Tissue Fractionation Studies

7. RELEASE OF BOUND HYDROLASES BY MEANS OF TRITON X-100*

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Recent studies have disclosed the probable existence of an intermediate class of cytoplasmic granules, apparently devoid of mitochondrial or microsomal enzymes, but containing several acid hydrolases, namely acid phosphatase, ribonuclease, deoxyribonuclease, cathepsin and β-glucuronidase (Appelmans, Wattiaux & Duve, 1955; Duve *et al.* 1955). Tentatively termed 'lysosomes', these particles have been shown to behave as essentially inert little bags which, when ruptured, release their enzyme content in soluble and fully active form.

As shown in previous work, disruption of the particles must precede any measurement of their total enzyme content. It can be accomplished by a variety of procedures, none of which, however, is entirely satisfactory from a practical point of view. The present paper describes a new method of activation which can be directly combined with the enzymic assays. It is based on the action of Triton X-100, a non-ionic detergent known to possess haemolytic properties (Glassman, 1950) and to cause the release of bound β-glucuronidase (Walker & Levvy, 1951, 1953; Walker, 1952).

METHODS

The mitochondrial fractions were isolated as described by Appelmans & Duve (1955). Free and total enzymic activities were measured according to Gianetto & Duve (1955) and Duve *et al.* (1955), with some modifications required by the presence of Triton X-100, which will be explained in the text. Triton X-100 was purchased from the Rohm & Haas Co., Philadelphia.

RESULTS

The influence of Triton X-100 on bound hydrolases was first investigated at a fixed concentration of 0·1% (v/v), known to be sufficient for the quantitative release of β-glucuronidase. A washed mitochondrial fraction, containing approximately 90% of the enzymes in bound, unreactive form, was used as experimental material. The assays were performed in the usual way, with the exception that 0·1% (v/v) Triton X-100 was present in the incubation mixture. Control experiments showed that the detergent was without inhibitory effect on any of the enzymes studied, but that it could interfere with the analytical procedures used in the nuclease and phosphatase assays. Filtrates from the nuclease measurements were found to become cloudy when diluted with distilled water prior to their spectrophotometric examination at 260 mμ. This cloudiness was probably due to the precipitation of insoluble components which had been solubilized by the detergent, and could be prevented by the use of 0·01% (v/v) Triton X-100 as diluent. Blank values were, however, higher than when the assays were carried out without detergent. For example, in the experiments of Fig. 1, the mean substrate and tissue blank values, expressed as readings at 260 mμ., were raised in the presence of 0·1% (v/v) Triton X-100 from 0·165 to 0·197 for ribonuclease, and from 0·086 to 0·102 for deoxyribonuclease, the difference due to the maximal enzymic activities being 0·231 and 0·180 respectively. In the phosphatase assays, the determinations of inorganic phosphate were complicated by the appearance of a precipitate in the filtrates upon addition of the molybdate reagent. However, this happened only when fairly large quantities of filtrate were analysed, and there was no interference if the concentration of Triton X-100 was reduced to less than 0·001% (v/v) in the final mixture.

By taking these precautions it was possible to complete the analyses satisfactorily, but the measurements lost somewhat in accuracy, especially with preparations of low activity. As shown by the results of Table 1, all five enzymes were quantitatively released in the presence of 0·1% (v/v) Triton X-100, and the activities measured by the new method compared favourably with those obtained after a preliminary treatment of the granules by any of the three procedures used in earlier investigations to release the bound hydrolases.

Several attempts were made to study in greater detail the influence of Triton X-100 on the liberation of lysosomal enzymes. The granules were either directly assayed for free activity in the presence of

* Part 6: Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). The absolute cytochrome *c* reductase values given in Table 1 of part 6 (Duve *et al.* 1955) were erroneously expressed in μmoles of cytochrome *c* reduced/min./g. of tissue, i.e. actually in half-units/g., according to the definition of units on p. 607.

Table 1. *Comparison of different activating procedures*

Enzymic activities of a washed mitochondrial fraction pretreated as described.

Activating procedure	Units/g. of original tissue* Acid phosphatase	Ribo-nuclease	Deoxyribo-nuclease	Cathepsin	β-Glucu-ronidase†
Waring Blendor (3 min.)	5·75	1·33	0·86	1·61	0·40
Freezing and thawing (12 times)	5·36	1·42	0·86	1·59	0·41
3 hr. at pH 5 and 37°	5·34	0·95	0·76	1·60	0·42
Exposure to Triton X-100 (0·1%)‡	5·75	1·60	0·81	1·61	0·42

* For definition of units, see Duve *et al.* (1955).
† Uncorrected for inhibition by sucrose (approximately 28%).
‡ Added together with the substrate at the beginning of assay.

increasing concentrations of Triton X-100, or were first suspended in ice-cold 0·25 M sucrose containing the detergent at different concentrations and then assays for free activity were made on suitable quantities of these suspensions. Since the effect of the detergent is irreversible, the determining factor in the latter procedure was the concentration of Triton X-100 in the original suspensions and not that in the more dilute assay mixtures. Free activities were measured as before (Gianetto & Duve, 1955; Duve *et al.* 1955), namely by incubating for 10 min. at 37° and pH 5 in the presence of the required substrates and of 0·25 M sucrose. The volumes were, however, varied according to the quantity of granules added, which itself depended on the expected activity, the aim being to keep the ratio of granules to detergent constant in each set of five assays performed at a given concentration of Triton X-100. This was done because preliminary results indicated that the activation was a function of this ratio as well as of the concentration in detergent.

