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COMPARISON OF THE ENZYME KINETICS AND IMMUNOLOGICAL PROPERTIES OF CATALASE IMMOBILIZED BY MICROENCAPSULATION AND CATALASE IN FREE SOLUTION FOR ENZYME REPLACEMENT

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SUMMARY

The enzyme kinetics and immunological properties of catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) immobilized by microencapsulation are studied and compared to catalase in free solution. In vitro studies show that the K_m for both forms of catalase is 0.55 M. The V of the microencapsulated catalase is 0.5 mM/min while that of catalase in free solution is 2.5 mM/min. The in vivo enzyme kinetics were studied using Feinsteins's acatalasemic mice which have a hereditary deficiency in catalase. These in vivo studies show that microencapsulated catalase is as effective as the same assayed amount of catalase in free solution in reducing total body substrates in the form of exogenous perborates. After injection, microencapsulated catalase with an in vivo half-life of 4.4 days is more stable than catalase in free solution which has an in vivo half-life of 2.0 days. In vitro studies show that the enclosing membranes of semipermeable microcapsules are impermeable to beef catalase antibodies. Repeated injection of beef catalase solution into acatalasemic mice sensitized the mice to a large dose of the enzyme in solution, but not to the same dose of enzyme immobilized within semipermeable microcapsules. Injection of catalase in free solution is removed very rapidly from the circulation of acatalasemic mice immunized to catalase. Catalase in free solution was not effective in reducing total body sodium perborate levels in immunized mice, while microencapsulated catalase acted efficiently when injected into the immunized mice.

INTRODUCTION

Enzymes can be immobilized by four major approaches: adsorbed, covalently bound, matrix-entrapped and microencapsulated [1]. Much detailed information has been written on this, examples include reviews [2–6] and books [7–9].

Microencapsulation is the immobilization of enzymes within semipermeable microcapsules which are spherical, ultrathin polymer membrane of cellular dimen-

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sions each enveloping a microdroplet of enzyme solution or suspension [6, 8, 10, 11]. Microencapsulated enzymes are retained within the spherical, ultrathin "microscopic dialysis bags", while permeant external substrates can diffuse rapidly into the microcapsules to be acted on by the enclosed enzymes. A large number of enzymes have been microencapsulated [8]. For example, microencapsulated catalase does not leak out, but acts efficiently on peroxide in vitro and in vivo [12–14]. The present study is a detailed analysis of the enzyme kinetics and immunological properties of microencapsulated catalase and catalase in solution, especially as a model system for enzyme replacement.

MATERIALS AND METHODS

Enzyme

Crystalline lyophilized beef catalase with an activity of 3000 units/mg was purchased from the Nutritional Biochemical Company.

Substrate

$NaBO_3 \cdot 4H_2O$ (Baker) was used as a substrate for catalase. It was made up to 1.5 mg/100 ml in distilled water and adjusted to pH 6.8 with concentrated HCl.

Animals

Pairs of acatalasemic C_s^b mice and normal C_s^a mice were obtained from Dr R. Feinstein at the Argonne National Laboratory, Chicago, Ill., and bred in this laboratory.

Preparation of semipermeable microcapsules

Semipermeable microcapsules were prepared by the updated method [8] of the original method for the preparation of collodion membrane microcapsules [10, 11]. 300 mg of catalase were dissolved in 6 ml of Tris-buffered hemoglobin solution containing 180 mg/100 ml 2-amino-2-(hydroxymethyl)-1,3-propanedial and 10 g/100 ml hemoglobin (Worthington). The solution was filtered with Whatman No. 42 to remove any undissolved particles. This final solution was microencapsulated following the standard procedure of interfacial coacervation to make microcapsules with a mean diameter of 80 μm [8]. The prepared microcapsules were washed in cold 0.9% saline until a completely clear supernatant was attained with no trace of catalase activity.

Permeability of microcapsules

The permeability characteristics of the microcapsules are determined using a rapid mixing stop-flow technique as described [15].

