OBSERVATIONS CONCERNING THE GROWTH AND METABOLIC ACTIVITIES OF MYXOCOCCI IN A SIMPLE PROTEIN-FREE LIQUID MEDIUM

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Although a great deal seems to be known concerning the mode of growth of myxococci upon solid media (for a summary of existing knowledge see, for example, Knaysi, 1944) there is surprisingly little information in the literature about their propagation and metabolic activities in homogeneous, chemically defined liquid media. Attention has recently been paid to their ability to grow upon and lyse eubacteria in suspensions of known electrolyte content (Singh, 1946). The lysis of dead eubacteria under these conditions seems to be chiefly due to exocellular proteolytic enzymes elaborated by the swarming myxococci, but Singh (1946) has proved that fully viable eubacteria are in certain instances also lysed. It is therefore possible that myxococci may sometimes produce true nonenzymic antibiotic substances, capable of killing viable eubacteria and thereby rendering them susceptible to lysis. In a search for these hypothetical antibiotic substances some typical myxococci have now been grown in a homogeneous protein- and polysaccharide-free medium of simple composition in which amino acids provided the sole source of both carbon and nitrogen. As anticipated, the occurrence of at least one antibiotic substance of considerable activity has been demonstrated, and its separation in the monamino-monocarboxylic acid fraction is described. Some observations, which appear to be new, concerning the peculiar mode of growth of myxococci in homogeneous liquid media are also recorded.

EXPERIMENTAL

1. *Cultures and method of assay for antibacterial potency.* Among a dozen cultures of myxococci and related chondrococci obtained from the Department of Soil Microbiology, Rothamsted Experimental Station, four of the former and one of the latter could be grown in the simple amino acid medium described in section 4. Only two of these, however, were of sufficient biochemical interest to warrant further investigation at the moment—namely, two distinct strains of *Myxococcus virescens* (Bergey *et al.*, 1939), one of which, strain A, produced an antibiotic substance, and the other, strain G, secreted an active gelatinase but no antibiotic substance.

Antibacterial activity was measured by the serial dilution method using *Staphylococcus aureus* (Heatley strain; N.C.T.C. no. 6571) as the test organism. The usual series comprised the following dilutions: 5, 14, 40, 70, 85, and 125, and was made up by adding 1.0, 0.3, and 0.1 ml of clear metabolic solution, re-

spectively, to 4-ml quantities of heart broth containing no added sugar, and then transferring 1.0, 0.8, and 0.5 ml, respectively, from the second tube to further 4-ml quantities of heart broth. One loopful (0.004 ml) of a well-shaken and 1,000-fold-diluted 20-hour culture of *S. aureus* in heart broth was used to inoculate each tube, and readings were made after 1 and 2 days' incubation at 37 C.

2. *Measurement of gelatinase activity.* Accurate determination of the absolute gelatin-liquefying power was not deemed necessary in this investigation (see Haines, 1932, for a discussion of the difficulties involved in such determinations). All that was required was a quick method for assigning the correct relative gelatinase activities to the various members of a series of preparations put up at the same time. The following method, which required no special media or apparatus, was found to be adequate: Ordinary sterile 12 per cent nutrient gelatin medium, as used for bacterial stab cultures (8-ml lots in 6-inch test tubes) was first completely liquefied by slowly heating to 45 C in a water bath. The flame was then removed and cold water added to the bath to reduce the temperature to 40 C. When the temperature had further fallen to 37 C by ordinary cooling, 1.0 ml of the clear metabolic liquid under test was added to 8 ml gelatin and the tube well shaken. Three drops of chloroform were then added, and the whole was well shaken again to saturate the gelatin with chloroform. The series of tubes so prepared (each specimen being tested in duplicate) was immediately transferred to the cold room for 15 minutes for the gelatin to set, then it was incubated at 24 C, and readings were taken after 6 and 24 hours. Good liquefaction required 6 hours or less; moderate liquefaction required overnight incubation. Control tubes containing 1.1 ml of uninoculated medium invariably remained firm even after several days' incubation.

