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Some Factors Affecting the Permeability of Large-Granule Membranes to Mouse-Liver Catalase *in vitro*

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Approximately 70% of the catalase in mouse liver is present in the large granules, the remainder being in the extraparticulate cytoplasm (Adams & Burgess, 1957). Adams & Burgess (1959a) showed that when slices from mouse liver were incubated at 38° in a phosphate medium, catalase migrated from the granules to the extraparticulate cytoplasm. Also, when granules were isolated and incubated at 38° in 0.25M-sucrose, catalase migrated into the surrounding medium. They produced evidence suggesting that no appreciable rupture of granules occurred under these conditions, and concluded that the migration was due to permeability of the intact granule membranes to catalase. In the same paper it was also shown that catalase

migration was greatly reduced when slices in phosphate, or preparations of granules in sucrose, were incubated at 20°. No catalase migrated from granules prepared and incubated in phosphate medium at 38°. It appeared therefore that the granule membrane permeability depended greatly on the applied conditions, and the purpose of the present paper is to study these conditions further.

EXPERIMENTAL

Animals. Young adult mice of the CBA strain (Committee on Standardized Genetic Nomenclature for Mice, 1960) were used. The diet of the animals consisted of commercial rat cubes and water (both without restriction). The catalase migration from granules prepared from the livers

of these mice was qualitatively similar to that observed with granules from the Swiss albino strain used previously (Adams & Burgess, 1959a). However, the granules from CBA mice retained more of their catalase after incubation in 0.25 M-sucrose than did those from the albino strain.

Media used for liver homogenates. The phosphate medium used in some experiments was that described by Adams & Berry (1956), with the addition of 0.01 M-ethanol in order to prevent losses of catalase through the formation of an inactive complex of catalase with hydrogen peroxide ('Complex II', Chance, 1950; Adams & Burgess, 1959b). A similar addition of ethanol was made to all the media used. Except where otherwise stated, all homogenates and media were brought to pH 7.0 as previously described (Adams & Burgess, 1959a). Triton X-100 (0.25%) was added to liver homogenates or to large-granule suspensions when it was required to disrupt the granules and liberate their catalase into solution.

Preparation of catalase fractions from mouse liver. Livers were homogenized with 12 strokes of a Ten Broeck grinder, 1 g. of liver in 9 ml. of medium being used (Adams & Burgess, 1957). This will be referred to as a '12-stroke homogenate'. Various preparations were made from this. In some the homogenates were centrifuged at 800 g for 10 min. to remove nuclei and debris, the granules therefore remaining in contact with the soluble components of the liver. In others large granules were separated from the nuclei-free suspension by centrifuging at 10 000 g for 20 min. The granules were resuspended in the appropriate volume of fresh medium.

Catalase measurements were made at various times on the following preparations: (1) 12-stroke homogenate (i.e. extraparticulate cytoplasmic fraction and unbroken granules); (2) extraparticulate cytoplasmic fraction; (3) granule fraction (by addition of Triton to a granule suspension); (4) total catalase (by addition of Triton to a 12-stroke homogenate, or to one free of nuclei and debris); (5) supernatant catalase, i.e. catalase present in the supernatant after centrifuging resuspended granules. Except where otherwise stated, the homogenates or granule suspensions were diluted (1:10) with the appropriate medium before incubation. In some experiments where this procedure was not practicable (e.g. in the measurement of total protein released from granules), the concentrated homogenates were incubated. Where the first procedure was followed, sufficient tubes were incubated to enable a pair to be removed from the bath for each estimation. In the second procedure portions of concentrate were removed and diluted for the catalase estimations.

Estimation of liver-catalase activity. Catalase was estimated as previously described (Adams, 1950; Adams & Burgess, 1959a, b) and the activities of the various fractions were referred to the nitrogen content of the whole homogenate from which they were prepared. Nitrogen was estimated by the Kjeldahl method.

Measurement of oxygen uptake. Oxygen uptake by homogenates was estimated with conventional Warburg manometers and is given as mm.³ of O₂/hr./mg. dry wt. (g_{O₂}). The total volume of fluid in the flasks was 3 ml. The amount of homogenate or granule suspension added was 0.5 ml. (equivalent to about 50 mg. of whole liver), made up in 0.25 M-sucrose buffered with 0.02 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris), pH 7.4. Cofactors added in some experiments were (final concentrations):

25 mM-KCl, 1 mM-adenosine triphosphate (ATP) and 1 mM-sodium malate. The volume was made up with water containing 0.01 M-ethanol. The centre well contained 40% NaOH to absorb expired CO₂. The incubation temperature was 38°.

Polyvinylpyrrolidone. The sample had a molecular weight of 11 000.

