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Catalase Activity in Pathogenic *Leptospira*

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SUMMARY: Catalase activity of an order comparable with that of aerobic bacteria was demonstrated in many strains of pathogenic leptospira. There was no correlation with serotype or virulence. Catalase was intracellular; its activity was increased by lysing the leptospire with bile salt or by freezing and thawing, and was inhibited by 0.001 M-KCN or heating. Its optimum pH was 7.0 and optimum temperature 37° whether the leptospire was grown at 30° or 37°. Ageing cultures showed increased catalase activity inversely proportional to the numbers of leptospira organisms, due to autolysis. Qualitative tests for peroxidase activity were negative. Positive qualitative tests for enzymic oxidizing systems were obtained with catalase-active and catalase-inactive leptospire in washed suspensions and when growing on solid media.

Leptospira need haemoglobin or a source of iron for satisfactory growth in Korthof medium (Faine, 1959). Fulton & Spooner (1956) demonstrated the absorption spectrum of cytochrome *c* in *Leptospira icterohaemorrhagiae* strain Jackson, but Czekalowski, McLeod & Rodican (1958) did not find cytochrome or catalase activity in various leptospira cultures. Assuming that haemoglobin or iron might be required for Fe-porphyrin-containing enzymes, qualitative tests for catalase and oxidizing enzyme activity were done on various cultures. Positive results led to the following studies.

METHODS

Sources and culture of Leptospira. Strains of *Leptospira* were obtained from the sources shown in Table 1.

Cultures were grown in 20-200 ml. volumes of modified Korthof medium containing haemoglobin from laked red cells, and pooled rabbit or sheep serum. Catalase activity due to the laked red cells was destroyed by heating the medium at 56° for 60 min. after Seitz filtration. The medium was solidified with 1.0 % (w/v) 'Difco' agar, when required (Kirschner & Graham, 1959). Incubation temperatures are given for each experiment. Cultures grown at different temperatures for comparison of enzyme activity were incubated in water-baths controlled to $\pm 0.5^\circ$.

Counting method. Leptospire were counted directly, as described previously (Faine, 1957).

Dry weight. In two separate experiments 8-day cultures at 30° were centrifuged at c. 9000 *g* for 30 min. in a 'Spinco' model L centrifuge, washed twice with glass-distilled water and heavy suspensions dried. Dry weights of 1.2 and 8.6 mg. were obtained from 1.6×10^9 and 1.17×10^{10} leptospire, respectively,

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corresponding to 75 and 73 $\mu\text{g.}/10^8$ leptospire. This is considerably more than 20–40 $\mu\text{g.}/10^8$ found by Fulton & Spooner (1956).

Catalase activity. (a) *Qualitative tests.* Approx. 2 cm. length of fluid or semi-solid culture was taken up in a capillary tube, followed by a similar length of 10 % H_2O_2 . Bubbles of gas were evolved immediately from active cultures.

(b) *Quantitative tests and units of measurements.* Most bacterial catalase activity has been measured manometrically (Fujita & Kodama, 1981) and expressed as:

$$\text{Kat. f. (Katalysefähigkeit)} = \frac{\text{reaction constant } k \text{ at pH } 6.8, 0^\circ}{\text{dry weight in g./50 ml. solution}},$$

$$\text{or as } Q_{\text{Kat}} = \frac{\mu\text{l. O}_2/30 \text{ min. at } 38^\circ}{\text{mg. dry weight}}.$$

Herbert (1955) states that manometric methods may be grossly erroneous, and recommends for whole cultures the method used here. It involves reaction between 1 ml. culture and a known amount of 0.01 M- H_2O_2 for known times, after which the reaction is stopped by adding 2 N- H_2SO_4 and the remaining H_2O_2 titrated iodimetrically. When activity was low the method was modified to cover reaction times up to 30 min. The rate of H_2O_2 destruction was frequently found to remain constant for at least 10 min. Comparisons of activity relate to reactions of the same duration. All tests were done at 25° at pH 6.8 unless otherwise stated. Because of the discrepancy between values for dry weights, results have been expressed as $\mu\text{l. O}_2/10^8$ leptospire; $\mu\text{l. O}_2$ was preferred to $\mu\text{g. H}_2\text{O}_2$ destruction to aid comparison with catalase activity in other bacteria.

