MICROBIAL OXIDATION OF NAPHTHALENE

I. Factors Concerning Salicylate Accumulation

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Little information has been contributed to the mechanisms of biological degradation of polycyclic hydrocarbons. The oxidation of naphthalene has received the most attention. Strawinski and Stone (1943, 1954) reported the isolation of substantial amounts of salicylic acid ("most" of the 2.9 mg per ml medium of ether extractable product) from the breakdown of naphthalene by *Pseudomonas aeruginosa*. Salicylic acid has been verified as an intermediate in naphthalene oxidation by Treccani (1953), Walker and Wiltshire (1953), and Murphy and Stone (1955). These latter systems were not entirely satisfactory for intermediary studies. Either the intermediate compound (salicylate) was recovered in small amounts; i.e., 23 mg from 2 L culture medium (Walker and Wiltshire, 1953), 0.6 mg per ml medium (Murphy and Stone, 1955), or the organism employed was incapable of growth when subcultured continuously in medium containing naphthalene as sole carbon source (Murphy, 1953; Murphy and Stone, 1955).

The present study was undertaken in order to isolate a naphthalene-oxidizing system which would be useful for investigating the mechanism of oxidation of polycyclic hydrocarbons. A procedure for isolating such a system and certain physiological factors concerned with the accumulation of the intermediate product, salicylic acid, are described.

METHODS

Three mineral salts-naphthalene media were employed during the course of this investigation. Medium M, essentially that of Murphy and Stone (1955), was composed of: NH_4Cl, 4 g; K_2HPO_4, 1.0 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7\ H_2O$, 0.5 g; $MnCl_2 \cdot 4\ H_2O$, 0.2 g; $FeSO_4 \cdot 7\ H_2O$, 0.05 g; $CaCl_2 \cdot 2\ H_2O$, 0.05 g; $CaCO_3$, 2 g; tap water, 1 L. Medium S, similar to that of Strawinski and Stone (1954), has the following composition: NH_4Cl, 2.5 g; KH_2PO_4, 0.5 g; Na_2HPO_4, 1.0 g; $MgSO_4 \cdot 7\ H_2O$, 0.5 g; $MnCl_2 \cdot 4\ H_2O$, 0.2 g; $FeSO_4 \cdot 7\ H_2O$, 0.05 g; $CaCl_2 \cdot 2\ H_2O$, 0.5 g; KI and $CuSO_4 \cdot 5\ H_2O$, trace; tap water, 1 L. Medium S2 contained the mineral salts, with the exception of phosphates, added in 500 ml tap water in the order and weight listed for medium S. Sufficient 25 per cent K_2HPO_4 solution was then added to give the desired phosphate concentration. The medium was adjusted to pH 8.0 and brought to a final volume of 1 L with tap water. One per cent sterile, finely pulverized naphthalene was added aseptically to each medium after autoclaving. Sterile naphthalene was prepared by storing the desired amounts of this bactericide in tightly stoppered test tubes for 18 to 24 hr. All physiological studies were carried out in medium S unless otherwise stated.

Enrichment procedures were carried out with 50 ml medium in 250-ml Erlenmeyer flasks. Physiological studies were conducted in 160 ml square milk dilution bottles containing 10 ml medium except where otherwise noted. Aeration was maintained throughout by incubating on a reciprocal shaker at a rate of 120 1.5 inch strokes per min. All experiments were carried out at room temperature.

Salicylic acid was determined colorimetrically according to the method of Murphy (1953), based on the color reaction obtained with ferric chloride. Spectrophotometric and chromatographic studies indicated that salicylic acid was the only product formed in the culture medium which gave this reaction.

Stock cultures, transferred every three months, were maintained on slants prepared by adding 2 per cent agar to medium M. The slants were sprinkled with sterile naphthalene after inoculation, incubated 48 hr, and refrigerated. Seed cultures were prepared by inoculating 10 ml medium S from a stock slant and incubating for 24 hr. Test media were inoculated with one per cent of the seed culture.

RESULTS

Enrichment and isolation. This procedure was designed to select organisms capable of (1) rapid growth, (2) acid accumulation during vigorous aeration, and (3) utilization of naphthalene as a sole carbon and energy source during prolonged serial transfer.

Accordingly, 200 g of recently cultivated soil were dried overnight at 37 C, lightly moistened and mixed well with 2 g of finely pulverized naphthalene. The soil was kept lightly moistened and loosely packed at all times. Four additional portions of naphthalene were added during the enrichment period. After seven weeks two flasks each of medium M and medium S were inoculated with 5 g portions of the enriched soil. The cultures were transferred (5 per cent inoculum) at least twice weekly through 8 serial transfers. Aliquots were then streaked in nutrient agar plates. After 72 hr incubation 50 colonies of varied types were inoculated into the medium from which they had been isolated. Thirty-two cultures, acid to bromcresol purple (pH 5.3) after 48 hr incubation, were selected for further study.