3. *The maintenance of fruiting cultures of myxococci on a solid medium of simple composition.* It is well known that myxococci do not in general form fruiting bodies on ordinary nutrient agar, and that the vegetative bacterial growth on this medium, being liable to autolysis within 10 to 15 days and being also tenaciously adherent to the agar before autolysis, is not very suitable for the inoculation of liquid media. Contrary to expectations aroused by the literature, the species here studied did not need special decoctions of dung or of eubacterial cells in order that a supply of long-lived cultures, containing both fruiting bodies and swarming rods, might be maintained. The organisms gave scanty but typical growth, with numerous fruiting bodies, on slopes of sucrose Czapek agar of the following composition: sucrose, 0.5 per cent; $NaNO_3$, 0.2 per cent; K_2HPO_4, 0.1 per cent; KCl, 0.05 per cent; $MgSO_4 \cdot 7H_2O$, 0.05 per cent; $FeSO_4 \cdot 7H_2O$, 0.001 per cent; and agar, 2 per cent. When an inoculation was made at the top of such a slope, swarming invariably took place at 24 C—most evenly when the surface of the agar was dry—with abundant production of tiny fruiting bodies, usually well behind the advancing edge of growth, which could easily be detached from the medium, either by striking gently with a loop or by shaking with a little sterile water. The bacterial growth was very thin indeed and traveled at so slow a rate that weeks elapsed before a 2-inch-long slope was covered. The practice has been to use almost the whole of the growth on a 3- or 4-week-old slope at one

time, in order to inoculate two or three tubes of liquid medium, each receiving the growth from an equal area on the slope. What was left of the growth was finally transferred to another sucrose Czapek agar slope. In this way an adequate supply of viable growth for the inoculation of liquid media at any time could be maintained. For larger volumes of liquid medium (100 ml or more) the whole growth from a slope was used as inoculum, after emulsification in a little sterile water.

4. *Composition of the simplest liquid medium which will support growth of myxococci.* Attempts to grow myxococci in liquid media with a sugar as the source of carbon, together with inorganic nitrogen, failed completely. Even with the amino acid medium described below, the addition of glucose or sucrose, sterilized separately, did not result in any increase of growth or production of characteristic metabolic products, such as an antibiotic substance or gelatinase. The sugars appeared to remain unfermented, even when good growth took place.

After many trials a basal medium was hit upon which would allow a just perceptible development of a film of growth of *M. virescens* A at 24 C, with a correspondingly small but detectable production of antibacterial activity in the metabolic solution, i.e., when 1.0 ml of the clear supernatant after a week's incubation was added to 4 ml of heart broth. This basal medium had the following composition: asparagine, 0.44 per cent; total acid hydrolyzate of casein free from polypeptides and carbohydrates (in powder form as supplied by Glaxo Laboratories, Ltd.), 0.06 per cent; K_2HPO_4, 0.4 per cent; NaCl, 0.2 per cent; and $MgSO_4 \cdot 7H_2O$, 0.02 per cent. It had a pH of 7.8, and had not the slightest inhibitory effect upon the growth of *Staphylococcus aureus* when it was added to heart broth to the extent of 30 per cent. In exploratory experiments 5-ml quantities of this filtered medium in 6-inch test tubes were sterilized by steaming, and after inoculation the tubes were well shaken and incubated in a sloping position for the reason mentioned in section 5b. To render the growth more evident, after decantation of the supernatant and washing once with distilled water, it was stained with filtered 0.1 per cent methylene blue for 10 minutes, again washed, and the tube allowed to dry at room temperature in an inverted position. The extent and intensity of the blue stain in the lower part of the tube was a measure of the filmlike growth of the myxococcus, all of which usually adhered to the glass. Although microcysts were formed in abundance in 14 days, the production of macroscopic fruiting bodies was observed under these conditions with *M. virescens* G only. The growth on submerged glass may therefore sometimes be different in appearance from that on agar.

Myxccocci may be maintained in serial cultivation in this liquid medium provided the sowing is made from a culture at least 2 weeks old and that the growth is first thoroughly broken up by shaking in order to obtain a suspension of free microcysts. When in doubt, a stained preparation should be made from a loopful of the well-shaken culture.