Chemicals. All chemicals were analytical grade or the purest obtainable. H_2O_2 solutions were diluted from 130 vol. H_2O_2 in 0.01 M-phosphate buffer at the pH stated.

RESULTS

Catalase activity in different Leptospira

Catalase activity was measured in 7-day cultures, all in the logarithmic or early stationary growth phase, grown at 30° in sheep-serum medium. The results showed there was a great variation in activity, but no correlation with serotype or strain (Table 1), except that the most recently animal-passaged strains of *Leptospira icterohaemorrhagiae* strain Field and *L. pomona* strain EP were more active than slightly attenuated strains. This was also observed in other experiments.

Effect of lysis. Bile salts lyse leptospire. Addition of 0.1–0.25 % (w/v) sodium deoxycholate to cultures or suspensions increased the catalase activity within 1 min. to a maximum after 5 min., thereafter decreasing slowly (Table 2). Deoxycholate alone did not destroy H_2O_2 , nor did lysis by deoxycholate of an inactive strain (*Leptospira icterohaemorrhagiae* Jackson) containing 5.6×10^7 leptospire/ml. increase its activity. Lysis by freezing and thawing leptospiral cultures or suspensions had similar effects.

Effect of antiserum. Rabbit antiserum (agglutination titre 1/12,000) against *Leptospira pomona* was added in a final 1/50 dilution to a 12-day culture of *L. pomona* strain EP G, containing 8.9×10^7 leptospires/ml. Catalase activity was measured 1, 6, 30 min. and 3 and 24 hr. later. The activity of the untreated cultures uncorrected for numbers of leptospires was

Table 1. *Catalase activity of various serotypes of Leptospira, with their sources*

Tests performed on logarithmic or early stationary-phase cultures grown at 30°.

Serotype	Strain	Source*	Virulence†	No. of leptospires/ ml. ($\times 10^6$)	μ l. O ₂ /10 ⁸ leptospires/ 10 min.
<i>L. icterohaemorrhagiae</i>	Jackson	1	NG	166	10
	Wijnberg	2	NG	81	100
	Field	1	NG	50	50
	Field F		NG	41	117
	Field K		AG	37	202
	Field L		AG	70	108
	Field M		AG	56	119
	Field Q		VG	67	125
	Field R		VGX	105	124
	Field S		VGX	26	264
	21	2	VG	107	110
	21 A		VGX	104	110
<i>L. canicola</i>	Aldgate	1	—	7	80
<i>L. autumnalis</i>		1	—	56	60
<i>L. australis A</i>		1	—	116	72
<i>L. pomona</i>	NZ	1	—	114	7
	4	1	—	136	78
	EP	3	AC	150	105
	EP B	3	AC	131	104
	EP D	3	VC	141	96
	EP F	3	VC	112	140
	EP G	3	VCY	88	230
<i>L. hebdomadis</i>		1	—	86	85
<i>L. sejroe</i>		1	—	30	400
<i>L. bataviae</i>	C 62	1	—	116	17

* 1 = Dr J. C. Broom, Leptospira Reference Laboratory, Wellcome Laboratories for Tropical Medicine, London; during 1953–5. 2 = Dr L. Kirschner, Leptospira Reference Laboratory, Otago University Medical School, Dunedin, in 1957. 3 = Mr J. M. Rudge, Wallaceville Animal Research Station, N.Z., in 1958. Where no source is given the sub-strain has been maintained by the author, serially passaged in guinea-pigs and re-isolated as indicated by the serial alphabetical reference letter; S is the most recently isolated.

† A, attenuated; C, for calf; G, for guinea-pig; N, non-virulent; X, passaged in guinea-pigs within previous 3 months; Y, passaged in calf c. 2 months previously; —, information not available.

160 μ l. O₂/ml./min. There was no great change in activity which increased c. 10% in the first minute, fell to 72% in 30 min. and to 56% in 24 hr., expressed as % activity of untreated culture. It was impossible to count the leptospires because of agglutination and lysis-ball formation.

