STUDIES ON THE LUMINOUS BACTERIA

I. Nutritional Requirements of Some Species, with Special Reference to Methionine

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Anyone who has had occasion to isolate luminous bacteria can readily understand why these organisms have received so much attention from microbiologists of the present and of the last centuries The aesthetic satisfaction derived from the contemplation of these beautiful organisms repays the investigator many fold for the hours spent in darkness and seclusion *vis a vis* some fetid putrefying fish. Due partly, no doubt, to this fact, a voluminous literature dealing with these bacteria has accumulated. For an excellent bibliography and a review of all the most important contributions to the knowledge of the luminous bacteria and to the understanding of the physiology of luminescence, reference need only be made to Pratje (1923) and Harvey (1940, 1941).

Owing to the recognition that the light-emitting process is a by-path of respiration, and to the convenience of using changes in the intensity of luminescence as an indicator of physiological events in the organisms, the luminous bacteria have acquired, in the last few years, a new importance in scientific research, as material for exceedingly interesting experiments on general physiological problems. (E.g., Johnson, 1938, 1939.) Little attention, however, has been devoted to the natural history of this group of bacteria since Beijerinck's studies (Beijerinck, 1889, 1912, 1916). Although innumerable species have been described, no thorough systematic study of the group has ever been undertaken with a view to clarifying their relationship among themselves or with other bacteria.

In the hope of contributing to the elucidation of some of the complex taxonomic and physiological problems involved in the sudy of this group of organisms and to the better understanding of some previously recorded observations on the behavior of the bacteria under certain conditions, a series of investigations has been carried out on the nutrition and metabolism of several species, the results of which seem to be of sufficient general interest to warrant their publication.

In the present paper, the results of experiments on growth requirements of a number of strains will be presented.

MATERIAL AND METHODS

The various strains of luminous bacteria used in the present studies were obtained as follows. A culture of *Photobacterium phosphoreum* and one of *Photobacterium sepiae* from Prof. A. J. Kluyver's collection at Delft, were kindly supplied by Dr. F. H. Johnson, as was the type strain of *Achromobacter harveyi*

and the strain identified by Dr. Johnson as *Photobacterium fischeri* and used by him in the redescription of that species (Johnson and Shunk (1936)). A culture of *Photobacterium splendidum*, probably representing the original, Beijerinck strain, was given to me by Dr. C. E. Clifton, who obtained it also in Delft. Beside the strain of *Photobacterium phosphoreum* already mentioned (to be referred to as the "Delft strain"), ten morphologically and physiologically similar strains were selected for experimentation from those isolated on various occasions at Pacific Grove and Berkeley, California. Of these, nine, including strain no. 2 and the strain used in previously reported experiments (Doudoroff, 1938) were isolated from decomposing flatfish and one (Strain 12) from dead squid.

The basal medium consisted of distilled water containing M/30 Sörensen KH_2PO_4-Na_2HPO_4 phosphate buffer mixture at pH 7.0; 3 per cent NaCl; 0.03 per cent NH_4Cl; 0.03 per cent $MgSO_4$; 0.001 per cent $FeCl_3$; 0.001 per cent $CaCl_2$; and 0.1–0.3 per cent of the organic carbon source by weight. No attempt was made to study in detail the mineral nutrition of the organisms, and traces of all essential elements were assumed to be present in the reagents used.

Synthetic organic compounds were used wherever possible. Glucose was autoclaved separately in distilled water, since it was found to give rise to exceedingly toxic products if autoclaved with phosphates. Thermo-labile compounds were sterilized separately by filtration through glass filters and added to the sterile basic medium.

The medium was dispensed in 4 ml. amounts in large pyrex test tubes to insure an adequate oxygen supply, no special means of aeration or agitation being used.