There was no evidence that the asparagine in the foregoing basal medium served as a nutrient or was fermented. The change in pH and the production of

ammonia was trifling even when the growth was good (see below). As far as could be judged, all metabolic solutions gave as intense a bluish-purple color with copper sulfate and caustic soda under the conditions of the biuret test as did the unsown medium. This color which is quite different from that given by polypeptides, was found to be due to asparagine and to no other constituent of the medium. The asparagine may therefore be regarded as an inert inorganic buffer, tolerated by myxococci, the casein hydrolyzate being the real nutrient. It is not yet known which amino acids are really essential for growth, since no artificial mixture of pure amino acids has so far proved adequate. Experiments along these lines are being continued.

Attempts to improve the medium so that it would support better growth of, and development of antibacterial activity with, *M. virescens* A succeeded only when casein hydrolyzate was substituted for all or part of the asparagine. For example, other things being equal, the film of growth was obviously many times thicker when the concentration of casein hydrolyzate was 0.85 per cent than when it was 0.06 per cent as in the basal medium. Not all pure amino acids are tolerated when added to the basal medium at a concentration of 0.4 per cent. Sodium glutamate was as readily tolerated as asparagine (glutamic acid is in fact the chief constituent of hydrolyzed casein). The basic amino acids arginine, histidine, and lysine were also tolerated, but glycine, alanine, or cysteine hydrochloride, separately or admixed, entirely prevented growth of *M. virescens* A when present in the medium at a concentration of 0.4 per cent. Finally, the addition of 5 mg of a vitamin mixture of the following composition—nicotinic acid, 1.5; riboflavin and pyridoxine, 1 each; calcium pantothenate and *p*-aminobenzoic acid, 0.5 each; aneurin, 0.4; and biotin, 0.00025 mg—to 100 ml of basal medium had no perceptible effect in stimulating growth or production of antibiotic substance.

5. *Physical factors influencing the growth of myxococci in liquid media.* When growth occurs from an inoculum of microcysts added to a clear, liquid, amino acid medium free from solid particles (see last section), there is, of course, no development of a turbidity, emulsifiable deposit, or surface pellicle as with eubacteria, but rather of a widespread and surprisingly coherent submerged film of bacteria. This film, the thickness of which depends on the concentration of nutrients in the medium, adheres rather tightly to the whole of, or to only a part of, the wet glass surface of the containing vessel, depending on its shape. Certain purely physical factors, which need not be considered in connection with the growth of ordinary eubacteria in liquid media, have therefore to be reckoned with. Some of these factors are:

(a) *Depth of liquid.* This is important, not so much because it affects the oxygen supply to the lower layers of liquid (for growth will occur quite readily at the bottom of a 6-inch test tube nearly filled with medium), but because it determines the speed at which the added microcysts will make stable contact with the submerged glass surface and thereafter serve as foci of growth. The following experiment showed that the microcysts tended to settle slowly: Several 6-inch test tubes half-filled with sterile medium were inoculated from slopes as described in section 3, and an even suspension of microcysts was produced in

each by vigorous shaking. They were incubated in the upright position for a day, then a number of them were inclined almost to the horizontal position, the rest being undisturbed, and the incubation was continued for 10 days. Only those tubes which had been inclined showed growth on the submerged wall of the tube as well as on the bottom. The upright tubes were then inclined and further incubated, but no great spread of growth took place, presumably because there were no more viable microcysts in suspension to settle on the extra, almost horizontal glass surface thus provided. It may be concluded that, if only a small inoculum is to be given, the layer of liquid medium should be as shallow as possible.

(b) *Availability of the submerged glass surface and the importance of the ratio of available surface to volume of medium.* Although myxococci can swarm vertically, i.e., against gravity, up an agar slope, none of the strains here studied could do so to any appreciable extent up a vertical, or steeply sloping, submerged glass surface. Two reasons for this may be advanced: (1) Few of the microcysts from the inoculum can stably settle on such inclined surfaces to provide foci of growth (see above). (2) Good growth from a given focus, even on a horizontal submerged glass surface, seems never to extend for more than a few mm in any direction. Hence the desirability of a massive inoculation with microcysts evenly distributed by shaking.