Estimation of protein. Nitrogen was determined by the Kjeldahl method. In earlier experiments we expressed protein nitrogen as the difference between the total and non-protein nitrogen. The non-protein nitrogen was estimated in the supernatant after centrifuging the precipitate formed by the addition of an equal volume of saturated trichloroacetic acid. Since the values for the non-protein nitrogen were small and relatively constant, separate measurements of it were discontinued in later experiments, and the total nitrogen was taken as being equal to the protein nitrogen.

RESULTS

Effect of cations on the migration of catalase from large granules. A homogenate was made in 0.25 M-sucrose and freed of nuclei and debris. It was diluted immediately with various volumes of isotonic solutions of calcium, magnesium, sodium or potassium chloride dissolved in water, and sufficient additional 0.25 M-sucrose was added to produce the standard 1:10 dilution. The resulting suspensions were incubated at 38° and estimations of catalase activity made at intervals until no

Fig. 1. Inhibition of catalase migration from large granules to supernatant by the addition of varying concentrations of salts to large-granule suspensions prepared in 0.25 M-sucrose and incubated at 38°. (a) NaCl; (b) CaCl₂; (c) KCl; (d) MgCl₂.

Table 1. *Rates of catalase and of protein release from granules during incubation of large-granule preparations in various media*

Granules were isolated and resuspended in the media indicated and incubated at 20° or 38° as shown. Catalase activities are given as arbitrary units/mg. of N. Protein values are in mg. of protein N/ml. of supernatant or of granule suspension.

Time of incubation (min.)	...	...	0			15			40		
(1) Granule suspension in 0.25M-sucrose, incubated at 20°											
Supernatant catalase			89	74	60	84	81	80	95	81	78
Total catalase			294	364	283	335	364	283	335	356	272
Nitrogen in supernatant			0	0	0	0.16	0.17	0.21	0.18	0.17	0.19
Nitrogen in granules			0.51	0.59	0.45	0.36	0.37	0.25	0.36	0.33	0.28
(2) Granule suspension in 0.25M-sucrose, incubated at 38°											
Supernatant catalase			89	76	60	154	138	114	221	186	174
Total catalase			294	387	264	350	378	248	350	365	252
Nitrogen in supernatant			0	0	0	0.26	0.19	0.20	0.25	0.24	0.22
Nitrogen in granules			0.51	0.59	0.40	0.34	0.37	0.21	0.29	0.35	0.19
(3) Granule suspension in phosphate medium, incubated at 38°											
Supernatant catalase			32	24	—	34	19	—	37	24	—
Total catalase			267	182	—	267	189	—	270	186	—
Nitrogen in supernatant			0	0	—	0.18	0.17	—	0.18	0.17	—
Nitrogen in granules			0.52	0.35	—	0.39	0.17	—	0.39	0.17	—

Table 2. *Catalase loss from granules prepared and resuspended in 0.25 M-sucrose and incubated for 2 hr. at 38° compared with that from granules centrifuged and again resuspended after 1 hr. incubation, and incubated for a further 1 hr.*

Catalase activities are expressed in arbitrary units/mg. of N.

Time of incubation (min.)	0	30	60	120
Supernatant catalase	38	172	165	170
Granule catalase	355	190	190	200
Total catalase	420	405	405	420

After 60 min. incubation samples were removed, the granules centrifuged and resuspended in 0.25 M-sucrose, and reincubated for a further 60 min.

Time of incubation (min.)	0	30	60
Supernatant catalase	7	11	14
Granule catalase	190	185	185
Total catalase	200	200	200

more migrated from the granules. This plateau was usually reached after 60 min. of incubation. The results (Fig. 1) show that all the cations inhibited catalase migration from the large granules and that calcium was the most effective. The total amount of catalase in the suspensions was unaltered during the incubation period. When the concentration of the bivalent cations was increased beyond 1 mM some coagulation of the granules occurred. In additional experiments sodium sulphate was substituted for sodium chloride. The

inhibition curves were closely similar, and so are not separately reported.

Comparison of protein and catalase migrations from large granules suspended in various media. It seemed possible that the migration of catalase from the large granules into the surrounding medium in sucrose homogenates was part of a general protein migration. Granules were isolated and resuspended in the original media in which they were prepared, and the amount of protein lost from them into the supernatant during incubation for 40 min. was compared with the catalase loss. In these experiments (Table 1) total and supernatant catalase were measured.

Influence of extraparticulate-catalase concentration on catalase migration. Under our conditions between 30 and 70% of the granule catalase does not migrate on incubation. The following experiments were carried out to determine whether the rate of loss of catalase from the granules depends on the extraparticulate-catalase concentration. A homogenate was freed from nuclei and debris and the granules were centrifuged and resuspended in 0.25 M-sucrose. They were incubated at 38° for 120 min. Catalase migrated from the granules for the first 30 min. After 60 min. part of the suspension was centrifuged and resuspended in fresh sucrose. Practically no catalase migrated after this period from either the original preparation or from the resuspended granules (Table 2), showing that the cessation of migration is an intrinsic property of aging granules and is not dependent upon rise in concentration of extraparticulate catalase.