All 32 cultures resembled *Pseudomonas aeruginosa* as described by Breed *et al.* (1948). Representative cultures were further characterized as *P. aeruginosa* according to the procedures listed by Haynes (1951). Twenty-seven of the 32 strains gave a positive $FeCl_3$ test for salicylic acid after 48 hr growth in medium M. One of these 32 strains (S1B) elicited a positive salicylate test in either medium. This strain was selected for further study.

Factors concerning salicylate production. It has been reported that aeration, high pH, metallic ions, and time affect the salicylate content of the culture medium during naphthalene oxidation (Strawinski and Stone, 1954; Murphy and Stone, 1955).

TABLE 1

Effect of aeration on salicylate accumulation

Reaction Vessel* (50 ml Medium)	Salicylic Acid	
	18 hr	101 hr
	(μg/ml)	(μg/ml)
Milk dilution bottle	69	492
250 ml flask	234	527
1 L flask	441	511

* All vessels shaken at 120 reciprocal cycles per min.

TABLE 2

Role of metallic ions in salicylate formation after 24 hr

Deficiency	Terminal pH	Salicylate
		μg/ml
None	3.8	629.5
Mg	4.6	343.2
Mn	4.0	611.0
Ca	3.9	88.0
Fe	4.2	35.2
I	4.1	686.0
Cu	4.1	673.5

In the present investigation attempts to vary the amount of dissolved oxygen in static cultures by increasing the atmospheric surface area per unit volume ratio were negative. All stationary cultures showed only a weak, transient salicylate formation. Shaken cultures were aerated at different rates by incubating 50 ml medium in vessels of different sizes. Although agitation aided the formation of salicylate, the degree of forced aeration was reflected only in the rate of salicylate production, but not in total salicylate accumulation (table 1). Other experiments indicated that shaking 10 ml of culture in milk dilution bottles maintained in an upright position was satisfactory for rapid conversion, and all further experiments were conducted in this manner.

The optimum initial pH for salicylate accumulation was pH 8.3 $\pm$ 0.4. These findings corroborate the data of Strawinski and Stone (1954) who reported that a high initial pH favored increased salicylate yields.

For experiments designed to determine the effect of mineral ion deficiencies all media were prepared with distilled water. The same apparent reaction pattern occurred in medium S prepared with tap or with distilled water. The effect of ion deficiencies was tested after three serial transfers so as to eliminate the role of residual ions. Deletion of calcium, iron, or magnesium ions resulted in a dramatic decrease in salicylate formation (table 2). With iron or calcium deletions the decrease in salicylate content of the medium was not reflected in the absence of a lowered pH during growth, indicating that in these media acid materials other than salicylate were formed. This does not necessarily follow for magnesium deficient medium. Iodine and copper deletions appeared to be slightly favorable for salicylate accumulation. It has been noted that in

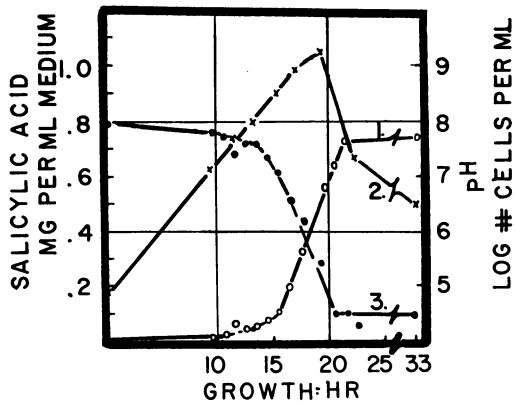

Figure 1. Salicylate accumulation during growth. Curve *1*, Salicylic acid concentration; Curve *2*, cell numbers; Curve *3*, pH.

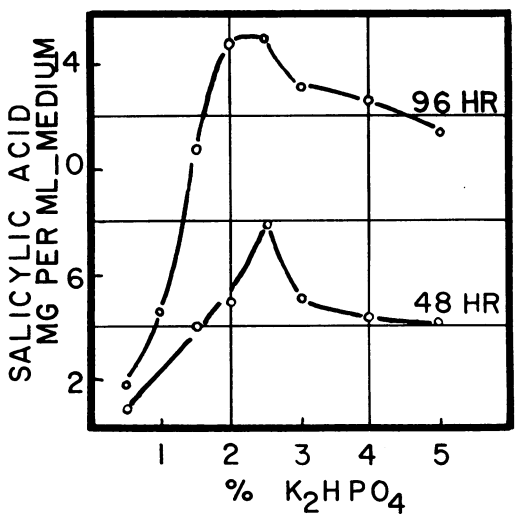

Figure 2. Effect of phosphate concentration on salicylate accumulation.

the cases of iron, calcium or magnesium deletions the yellow-orange pigment which normally was formed in the medium was decreased in amount so as to essentially parallel the salicylate concentration.