It follows that if the medium is contained in an ordinary flat-bottomed Erlenmeyer flask or in a cylindrical vessel such as the Glaxo container formerly used for penicillin production (Clayton *et al.*, 1944), the only part of the submerged glass available for supporting growth is the floor of the vessel. With such a cylindrical container 17 cm in diameter, containing liquid to a depth of 1 cm (a shallower layer than this is scarcely desirable), the horizontal floor area available for supporting growth of the myxococcus is 230 sq cm, the submerged vertical walls not so available occupy 54 sq cm, and the available surface per unit volume (hereafter referred to as A_v and expressed as sq cm per ml of liquid medium) is 1 sq cm, or $A_v = 1$. With a vessel of this type there is no practicable means of increasing A_v, and any increase in the volume of the medium will lower it. A_v becomes very small indeed in the case of an upright test tube half-filled with medium, but approaches unity if the test tube is held almost horizontally, when the greater part of the submerged glass becomes available to the myxococcus. The following device has been employed to increase A_v to 1.5 with a vessel holding 100 ml of medium: A liter, round-bottomed, spherical bolthead flask 13 cm in diameter contained liquid to a depth of 2.5 cm, giving roughly 100 sq cm of submerged glass, all of which is available to the organism (as proved by experiment) since the upward slope is never great. Extra available glass surface was supplied by the introduction of three 7.6-by-2.5-cm microscope slides, arranged almost horizontally below the surface of the liquid, two whole slides being side by side and the third, broken into two pieces, inserted below them. Naturally only the upper surface of the slides is available to the myxococcus, so that the extra surface available is $3 \times 7.6 \times 2.5 = 57$ sq cm. In using this arrangement it is best to sterilize the flask containing medium with the slides inside, inoculate, shake well, and then quickly arrange the slides in the

desired position by means of a sterile glass rod. One further advantage is that slides may be withdrawn aseptically from time to time and the growth upon them, which adheres to the glass so tenaciously that fixation is not required, examined microscopically after washing and staining with methylene blue, as described in section 4. The film of growth is usually rather too thick for profitable examination with the higher magnifications, but this difficulty might be overcome by cutting down the casein hydrolyzate in the medium to the absolute minimum.

It should be added that one strain, *M. virescens* G, did sometimes form very small elements of surface growth, apparently supported by surface tension, and occasionally also patches of growth spreading downwards from the surface over

TABLE 1

Antibacterial activity of metabolic solutions of M. virescens A at various stages of incubation

PERIOD OF INCUBATION	LIMITING DILUTION OF CLEAR METABOLIC SOLUTION IN HEART BROTH COMPLETELY INHIBITING S. AUREUS AT 37 C FOR:	
	1 day	2 days
days		
0	No effect	
2	No effect	
3	5	No effect
5	14, 40	14, 14
6	40, 40	14, 14
7	40	14
8	14, 40	14, 14
10	14, 40	14, 14
13	14	5
19	5	No effect
23	5, no effect	No effect
27	No effect	No effect

The medium contained casein hydrolyzate, 0.1%, and asparagine, 0.4%, and was distributed in 50-ml lots in a large number of 250-ml Erlenmeyer flasks, which were incubated side by side at 24 C.

the submerged vertical glass wall of a Glaxo container. The total area of such additional growth was, however, quite small in comparison with the growth on the floor of the vessel. As shown in the next section, the value of A_v ought to be as large as possible, not only for the maximum amount of growth but also to ensure the maximum metabolic activity.

6. *Production of antibiotic substance by M. virescens A.* The following facts have been established concerning this substance (or substances):

(a) The data summarized in table 1 show that it is not a stable product of metabolism, since it tends to disappear if the incubation is unduly prolonged—beyond 10 days.

(b) The better the growth, i.e., the greater the concentration of casein hy-

drolyzate in the medium, the better the production of antibiotic substance (table 2).

(c) For a given concentration of casein hydrolyzate in the medium, the greater the value of A_v (area of available submerged glass surface per unit volume of medium) the greater the concentration of antibiotic substance formed (table 3).

