compatible with the hypothesis that increase of β -glucuronidase is correlated with cell growth; they show that the increase in enzyme activity, at least in some cases,

coincides with atrophy and involution of the tissue.

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