Properties of a Deoxyribonucleoprotein Complex Derived from *Bacillus subtilis**

N. V. Bhagavan and William A. Atchley

ABSTRACT: Protoplasts from *Bacillus subtilis* were extracted with 1.0 M NaCl to give a crude extract rich in deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein. Lowering the NaCl concentration failed to precipitate any deoxyribonucleoprotein (DNAP), but addition of 0.01 M $MgCl_2$ to an otherwise salt-free crude extract precipitated all the DNA present as a fibrous DNAP complex. The DNAP solubility was greater at both higher and lower concentrations of $MgCl_2$. Deoxyribonucleoprotein obtained by precipitation with $MgCl_2$ contained 43% DNA, 5% RNA, and 52% protein. The nitrogen to phosphorus ratio was 3.8. The ultraviolet absorption spectrum showed a maximum at 258 mμ and a minimum at 238 mμ; the extinction coefficient, based on 1% DNA, was 197 at 258 mμ. About 40% of the protein present in the reprecipitated DNAP fraction was soluble in 0.25 N HCl. Electrophoresis of the acid-soluble extract revealed two basic proteins migrating toward the cathode. Amino acid analysis showed no cystine or tryptophan. The ratio of basic amino acids to total acidic amino acids (free and amide forms) was 0.62. These basic proteins isolated from *B. subtilis* DNAP are in many ways similar to histones.

If the salt of a monovalent metallic cation, such as NaCl, is added in increasing concentrations to a crude solution of mammalian deoxyribonucleoprotein (DNAP),[1] the solubility of the DNAP falls initially and DNAP is precipitated. Above a critical concentration of salt the DNAP regains solubility, and at sufficiently high salt concentrations the DNAP is completely soluble. If the salt of a divalent metallic cation, such as $MgCl_2$, is added to mammalian DNAP, one sees the same reversing change in solubility. The solubility curves associated with the salts of monovalent cations and divalent cations, however, are easily distinguished since they show both qualitative and quantitative differences (Atchley and Bhagavan, 1962, 1964).

In similar studies of a crude extract of *Bacillus subtilis*, NaCl precipitated no DNAP whatsoever. Appropriately dilute concentrations of $MgCl_2$, however, precipitated a DNAP complex containing virtually all of the deoxyribonucleic acid (DNA) present; raising the concentration of $MgCl_2$ increased the solubility of the bacterial DNAP in the same fashion as for mammalian DNAP. In the DNAP precipitate large amounts of a basic protein were associated with the DNA. This report deals with the DNAP prepared from *B. subtilis* and with the basic proteins extracted from the DNAP.

Methods

Culture of Organisms. B. subtilis, strain SB19, was grown aerobically in Difco Antibiotic Medium 3 at 37° for 10–11 hours while being shaken vigorously. The cells were harvested in a refrigerated Sharples Super centrifuge. The yield was about 2 g of wet cells per liter of culture.

Lysis of Cells. The harvested cells were washed twice in 0.15 M NaCl–0.015 M trisodium citrate, *p*H 7.0, by centrifugation at 20,000 × *g* for 15 minutes in a Spinco Model L ultracentrifuge. The cells were suspended in the proportion of 1 g of wet cells to 4 ml of saline-citrate solution and warmed to 37°; lysozyme (crystalline, egg white, Armour) was then added to a final concentration of 1 mg/ml (Nester and Lederberg, 1961). Incubation at 37° and gentle agitation for 30 minutes yielded protoplasts, identified by phase-contrast microscopy. The viscous preparation was chilled immediately by adding 20 volumes of cold saline-citrate solution and centrifuged at 30,000 × *g* for 30 minutes. The pellet was washed twice with saline-citrate solution.