Catalase activity

The method of Feinstein [16] using $NaBO_3$ as substrate was used for determining catalase activity (expressed as perborate units per ml of blood or per g body weight). 8 ml of 1.5% $NaBO_3 \cdot 4H_2O$ previously adjusted to pH 6.8 with concentrated HCl are added to a flask containing 1.5 ml of 0.067 M phosphate buffer, pH 6.8. After 20 min incubation at 37 °C, 0.5 ml of each enzyme source blank or control is then added and mixed by shaking. After exactly 5 min, 10 ml of 1 M H_2SO_4 are added

and the flasks titrated with 0.05 M $KMnO_4$. The results are expressed as perborate units per ml or g enzyme source.

Blood catalase determination

For the determination of blood catalase levels, blood drawn from the orbital sinus was diluted in cold saline and assayed as above.

Total body catalase

Total body catalase was measured by blending the carcass of each mouse in four parts of cold saline in a Waring blender and then filtering it through gauze. The filtrate was diluted with saline and assayed as above for catalase activity.

Total body $NaBO_3$

Total body $NaBO_3$ was measured by blending the carcass of each mouse in four parts of 0.05 M H_2SO_4. After filtering through gauze, the filtrate was precipitated with 10% trichloroacetic acid and the supernatant assayed for $NaBO_3$.

Instead of using K Mn O_4 titration for the determination of the total body $NaBO_3$ as described in the earlier study [12], a more sensitive colorimetric method [18] was modified slightly and used in the present experiments. The procedure was as follows: each 4 ml of standard $NaBO_3$ solution was mixed with 3 ml of saturated $Ti(SO_4)_2$ and read on a Bausch and Lomb colorimeter at 420 nm in order to get a standard curve. 4 ml of the supernatant for the assay of total body $NaBO_3$ was added to 3 ml of saturated $Ti(SO_4)_2$ and read on the colorimeter at 420 nm to determine the $NaBO_3$ concentration.

Immunization

Acatalasemic mice C_s^b, normal mice C_s^a and rabbits were immunized according to the approach of Feinstein et al. [17] over a period of 90 days with the modifications that in the present study crystalline lyophilized beef catalase was used. Injections, each consisting of Freunds's adjuvant and catalase solution (0.1 ml containing 4.8 perborate units of catalase), were given. Another group of acatalasemic mice received repeated injections of microencapsulated catalase with the same dosage, schedule and duration as above.

Antibody detection

The Ouchterlony agar gel double-diffusion technique [19] for antigen–antibody precipitation reactions was used to detect the presence and concentration of antibodies.

Permeability of microcapsules to antibodies

Microcapsules, both with and without catalase inside were incubated with different antibody titers at 37 °C for 24 h. The microcapsules were washed and their content examined for the presence of antibodies, using ^{125}I-labelled antibodies [20] or antigen–antibody complexes by microcomplement fixations [21].

Catalase and $NaBO_3$ doses

In the in vivo studies of non-immunized mice, each animal received one of the following intraperitoneal injections: (1) saline as control; (2) catalase in free solution

(12 perborate units/g body weight); or (3) catalase-loaded microcapsules (assayed activity of 12 perborate units/g body weight). This is followed by a subcutaneous injection of 0.014 mM $NaBO_3$ per g body weight. At predetermined intervals the animals were sacrificed and assayed for total body $NaBO_3$ as described above. For the in vivo immunological studies, in the first series of studies, the same intraperitoneal injections as described above were followed. In the second series of studies, each of the intraperitoneal injections contained only one-third of the amount of catalase used for the first series.

RESULTS AND DISCUSSION

Permeability characteristics

In the first set of in vitro experiments, collodion membrane semipermeable microcapsules were prepared and the permeability of $NaBO_3$ measured using a rapid mixing stop-flow apparatus. Table I shows the permeability constants and the $T_{\frac{1}{2}}$ for equilibration of $NaBO_3$ and H_2O_2 across the microcapsule membrane.

TABLE I

COLLODION MICROCAPSULES: PERMEABILITY DATA

Solute	Half-time for equilibration ($T_{\frac{1}{2}}$, s)	Permeability coefficient* (P, cm/s)	Solute permeability coefficient** (w, moles/dyne per s)
$NaBO_3 \cdot 4H_2O$	6.0	$1.89 \cdot 10^{-4}$	$7.38 \cdot 10^{-15}$
H_2O_2	3.8	$2.24 \cdot 10^{-4}$	$9.14 \cdot 10^{-15}$

* From $ds/dt = PA(C_o - C_i)$ where ds/dt = rate of movement of solute; A, membrane area; $(C_o - C_i)$, concentration gradient.

