CHAPTER I

INTRODUCTION

The study of algae has entered on a new phase through the extensive utilization of cultures for various purposes. As was the case in bacteriology and mycology, a much greater development of this mode of investigation may be expected in the near future. Progress in the study of algae will largely depend on the use of the existing methods of culture and their successful modification for special purposes.

The technique of obtaining pure cultures of algae has been considerably simplified and improved during the last few years. There is actually nothing new in the methods now adopted, but a few devices and accumulated experience render success more likely than it was ten years ago and result in a saving of time and material.

For the better understanding of the nature of the problems which can already be solved and to do justice to the scientists who prepared the way, the historical development will be traced.

Famintzin (1871) was probably the first to emphasize the possibility of ascertaining the nutritive needs of an alga with the aid of solutions of inorganic salts, a line of investigation pursued by Molisch (1895, 1896) and Benecke (1898) with considerable success. As a result the correspondence, in this respect, between algae and vascular plants was recognized, except that some of the former may not need calcium. It was only later realized that additional chemical elements (e.g. manganese) are necessary for algae as for flowering plants in addition to the ten long claimed as sufficient. But up to the present our knowledge on this point is far from satisfactory.

Miquel (1890–92, etc.) developed devices for cultivating diatoms in artificial media without securing pure cultures devoid of bacteria, which were first claimed by Richter (1903) and Chodat (1904).

The first step in this direction with respect to other algae was taken by Beijerinck (1890, 1893), who, adopting the technique devised by Robert Koch for bacteria ten years earlier, used gelatine for fixing germs at definite places. Klebs (1896, p. 184), however, doubts whether Beijerinck's cultures were really free from bacteria. Since the publication of Beijerinck's classical paper the methods of growing algae have undergone much change. Gelatine has proved unsatisfactory, since it is readily disintegrated by bacteria, which are always far more numerous than algal cells. The introduction of agar-agar instead of gelatine by Tischutkin (1897) marked an important step forward. A similar method was proposed by Marshall Ward (1899), who did not know of the work of Tischutkin and did not undertake any investigations as the latter did. It is unfortunate that Tischutkin's paper remained largely unknown, since it could have accelerated progress in culturing algae.

Chodat (1900, 1904, 1909, 1913) also used agar, but employed Erlenmeyer flasks instead of the Petri dishes used by Tischutkin and Marshall Ward. This complicated the technique without contributing any advantage. His method has even been used as late as 1926 by Bristol-Roach in her otherwise very able experiments. As recently as 1932 Skinner allowed the agar medium, with contained algal cells, to solidify in test-tubes, which must be broken to remove the algae. The writer has returned to Tischutkin's method (Pringsheim, 1912 and later), which has been further developed as described in the subsequent pages.

Another line of development originated from Klebs's often cited *Bedingungen der Fortpflanzung bei einigen Algen und*

Pilzen (1896). He made no effort to obtain bacteria-free cultures, because he did not believe that this could be achieved or, at any rate, not without great trouble. He therefore used large species which could be handled by simple methods, and cultivated them as far as possible in media composed solely of water and inorganic salts. It is now known that he underestimated the part played by micro-organisms in the results thus obtained, although some of these were of the greatest importance for further progress.

An important achievement of Klebs's was the use of zoospores as the starting-point for cultures of a single species. Immediately after their release from the parent cells these, as we now know, are free from contamination by micro-organisms, especially bacteria, which usually adhere to the surface of an alga. Zoospores are often the only means of obtaining pure cultures. Klebs defined the conditions for the formation of zoospores, to which Gerneck (1907) added further data. The latter was the first to rear bacteria-free cultures from single zoospores. Like his teacher Chodat (1909) and like Beijerinck, he worked with small species of algae, which can be treated almost like bacteria and yeast, the method of cultivation of which was already fairly well known through the researches of L. Pasteur, R. Koch and E. Ch. Hansen. This choice of small species, which would grow in the media used by Chodat and his collaborators, restricted the selection to those which are adapted to a habitat rich in organic compounds. This restriction was more extreme than was realized at the time, and exaggerated conclusions were deduced from the results. Furthermore, the conditions in such cultures contained within a compact block of agar were far more unfavourable than was at first apparent, so that only few species could withstand them. Finally, the conditions in Chodat's cultures were so different from those in nature that the cells were morphologically abnormal.

Despite these restrictions the experimental work of Chodat's Geneva school proved to be very noteworthy and even invaluable.

