ORGANIC GROWTH ESSENTIALS OF THE AEROBIC NONSULFUR PHOTOSYNTHETIC BACTERIA[1]

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Ever since the nonsulfur purple and brown bacteria were defined as a taxonomic entity—the *Athiorhodaceae*—it has been recognized that one of their distinctions from other photosynthetic bacteria was their failure to grow in inorganic media even in the presence of a suitable carbon-hydrogen-energy source ("substrate"); they had additional nutritional requirements which were met by inclusion in the medium of a small amount of yeast extract or peptone (van Niel, 1941, 1944). Despite this experimental imprecision, studies of these bacteria contributed data indispensable in erecting a satisfactory theory of the mechanism of photosynthesis (van Niel, 1941, 1944; Franck and Gaffron, 1941; Rabinowitch, 1945). The facultative aerobes in this group can grow aerobically in the dark—an indication of the manner in which the photosynthetic and nonphotosynthetic ways of life may have been linked phylogenetically. This fundamentally important type of evolutionary transition has been studied intensively from the nutritional standpoint only in algae, algal flagellates, and their colorless counterparts (Lwoff, 1944). It was evident from these considerations that the identification of the essential nutrients for *Athiorhodaceae* was of extraordinary interest from the standpoint of comparative cell physiology.

To summarize the results of the present study: of 124 aerobic isolates tested, 121 grew in synthetic media containing thiamine, biotin, *p*-aminobenzoic acid, nicotinic acid, or an appropriate combination of these. Different strains assigned by other criteria to the same species were remarkably similar in vitamin requirements, and each of the 5 species studied had a different pattern of vitamin requirements.

The technique for an investigation of unidentified growth factors should be rigorous enough to detect with certainty new vitamins of even greater ubiquity and potency than *p*-aminobenzoic acid, biotin, and folic acid. Procedures were developed which seemed adequate for the purpose and were convenient. As some of these methods may be unfamiliar in detail, an extended description follows.

To obtain large numbers of cells with a minimum of manipulation and a minimal carry-over of nutrients, and to exercise closer control over the purity of cul-

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tures, it was considered desirable to inoculate flask cultures from agar slants. All but 5 or 6 of the isolates grew well on moist slants, the greater number growing very heavily. Screw-capped tubes proved excellent for minimizing drying of the slants. After seeding, the plastic caps could be closed tightly without noticeable hindrance to growth. When water of syneresis was insufficient, a few drops of distilled water (likewise sterilized in screw-capped tubes) were added. It was found desirable to remove the liners from the caps in order to avoid unintentional sealing and consequent building up of pressure differences when the caps were screwed on loosely for sterilization. The experiments were conducted with 25-ml Erlenmeyer flasks containing 10 ml of medium and capped with 10-ml beakers; 50-ml flasks supported growth no better. Many flasks had their rims ground down on a fine emery wheel to enable the beakers to fit easily.

Inoculation pipettes were sterilized by autoclaving in individual pyrex tubes plugged at both ends with cotton that had been sterilized repeatedly. Dry sterilization led to the separation of tarry breakdown products from the cotton. The autoclaved pipettes in their tubes were dried at temperatures below 80 C. By this procedure the pipettes were not subjected to contact with metal containers, with risk of toxicity and of interference with experiments on trace element requirements.

All media were autoclaved 10 minutes at 118 to 121 C. In preliminary trials, a disquieting number of air-borne contaminations were observed in the beaker-capped flask cultures. These contaminations were traced to the overrapid influx of air into flasks cooling after autoclaving and were eliminated by allowing the flasks after sterilization to cool in the autoclave over a period of at least 5 hours, with the exhaust valve kept shut. Then, if a vacuum was still present, air was admitted very gradually. Contaminations of agar slants were similarly minimized by allowing the melted agar to cool in the autoclave in a slanted position and by then screwing the caps on tightly as the tubes were removed.

