THE INTRACELLULAR CATALASE OF *MICROCOCCUS LYSODEIKTICUS*

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INTRODUCTION

As has been observed by PENROSE AND QUASTEL[1], GALE AND EPPS[2], and later HERBERT AND PINSENT[3], lysis of *M. lysodeikticus* with lysozyme results in an increase in catalase activity of the suspensions. Lysis of cells in general leads to changes in enzymic activity. An increase in activity is usually attributed to removal of a permeability barrier, thus facilitating access of substrate to the enzyme. This idea has been used for catalase in *M. lysodeikticus*[1]. In addition it has been noted that the pH for maximum activity of several enzymes differs for intact cells and soluble cell-free preparations. This has been interpreted by GALE[4] as evidence for the existence of a difference between the internal pH of a cell and the pH of its external medium, possibly maintained by barriers impermeable to hydrogen and buffer ions.

Besides the effective concentration of substrate and pH at the enzyme site, another factor of major importance could be that lysis may result in a change in physical state of the enzyme. Thus KAPLAN[5] has suggested that the increase in catalase activity of yeast following treatment of cells with a range of chemical and physical agents is due to this cause. The agents are pictured as causing, directly or indirectly, a desorption of the partially unfolded enzyme from an intracellular interface, the desorbed enzyme then being in a more active configuration.

In the present investigation, a study has been made on the catalase activity of *M. lysodeikticus*. This organism has several advantages when compared with yeast. By the action of lysozyme, the bacteria can be conveniently and completely lysed. In addition, if the lysozyme treatment is carried out in a medium of high osmotic pressure, the cell wall is removed whilst the resulting protoplast remains intact. In this way one possible permeability barrier is removed and free access of agents to the plasma membrane is ensured.

EXPERIMENTAL

Preparation of bacteria

M. lysodeikticus of the same strain as studied previously[6] was grown by surface culture on a beef tryptic digest agar medium contained in 20 oz. bottles. After growth for about 20 hours at 37°, the organisms were harvested, filtered through glass wool and washed 3 × by centrifugation in distilled water. Finally they were resuspended at about 20 mg dry wt/ml in distilled water. Bacterial dry weights were determined by spectrophotometric measurement of the optical density at 500 mμ of suitable dilutions.

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Preparation of protoplasts and lysed bacteria (lysates)

In general, protoplasts and lysates were prepared by lysozyme treatment of bacterial suspensions at a concentration of 10 mg bacterial dry wt/ml. Lysozyme treatment was carried out at 20° in 0.03 *M* phosphate buffer (pH 6.5). A ratio of 20 μg of lysozyme to 1 mg dry weight bacteria was employed. Stabilisation of protoplasts against lysis was achieved by the presence of 1.0 *M* sucrose in the reaction medium[7]. With these conditions, destruction of the cell walls was complete after 30 minutes.

Measurement of catalase activity

Appropriate dilutions of bacteria, protoplasts and lysates were made in 1.0 *M* sucrose containing 0.03 *M* phosphate buffer, pH 6.5, for estimation of catalase activity. Bacterial preparations were used at concentrations up to 0.01 mg bacterial dry wt/ml and usually at 0.005 mg/ml. With a final concentration of substrate of 0.089 *M* activities were directly proportional to the bacterial concentration.

Measurement of catalase activity was made by standard Warburg manometric techniques. Activities were taken from the linear slopes of the oxygen evolution–time curves, involving measurement of oxygen evolved over usually 1 to 5 min. Consequently the estimation concerns the overall reaction including the formation of the inactive ES_{II} complex of CHANCE[8] and some irreversible inactivation by peroxide[9]. The problems involved have been previously discussed in connection with yeast catalase[5,10]. Measurements of activation energies for the overall catalase – H_2O_2 reaction were made by the "short method" of FRASER AND KAPLAN[10], *i.e.* by determining the activity–enzyme concentration curves at two temperatures, 7° and 25°. Catalase activity of preparations is unaffected by the presence of lysozyme or 1 *M* sucrose and is identical at a given pH for the different buffer systems used. In these measurements and in the treatment with various agents, appropriate combinations of A.R. grade citric acid, disodium phosphate, monosodium phosphate and borax were used at a concentration of 0.03 *M* to maintain pH stability in the range 4 to 9.