(d) The antibacterial effect is not due to an enzyme since solid products of considerable activity have been obtained which are soluble in ethyl or butyl

TABLE 2

Effect of casein hydrolyzate on antibacterial activity of metabolic solutions of M. virescens A

CONCENTRATION OF CASEIN HYDROLYZATE	ANTIBACTERIAL TITER (AS IN TABLE 1)	
	1 day	2 days
%		
0.06	5	5
0.1	14	14
0.33	70	40
0.5	200	85
0.85	200	70

The experiments were conducted with 5-ml quantities of medium in sloping 6-inch test tubes which were incubated for 11 days at 24 C. When the initial concentration of casein hydrolyzate was below 0.5%, asparagine was added to the medium to bring the total concentration of amino acids up to 0.5%.

TABLE 3

Effect of volume of liquid medium in a Glaxo container 17 cm in diameter upon production of antibiotic substance from 0.1 per cent casein hydrolyzate

VOLUME OF MEDIUM	A_v (AVAILABLE SURFACE/VOLUME)	ANTIBACTERIAL TITER (AS IN TABLE 1)	
		1 day	2 days
ml			
200	1.1	40	14
300	0.75	14	5
400	0.6	14	5
600	0.4	5	No effect
900	0.25	No effect	

The incubation period was 11 days in each instance. The floor of the vessel was covered with growth always, but not the vertical walls.

alcohol (see next section) and which have no proteolytic action at all. This was shown very simply by adding Esbach's protein reagent to those *S. aureus* assay tubes which remained clear after a week's incubation at 37 C. Those to which bactericidal metabolism solution, containing also protease, had been added gave no precipitate, but those to which a solution of the alcohol-soluble product had been added gave heavy precipitates, just as with sterile uninoculated heart broth itself.

(e) Seitz filtration removed part but not all of the antibacterial substance from an active metabolic solution. This substance was far more stable to heat at 60 C than the gelatinase also present in the metabolism solution (see section 8).

7. *Preparation of a crude solid product containing the antibacterial activity.* The antibiotic substance rapidly disappeared from the separated metabolism solution unless the latter was stored in the cold room. It appeared to be insoluble in the usual nonhydroxylic organic solvents at all pH values (or else inactivation took place during the extraction process), nor could it be eluted from charcoal without inactivation. The only feasible mode of extraction so far discovered was to evaporate the filtered metabolic solution to small volume *in vacuo* below 45 C, then immediately to take it down to dryness in a vacuum desiccator over conc. H_2SO_4, and to extract the solid so obtained with 100 parts of absolute ethanol at 55 to 60 C for an hour, with stirring from time to time to reduce the original gummy material to a state of fine division. The whole was then filtered and the filtrate evaporated to dryness in a vacuum desiccator at room temperature. In a typical experiment the contents of a Glaxo container, which originally contained 300 ml of medium (casein hydrolyzate, 0.33 per cent; asparagine, 0.3 per cent), were filtered after 12 days' incubation when the 1-day *S. aureus* titer was 70. The solid product obtained by evaporation of the metabolic solution weighed 2.8 g, and a 1 per cent aqueous solution of a small portion of it, after heating at 65 C for 6 minutes to kill extraneous eubacteria, had roughly the expected antibacterial activity (1-day titer = 40). An alcohol extraction, conducted on 200 mg as described above, yielded 13 mg of colorless solid, which gave the ninhydrin and other reactions for amino acids. One part of this solid in 12,500 parts of heart broth completely inhibited the growth of *S. aureus* for 2 days under the ordinary assay conditions. But little activity remained in the alcohol-insoluble residue, so considerable inactivation had taken place during the alcohol extraction. The active product, like the original metabolic solution, had no detectable inhibitory action on gram-negative bacteria.

The optimum temperature for the alcohol extraction seems to be 55 to 60 C. When boiling alcohol was used, the yield was greater but the product was, weight for weight, considerably less active, whereas an extraction at 37 C gave a smaller yield of a product with about the same activity. The further purification of the antibiotic substance is in progress. No advantage accrues by the use of dry butanol in place of ethanol, and wet butanol causes complete inactivation.