The first to review the whole situation was Küster (1907), whose book on the technique of the culture of micro-organisms contains a very valuable account of the different media and devices used in experimental work on algae, as well as of the influence of external conditions on their morphology and reproduction. In the later editions, however, the author's lack of detailed familiarity with this field of research makes itself felt. Moreover, the Klebs school to which he belonged had not properly appreciated that bacteria-free cultures were essential for many of the problems to be investigated.

Meanwhile, a different method of isolating single cells for pure cultures was gradually developed. Klebs (1896, p. 184) had already picked up zoospores and flagellates with the help of capillary pipettes. Zumstein (1900) was apparently the first to obtain bacteria-free cultures by means of this technique, which he combined with another means of getting rid of contaminating bacteria, namely, making the medium as acid as was compatible with the growth of the alga concerned. This of course was only possible with acid-tolerant forms, such as *Euglena gracilis*, which Zumstein was cultivating. The same combination of methods was used by Ternetz (1912) and by Pringsheim (1921*b*, 1934*a*) in dealing with *Euglena gracilis*, *Astasia ocellata* and *Chilomonas paramecium*.

The pipette method was first recommended by Pringsheim (1921*a*, p. 402) and improved by Lwoff (1923, 1929). By isolating single cells in a sterile medium and repeating this procedure many times the ratio of number of bacterial to algal cells is gradually reduced until individual cells of the alga could be transferred to a sterile medium and allowed to multiply.

Similar methods had formerly been used for ciliates. The pipette method thus developed into the washing method.

Further improvement of the cultural technique was achieved by introducing several minor but useful devices, and by applying the experience that had meanwhile been gained as to the factors necessary for the existence of forms showing special adaptations or specially sensitive to changes in the environment. The work of Bouilhac (1897), Chodat (1904) and Pringsheim (1913) led progressively to the attainment of bacteria-free cultures of Cyanophyceae, the last-named undertaking with such cultures a study of the physiology of their nutrition. Pringsheim (1912, 1918) obtained the first healthy cultures of Conjugales, which his pupil Czurda (1926) succeeded in growing on agar free from bacteria. The first colourless alga to be grown in pure culture was *Polytoma* (Jacobsen, 1910), whilst Pringsheim (1920, 1921 *b*) was able to improve the method and to use it on a larger scale through the discovery that these and other forms were acetate organisms (Pringsheim, 1921 *b*, 1935).

Organic compounds were first used in algal cultures by Bouilhac (1897, 1898) and Treboux (1905), who employed sugars and organic salts respectively. The first more elaborate study as to the food value of various organic substances was probably that of Matruchot and Molliard (1902) on *Stichococcus*.

As a step towards the understanding of the ecology of algae, an experiment first carried out by Beijerinck (1901) is important. He found that certain Cyanophyceae, such as species of *Nostoc*, *Anabaena* and *Cylindrospermum*, usually develop when small quantities of soil are covered with a relatively large amount of water, only enriched by a low concentration of phosphate. He called these Nostocaceae oligonitrophilic and suspected them of utilizing atmospheric nitrogen, a conjecture which has since proved correct. When

larger amounts of soil or complete nutrient solutions are employed, Oscillatoriae, diatoms or other algae are mostly stimulated to active multiplication.

Jacobsen (1910), on the other hand, demonstrated the effect of the addition of a rich supply of organic substances to cultures of soil algae. Volvocales, such as *Carteria*, *Chlamydomonas* and *Sphondylomorum*, showed vigorous development in the presence of protein if the cultures were illuminated, while in the dark *Polytoma* appeared. It was on the basis of such cultures that Pringsheim (1921 *b*, 1935) built up his theory of acetate organisms.

In relation to the improvement of algal media, the utilization of soil extracts (Pringsheim, 1912, p. 326; 1936 *a*; Mainx, 1927 *b*, p. 323), the effective components of which are probably soluble iron compounds, and of peat extract (Wettstein, 1921), which is valuable for its acidity and low salt concentration, is noteworthy. These substances can be used for regulating and buffering the *p*H of the medium. Uspenski (1925, 1927) has made a special investigation of the influence of iron supply in algal cultures; this has been supplemented by Pringsheim (1930, 1936 *a*).

If algae are to be kept under constant conditions or to be maintained for more than a few months, daylight is insufficient, because it is too variable and, during the winter, too feeble. Hartmann (1921) remedied the defects of electric illumination by allowing the rays to pass through a screen of cold water, thus eliminating the infra-red radiation which injures the algae by heating the cultures and is also harmful through its desiccating effect on the media. Pringsheim (1926 *a*, p. 293), in summarizing his own experience and that of his collaborators, described an improved model of the 'artificial sun' of Hartmann.