A potential source of error was that resulting from inapparent bacterial growth in components of culture media supplied from stock solutions. Even a scarcely perceptible growth of microorganisms in a solution, followed by their death and lysis and by the restoration of clarity to the solution, might furnish a significant residuum of growth factors. It was inconvenient to weigh out every component of the medium for each experiment, and especially inconvenient for trace elements. Satisfactory preservation of highly putrescible solutions of yeast extract and peptones could be achieved by storing such solutions in glass-stoppered pyrex bottles, adding at least 1 per cent of a 1:1 mixture of redistilled CCl_4 and toluene, and keeping at 6 C. These solvents were completely removed on autoclaving. Constant vigilance had to be exercised to detect and prevent microbial spoilage of solutions. For instance, solutions in pyrex bottles of salts of boron and molybdenum acidified with HCl, but when kept at room temperature, eventually became contaminated despite the presence of a preservative. Subsequent experiments revealed that these salts had a favorable assortment of trace element impurities—perhaps the reason they were good substrates for the germination of

food-laden bacterial and fungal spores. After extensive trials[3] it appeared likely that a 3:1 mixture of *n*-butyl chloride and CCl_4 formed a more effective volatile preservative.

The following simple inoculation procedure reduced carry-over effects to negligible proportions. A loopful of slant growth was suspended in 20 ml of the basal medium in a "dilution" flask, and each experimental flask received one drop of this supension. From time to time carry-over effects were estimated by suspending a loopful of culture from a flask culture into a fresh dilution flask and by inoculating a duplicate series of flasks. No significant carry-over effects were observed. It was sometimes necessary to seed a hundred or more flasks from one dilution flask, using a single pipette. The risk of contamination incurred by this eggs-in-one-basket procedure was effectively minimized by avoidance of the violent air currents generated by a flame. The flame was used only for sterilizing the wire loop; the preparation of the cell suspension and the subsequent pipetting proceeded with the flame turned off.

All cultures were grown at room temperature (22 to 30 C) under 40-watt tungsten lamps at a distance of 30 to 60 cm that was arranged to furnish fairly even illumination. The illumination did not appear critical except in respect to the danger of overheating the cultures, and appeared adequate for even relatively dense cultures. As many of the bacteria did not grow well above 31 C, overheating was a serious problem when large numbers of flasks were used at one time during the summer.

Agar slants. Many different media proved suitable for the maintenance of cultures. The substrate was adequately supplied as lactate, 0.2 to 0.4 per cent, or malic acid (natural or synthetic), 0.1 to 0.4 per cent; occasionally Na-acetate. $3H_2O$, 0.05 to 0.1 per cent, or Na-butyrate, 0.04 per cent, was added to malate and lactate media. Media not containing malate contained Na_3-citrate $.2H_2O$, 0.025 to 0.1 per cent, to ensure full availability of heavy metals and calcium. The unidentified requirements were adequately supplied as trypticase (Baltimore Biological Laboratories), 0.1 to 0.2 per cent, or thiopeptone (Wilson), 0.1 per cent. Yeast extract (Difco) was inhibitory to many strains; trypticase was noninhibitory and permitted extremely good growth. The remainder of the medium consisted of agar, 1.5 per cent; small amounts of K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $(NH_4)_2HPO_4$, and Fe, 0.1 to 0.4 mg per cent; and Mn, 0.05 to 0.2 mg per cent. The pH was adjusted to 6.5 to 6.8. A few isolates designated "*Rhodovibrio*," which appeared to be microaerophilic, and a few isolates of *Rhodospirillum rubrum*, which, although uninhibited by air, seemed unusually sensitive to inhibitory substances or were exacting for other reasons, were grown on the same media rendered semisolid by decreasing the agar to 0.2 to 0.4 per cent.

The growth of slant cultures was usually heavy in 24 to 48 hours. They were then stored in the dark at 6 C. They remained satisfactorily viable for at least a month; indeed many strains grew appreciably during such storage. Cultures

[3] Bjerknes, Clara A., and Hutner, S. H. Presented before the New York City Branch of the Society of American Bacteriologists, April, 1946.

older than a month were not, however, used for inoculating experimental flasks. There was no obvious impairment of photosynthetic ability as a result of this treatment.