** From $Js = ds/dt$. $1/A = wRT(C_o - C_i)$ when volume flow is kept at zero. R is the gas constant and T the absolute temperature.

In vitro enzyme kinetics

Enzyme kinetics (K_m and V values) were determined for both catalase in free solution and for microencapsulated catalase and Lineweaver–Burk kinetics were observed. The results show that the K_m of 0.55 M is the same in both cases; the V of the intact catalase-loaded microcapsules is 0.50 mM/min while that for the catalase in free solution is 2.5 mM/min (Table II).

TABLE II

ENZYME KINETICS OF CATALASE PREPARATIONS

Catalase	V (mmoles/ml per min)	K_m (M)
In free solution*	2.50	0.55
Microencapsulated	0.50	0.55

* Catalase in free solution obtained from homogenized catalase-loaded microcapsules.

The differences in V between catalase in free solution and microencapsulated catalase may be the result of a number of factors. The permeability barrier of the microcapsule membrane to $NaBO_3$ may limit the amount of substrate available to the enzyme at any given time. Using the equation of Levine and LaCourse [22], and taking into account the membrane permeability barrier in addition to the enzyme activity, one arrives at the result that the internal concentration of $NaBO_3$ at a steady state is significantly lower than the value for the external $NaBO_3$ concentration. More detailed analysis has been carried out by Vieth et al. [23], who show that there is a significant immobile "boundary layer" around the microcapsule and that this boundary layer decreases with increasing stirring and mixing; in addition, there is the factor of membrane barrier to the free diffusion of substrate. A third factor is a radial gradient existing inside the microcapsule with the concentration of substrate highest close to the surface and decreases towards the center. All these factors help to explain the difference in the observed in vitro enzyme kinetics between the microencapsulated enzyme and the enzyme in free solution.

Characteristics of acatalasemic C_s^b mice

Experiments showed that the blood and total body catalase level of the acatalasemic C_s^b mice bred in this laboratory from the original Feinstein strain, were 1–2% and 15%, respectively, that of the normal C_s^a mice, the same values as obtained by Feinstein et al. [24]. The LD_{50} of H_2O_2 for the C_s^b and C_s^a mice bred in this laboratory was also in agreement with Feinstein et al. [24].

Recovery of injected $NaBO_3$

Fig. 1 represents the time course of removal of injected $NaBO_3$ from acatalasemic mice and from normal mice. In addition, it was also observed that while the normal mice showed no outward symptoms after the injection of $NaBO_3$, the acatalasemic mice were in some distress. The eyes of these acatalasemic mice turned from red to dark brown due to methemoglobin formation and they became increasingly immobile and by 20 min most of them were flaccid and in respiratory distress. Acatalasemic mice given prior intraperitoneal injections of either catalase in free solution or microencapsulated catalase did not suffer any distressful symptoms and were able to remove the $NaBO_3$ in a manner similar to the normal mice.

These in vivo studies demonstrate that subcutaneously injected $NaBO_3$ is removed very slowly from the bodies of the acatalasemic mice, whereas this is removed significantly faster in the normal mice. In addition, the acatalasemic mice, unlike the normal mice, developed symptoms of respiratory distress associated with methemoglobin formation. Injection of catalase solution or an equivalent amount of microencapsulated catalase intraperitoneally into the enzyme-deficient mice protected the animals from the adverse effects of $NaBO_3$ injection. Quantitative analysis shows that the time course of removal of $NaBO_3$ from the bodies of these animals is also significantly increased. It would appear therefore that the protecting effect of the catalase solution and the microencapsulated catalase is due to their removal of the injected peroxide.

