THE NUCLEIC ACIDS OF *RICKETTSIA BURNETI*.

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IN recent years much interest has centred about the structure and biological distribution of the nucleic acids. There is evidence to show that these are concerned in protein synthesis and are intimately linked with the genetic mechanism. In a few instances they have been shown to possess specific biological properties, one of the most remarkable being the parts played by deoxypentose nucleic acids in inducing the type transformations of pneumococci and *Bact. coli* (Avery, Macleod and McCarty, 1944; Boivin, Delaunay, Vendrely and Lehoult, 1945). General acceptance of the tetranucleotide hypothesis, originally put forward by Levene (Levene and Bass, 1931), had led to the belief in common structures for all ribonucleic acids (RNA) and all deoxypentose nucleic acids (DNA). Such uniformity of structure was difficult to reconcile with the existence of specific biological properties pertaining to individual nucleic acids. Although Gulland and his co-workers (Gulland, 1947) had thrown considerable doubt on the tetranucleotide hypothesis, proof of its incorrectness had to await the development of new methods, based on chromatography and spectrophotometric analysis, which permitted the accurate analysis of the nucleotide composition of nucleic acids from many sources. In addition to disproving the tetranucleotide hypothesis, the application of these methods has led to the recognition of 5-methyl cytosine as a constituent of DNA from higher animals and plants (Wyatt, 1951*a*), but which is absent in the DNA from bacteria and viruses. It has also been shown that the composition of a deoxypentose nucleic acid is characteristic of the organism from which it is derived (Chargaff, 1950; Wyatt, 1951*b*). This is also true of the ribonucleic acids from the plant viruses, in which it is even possible to demonstrate small differences in composition of ribonucleic acids from different virus strains (Markham and Smith, 1950, and unpublished). These results have stimulated interest in the compositions of nucleic acids from as wide a range of biological sources as possible, and in this paper we describe the analysis of the nucleic acids from a rickettsia, *Rickettsia burneti*.

In size and morphological complexity the rickettsiae occupy a position between bacteria and the larger animal viruses. Chemical analyses of the isolated rickettsial organisms have been limited to *Rickettsia prowazeki*, which has been shown to contain protein, nucleic acid, carbohydrate, lipid and phospholipid. The published quantitative analyses show differences which probably reflect to some extent variations in the procedures used for the purification of the organisms. Cohen and Chargaff (1944) found the dried rickettsiae to contain 13·2 per cent lipids, of which 9·5 per cent was acetone-soluble and 3·1 per cent phospholipids, 12·2 per cent nitrogen and 0·93 per cent phosphorus. The

analyses of Tovarnickij, Krontovskaja and Ceburkina (1946) gave 46·6 per cent lipids (of which 29·7 per cent was neutral fat and 15·8 per cent phospholipids) and only 30·2 per cent protein. The discrepancy between the lipid contents given by the two analyses is almost certainly in part due to the fact that Cohen and Chargaff used ether treatment to remove host material, whereas Tovarnickij and co-workers obtained their preparations entirely by differential centrifugation. Both groups of workers demonstrated nucleic acid in the rickettsiae. By sonic disintegration of the organisms followed by centrifugation and acid precipitation of the supernatant liquid Cohen (1946) isolated nucleic acid amounting to 2–2·5 per cent of the dry weight; this was identified as DNA but the method used is not stated. Tovarnickij and co-workers isolated from their preparation 12 per cent. of nucleic acid which gave a positive Feulgen reaction, but no other properties of this nucleic acid are given. These analyses show that DNA is a constituent of rickettsiae, but the methods of isolation and analysis do not exclude the possible presence of RNA. For example, Tovarnickij and co-workers extracted the nucleic acid by the method used by Taylor and Sharp (1943), which consists of treatment with 5 per cent sodium hydroxide at 0–5° for 2 hr. and is sufficient to bring about appreciable hydrolysis of RNA to nucleotides.

With the aid of chromatographic techniques we have examined quantitatively the nucleic acid composition of *Rickettsia burneti*. This rickettsia was first investigated in Australia by Burnet and Freeman (1937), and independently in the United States by Davis and Cox (1938). It causes a pneumonia in man known as Q fever, but also infects cattle, sheep and goats. *R. burneti* resembles other pathogenic rickettsiae in its morphology, staining reactions, behaviour in experimental animals, and ability to multiply in an arthropod host (the tick). It differs from other pathogenic rickettsiae, however, in its ability to pass through filters with an average pore diameter as small as 400 mμ. Microscopically it is pleomorphic, but is frequently seen as a short rod 200–300 mμ. in diameter and 600–800 mμ. long. It is also more resistant than other rickettsiae to certain chemical and physical agents, and Philip (1948) has suggested that all these differences merit a separate genus *Coxiella* within the family *Rickettsiaceae*. *R. burneti* grows very profusely in the yolk sac of the fertile hen's egg, from which purified rickettsial suspensions may be prepared. In addition it was possible to isolate and analyse the DNA from the host for direct comparison with that of the rickettsia.