Influence of sucrose concentration in the medium on catalase migration. Livers were dissected from four animals, minced finely with scissors and carefully mixed together. The total was then divided approximately into four parts. The four samples were homogenized in 0.25M-, 0.45M-, 0.65M- and 0.88M-sucrose respectively, and samples of the resulting homogenates were incubated at 38°. The results appear in Fig. 2.

Effect on catalase migration of the addition of polyvinylpyrrolidone to 0.25M-sucrose. Many attempts have been made to isolate mitochondria with minimum damage to the membranes. Among the methods used have been the addition of relatively inert substances of high molecular weight to the media, such as polyvinylpyrrolidone (Novikoff,

1956) and dextran (Birbeck & Reid, 1956). It seemed desirable therefore to examine the effects of such substances on our preparations.

The livers from four mice were prepared as in the last experiment, and homogenized in 0.25M-sucrose and 0.25M-sucrose made up to 2, 4 and 7% (w/v) with respect to polyvinylpyrrolidone. Samples of the homogenates were incubated at 38° and the effect on catalase migration is illustrated in Fig. 3. Over the first 60 min. of incubation catalase migration progressively decreased as the concentration of polyvinylpyrrolidone increased.

Fig. 2. Effect of sucrose concentration in the medium on catalase migration from large granules incubated at 38°. (a) 0.25M; (b) 0.45M; (c) 0.65M; (d) 0.88M. Δ , Total catalase; $\circ$, granule catalase; $\bullet$, extraparticulate cytoplasm catalase.

Fig. 3. Effect of the addition of polyvinylpyrrolidone to the 0.25M-sucrose medium on catalase migration from large granules incubated at 38°. Concentrations of polyvinylpyrrolidone: (a) nil; (b) 2%; (c) 4%; (d) 7%. Δ , Total catalase; $\circ$, granule catalase; $\bullet$, extraparticulate cytoplasm catalase.

Table 3. *Effect of the addition of respiratory cofactors (25 mM-potassium chloride, 1 mM-adenosine triphosphate and 1 mM-sodium malate) on catalase loss from isolated granules*

Granules were prepared and resuspended in 0.25M-sucrose and incubated at 38°. Catalase activities are expressed in arbitrary units/mg. of N.

Time of incubation (min.) ...	0	30	60	90	$q_{0.5}$
No cofactors					
Supernatant catalase	35	200	225	222	4.0
Total catalase	370	375	380	390	—
With cofactors					
Supernatant catalase	42	140	190	195	22.3
Total catalase	350	375	400	370	—

Effect of addition of respiratory cofactors to large-granule suspensions on catalase migration. It was thought possible that the permeability of the large-granule membranes to catalase would be affected by the degree to which the granules were able to respire and metabolize. Liver homogenates prepared in 0.25M-sucrose were incubated in Warburg vessels in the presence of sodium malate, ATP and KCl. These homogenates respired very much more rapidly than those to which no cofactors were added (Table 3), but the rate of appearance of catalase in the medium was only slightly decreased.

DISCUSSION

Adams & Burgess (1959a) concluded that catalase was capable of migrating through the intact membranes of large granules, and Adams (1960) suggested that this migration occurred *in vivo* as part of the mechanism regulating intracellular-catalase distribution. In view of the fact that catalase is a comparatively large molecule, it seemed desirable to seek further evidence that migration from large granules was not simply the result of damage to the membranes. In experiments with 0.25M-sucrose as the basic medium, the addition of small concentrations of cations greatly decreased the catalase migration. The order of inhibitory activity was

mM- Ca^{2+} ions produced 80% inhibition. On the other hand, increasing the sucrose concentration from 0.25 to 0.88M progressively increased the rate of catalase migration. The addition of respiratory cofactors to 0.25M-sucrose appeared to decrease catalase migration by only a slight amount. These results seem to confirm that the loss of catalase from large granules *in vitro* is a true migration of enzyme through the intact membranes and they show that permeability is greatly influenced by the composition of the surrounding medium.

There was a rapid protein loss from large granules during incubation, but the conditions under which this occurred differed markedly from those governing catalase loss. Thus the protein loss occurred during the first 15 min. of incubation, whereas considerable amounts of catalase migrated during the 15–40 min. period. Also, protein loss occurred in both phosphate medium and 0.25M-sucrose incubated at 38°, and in 0.25M-sucrose incubated at 20°. As previously found (Adams & Burgess, 1959a) no catalase migrated from granules incubated in phosphate medium, and very little from granules incubated in 0.25M-sucrose at 20°. The results seem consistent with the supposition that protein was lost by elution from the granule surface.

