

Azelaic Acid Utilization by a *Pseudomonas*

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SUMMARY

A *Pseudomonas* isolate which utilizes azelaic acid as the sole carbon source was isolated from garden soil. Two different variants of the micro-organisms were obtained from the original culture in azelaic acid medium. One variant (s), exhibited uniform turbidity which clarified rapidly after maximal growth was obtained. The second variant (τ) grew in aggregates and clumps. Both variants gave normal growth curves with a maximal stationary phase in medium with glucose or in azelate medium with high osmotic pressure. The double role of azelaic acid as a source of carbon and as a harmful agent is discussed. It was concluded that when the concentration of azelate is low enough and its action not prolonged it caused cytological disturbances which were not easy to observe (variant s). But the prolonged action of azelaic acid resulted in phenotypical changes that were partially inheritable even in its absence (variant τ).

INTRODUCTION

Bacteria which utilize azelaic acid as the sole carbon source have not been described heretofore. Like other long chain dicarboxylic acids, azelaic acid may be a by-product of oxidation of fatty acids in mammalian systems (Verkade, 1938). It is also possible that azelaic acid can arise from alkanes. Alkanes are known to serve as a source of six and seven carbon dioic acids (Colla & Treccani, 1960) or ten and more carbon dioic acids (Kester & Foster, 1963). The capacity to oxidize azelaic acid by bacteria is interesting from the point of view of the carbon cycle in nature, and also because of the fact that this nine carbon compound is one of the precursors of biotin in the growth of some micro-organisms (Wright, Cresson & Driscoll, 1955). The aim of the present work was to isolate bacteria able to utilize azelaic acid and to establish the best conditions for investigations of azelaic acid degradation.

METHODS

Organisms. The original culture of *Pseudomonas* sp. strain w was isolated in our laboratory from garden soil by the enrichment culture technique. Organisms were maintained on agar slopes of the following composition (% w/v): $(\text{NH}_4)_2\text{SO}_4$, 0.1; K_2HPO_4 , 0.05; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002; NaCl, 0.01; azelaic acid, 0.1, 0.2, or 0.3; agar, 1.2; distilled water. This and all subsequent media were adjusted to pH 7.0 with dilute NaOH before final sterilization. Isolate w was sub-cultured every week.

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Media. Azelaic acid liquid medium unbuffered: the same composition as azelaic acid solid medium. Azelaic acid liquid medium buffered: prepared with phosphate buffer: KH_2PO_4 , 4.0 g. and K_2HPO_4 , 13.6 g. per 1000 ml. instead of distilled water. Special media for identification purposes (% w/v): carbohydrate media: synthetic liquid medium with Durham tubes: $\text{NH}_4\text{H}_2\text{PO}_4$, 0.1; KCl, 0.02; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02; carbohydrate, 0.5; distilled water. Peptone-carbohydrate semisolid medium of Hugh & Leifson (1953): peptone, 0.2; NaCl, 0.5; K_2HPO_4 , 0.3; agar, 0.3; bromthymol blue, 0.003; carbohydrate, 1.0; distilled water. King, Ward & Raney (1954) medium A for enhancement of pyocyanin and pyorubrin: bacto peptone, 2.0; bacto agar, 1.5; glycerol, 1.0; K_2SO_4 (anhydr.), 1.0; MgCl_2 (anhydr.), 0.14; distilled water. King *et al.* medium B for enhancement of fluorescin: proteose peptone, 2.0; bacto agar, 1.5; glycerol, 1.0; K_2HPO_4 (anhydr.), 0.15; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.15; distilled water.

Isolation and maintenance of different variants of micro-organisms. Variants s and t (transition) were obtained by subcultivation from growth of isolate w in azelaic acid liquid medium, to azelaic acid agar plates. Both variants were un-stable on azelate medium after several transfers changing partially to the opposite variant. The variant s was purified several times by single colony selection and has been maintained on nutrient agar slopes. Variant t was more stable in azelate medium and in such a medium has been cultivated.