MATERIALS AND METHODS.

Preparation of rickettsial suspensions.

The procedure used for the preparation of purified rickettsial suspensions was based on Wolfe and Kornfeld's modification of Plotz's method (Wolfe and Kornfeld, 1949; Plotz, 1943). In this method non-rickettsial material is removed from crude formaldehyde-treated yolk-sac suspensions by centrifugation and treatment with ethyl ether.

Fertile hen's eggs were incubated at 38° for 6 days. The yolk sacs were then inoculated with a stock suspension of the Henzerling strain of *R. burneti* diluted in 10 per cent horse serum broth. This strain had already been adapted to growth in eggs. After incubation for 5–6 days at 35°, when about one-third of the embryos had died, the yolk sacs from all the eggs were harvested and stored

at — 25°. When smears showed bacterial contamination or only small numbers of rickettsiae the yolk sacs were discarded.

When 300–400 g. of infected yolk sacs had accumulated these were thawed, weighed, rapidly homogenized in a mechanical blender and made up to 20 per cent w/v in a diluent with the following composition: 0·85 per cent NaCl in distilled water, 4 parts; Sörenson's buffer pH 7·0, 1 part; the whole solution containing 0·5 per cent formalin. After standing at 2° for 48 hr. the suspension was centrifuged at 3600 r.p.m. (approx. 4500 g.) for 1 hr. (or at 11,000 r.p.m. for 30 min.). The pellet was re-suspended in the original volume of diluent and vigorously shaken for 1 min. with an equal volume of ethyl ether. After separation overnight in the cold room, the ether layer and the interphase, where most of the cellular debris had accumulated, were discarded. The aqueous phase, containing most of the rickettsiae, was centrifuged again as before. The pellet was then re-suspended in 1/5 the original volume of diluent, the suspension shaken with half its volume of ether, and left at 2° overnight. The aqueous phase was then repeatedly shaken with 1/4 vol. of ether and allowed to separate at room temperature for about 1 hr. After 3 or 4 ether treatments no interphase layer could be seen. The rickettsiae in the aqueous phase were then sedimented by centrifuging for 30 min. at 12,000 r.p.m. in a Sorval S.S.1 angle centrifuge, and washed by centrifugation 3 times in the buffered saline containing formalin.

From examination of stained smears the final preparations obtained by this method appear to consist entirely of rickettsiae, and this has also been confirmed by electron microscopy. The repeated washing by centrifugation would remove all extraneous particles of low molecular weight, and any material that might be loosely adsorbed on the surface of the organisms. However, there remained the possibility that the preparation might contain host particles of rickettsial size, indistinguishable microscopically from the organisms. In view of this, yolk sacs inoculated with 10 per cent horse-serum broth alone were submitted to the purification procedure described above. The final deposit, which was not visible to the naked eye, was made up to a volume equivalent in terms of starting material to that of the rickettsial suspensions, and the ultra-violet absorption spectrum of each examined in the Beckman photoelectric spectrophotometer. The absorption curve of the rickettsial suspension (Fig. 1) shows a peak at 255 mμ. due largely to the nucleic acid content, while the material isolated from normal eggs has only a small peak at 280 mμ., presumably due to aromatic amino-acids contained in protein, and is less than 1 per cent that of the rickettsial suspensions.

Analysis of the nucleic acids.

The isolation of purified nucleic acids from rickettsiae would not have been possible without the use of larger quantities of the organisms than could conveniently be prepared. However, the information required, namely the proportions of the component nucleotides in the nucleic acids, could be obtained by a simple direct method. This consisted of the extraction of the total nucleic acid components, the hydrolysis of these to the pyrimidine and purine bases, and their chromatographic separation and estimation, together with a similar extraction and analysis of the DNA after removal of RNA by hydrolysis with alkali.

