DIFFERENTIAL RELEASE OF ALKALINE DEOXYRIBONUCLEASE AND OF GLUTAMATE DEHYDROGENASE FROM RAT-LIVER MITOCHONDRIAL FRACTIONS

by

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In experiments performed more than ten years ago in this laboratory, strong indications were obtained that in rat liver DNase I is essentially localized in the mitochondria, in contrast with DNase II, which is a lysosomal enzyme. Except for a 10 percent excess of activity in the microsomal fraction, DNase I was found to be distributed almost exactly like cytochrome oxidase, GDH, and other mitochondrial enzymes, between fractions isolated from whole tissue homogenates by differential centrifugation (Beaufay *et al.*, 1959a), and between subfractions separated from mitochondrial fractions by means of density gradient differential sedimentation and of isopycnic centrifugation in gradients of sucrose with either H_2O or D_2O as solvent (Beaufay *et al.*, 1959b). The variety of fractionation conditions under which DNase I remained closely associated with known mitochondrial enzymes was such as to enforce the conclusion that DNase I truly belongs to the mitochondria.

It was also found that DNase I is largely latent in intact mitochondrial fractions and that its mode of release under various conditions resembles that of GDH much more than it does that of enzymes not located in mitochondria, such as the lysosomal hydrolases (Baudhuin, 1959). However several differences were also observed. The free activity of DNase I in fresh mitochondrial preparations was 21.1 $\pm$ 5.4 (S.D.) % of its total activity, as against 9.0 $\pm$ 5.0 for GDH. After a one-

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Abbreviations : DNase I = alkaline deoxyribonuclease; DNase II = acid deoxyribonuclease; GDH = glutamate dehydrogenase; MAO = monoamine oxidase; BSA = bovine serum albumin.

hour exposure of the particles to a critical concentration of Triton X-100 (1 mg/ml, with 25 mg/ml of mitochondrial protein), the free activity of DNase I rose from 25.1 to 58.0, that of GDH from 9.3 to 20.4 % of their respective total activities. As shown in Fig. 1, the two enzymes differed particularly in their mode of release from particles subjected to decreasing concentrations of sucrose.

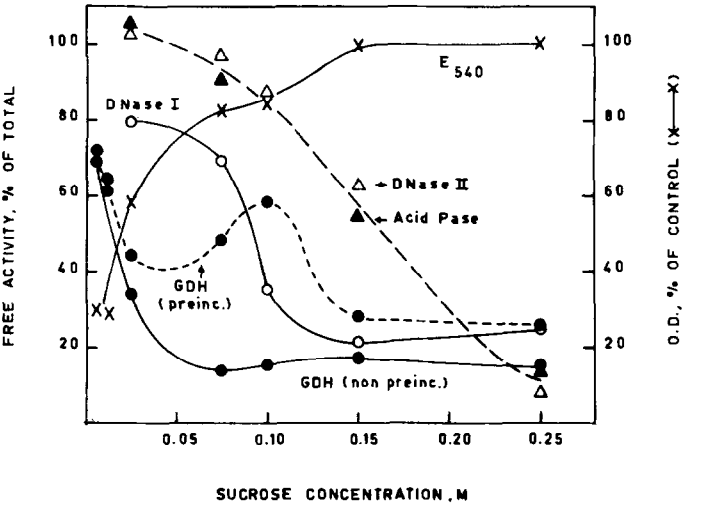

Fig. 1. Osmotic release of latent enzymes. Mitochondrial fraction from rat liver exposed for 15 min. at 0° to sucrose concentration shown in abscissa and then returned to 0.25 M sucrose. Assays for free and total activities of GDH (Bendall and de Duve, 1960), DNase II (de Duve *et al.*, 1955), acid phosphatase (Appelmans and de Duve, 1955). DNase I measured in 20 min. incubation at 37° according to Beaufay *et al.* (1959a), in the presence of 0.25 M sucrose. Dotted line shows free GDH activity as measured after a 20 min. incubation under conditions of assay of DNase I. E_{540} is optical density of suspension at 540 nm.

These experiments were beset with technical difficulties which prevented unequivocal interpretation of the results. DNase I, when released in free form, was found to be very unstable. With the methods then in use in our laboratory, the free activities of the two enzymes were measured under very different conditions. Whereas free GDH was assayed in a few minutes at 25°, the determination of free DNase I required a 20 min. incubation at 37°, which in itself was sufficient to cause an approximately 10 % release of latent enzyme activity.

The influence of the latter conditions on free GDH was studied in the experiment of Fig. 1, yielding results that, although they appeared erratic, indicated strongly that a considerable additional release of GDH obtains in particles subjected to an osmotic shock, if they are then incubated for 20 min. at 37° in the medium used for the assay of DNase I. Finally, attempts to solve the problem by measuring unsedimentable instead of free activities were complicated by what appeared to be adsorption artifacts. Publication of the results of these activation experiments was therefore deferred until clearer evidence of their significance could be obtained. For other reasons, work on this subject was discontinued.

