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Studies on Sulphatases

8. THE ARYLSULPHATASE OF A STRAIN OF *ALCALIGENES METALCALIGENES* ISOLATED FROM INTER-TIDAL MUD

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Karunairatnam & Levvy (1951) showed that the β-glucuronidase activity found in sheep rumen was due to micro-organisms, and Marsh, Alexander & Levvy (1952) suggested that the high β-glucuronidase activity found in the omasum, rumen, caecum and colon of many animals might have a similar origin. It was possible that the high arylsulphatase activity of the visceral humps of marine molluscs (Dodgson, Lewis & Spencer, 1953) was due to the presence of micro-organisms, although arylsulphatase activity in bacteria is not common. Barber, Brooksbank & Kuper (1951) found activity in only two of a large number of strains of *Staphylococcus pyogenes*. Whitehead, Morrison & Young (1952) detected the enzyme in certain salmonellae and mycobacteria after studying 212 strains from a wide range of bacterial species, while Hare, Wildy, Billet & Twort (1952) found activity in only two out of ninety-nine strains of anaerobic cocci. Harada (1948) noted arylsulphatase activity in strains of *Streptococcus alcalophilus* isolated from urine and soil.

We have been unable to detect any significant arysulphatase activity in bacteria isolated from the visceral humps of a number of different marine molluscs but have noted appreciable enzyme activity in two micro-organisms isolated from certain inter-tidal muds. The least active of these organisms, a yeast identified as *Trichosporon cutaneum*, has not been further investigated but the other, a bacterium of the genus *Alcaligenes*, is interesting, since it is an example of an arylsulphatase with an optimum pH well on the alkaline side of neutrality. A preliminary account of this work has already been given (Dodgson, Melville, Spencer & Williams, 1954).

MATERIALS AND METHODS

Arylsulphatase substrates. The following compounds were prepared: potassium *p*-acetylphenyl sulphate (Dodgson & Spencer, 1953), tripotassium phenolphthalein disulphate (Whitehead *et al.* 1952); potassium *p*-nitrophenyl sulphate (Burkhardt & Lapworth, 1926) and dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, Roy, 1953*a*).

Detection of arylsulphatase activity. Barber *et al.* (1951) studied arylsulphatase activity in bacteria by detection of phenolphthalein liberated from phenolphthalein disulphate which was incorporated in the medium. This principle was used in the present work but the organisms were grown on solid media, either nutrient agar (1% (w/v) 'Difco' peptone (Difco Laboratories Inc., Detroit, U.S.A.), 0·3% (w/v) 'Difco' beef extract, 0·5% (w/v) NaCl and 1·5% (w/v) 'Difco' agar) or sea-water agar (Zobell, 1946). Arylsulphatase-producing colonies could be subcultured without difficulty after detection by exposure to NH_3 providing subculturing took place at once, but re-incubation of plates subsequent to treatment with NH_3 led to the destruction of the organisms (cf. Bray & King, 1943). The method was tested using the known arylsulphatase producers, *Mycobacterium piscium* and *Myco. ranae* (Whitehead *et al.* 1952).

Determination of arylsulphatase activity of growing cultures. The method of Whitehead *et al.* (1952) was modified. Centrifuge tubes graduated at 7 ml. were dried at 105°, weighed and 0·7 ml. of 0·01 M tripotassium phenolphthalein disulphate was added to each tube. Control tubes containing 0·7 ml. water were prepared. The tubes were plugged with cotton wool and autoclaved for 20 min. at 20 lb./sq.in., no destruction of substrate occurring under these conditions. Sterile nutrient broth (1% (w/v) 'Difco' peptone, 0·3% (w/v) 'Difco' beef extract and 0·5% (w/v) NaCl) was added to each tube to give a total volume of 7 ml. and after incubation overnight at 37·5° any tubes showing signs of growth were rejected. The remaining tubes were inoculated with 2 drops of a culture of the organism and incubated at 25 and 37·5°. Tubes were tested at intervals of 2, 4, 8 and 16 days as follows: the volume of liquid in each tube was adjusted to 7 ml. with water to compensate for loss due to evaporation, and the bacteria were separated by centrifuging. A portion (5 ml.) of each clear supernatant was mixed with 5 ml. of glycine buffer, pH 10·8 (Talalay, Fishman & Huggins, 1946) and the red phenolphthalein colour measured spectrophotometrically (Hilger Uvispek) at 560 mμ. using the control tubes as blanks. A calibration curve was prepared using varying concentrations of phenolphthalein in sterile medium. The bacterial cells were washed with water, centrifuged and finally dried overnight at 105° for dry-weight determination. It was possible that the organisms attacked the liberated phenolphthalein; to check this, tubes containing 0·7 ml. of a solution of phenolphthalein (100 μg./ml. in 10% (v/v) ethanol) were treated as above and the amount of phenolphthalein remaining at the end of 16 days was measured.

