STUDIES ON THE NUCLEOPROTEINS FROM HUMAN VIRULENT TUBERCLE *BACILLI* AND ATTENUATED BOVINE TUBERCLE *BACILLI* (BCG)

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SUMMARY

Nucleoproteins were extracted from bovine tubercle *bacilli* (BCG) and a human virulent strain of tubercle *bacilli*, E9656. These preparations could be separated by zone electrophoresis in glass beads into two RNAP zones one of which coincided with that of the DNAP. The two nucleoproteins cross-reacted in the OUCHTERLONEY[12] diffusion technique using a horse antiserum to E9656 human strain tubercle *bacillus**.

INTRODUCTION

The presence of nucleic acids in tuberculin (PPD) from human virulent tubercle *bacilli* was demonstrated by SPIEGEL-ADOLF AND SEIBERT[1]. Later nucleic acids were identified in the culture medium by electrophoretic separation[2], and the same authors showed that considerable amounts of DNA were present. Nucleic acids were also isolated from the tubercle *bacillus* by various workers[3,4].

However, it was not until 1949 that any detailed chemical studies on mycobacterial nucleoproteins were carried out. CHARGAFF AND SAIDEL[5], using an avian strain of tubercle *bacilli* isolated a purified nucleoprotein which was mainly of the DNA type although RNA was also identified in the crude nucleoprotein. TSUMITA AND CHARGAFF[6] have recently published an article on the composition of the DNA and the DNAP from bovine tubercle *bacilli* (BCG). This work is mainly concerned with the isolation of DNAP and the study of the preparation, composition, and nucleotide arrangement of the nucleic acid and the investigation of the protein moiety.

The aim of the present work was to investigate the possibility of obtaining nucleoproteins from virulent human tubercle *bacilli* and bovine tubercle *bacilli* (BCG), with a view to employing them as antigens. The experiments described here concern the extraction, composition, and some physico-chemical properties of these nucleoproteins.

* Abbreviations: DNA, desoxyribonucleic acid; DNAP, desoxyribonucleoprotein; RNA, ribonucleic acid; RNAP, ribonucleoprotein.

MATERIALS AND METHODS

Growth of bacilli

Bacillus Calmette-Guerin (BCG)* and tubercle *bacilli* (E9656) virulent human strain** were grown for 3 weeks on Sauton medium at 37°. At the end of this period the *bacilli* were killed by adding 2 % phenol and harvested after 2 days; finally they were well washed with distilled water.

Isolation of nucleoprotein

All extractions were carried out at about +2°. 3 portions of 30 g wet weight *bacilli* were triturated in a mortar with 60–70 g pyrex glass powder grade 4 and a small amount of distilled water to make a paste. After about 10 min trituration more water was added to make a suspension and the mixture was centrifuged at 700 × *g* for 30 min at +2°. The supernatant was removed and the residue washed once with distilled water, the second supernatant being recovered after re-centrifugation. The pooled supernatants were centrifuged at 10,000 × *g* at room temperature to remove bacterial debris and then filtered through a Membrane filter No. 5*** overnight at +4°. Most of the nucleoproteins were retained on the filter, whilst most of the proteins, polysaccharides, and low molecular weight compounds were found in the filtrate. The residue on the filter was dissolved in water and centrifuged at 31,000 × *g* to remove glycogen[5]. The supernatant obtained after centrifugation was dialyzed for at least 24 h against distilled water containing merthiolate before lyophilization. The resulting powder constituted the crude nucleoprotein. An attempt was made to purify these nucleoproteins according to CHARGAFF AND SAIDEL[5], who observed that the conjugated protein was not precipitated by half-saturation with ammonium sulphate. The same volume of saturated ammonium sulphate was added to a solution of the crude nucleoproteins at pH 7 with stirring. The mixture was kept overnight at +4° and then centrifuged at 10,000 × *g* for 20 min. The crude BCG nucleoprotein yielded a fairly heavy precipitate which was shown to be rich in lipids; this was demonstrated by staining with sudan black. The supernatant still remained rather turbid even after repeated centrifugation. The E9656 solution also yielded a precipitate with ammonium sulphate which contained lipid, although not a very large amount, and the supernatant was only slightly turbid. The supernatants were dialysed against distilled water until there was no more ammonium sulphate present, and then lyophilized. The crude and "purified" nucleoproteins were then investigated chemically, by zone electrophoresis, ultracentrifugation, and agar diffusion.