Recent interest in the presence of genetically active DNA in mitochondria has prompted us to reexamine the problem of the osmotic release of DNase I from mitochondrial fractions. A technique was devised whereby the assays of free and total DNase I and GDH activities could be done under closely similar conditions.

To this end, DNase I was assayed at 25° and in a medium containing all the ingredients present in the assay medium of GDH, except NAD and nicotinamide, which interfere with the spectrophotometric determination of the oligonucleotides formed from DNA. This medium included 1.125 mg/ml of denatured DNA (dissolved in distilled water, heated for 5 min. at 95° and cooled rapidly in ice), 20 mM glycyl-glycine buffer pH 7.7, mM $MgCl_2$, 0.4 mM NaCN, 15 mM sodium glutamate, 0.25 M sucrose, 1.6 % bovine BSA (P.3.D., Poviet Producten N.V., Amsterdam, The Netherlands), and, when total activities were measured, 0.1 % Triton X-100. Incubation was carried out for 2 and 22 min. at 25° in a total volume of 1 ml, the reaction was stopped with 10 ml ice-cold 0.75 M perchloric acid, the mixtures were filtered after 10 min. at 0°, and the optical density of the filtrates was read at 260 nm without further dilution. The difference in O.D. between the two samples is related to the enzyme concentration by a slightly sigmoid curve (Beaufay *et al.*, 1959a) which is quite reproducible and can serve as reference for conversion of the results to arbitrary enzyme units. The blanks are high with this technique, but the presence of BSA in the medium and the use of a sensitive spectrophotometer decrease the dispersion and make the assay reasonably accurate and reliable.

For the measurement of free and total GDH, the samples were first incubated for 20 min. at 25° exactly as in the assay of DNase I. They were then appropriately diluted with the same medium, containing in addition 30 mM nicotinamide. The reaction was started by the addition of 1.4 mM NAD^+ and the increase in O.D. at 340 nm was recorded for a few minutes. Thus the free GDH activities measured corresponded to those present *at the end* of the free DNase assay.

The osmotic treatment of the particles was done as in earlier experiments, but in the presence of 1.6 % BSA. After isolation according to de Duve *et al.* (1955), the M+L fraction of these authors was diluted to the desired sucrose concentration with an appropriate solution of sucrose-BSA. After standing for 15 min. at 0°, they were then returned to 0.25 M sucrose-1.6 % BSA, and assayed as described above.

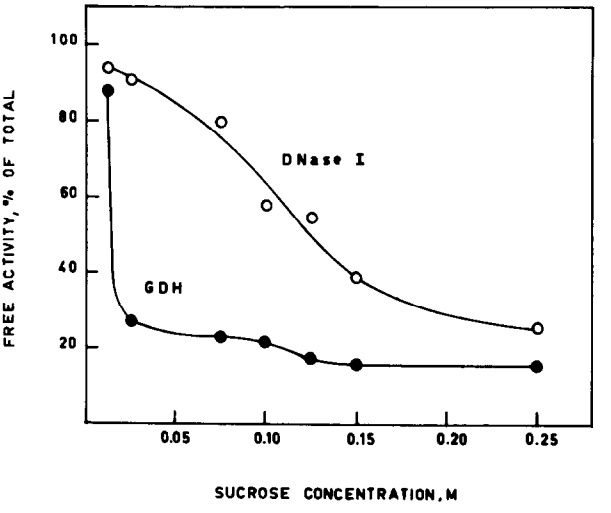

Fig. 2. Osmotic release of latent enzymes. Experimental procedure as for Fig. 1, but on particles subjected to osmotic shock in the presence of 1.6 % BSA. Both enzymes measured at 25° as described in text. Free GDH assayed after 20 min. incubation under conditions of assay of DNase I.

In Fig. 2 are shown the results of an osmotic activation experiment performed with the new technique. They confirm in a more definitive fashion the findings of Fig. 1, indicating that DNase I can be released almost entirely by exposure of the particles to concentrations of sucrose insufficiently low to

affect significantly the accessibility of glutamate dehydrogenase to its substrate(s). We have also found that a considerable fraction of the DNase I released in this manner is truly unsedimentable in the treated suspensions, whereas the amount of GDH liberated in soluble form is very small. The presence of 1.6 % BSA throughout all manipulations played an important role in the success of the new experiments. Apparently, BSA protects osmotically shocked mitochondria against subsequent damage leading to the release of GDH. When the particles were incubated as described above, but in a medium containing no or 0.2 % BSA, results similar to those depicted in Fig. 1 for the incubated suspension (dotted GDH curve) were obtained. In particular, the hump in the activation curve of GDH around a sucrose concentration of 0.1 to 0.125 M was observed several times.