Determination of arylsulphatase activity of bacterial preparations. The problems arising from the use of phenolphthalein diphosphate in studying the kinetics of phosphatases have been outlined by Huggins & Talalay (1945). Similar considerations apply to the use of phenolphthalein disulphate as a substrate for arylsulphatase and for this reason other substrates were used for studying the properties of the bacterial enzyme. When potassium *p*-acetylphenyl sulphate was used as substrate, enzyme activity was measured by the method of Dodgson, Spencer & Thomas (1953) while in experiments with potassium *p*-nitrophenyl sulphate the liberated *p*-nitrophenol was determined in substantially the same way, the final spectrophotometric measurement being made at 400 mμ. (Dodgson & Spencer, 1953). When the substrate was nitrocatechol sulphate the method of Roy (1953*a*) occasionally failed to give satisfactory duplicate determinations and was modified by omitting the precipitation of protein with phosphotungstic acid. The incubation mixture contained 0·2 ml. buffered substrate and 0·2 ml. buffered enzyme. After incubation, 2 ml. of the alkaline quinol mixture was added and, after centrifuging, the intensity of the red colour produced was measured at 515 mμ., the wavelength of maximum absorption of nitrocatechol under these conditions ($E_{max.}$ 11300). In all cases sodium salts were used in preparing the buffers.

Nitrogen estimation was by the micro-Kjeldahl method (Markham, 1942).

RESULTS

Examination of mollusc suspensions for arylsulphatase-producing bacteria. Molluscs were collected locally. The visceral humps were dissected out, washed in sterile sea-water and suspended in the same medium using a sterile glass homogenizer (Potter & Elvehjem, 1936) under aseptic conditions to give a 10% (wet wt./v) suspension. A loose-fitting pestle was used in the homogenizer and the time was limited to 1 min. in order to break up the tissues without appreciably destroying the bacteria present. Plates prepared as described earlier were streaked with these undiluted suspensions and then incubated at 25° under both aerobic and anaerobic conditions. Good growth occurred with both ordinary and sea-water (Zobell, 1946) nutrient media under aerobic conditions but anaerobic growth was poor. In general, no arylsulphatase-producing organisms were isolated from molluscs collected in various localities on the Bristol Channel. Occasionally weak arylsulphatase activity was detected but this invariably disappeared on subculturing and was probably due to the molluscan arylsulphatase present in the initial streaks. Enrichment of the culture media by addition of mollusc suspensions in which arylsulphatase activity had been destroyed either by boiling or by incubation of sterilized filtrates for 3 weeks at 37·5° (see Dodgson, Lewis & Spencer, 1953) did not result in the isolation of arylsulphatase-producing organisms.

Arylsulphatase producers in inter-tidal mud. Other possible marine sources of arylsulphatase-producing bacteria were examined under aerobic and anaerobic conditions using nutrient agar and sea-water agar plates. Agar-digesting organisms isolated according to Stanier (1941) from the red seaweed *Corallina* did not attack phenolphthalein disulphate. Samples of undiluted inter-tidal mud collected from Sully, Glamorgan, gave organisms with arylsulphatase activity when grown aerobically on both nutrient and sea-water agar, although some colonies lost their activities after subculturing. Anaerobic growth was poor. Occasional activity was found in cultures from mud collected from Barry harbour but in general, mud samples from a number of other areas of the Bristol Channel

Table 1. *The hydrolysis of phenolphthalein disulphate by certain micro-organisms*

Growth under the conditions outlined in the text for 16 days. In the recovery controls, organisms were grown without phenolphthalein disulphate but in the presence of 70 μg. phenolphthalein.