RESULTS

Of 17 isolates previously identified as *Rhodospirillum rubrum*, 15 required biotin; 2 did not grow in the synthetic medium. Among the isolates growing in synthetic media were the Esmarch, Muller, and Lister strains. The isolates not growing in synthetic media were those designated at no. 5 (E III 2.1.I.b)and no. 8 ("Giesberger").

All 34 isolates of *Rhodopseudomonas palustris* required *p*-aminobenzoic acid and grew readily in synthetic media. Isolates used by Gaffron were included in this collection.

TABLE 1

Basal medium for identification of vitamin requirements

	g		mg
K_2HPO_4	0.05	B	0.005
KH_2PO_4	0.05	Ca	0.5
$(NH_4)_2HPO_4$	0.08	Cu	0.001
$MgSO_4 \cdot 7H_2O$	0.02	Fe*	0.2
Lactate	0.3	Ga	0.001
Na-acetate$\cdot 3H_2O$	0.1	Mn†	0.1
Na_3-citrate$\cdot 2H_2O$	0.1	Zn	0.2

Distilled water to 100 ml

pH adjusted to 6.6 to 6.8 with NaOH

Vitamins supplied when necessary as follows: thiamine 0.1 mg, nicotinic acid 0.1 mg, *p*-aminobenzoic acid 0.01 mg, and biotin‡ 0.4 μg per cent. Elements obtained as metals were dissolved in a small amount of HCl, HNO_3, or aqua regia.

* Westinghouse high purity iron supplied through the courtesy of Mr. E. B. Ashcraft.

† Electrolytic manganese kindly furnished by the U. S. Bureau of Mines.

‡ Gift of Merck and Co., Inc.

In the early experiments with lactate media, 14 of the 15 isolates of *Rhodopseudomonas capsulatus* required thiamine alone. The same batches of media had been successfully used in detecting the biotin and nicotinic acid requirements of *R. gelatinosa* and *R. spheroides*. On rechecking these early results with purer media containing synthetic malate, however, thiamine alone was inadequate for 2 of the 3 isolates tested; biotin plus nicotinic acid was also necessary. Further work is under way to determine in what manner the vitamin requirements of strains of this species vary with the other constituents of the medium. The strain that in many experiments did not grow in synthetic media was the one designated as no. 26 ("*Streptococcus varians* C10").

All 20 isolates of *Rhodopseudomonas gelatinosa* required biotin + thiamine, and all 17 isolates of *R. spheroides* required biotin + thiamine + nicotinic acid. Among these isolates was one designated as "*Streptococcus varians* (original)" and two designated as "*Phaeomonas*."

It is evident that success was obtained in determining the requirements of the aerobic strains employed by other workers in studies of photosynthesis.

Twenty-one miscellaneous unclassified isolates were assigned to one or another of the 5 species on the basis of vitamin requirements; a superficial examination showed no obvious discrepancies between the new taxonomic criterion represented by the growth factor requirements and the original criteria for these species. The diagnostic characters employed by van Niel (1944) to delimit these species were morphological (shape and arrangement of cells, color, and mucus formation, all at different pH's), and biochemical (liquefaction of gelatin and utilization of various oxidation substrates). The results are summarized in table 2.

TABLE 2

Vitamin requirements of the aerobic nonsulfur photosynthetic bacteria

	BIOTIN	*p*-AMINO-BENZOIC ACID	THIAMINE	THIAMINE + BIOTIN	THIAMINE + BIOTIN + NICOTINIC ACID
Rhodospirillum rubrum	+				
Rhodopseudomonas palustris		+			
Rhodopseudomonas capsulatus			+*		+*
Rhodopseudomonas gelatinosa				+	
Rhodopseudomonas spheroides					+

* Some strains.