The action of polyvinylpyrrolidone on the system (Fig. 3) was rather complex. For the first 60 min. of incubation the rate of catalase migration was progressively decreased as the concentration of polyvinylpyrrolidone in the medium increased. However, in the sucrose media studied, and also with the sucrose media to which cations had been added, catalase migration virtually ceased after 60 min. of incubation. When polyvinylpyrrolidone was present in the medium, the rate of catalase migration between 60 and 120 min. at all concentrations was equal to or greater than the rate from 0 to 60 min. It has always been difficult to explain why in plain sucrose media catalase should migrate steadily for 60 min. and then cease while a large amount is still present in the granules. The experiments given in this paper (e.g. Fig. 2) show that the proportion of catalase migrating differs considerably with the composition of the medium, and also is not influenced by the rise in extraparticulate-catalase concentration. In any event the cessation of catalase migration in this way would not be expected if the catalase release is due to membrane damage. Since it appears that granule membranes are in better condition in media containing polyvinylpyrrolidone, it also seems unlikely that the release of catalase during 60–120 min. of incubation in these media is due to membrane damage. Most probably the effect of polyvinylpyrrolidone is to slow the general rate of migration. In liver slices, for example (Adams & Burgess, 1959a), catalase migrates steadily from the granules during the 2 hr. of incubation, and more slowly from 2 to 4 hr. It was thought, however, that to incubate granules for more than 2 hr. would be very likely to produce serious membrane damage, which would render the interpretation of the results very difficult.

One other point of interest, shown in Fig. 3, is that the catalase activity present in the medium immediately after liver homogenization decreased steadily as the proportion of polyvinylpyrrolidone in the medium increased. About 90 catalase units were present in 0.25M-sucrose, but only about 40 in 0.25M-sucrose with 7% of polyvinylpyrrolidone. There seem two possible explanations for this. First, polyvinylpyrrolidone may assist in the preservation of structures present in the cytoplasmic reticulum, with the result that part of the extraparticulate catalase sediments with them. Or, secondly, there may be catalase-containing particles which are stable only in sucrose-polyvinylpyrrolidone solutions. In this case the extraparticulate catalase present in plain sucrose or phosphate media would contain some enzyme derived from particles. It is known (Thomson & Klipfel, 1958) that, in the rat, catalase activity is distributed over a range of different articles in the

large-granule fraction. However, increasing the sucrose concentration to 0.88M (Fig. 2) did not appear to decrease the catalase activity found in the extraparticulate fraction immediately after the preparation of the homogenate.

SUMMARY

1. The addition of cations to suspensions of liver large granules in 0.25M-sucrose decreases the catalase migration from them during subsequent incubation at 38°.

2. The addition of polyvinylpyrrolidone to such suspensions also decreases the catalase migration.

3. Catalase migration from granules is increased as the sucrose concentration in the medium is increased.

4. The addition of respiratory cofactors to granule preparations in 0.25M-sucrose has little or no effect on catalase migration.

5. When granules are incubated in either a phosphate medium or in 0.25M-sucrose, 30–50 % of their protein rapidly appears in the medium. This is probably due to an elution of material from

the granule surface, whereas the catalase loss appears to occur by migration through the granule membranes.

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Biochemical Changes in the Tissues of Animals Injected with Iron: Acid Phosphatase and other Enzymes

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Iron occupies a unique place among heavy metals, both in the large amount normally present in the body and in the special mechanism available for its storage in tissues as ferritin and haemosiderin. Syndromes associated with the storage of excessive quantities of iron are of clinical importance but have not been studied biochemically. The availability of iron for parenteral administration, in the form of an iron-dextran complex which displays low toxicity, makes it possible to increase by 100 times or more the total body iron of mice, rats and other animals. Apart from some reduction in growth rate there is no obvious adverse effect on the health and well-being of the animal, which can live its normal span (Golberg, Smith & Martin, 1957a). In the course of time, however, the rat which is rendered siderotic in this way develops many of the manifestations of vitamin E deficiency and there is strong evidence that the stored iron reacts with, or otherwise renders in-

adequate, the tissue reserves of antioxidant (Golberg, Smith & Martin, 1957b; Golberg & Smith, 1958). It has also been shown that the siderotic liver, although histologically intact, is particularly susceptible to toxic and adverse nutritional influences (Golberg & Smith, 1960).

Biochemical study of the grossly siderotic liver has revealed enhanced activities of some enzymes, with acid phosphatase showing an outstandingly large increase of an apparently permanent nature. An increase of this kind was observed (van Duijn, Willighagen & Meyer, 1958; van Duijn & Willighagen, 1959) for animals injected with polymers such as high-molecular-weight dextran or polyvinylpyrrolidone. With iron-dextran, enhanced liver acid-phosphatase activity appears to be attributable to excessive iron deposition, since moderate doses of iron, still well above the therapeutic doses in man, have no such effect.