Extraction of DNAP. The protoplasts were extracted with 1 M NaCl which had been adjusted to *p*H 8.0 to inhibit DNAase II (Catcheside and Holmes, 1947; Maver and Greco, 1949b) and cathepsins (Maver and Greco, 1949a). All steps were carried out at 4°. In the standard procedure the protoplast pellet was suspended in 10 volumes of 1 M NaCl and left for 4 hours, following which the extract was clarified by centrifugation at 30,000 × *g* for 30 minutes. The supernatant was dialyzed for 16 hours against 0.7 mM phosphate buffer, *p*H 8.0. The first two changes contained 0.001 M ethylenediaminetetraacetic acid (EDTA) to block the action of deoxyribonuclease I; the final two changes (all containing more than 10 volumes) were free of EDTA to prepare for the subsequent fractionation by $MgCl_2$. No precipitate appeared during

* From the Cancer Research Institute and the Department of Medicine, University of California School of Medicine, San Francisco. *Received September 30, 1964.* Supported by a grant (CA-05060) from the U.S. Public Health Service, by the Martha and Spencer Love Foundation, the Robert Lehman Fund, and a Various Donors Fund.

[1] Abbreviation used in this work: DNAP, deoxyribonucleoprotein.

dialysis. The dialyzed preparation was designated crude extract.

Solubility Studies. The solubility of DNAP in salt solutions was determined by a previously described method (Bernstein and Mazia, 1953; Atchley and Bhagavan, 1964). In brief, the absorbancy of the supernatant at 260 mμ was used as an index of the amount of DNAP precipitated.

Isolation of DNAP Fraction. In order to prepare the DNAP fraction in bulk from *B. subtilis*, an equal volume of 0.02 M $MgCl_2$ was added with constant stirring to the crude extract after it had been adjusted to *p*H 6.5. The solutions first became viscous and then developed a precipitate of short ropelike fibers. The precipitate was collected by centrifugation at 10,000 × *g* for 10 minutes, washed twice with 0.01 M $MgCl_2$, dissolved in saline-citrate solution, *p*H 8.0, and dialyzed against 0.7 mM PO_4 buffer, *p*H 8.0. Any insoluble material present after dialysis was removed by centrifugation at 56,000 × *g* for 30 minutes. The process of precipitation by $MgCl_2$ was then repeated.

Extraction of Acid-soluble Proteins. To obtain the acid-soluble basic proteins, the precipitated DNAP was extracted with 0.25 N HCl for 18 hours while being gently stirred. The extract, clarified by centrifugation at 10,000 × *g* for 20 minutes, was dialyzed against 0.25 N HCl for 24 hours to remove material of low molecular weight. It was then adjusted to *p*H 7.0 with dilute NaOH, and any precipitate was removed by centrifugation. The acid-soluble proteins were precipitated in the cold overnight with seven volumes of ethanol (Butler and Godson, 1963); they were then dissolved in water and lyophilized.

Chemical Determinations. DNA was measured by the diphenylamine reaction of Dische (1955), with calf thymus DNA (Worthington Biochemical Corp.) as reference standard. The blue color was developed overnight at 37° (Patterson and Dackerman, 1952) and related to the difference in absorbance at 595 and 650 mμ. RNA was fractionated by the method of Ogur and Rosen (1950) and measured by the orcinol method of Dische (1955), with yeast RNA (Worthington) as standard. Protein was measured by the biuret reaction (Kabat and Mayer, 1961), with crystalline bovine serum albumin (Armour) as standard. Amino acid analyses were done by the method of Spackman *et al.* (1958) at the Oxford Laboratories, Redwood City, Calif. Hydrolysis in 6 N HCl was carried out for 22 hours at 110°. Tryptophan, since it is destroyed by acid hydrolysis, was measured separately by both a colorimetric method (Bates, 1937), with DL-tryptophan as standard, and by an ultraviolet spectrophotometric method (Beaven and Holiday, 1952). Nitrogen analyses were carried out by a micro-Kjeldahl procedure (Kabat and Mayer, 1961), and phosphorus was measured by the method of Skidmore *et al.* (1964). Ribonuclease activity was determined by the procedure of Kalnitsky *et al.* (1959).

Electrophoresis. Electrophoretic analysis was performed on cellulose acetate (Consden and Kohn, 1959) at a constant voltage of 150 v, with barbital buffer, *p*H 8.6, $\mu = 0.075$. Ponceau S was used to stain the protein bands. Reference points were established by the simultaneous application of lysozyme (crystalline, egg white, Armour) and ribonuclease (crystalline, salt free, Worthington).

