LXXIV. THE OCCURRENCE OF NUCLEIC ACID DERIVATIVES IN NITROGEN-FIXING BACTERIA.

By FLORENCE ANNIE Mockeridge.

From the Department of Biology, University College of Swansea.

(Received April 8th, 1924.)

The occurrence of nucleic acid in the cells of the tubercle bacillus is a well-known fact, for Ruppel [1898] separated an acid under the name of tuberculinic acid, which has been shown [Levene, 1901, 1904; Johnson and Brown, 1922, 1, 2], to have all the properties of a nucleic acid, and which apparently constitutes from 2.0 to 3.5% of the weight of the organism [Long, 1920]. Very little work has been done with regard to the appearance of this acid in other bacteria, but attention was directed to the possibility of its occurrence in the nitrogen-fixing bacteria by the fact that the products of these bacteria have a growth-promoting effect on plants [Bottomley, 1917], an effect which is shared by yeast [Mockeridge, 1924] and by extracts of soils and manurial materials which all contain nucleic acid or its derivatives [Bottomley, 1919; Mockeridge, 1920]. In view of the constant occurrence of these substances in all other materials which are known to have this growth-promoting effect on plants, it was decided to make a preliminary investigation of Azotobacter chroococcum in order to determine whether it contained any nucleic acid or derived substance. For this purpose as large a quantity of the organism as possible was required, and it was obtained by growth on mannitol agar. A medium consisting of nutrient materials in the following proportion was prepared:

- Mannitol: 1 g.
- Dipotassium phosphate: 0.2 g.
- Magnesium sulphate: 0.02 g.
- Calcium carbonate: 0.2 g.
- Agar-agar: 2 g.
- Distilled water: 100 cc.

This nutrient agar was poured into six-inch Petri dishes, which were then sterilised in an autoclave at a temperature of 120° for half-an-hour. When cool and solidified, the medium was inoculated with a suspension in sterile distilled water of Azotobacter from a colony of recent isolation from soil. The plates were then incubated at a temperature of 26° for about 14 days, at the end of which period the surface of the medium was generally covered with a mass of the bacteria of the typical coccus form, embedded in wide slime capsules often several times their own diameter, forming large masses of mucilaginous material which could be readily scraped off the surface of the agar and could thus be obtained free from nutrient materials. The available
incubating space was utilised many times over in this way, in order to obtain sufficient material for examination, and several successive fresh isolations of *Azotobacter* from soil were made for the purpose, since it was always found that the organism grew most rapidly when only recently isolated.

The thick gelatinous culture thus obtained was examined, in the first place, in three different ways. One portion was sterilised in the autoclave at a temperature of 140°, and then extracted with a 10% solution of caustic soda. A second portion was extracted at once, without previous sterilisation, with a similar caustic soda solution, and the third portion was merely shaken with distilled water. This last method has so far given negative results, but it is proposed to repeat it on the much larger scale which is obviously required in order to extract the small quantities in which these materials occur in the slime capsule, if they are to be found at all.

Except for the fact that the first portion of the bacterial material received the preliminary treatment of being heated in the autoclave to a temperature of 140° for two successive periods of one hour each, separated by a two hours' interval, the remaining two portions were treated similarly. The gelatinous material, autoclaved or otherwise, was covered with a very large excess of 10% caustic soda solution, placed in a stoppered bottle and shaken in a shaking machine for about 24 hours, in order thoroughly to extract the required materials. It was then almost neutralised with hydrochloric acid, and allowed to stand for a few hours, in order that the bacteria might settle out as much as possible. The supernatant liquid was decanted off and centrifuged in order to get rid of the remainder of the bacteria, and as a last resort the clear liquid was passed through a Berkefeldt filter under pressure. Microscopical examination proved that this liquid was free from bacteria, so it was acidified with acetic acid in order to precipitate any protein. The slight precipitate was removed by filtration, the whole filtrate just neutralised with caustic soda, and concentrated in vacuo to a small volume. Concentrated hydrochloric acid was added in sufficient quantity to render the liquid strongly acid, and the whole poured into about five times its total volume of absolute alcohol. A slight flocculent precipitate separated out on long standing, and this was removed by filtration. From the method of preparation it was probably nucleic acid, but the quantity was too small for further examination.