During recent years other authors have endeavoured to summarize, wholly or in part, the methods used for algal

culture and the results obtained. Kufferath's book (1930) is of little value, but Vischer's instructions (1937) for the culture of Heterokontae might serve as a model for such treatises. The papers of Lwoff (1932), Provasoli (1937–8) and Hall (1939) on the nutritional needs of algae and Flagellata supply a useful basis on which the future science of algal nutrition can be built.

CHAPTER II

SELECTION AND PREPARATION

1. CHOICE OF MATERIAL

When selecting algal material to be raised for a morphological or physiological study, one may proceed in various ways. A start may be made by finding out what will grow from among a mixture of forms, such as are found in a pool, ditch, etc.; or a search could be made for a population practically composed of one species. Both methods may give valuable results, but both tend to afford only such species as will readily grow under certain conditions.

For several years now I have adopted a third procedure, which affords most interesting results and consists in picking out single specimens from a mixture, with a view to analysing the algal flora of a certain habitat or to the isolation of certain species belonging to a definite taxonomic group (cf. also Chu, 1942). In this way, even in a small pond, a multitude of species may be found, the existence of most of which is usually unnoticed owing to the competition of others and the lack of food for all.

This can be demonstrated by subjecting a natural mixture of species, such as would be present in nature, to various conditions. If, for example, we take a seemingly barren mud from the bank of a river and add to different portions water only, Beijerinck's solution, and water with a small piece of cheese respectively, we should find after some weeks practically no growth in the first, a few filamentous and unicellular species in the second, and a rich and varied flora comprising some unexpected species in the third.

In another set of experiments mixed material from a small

garden pond was analysed by subjecting it to varying treatment. Some thirty species of green and unpigmented algae were isolated, most of which had not been recognized by mere microscopic investigation. Certainly more than double the number would have been found, if more time could have been spent on the task.

When analysing an algal flora, certain forms are found not to grow well in the usual inorganic media, even when various concentrations and *p*H values are employed, while others will not grow at all in them. When that is so, the soil-and-water culture method (p. 16), first devised for cultivating heterotrophic flagellates, is useful.

2. Preparatory Cultures

It is often advisable to undertake preparatory cultures before attempting pure ones. There are manifold reasons for this procedure:

By cultivating a species for some time under various circumstances, we gain experience concerning the conditions suitable for its existence and multiplication.

We can pick up single cells and allow them to grow, independently of other organisms which might obscure the results. We are thus able to start purification with healthy and relatively pure material. There is also the possibility of detecting interesting species in a provisional rough or enrichment culture (p. 11) which would have been overlooked if we had placed reliance on material from nature only.

Pure cultures are sometimes only obtained after a tedious and wearisome procedure. If material freshly brought from its natural habitat is used, the species required to be raised in pure culture may be lost before success is attained. If we start with a healthy culture, however, successive attempts are possible, and we can learn from failures until our efforts are crowned with success.

At any rate the time spent in growing a species in the laboratory and observing it more closely than would have been possible in the natural habitat is not lost, even if a pure culture is not achieved. Such cultures, especially when originating from single cells, may throw light on certain characteristics, which had not been previously noticed, and may sometimes even prove more helpful than pure cultures.

The following matter deals first with the employment of preparatory mineral mixtures, then with that of special enrichment cultures, and finally with the soil-and-water culture method.

A. *Preparatory cultures in nutritive solutions.* The use of solutions of inorganic salts in preparatory cultures is advisable for various reasons. Their composition can often be made to approximate to that of the natural medium. Such solutions, especially if used in a highly dilute form, have the effect of securing multiplication of species which thrive in pure waters or other localities devoid of organic matter.

If the solutions of Knop, Detmer or Beijerinck are added to a mixture of aquatic or soil algae, species will, after some time, be found growing in abundance which were only present in small numbers and would scarcely have appeared if pure water had been used. The material thus obtained is suitable for further cultural treatment (Lund, 1942, p. 273; John, 1942).

Inorganic solutions favourable to the growth of a species increase the number of individuals, while bacteria and other unwelcome organisms are diminished in comparison to the original mixture. This is advantageous in starting pure cultures, especially when algal cells are hidden between collections of mineral particles, detritus and organic residue, usually called mud. Moreover, they may have been present as resting stages which might easily be overlooked.

When using this method, it must not be forgotten that this