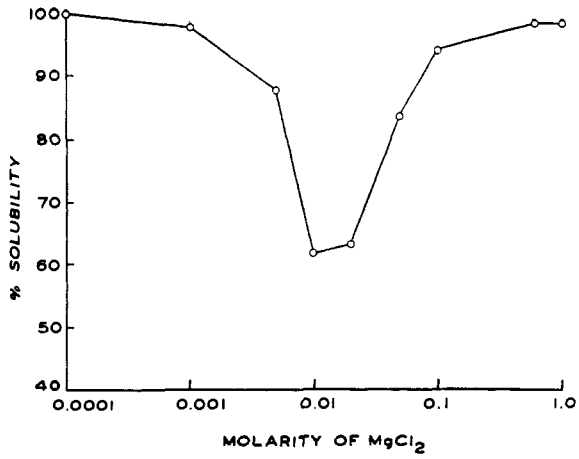

FIGURE 1: Solubility of DNAP contained in crude extract of *B. subtilis* protoplasts in 0.7 mM phosphate buffer in the presence of $MgCl_2$.

Results

Nature of the Crude Nucleoprotein Extract. In a typical extraction of the protoplasts from 8.6 g (wet wt) of *B. subtilis* with 1 M NaCl as the solvent, the yield per gram of cells was 1.85 mg of DNA, 1.17 mg of RNA, and 4.71 mg of protein, providing a ratio of 1.0:0.6:2.5 (w/w). In the crude extracts of six consecutive batches of *B. subtilis* the ratio of DNA/RNA/protein averaged 1.0:0.8:3.4. Efforts to extract DNAP with a low ionic strength solvent were abandoned because of the low yield of DNA and excessively high yield of RNA and protein.

Solubility of DNAP Contained in the Crude Extract. In the range of 0.0001–1.0 M, NaCl did not precipitate any detectable DNAP. This is in marked contrast to certain mammalian preparations, in which 80–100% of the DNA is precipitable as DNAP by appropriate concentrations of NaCl (Atchley and Bhagavan, 1964).

Addition of $MgCl_2$ to the crude extract gave a solubility curve for the DNAP contained in the extract that was similar to the reversing curve characteristic of mammalian DNAP (Figure 1). As the concentration of $MgCl_2$ increased, the DNAP became progressively less soluble until minimal solubility was reached at 0.01 M. At higher concentrations of $MgCl_2$ the DNAP solubility rose rapidly, so that at 0.1 M $MgCl_2$ about 95% of the DNAP was soluble. At 0.01 M $MgCl_2$ no DNA was detectable in the supernatant by the diphenylamine reaction, indicating that all of the DNA was precipitated in the DNAP complex described below. (The absorbance of the supernatant was ascribed primarily to ribonucleoproteins.)