DISCUSSION

The uniformity of the vitamin requirements suggested that there might have been some duplication of strains. There was, however, considerable color and morphological variation among isolates within each species—a good indication of heterogeneity. Also many of the isolates were obtained from different localities and by different enrichment procedures. Most of the isolates studied here were obtained by Foster (1944), who used a variety of alcohols as enrichment substrates. In keeping with the taxonomic scheme proposed by van Niel (1944), *Rhodopseudomonas palustris* and *Rhodospirillum rubrum* thus form two distinct and rather homogeneous species, standing apart from each other and from the group represented by the rather similar *Rhodopseudomonas capsulatus*, *R. gelatinosa*, and *R. spheroides*. It is remarkable that no isolates were found without vitamin requirements, and that each of the 5 species should have a different vitamin requirement, considering how few growth factors were involved. The results thus furnish a neat example of how nutritional data may support taxonomic surmises based on other biochemical and morphological criteria. It will be of interest to determine whether the vitamin requirements are the same for cells grown in the dark, or in anaerobiosis. The problems of the nutrition of the obligate anaerobes and of the more exacting aerobic strains remain for the future.

The absence of any amino acid requirement, despite the presence of so many

vitamin requirements, is an unusual state of affairs in microbiology. The stimulating effect of protein hydrolyzates, noted earlier (Hutner, 1944), disappeared when basal media were developed that contained good substrates and adequate amounts of trace elements.

It proved very difficult to duplicate in synthetic media the favorable effects of amino acids and protein hydrolyzates. The conclusion became inescapable after very many experiments that the limiting factors for growth, in the absence of compounds of biological origin, were likely to be essential elements as yet unidentified but occurring as extraordinarily potent impurities in the usual cp grades of trace elements.

Trace element requirements. Paradoxically, organisms with simple growth requirements present difficulties perhaps not fully appreciated by workers whose principal experience has been with forms having complicated organic requirements. Many compounds of biological origin commonly used in culture media, particularly those with metal-complex-forming groups, are heavily contaminated with essential trace elements; asparagine, glutamate, and sugars are noteworthy in this respect. Nearly all the photosynthetic bacteria here studied could be grown in media containing as sole organic constituents synthetic vitamins contributing an insignificant amount of trace elements, plus carbon-hydrogen-energy sources in the form of fatty acids or alcohols rendered metal-free by distillation. Hence in replacing biological materials such as yeast extract, peptones, natural amino acids, and protein hydrolyzates with purified synthetic compounds, it became necessary to make a special effort to provide adequate amounts of the trace elements required. The success of the medium described in table 1 was shown by later experiments to depend on some largely unforeseen factors:

(1) The *lactate* (prepated by neutralizing reagent lactic acid with ordinary reagent grade NaOH) was heavily contaminated with favorable trace elements. The source of this contamination was probably not only attributable to the biological origin of the lactic acid, but also to the calcium or zinc salts through which the lactic acid was purified and to the NaOH used for neutralization. Commercial salts of calcium and zinc, and ordinary NaOH appeared to be good carriers of essential trace elements. Lactate was utilized with a readiness unsurpassed by any other compound tested and, unlike the fatty acids, was completely devoid of inhibitory properties, even in relatively high concentrations (0.3 to 0.5 per cent) and through a wide pH range (6.3 to >8.5).

(2) The *trace element supplement* supplied the elements known or suspected to be essential in amounts clearly in excess of the true quantitative requirements for these elements. Later work with purified media indicated that these excessive concentrations of heavy metals were really necessary to provide adequate amounts of the essential but unidentified elements with which they were contaminated. The iron, zinc, and manganese were especially favorable, and could be increased manyfold without toxicity and with some betterment of growth as long as they did not give rise to precipitates; in fact they appeared to protect the bacteria against toxic heavy elements. This effect was shared to a greater or lesser extent by samples of several other elements, among them cobalt, vanadium,

molybdenum, nickel, iridium, and rhenium. Gallium and scandium and the usual trace elements were not limiting factors in these experiments.

With the increasing use of glass, stainless steel, and "de-ionized" water in the chemical industry, it is likely that in the future the chemicals used by the microbiologist will be much purer than those now prevailing. Hence the problem of trace element requirements will become more troublesome until much more information is gained about these elements. Since there appear to be great variations in the amount of essential trace elements fortuitously present in reagent grade chemicals, different laboratories studying the same organisms grown in simple media are likely to have difficulty in reproducing one another's results. A case in point is provided by *Azotobacter*, as reported by Burk and Burris (1941). Hence, in order to enhance the reproducibility of this work, great attention had to be paid to the trace element requirements. These studies are continuing. The necessity of further information in this direction was emphasized by Emerson and Lewis (1939), who found that the efficiency of photosynthesis in *Chlorella* was directly dependent on the supply of essential but poorly identified trace elements.