The filtrate from this precipitate was accordingly further examined for nucleic acid derivatives. For this purpose it was just neutralised with caustic soda, and concentrated in vacuo to a small bulk in order to remove the alcohol. A small portion of the liquid was tested for the presence of phosphoric acid by both magnesia mixture and ammonium molybdate, with positive results in both cases. A further portion was submitted to Molisch’s test, which proved the presence of carbohydrates, and that these were reducing sugars was shown by a slight reduction of Fehling’s solution. The liquid also responded to the phloroglucinol and orcinol tests for pentoses, so that part, at least, of the carbohydrates present consisted of these sugars.
Separation of the Purine Bases.

The remainder of the liquid was used for the separation of the purine and pyrimidine bases, the methods followed being those of Jones [1914], with slight modifications. On account of the large amount of chloride present, the use of a silver salt was avoided, the purine compounds being precipitated by the use of a copper salt, while an attempt was made to separate the pyrimidine derivatives by the use of a mercuric salt. In order to accomplish the desired results, the liquid was brought to the boil, and concentrated ammonia added, the whole being kept briskly boiling all the time, until no further precipitate formed. This precipitate would contain all the phosphoric acid, together with guanine, if present. The liquid was boiled for a short time after the addition of the ammonia, and the precipitate was then removed by filtration, washed with dilute ammonia several times, and the filtrate and washings added together. The precipitate was warmed with dilute caustic soda solution in order to dissolve any guanine present, while the phosphoric acid remained unaffected. This insoluble material was filtered off, and to the filtrate was added dilute acetic acid, in order to precipitate any guanine. An appreciable precipitate was obtained, which was filtered off, washed with dilute acetic acid, and submitted to various tests indicated below in order to identify the material.

The combined filtrate and washings from the guanine and phosphoric acid precipitate were acidified with dilute sulphuric acid. Crystalline copper sulphate was added in large excess, and the liquid brought to the boil; the addition of a saturated solution of sodium bisulphite then resulted in the formation of a white precipitate. This reagent was therefore continually added in successive small quantities until the precipitate formed began to show a reddish tinge. Boiling was then continued rapidly for a short time, and the white precipitate of copper-purine filtered off, washed with water slightly acidified with sulphuric acid, suspended finally in hot water and decomposed with sulphured hydrogen. The black precipitate of copper sulphide was removed, using a filter pump to accelerate the process, washed with water, and the combined filtrate and washings concentrated in vacuo, filtered, and used for the preparation of salts of adenine for purposes of identification.

Separation of Pyrimidine Derivatives.

The filtrate from the copper-purine compound was then examined for the presence of pyrimidine bases. The liquid was first treated with sulphured hydrogen, the precipitate of copper sulphide filtered off, and the sulphuric acid removed with baryta. The filtrate was concentrated somewhat, after which a solution of mercuric sulphate was added and the whole acidified with sulphuric acid. After standing for 24 hours the very slight precipitate was filtered off, washed with water slightly acidified with sulphuric acid, suspended in hot water and decomposed with sulphured hydrogen. The precipitated mercuric sulphide was removed, and the filtrate concentrated in vacuo.

An attempt was made to precipitate cytosine in form of the picrate by the addition of a hot saturated solution of picric acid. After standing for
NUCLEIC ACID FROM BACTERIA

24 hours no precipitate had appeared, so the liquid was freed from picric acid by shaking in a separating funnel with sulphuric acid and ether, after which the sulphuric acid was removed with baryta. The filtrate from the barium sulphate was again concentrated, and set aside in order to allow of the separation of any uracil present. After some days no deposit had appeared, so the liquid was subjected to the colour test of Wheeler and Johnson, with positive results. Apparently, therefore, the pyrimidine derivatives, though present, occurred in such small quantity as to fail to separate out. Whether they do actually occur in less quantity than the purines, or whether they had been lost during the process of separation, is a matter for future investigation.

Examination of the Purine Bases.