Transfers of the organisms from tube to tube were made with a platinum loop of 0.003 ml. capacity, except in experiments with *P. phosphoreum*, where 0.05 ml. of the culture was used for inoculation. This large inoculum was selected for three reasons. Firstly, the development of this species was quite slow in synthetic media, and a great deal of time could be saved by using an inoculum which would give rise to an earlier development of visible turbidity. Secondly, results obtained with smaller inocula were often inconsistent, due, apparently, to the very rapid senescence and autolysis of the bacteria, especially in carbohydrate-containing media, after the "maximum stationary phase of development" was attained. Thirdly, it appeared that in many cases an "adaptation" of the culture, involving the selection of individuals possessing special capabilities was necessary for the development of cultures upon transfer to a medium of a different composition from that to which the bacteria had become accustomed; and a larger inoculum would provide greater chances of obtaining "adaptable" cells (Doudoroff, 1940). Since the use of heavy inocula involves a great "carry-over" of any added substances from one culture to the next, no synthetic medium was regarded as satisfactory unless the bacteria could be cultured with it through at least seven serial transfers.

Whenever an attempt at substitution of one of the constituents of a satisfactory medium by another resulted in the failure of the organisms to develop upon initial transfer, the experiment was repeated, using a series of concentra-

tions of the new factor and, in some cases, different initial pH values of the basal medium. Furthermore, the bacteria were allowed to develop in a medium containing a satisfactory nutrient factor together with the one under investigation and again transferred to the altered medium. Such cultures not only served as tests for the possible toxicity of the new factor, but in some cases apparently favored the "adaptation" of the organisms to the new conditions. Tubes showing no development were usually kept for from fifteen days to a month before being discarded.

NUTRITION OF P. FISCHERI, P. SPLENDIDUM, P. SEPIAE AND A. HARVEYI

All of the above species developed readily in the synthetic basal medium with glucose as sole carbon source, with mean division times from 100 to 150 minute at 18–20°C. Growth was considerably slower with synthetic glycerol[1] in place of glucose, the division rate being approximately one third of that observed with glucose. On first transfer to synthetic medium with glycerol, *A. harveyi* developed still more slowly, but after two subcultures in this environment a constant rate of reproduction comparable to that found in other species was established. With alanine as the only substrate, *A. harveyi* propagated quite readily, *P. sepiae* and *splendidum* very slowly, and *P. fischeri* not at all.

Unlike *P. phosphoreum*, which always showed fairly good luminescence in synthetic media, the other species emitted very little light or none at all, retaining, however, their ability to luminesce upon return to complex media.

NUTRITION OF P. PHOSPHOREUM

In contrast to the other species studied, most strains of *P. phosphoreum* did not develop readily in the basal medium with a single carbon source.

Experiments with strain no. 2

Strain no. 2 was used for a series of investigations which were carried out over a two-year period. This strain did not propagate in the basal medium with either glucose, glycerol, or alanine as carbon source, unless a mixture of amino acids was also added. By the process of elimination, it was found that the effect of the mixture could be duplicated by the use of a single amino acid: dl-methionine. Methionine could not be replaced by any other single pure compound or any of several hundred combinations tried.

With glycerol as chief carbon source, distinct turbidity due to bacterial development could be detected when a concentration of methionine corresponding to 0.2 mg. per liter of culture medium was used. With higher concentrations, more growth was obtained, maximum development occurring with from 3 to 5 mg. per liter. Greater amounts (up to 20 mg. per liter) had neither a more favorable nor any unfavorable effect. Approximately the same concentrations of methionine were effective with glucose, lactate, succinate, fumarate, or alanine as chief carbon sources in place of glycerol.

It should be pointed out that ordinary samples of leucine, which contain

[1] Synthetic glycerol was kindly furnished by the Shell Development Company.

methionine as an impurity, could be used in place of methionine, as well as one sample of tryptophane (which theoretically should not be so contaminated). However, neither synthetic dl-leucine nor methionine-free l-leucine, nor other samples of tryptophane tested had any activity. An interesting complementary effect of leucine and methionine came to light in these experiments. Leucine, which, in itself, is apparently unsatisfactory either as carbon or nitrogen source to the organisms, in some manner "protected" methionine, so that the addition of 0.01 per cent dl-leucine to the basic medium with glycerol made it possible to obtain maximum growth with as little as 0.3 mg. of methionine per liter.

The following substances could not be substituted for methionine either alone or in any combination so far tried: sulfur, sulfide, sulfate, thiosulfate, thiourea, thioglycollate, cystine, S-methyl-cysteine, benzyl hemocysteine, homocystine, glutathione, thiamine, glycine, alanine, serine, threonine, α amino-n-butyric acid, valine, leucine, norleucine, isoleucine, aspartic acid, asparagine, glutamic acid, arginine, tyrosine, phenylalanine, tryptophane, proline, choline, as well as a number of organic acids, alcohols, and recognized growth-promoting substances.