Chemical analysis

Protein, RNA, and DNA were estimated by the Folin or biuret[7,7a] orcinol[8] and diphenylamine[9] methods respectively. Phosphorus was determined according to Allen[10] and nitrogen by the micro-Kjeldahl method[11].

Ultracentrifugal analysis

These analyses were kindly carried out by Engineer A. BIRCH-ANDERSEN and

* BCG *bacilli* were obtained from Dr. K. TOLDERLUND, State Serum Institute, Copenhagen, Denmark.

** The BCG and E9656 *bacilli* were grown on Sauton medium in the Tuberculin Department of State Serum Institute: Head of Department, Engineer M. MAGNUSSON.

*** Obtained from Membranefilter Gesellschaft, Sartorius-Werke, Göttingen, Germany.

Mr. CH. LARSEN of the State Serum Institute in the analytical Spinco Model E. The nucleoproteins were dissolved in phosphate buffer pH 7.3 to give a 0.6 % solution.

Agar-diffusion was carried out according to the technique of OUCHTERLONEY[12].

Zone electrophoresis of the nucleoproteins

Separation of the nucleoproteins was first attempted using starch and starch-gel as supporting media. Neither of these two materials was suitable since the nucleoproteins did not migrate in these carriers, but the DNA, RNA, and protein were recovered at the start after electrophoresis in Tris buffer pH 7.4, $\mu = 0.02$, and veronal buffer pH 8.6, $\mu = 0.01$. Topz ballotini glass beads (0.1 mm) were finally chosen as the supporting medium. It was first necessary to wash the glass beads with 1 l veronal buffer pH 8.6, 1 l physiological saline, 2 l 0.01 *N* sodium hydroxide and then with distilled water to pH 7.0, since it was found that the nucleoproteins would not migrate unless the glass beads were treated in this way. The buffer employed for electrophoresis was tris(hydroxymethyl)aminomethane (Tris) pH 7.4, $\mu = 0.02$. The glass bead-block was prepared as described previously by RHODES *et al.*[13].

20 mg of the nucleoprotein was dissolved in 0.1 ml water and dry glass beads were added to make a paste which was placed in a small transverse trough about 4 cm from the cathode end of the block. After laying a piece of dry paper under the lid, the latter was sealed down with a strip of plaster and the current was applied for 41 h at 300 V, and 1.5 mA, at $+4°$.

After electrophoresis the glass block was cut into 30 × 1 cm segments each of which was eluted with 5 ml water, the glass beads being centrifuged down at 700 × *g*. Protein, RNA, and DNA in the supernatants were estimated by the Folin[7,7a], orcinol[8], and diphenylamine[9] methods respectively.

RESULTS

Table I illustrates a typical chemical analysis of the crude BCG and human strain E9656 nucleoproteins, and of the "purified" nucleoproteins. The latter were prepared by the addition of an equal volume of saturated ammonium sulphate to a solution of the crude nucleoprotein, as described under MATERIALS AND METHODS.

The solution of the BCG nucleoprotein was turbid, probably due to the lipid

TABLE I

CHEMICAL ANALYSIS OF THE BCG AND HUMAN STRAIN E9656 NUCLEOPROTEINS AFTER LYOPHILIZATION

Substance	*E9656*		*BCG*	
	Crude (%)	*"Purified"* (%)	*Crude* (%)	*"Purified"* (%)
Protein (Biuret)	40.1	30.8	(> 100) (turbid lipid ?)	71.3
DNA Dische	24.0	25.2	24.0	18.2
RNA	9.6	7.2	8.2	6.0
P	5.4	5.3	1.6	1.2
N	6.0	9.1	3.2	8.0

present, which may account for the excessively high protein value found with the biuret reagent.

The nucleoproteins showed an absorption maximum at 260 mμ and a minimum at 235 mμ. The curves are shown in Fig. 1 and 2. Both indicate that some of the nucleic acid has been lost in the "purification" procedure since otherwise the maximum at 265 mμ would be considerably higher.