Organism	Temperature of growth (°)	Total phenolphthalein liberated (μg.)	Phenolphthalein liberated/mg. dry wt. of organism (μg.)	Recovery of added phenolphthalein (%)
Myco. piscium	37·5	237	39	95
	25·0	114	12	98
Myco. ranae	37·5	31	4	92
Alcaligenes metalcaligenes	25·0	395	72	83
Trichosporon cutaneum	25·0	38	6	82

revealed no arylsulphatase producers. Arylsulphatase-producing organisms from Sully mud grown on nutrient agar were repeatedly subcultured on this medium and two aerobic micro-organisms capable of attacking arylsulphates were isolated and identified. One of these organisms was a yeast, *Trichosporon cutaneum*, the other a bacterium of the genus *Alcaligenes*. Bacteriological identification tests (Bergey, 1948) showed the bacterium to be a strain of *A. metalcaligenes*. Attempts were made to estimate the arylsulphatase activity of these organisms but the results were obscured by the fact that both were capable of destroying phenolphthalein. However, the enzyme activity of *Trichosporon* was comparable to that of *Myco. ranae* while that of *Alcaligenes* was greater than that of *Myco. piscium* (Table 1).

Table 2. *Distribution of arylsulphatase activity in cultures of* Alcaligenes metalcaligenes *and in extracts obtained after partial destruction of the cells in a bacterial mill*

The cells from 1·6 l. of the bacterial culture were suspended in 40 ml. distilled water for crushing. Enzyme activities were measured at 37·5° over a period of 18 hr. in the presence of 0·006 M potassium *p*-acetylphenyl sulphate in 0·5 M sodium acetate at pH 6·5. Results are the average of two experiments.

Fraction (from 1·6 l. of bacterial culture)	Enzyme activity (μg. *p*-hydroxyacetophenone liberated)	
	Whole fraction	Per mg. N
Culture fluid	1670	0·62
Cells	4352	53
Supernatant from crushed cells	2300	90
Debris from crushed cells	2514	45

Table 3. *The yields and activities of concentrates of the arylsulphatase of* Alcaligenes metalcaligenes

The powders were suspended in a suitable volume of 0·1 M sodium phosphate, pH 8·75 and enzyme activities were measured over 1 hr. using 0·4 ml. enzyme suspension and 0·4 ml. 0·006 M potassium *p*-acetylphenyl sulphate dissolved in the same buffer. The results are the average of ten 16 l. culture experiments.

Fraction	Yield (g.)	Arylsulphatase activity (μg. *p*-hydroxyacetophenone liberated)	
		Whole fraction	Per mg. N
Whole culture (16 l.)	—	353 000*	—
Powder *A*	5	210 000	370
Powder *B*	0·67	108 540	1190

* Measured over 18 hr. in 0·1 M sodium phosphate, pH 8·75.

Preparation of an enzyme concentrate from Alcaligenes. Attempts were first made to extract the enzyme from the bacterial cell. The organism was grown aerobically in the nutrient broth at room temperature or 25° for 7 days, when the cells were spun down at 0°, washed with a little ice-cold water and the washings added to the supernatant. Measurement of arylsulphatase activity of the supernatant using potassium *p*-acetylphenylsulphate as substrate showed that about one-third of the total activity was associated with the cell-free culture fluid. The bacterial mass was suspended in ice-cold distilled water and ground for 2 hr. in a bacterial mill (Booth & Green, 1938), during which time the temperature of the suspension increased to 10°. The resultant suspension was centrifuged at 0° and the total arylsulphatase activity and the activities of the debris and supernatant were measured (Table 2). The bacterial mill released only about 50% of the enzyme of the cell into solution. Acetone was more effective in rupturing the bacterial cells and an acetone-dried preparation of the bacterial mass retained about 90% of the original arylsulphatase activity, 70% of the activity of the powder being soluble in water.

On a larger scale, *Alcaligenes* was grown aerobically in large flasks each containing 16 l. of the nutrient broth, the pH being adjusted to 7·4–7·6 with N-NaOH. The cultures were aerated by means of a sterile air-stream and incubated at room temperature or at 25°. After 6 days the cells were spun down at 0°, washed with ice-cold water and again separated at 0°. No attempt was made to recover that part of the enzyme activity which was associated with the culture fluid in view of the low enzyme concentration in terms of nitrogen content (Table 2). The cells were suspended in ice-cold acetone using a Townson & Mercer macerator, filtered at the pump and washed well with ice-cold acetone. After drying *in vacuo*, a stable light-coloured powder remained (powder *A*), the average yield and activity of which is recorded in Table 3. The powder (5 g.) was suspended in ice-cold water (75 ml.) using the Townson & Mercer macerator and allowed to stand at 0° for 90 min. The

suspension was clarified by centrifuging at 0° (24000 *g*) and the debris resuspended in 60 ml. of ice-cold water. After centrifuging, the two clear supernatants were combined and treated with ice-cold acetone (Askonas, 1951) until the concentration of acetone was 85% (v/v). The resulting precipitate was separated at the pump, washed well with ice-cold acetone and dried *in vacuo* (powder *B*). The yield and activity of this powder is recorded in Table 3. The concentrate is stable when stored in a stoppered bottle at 0°.