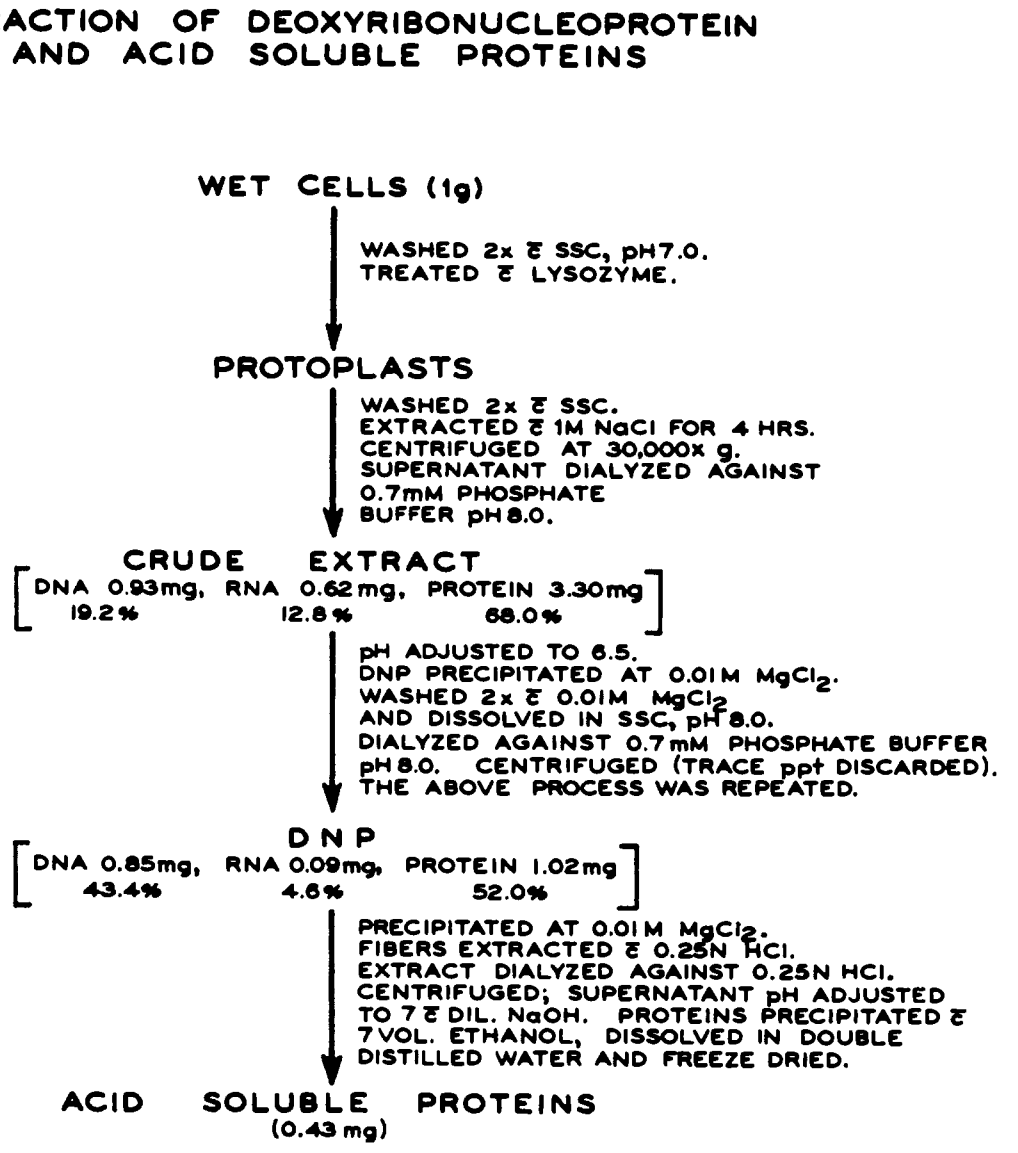

FIGURE 2: Steps in the preparation and purification of DNAP and acid-soluble proteins from *B. subtilis* protoplasts.

Preparation, Purification, and Properties of the DNAP Fraction. Information gained from the solubility study was used in preparing a relatively more purified DNAP fraction from the crude extract. In brief, the DNAP contained in the crude 1 M NaCl extract of the protoplasts was precipitated twice by the addition of 0.01 M $MgCl_2$. A typical flow diagram is shown in Figure 2. Each gram of wet cells (in a batch of 20 g) in the crude extract yielded 0.93 mg of DNA, 0.62 mg of RNA, and 3.30 mg of protein. Considerable purification of the DNAP was effected by precipitation with $MgCl_2$, with very little loss of DNA. It is evident that in the reprecipitated DNAP, protein was present slightly in excess of the DNA, while in the removed material it was considerably in excess of the RNA. Removal of the remaining RNA was not possible either by further precipitation with $MgCl_2$ or by treatment with crystalline ribonuclease.

When precipitation of the DNAP by 0.01 M $MgCl_2$ was carried out at *p*H 6.5, the DNAP appeared as short fibers, which by phase-contrast microscopy appeared to be twisted in a ropelike fashion (Figure 3). Individual strands revealed a clear-cut periodicity, the significance of which was not clear. When precipitation was carried out at *p*H 8.0, the product was granular. Solutions of the reprecipitated DNAP were clear, colorless, and viscous. The ultraviolet absorption spectrum of a typical preparation dissolved in saline-citrate solution at *p*H 6.95 is shown in Figure 4. Maximum absorption was at 258 mμ and minimum absorption at 238 mμ. The extinction coefficient was 197 at 258 mμ if calculated for a solution of DNAP containing 10 mg of DNA per ml. The ratio of absorbance at 280 mμ to the absorbance at 260 mμ ranged from 0.58 to 0.60. The nitrogen to phosphorus ratio was 3.75.