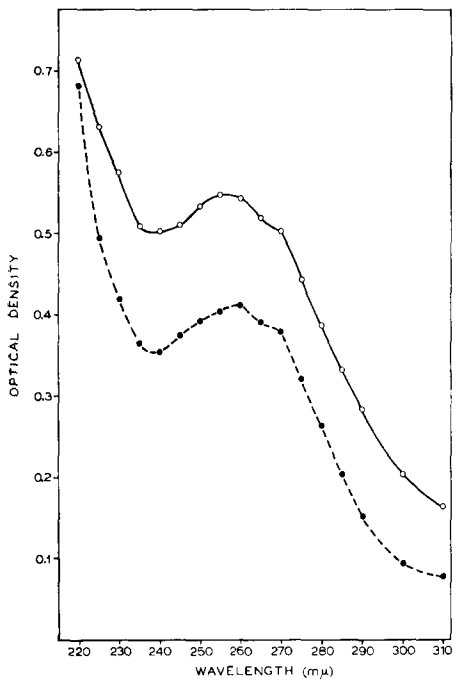

Fig. 1. u.v. spectrum of BCG nucleoproteins, 0.1 mg/ml in water: A = crude nucleoprotein; B = "purified" nucleoprotein.

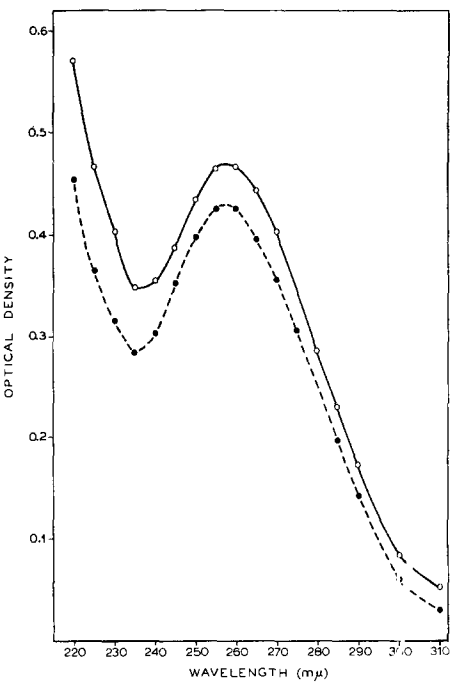

Fig. 2. u.v. spectrum of nucleoproteins from human strain E9656, 0.1 mg/ml in water: A = crude nucleoprotein; B = "purified" nucleoprotein.

Only one sedimentation peak is evident in the analytical ultracentrifuge (see Fig. 3 and 4), the crude nucleoprotein from the human virulent tubercle *bacillus* strain E9656 giving a value of $S_{20} = 7.4 \cdot 10^{-13}$ and the crude BCG nucleoprotein $S_{20} = 8.8 \cdot 10^{-13}$. The very sharp line shown by the BCG nucleoprotein may conceivably be due to the lipid present.

The results of zone electrophoresis of the BCG and E9656 nucleoproteins in glass beads are illustrated in Figs. 5 and 6. The curve for RNAP in both cases shows a major band and a second band of higher mobility in the same position as the DNAP. Protein was found across the whole block, but the main portion appears to be bound as RNA protein of slower mobility. The separation of the DNAP from the first RNAP is very clear-cut; the proportion of DNAP to RNAP in the second band being about 4 or 6:1, whereas there is about twice as much protein in the slower moving RNP band than in the faster moving RNAP zone. The absorption, in the diphenylamine test, which is apparent at the start in the electrophoresis pattern of the BCG nucleoprotein (Fig. 6),

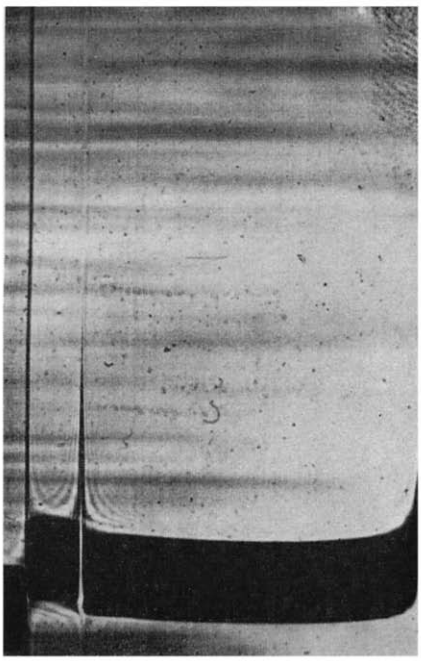

Fig. 3. Ultracentrifuge pattern of crude nucleoprotein from human virulent tubercle *bacilli*. 0.6% phosphate buffer, pH 7.3. Rotor speed 36,000 r.p. m. Model E Spinco analytical ultracentrifuge. Exposure 44 min after maximum speed. Angle 60°. $S_{20} = 7.4 \cdot 10^{-13}$.