Morphological and cultural characteristics. Organisms were stained by Gram's method and for flagella by Leifson's method after incubation for 1 day at 22° or 30° on nutrient agar. Their size in hanging drop, on nutrient agar, and in azelate culture during 14 days of incubation was measured. Observations were made on the first subculture after isolation or re-isolation.

Physiological tests. Most of the physiological tests were made according to procedures given by the *Manual of Microbiological Methods* (1957). A standard inoculum for all tests was one loopful of 1-day nutrient broth culture. All media were incubated at 22°. The ability of strain w to grow in nutrient broth at 42°, 37°, 30°, 25°, 10°, 6° and 1° was noted, the incubation periods extending to 1 month at the lower temperatures.

The medium of Hugh & Leifson (1953) in sealed and unsealed tubes was used to distinguish oxidation from fermentation of carbohydrates.

The diffusible pigment production was examined with the media of King *et al.* (1954); oxidase activity was checked according to the method of Gaby & Hadley (1957).

Growth measurements. Growth of shaken cultures incubated at 30° was followed turbidimetrically by using a Bausch & Lomb 340 colorimeter. The correlation between percentage of extinction (wavelength 660 m μ) and dry weight of washed organisms of strain w was determined. A standard inoculum for all growth tests was 0.2% (v/v) of 1-day culture.

Changes in pH value of cultures. Readings of the pH value of the samples were made with a Beckman Model G pH meter.

Determination of acids. A partition chromatographic method for the determination of the total amount of acids in the supernatant solutions of cultures after bacterial centrifugation was used. The method used was essentially that of Isherwood (1946); silica gel was prepared from Mallinckrodt silicic acid. Acids after

different periods of bacterial growth were isolated and titrated with dilute standard NaOH. Total amount of acids in equivalents of azelaic acid is given. The average error of estimations was 2%.

RESULTS

Morphological and cultural characteristics

Cell morphology on nutrient agar. Pseudomonas strain w showing monotrichous rods, $0.6 \times 1.8 \mu$, occurring singly and in pairs. Characteristic arrangement of organisms side by side was observed. Gram negative.

Variant s: like strain w.

Variant r: monotrichous rods, 0.6×1.6 to 8.0μ , occurring singly, in pairs and in very tight aggregates.

Cell morphology in azelaic acid medium. Strain w: exact observations lacking.

Variant s: in shaken and unshaken 1-day cultures a tendency for longer rods to develop (length, 3.2μ) was observed. In 2-day cultures single organisms (length, 1.8μ) and small amount of aggregates were noted. In older cultures the degree of aggregates fluctuated with the time of incubation.

Variant r: in 1–2-day shaken cultures a great variety of forms was regularly seen: short rods (length, 0.8μ), curved filaments (length, 16μ) and hundreds of small rods in very tight aggregates. In older cultures fewer filamentous forms were noted. In unshaken cultures length of filaments varied around 65μ ; filaments were seen dividing into coccoidal forms; a large number of aggregates was always seen.

In buffered azelaic acid medium both variants s and r grew alike. In shaken culture the organisms were single and after the second day of incubation short organisms only were observed (s, length, 0.6 – 1.6μ ; r, length, 0.6 – 1.6μ and exceptionally up to 4.8μ). In unshaken s and r cultures during the first 4 days of incubation single rods and chains were observed. After 5 days there were also curved filaments (length, 50μ) without transverse walls. In older cultures single organisms, swollen organisms, chains, curved filaments, filaments with granulated plasma, and rarely aggregates were seen.

Morphology of organisms grown in azelaic acid medium supplemented with 5.8% NaCl. Organisms of both variants s and r in medium with high osmotic pressure grew like organisms in buffered medium. Round swollen forms ('spheroplasts') were also seen.

Colonial morphology on nutrient agar. Strain w: 48 hr colonies were circular, about 2 mm. in diameter. On further incubation, these colonies became larger, about 5 mm., elevated and with filamentous margins. The surface was smooth, glistening, the centre of the colonies yellow, the periphery transparent.