Action of lytic and physical agents

The effect of a number of lytic agents upon the catalase activity of intact organisms, protoplasts and lysates was studied. Treatment with the detergents sodium dodecyl sulphate (SDS) and dodecyl-trimethyl-ammonium bromide (DTAB) and with polymyxin E was carried out for 30 minutes at both 25° and 37° on preparations corresponding to 0.5 mg bacterial dry wt/ml. The action of 1% phenol, 0.76 *M* butanol and aqueous suspensions of chloroform and toluene was studied similarly at 37°. As well as assay of catalase activity, changes in turbidity of bacterial and protoplast suspensions on treatment were measured spectrophotometrically at 500 mμ. Further, to assess damage to the plasma membrane, the leakage of cell solutes was followed spectrophotometrically by the determination of the 260 mμ absorption of cell and protoplast free centrifugates. This leakage is associated with disruption of the permeability barriers of the cell[6]. Centrifugations of these suspensions were carried out at 5° in order to eliminate mechanical damage to the protoplasts[11]. The occurrence of irreversible changes produced by extremes of pH in the permeability barriers of the cell was investigated by suspending intact bacteria at 10 mg bacterial dry wt/ml in buffers over a range of pH 4–9 at 25°. After 30 minutes, the suspensions were returned to pH 6.5 by a 1 in 20 dilution into the pH 6.5 buffer and samples taken at intervals during 5 hours were centrifuged for 260 mμ absorption measurements on the supernatant fluid.

In experiments to dermine the stability to pH of catalase in intact cells, protoplasts and lysates, preparations were made at 0.5 mg bacterial dry wt/ml at the required pH. Samples were then diluted 100 × at intervals with pH 6.5 buffer for catalase determination. Similarly, the effect of temperature on catalase activity was followed on 0.5 mg bacterial dry wt/ml suspensions of bacteria, protoplasts and lysates incubated at 55°, 65° and 70° in buffer at pH 6.5. At intervals, aliquots were withdrawn and diluted for determination of catalase activity. Where the influence of pH on catalase activity was studied, samples were suspended and determined in buffer at the appropriate pH.

Viability

The viability of bacteria after treatment with the different agents was determined by plating out 1 in 100 dilutions of the suspensions on to agar medium. The presence or absence of growth was noted after incubation for 24 hours at 37°.

RESULTS

Catalase activity of intact cells, protoplasts and lysates

The catalase activities of intact bacteria, protoplasts and lysates of *M. lysodeikticus*

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determined at pH 6.5 are compared in Table I. A significant increase in activity (*ca.* 4 ×) is found on complete lysis with lysozyme, whereas removal of the cell wall alone (protoplast formation) results in but a slight increase in catalase activity. This small increase (*ca.* 1.3 ×) can be accounted for by the presence of variable quantities of disrupted protoplasts formed either during their preparation or during the measurement of catalase activity. WEIBULL[11] has noted that protoplasts of *B. megaterium* are sensitive to oxygen and, since oxygen evolution is used to measure catalase activity, it is perhaps surprising that the damage to protoplasts is not more extensive.

Table II records similar data for bacterial suspensions treated with a series of lytic agents. Under the conditions employed, the agents produced no reduction of catalase activity in lysates. After treatment with chloroform, toluene, phenol, butanol and DTAB, the intracellular catalase expressed an activity equal to that of lysates. Slightly lower relative activities were observed with polymyxin E and SDS. Removal of the treated cells by centrifugation showed that the entire catalase was always retained in the cell fraction and no catalase escaped to the supernatant. Further the catalase of lysates was not found to be localized on any particular cell fraction. Lysates at pH 4.7 and 6.5 have been centrifuged at up to 100,000 × *g*, sufficient to sediment all particulate material from most cell homogenates. Full catalase activity remained in the supernatant.

