From the results of the studies summarized in Tables I-V of this paper and those previously reported, indicating aminonucleoside-inhibition of partially purified submitochondrial preparations of ATPase(2), it now seems quite evident that the inhibitory effects of aminonucleoside are dependent upon activation and structural state of the mitochondria. Not so clear, however, is the significance of the inhibitory effect, particularly in view of aminonucleoside-activation of the ATPase of freshly prepared kidney mitochondria, assayed in the absence of commonly added activating agents, or even in the presence of Mg^{++} when mitochondrial ageing is for periods of less than 10 minutes. From these studies, however, fairly well-defined requirements for aminonucleoside activation and inhibition have emerged. Thus aminonucleoside-inhibition of normal rat kidney mitochondrial ATPase is readily demonstrable with mitochondrial preparations in which latent ATPase has been released either by ageing, disruption of the mitochondria, or the presence of activating agents. Generally speaking, mitochondria subjected to such procedures become loosely coupled or even completely uncoupled in respect to phosphorylating capability. Activating effects of aminonucleoside, on the other hand, appear to be more readily demonstrable with freshly prepared mitochondria, which exhibit relatively low ATPase activity and are tightly coupled in respect to phosphorylating capability. Since the latter might therefore be considered to more nearly represent the normal state, effects of aminonucleoside thereon are perhaps of greater physiological significance.

Summary. 1. *In vitro* effects of aminonucleoside on ATPase activity of normal rat kidney mitochondria, assayed in the presence and in the absence of a wide variety of activating agents, have been studied. 2. Results clearly indicate dependence of aminonucleoside inhibition of rat kidney mitochondrial ATPase on activation and structural state of the mitochondria. 3. ATPase activity of freshly prepared normal rat kidney mitochondria, assayed in the absence of commonly added exogenous activating agents, is stimulated by aminonucleoside. The latter is more readily demonstrable with kidney mitochondrial preparations showing relatively low initial ATPase activity and appears to be polyphasic in response to increasing aminonucleoside concentration.

We are indebted to Dr. J. M. Ruegsegger, Lederle Medical Research Section, American Cyanamid Co., for aminonucleoside used in this study.

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Some Biochemical and Serologic Properties of the Pneumococcal C Polysaccharide.* (28578)

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The appearance of C-reactive protein (CRP) in serum of humans is one of the acute phase phenomena that occur in response to many forms of stress. Although CRP is antigenically distinctive and can be detected conveniently by a specific antise-

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rum, the initial demonstration of the protein was made by precipitation with the C (noncapsular) polysaccharide of pneumococci. Since the nature of the C-CRP complex is of considerable biologic interest, the studies reported here were carried out in an attempt to characterize chemically the serologically active pneumococcal fraction and to determine the reactive groups responsible for its complexing with CRP. Goebel *et al.*(1) had previously shown that C polysaccharide contained hexosamine, phosphorus, and an U-V absorbing material, possibly a pyrimidine. McCarty(2) reported that C substance was solubilized from the C-CRP complex in 75% saturated ammonium sulfate. Based on these observations, serologically active C polysaccharide was isolated from pneumococci, as "homogeneous" preparations for further analysis.

Materials and methods. Rough strain ATCC 11733 was grown 14 hours at 37° in 30 to 250 liters of trypticase soy broth with 0.5% glucose, buffered initially at pH 7.8 with McIlvain's phosphate-citric acid. During growth pH 7.2 ± 0.2 was maintained by addition of alkali. Growths were harvested by Sharples centrifugation, washed once in phosphate buffered saline and the packed cells stored at −15°. When needed, the cells were thawed rapidly, suspended in 20 volumes of distilled water and disrupted in a Raytheon sonic oscillator (250 W-10 KC max. breakage) for 1½ hours at 8°. At the end of this time microscopic examination showed only Gram-negative amorphous material. The sonicates were centrifuged 1 hour at 5000 rpm and 4° yielding a sediment, Fraction 1, which was frozen at −15° until further use. The yellow translucent supernate was made 75% saturated with ammonium sulfate for 2 hours at 24°; the precipitate which formed, Fraction 2, was recovered by centrifugation and frozen at −15°. The soluble phase was designated Fraction 3.

Fraction 1 was extracted with 20 volumes of 5% trichloracetic acid and the extract then dialyzed against distilled water. Polysaccharide was precipitated from the dialysate by addition of 4 volumes of ethanol containing 1% sodium acetate, redissolved in distilled water, reprecipitated 4 times, dialyzed against distilled water and lyophilized.

Fractions 2 and 3 were dialyzed until ammonium sulfate was removed and then "deproteinized," by the Sevag method using chloroform and octyl alcohol. Ethanol precipitation and lyophilization of the polysaccharide were carried out as described for Fraction 1.