Properties of the enzyme concentrate. When the arylsulphatase activity was measured over 1 hr. at 37·5° with potassium *p*-acetylphenyl sulphate (0·003 M) as the substrate, the optimum pH of the enzyme in 0·1 M phosphate was 8·75 (Fig. 1). This high optimum pH was not an artifact arising from the acetone treatment since an aqueous extract of the bacteria prepared by crushing the cells in a bacterial mill (Booth & Green, 1938) showed the same optimum pH. Maximum activity of the powder occurred at a substrate concentration of 0·003 M in 0·1 M phosphate at pH 8·75, the enzyme being inhibited by excess substrate (Fig. 2). Using these optimum conditions the effects of enzyme concentration, time of incubation and time of incubation before the addition of substrate, on the activity of the enzyme were studied (Figs. 3 and 4). The enzyme was rapidly inactivated at temperatures greater than 50° and at pH lower than 5.

Using potassium *p*-nitrophenyl sulphate (0·0015 M in 0·1 M phosphate) maximum activity was again obtained at pH 8·75 (Fig. 1). At this pH the optimum substrate concentration was 0·0015 M

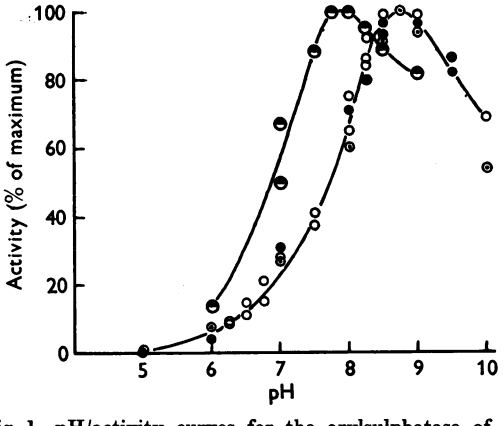

Fig. 1. pH/activity curves for the arylsulphatase of an acetone-dried concentrate of *A. metalcaligenes* (powder *B*) using different substrates in the presence of 0·1 M phosphate buffer. The concentration of the enzyme solution was 0·1% (w/v) when nitrocatechol sulphate was the substrate and 0·05% with other substrates. ⊖—⊖, nitrocatechol sulphate (0·015 M); ●—●, *p*-nitrophenyl sulphate (0·0015 M); ○—○, *p*-acetylphenyl sulphate (0·003 M); ⊙—⊙, *p*-acetylphenyl sulphate (0·003 M) acted upon by an aqueous extract of the crushed bacteria (see text). Incubation was for 1 hr. at 37·5°.

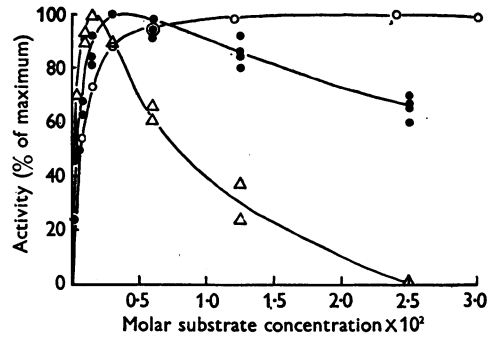

Fig. 2. Substrate concentration/activity curves for the arylsulphatase of *A. metalcaligenes* (powder *B*) acting on different substrates in the presence of 0·1 M phosphate buffer adjusted to the appropriate optimum pH. Incubation was for 1 hr. at 37·5°. The concentration of the enzyme solution was 0·1% (w/v) when nitrocatechol sulphate was the substrate and 0·025% with other substrates. ○—○, nitrocatechol sulphate (pH 8·0); ●—●, *p*-acetylphenyl sulphate (pH 8·75); △—△, *p*-nitrophenyl sulphate (pH 8·75).