TABLE I

CATALASE ACTIVITY OF INTACT CELLS, PROTOPLASTS AND LYSATES OF *M. lysodeikticus*, DETERMINED AT pH 6.5

Averages and standard deviations of 18 samples

Preparation	*Catalase activity*	
	A (*μlO_2 evolved/min/mg dry wt*)	*B* (*Relative activity referred to intact cells*)
Intact cells	5,900 ± 600	—
Protoplasts	7,800 ± 700	1.3
Lysates	23,500 ± 2,500	4.0

TABLE II

CATALASE ACTIVITY OF *M. lysodeikticus* CELLS TREATED WITH VARIOUS AGENTS AT 37° C FOR 30 MIN AND AT pH 6.5 RELATIVE TO ACTIVITY OF UNTREATED CELLS

Treatment carried out at 0.5 mg bacterial dry wt/ml.

Agent	*Relative activity referred to intact cells*
Chloroform suspension	3.9
Toluene suspension	3.7
0.21 *M* Phenol	4.0
0.76 *M* Butanol	3.4
10^{-3} *M* Sodium dodecyl sulphate	2.8
10^{-3} *M* Dodecyl-trimethylammonium bromide	3.8
10^{-4} *M* Polymyxin E	2.6

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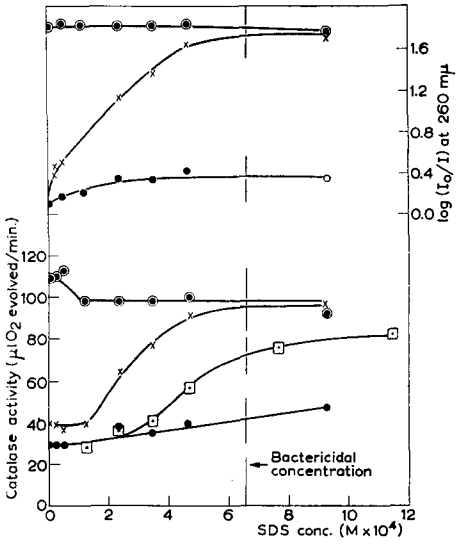

Fig. 1. Effect of SDS on *M. lysodeikticus*. Lower curves show the catalase activity after treatment for 30 min at 25° of bacteria (●), protoplasts (×) and lysates (◉), and after treatment for 30 min at 37° of bacteria (⊡). Upper curves show the O.D. at 260 mμ of the corresponding supernatant solutions centrifuged after treatment for 30 min at 25° C.

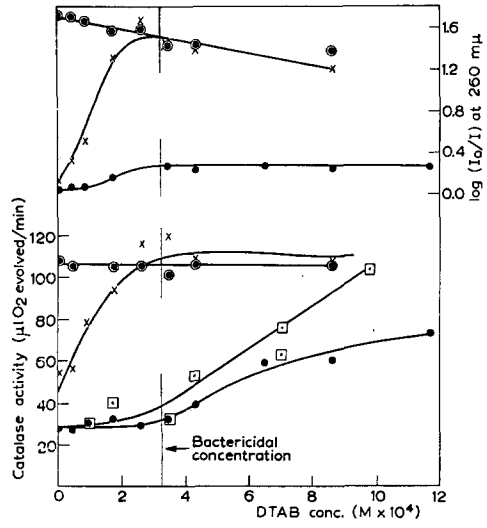

Fig. 2. Effect of DTAB on *M. lysodeikticus*. Lower curves show the catalase activity after treatment for 30 min at 25° of bacteria (●), protoplasts (×) and lysates (◉), and after treatment for 30 min at 37° of bacteria (⊡). Upper curves show the O.D. at 260 mμ of the corresponding supernatant solutions centrifuged after treatment for 30 min at 25° C.

Effects of surface active agents and polymyxin E

Figs. 1 and 2 show the change in catalase activity and release of soluble cell constituents absorbing at 260 mμ for intact bacteria, protoplasts and lysates on treatment with SDS and DTAB.

