THE ISOLATION OF BACTERIAL NUCLEIC ACIDS USING CETYLTRIMETHYLAMMONIUM BROMIDE (CETAVLON)

by

A. S. JONES*

Chemistry Department, The University, Edgbaston, Birmingham (England)

Recently much work has been carried out on the composition of bacterial nucleic acids (CHARGAFF *et al.*[1,2]; SMITH AND WYATT[3]). It has been found, however, that the isolation of pure nucleic acids from bacterial cells is a rather difficult operation. The classical salt extraction and dilution technique of MIRSKY AND POLLISTER[4] has not found general application in the field of bacterial nucleic acids due to the fact that many bacterial nucleoproteins could not be precipitated from solution in 1 *M* sodium chloride by dilution with water (CHARGAFF AND SAIDEL[1], OVEREND, STACEY, WEBB AND UNGAR[5]). The method of HAMMARSTEN[6] has been used to isolate nucleic acid from *Mycobacterium phlei* (SNELLMAN AND WIDSTRÖM[7]), but in the present work this procedure gave gelatinous precipitates which were worked with difficulty. The present investigation was carried out, therefore, in order to devise for the isolation, under mild conditions, of bacterial nucleic acids, a new method which might be more convenient and of wider application than those used at present. A short account of such a method has already been published (JONES[8]); this communication gives details of the procedure adopted.

EXPERIMENTAL

Source of organisms

Mycobacterium tuberculosis (human strain). This was a mixed strain kindly supplied by "The Ministry of Agriculture and Fisheries Veterinary Laboratories, Weybridge". The cells were removed from the culture medium and were immediately extracted with urea at 37° C.

Mycobacterium phlei Söhn. (National type culture collection). The organism was grown on the following complex medium. Lab. lemco, 1 %; Oxoid bacteriological peptone, 1 %; sodium chloride, 0.5 % and glycerol 5 %. The cells were harvested after 6–10 days incubation at 37° C.

Sarcina lutea was grown on solid complex medium of the following composition: Lab. lemco, 1 %; Oxoid bacteriological peptone, 1 %; sodium chloride, 0.5 %; glucose, 2 % and agar, 2 %. Cells were harvested after 48 hours incubation at 37° C.

Analytical methods

Nitrogen estimations were carried out by the micro-Kjeldahl method of MA AND ZUAZAGA[9] using the distillation apparatus of MARKHAM[10]. *Phosphorus* was estimated by the method of JONES, LEE AND PEACOCKE[11]. *Pentose* estimations were carried out by the method of EULER AND HAHN[12] using a purified sample of yeast nucleic acid as standard.

Extraction of nucleic acids from bacterial cells

In the case of *Myco. tuberculosis*, the cells were readily extracted with saturated aqueous urea solution at 37° C (BALDWIN, ILAND, GILBERT AND JONES[13]). The urea

* Beit Memorial Research Fellow.

References p. 612.

extract contained only a small amount of nucleic acid, but, upon re-extraction of the cell residues remaining after urea extraction, with water at 0° C, a considerable proportion of the nucleic acid diffused into solution. The aqueous extraction of urea-extracted cells of *Myco. tuberculosis* provided a convenient method for obtaining nucleic acid from this organism. This effect of urea extraction was not obtained with *Myco phlei* or *Sarcina lutea*, however, and nucleic acid could only be extracted from intact cells of these organisms by relatively drastic methods, such as extraction with sodium cholate at 60° C (HENRY AND STACEY[14], JONES, MUGGLETON AND STACEY[15]). These organisms were therefore disintegrated by shaking with glass beads, prior to their extraction with dilute sodium citrate or sodium arsenate at 0° C.