Preparation and Properties of Acid-soluble Proteins

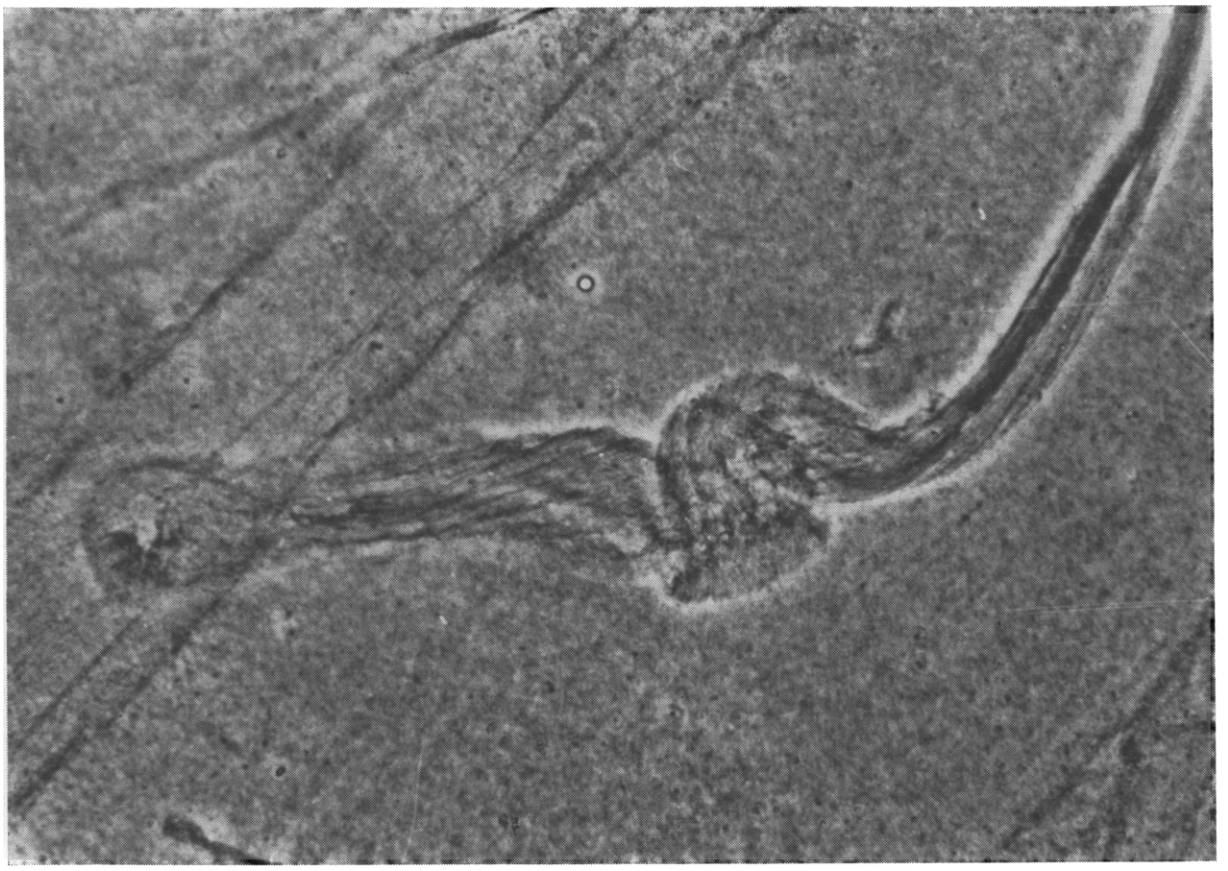

FIGURE 3: Photomicrograph (phase contrast) of DNAP fibers precipitated by $MgCl_2$ from a crude extract of *B. subtilis* protoplasts; ×160.

from DNAP. To determine if the reprecipitated DNAP fraction contained proteins resembling histones, the DNAP was again precipitated with 0.01 M $MgCl_2$ and the fibers were extracted with 0.25 N HCl. Figure 2 gives the details of a typical extraction and shows that 43% of the total protein contained in the reprecipitated DNAP fraction was acid soluble.