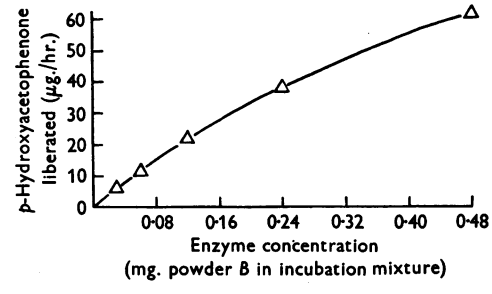

Fig. 3. Enzyme concentration/activity curve for the arylsulphatase of *A. metalcaligenes* (powder *B*) acting on 0·003 M potassium *p*-acetylphenyl sulphate in 0·1 M phosphate, pH 8·75 at 37·5° for 1 hr.

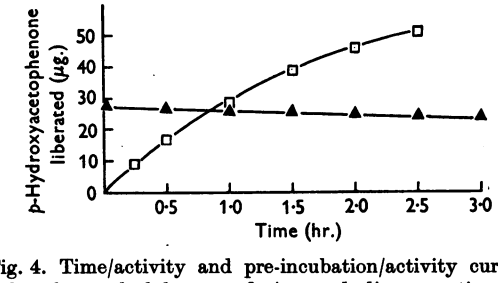

Fig. 4. Time/activity and pre-incubation/activity curves for the arylsulphatase of *A. metalcaligenes* acting on 0·003 M *p*-acetylphenyl sulphate in 0·1 M phosphate, pH 8·75. Each incubation mixture contained 0·18 mg. of powder *B*. In the pre-incubation–activity experiment the enzyme preparation was incubated at 37·5° for varying periods before addition of substrate and was subsequently incubated for 1 hr. at 37·5°. □—□, time/activity; ▲—▲, pre-incubation/activity.

(Fig. 2). With increasing substrate concentration, enzyme activity declined rapidly until at a concentration of 0·025M activity was nil. Using the optimum concentration of nitrocatechol sulphate (0·015M in 0·1M phosphate, see Fig. 2) maximum activity was obtained at about pH 8·0 (Fig. 1).

Presence of other enzymes in the concentrate. Powder *B* showed no β-glucuronidase activity when tested against 0·004M *p*-chlorophenyl glucuronide (Dodgson *et al.* 1953*a*) in 0·1M phosphate at pH values 6–9 and in 0·5M acetate at pH 6. Using the method of Dodgson & Spencer (1954) no glycosulphatase activity was observed at pH values 5–8 towards 0·005M potassium glucose 6-sulphate in 0·5M acetate.

Table 4. *Recovery of added phenolphthalein from growing cultures of various* Alcaligenes *organisms*

Organisms obtained from the National Collection of Type Cultures were grown at 25° for 7 days under the conditions outlined in the text.

Organism (National Type Culture designation)		Recovery of added phenolphthalein (%)
6535	*Alcaligenes bookeri*, Beef	60
712	*A. faecalis*, Castellani	48
415	*A. faecalis*, 439	98
1347	*A. faecalis*, Bedson	100
6572	*A. faecalis*, Edwards II	94
8763	*A. faecalis*, 2792	80
8769	*A. faecalis*, 15204	60
8840*	*A. faecalis*, 43024	100
5043	*A. haemolysans*, Baumann	34
3233	*A. subviscosum*, ATCC 337	40
7249	*Alcaligenes* sp. F/16 Freeman	26
7570	*Alcaligenes* sp. Wheatley	36
7991	*Alcaligenes* sp. 21919	6
7992	*Alcaligenes* sp. 21445	6
7993	*Alcaligenes* sp. 21447R	32

* This organism showed very feeble arylsulphatase activity towards phenolphthalein disulphate.

The arylsulphatase activity of related organisms. Other species were examined for arylsulphatase activity. One strain each of *Alcaligenes bookeri*, *A. haemolysans*, *A. subviscosum*, seven strains of *A. faecalis* and five unidentified species were examined but only one strain showed any activity. Recovery of phenolphthalein from growing cultures of the majority of the organisms was poor (Table 4), and it was possible that arylsulphatase activity was being masked by the ability of the organism to destroy the liberated phenolphthalein. However, acetone-dried preparations of two of these organisms (nos. 712 and 7991, see Table 4) showed no arylsulphatase activity at pH values 5, 7 and 9 in 0·1M phosphate using 0·001M tripotassium phenolphthalein disulphate or 0·003M potassium *p*-acetylphenyl sulphate as substrates, although neither acetone-dried preparation was able to metabolize phenolphthalein. The one organism (no. 1347) which did have arylsulphatase activity did not destroy phenolphthalein.