Precipitation of the Bacterial Nucleoproteins

Since the nucleoproteins of the three organisms used in this work, were not precipitated by the dilution of a 1 *M* sodium chloride solution, other methods for the precipitation of the nucleoprotein were tried. They were easily precipitated by adjusting the solutions to pH 4.5, but it was considered to be desirable to avoid the use of acid conditions due to possible degradation of the nucleic acids. The precipitate which was obtained by the addition of calcium chloride solution was difficult to separate, but it was found that copper sulphate gave a nucleoprotein-containing precipitate with which it was convenient to work. Using the latter reagent as a precipitant for the nucleoprotein, the following method was employed to isolate the nucleic acid from *Myco. phlei*.

Living cells of *Myco. phlei* (dry weight 1.4 g), disintegrated in the presence of sodium citrate (0.1 *M*) were extracted at 0° C with 0.1 *M* sodium citrate, pH 7.5 (200 ml) for 48 hours. The cell debris was removed (Sharples super centrifuge), the supernatant liquid treated with ethanol (3 vols) and the fibrous material thus precipitated, removed mechanically. The granular material was collected at the centrifuge, redissolved in 1 *M* sodium chloride and ethanol (3 vols) added when a further quantity of fibrous material was obtained. This material was dissolved in 1 *M* sodium chloride, purified by reprecipitation with ethanol, the fibres collected, redissolved in 1 *M* sodium chloride, and 2% copper sulphate solution added until precipitation was complete. The precipitate was removed at the centrifuge, washed with water, dissolved in 1 *M* ammonium chloride (adjusted to pH 8 with 0.2 *M* borate buffer, pH 9) (30 ml) and dialysed against the same solution until no more copper was removed. The solution was centrifuged and to the supernatant liquid (35 ml) there was added ethanol (3 vols). The fibrous material was collected, dissolved in 1 *M* sodium chloride and protein removed by repeated shaking with chloroform and octanol (9:1). The nucleic acid was precipitated from the aqueous solution by the addition of ethanol (3 vols), redissolved in distilled water, the solution dialysed against distilled water until free from sodium chloride, and dried from the frozen state. The nucleic acid was obtained as a slightly green fibrous product (39 mg) which had N, 11.1%; P, 6.2% and pentosenucleic acid, 13.4%.

This method was effective for the isolation of bacterial nucleic acids, but it was found to be extremely difficult to remove the copper by dialysis. Furthermore, the analytical data indicated that there was present a considerable amount of impurity in the final product.

The most convenient method for the precipitation of bacterial nucleoproteins was found to be by the use of the cationic detergent, cetyltrimethylammonium bromide (Cetavlon). The complex formed by this substance with nucleoproteins was insoluble in

References p. 612.

dilute salt solutions (< 0.3 *M*) but soluble in 1 *M* sodium chloride. Thus this complex could be purified by repeated precipitation from solution in 1 *M* sodium chloride by dilution with water. This reagent has been used to precipitate the nucleoproteins of *Myco. tuberculosis*, *Myco. phlei*, and *Sarcina lutea* and thus to isolate the nucleic acids from these organisms.

The isolation of nucleic acid from Myco. tuberculosis

Myco. tuberculosis (*ca.* 1 kg of moist cells) was extracted with urea at 37° C for several days. The urea extract was removed and the residual cells extracted repeatedly with water. To the combined extracts (3 litres), 2% Cetavlon solution was added until precipitation was complete. The precipitate was removed, dissolved in 1 *M* sodium chloride (300 ml) and the viscous solution diluted with two volumes of water. The fibrous precipitate was separated at the centrifuge and redissolved in 1 *M* sodium chloride. This procedure was then repeated a further three times. Protein was removed by shaking the solution of the nucleoprotein-Cetavlon complex in 1 *M* sodium chloride, with chloroform and octanol (9:1) until no more protein-chloroform gel was formed. This process simultaneously removed the Cetavlon. The nucleic acid was then precipitated with ethanol, dissolved in 1 *M* sodium chloride, and reprecipitated with ethanol. The fibrous precipitate was washed with 85% ethanol (5 times), to remove most of the sodium chloride, dissolved in distilled water and the solution dialysed against distilled water at 0° C. Upon freeze-drying, the nucleic acid was obtained as a white fibrous solid (400 mg) which contained N 13.5%; P, 7.9% and pentosenucleic acid, 32.2%.