Survival of catalase in vivo

After intraperitoneal injection of catalase, the in vivo enzyme activity was

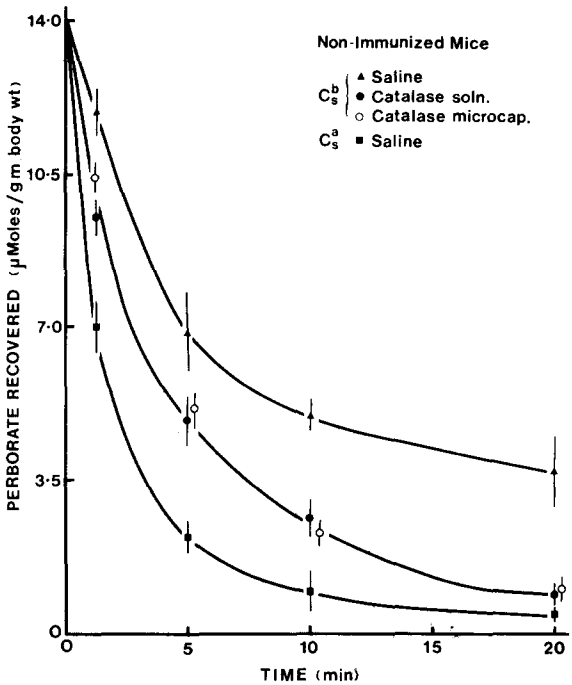

Fig. 1. Time course of fate of subcutaneously injected $NaBO_3$ in acatalasemic (C_s^b) mice and normal (C_s^a) mice. Immediately before the perborate injection, each animal received one of the following intraperitoneal injections: saline, catalase solution or microencapsulated catalase. After varying time intervals, the mice were sacrificed and assayed for total $NaBO_3$ remaining in the body. Each point and bar represents the mean and standard error from five animals.

measured by the ability of the animal to remove injected $NaBO_3$. As shown in Fig. 2, the microencapsulated catalase had an in vivo half-life of 4.4 days whereas the half-life of the catalase in free solution was only 2 days.

There are at least two possible explanations for the significantly longer duration of in vivo action for the microencapsulated catalase. First, the results in Fig. 3 show that after intraperitoneally injection of catalase solution, the enzyme appears quickly in the blood stream; whereas, the enzyme does not enter the blood stream when injected as the microencapsulated form. Earlier studies [8] have also shown that microcapsules containing radioactive labelled proteins are located in the peritoneal cavity even up to one month after injection. It would thus appear, that after intraperitoneal injections the beef catalase in solution entering the blood stream is removed as a foreign protein by the reticuloendothelial system of the mice, whereas the microencapsulated catalase stays in the peritoneal cavity. The second explanation is that catalase microencapsulated together with a high concentration of hemoglobin is more stable than catalase in free solution [13], thus, microencapsulated catalase remains stable for at least three months when kept at 4 °C, whereas the activity of the enzyme in free solution deteriorates rapidly. At a temperature of 37 °C the microencapsulated catalase is also more stable than the enzyme in free solution [13], especially if cross-linked with glutaraldehyde after microencapsulation.

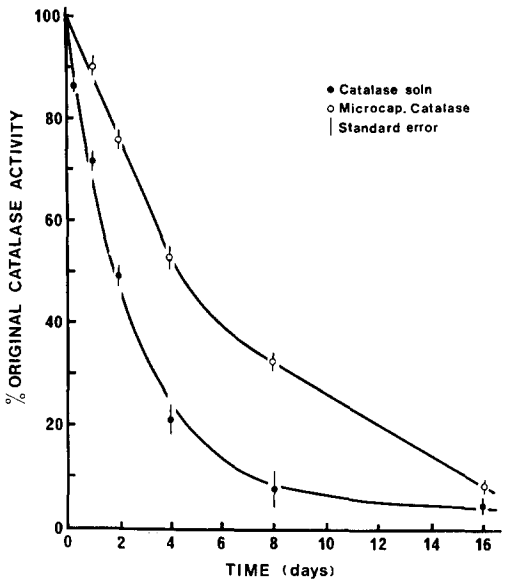

Fig. 2. In vivo activity of catalase in solution and of microencapsulated catalase after intraperitoneal injection. The in vivo activity of the injected catalase preparations at different time intervals after injection is represented as a percentage of the initial activity injected at time zero. Each point and bar represents the mean and standard error from five animals.