Variant s: like strain w, only the margin of colonies was entire or undulate.

Variant r: circular yellow colonies with a curled appearance. On prolonged incubation, smooth and transparent outgrowths projected from the margins.

Colonial morphology on azelate agar. Strain w: 48 hr colonies were circular, about 2 mm. in diameter. After incubation for 14 days, colonies became about 8 mm. in diameter, raised and with entire margin; on further incubation the margin became arborescent. The surface was smooth, glistening, gold-yellow. Some colonies developed smooth secondary colonies on their surfaces.

Variant s: like strain w, but without the smooth secondary colonies. In older colonies concentric rings with different transparency were also observed.

Variant τ: as on nutrient agar.

Appearance in liquid media. Strain w: in broth, broth + yeast extract and in peptone water, strain w produced strong turbidity, sediment and pellicle. Formation of the pellicle in azelate cultures was variable. Growth in shaken azelate cultures was at first uniform. After a few transfers in some flasks smaller and bigger grains became visible. Aeration by bubbling stimulated the production of clumps. From these cultures, variants s and τ were re-isolated. Near the end of the exponential phase of well growing cultures, the supernatant fluid became yellow. A depression of the surface tension paralleled the growth of the bacteria.

Variant s: uniform heavy turbidity in unshaken and in shaken azelate cultures, with yellow colour of supernatant fluid near the end of the growth.

Variant τ: turbidity, clumps and pellicle in unshaken azelate cultures; turbidity, smaller and larger grains in shaken culture. As in variant s, the supernatant fluid of well-developed cultures became yellow.

Physiological characteristics of strain w

Gelatin liquefaction—negative.

Nutrient gelatin liquefaction—negative.

Action on litmus milk—alkalization without litmus reduction.

Reduction of nitrate to nitrite—nitrate reduced in 24 hr to nitrite, followed by nitrite reduction to ammonia and nitrogen.

Ammonia production—positive.

Hydrogen sulphide formation—slightly positive.

Indole formation—positive after 24 hr incubation at 30°.

Production of acid from carbohydrate in synthetic media:

- (a) Acid from: D + xylose (1·5*); D + glucose (3·0*);
 D + galactose (3·0*); D - levulose (2·0*);
 D + maltose (3·0*).

- (b) Small amount of acid from: D + mannose (1·0*); D + lactose (1·0*);
 (*decrease of pH value during 10 days of incubation.)

- (c) No acid from: L - xylose; L + arabinose; D - arabinose;
 L sorbose; sucrose; D + melibiose;
 D + trehalose; D + raffinose; L + rhamnase.

No gas was produced from any above-mentioned sugars in synthetic media or in media with the addition of peptone. No acid was produced in sealed tubes in the medium of Hugh & Leifson (1953).

Starch hydrolysis—negative.

Growth in Koser's citrate medium—positive.

Voges-Proskauer and methyl red tests—negative.

Greenish yellow pigment on the medium for enhancement of fluorescein (King *et al.* 1954).

Oxidase production—positive.

Temperature range of growth 6–35°; no growth at 37°.

Azelaic acid bacteria strain w is considered to be a *Pseudomonas* sp. because it: (1) is Gram-negative and motile with a single polar flagellum; (2) produces a greenish yellow water-soluble pigment; (3) produces acids oxidatively from carbohydrates.

Morphological and physiological characteristics of this organism suggest that the isolated strain belongs to a species never before described in the literature.

Growth of Pseudomonas strain w in defined medium with azelaic acid

To establish the growth curve of the *Pseudomonas* strain w several nephelometer readings of the shaken culture growing with 0.3% azelaic acid were made. Simultaneously samples from the same culture were centrifuged to obtain the dry weight of organisms in the sample. Results from this experiment show rapid growth of micro-organisms and unusually rapid decrease of cultural turbidity (Fig. 1). During this growth slightly acid (pH 6.7) medium became strongly alkaline (pH 9.4).