Assay of serologic activity. The serologic test reagents used were: rabbit antisera to pneumococcal types 14, 19, and 26; horse antiserum to type 3 pneumococci, and human sera obtained 24 hours following surgery. The human post-operative sera gave strongly positive (4+) reactions for CRP with commercial CRP-antiserum(3) and were obtained from patients whose preoperative sera gave no precipitate either with CRP-antiserum or the bacterial fraction to be tested. Each bacterial fraction was tested for ability to produce precipitation with the above sera.

Samples (0.9 ml) of each serum were mixed with 0.1 ml of the fraction to be tested, diluted serially in saline containing 0.001 M $CaCl_2$. Mixtures were incubated at 37° for 2 hours and overnight at 4°, centrifuged, and the supernate of the human serum tested for presence of CRP with CRP-antiserum. Control mixtures containing 0.9 ml of test serum and 0.1 ml of saline were similarly treated.

Ultracentrifugation studies. A 1% solution of C substance (Fraction 3) was spun at 59,780 rpm in a Spinco ultracentrifuge and photographed at 32-minute intervals, using a Schlieren optical system. It was observed visually for the first 32 minutes.

Electrophoresis. 0.1 ml samples of a 2% solution of C substance were electrophoresed on paper strips 30 × 3 cm in Durrum cells using phosphate buffer 0.01 ionic strength pH 7 with a constant current of 5 ma, 83 volts, for 3 hours. The strips were dried, the material located by U-V absorption, eluted with buffered saline and assayed for serological activity with human CRP positive sera as described above.

Chemical analysis. Nucleic acid and nucleotides. The serologically active fraction, which was homogeneous in the ultracentrifuge and on paper electrophoresis, was

treated with DNA-ase and RNA-ase as follows: In each of 3 tubes was placed 3 ml of a 0.2% solution of C polysaccharide in saline containing 0.1% $MgCl_2$. Saline (0.1 ml) was added to the first tube, 0.15 mg DNA-ase in 0.1 ml saline was added to the second tube and 0.15 mg RNA-ase in 0.1 ml saline was added to the third tube. Each mixture was dialyzed against 500 ml of 0.85% NaCl solution (containing 0.1% $MgCl_2$) at 37° for 2 hours, then dialyzed in the cold against 500 ml of saline for 24 hours. The U-V absorption curve of each dialyzed mixture was determined in a Beckman automatic spectrophotometer and serological activity was determined by titration against CRP positive human serum described above. Nucleic acid was prepared from pneumococci according to the method of Colowick and Kaplan(4). Yeast RNA and thymus DNA were obtained commercially. Each was tested for its ability to precipitate CRP. Isolation of RNA nucleotides from C substance was attempted, using the method of Astrachan and Volkin (5). The silver nitrate, bromphenol blue reagent was used to test for purines and cytidine. C substance (100 mg) was hydrolyzed in 6 ml of 6 N HCl for 6 hours at 110°, evaporated to dryness, redissolved in water, then chromatographed on Whatman No. 4 paper using butanol, acetic acid, water (120:30:50) in an ascending technique at room temperature. The U-V absorbing band was eluted and nucleotide identified by absorption spectrum and chromatography using the following solvents: butanol, acetic acid, water (120:30:50); n-butanol, pyridine, water (65:65:65); and 0.1 M phosphate pH 6.8, ammonium sulfate, n-propanol (100:60:2). Commercially obtained RNA nucleotides were included as controls. A 1% solution of C substance was treated with streptomycin (2.5 mg/ml), the precipitate recovered by centrifugation and the supernate assayed for serological activity. The precipitate was redissolved in 1 M NaCl.

Lipids were tested by staining with Sudan black and Oil red, uronic acid by the Dische carbazole reaction(6), sialic acid by the diphenylamine reaction(7), nitrogen by a micro-Kjeldahl method(8), phosphorus by the Fiske-Subbarow method(9), reducing value by the anthrone(10), Benedict's quantitative method(11), and phenol-H_2SO_4(12). Hexosamine was determined by Elson-Morgan method(13), biuret(14), methyl pentose(15), and muramic acid(16) by methods indicated.