Although the addition of mixtures of amino acids to the medium increased the growth rate of the organisms considerably, the same amino acids added singly often had a distinctly inhibitory effect. Thus, the addition of 2 mg. of homocystine or S-methyl-cysteine per liter of glycerol-methionine medium either considerably delayed or completely prevented development, whereas 5 to 10 mg. per liter could be easily tolerated if, in addition, a little peptone or a mixture of glycine, alanine, leucine and asparagine was added. α amino-n-butyric acid was inhibitory in even lower concentrations unless traces of peptone were present.

With methionine as accesory factor, glucose, glycerol, lactate, pyruvate, succinate, fumarate, alanine, aspartic acid and asparagine were found to be satisfactory carbon sources. On the other hand, formate, acetate, malate, glycine and leucine appeared to be unsatisfactory. Cultures developing with glucose and, to a lesser degree, with glycerol tend to become acid, and the organisms in such cultures show senescence and autolysis sooner than those developing with substrates such as organic acids and amino acids, the utilization of which usually results in the increased alkalinity of the medium.

When serial transfers were made in media with certain carbon sources, a very long "lag phase" preceding visible development of the bacteria was often encountered, even though the organisms were transferred to the same medium in which they had propagated. Indeed, occasionally, subcultures failed completely, although the bacteria were still luminous and viable in peptone media at the time of inoculation. Such behavior of the bacteria was most striking in media with pyruvate, lactate and fumarate as chief carbon sources, but was also observed in cultures with succinate, aspartic acid, and asparagine.

The lag period observed in such instances could be greatly reduced or eliminated if a larger inoculum was used or if a trace of glycerol was added to the fresh medium. It was further found that, in the case of cultures grown with pyruvate, the introduction together with the inoculum of some of the culture medium in which the bacteria had developed, freed of the organisms by centrif-

ugation or filtration, had a similar effect to that of using a large inoculum. The stimulating factor or factors were absent from the neutral distillate of such a medium but could be recovered in the steam-volatile acid fraction as well as in the residue after steam distillation. Since formic, acetic, lactic and succinic acids were found to be among the products of fermentation by *P. phosphoreum*, these compounds were tested for growth-promoting activity. Indeed, the addition of 0.002 per cent of sodium formate, acetate, lactate, succinate, or fumarate hastened development with pyruvic acid as chief carbon source. Varying the CO_2 partial pressure by the addition of bicarbonate to the medium, of CO_2 to the gas phase, or both, did not seem to affect the growth. With lactate as chief carbon source, fumaric, succinic and aspartic acids exhibited growth-stimulating properties, although not as marked as those of glycerol. To a lesser degree, pyruvic, lactic, formic, acetic, and aspartic acids stimulated development in a fumarate-containing medium.

Experiments with eight similar strains

Eight other strains of *P. phosphoreum*, tested for their ability to grow on synthetic media with and without methionine after more or less prolonged cultivation on peptone-glycerol agar, developed in the presence, but not in the absence of methionine. On first transfer to synthetic media, two of these developed with alanine and methionine but not with glycerol and methionine, two others grew in the latter but not in the former medium, while the other four developed with either carbon source supplemented with methionine. After developing in a synthetic medium, all strains were found to be capable of using both alanine and glycerol as chief carbon sources, but still unable to grow in methionine-free medium.

Experiments with strain no. 12

In contrast to the previously discussed strains, strain no. 12, a particularly hardy and rapidly growing culture of *P. phosphoreum*, was found to be capable of developing without methionine in the basal medium with glucose, glycerol, or alanine as sole carbon sources. However, in the first transfers to synthetic medium, methionine had a strikingly stimulating effect, which became somewhat less pronounced after prolonged cultivation in methionine-free media. With glucose as carbon source, it could be shown that transfers made from the logarithmic or the very early stationary phases of development were less affected by methionine than those made from the later stationary phase. In fact, if the transfers were made at a properly selected time, the organisms did not develop at all without added methionine, while as little as 0.1 mg. of this substance per liter was sufficient to initiate growth. Transfers made from the senescent phase could no longer develop in media with methionine unless peptone was also added.