Various tests were then carried out in order to identify the two materials separated. A small portion of the precipitate which was suspected to be guanine was warmed on porcelain with a drop of nitric acid. When evaporated to dryness it left a muddy yellow spot which became brownish-red on the addition of sodium hydroxide: a reaction which indicated the presence of guanine. A small portion of the precipitate was treated with hot sulphuric acid and the remainder was dissolved in hydrochloric acid, and the latter solution allowed to stand for a considerable time in a desiccator. A mixture of needle-shaped and long tetrahedral crystals of the hydrochloride was thus obtained eventually, and from this chloride were prepared the picrate, by the addition of picric acid to the solution, and the dichromate, by the similar addition of a solution of chromic acid. The former salt separated out as a mass of long, fine, thread-like needles, which had a woolly appearance in bulk, and which dried to a felt-like mass. On heating, this salt became orange-red, and finally decomposed without melting at 190°. The latter salt appeared very slowly in the form of truncated prisms, bright orange in colour, which changed to a dark violet when the salt was heated to 100°. The solution in hot sulphuric acid was cooled rapidly, when the sulphate separated out in the form of needle-shaped crystals. The formation of these salts, together with the method of preparation of the material, the fact that it formed a gelatinous precipitate with ammoniacal silver nitrate, dissolved in hydrochloric acid and was thrown out of solution by ammonia, afforded sufficient evidence to identify the substance as guanine.

The material which was separated out by precipitation with copper sulphate and sodium bisulphite, and which was in all probability adenine, was tested first of all for the formation of a picrate. To a portion of the liquid, picric acid in saturated solution was added, and a very little ammonia, when a precipitate consisting of clusters of fine needles was at once obtained. When redissolved and recrystallised this appeared in the form of somewhat elongated prisms which were dried and found to melt with decomposition at about 277°, the approximate melting point of adenine picrate. Another portion of the material was tested with ammoniacal silver nitrate, when a gelatinous precipitate appeared; to yet another was applied Kossel’s test for purines,
which gave a positive result; a drop of ferric chloride was added to a fourth portion, resulting in a deep red colour, unchanged by heating. From the remainder of the liquid salts were prepared; the hydrochloride, by the addition of hydrochloric acid, separating in flat, deliquescent prisms; the dichromate, by the addition of chromic acid, forming six-sided plates; and the double salt with gold chloride, occurring as long, orange-coloured prisms. All these reactions confirmed the material as adenine.

DISCUSSION.

Exactly similar materials were isolated from the Azotobacter growth, whether the organisms had been autoclaved or not, the only difference being that in the case of the autoclaved material a bigger yield of the organic bases was obtained. It is clear, therefore, that whether or not nucleic acid occurs as such within the Azotobacter cells, there are elaborated within the organisms all the essential radicles for its formation, viz.: phosphoric acid, a carbohydrate, two purine bases, adenine and guanine, and also pyrimidine bases.

Apart from any bearing that this result of the work may have upon the question of auximones, it is of interest from the point of view of the actual organisation of the bacteria themselves, for the fact that there are to be extracted from these cells substances which must be regarded as essentially bound up with a nucleus is a strong point in favour of the occurrence within the bacteria of at least the rudiments of a nucleus. It is intended to carry out quantitative work in the hope of obtaining some idea of the extent to which actual nuclear material can be regarded as being present in these lowly organisms. It is also hoped to identify the pyrimidine derivatives present, and so to ascertain whether this organism is similar to the tubercle bacillus in the occurrence of thymine [Johnson and Brown, 1922].

From the point of view of the metabolism of these bacteria it is of interest to note that these relatively complex nitrogenous substances have been synthesised by the organisms from elementary nitrogen, since the bacteria are cultivated in a medium absolutely free from combined nitrogen, their only source of this element being the atmosphere. A similar investigation of other nitrogen-fixing bacteria will be made in due course.

The author wishes gratefully to acknowledge the receipt of a grant from the Royal Society for the purchase of apparatus by the aid of which this investigation was carried out.

REFERENCES.

Johnson and Brown (1922, 1). J. Biol. Chem. 54, 721.
----- (1922, 2). J. Biol. Chem. 54, 731.
Jones (1914). The Nucleic Acids.