Table I lists the amino acid composition of the acid-soluble proteins extracted as described. The ratio of basic amino acids (arginine, histidine, and lysine) to acidic amino acids (aspartic and glutamic) was 0.62, a ratio which must include the amides asparagine and glutamine in the total aspartic and glutamic acids. No peak suggesting the presence of ϵ-*N*-methyllysine was seen (Murray, 1964). It is to be noted that no cystine or methionine was found and that tryptophan was not detectable by either colorimetric or spectrophotometric method.

The isoelectric point of the acid-soluble proteins was determined approximately by electrophoresis on cellulose acetate at *p*H 8.6. As shown in Figure 5, electrophoresis resolved the acid-soluble proteins into two components, both of which migrated toward the cathode, indicating isoelectric points greater than *p*H 8.6. The simultaneous electrophoresis of ribonuclease and lysozyme, the isoelectric points of which are approximately *p*H 9.5 and 11.0, respectively (Anderson and Alberty, 1948), was of additional help in determining the basic nature of the acid-soluble proteins, for one component of the latter migrated toward the cathode at a rate similar to that of ribonuclease and

TABLE I: Amino Acid Analysis of Acid-soluble Proteins.[a]

Amino Acid	Amount	Amino Acid	Amount
Lysine	6.5	Alanine	9.8
Histidine	0.9	Half-cystine	None
Arginine	6.8	Valine	5.4
Methionine sulfoxides	1.1	Methionine	None
		Isoleucine	5.2
Aspartic acid	13.3	Leucine	8.5
Threonine	5.7	Tyrosine	3.5
Serine	7.7	Phenylalanine	4.2
Glutamic acid	9.8	Tryptophan[b]	None
Proline	Trace		
Glycine	11.6		

[a] As moles per 100 moles of all amino acids. [b] Measured by colorimetric and spectrophotometric methods.

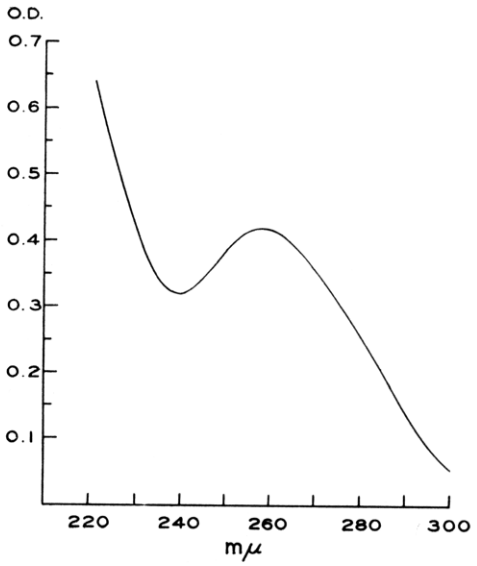

FIGURE 4: Ultraviolet absorption spectrum of purified DNAP (49 μg/ml).

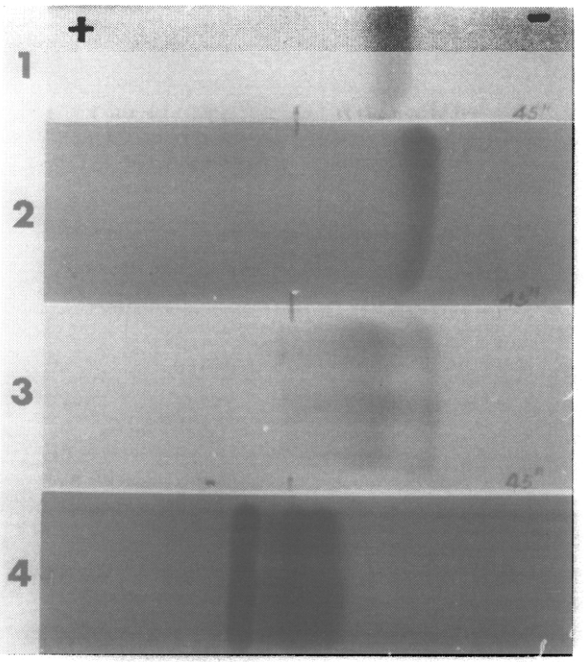

FIGURE 5: Electrophoretic analysis of the acid-soluble protein extracted from purified DNAP. The strips contain, from the top downward, (1) ribonuclease, (2) lysozyme, (3) acid-soluble proteins, and (4) normal human serum.

one at a rate similar to that of lysozyme. Analysis of the acid-soluble protein mixture showed it to be devoid of ribonuclease activity.