The tested strain never grew similarly in the subsequent experiments, in the same or in other growth conditions (different temperature, different amount or age of the inoculum). When different concentrations of azelaic acid were used (0.1, 0.2, 0.3%), repeatable results were obtained solely with 0.1% azelaic acid (Fig. 2).

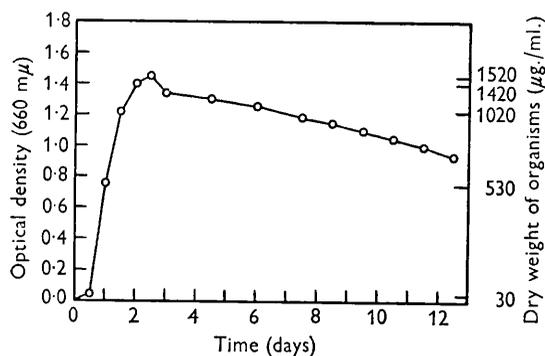


Fig. 1

Fig. 1. Growth curve for strain w after ten transfers through azelate medium. Growth in medium with 0.3% of azelaic acid.

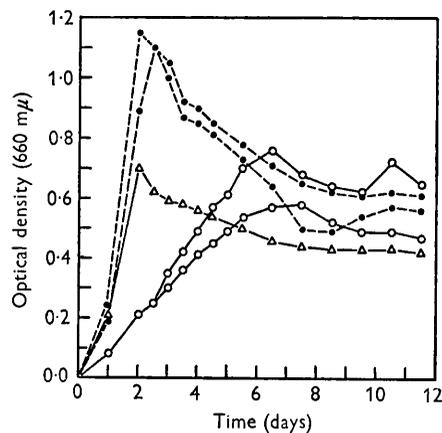


Fig. 2

Fig. 2. Growth curve for strain w after twenty transfers through azelate medium. Growth in medium with different concentrations of azelaic acid: ○—○, 0.3% azelaic acid; ●—●, 0.2% azelaic acid; △—△, 0.1% azelaic acid.

Microscopical observations of growing cultures revealed a large amount of aggregates of cells in flasks with low turbidity. Subculturing of strain w on plates gave two types of colonies from which variant s and variant r were re-isolated. Growth of both variants was determined in buffered and unbuffered medium containing 0.1% azelaic acid. The aggregates of variant r strongly influenced the nephelometer readings in unbuffered medium. Addition of the mono- and dibasic potassium phosphates in amounts mentioned in 'Methods' gave very rapid growth. The slope of the growth curve after maximal growth was not so sharp as usual for both variants (Fig. 3).

Small changes of pH value during the first 2 days of incubation of unbuffered cultures (pH 6.7-7.3) did not indicate that the alkalinity was the factor inhibiting growth.

Total acid utilization in culture medium was approximately the same for both strains (Fig. 3).

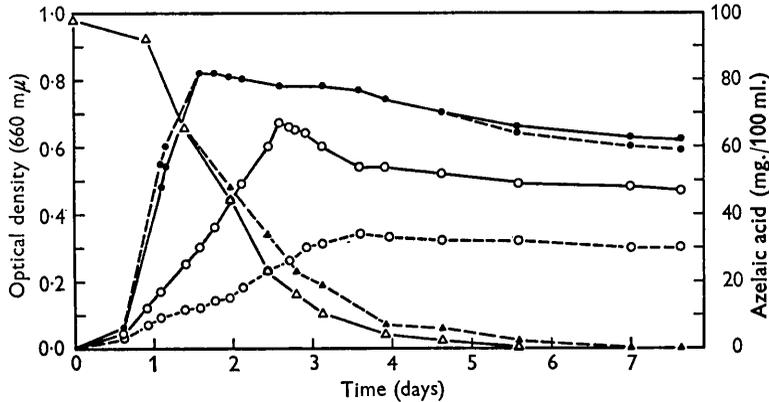


Fig. 3. Growth and azelaic acid utilization by variants *s* and *r* in medium with 0.1 % of azelaic acid. ○—○, Growth of variant *s*; ○--○, growth of variant *r*; ●—●, growth of variant *s* in buffered medium; ●--●, growth of variant *r* in buffered medium; △—△, azelaic acid uptake by variant *s*; ▲--▲, azelaic acid uptake by variant *r*.