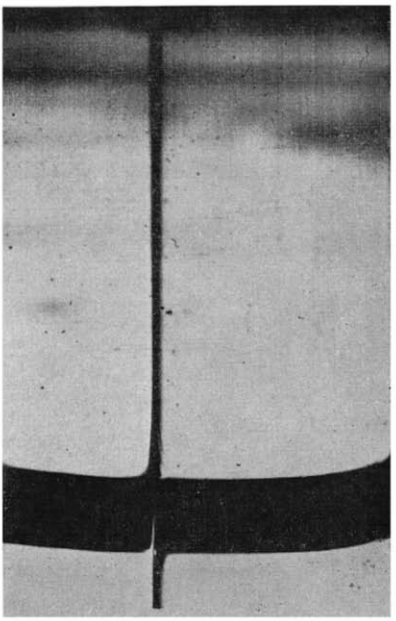

Fig. 4. Ultracentrifuge pattern of crude nucleoprotein from BCG 0.6% in phosphate buffer pH 7.3. Rotor speed 36,000 r.p.m. Model E Spinco analytical ultracentrifuge. 33 min after maximum speed. Angle 70°. $S_{20} = 8.8 \cdot 10^{-13}$.

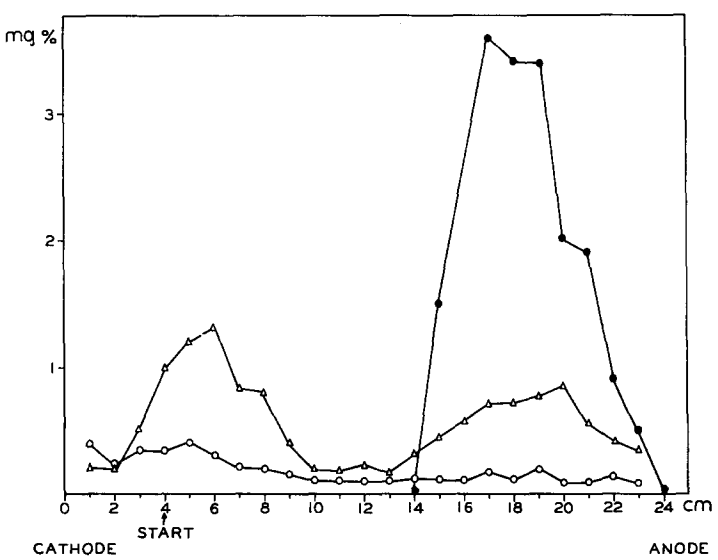

Fig. 5. Zone electrophoresis of crude nucleoprotein from human virulent tubercle *bacilli*. 20 mg in 0.1 ml Tris buffer, 0.01 μ, pH 7.3. Electrophoresis at 350 V, 1.5 mA, 41 h: △—△ RNA; ●—● DNA; ○—○ Protein.

is no doubt due to the lipid material present, since there is no typical diphenylamine colour in this region.

The "purified" nucleoproteins, which had been prepared by the addition of saturated ammonium sulphate, gave essentially the same type of result after electrophoresis as those illustrated in Figs. 5 and 6, namely, in both cases the DNAP again migrated with the faster moving RNAP, whilst the main portion of the protein was recovered with the slower moving RNAP. The ratio of DNA to RNA in the second zone was about 6:1, whereas there was now about 4–6 times as much protein in the first RNA zone. Since in all other respects the separations were similar to those of the crude nucleoproteins the figures for the "purified" proteins are not shown.