Effect of formalin. Formalin (c. 0.2%, v/v) interfered with H₂O₂ titrations,

but satisfactory controls were obtained when formalin-killed suspensions were washed free from formalin and the dead leptospires tested. A live twice-washed suspension of *Leptospira pomona* strain EP G in 0.01M-phosphate buffer (pH 6.8) catalysed 205 μ l. $O_2/10^8$ leptospires/10 min. A final 1% (v/v) formalin was added to the suspension, which was centrifuged for 10 min., washed and resuspended. The dead leptospires gave 200 μ l. $O_2/10^8$ leptospires/10 min. Thus formalin does not affect intracellular catalase.

Table 2. *Increase in catalase activity of leptospires after lysis*

Whole cultures were lysed with final 0.1% (w/v) deoxycholate or by repeated freezing to -70° and thawing rapidly 8 times, or by deoxycholate added to frozen and thawed culture. Numbers are % activity of unlysed culture.

			Min. between lysis and test									
			1	5	10	15	30	240	15	15		
			Lysed by									
Culture	Age (days)	No. (× 10 ⁶) leptospire/ml.	Deoxycholate						Freezing and thawing	Freezing and thawing + deoxycholate after 15 min.		
<i>Leptospira pomona</i> 4	7	98	126	182	161	—	163	126	—	—		
<i>Leptospira pomona</i> 4	4	80	240	220	—	190	—	—	—	—		
<i>Leptospira pomona</i> EP G	12	89	—	—	—	210	—	—	165	197		

Comparison of qualitative and quantitative tests. There was a broad correlation between strongly positive tests in capillary tubes and high activity in quantitative tests, and vice versa. Numbers of leptospires are not considered in qualitative tests. In several experiments there was poor correlation even when the length of the column of bubbles in the capillaries was measured after set times and corrected for numbers of leptospires.

Optimum pH. Figure 1 shows catalase activities when a culture of *Leptospira pomona* strain EP G was tested in 0.01M-phosphate buffer at the pH values shown. Addition of the sample of culture (pH 7.2) did not alter the pH value during the test. The optimum pH was c. 7.0.

Optimum temperature relative to temperatures of incubation. *Leptospira pomona* strain EP was grown in bottles of the same medium simultaneously at 30° and 37° . Samples were removed after incubation for 2, 5, 7, 12, 16 and 20 days, the leptospires counted and catalase activity tested at 25° , 30° , 37° and in some tests 40° . Cultures grown at 30° were also lysed with deoxycholate and similarly tested at 25° , 15 min. later. A typical finding in a 12-day culture is given in Fig. 2 showing that the optimum temperature was 37° , whether grown at 30° or 37° .

Catalase activity and age of culture. The relationship between catalase activity and the age of the culture is shown in Fig. 3, using information ob-

tained from the previous experiment. Changes in catalase activity paralleled and followed in time changes in the numbers of leptospires in the culture. This effect probably is due to lysis of leptospires, seen especially at 37° (Fig. 8A). Also the 'lysis ratio' (the ratio of activities of lysed: unlysed cultures/ 10^8 leptospires) was relatively constant during the 'stationary' growth

Fig. 1. Optimum pH of catalase. *Leptospira pomona* strain EP G was tested in 0.01 M-phosphate buffer at the pH shown. Activity is expressed as a percentage of the maximum, at pH 7.0.

Fig. 2. Optimum temperature of catalase. Activity of 12-day *Leptospira pomona* strain EP G cultures grown at 30° and at 37° and tested at 25°, 30°, 37° and 40°, expressed as a percentage of the maximum, at 37°. ×—×, grown at 37°; ○—○, grown at 30°.

Fig. 3. Catalase activity and age of culture. Catalase activity at 25° over 10 min. in comparable cultures of *Leptospira pomona* strain EP G grown in the same batch of sheep-serum medium at 37° (A) and 30° (B). Aliquots of each culture were tested after 2, 5, 7, 12, 16 and 20 days incubation. Additional samples of cultures at 30° were tested 15 min. after lysis with 0.1% (w/v) deoxycholate. The 'lysis ratio' is the ratio of activity after lysis: activity before lysis/ 10^8 leptospires. ○—○, log₂ no. $\times 10^8$ of leptospires; +—+—+, log₂ μ l. O₂/ml. culture; ●—●—●, log₂ μ l. O₂/ml. culture after lysis; ×—×, μ l. O₂/10⁸ leptospires; ●—●, μ l. O₂/10⁸ leptospires after lysis; ▲—▲, lysis ratio.

phase (Fig. 3B) indicating that there was a greater increase in activity on lysis with greater numbers of leptospire, and vice versa. The fluctuation in numbers during the 'stationary' phase was frequently observed in cultures grown at 30°.