Some characteristics of s and r variants of azelaic acid bacteria

From the above-described experiments, the question arose as to whether or not variant *r* retains its special features after several cell divisions in buffered azelate medium. At the same time, the data indicated that the filtrate of the old culture of the tested micro-organisms contains some lytic enzymes which dissolve damaged bacterial cell walls.

Two 25 ml. samples of the culture of each variant at the maximal stationary growth phase in buffered medium were centrifuged and washed several times. The organisms were then placed in 45 ml. Ringer's solution in Erlenmeyer flasks and the mixture made homogeneous by mechanical shaking. After shaking for 4 hr, 5 ml. water was added to one flask and 5 ml. sterile filtrate of the old culture of the same bacteria was added to the second flask. Measurements of extinction of the fluid were made until a uniform suspension of organisms was achieved.

Overall homogenization of variants *s* and *r* took 76 and 112 hr respectively (Fig. 4). The addition of the filtrate of old culture strongly influenced the attainment of homogeneity, the rate of which was enhanced by the filtrate and was the same for both variants. If azelaic acid is the cause of the partially inheritable changes of outside layers of bacteria, then perhaps when unprotected by the normal cell walls, micro-organisms form aggregates, undergo lysis, and die rapidly in hypotonic solution. For this reason, both strains were inoculated into the medium with high osmotic pressure.

Growth in the medium with 0.1 % azelaic acid and 5.8 % NaCl started after incubation for 48 hr and continued slowly during the next few days. The growth

curve of both strains was 'normal' (Fig. 5) and turbidity was homogeneous. Cultures without NaCl served as controls and showed that both variants in the medium with azelaic acid retained their characteristics.

No abnormal damaging of microbial cells and no differences between variants *s* and *t* in defined medium with glucose were observed. Maximal turbidity of cultures with glucose (added in an amount equivalent to the amount of carbon supplied by 0.1% azelaic acid) was stable, but one half that in azelaic acid culture of variant *s* (Fig. 6). The lower final acidity of the culture with glucose (pH 4.1–4.3) could be the growth-inhibiting agent.

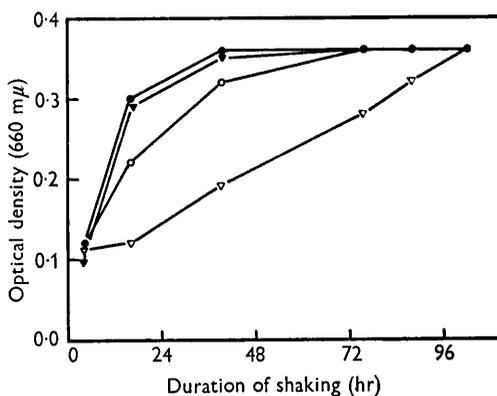


Fig. 4

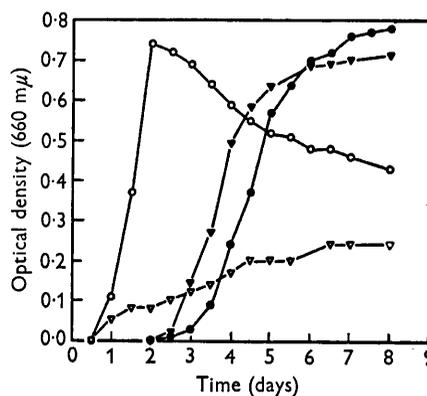


Fig. 5

Fig. 4. Homogenization by mechanical shaking of variants *s* and *t* in Ringer's solution with and without sterile filtrate of an old culture. ○—○, Variant *s* in Ringer's solution; ●—●, variant *s* in Ringer's solution with filtrate of an old culture; ▽—▽, variant *t* in Ringer's solution; ▼—▼, variant *t* in Ringer's solution with filtrate of an old culture.