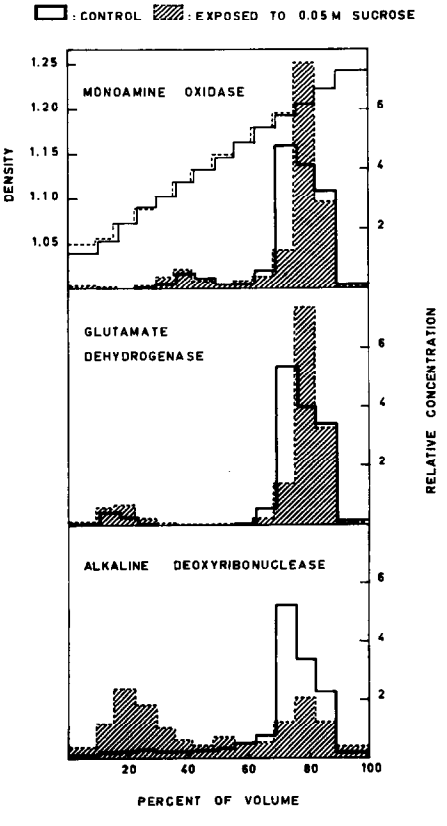

Fig. 3. Density equilibration of mitochondrial fraction in linear sucrose gradient containing 1.6 % BSA. Experiments performed according to Beaufay *et al.* (1964), with particles initially layered above gradient. Ordinate is concentration in fraction relative to average concentration throughout gradient (sum of recovered activities divided by total volume). Solid line : control. Shaded histogram : fraction exposed for 15 min. at 0° to 0.05 M sucrose in the presence of 1.6 % BSA, and then returned to 0.25 M sucrose-1.6 % BSA. Recoveries in gradient were 115 and 119 % for MAO, 99 and 97 % for GDH, 108 and 65 % for DNase I, in control and osmotically treated particles respectively. Loss of DNase I presumably affects soluble enzyme selectively. Therefore, its release is likely to be greater than shown on normalized graph.

The results of Fig. 3 show that exposure of a mitochondrial fraction to 0.05 M sucrose affects the behavior of DNase I much more strongly than those of GDH and of MAO, upon centrifugation of the particles through a sucrose gradient containing 1.6 % BSA to their density equilibrium position. Whereas in the control suspension practically all the DNase I bands together with the two other enzymes, in the treated suspension, a large fraction of this enzyme remains in soluble form in the top layer. In contrast, GDH and MAO behave very similarly in the two preparations. The bulk of both enzymes remains particle-bound after exposure to hypotonic sucrose. This treatment does, however, appear to affect their density distribution, which has become sharper, with a peak around the second equilibrium position of mitochondria in sucrose gradients (Beaufay *et al.*, 1964). In both suspensions, small amounts of soluble GDH are found in the top layer, and a small fraction of MAO bands around a density of 1.12, characteristic of the outer mitochondrial membrane.

The most likely interpretation of these results is that DNase I is located in the space between the two mitochondrial membranes. It is consistant with the evidence indicating that the enzyme truly belongs to the mitochondria and with the observation that its release is practically complete after exposure to 0.05 M sucrose solutions, which have about the same osmolarity as the 20 mM phosphate buffer shown by Parsons *et al.* (1966) to cause a selective rupture of the outer mitochondrial membrane. The integrity of the inner membrane under these conditions would account for the lack of release of GDH. Arguing against this interpretation is the fact that MAO, a marker of the outer mitochondrial membrane (Schnaitman and Greenawalt, 1968), is not significantly detached by the osmotic treatment. However, Parsons *et al.* (1966) have emphasized that the amounts of outer membrane that are actually freed from the mitochondria after exposure to hypotonic phosphate are very small.

Considered in the framework of this interpretation, the hump seen in the release curve of GDH around 0.1 - 0.125 M sucrose under conditions of inadequate protection (Fig. 1) suggests that the inner membrane is more sensitive to osmotic shock in the range of osmotic pressure where breakage of the

outer membrane occurs. The reason for this is not obvious, but could be related to differences in the mechanism of rupture of the outer membrane depending on the sudenness and magnitude of the change in osmotic pressure. With a relatively mild osmotic shock, pressure of the swelling matrix against the outer membrane could be the main factor involved, as suggested by Parsons *et al.* (1966). With a more drastic osmotic change, the uptake of water into the intermembrane compartment which is believed to represent the sucrose space of the mitochondria could be mainly responsible for the breakage of the outer membrane, and unfolding of the inner membrane could take place independently of changes affecting the outer membrane.

We cannot rule out the alternative interpretation that DNase I is located in granules different from those that contain the bulk of GDH. The only argument supporting such a hypothesis is the presence of larger amounts of DNase I than of typical mitochondrial markers in the microsomal fraction. However, other mitochondrial enzymes, in particular MAO (Beaufay *et al.*, 1959a), also occur in the microsomal fraction in amounts greater than those of cytochrome oxidase or GDH found in this fraction. The significance of the microsomal DNase I is presently under investigation.

Whatever their final interpretation, our results demonstrate clearly that DNase I is not present in the mitochondrial matrix where GDH and the mitochondrial DNA are located. This point is obviously of interest.

Acknowledgement

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