An unexpected observation was made with one of the cultures of this strain that had been grown for some time with alanine as sole carbon source. Methionine, added to the alanine medium, was in some cases inhibitory to development

in the same concentrations in which it exerted a stimulating effect on the same culture transferred to media with glycerol or glucose. This inhibitory effect, demonstrated by a prolonged lag phase, was noted only if the transfers were made during the logarithmic phase or before maximum development had been reached and not in the case of transfers made from older cultures.

Experiments with the Delft strain

In the first attempts to cultivate the "Delft strain" of *P. phosphoreum*, in synthetic media, good growth was obtained in the basal medium with either glucose or glycerol (but not with alanine) as chief carbon source, provided methionine was also added. From such cultures, the organisms could be transferred to the alanine-methionine medium, in which the bacteria propagated readily. Attempts to cultivate the organisms in methionine-free media were generally unsuccessful, with a few interesting exceptions. As a rule, the amount of growth was found to depend on the concentration of methionine in a manner similar to that observed with strain no. 2. Also, as with strain no. 2, cystine, S-methyl-cysteine, glycine, alanine, leucine, asparagine, proline, choline and, in addition, creatine could not replace methionine. However, homocystine, which was apparently unsuitable to the other strain, was found to be a good substitute for methionine with the Delft strain, provided the bacteria were first allowed to grow in the basal medium containing glucose and both methionine and homocystine. Surprisingly, it was found that from the media in which homocystine was used in place of methionine, the bacteria could now be transferred to media containing glucose or glycerol alone, and could be cultivated indefinitely in such media without any accessory growth factors. The first transfers from homocystine-containing to homocystine-free media sometimes gave rise to imperfectly developing cultures, which showed but slight turbidity followed by rapid autolysis. However, transfers to the same medium from such abortive cultures at the peak of their development gave rise to vigorously growing sub-cultures. It must be added that after several unsuccessful attempts a single culture requiring no accessory factors was also obtained by heavily seeding a methionine-free medium containing alanine with bacteria from an alanine-methionine culture which had not yet reached the stationary phase of development. Growth was very slow at first, but after several transfers the rate of development increased. It therefore seems probable that cells not requiring any accessory factors occur occasionally in all cultures of the bacterium. A conceivable explanation for the "weaning" of the bacteria from accessory growth factors by the intermediate substitution of homocystine for methionine could be offered on the assumption that the latter compound serves several purposes to the organisms, not all of which (*e.g.*, source of methyl group) can be served by the former. An "adaptation" of the culture either through "acclimatization" of the cells or the selection of those least dependent on methionine might thus have been accomplished by cultivation in a partially deficient medium.

The cultures "adapted" to growth without accessory factors were benefited by the addition of methionine or homocystine. As with strain no. 12, the

growth-promoting effect of methionine was most pronounced if transfers were made from cultures in the stationary phase of development.

It here seems appropriate, although not altogether pleasant, to record some further observations on the somewhat paradoxical behavior of some cultures of this strain under certain conditions, which at present seem further to confuse the already somewhat complex picture of the growth requirements of the species.

Although the "Delft strain" could tolerate considerably higher concentrations of homocystine than strain no. 2, amounts greater than 5 mg. per liter of glucose methionine medium were found to be somewhat inhibitory to its development. A "detoxification" similar to that observed with strain no. 2 and a marked increase in the rate of growth could be accomplished by adding a mixture of small amounts of glycine, alanine, leucine, asparagine and proline. However, after the organisms had become accustomed to homocystine and this compound was substituted for methionine as the accessory factor, the same mixture of amino acids exhibited a somewhat retarding action on growth in glucose-homocystine medium.