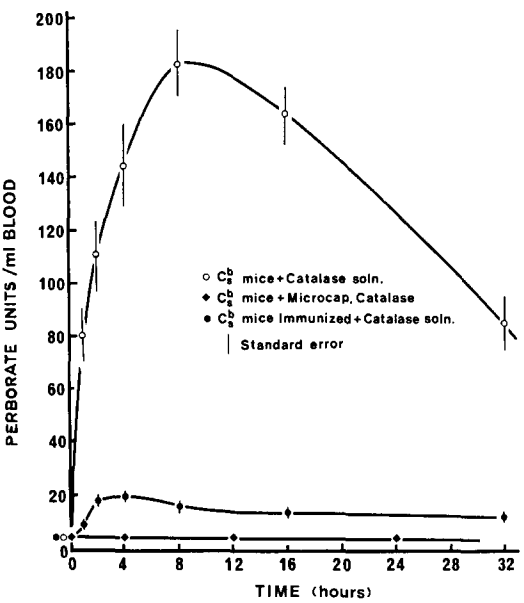

Fig. 3. Time course of appearance of intraperitoneally injected catalase in the blood stream of acatalasemic C_s^b mice and acatalasemic mice immunized to catalase. To avoid severe anaphylactic reactions, only one-third the usual dose of catalase was used in this experiment. Note that intraperitoneally injected catalase solution appeared in the blood stream; whereas intraperitoneally injected microencapsulated catalase did not enter the blood stream. In C_s^b mice immunized to catalase, the intraperitoneally injected catalase solution which enters the blood stream is removed much more rapidly from the circulation.

Production of antibodies

The Ouchterlony double-diffusion technique showed that antibody titers were produced in acatalasemic mice (C_s^b) and rabbits immunized with beef liver catalase in free solution. The same test showed that no detectable antibody titers were observable in animals receiving repeated injections of microaencapsulated catalase (Fig. 4).

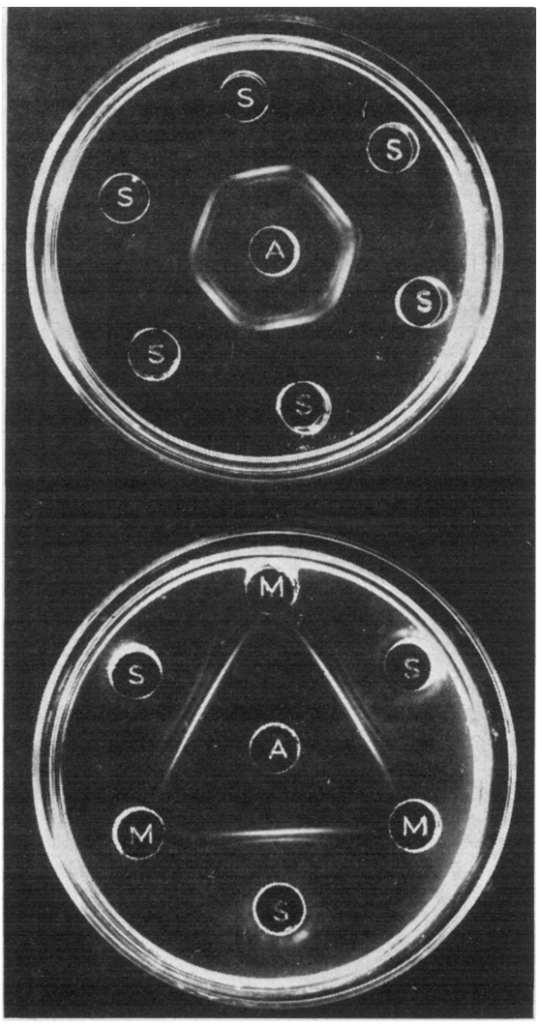

Fig. 4. Ouchterlony double-diffusion technique for the detection of antigen–antibody precipitating complexes. The antigen (A), catalase solution, is placed in the center hole. The serum from acatalasemic mice immunized to catalase solution is represented by S. The serum from acatalasemic mice which have received repeated injections of microencapsulated catalase is represented by M.

Permeability of microcapsules to antibodies

The antibodies were incubated with catalase-loaded microcapsules or with control microcapsules. No antibodies were detected inside the microcapsules by either

the radioactive labelled antibody technique or the microcomplement fixation technique, suggesting that no antibodies had entered the microcapsules. This corresponded to earlier permeability studies which showed that semipermeable microcapsules were not permeable to macromolecules [8, 10–12, 15].