The isolation of the nucleic acid of Myco. phlei

Cells of *Myco. phlei* (dry weight 14 g) were disintegrated in the presence of sodium arsenate (0.01 *M*) by shaking a thick suspension of the organisms with ballotini glass beads (0.8–1 mm dia.) for 3 hours. The solution was diluted ten times and allowed to stand at 0° C for 24 hours. The suspension was decanted from the glass beads and solid Cetavlon added with stirring until precipitation was complete. The precipitate was washed with water and purified by four reprecipitations from 1 *M* sodium chloride solution by dilution with water. The complex was finally dissolved in 1 *M* sodium chloride and the protein, cell debris and Cetavlon removed by repeatedly shaking the solution with chloroform and octanol (9:1). The nucleic acid was precipitated by the addition of ethanol (3 vols.), redissolved in 1 *M* sodium chloride, reprecipitated with ethanol, sodium chloride removed by repeated washing with 85% ethanol (*ca.* 15 times) and the NA dissolved in distilled water. The solution was freeze-dried to give a white fibrous product (200 mg) containing N, 12.2%; P, 7.6% and pentosenucleic acid, 11.2%.

The isolation of the nucleic acid of Sarcina lutea

Cells of *Sarcina lutea* (dry weight, 16.4 g) were disintegrated by shaking with glass beads, extracted with sodium arsenate solution and the nucleoproteins precipitated with Cetavlon as previously described. The nucleoprotein was purified by repeated reprecipitations from 1 *M* sodium chloride solution by dilution with water and the solution treated with chloroform and octanol in the usual way. The nucleic acid was precipitated and reprecipitated from 1 *M* sodium chloride solution, with ethanol, the precipitate washed with 85% ethanol, and dissolved in distilled water. The solution was dialysed

References p. 612.

against distilled water and freeze-dried to give the nucleic acid as a white solid (230 mg) N, 12.0%; P, 7.3%; pentosenucleic acid, 59%.

Estimation of Cetavlon in nucleic acid preparations

The amount of Cetavlon remaining in the nucleic acid preparations was estimated by microbiological assay. The bacteriostatic activity of Cetavlon (1: 256,000) against *Staphylococcus aureus* in a nutrient broth medium containing 4% sodium chloride was found to be unaffected by the presence of 0.1% nucleic acid which had been isolated without the use of Cetavlon. A 0.1% solution of the nucleic acids isolated by the Cetavlon method was without bacteriostatic activity against staphylococcus aureus, thus indicating that there was less than 0.4% Cetavlon in the nucleic acid preparation.

The reaction of acidic polysaccharides with Cetavlon

Acidic polysaccharides are frequently found in bacterial cells and as these substances may precipitate with Cetavlon it was possible that they would occur as impurities in the nucleic acids isolated by the above method. It was of interest, therefore, to investigate the action of Cetavlon on certain acidic polysaccharides. The results recorded in Table I were taken immediately after dilution of the solutions. After standing for several hours at 0° C precipitates slowly formed in some solutions which hitherto had been clear.

TABLE I

Polysaccharide	*Pptn. with Cetavlon*	*Pptn. when 1 M NaCl solution of complex was diluted to —*	
		(a) 0.3 Molar	*(b) 0.1 Molar*
Rhizobium radicicolum	+	—	+
Azoterbacter cröococcum	+	—	+
Pneumococcus type II	+	—	—
Gum arabic	—	/	/
Arabic acid	+	—	+
Starch glycollate	+	±	+
Gum tragacanth	+	—	—
Luteic acid	+	—	—
Quince gum	+	—	+
Sodium alginate	+	+	+

DISCUSSION

The bacterial nucleic acids isolated by the use of Cetavlon were white, fibrous substances which gave highly viscous aqueous solutions. It did not appear, therefore, that the method of isolation had caused any extensive degradation. It was interesting to note that the nucleoproteins of the bacteria examined were soluble in dilute salt solutions ($< 0.3\ M$) thereby differing from the desoxypentosenucleoproteins of certain higher organisms such as herring roe, calf thymus and wheat germ. The Cetavlon complexes of the bacterial nucleoproteins were insoluble in dilute sodium chloride solution but soluble in $1\ M$ sodium chloride. In this respect, therefore, the Cetavlon-bacterial nucleoprotein complex was rather similar to the desoxypentosenucleoproteins of animal tissues.