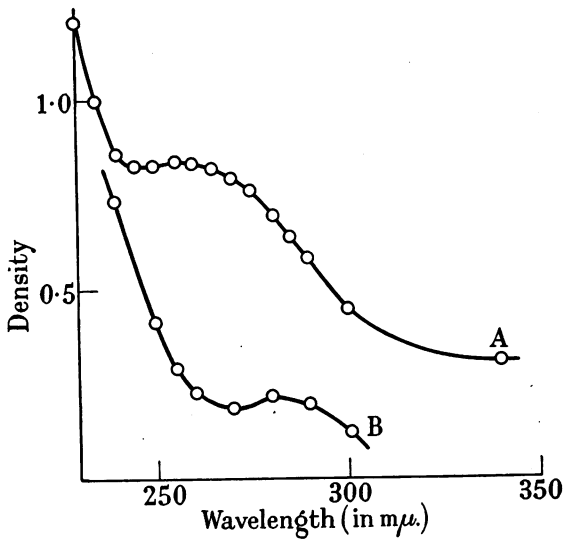

FIG. 1.—Ultra-violet absorption spectra of rickettsial suspension containing 88 μg./ml. (curve A), and material obtained by the purification procedure from normal yolk sacs (curve B). In terms of starting material A is at a dilution 20 times that of B.

Total nucleic acids.—By three successive treatments with 5 per cent trichloracetic acid at 90° the total nucleic acid components were extracted from 5–10 mg. of rickettsiae. The extract was evaporated to dryness and hydrolysed in 72 per cent w/w perchloric acid at 100° to liberate the purines and pyrimidines (Marshak and Vogel, 1950). Marshak and Vogel give the duration of the hydrolysis as 1 hr., but we have found that in the presence of small amounts of protein it is necessary to extend this to 2 hr. in order to ensure complete hydrolysis of the pyrimidine nucleotides from RNA. The increase in the hydrolysis time is not accompanied by any destruction of the purine and pyrimidine bases. The hydrolysate was diluted with an equal volume of water, centrifuged (to remove carbon derived from the sugars), and aliquots placed on a paper chromatogram which was run with 65 per cent *iso*propanol in water, 2N with respect to HCl (Wyatt, 1951*b*). This solvent resolves the purines and pyrimidines which were detected on the paper by the photographic method of Markham and Smith (1949), eluted in 0·1N HCl and estimated spectrophotometrically.

Deoxypentose nucleic acid.

Wyatt (1951*b*) has shown that treatment with N NaOH at 37° does not alter the quantitative composition of DNA. This was therefore used for the removal of RNA as nucleotides, preliminary to obtaining the quantitative composition of the rickettsial DNA (Schmidt and Thannhauser, 1945). The rickettsiae (8–18 mg.) were incubated with N NaOH at 37° for 12 hr. Addition of glacial acetic acid to pH 4 and one volume of ethanol precipitated the DNA together with some protein, leaving the ribonucleotides from RNA in solution. The precipitate was collected by centrifugation, washed with 50 per cent ethanol at

pH 4, extracted with hot trichloroacetic acid, hydrolysed and chromatographed as described above.

For the estimation of 5-methyl cytosine in DNA we used the technique described by Wyatt (1951*a*).

Isolation of DNA from normal chick embryos.

Mirsky and Pollister (1946) have isolated deoxyribonucleoproteins from several animal tissues by the removal of the bulk of the cytoplasmic components including the ribonucleoproteins by extraction with 0·14M NaCl, followed by extraction of the deoxyribonucleoproteins with M NaCl. This method was not very satisfactory for the isolation of DNA in the deoxyribonucleoproteins from chick embryos, apparently because of dissociation of the nucleoproteins.

The following method was found more useful: Normal chick embryos harvested after 12 days' incubation were mixed with ethanol in a mechanical blender (2 ml. ethanol/g. fresh wt.). The ground tissue was collected by centrifugation and extracted again with ethanol and with ethyl ether until most of the fats had been removed. It was then dried at 50° and incubated in a small volume of N NaOH at 37° overnight. The solution was centrifuged to remove material insoluble in the alkali, and the supernatant brought to pH 4 with acetic acid and one volume of ethanol added. After standing, the precipitate containing the DNA was collected by centrifugation and extracted with water brought to pH 8 with sodium bicarbonate. Protein was removed from the extract by gel formation on shaking with a chloroform-octanol mixture (chloroform 8 parts, octanol 1 part, v/v; Sevag, Lackman and Smolens, 1938). The nucleic acid was precipitated with ethanol and acetic acid and dried at 110°.

Nitrogen.

Nitrogen was estimated by a micro-Kjeldahl method.

Phosphorus.

Phosphorus was determined by the colorimetric method of Allen (1940).