Discussion

With DNA established as the major repository of genetic information, there is growing interest in the macromolecular events responsible for the control of gene action and hence for growth and differentiation. Attention has recently been focused on histones as gene regulators, for they are able to alter the rate of certain nuclear metabolic reactions (Allfrey *et al.*, 1963; Allfrey and Mirsky, 1962; Huang and Bonner, 1962; Stedman and Stedman, 1943). Histones and protamines are now accepted components of animal cells and many plant cells. Efforts to isolate similar basic proteins from bacteria, however, have failed to reveal histone-like proteins clearly associated with DNA (Butler and Godson, 1963; Masui *et al.*, 1962; Tsumita and Chargaff, 1958; Wilkins and Zubay, 1959; Zubay and Watson, 1959), with the exception of the studies of Palmade *et al.* (1958), who found basic proteins associated with a DNAP fraction from *Escherichia coli*. Cruft and Leaver (1961) described basic proteins extracted from intact *Staphylococcus aureus*, but the relationship of the proteins to DNA was not apparent. The present report is of some pertinence to the problem of bacterial histones, for it describes the simple preparation from *B. subtilis* protoplasts of a reprecipitated DNAP fraction which contains large amounts of basic proteins resembling histones.

Low ionic strength solvents, such as 0.7 mM phosphate buffer, extracted only low yields of DNA. It has been shown that 1 M NaCl causes dissociation of DNAP derived from higher organisms (Chargaff, 1955); however, there is some evidence to suggest that bacterial DNAP may be more resistant to cleavage by strong salt (Chargaff and Saidel, 1949; Jones and Marsh, 1954; Masui *et al.*, 1962; Tsumita and Chargaff, 1958), although it is not clear in the work cited whether the DNAP actually included nucleohistone. The composition of the 1 M NaCl extract of *B. subtilis* protoplasts was fairly constant, with DNA/RNA/protein at about 1.0:0.8:3.4, which is similar to the 1.0:1.0:3.0 found by Spiegelman *et al.* (1958) for "nuclear material" of *Bacillus megaterium*, an organism closely related to *B. subtilis*.

Precipitation of a DNAP fraction from the crude or partially purified extract did not occur with NaCl, in contrast to the findings with DNAP from higher organisms. Jones (1953) also noted the saline solubility of DNAP from *Mycobacterium tuberculosis*, *M. phlei*, and *Sarcina lutea* extracts, although Palmade *et al.* (1958) were able to precipitate DNAP of *E. coli* by dialysis against 4% NaCl. $MgCl_2$, however, was very effective in precipitating DNAP, thus making possible a considerable concentration of DNA. The ratio of DNA/RNA/protein for the reprecipitated DNAP fraction was 1.0:0.1:1.2, similar to the ratio found for the "nuclear material" of *B. megaterium* by Butler and Godson (1963). The nitrogen to phosphorus ratio of the reprecipitated *B. subtilis* DNAP fraction was 3.8, compared with the value of 3.6 found for DNAP from *E. coli* (Palmade *et al.*, 1958; Zubay and Watson, 1959), 3.8 for DNAP from avian tubercle bacilli

(Chargaff and Saidel, 1949), and 3.7 for DNAP from calf thymus (Zubay and Doty, 1959).

When the acid-soluble proteins were analyzed by electrophoresis, they resolved into two basic components. Amino acid analysis of the basic proteins revealed the absence of detectable cystine or tryptophan, deficits which are hallmarks of histone (Phillips, 1962). The ratio of basic amino acids to acidic amino acids was less than might be expected for histones (Phillips, 1962); however, some of the aspartic and glutamic acid residues could well have existed, prior to hydrolysis, as asparagine or glutamine.

It can be concluded that the acid-soluble proteins extracted from the reprecipitated DNAP fraction of *B. subtilis* are similar to histones, since they are associated with DNA, are basic, and lack cystine and tryptophan. It is possible that they were associated in the intact cell with RNA, but this seems unlikely since they constituted at least 43% of the purified DNAP, of which RNA comprised only 5%.

Acknowledgment

The authors are grateful to Lynne Mahoney and Sarah Ebers for expert technical assistance and to David A. Wood, M.D., Director of the Cancer Research Institute, for continued interest and support.