Although growth proceeded essentially logarithmically almost from the time of inoculation, salicylate accumulation and the concomitant change in pH did not occur until 10 to 15 hr after inoculation (figure 1). Salicylic acid formation, pH change, and growth then pursued parallel rates until the pH level reached 4.0 to 4.5. These data suggested that at low pH levels the organism was no longer capable of growth or acid production. Accordingly, media having

TABLE 3

Salicylate accumulation in 2 per cent phosphate medium

Hr Growth	pH*	Salicylic Acid*
		mg/ml
0.0	7.5	0.00
21.0	6.9	2.42
28.5	6.5	4.67
36.5	6.4	6.74
40.5	6.2	8.20
44.5	6.0	9.34
48.5	6.6	2.29
52.5	6.8	0.51

* Average of triplicate samples.

greater buffering capacities (medium S2) were prepared. At 2.0 to 2.5 per cent K_2HPO_4 maximum yields were attained (figure 2). It must be mentioned that these data were not corrected for evaporation during 4 days incubation, and so represent more than 100 per cent conversion of naphthalene to salicylic acid.

Subsequent data, in medium containing 2.0 per cent dibasic potassium phosphate, indicated that at 45 hr salicylic acid accumulation reached maximum (table 3) and then rapidly disappeared. Complete conversion of naphthalene would produce 10.8 mg salicylic acid per ml medium.

The discrepancy observed, in that after 4 days salicylate still was present in one case but disappeared in similar experiments, was apparently a factor of temperature of incubation. Room temperature for the data in figure 2 varied from 20 to 25 C. The data presented in table 3 were obtained at temperatures varying from 33 to 37 C.

DISCUSSION

It was apparent from reports of previous investigators that some difficulty is encountered in isolating cultures capable of utilizing naphthalene as sole carbon source and of accumulating substantial amounts of intermediate products. The present investigation indicates that if the enrichment procedure was directed toward isolating organisms capable of acid production under vigorously aerobic conditions, and if enrichment cultures were carried through numerous transfers, these difficulties were resolved. Of 50 colonies selected from enrichment cultures 32 produced acid from naphthalene. Of these, 27 oxidized naphthalene to salicylic acid under proper cultural conditions. These cultures were

maintained in a mineral salts-naphthalene medium for an indefinite number of transfers.

Iron or calcium deficiencies interfered with salicylate production but did not prevent the occurrence of a decrease in pH as normally encountered in the complete medium. It may be possible that an unknown acidic substance other than salicylate accumulated. The role of iron, calcium and magnesium is obviously of interest considering their importance in mediating salicylate formation.

The yellow pigmented material failed to appear in iron or calcium deficient media. However, in complete medium the appearance of the pigment followed a pattern similar to the iron and magnesium deficient cultures of Murphy and Stone (1955). The pigment from 24-hr cultures, however, was strongly acidic in nature, unlike the naphthoquinone pigment reported by Murphy and Stone (1955). The exact nature of this material remains to be determined. It might be mentioned that although in naphthalene medium the yellow pigment is produced to the exclusion of the typical blue-green pigment of *Pseudomonas aeruginosa*, the normal blue-green discoloration occurred in medium S agar in which salicylate, rather than naphthalene, served as the sole carbon source.

Although the organism utilized salicylate for growth, this product accumulated during oxidation of naphthalene indicating that the culture preferentially oxidized naphthalene. Upon exhaustion of naphthalene, salicylate was dissimilated provided conditions were favorable. Murphy and Stone (1955) reported that at maximum salicylate accumulation in the medium there was a decrease in cell count, followed by a period of more rapid growth and salicylate disappearance, suggesting a selective process. It appears unlikely that this phenomenon occurred in the present investigation in view of the rapid dissimilation of salicylate after naphthalene has been exhausted.

In view of the plasticity of the metabolism of the bacterial cell, it seems possible that the described system may be instrumental in providing better insight into the mechanism of degradation of polycyclic hydrocarbons than has been obtained from studies with animal tissues. It is of interest to note that Boyland (1950) has called attention to the possibility that carcinogenesis and metabolism of carcinogenic hydrocarbons are associated. Although naphthalene has not been implicated in carcinogenesis, it is the least complex member of the series of polycyclic hydrocarbons, some of which have been implicated as carcinogenic agents.

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SUMMARY

A procedure for the isolation of organisms capable of oxidizing naphthalene almost entirely to salicylic acid was described.

Salicylate accumulation was dependent upon forced aeration, initial pH near 8, and buffering capacity of the medium (2.0 to 2.5 per cent K_2HPO_4). In the absence of iron or calcium ions salicylate accumulation was reduced by approximately 90 per cent. Magnesium deficiency interfered less drastically with salicylate production.

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