Despite these precautions, it was not possible to attain a satisfactory degree of accuracy in the activity measurements, which were limited by the combined restrictions imposed by the assay conditions and by the presence of Triton X-100. As shown before, these conditions must be strictly adhered to in order to prevent breakage of the granules during the assay, but are far from conducive to optimum accuracy, especially since the measured activities are usually low and the blanks relatively high. In addition, the release of the bound hydrolases under the influence of the detergent proved a delicate phenomenon in itself, occurring in an almost all-or-none fashion as soon as the detergent concentration exceeded a certain level. The latter was about 0·035% (v/v) for a concentration in granules corresponding to approximately 0·1 g. of original liver/ml., but varied somewhat from one preparation to the other, making it very difficult to obtain detailed activation curves.

The results of two such experiments are represented graphically in Fig. 1. It is clear, in spite of the considerable scattering of the experimental points, that the activation of all five hydrolases occurs roughly in the same range of detergent concentration. Some fairly large differences are noticeable in the narrow critical region, but these were not reproducible in other experiments.

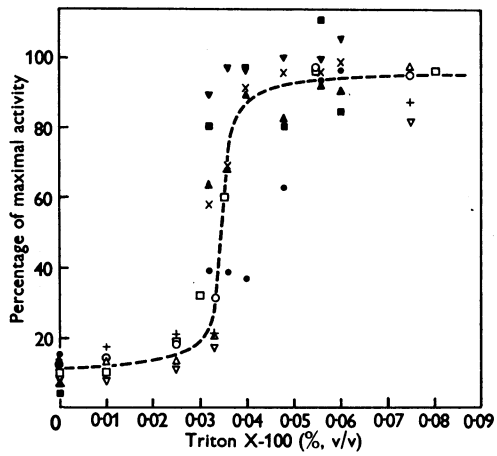

Fig. 1. Activation by Triton X-100. Expt. 1 (open points): all incubated mixtures contained the required substrate and buffer, 0·25 M sucrose, Triton X-100 at the concentration indicated, and an amount of large granules corresponding to 0·1 g. of original liver/ml. The total volume was varied according to the predicted activity of the enzymes. The tests were run in 10 min. at 37°. Expt. 2 (filled-in points): free activity assays were performed in the usual manner on appropriate quantities of mitochondrial suspensions containing the equivalent of 0·15 g. of original liver/ml. and Triton X-100 at the concentration indicated. Acid phosphatase: + (Expt. 1), × (Expt. 2); ribonuclease: △, ▲; deoxyribonuclease: ▽, ▼; cathepsin: □, ■; β-glucuronidase: ○, ●. In both experiments, acid phosphatase and ribonuclease were measured simultaneously as described by Duve *et al.* (1955). All results are expressed in percentage of the activities measured in the presence of 0·15% (v/v) Triton X-100.

DISCUSSION

Compared with the procedures used previously to release bound hydrolases, the method described in this paper offers considerable advantages, in that it can be used with minimal quantities of material, requires no preliminary treatment of the granules and ensures complete activation without denaturation or inhibition of the enzymes. These advantages more than make up for the loss in accuracy which may affect some of the measurements, and justify the use of the method for routine determinations of total activity.

The results obtained on the graded release of the hydrolases may be added to the previous data, which indicated a parallel liberation of the five enzymes under the influence of a variety of agents (Gianetto & Duve, 1955; Duve *et al.* 1955). Such data form the main basis of the hypothesis that the enzymes may all belong to a single homogeneous population of granules. Since, however, the papers just mentioned contain evidence conflicting with this hypothesis, additional information would have been particularly welcome. It is unfortunate, therefore, that the conditions of the experiments did not allow a sufficient degree of accuracy to verify the significance of the finer differences which were occasionally observed. As mentioned in the Results section, these differences were not reproducible, and the fact that they occurred in the critical range of detergent concentration renders their significance doubtful. It must indeed be remembered that the granules were not in exactly the same environment during the assays, which, of necessity, had to be performed with different substrates. It is quite possible that these differences were sufficient to modify the degree of activation of such delicately balanced systems as must obtain in the neighbourhood of the critical detergent concentration. It may be significant in this respect that the two enzymes which were actually assayed together in the same mixture, namely acid phosphatase and ribonuclease, did show closely similar activities at all concentrations of detergent.

SUMMARY

1. The bound forms of acid phosphatase, ribonuclease, deoxyribonuclease, cathepsin and β-glucuronidase were released quantitatively in preparations from rat liver exposed to 0·1% (v/v) Triton X-100. This detergent had no inhibitory effect on the enzymes.

2. The above findings form the basis of a method whereby total activities of lysosomal enzymes can be assayed directly, without preliminary treatment of the liver preparations, simply by running the assays in the presence of 0·1% (v/v) Triton X-100.

3. In mitochondrial preparations exposed to increasing concentrations of Triton X-100, the enzymes were released in a fairly abrupt fashion when the detergent concentration exceeded a certain critical level, which was about 0·035% (v/v) for an amount of granules corresponding to 0·1 g. of original tissue/ml. All five enzymes were liberated in a roughly parallel manner, but the scattering of the results was such that finer differences could not be excluded.

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Tissue Fractionation Studies

8. CELLULAR LOCALIZATION OF BOUND ENZYMES

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One of the difficulties in interpreting the results of tissue-fractionation experiments arises from the cellular heterogeneity of the starting material. In liver, accessory cell types represent only a small proportion of the total mass of the organ, but they could nevertheless be a source of complication if they should contain some specific systems. The recent identification of a special group of cytoplasmic granules (lysosomes), showing atypical distribution patterns and associated with only a small fraction of the total nitrogen (Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955), has made