As stated earlier, even after the "adaptation" of cultures to an existence in an environment containing neither methionine nor homocystine, the addition of these compounds stimulated their growth. Seemingly anomalous results, however, were occasionally obtained in experiments in which the effect of different concentrations of methionine on the development of such cultures was tested. Without added methionine, the cultures grew somewhat more slowly, but to as great an extent as those receiving 3 to 5 mg. of methionine per liter. Those, however, developing with low concentrations of methionine (0.1–0.5 mg. per liter) grew rapidly at first, but showed considerably less turbidity at the peak of development, the magnitude of the maximum crop depending on the concentration of the accessory factor. Although usually the bacteria underwent a rather rapid autolysis after the stationary phase was reached, on one occasion a rapid but slight development in a methionine-poor medium was followed at first by the usual stationary phase and partial autolysis, and then by a second period of growth, leading to turbidity as heavy as that obtained in cultures under optimal conditions. Two possible interpretations of the peculiar response of the bacteria to low concentrations of methionine, which seems to controvert the general belief that half a loaf is better than no bread at all, may be suggested: a) The addition of small amounts of methionine to the medium may result in the preferential propagation of cells incapable of growing without this factor and a subsequent change in the external environment due to the development of such cells, which, in turn, inhibits the multiplication of those cells for which methionine is unnecessary; or, more probably, b) The increased rate of development in a medium containing little methionine leads to the exhaustion or destruction of some internal mechanism in the cells which is necessary for propagation in the absence of the accessory factor. The lack of sufficient methionine, possibly coupled with some external changes brought about by the development of the culture, may then become limiting to growth. Somewhat

in contradiction to either of the above hypotheses was the result of a single experiment, in which a culture which had been adapted to growth without methionine was allowed to develop in the presence of a sufficient amount of this substance to permit maximum growth, and then transferred back to methionine-free medium, wherein profuse growth was observed.

DISCUSSION

In view of the demonstrated ability of at least some strains of all of the luminous bacteria studied to grow in synthetic media with a single carbon source, the distinction made by Beijerinck (1916) between types requiring peptone and those requiring both peptone and carbohydrate loses most of its significance. At first, a clear-cut physiological differentiation of *P. phosphoreum* from the other species studied seemed possible on the basis of its requirements for methionine, but the cases of adaptation of some strains to media without this compound have made clear the illusory nature of such criteria. The danger of relying on nutritional requirements for the identification of species was clearly shown by the many cases of "adaptation" of various strains to different substrates.

The experiments on the role of methionine certainly do not clarify the function of this compound in the metabolism of *P. phosphoreum*, and it seems likely that methionine may serve more than one purpose to the organisms. (For known functions, see Toennis, 1937; White, 1941; Lewis, 1941; also Harris and Kohn, 1941.) It seems clear that complex media are preferred by this species to those in which the minimum requirements are just satisfied, since the addition of a variety of non-essential substances serves not only to hasten development, but in some way to counteract the inhibitory action of compounds which are toxic when added singly. Cultures growing in the simplest media may, therefore, be thought of as artifacts; and the remarkable adaptability of the organisms, coupled with their sensitivity to external environmental factors leads to the tentative conclusion that each separate culture may be regarded as a delicately balanced system of living cells and their environment, each in a constant state of change and flux. As a further example of the changes in nutritional requirements of one of the strains of *P. phosphoreum* used in these studies, it need only be recalled that dissociates apparently unable to synthesize riboflavin were obtained in previous experiments, as well as "atavistic" back-dissociates from such deficient variants (Doudoroff 1938).

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SUMMARY

1. *Photobacterium fischeri*, *P. splendidum*, *P. sepiae*, and *Achromobacter harveyi* were found to be capable of developing in inorganic media with simple organic compounds as the sole carbon source, while most strains of *Photobacterium phosphoreum* did not grow unless methionine was added as an accessory factor.

2. In experiments with one strain of *P. phosphoreum*, no compound or combi-

nation of compounds tried could replace methionine. Among the ten other strains tested, one was found for which no accessory factor whatsoever was essential, although methionine had a growth-promoting effect. Cultures of another strain studied could be "weaned" from methionine to accept homocystine as substitute, or even to develop in the absence of any accessory factor.

3. The complementary action of leucine and methionine, the "detoxification" of media to which certain inhibitory compounds were added by the addition of other compounds, and the stimulating effect of a variety of substances on the initiation of growth in certain media were observed in the studies with *P. phosphoreum*.

4. Seemingly anomalous effects of methionine and of mixtures of amino acids on growth, and the occurrence of "abortive" cultures were occasionally observed under certain conditions.

5. It would seem that a great many factors, including the previous history of the bacteria and the changes in both the organisms and their environment resulting from their growth play an important part in determining the course of development of each culture.

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