Intracellular situation of catalase

The above experiments suggest that catalase is intracellular. Direct evidence for this was sought in two groups of experiments.

Action of deoxycholate following formalin treatment. If catalase were intracellular an increase in catalase activity following treatment with deoxycholate would not be expected in formalin-fixed organisms, which are not lysed by deoxycholate. Leptospire killed with 0.2 % (v/v) formalin for 30 min. were washed free of formalin. A final 0.1 % (w/v) concentration of deoxycholate was added to half the suspension, and both halves tested for catalase activity 15 min. later. There was no increase on addition of deoxycholate to the formalinized suspension.

Activity in centrifuged preparations. In four separate experiments attempts were made to compare the catalase activities of whole cultures, washed suspensions and culture supernatant fluids. Leptospire were centrifuged from their culture media, washed and resuspended in Korthof medium or in 0.01 M-phosphate buffer (pH 7.2). Supernatant fluids contained $3-20 \times 10^8$ leptospire/ml. The sum of the catalase activities in the suspension and the supernatant fluid always exceeded the activity of the original culture, probably owing to lysis following damage to the leptospire during centrifuging and washing.

Inhibition of catalase activity

Heat inactivation. Samples of an 11-day culture of *Leptospira pomona* strain EP G grown at 37° and containing 9×10^7 leptospire/ml. were heated in water baths at 40°, 42.5°, 48°, 52°, 54.5°, 56° and 60°. Samples (1.0 ml.) were removed after 2.5, 5, 10, 15 or 20 and 30 min. and catalase activity at 25° for 10 min. measured. The results expressed as % activity of the unheated culture (100 μ l. $O_2/10^8$ leptospire/10 min.) are shown in Fig. 4.

Inhibition by KCN. Catalase activity was measured over 10 and 20 min. in three samples of 5-day culture of *Leptospira icterohaemorrhagiae* strain Field containing 7.8×10^7 leptospire/ml. A final concentration of 0.001 M-KCN was added to the first sample at the beginning of the experiment, and to the second after 10 min.; none was added to the third sample. KCN alone did not interfere with the H_2O_2 titrations. There was 95 % inhibition of catalase activity by KCN (Fig. 5).

Tests for peroxidase activity

Qualitative benzidine, guaiacol and pyrogallol tests for peroxidase were negative with catalase-active cultures of leptospire but were positive with blood and with milk. Leptospiral cultures turned 0.01 M-dihydroxyphenyl-alanine (dopa) black. Tests were made to show whether the blackening of dopa was due to dopa-oxidase, peroxidase or tyrosinase. Final concentrations of

0.002 M-dopa or 0.002 M-tyrosine were incubated at 37° for 6 hr. with washed buffered (pH 7.2) suspensions of catalase-active suspensions of *Leptospira pomona* strain EP G, with and without 0.02 M-H₂O₂ and 0.001 M-KCN. Control tubes were prepared without suspensions. KCN blackened dopa in controls and tests. The suspensions turned dopa but not tyrosine black, with or without H₂O₂. In another experiment washed buffered suspensions of catalase-inactive *L. icterohaemorrhagiae* strain Jackson and catalase-active *L. pomona* strain 4 and *L. pomona* strain EP G were incubated at 37° with a final concentration of 0.005 M-dopa, with and without 0.2 M-H₂O₂ and 0.001 M-NaN₃. Black precipitates were formed by all suspensions with or without H₂O₂, in tubes with dopa without NaN₃. NaN₃ produced a pale brown colour in control tubes with dopa, with or without organisms. A freshly prepared 1 % solution of tetramethyl-*p*-phenylenediamine-HCl was poured over cultures of several serotypes grown on plates of solid medium ('oxidase' test). The leptospiral growth became dark purple within 5–10 min., especially at the periphery, with all serotypes. The medium remained faintly purple. These tests indicate that both catalase active and catalase-inactive strains of *Leptospira* contain enzymic oxidizing systems, not peroxidase, dopa-oxidase or tyrosinase.