References p. 612.

Nucleoproteins were not the only substances found in a bacterial extract which were precipitated by Cetavlon; a great many acidic polysaccharides were readily precipitated by this reagent. The Cetavlon complexes of most of the polysaccharides differed from those of the nucleoproteins in being soluble in 0.3 *M* sodium chloride. Therefore, much polysaccharide material was removed from the precipitated nucleoprotein by repeated reprecipitation at 0.3 *M* sodium chloride concentration. It may be noted, however, that the nucleic acids isolated by the above method had rather low nitrogen and phosphorus values. Therefore, they may have still contained polysaccharide impurities. Work in progress at present has resulted in the separation of carbohydrate material from these nucleic acids.

Many of the nucleic acids isolated from bacterial sources gave low nitrogen and phosphorus values, *e.g.* desoxypentosenucleic acid from avian tubercle bacilli (N, 10.6%, 13.3%; P, 6.3%, 7.4%) (CHARGAFF AND SAIDEL[1]; VISCHER, ZAMENHOF AND CHARGAFF[16]) and desoxypentosenucleic acid of *Haemophilus pertussis* (OVEREND, STACEY, WEBB AND UNGAR[5]) (N, 10.83%; P, 6.36%). It appears, therefore, that certain bacterial nucleic acids are contaminated with impurity such as polysaccharide, or that the composition of these nucleic acids differs from that required for the accepted polynucleotide structure. Thus, although the nucleic acids isolated by the use of Cetavlon may still contain impurities, the method compares favourably with any existing mild method for the isolation of bacterial nucleic acids. The Cetavlon method has the great advantage of simplicity and appears to be of fairly general application.

It was apparent that both desoxypentosenucleoproteins and pentosenucleoproteins were precipitated by Cetavlon as the nucleic acids isolated were in all cases mixtures of the two types. The pentosenucleic acid content of the final product varied quite widely in different preparations from the same organism. This was probably due to the fact that no attempt was made to ensure that all the cells were harvested at the same phase of growth for each preparation. A method for the separation of the two types of nucleic acid is described in the following paper.

The author wishes to thank Professor M. STACEY, F.R.S. for his interest in this work, Mr K. G. HIGGINS for technical assistance, and the Medical Research Council for financial assistance.

SUMMARY

Nucleic acids have been isolated from *Myco. tuberculosis*, *Myco. phlei* and *Sarcina lutea* by a new mild method. The nucleoproteins were extracted from disintegrated cells of the organisms, or in the case of *Myco. tuberculosis* from urea-extracted cells, and then precipitated with cetyltrimethylammonium bromide (Cetavlon). The Cetavlon-nucleoprotein complex was dissolved in 1 *M* sodium chloride and reprecipitated by dilution with two volumes of water. This purification procedure was repeated several times. The protein and Cetavlon were removed by repeatedly shaking a solution of the complex in 1 *M* sodium chloride with chloroform and octanol (9:1). The nucleic acids were obtained as white fibrous solids which were mixtures of desoxypentosenucleic acid and pentosenucleic acid.