A potential source of error remaining to be evaluated was that forthcoming from the presence in the medium of citrate—a biological product. The citrate filled an indispensable function; by forming soluble co-ordinate complexes with essential metallic elements, it kept them from becoming unavailable by precipitation as highly insoluble phosphates and hydroxides. This need is not usually obvious when the complex media are used, as certain amino acids are efficient compex-formers (Johnson, 1943; Smythe and Schmidt, 1930). The need for complex-formers of the citrate type was intensified for the nonsulfur photosynthetic bacteria by the high calcium requirement exhibited by many of them, the absence of amino acids from the media, and the alkalinization of the media when they were grown on media containing utilizable organic acids. Fortunately, synthetic malic acid, a fairly good complex-former, was available and was well tolerated by the bacteria in experiments to determine whether the citrate was contaminated by any vitamins. Tests on 5 isolates of each of the 5 species yielded with malate the same results as were earlier found with lactate-citrate media, except for the instance already mentioned of certain isolates of *R. capsulatus*. At any rate, the citrate did not harbor any vitamins needed by the bacteria.

The calcium requirement. There was considerable variation in the calcium requirement from one strain to another. For some it was indispensable, e.g., certain strains of *Rhodopseudomonas capsulatus* and *Rhodospirillum rubrum;* yet for others it was not clearly demonstrable, as with certain strains of *R. palustris*. No calcium requirement was noted for *Protaminobacter albus* and *Chlorella* grown in the same media. Yet many quantitative experiments demonstrated that, when a calcium requirement was demonstrable at all, it was rather high, about 0.5 mg per cent. It was eventually realized that in order to judge the genuineness of the calcium requirement many factors had first to be evaluated; among the foremost, the impurities in calcium. At least 20 elements tend to be

coprecipitated with calcium, and, as commercial calcium is itself in the last analysis the product of a biological enrichment from sea water, there is ample opportunity for contamination to occur; indeed certain rare earth elements are detectable in all calcium compounds (Sandell, 1944). A similar problem had been noted for *Chlorella:* Emerson and Lewis (1939) noted that photosynthesis was higher in media containing calcium carbonate, yet Trelease and Selsam (1939) obtained excellent growth in the absence of calcium. The disputed role of calcium in the metabolism of *Azotobacter* represents another parallel problem.

It may be gathered from the foregoing discussion that the identification of the organic requirements for the nonsulfur bacteria is but a prelude to the attack on the far more difficult problem of the inorganic requirements for growth and, more narrowly, for photosynthesis. These bacteria are, as pointed out by Foster and by van Niel, excellent subjects for such studies. Their rapid, heavy growth at room temperature, their utilization of distillable organic substrates, and their indifference to aeration in the presence of light and suitable substrates—all contribute to this suitability. In the later phases of this work, growth in synthetic media equaled that in media containing bacteriological peptones or yeast extract, but this goal has not yet been achieved with certain more highly purified synthetic media—an indication, if one were needed, that much remains to be done before the optimal conditions for growth of even the least exacting organism can be accurately defined.

SUMMARY AND CONCLUSIONS

Investigation of 124 isolates of aerobic nonsulfur purple and brown bacteria revealed that *Rhodospirillum rubrum* required biotin; *Rhodopseudomonas palustris*, *p*-aminobenzoic acid; *Rhodopseudomonas capsulatus*, thiamine and, in certain media, biotin + nicotinic acid in addition to thiamine; *R. gelatinosa* thiamine + biotin, and *R. spheroides* thiamine + biotin + nicotinic acid. One strain of *R. capsulatus* and 2 strains of *Rhodospirillum rubrum* appeared to have additional requirements aside from the carbon-hydrogen-energy source.

By the provision of suitable trace elements it was possible to grow the bacteria in media containing no organic constituents of biological origin. Some strains had an ostensible requirement for calcium.

The necessity of a better knowledge of inorganic requirements was emphasized.

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