At low concentrations of the detergents, little change in catalase activity was observed for intact cells treated at 25° for 30 min whilst, at the higher concentrations, the catalase activity increased, but did not reach that of lysates. At 37° both detergents enhanced the catalase activity, which at high detergent concentration approached that of the lysates. Treatment of protoplasts with the detergents showed that with DTAB the catalase activity increased rapidly and reached that of the lysates. For SDS initially no change in activity occurred but at higher concentrations the catalase activity again reached that of the lysates. The minor differences in behaviour are probably associated with the different charges on the detergent ions. With both detergents, protoplasts reached the maximum catalase activity at detergent concentration just sufficient to cause complete sterilization of the bacterial suspensions. Intact bacteria themselves, however, showed no great increase in catalase activity at these concentrations when assayed 30 minutes after treatment at 25°.

Examination of the amounts of soluble cellular constituents released by the detergents revealed that for both bacteria and protoplasts, maximum leakage was reached at the minimum bactericidal concentrations. With bacteria the maximum optical density at 260 mμ did not reach that of lysates owing to the presence of the cell wall which prevents the release of 260 mμ absorbing intracellular macromolecules.

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For protoplasts, however, the optical densities at 260 mμ rapidly reached those of the lysates, suggesting complete disruption of the protoplasts. This was confirmed by the complete loss of turbidity, measured at 500 mμ, of protoplasts treated with these minimal bactericidal concentrations of the two detergents. Precipitation of 260 mμ absorbing material derived from lysates and protoplast suspensions owing to interaction with DTAB caused the optical density at 260 mμ to decrease approximately linearly with further increase of DTAB concentration.

Essentially similar results were obtained using the cyclic polypeptide antibiotic, polymyxin E, as shown in Fig. 3. Thus the effect on catalase activity of treatment with polymyxin E is analogous to that with the detergents. However, whilst the catalase activity of protoplasts reached that of lysates, complete release of 260 mμ absorbing solutes to the lysate level did not occur. Parallel measurements of the turbidity at 500 mμ indicate that lysis of protoplasts was only partial. As with DTAB, there was evidence of a direct reaction between polymyxin E and 260 mμ absorbing cellular constituents so that the optical density at this wavelength decreased for protoplasts and lysates with increasing concentration. It is known that cationic detergents[12] and polymyxin E[13] form insoluble complexes with nucleic acids.

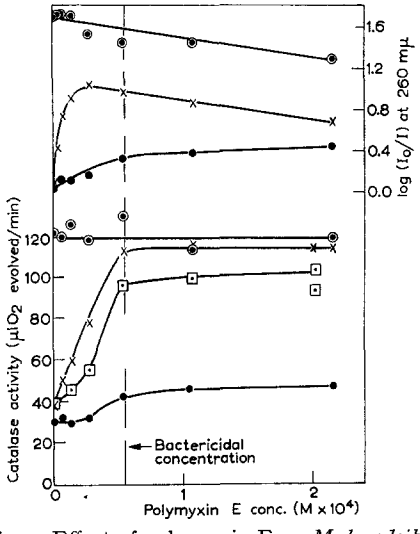

Fig. 3. Effect of polymyxin E on *M. lysodeikticus*. Lower curves show the catalase activity after treatment for 30 min at 25° of bacteria (●), protoplasts (×) and lysates (◉), and after treatment for 30 min at 37° of bacteria (⊡). Upper curves show the O.D. at 260 mμ of the corresponding supernatant solutions centrifuged after treatment for 30 min at 25° C.

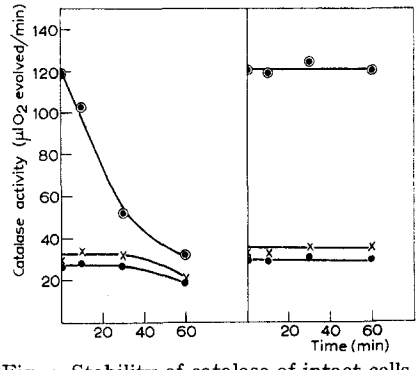

Fig. 4. Stability of catalase of intact cells (●), protoplasts (×) and lysates (◉) with time at pHs 4.25 (left) and 4.65 (right).