Effects of catalase on immunized acatalasemic mice

When catalase in free solution was given intraperitoneally, the mice went into shock and 80% of them died within the first 10 min (Fig. 5). Death was accompanied by violent gasping, convulsions, and collapse similar to that observed in anaphylactic shock in mice [26]. Injection of microencapsulated catalase did not produce any adverse reaction. Fig. 5 shows that unlike the catalase solution, the microencapsulated

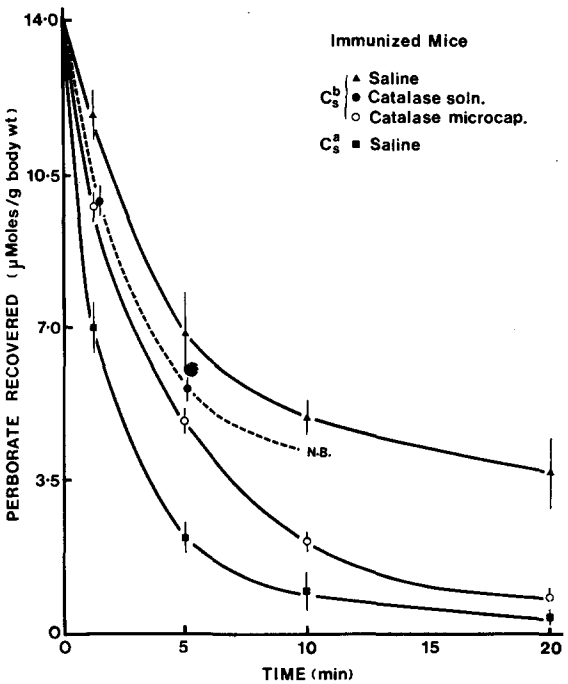

Fig. 5. Time course of removal of injected $NaBO_3$ in immunized normal (C^a) mice and in immunized acatalasemic (C_s^b) mice. N.B., death of immunized mice from anaphylaxis after injection of catalase solution.

form acted to reduce the total body perborate to 9.5 ± 4.5%. In other experiments the dose of catalase in free solution or of catalase-loaded microcapsules was reduced to one-third of the usual amount. With the lower dosage the microencapsulated catalase continued to work effectively. In the case of injection of the free catalase, although the mortality rate dropped from 80 to 20%, the rate of removal of total body perborate was not as efficient as that for the microencapsulated catalase.

Appearance of injected catalase in the bloodstream of immunized and non-immunized acatalasemic mice following the intraperitoneal injection of catalase in

free solution or in the microencapsulated form is shown in Fig. 3. The peak catalase activity in the blood reached 182.8 perborate units/ml 20 h after the injection of catalase solution into non-immunized acatalasemic mice. The blood catalase level reached a peak of only 19.6 perborate units/ml of blood 4 h after the injection of catalase solution into immunized acatalasemic mice. Injection of microencapsulated catalase into non-immunized or immunized acatalasemic mice did not result in any significant changes in the blood catalase levels when followed for up to three months.

GENERAL DISCUSSION

A large number of inborn errors to metabolism is associated with an enzyme defect [27]. Studies carried out to investigate the treatment of hereditary enzyme deficiency diseases include two major groups [28, 29]: (1) "Environmental engineering" which is an approach which aims at restoring the metabolic balance of the body by nutritional or therapeutic management of the disorder without getting down to actual replacement of the defective enzyme. This concept of environmental engineering has been carried out in the form of substrate restriction as in the dietary control of phenylketonuria; product replacement as in the replacement of thyroid or glucocorticoid hormones; or other approaches. (2) "Genetic engineering" which attempts to restore a balance by replacing the enzyme deficiency either by attempting to correct the gene defect, or by enhancement of the enzyme activity. Enhancement of the enzyme has been carried out by a number of approaches: coenzyme supplement, where the deficient activity of an enzyme may require a vitamin-containing coenzyme for supplement; induction of enzyme, as in the induction of liver glucuronyl transferase by phenobarbital; kidney transplantation as in the case of Fabry's disease; leukocyte transfusion and human plasma infusion; direct injection of the deficiency enzyme obtained from heterogeneous sources, for example α-glucosidase and arylsulfatase A is another possible approach. There are, however, a number of problems which are encountered with the injection of heterogeneous enzymes: (a) the enzyme must be able to locate at the site of action; (b) the enzyme has to be available in a highly purified and non-toxic form; (c) the heterogeneous enzyme may result in immunological and hypersensitivity reactions; (d) the enzyme has to act for a sufficient length of time. It has been suggested [8, 10, 11] that some of these problems might be avoided by using semipermeable aqueous microcapsules of cellular dimensions containing the deficient enzyme. Provided they can be put in a place where the deficiency is manifested, then the enclosed enzyme does not leak out to become involved in immunological or hypersensitivity reactions, but can act on permeant substrates which readily diffuse into the microcapsules.