DISCUSSION

It seems clear that the bacteria present in the digestive organs of marine molluscs contribute little or nothing towards the high arylsulphatase activity which is found in these organs. Indeed the present work emphasizes the findings of earlier workers that arylsulphatase activity is not common in bacteria; a point which contrasts sharply with the widespread occurrence of the enzyme in the animal world. However, Whitehead *et al.* (1952) have pointed out the difficulties involved in the detection of arylsulphatase in micro-organisms which are capable of metabolizing phenolphthalein, and the present findings show the need for suitable recovery control experiments in such investigations.

There appears to be no relationship between the various types of organisms which possess arylsulphatase activity, although it is interesting that the two most active organisms examined to date, *Myco. piscium* and *A. metalcaligenes*, were both isolated from a marine environment. However, the high activities observed in these bacteria are somewhat artificial, since enzyme activities were measured at a temperature much higher than that of their normal habitat, and the enzyme from *Alcaligenes* is about 4 times more active at 37·5° than at 10° when measured over a period of 1 hr. *Myco. piscium* liberates more phenolphthalein at 37·5° than at 25° (Table 1), despite the fact that the organism grows better at 25°. The difference may be accounted for by the increased activity of the enzyme at 37·5° rather than an increased production of arylsulphatase at this temperature.

The high optimum pH value of the enzyme of *A. metalcaligenes* may possibly be related to the ability of this organism to produce considerable amounts of alkali. During the growth of the organism the pH of the culture fluid increased from 7·4–7·6 to 8·4–8·7 in 6 or 7 days. The inter-tidal mud in which the organism flourishes has a pH of 8·1–8·2, which is higher than the normal pH of the sea-water in this area (7·5). A comparison of the optimum pH of the enzyme of *A. metalcaligenes* with those of arylsulphatases from other sources is not strictly valid, since these have been mainly studied in the presence of acetate rather than phosphate buffers. Such evidence as is available indicates that in general the optimum pH of the various arylsulphatases is higher in the presence of phosphate (Table 5). The *Alcaligenes* enzyme has optimum activity against nitrocatechol sulphate at a pH lower than that for the other substrates tested; this is in agreement with the findings for arylsulphatases

Table 5. *The* pH *optima of arylsulphatases of different origin*

Optimum concentrations of the sulphates indicated were used in the presence of 0·5 M acetate or 0·2 M phosphate buffers unless otherwise stated.

	Optimum pH using					
	p-Acetylphenyl sulphate		*p*-Nitrophenyl sulphate		Nitrocatechol sulphate	
Source of enzyme	Acetate	Phosphate	Acetate	Phosphate	Acetate	Phosphate
Alcaligenes metalcaligenes	—	8·75*	—	8·75*	—	8·0*
Aspergillus oryzae	6·3	7·6	6·3	—	5·8	—
Rat liver	7·2	8·2	—	—	6·0	—
Human liver	6·9	7·3	7·3	—	6·0	—

* In 0·1 M phosphate buffer.

from other sources which have been studied in this laboratory (Table 5).

Recent investigation of arylsulphatase activity in the livers of rat, ox, mouse, rabbit and man in this laboratory (Dodgson, Spencer & Thomas, 1954; cf. Roy, 1953*a*, *b*) has shown the presence in these tissues of at least two arylsulphatases, which were distinguished by their widely different affinities for nitrocatechol sulphate on the one hand and potassium *p*-acetylphenyl sulphate and potassium *p*-nitrophenyl sulphate on the other. It was possible that two similar enzymes were present in the concentrates from *A. metalcaligenes* but the ratio of activities towards the three substrates under the respective optimum conditions was the same for the culture fluid and powders *A* and *B*. The activity towards nitrocatechol sulphate was about one-fifth of the activity towards the other two substrates when measured on a molar basis.

The importance of arylsulphatase to *A. metalcaligenes* is obscure. The organism grows well in absence of arylsulphate and the enzyme activity is in no way diminished. An attempt has been made to detect spectroscopically the presence of natural substrates of the enzyme in the inter-tidal mud in which the organism flourishes but results obtained so far have been inconclusive.

SUMMARY

1. The high arylsulphatase activity of the visceral humps of marine molluscs is not due to micro-organisms.

2. A strain of *Alcaligenes metalcaligenes* isolated from inter-tidal mud possesses considerable arylsulphatase activity but is free from glycosulphatase and β-glucuronidase.

3. A concentrate of the *Alcaligenes* enzyme has been prepared and some of its properties are described.

4. A number of other bacteria of the genus *Alcaligenes* showed no arylsulphatase activity when tested against phenolphthalein disulphate.

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