Stability of bacterial catalase to pH

Fig. 4 shows the data obtained on the stability of catalase in intact cells, protoplasts and lysates at pH 4.25 and 4.65. Between pH 4.65 and 9 no change was observed for periods up to 1 hour. At pH 4.25 the catalase in lysates was rapidly and irreversibly inactivated. In contrast, no significant fall in activity was shown by the bacterial and protoplast suspensions on treatment at this pH for periods up to 30 minutes. After this, a slow inactivation occurred.

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Variation of catalase activity with pH

As shown in Fig. 5, the catalase activity of intact cells is constant over the pH range 4.4 to 9, while below this range there was a rapid decrease. In contrast, the catalase activity of lysates decreased slowly below pH 9, then rapidly below pH 6 to reach a value equal to that of intact cells at pH 4.7. The pH-activity curve for protoplasts resembles closely that of the intact bacteria. The presence of about 5–10% of lysed protoplasts, formed during both the preparation of the protoplasts and the determination of catalase activity, would account quantitatively for the differences in the two curves. Bacterial cells treated with $10^{-3}M$ DTAB at 37° gave a pH/activity curve identical to that of the lysates even though centrifugation experiments showed no catalase was released from the treated cells.

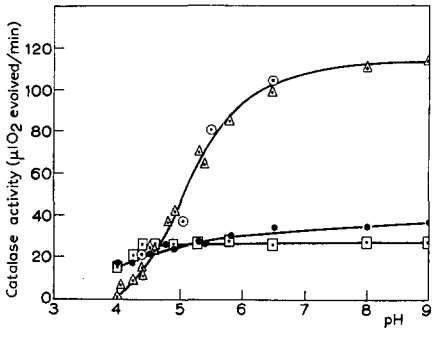

Fig. 5. Effect of pH on catalase activity of suspensions of intact cells (⊡), DTAB-treated cells (◉), protoplasts (●) and lysates (△).

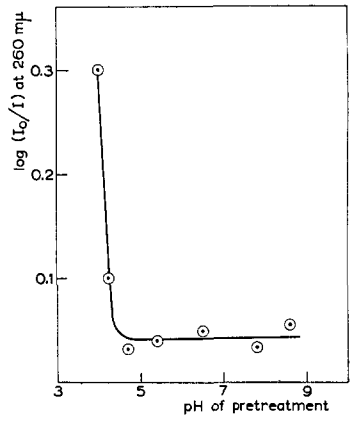

Fig. 6. Change in O.D. at 260 mµ of supernatants from centrifuged preparations of intact cells pretreated at different pH values for 30 min and then returned to pH 6.5.

No correction has been applied for irreversible inactivation of catalase at low pH (*cf.* Fig. 4). The time taken to assay the catalase activity (about 20 minutes) would suggest that this correction becomes significant only for lysates at and below pH 4.25.

The results of experiments to determine the occurrence of changes produced by pH in the permeability properties of bacteria are given in Fig. 6. Here the leakage with time at pH 6.5 of 260 mµ absorbing material has been measured after intact cells had been held for 30 minutes at a different pH. The points shown were obtained by extrapolating the linear optical density at 260 mµ–time curves back to zero time as an indication of the degree of damage to cell permeability barriers. It is evident that between pH 4.4 and 9 there occurs little change, but below pH 4.4 there is a sudden rapid increase in the material leaking from the cell. It is likely therefore that a pH below pH 4.4 produces far-reaching changes in the cell membrane properties.