References

Allfrey, V. G., Littau, V. C., and Mirsky, A. E. (1963), *Proc. Natl. Acad. Sci. U.S. 49*, 414.

Allfrey, V. G., and Mirsky, A. E. (1962), *Proc. Natl. Acad. Sci. U.S. 48*, 1590.

Anderson, E. A., and Alberty, R. A. (1948), *J. Phys. Colloid Chem. 52*, 1345.

Atchley, W. A., and Bhagavan, N. V. (1962), *Federation Proc. 21*, 370.

Atchley, W. A., and Bhagavan, N. V. (1964), *Arch. Biochem. Biophys. 106*, 505.

Bates, R. W. (1937), *J. Biol. Chem. 119*, vii.

Beaven, G. H., and Holiday, E. R. (1952), *Advan. Protein Chem. 7*, 319.

Bernstein, M. H., and Mazia, D. (1953), *Biochim. Biophys. Acta 11*, 59.

Butler, J. A. V., and Godson, G. N. (1963), *Biochem. J. 88*, 176.

Catcheside, D. G., and Holmes, B. (1947), *Symp. Soc. Exptl. Biol. 1*, 225.

Chargaff, E. (1955), *in* The Nucleic Acids, Vol. I, Chargaff, E., and Davidson, J. N., eds., New York, Academic, p. 307.

Chargaff, E., and Saidel, H. F. (1949), *J. Biol. Chem. 177*, 417.

Consden, R., and Kohn, J. (1959), *Nature 183*, 1512.

Cruft, H. J., and Leaver, J. L. (1961), *Nature 192*, 556.

Dische, Z. (1955), *in* The Nucleic Acids, Vol. I, Chargaff, E., and Davidson, J. N., eds., New York, Academic, p. 285.

Huang, R. C., and Bonner, J. (1962), *Proc. Natl. Acad. Sci. U.S. 48*, 1216.

Jones, A. S. (1953), *Biochim. Biophys. Acta 10*, 607.

Jones, A. S., and Marsh, G. E. (1954), *Biochim. Biophys. Acta 14*, 559.

Kabat, E. A., and Mayer, M. M. (1961), Experimental Immunochemistry, 2nd ed., Springfield, Ill., Charles C Thomas.

Kalnitsky, G., Hummel, J. P., and Dierks, C. (1959), *J. Biol. Chem. 234*, 1512.

Masui, M., Iwata, T., Ishimitsu, A., and Umebayashi, Y. (1962), *Biochim. Biophys. Acta 55*, 384.

Maver, M. E., and Greco, A. E. (1949a), *J. Biol. Chem. 181*, 853.

Maver, M. E., and Greco, A. E. (1949b), *J. Biol. Chem. 181*, 861.

Murray, K. (1964), *Biochemistry 3*, 10.

Nester, E. W., and Lederberg, J. (1961), *Proc. Natl. Acad. Sci. U.S. 47*, 52.

Ogur, M., and Rosen, G. (1950), *Arch. Biochem. 25*, 262.

Palmade, C., Chevallier, M.-R., Knobloch, A., and Vendrely, R. (1958), *Compt. Rend. 246*, 2534.

Patterson, E. K., and Dackerman, M. E. (1952), *Arch. Biochem. Biophys. 36*, 97.

Phillips, D. M. P. (1962), *Progr. Biophys. Biophys. Chem. 12*, 211.

Skidmore, W. D., Duggan, E. L., and Gonzales, L. J. (1964), *Anal. Biochem. 9*, 370.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Spiegelman, S., Aronson, A. I., and Fitz-James, P. C. (1958), *J. Bacteriol. 75*, 102.

Stedman, E., and Stedman, E. (1943), *Nature 152*, 556.

Tsumita, T., and Chargaff, E. (1958), *Biochim. Biophys. Acta 29*, 568.

Wilkins, M. H. F., and Zubay, G. (1959), *J. Biophys. Biochem. Cytol. 5*, 55.

Zubay, G., and Doty, P. (1959), *J. Mol. Biol. 1*, 1.

Zubay, G., and Watson, M. R. (1959), *J. Biophys. Biochem. Cytol. 5*, 51.