Dry weight.

All dry weights were determined after drying at 110°.

Qualitative amino acid analysis.

The rickettsiae (1·76 mg.) were hydrolysed in 6N HCl for 6 hr. at 120°. After removal of HCl by vacuum distillation the amino acids in the hydrolysate were separated by two-dimensional paper chromatography using butanol-acetic acid-water and phenol-water-ammonia as solvents. They were detected by the usual ninhydrin method.

RESULTS.

Composition of the rickettsial nucleic acids.

Three separate preparations of *R. burneti* were used in these analyses. A portion of the rickettsial suspension from preparation 1 was dialysed against distilled water and made up to a known volume for the determination of dry weight, nitrogen and phosphorus. The total yield from 344 g. of yolk sacs (wet

weight) was 44 mg. (dry weight). This contained 13·2 per cent N and 1·8 per cent P.

The purine and pyrimidine composition of the total nucleic acid fraction was estimated in two preparations (1 and 2), while the composition of the DNA from all three preparations was determined. Adenine, guanine, cytosine, uracil and thymine were identified on the chromatograms of the total nucleic acid bases. Both RNA and DNA thus appeared to be present, and this was confirmed by the complete removal of uracil together with a portion of the adenine, guanine and cytosine after hydrolysis with N NaOH at 37°. The compositions of the DNA from all three preparations were identical within the limits of experimental error. It was thus possible from the amount of thymine in the total nucleic acid fraction to calculate the quantities of adenine, guanine and cytosine accounted for as DNA, and hence, by subtraction, obtain the quantities of the bases present in the RNA. (It should be pointed out here that the adenine fraction of the RNA as estimated would include any adenine present as free adenylic acid or its derivatives.) Expressed on a dry weight basis the amounts of DNA in the two preparations are identical but the RNA content shows some variation (Table I).

TABLE I.—*The Nucleic Acid and Phosphorus Content (per cent dry weight) of Preparations of* Rickettsia burneti.

	Preparation 1.	Preparation 2.
DNA	9·8	9·5
RNA	2·95	5·5
P	1·81	..
P accounted for as nucleic acid	1·30	..

5-methyl cytosine could not be detected on the chromatograms of the DNA hydrolysates run in the *iso*propanol-HCl solvent, nor was this substance observed when the area corresponding to the position of this base was eluted and the concentrated eluent chromatographed using a second solvent. On elution and spectrophotometric examination, the area corresponding to 5-methyl cytosine on the second chromatogram showed no ultra-violet absorption characteristic of this substance. This enabled a definite limit to be set to the amount of 5-methyl cytosine that would have passed undetected. The compositions of DNA from the three preparations of rickettsiae are given in Table II.

TABLE II.—*Ratios of Purines and Pyrimidines in Deoxypentose Nucleic Acids from* Rickettsia burneti *and Chick Embryos.*

	R. burneti DNA.			Embryo DNA.			Significance of the difference between the two means (P.).
	Number of preparations analysed.	Mean molar values.*	S.E.	Number of preparations analysed.	Mean molar values.*	S.E.	
Adenine	3	**1·18**	±0·012	3	**1·17**	±0·018	0·5 –0·7
Guanine	3	**0·90**	±0·015	3	**0·96**	±0·012	0·02–0·05
Cytosine	3	**0·88**	±0·020	3	**0·82**	±0·004	0·02–0·05
Thymine	3	**1·04**	±0·010	3	**1·06**	±0·009	0·2 –0·3
5-methyl cytosine	2	**<0·008**		2	**0·037**		

* The molar values are calculated such that those for the 4 major bases (adenine, guanine, cytosine and thymine) total 4.

Deoxypentose nucleic acid from chick embryos.

During growth in the fertile egg the rickettsiae proliferate in the cells of the of the yolk sac. This would therefore have been the most suitable material for the preparation of host DNA for comparison with that of the rickettsiae. However, only small amounts of DNA could be isolated from yolk sacs, whereas appreciable yields were obtained from the whole embryo. DNA from different tissues of the same species have been found to show the same composition (Chargaff, 1950 ; Wyatt, 1951*b*). There is no reason, therefore, to suspect that the DNA of the yolk sac differs in composition from that isolated from the whole embryo.

Three preparations of chick embryo DNA were analysed, and in Table II the results are compared with those of the DNA from rickettsiae. The small differences in the proportions of adenine, guanine, cytosine and thymine between the two nucleic acids are on the borderline of significance. The nucleic acids differ, however, in their content of 5-methyl cytosine. While the embryo DNA contains a proportion of 5-methyl cytosine equal to 4·2 per cent of the cytosine, in the rickettsia DNA this base could not be detected, and must be less than 0·98 per cent of the cytosine.