Activation energies for the overall catalase/H_2O_2 reaction

In Table III, the activation energies for the overall catalase/H_2O_2 reactions are recorded. For intact organisms the activation energy was close to 9.0 ± 1.5 kcal/mole at both pH 4.6 and 6.5, whereas that for lysates changed from 8.1 ± 1.5 kcal/mole

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TABLE III

ACTIVATION ENERGIES FOR THE OVERALL SUBSTRATE–ENZYME REACTION FOR INTACT BACTERIA, PROTOPLASTS AND LYSATES AT pH 4.65 AND 6.5

Substrate concentration 0.089 *M*

Preparation	*Activation energy (kcal/mole, ± 1.5 kcal/mole)*	
	pH 6.5	pH 4.65
Intact bacteria	9.2	8.9
Protoplasts	6.2	8.9
Lysates	3.0	8.1
Bacteria treated with:		
Toluene	2.1	
Chloroform	1.5	
DTAB	1.5	

at pH 4.6 to 3.0 ± 1.5 kcal/mole at pH 6.5. The values obtained for protoplasts, namely 9.0 and 6.2 kcal/mole respectively, were subject to a much greater experimental error since relatively small changes in the degree of lysis during preparation and measurement affect the results very strongly. Organisms treated with DTAB, toluene and chloroform, in common with lysates, had low activation energies (about 2.0 kcal/mole) at pH 6.5.

Effect of temperature on catalase activity

At 55° the catalase activity was found to be unchanged during 80 minutes for lysates and intact bacteria, while the activity of protoplasts increased rapidly during 15 minutes and was thereafter unchanged. Similarly at 65°, lysates possessed unchanged catalase activity during 60 minutes whereas both bacteria and protoplasts showed an increase in activity during incubation at this temperature. In particular the catalase activity of protoplast suspensions closely approached that of lysates within 15 minutes, owing to complete lysis.

Fig. 7 shows the time course of catalase activity of suspensions of bacteria, protoplasts and lysates incubated at 70° C. Lysates exhibited a rapid fall in catalase activity.

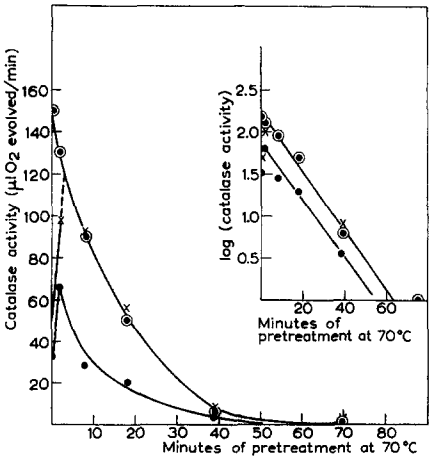

Fig. 7. Effect of heating at 70° on catalase activity of intact cells (●), protoplasts (×) and lysates (◉). Inset shows the logarithmic decrease in activity with time.

The activity of protoplasts rapidly increased initially and then decreased in a manner apparently identical to the lysates. Bacterial suspensions also showed an initial enhanced activity followed by inactivation. As with heat inactivation of most proteins, the decrease in catalase activity followed a logarithmic plot (inset Fig. 7) the rate of inactivation being the same in each case.

DISCUSSION

If it be assumed that the catalase of *M. lysodeikticus* is in the same physical state

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in intact cells and lysates, then there are two factors of major importance in determining its enzymic activity. These factors are the effective concentration of substrate and the pH at the enzymic site, both of which may be affected by permeability barriers in the intact cell. Since, when allowance is made for a small degree of lysis of protoplasts, intact bacteria and protoplasts possess identical catalase activity, it is unlikely that the cell wall itself forms such a barrier. It is only with lysates that the effective substrate concentration and pH are known with certainty.

KAPLAN[5] has claimed to have shown that, with yeast cells, penetration of H_2O_2 into the cells is not a rate-limiting step. There is evidence to suggest that this applies also in the present case. Thus at pH 4.7, where there is no irreversible inactivation, catalase exhibits both identical activity and identical activation energy in bacteria, protoplasts and lysates. Since in lysates permeability barriers have been eliminated, the importance of restricted access of substrate in the intact cells is doubtful.

There is a marked contrast between the sensitivity to pH of the catalase activity of lysates and the behaviour of bacteria and protoplasts, which show a constant catalase activity over the pH range 4.4 to 9. The most probable explanation is that the constant level of catalase activity of bacteria and protoplasts is due to the non-equilibration of the internal environment of the cells to the pH of the external medium. The alternative hypothesis that the internal pH of the cells follows that of the external medium involves an unlikely implication; namely that the effect of pH upon the permeability of cells to substrate must follow a precisely inverse relationship to the pH/activity curve of lysates, such that the observed activity for intact cells remains constant. When the specific permeability properties of the bacterial cells are broken down, *e.g.* by treatment with DTAB, the pH-activity curve is identical with that of lysates although no catalase has left the cells.