8. *Gelatinase production by myxococci in a protein-free medium.* Like certain eubacteria, species of *Myxococcus*, particularly *M. fulvus* and *M. virescens*, secrete gelatinase in a protein-free liquid medium, in this instance the amino acid medium previously described. The enzyme, as produced by the strains studied in this investigation, was very heat-labile, and at pH 7 to 8 was destroyed by heating the metabolic solution for 10 minutes at 60 C. Metabolic solutions of *M. virescens* G which were active in liquefying gelatine in 6 hours at 24 C under the conditions described in section 2 were also capable of clearing suspensions of killed gram-negative eubacteria (rendered nonviable by heat, by chemical poisons such as chloroform, or by age in the ordinary way) of con-

siderable opacity; e.g., an almost water-clear liquid resulted from the incubation for 6 hours at 37 C of a mixture of equal parts of the metabolic solution and a distilled water suspension of *Escherichia coli*, killed at 100 C, of opacity of 400 to 800 *m* according to Peskett's (1927) $BaSO_4$ standards. There was, however, no visible effect during the same length of time upon a suspension of viable coliform bacteria, freshly prepared from a young nutrient agar slope. The lytic enzyme was truly exocellular since it was found in a Seitz filtrate of a strongly proteolytic metabolic solution.

There remains the further question whether myxococci, like eubacteria, secrete gelatinase only in a medium containing calcium ions (Haines, 1931, 1932, 1933). The casein hydrolyzate used in this research was not quite calcium-free, nor has any simple method been yet devised for removing the calcium from it without impairment of its nutrient value toward myxococci. The latter have, however, been grown in washed and heat-killed suspensions of *Pseudomonas fluorescens* in distilled water, which were presumed to be calcium-free because the organism had been maintained for many subcultures in a liquid synthetic glucose nitrate Czapek-Dox medium (at pH 7) containing no calcium, since all the inorganic salts used were the purest obtainable. The metabolic solutions so prepared undoubtedly contained gelatinase, and there was no increased production of the enzyme observed when calcium chloride was added to the distilled water suspension of the washed pseudomonads before inoculation with microcysts of *M. virescens*. No case could therefore be made for any important role played by the calcium ion in the production of exocellular myxococcal proteinase.

DISCUSSION

A consideration of the solubilities of amino acids in absolute alcohol (Cohn and Edsall, 1943) shows that the antibiotic substance must have been extracted by this solvent together with certain monamino-monocarboxylic acids, chiefly valine and the leucines, which together constitute nearly 20 per cent of the total amino acids of hydrolyzed casein. At 25 C, for example, the volume of alcohol used in the extraction (20 ml) would dissolve about 2 to 3 mg each of valine and the leucines, and no doubt the solubilities of these amino acids are not less than this at 55 C. It is highly probable, therefore, that the greater part of the 13 mg of active solid obtained by alcohol extraction was in fact made up of valine and the leucines; hence the activity of the antibiotic substance, when obtained in the pure state, ought to be many times greater than the best value recorded in this paper.

Although the limited survey of the antibacterial activities of myxococci here described has yielded disappointing results, in that only one antibiotic substance has come to light, yet it might be well worth while to extend the study to include other genera in the great group of myxobacteria. The soluble proteolytic and bacteriolytic enzyme elaborated by myxococci also deserves further study since it may not be identical with the corresponding eubacterial enzyme. In particular, the inorganic ions required for its formation should be determined since they may be different from those required for the production of eubacterial proteinase.

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SUMMARY

Two strains of *Myxococcus virescens* will grow in a simple liquid medium containing the amino acids of a total acid hydrolyzate of casein as the sole source of carbon and nitrogen. They then form a coherent film in contact with all or part of the submerged glass surface, depending on the shape of the vessel, but are not able to spread vertically to any appreciable extent on wet glass. Gelatinase is secreted under these conditions, and in one instance a nonenzymic, alcohol-soluble, chemically labile antibiotic substance was produced also, active against gram-positive eubacteria only. Further quantities of asparagine, sodium glutamate, or the basic amino acids are tolerated by this myxococcus, when they are added as supplements to the casein hydrolyzate medium, but other amino acids such as glycine, alanine, or cysteine, if present in sufficient concentration, are definitely inhibitory to its growth. The antibiotic substance is associated with the valine and leucine components of the monamino-monocarboxylic acid fraction.

The production of gelatinase by myxococci probably does not need the presence of calcium in the medium as with eubacteria. Myxococcal metabolic solutions which contain gelatinase, but no antibiotic substance, are also markedly bacteriolytic toward nonviable gram-negative bacteria, but have no corresponding action on viable bacteria.

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