Fig. 5. Growth curves for variants *s* and *t* in the medium with 0.1% of azelaic acid and different concentrations of NaCl. ○—○, growth of variant *s* in medium with 0.01% NaCl; ●—●, growth of variant *s* in medium with 5.8% NaCl; ▽—▽, growth of variant *t* in medium with 0.01% NaCl; ▼—▼, growth of variant *t* in medium with 5.8% NaCl.

Effect of cultures of azelate-grown pseudomonads on the growth of Streptococcus lactis

In a preliminary experiment, it was observed that autoclaved supernatant fluid from old cultures of azelate-grown pseudomonads had a growth-stimulating effect on *Streptococcus lactis* under conditions where lipoic acid was the limiting factor for growth. This suggested the possibility that azelaic acid or a metabolite of it served as a precursor of lipoic acid. To test this hypothesis a bioassay of supernatant fluids from cultures of azelate-grown pseudomonads which had been incubated with 0.1% azelaic acid or with glucose, was made. In both cultures of azelate-grown pseudomonads the total amount of carbon was the same before inoculation. Samples were taken with sterile precautions after different incubation times, centrifuged and the clear supernatant solutions autoclaved for 15 min. at 120°. At the same time the extinction and the pH value were taken as a measure of growth. After 5 days, when the last sample for the bioassay was prepared, the remaining amounts of the cultures were autoclaved at 120° for 3 hr with sulphuric acid at pH 1.5, and the hydrolysates

neutralized and used for additional tests. The assay procedure was that described by Reed, DeBusk, Johnston & Getzendaner (1951). Nine ml. of assay medium was supplemented with 1 ml. of (1) supernatant solutions, (2) hydrolysates of culture, (3) sodium acetate solutions. The growth response of *Streptococcus lactis* 9936 group N was tested as a function of the various supplements. A stimulating effect of the supernatant fluid from the azelate-grown pseudomonads culture was observed, which increased with age of the culture. The supernatant solutions from the culture of bacteria utilizing glucose were inactive (Fig. 7). The hydrolysates from both cultures were active and the extinctions of the *S. lactis* cultures were 0.25 and 0.21, respectively.

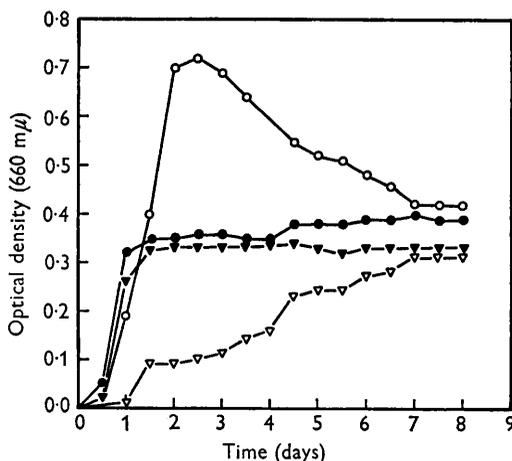


Fig. 6

Fig. 6. Growth curves for variants s and r in medium with 0.1% of azelaic acid and in medium with 0.1436% of glucose. ○—○, Growth of variant s in medium with azelaic acid; ●—●, growth of variant s in medium with glucose; ▽—▽, growth of variant r in medium with azelaic acid; ▼—▼, growth of variant r in medium with glucose.