A strain of mice deficient in the enzyme catalase was developed by Feinstein et al. [25] and shown to be a good model for the study of enzyme replacement therapy for enzyme deficiency diseases. Using the Feinstein acatalasemic mice, we have carried out studies which demonstrated the feasibility of using catalase-loaded semipermeable microcapsules for enzyme replacement therapy in acatalasemic mice [12]. This has stimulated more detailed studies on microencapsulated catalase [8, 13, 14]. The problem of immunological and hypersensitivity reaction due to repeated injections of a foreign protein is demonstrated in the present study. The immunized acatalasemic mice when challenged with catalase in free solution responded with acute hypersensitivity

reaction .The sequence of events from the injection of the challenging dose to the death of the animals within 20 min corresponded to that described for anaphylaxis in the mice [26]. By decreasing the challenging dose to one-third, it is possible to inject the catalase solution without having any fatal effect in the animal. This way it is then possible to study the fate and the action of the injected catalase solution. Thus, when this smaller dose of catalase was given to the non-immunized mice and the immunized mice, catalase in free solution diffused slowly from the peritoneal cavity into the blood stream, and reached a high concentration in the blood stream of the unimmunized mice; in the case of the immunized mice, the catalase diffusing into the blood stream is rapidly removed so that the catalase level in the blood stream only reaches a level of about 5% of that in the non-immunized animal, Fig. 4. It appears that in the immunized acatalasemic mice, as soon as the catalase gets into the blood stream from the peritoneal cavity, it combines with the antibody to form a complex which is rapidly removed by the reticuloendothelial system.

Microencapsulated catalase when injected intraperitoneally is efficient in replacing the deficient catalase in the enzyme-deficient animal. It is more stable than the enzyme in free solution and stays at the site of injection without leaking into the circulation. It does not, unlike the enzyme in free solution, cause immunological or hypersensitivity reactions. Unlike the enzyme in free solution, the microencapsulated form when injected into immunized acatalasemic mice still continues to function efficiently in replacing the deficient enzyme to remove injected $NaBO_3$.

It should be emphasized that this is an experimental model demonstrating the feasibility of using microencapsulated enzyme for the supplement of enzyme deficiency conditions. However, in a practical way, the intraperitoneal injection of microencapsulated catalase would not necessarily be the form of administration. Other ways of administration could also be used [8]. Thus it has earlier been demonstrated [30] that microencapsulated enzymes could be placed in an extracorporeal shunt chamber in which the microcapsules are retained by screens which allow blood from the body to recirculate continuously through the shunt, thereby allowing the microencapsulated enzyme to act on blood-borne substrate. In this way the microencapsulated enzyme does not enter the body and can be replaced or supplemented when required. Using the same approach, microencapsulated catalase has been placed in an extracorporeal shunt through which peritoneal dialysis fluid recirculates [12]. In this way it has been demonstrated that it can efficiently remove $NaBO_3$ from acatalasemic mice. Other approaches have also been used; for instance, microencapsulated urease has been administered orally to work in the digestive tract on substrates diffusing into the gastrointestinal tract [8]. Furthermore, microencapsulated catalase has also been applied directly to oral lesions in acatalasemic mice and was found to act efficiently [14].

This experimental model using acatalasemic mice might be extended to certain inborn errors of metabolism where the deficient enzyme is one which manifests itself in a disturbance of substrate concentration in the plasma such as pseudocholinesterase deficiency. On the other hand, there are many conditions where the defective enzyme has to be located at specific intracellular sites, as in the glycogen storage diseases. In these cases, a different experimental animal model system would be required, and the administration of enzymes would have to be in such a way that the enzyme could be located at the desired intracellular sites. This may require the investigation

of microcapsules or other types of immobilized enzymes with different physicochemical properties [8] to select those which would allow the microcapsules to locate at the desired site. The experimental model using microencapsulated enzymes have also been tested in the experimental use of microencapsulated asparaginase for the suppression of asparagine-dependent tumors [8, 31, 32].

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