The amino acids of R. burneti.

Qualitative paper chromatography showed the presence of alanine, arginine, aspartic acids, cystine, glutamic acid, glycine, histidine, leucines (probably an unresolved mixture of leucine and isoleucine), lysine, methionine, phenylalanine, proline, serine, valines, and a trace of threonine. Tyrosine could not be detected on the chromatogram, suggesting that it cannot be present in more than about 0·05 per cent by weight. It is probably present in small quantities as the rickettsiae give a faint reaction with Millon's reagent. Under the conditions used for the formaldehyde inactivation of the organisms only the free amino groups in the proteins would combine and this reaction would be incomplete. There is little reason to believe that the low tyrosine content is due to the formaldehyde treatment.

DISCUSSION.

The bacteria which have been examined contain both RNA and DNA, and this is generally believed to hold for all bacteria. The early investigations on the nucleic acids of viruses, largely based on colour reactions of the sugars, showed that plant viruses contained ribonucleic acid and animal viruses deoxypentose nucleic acid. It now appears that the viruses may be divided into three groups on the basis of their nucleic acids ; the plant viruses (at least those which can be purified), and possibly certain animal viruses, which contain only RNA ; the bacteriophages (Cohen and Arbogast, 1950) and the insect polyhedral viruses (Bergold, 1947) containing only DNA ; and some animal viruses such as influenza and vaccinia which possess both types of nucleic acid (Knight, 1947 ; Wyatt, unpublished). In containing both RNA and DNA *R. burneti* resembles the bacteria and the larger animal viruses.

The quantitative compositions of deoxypentose nucleic acids from some bacteria and viruses have been studied by Smith and Wyatt (1951). The range of compositions among microbial DNA is very much wider than that of DNA from higher animals. However, DNA from different strains of the same bacterium

or virus show very similar compositions. In contrast to that from animals and plants, the DNA from the micro-organisms studied contains no 5-methyl cytosine. This is also true of *R. burneti*, and possibly the absence of this pyrimidine is a characteristic of all microbial deoxypentose nucleic acids.

For reasons stated elsewhere in this paper we do not believe that our rickettsial preparations were contaminated to any measurable extent with host cell material. It is interesting then that in the proportions of the four major bases (adenine, guanine, cytosine and thymine) the rickettsia DNA is almost identical with the host DNA; the two nucleic acids differ only in that one contains 5-methyl cytosine and the other apparently does not. In the case of the bacteriophages T2 and T5 and their host *Bact. coli* the deoxypentose nucleic acids of host and virus differ strikingly in composition (Smith and Wyatt, 1951). There is no reason to suppose that the DNA present in any cell consists of a single molecular species, and the nucleic acid as analysed may be an aggregate of several different types of molecule with differing compositions. In the embryo nucleic acid, for instance, it is possible that only one or a small number of these contains the 5-methyl cytosine. It is not possible therefore to dismiss the possibility that host nucleic acid may be used directly in the proliferation of the rickettsiae.

It would, of course, be desirable to study the nucleic acid composition of this organism grown in a host with a nucleic acid composition different from that of the chick embryo. This might be possible by growth in the mouse lung, although for technical reasons it is less easy to isolate sufficient quantities of washed rickettsial suspensions from this source.

SUMMARY.

Suspensions of *R. burneti* have been prepared which are believed to contain little, if any, material derived from the host cell. The compositions of the nucleic acids present in the rickettsiae in these preparations have been examined quantitatively, using paper chromatography and spectrophotometric methods.

R. burneti contains 9·7 per cent (dry weight) deoxypentose nucleic acid and about 4·3 per cent ribonucleic acid. Different preparations have the same content of DNA but the amounts of RNA differ. The nucleic acids do not account for all the phosphorus in the organisms.

The purine and pyrimidine composition of the rickettsial DNA has been determined. No 5-methyl cytosine could be detected in this nucleic acid.

Deoxypentose nucleic acid has been isolated from normal chick embryos and its purine and pyrimidine composition determined. The proportions of adenine, guanine, cytosine and thymine in this nucleic acid do not differ appreciably from that of the rickettsial DNA. The embryo nucleic acid, however, contains 5-methyl cytosine amounting to 4·3 per cent. of the cytosine content.

The amino-acids present in the rickettsiae have been identified by paper chromatography.

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