RÉSUMÉ

Des acides nucléiques ont été isolés par une méthode douce nouvelle, à partir de *Myco. tuberculosis* *Myco. phlei* et *Sarcina lutea*. Les nucléoprotéines furent extraites de cellules désintégrées ou, dans le cas de *Myco. tuberculosis*, de cellules extraites à l'urée; elles furent ensuite précipitées par le bromure de cétyl-triméthyl-ammonium (Cetavlon). Le complexe nucléoprotéines-Cetavlon, dissous dans une solution 1 *M* de chlorure de sodium, a été reprécipité par dilution avec deux volumes d'eau. Ce

References p. 612.

procédé de purification a été répété plusieurs fois. La protéine et le Cetavlon ont été éliminés par agitation répétée de la solution du complexe dans du chlorure de sodium 1 *M* avec un mélange chloroforme-octanol (9:1). Les acides nucléiques, obtenus sous forme de solides blancs fibreux, étaient des mélanges d'acides désoxypentose- et pentosenucléique.

ZUSAMMENFASSUNG

Es wurden Nukleinsäuren aus *Myco. tuberculosis*, *Myco. phlei* und *Sarcina lutea* mit einer neuen milden Methode isoliert. Die Nukleoproteine wurden extrahiert aus zerstörten Zellen der Organismen oder, im Fall von *Myco. tuberculosis*, aus mit Harnstoff extrahierten Zellen und dann mit Cetyltrimethylammoniumbromid (Cetavlon) gefällt. Der Cetavlon-Nukleoproteinkomplex wurde in 1 *M* Natriumchlorid aufgelöst und durch Verdünnung mit zwei Volumen Wasser wieder ausgefällt. Dieses Reinigungsverfahren wurde mehrere Male wiederholt. Das Protein und das Cetavlon wurde durch wiederholtes Schütteln einer Lösung des Komplexes in 1 *M* Natriumchlorid mit Chloroform und Oktanol (9:1) entfernt. Die Nukleinsäuren wurden als weisse faserige Massen erhalten, die Mischungen von Desoxypentosenukleinsäure und Pentosenukleinsäure darstellen.

REFERENCES

1 E. CHARGAFF AND HELEN F. SAIDEL, *J. Biol. Chem.*, 177 (1949) 417.
2 E. CHARGAFF, S. ZAMENHOF, G. BRAUVERMAN AND L. KERIN, *J. Am. Chem. Soc.*, 72 (1950) 3825.
3 J. D. SMITH AND G. R. WYATT, *Biochem. J.*, 49 (1951) 144.
4 A. E. MIRSKY AND A. W. POLLISTER, *Proc. Natl Acad. Sci. U.S.*, 28 (1942) 344.
5 W. G. OVEREND, M. STACEY, M. WEBB AND J. UNGAR, *J. Gen. Microbiol.*, 5 (1951) 268.
6 E. HAMMARSTEN, *Biochem. Z.*, 144 (1924) 383.
7 O. SNELLMAN AND G. WIDSTRÖM, *Arkiv. Kemi Mineral. Geol.*, 19 A (1945) No. 31.
8 A. S. JONES, *Chemistry and Industry*, (1951) 1067.
9 T. S. MA AND G. ZUAZAGA, *Ind. Eng. Chem. Anal. Ed.*, 14 (1942) 280.
10 R. MARKHAM, *Biochem. J.*, 36 (1942) 790.
11 A. S. JONES, W. A. LEE AND A. R. PEACOCKE, *J. Chem. Soc.*, (1951) 623.
12 H. V. EULER AND L. HAHN, *Svensk. Kemi. Tid.*, 58 (1946) 251.
13 C. N. ILAND, G. A. GILBERT, A. S. JONES AND R. W. BALDWIN, *Proc. 5th International Congress for Microbiology*, Rio de Janeiro, Brazil (1950), 201.
14 H. HENRY AND M. STACEY, *Proc. Roy. Soc., B*, 133 (1946) 391.
15 A. S. JONES, P. W. MUGGLETON AND M. STACEY, *Nature*, 166 (1950) 650.
16 E. VISCHER, S. ZAMENHOF AND E. CHARGAFF, *J. Biol. Chem.*, 177 (1949) 429.

Received October 30th, 1952