Fig. 4

Fig. 5

Fig. 4. Relationships of time and temperature required for inactivation of catalase. Samples of an 11-day whole culture of *Leptospira pomona* strain EP G, growth at 37° were heated at 40°, 42.5°, 48°, 52°, 54.5°, 56° and 60°, within $\pm 0.5^\circ$, for 2.5, 5, 10, 15 or 20 min. Catalase activity over 10 min. at 25° is expressed as % activity of the unheated culture.

Fig. 5. Inhibition of catalase by KCN. Catalase activity of a 5-day culture of *Leptospira icterohaemorrhagiae* strain Field containing 7.8×10^7 leptospires/ml. after reaction for 10 and 20 min. ○—○, without KCN; ●—●, 0.001 M-KCN added before reaction; x—x, 0.001 M-KCN added at 10 min.

DISCUSSION

Quantitative studies of enzymes in *Leptospira* involve special technical difficulties. The relatively slight and slow growth and the low activity of whole cultures necessitates the use of large quantities of medium, usually containing

10 % (v/v) rabbit serum, which are beyond the resources of most laboratories. The fragility of leptospire adds to difficulties in centrifugation when damage or lysis, accompanied by changes in enzyme activity, are to be avoided. Also, because of the same difficulties, a quantitative basis other than dry weight or N content is desirable. Expression of enzyme activity in terms of number of organisms is feasible and has direct biological significance. Direct counting methods cover the widest range, are suitable for use under 'unsterile' conditions, yield immediate and reasonably accurate results in the absence of clumping, and differentiate between motile and non-motile organisms. Plate counts are slow and of limited use with live cultures or suspensions, as not all strains of *Leptospira* in this laboratory will grow on solid medium in plates. Opacity methods are suitable only for cultures free from clumps or deposit, containing more than $c. 10^6$ – 10^7 leptospire/ml.

Leptospire treated with deoxycholate (Czekalowski & Eaves, 1955) or taurocholate (Babudieri, 1948) first lose their axostyles. The cytoplasm becomes transparent, the spiral structure is lost and the organism dissolves. The rapid increase in activity following lysis is consistent with an initial increase in permeability to H_2O_2 followed by liberation of enzyme from the disrupted organism. It follows that ageing cultures contain soluble catalase derived from autolysing cells. On the other hand, the finding that antiserum which caused agglutination-lysis did not alter catalase activity greatly is compatible with the survival of leptospire in the presence of 'lytic' antiserum. The observation is also evidence that 'lysis' of leptospire by antiserum is different from true lysis by chemical or physical agents, and from immune lysis in other bacteria (Lawrence, 1955).

Catalase activity among strains of *Leptospira* tested was variable and apparently unrelated to serotype or virulence. All the known virulent cultures were active, the most active being the most recently passaged strains in two cases. On the other hand, of well-known non-virulent strains *Leptospira icterohaemorrhagiae* strain Wijnberg was active, while *L. icterohaemorrhagiae* strain Jackson was not. 'Jackson' was one of the inactive strains tested by Czekalowski, McLeod & Rodican (1958); their strain was, however, 'antigenically degraded' and serologically unrelated to typical *L. icterohaemorrhagiae*, while the strain in these experiments is serologically typical (Faine, 1957).

If catalase activity of the order found for highly active strains were expressed as $Q_{K_{M}}$ it would be $c. 5$ – $10,000$, similar to that of the most active aerobic bacteria (Fujita & Kodama, 1981) and consistent with aerobic metabolism. The general properties of leptospiral catalase are essentially similar to other bacterial catalase.

Fulton & Spooner (1956) observed the absorption spectrum of cytochrome *c* in *Leptospira icterohaemorrhagiae* strain Jackson. The results of the dopa, tyrosinase and 'oxidase' qualitative tests are consistent with the presence of a cytochrome system in all *Leptospira* which are aerobic organisms, although Czekalowski *et al.* (1958) were equivocal about results of 'oxidase' tests. Catalase, however, appears to be irregularly distributed throughout the genus, without relation to serotype or virulence. Growth of leptospire on

solid media in Petri dishes permits the use of qualitative biochemical tests suitable for routine laboratory use. Although the results of the tests used here did not correlate with serotype or virulence, other biochemical tests applicable to plate cultures may aid in diagnosis or taxonomy.

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