Furthermore, it is suggested that the pH at which lysates exhibit the same activity as intact cells (pH 4.7) may be the effective pH at the site of intracellular catalase. It has been shown that catalase is stable at this pH. At pH 4.25 however, the catalase activity of lysates is rapidly destroyed, while that of intact cells and protoplasts decreases only after 30 minutes. The fact that the latter remain unaffected for but a short period indicates that at this pH the barrier to pH equilibration is only temporary. The results on the leakage of cell constituents with pH, shown in Fig. 6, indicate that irreversible damage to the permeability barriers of the organism occurs below pH 4.4. This acid damage accounts also for the decrease in catalase activity below pH 4.4 on the pH-activity curves of bacteria and protoplasts.

Further evidence for the constancy of pH at the site of intracellular catalase despite change in pH of the external medium is given by the equality of the overall activation energies of intact cells determined at pH 4.65 and at pH 6.5. In contrast, the activation energy for the catalase reaction of lysates increases from 3 kcal/mole at pH 6.5 to, at pH 4.65, the value obtained with intact cells. Similarly, bacteria treated with lytic agents, unlike intact cells, have at pH 6.5 low activation energies characteristic of lysates at this same pH. Additional experiments to measure the endogenous respiration of *M. lysodeikticus* at different pH suggest that this property also is independent of external pH over a wide range.

The quantitative study of the effect of concentration of DTAB, SDS and polymyxin E upon the catalase activity of bacteria and protoplasts again demonstrates that the increase in activity produced at pH 6.5 is associated with disruption

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of the permeability membrane. With intact bacteria, time and temperature of treatment are much more important in the magnitude of the effects, presumably owing to protection by the cell wall. However, the effects on the catalase activity are associated with changes produced in the properties of the plasma membrane.

Therefore, it is concluded that the intracellular catalase of *M. lysodeikticus* acts at a pH of approximately 4.7. Provided the permeability barriers of the cell remain intact, this pH is not affected by changes in the pH of the external medium. Changes in catalase activity produced by lysis or the action of agents which damage the plasma membrane result from the breakdown of the permeability barriers and consequent equilibrium of the pH inside the cell with that of the external medium. It is not necessary to invoke a change in state of the catalase, *e.g.* desorption from an interface[5], to account for the increase in activity.

It should be emphasised that the present results cannot be generalized to apply to other organisms where the catalase system may possess different properties. Indeed, a difference in behaviour between the catalase of yeast and of *M. lysodeikticus* is evident from the action of ultraviolet light. Irradiation of yeast suspensions with ultraviolet light has been shown to produce a marked increase in catalase activity which is maximal at doses sufficient to cause sterilization[14]. However, with *M. lysodeikticus* there is no increase in catalase activity at doses up to 20 × that required for sterilization. Prolonged irradiation leads to inactivation of catalase in both systems.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. J. H. SCHULMAN, O.B.E., and Dr. J. G. KAPLAN for their interest. Grateful acknowledgement is made to the Royal Commission for the Exhibition of 1851 for an Overseas Fellowship (M.J.F.) and to the Commonwealth Scientific and Industrial Research Organisation for an Overseas Studentship (A.R.G.).

SUMMARY

The activity of catalase in *M. lysodeikticus* and its protoplasts and lysates has been investigated over the pH range 4–9. The results suggest that the intracellular catalase is protected from change in the pH of the external environment by a barrier impermeable to the H^+ ion. The effect of a series of lytic agents upon the catalase activity indicates that this barrier is the plasma membrane of the organism.

The changes with pH of the activation energy of the overall catalase–H_2O_2 reaction in the three bacterial preparations provide support for this interpretation. It is suggested that the intracellular bacterial catalase is operating at an effective pH of 4.6–4.8.

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Received November 3rd, 1956