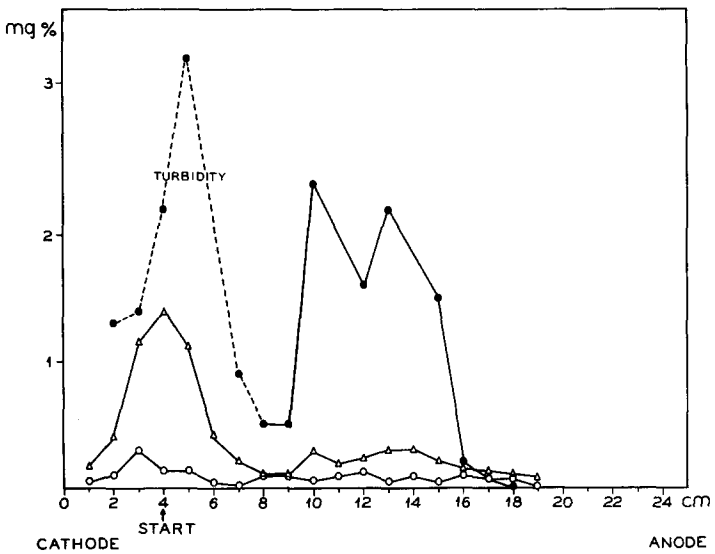

Fig. 6. Zone electrophoresis of crude nucleoprotein from BCG. 20 mg in 0.1 ml Tris buffer, 0.02 μ, pH 7.3. Electrophoresis at 350 V, 1.5 mA, 41 h: △—△ RNA; ●—● DNA; ○—○ Protein.

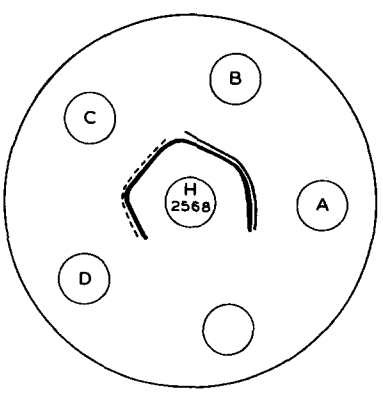

Fig. 7. Agar diffusion of nucleoproteins from BCG and human virulent strain E9656 in 1% agar. All nucleoproteins were dissolved in phosphate buffer pH 7.3 to give a solution containing 1 mg/ml: A = BCG crude nucleoprotein; B = BCG "purified" nucleoprotein; C = TB crude nucleoprotein; D = TB "purified" nucleoprotein; H2568 = horse antiserum to phenol killed E9656 strain human tubercle *bacillus*.

Immunologically the nucleoproteins are similar since they all cross-reacted when tested against horse antiserum to phenol killed human tubercle *bacilli* E9656 (see Fig. 7). The two fainter lines in C and D are probably similar to the second line in A and B but are not so clearly visible due to lack of that antigen in the nucleoprotein.

DISCUSSION

These results indicate that the nucleoprotein preparations from BCG and human tubercle *bacilli* E9656 show qualitatively the same type of result in zone electrophoresis in glass beads, in the ultracentrifuge, and in agar diffusion. The mycobacterial nucleoproteins described here can be separated into two RNAP zones and one DNAP zone in glass beads. The fact that these preparations could not be separated in starch and that the glass beads had to be pre-washed before use suggests that the glass beads might be acting as an ion-exchanger. Nucleoproteins isolated from *E. coli* have been separated by electrophoresis in starch, by PARDEE *et al.*[14], into 2 RNP zones and one DNAP zone in much the same way as in the experiments illustrated in Figs. 5 and 6. The DNAP was also found to have the same mobility as the faster moving RNP fraction. These workers were dealing with particulate DNAP and RNAP as shown by their sedimentation data in the ultracentrifuge of 40 S, 29 S, and 5 S whereas the preparations described here are in all probability no longer in the particulate form due to the method of preparation.

The fact that only one peak is visible in the ultracentrifuge might substantiate the view that the nucleoproteins described here are not in the particulate form. On the other hand, all the components may have similar molecular weights which would cause them to sediment simultaneously. Yet another interpretation of the results could be that the ribonucleoproteins are in some way attached to higher molecular weight components, such as DNA. It has been demonstrated that purified RNAP isolated from various sources does contain two sedimenting components[15–17] having molecular weights of $1.7 \cdot 10^6$ and $0.7 \cdot 10^6$. However, although there is evidence for two RNAP components after zone electrophoresis of the nucleoproteins described here no corroborative results were found in the ultracentrifuge.

It is perhaps not so surprising that the nucleoproteins from BCG and human virulent tubercle strain (E9656) cross react in agar diffusion against a horse antiserum to phenol killed E9656 tubercle *bacilli*. JENSEN AND LIND[18] and LIND AND TOLDERLUND[19] have shown that there is no difference in the specificity between PPD's from human and bovine type *bacilli* and BCG tested in human beings and guinea pigs. Other authors have also published similar findings[20–23].

Two types of antibodies to the nucleoproteins can be shown by the OUCHTERLONEY[12] technique. It is not known whether the proteins attached to the RNA or DNA give rise to these antibodies or whether they are directed against the RNAP and DNAP complex itself.

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