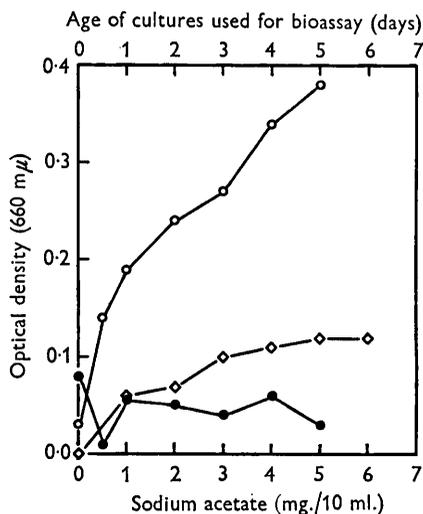


Fig. 7

Fig. 7. Growth of *Streptococcus lactis* after 18 hr incubation in Guirard's medium supplemented with sodium acetate or the supernatant from azelate bacteria cultures. ◇—◇, Medium with Na acetate; ○—○, medium with supernatant from culture with azelaic acid; ●—●, medium with supernatant from culture with glucose.

This insignificant difference in the growth-stimulating activity of acid-hydrolysed cultures does not prove the hypothesis that azelaic acid has a special role in the biosynthesis of the growth factor determined. On the basis of this experiment one can only suspect the greater permeability of the cell walls (perhaps in damaged bacteria) of the micro-organisms growing at the expense of azelaic acid.

DISCUSSION

The measurement of the extinction of cultures of azelate-grown pseudomonads revealed such a great irregularity of growth that it was decided to examine more carefully the morphology of the bacteria concerned, especially those from media containing azelaic acid. The smooth secondary colonies situated on the primary

colonies and the outgrowths of strain w resembled the variations in *Lactobacillus casei* described by de Klerk & Coetzee (1962*a, b*). However, neither of the isolates of azelate bacteria (s and τ variants) could be identified as a smooth or a rough form. Also Tween 80, a smooth-colony-inducing agent, added to the culture of variant τ did not change the features of that form. On the other hand, the τ variant of azelate-grown bacteria and the L-type transition form described by Klieneberger-Nobel (1960) have some characteristics in common: the dark centre and a lighter periphery of the colony and growth in liquid media in the form of clumps.

The observations on the release from the pseudomonads growing on azelate of a growth factor for *Streptococcus lactis*, and the release of the pigment indicated that some factor damaged the pseudomonads such that the cell walls become more permeable. Damaged walls may also be more easily digested by lytic enzymes. The observations on the formation of bacterial aggregates and the decrease of the surface tension from the beginning of growth were indications that azelaic acid itself was harmful for microbes which probably release fatty acids from their envelope. The cell-wall autolytic activity increasing toward the end of the log. phase of *S. faecalis* is described by Strampp, Conover & Shockman (1963). Weidel, Frank & Leutgeb (1963) used sodium dodecylsulphate to suppress the damaging effect of lytic enzymes during preparation of the wall of some Gram-negative bacteria. This compound was inactive in our experiments. Also uranyl nitrate, a protecting agent against lysis by anionic and cationic detergents (Razin & Argaman, 1963), was without effect on the growth curve of azelate-grown pseudomonads. It remains to be tested whether or not high concentrations of salts in the culture medium will protect damaged bacteria against bursting in hypotonic solution. Both buffered and NaCl media prevented visible damage of the pseudomonads when aggregated. Both media induced the bacteria to produce aberrant forms in unshaken culture. The data presented in this work are not sufficient to explain changes in the walls and death of tested bacteria under the influence of azelaic acid and eventually under the influence of some metabolic products. For such an explanation, additional research would have to be done, taking into consideration the effect of inorganic cations which, when present in the medium in large amounts, can act by decreasing the negative charge at the cell surface (Voss, 1963). It seems, however, that the results described above prove the double role of azelaic acid: (1) as a source of carbon, (2) as a harmful agent. When the concentration of azelate is low enough and its action not prolonged, it causes cell disturbances not easy to observe (variant s). But the prolonged action of azelaic acid results in phenotypical changes, partially inheritable even in its absence (variant τ).

Pseudomonas sp. strain w has been deposited with the American Type Culture Collection, Washington, D.C., U.S.A.

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