Chromatographic studies of constituent sugars. Samples of C substance ranging from 5 mg to 100 mg were hydrolyzed in 6 N HCl at 100° and in 2 N HCl at 105° for 0.5, 2, 4, and 6 hours, evaporated to dryness, redissolved in distilled water and brought to dryness 3 times over NaOH. The hydrolysates were redissolved in minimal amounts of water and chromatographed on Whatman No. 4 paper at room temperature using 4 different solvent systems: isopropanol, water (160:40); ethyl acetate, pyridine, water (120:50:40); n-butanol, pyridine, water (80:80:40); and isopropanol, N-butanol, water (140:20:40). Both ascending and descending systems were used. In each system authentic samples of glucosamine, galactosamine, glucose, galactose, mannose, fucose and muramic acid were run individually and as mixtures. Glucosamine-galactosamine mixtures were separated by descending chromatography in which a filter paper pad was sewn to the bottom of the sheet of filter paper containing the sample. Detection reagents were aniline hydrogen phthalate, tetrazolium red, ninhydrin, benzidine reagents, the Elson-Morgan hexosamine reagent, and the ammonium molybdate reagent for phosphate. In all paper chromatography experiments the reagent formulas and development techniques were those of Smith(17). Glass paper chromatography was also used for identification of amino sugars(18), as was ninhydrin-pyridine conversion to pentose(19). When the 100 mg sample was chromatographed, the positions of the sugars were determined by developing strips of paper cut from each side of the sheet, the corresponding sugar band eluted, dried, weighed, and rechromatographed to confirm its identity. Glucosamine, galactosamine and muramic acid were also determined by use of the Beckman Spinco model 120 amino acid analyzer as described below.

Amino acids. Samples (2 mg) of C substance were hydrolyzed in 6 N HCl for 24

hours at 110°, evaporated to dryness, residual HCl removed as described above and determination of amino acids carried out on the Beckman Spinco model 120 amino acid analyzer. Amino acids were also determined by 2-dimensional paper chromatography.

Inhibition studies of C-CRP reaction. N-acetyl glucosamine, N-acetyl galactosamine, muramic acid and uridine monophosphate were tested for ability to inhibit the C-CRP reaction. In each case 1 ml aliquots of CRP positive (4+) serum were treated with the different compounds, in amounts ranging from 0.063 mg to 2 mg, in 0.1 ml buffered saline, and incubated for 1 hour at 37° following which 0.1 mg of C substance in 0.1 ml of saline was added to each tube. The mixtures were then incubated 2 hours at 37° and overnight at 4°. Controls were mixtures of serum plus saline, serum plus test substance alone and serum plus 0.1 mg C substance. Following incubation, all tubes were examined for the presence of precipitate and the supernates tested with CRP-antiserum for presence of CRP. In addition, 25 mg of C substance was dissolved in 25 ml of 0.1 N HCl and heated to 100°; 5 ml samples were taken at the end of 2, 5, 10 and 15 minutes of heating. Each sample was then dialyzed against 1 liter of distilled water. Following dialysis, each dialysate was tested for serological activity. The dialyzable fraction was reduced in volume to 1 ml, of which 0.1 ml was tested for inhibitory activity as described above, using similar controls. The absorption spectrum of the dialyzable fraction was determined and samples were chromatographed on paper. The U-V absorbing spots were located and separate papers treated with ninhydrin and the Elson-Morgan hexosamine reagent. Subsequently, the concentrated hydrolysates were further hydrolyzed in 6 N HCl for 4 hours at 110°, and HCl removed as described above. These materials were rechromatographed, and tested for nucleotides, sugars and phosphate.

Results. Practically all (90%) of the serologically active C polysaccharide was found in the sonicate of pneumococci which did not precipitate at 75% saturation with ammonium sulfate, Fraction 3. Total yield was 29 mg/g of dried wt. of whole cells. The TCA extract of the sediment obtained by centrifugation at 5000 RPM after sonication (Fraction 1) contained trace amounts of activity (2.5 mg C substance/gram dried wt. of lyophilized residue). The polysaccharide which precipitated at 75% saturation with ammonium sulfate (Fraction 2) was serologically inactive. The active fraction was capable of removing all CRP from positive (4+) human serum when added in amounts ranging from 12.5 μg to 50 μg/ml; 200 μg/ml completely inhibited formation of a precipitate. It gave a precipitate with rabbit and horse antipneumococcal sera and with human CRP-positive sera. In trials in which heat (100°) and acid were used to remove residual protein, it was found that the resultant polysaccharide did not react with rabbit "acute phase" serum and was incapable of removing all CRP from human CRP-positive serum. Material deproteinized by the Sevag technique presumably maintained a more native state.

Ultracentrifugation showed a single peak formed by material having an S_{20w} value of 3, with some suggestion of the presence of a small amount of material behind the 3 S peak (Fig. 1). The material moved as a single U-V absorbing band in paper electrophoresis, travelling rather rapidly towards the anode. When this band was eluted, the material was found to be serologically active against human CRP positive serum. The remaining areas of the paper strips were eluted and this eluate showed no serological activity nor U-V absorption. The active material recovered from the paper had the same serologic titer as the starting material and the shape of the absorption curve was the same. The errors inherent in these measurements are such that the presence of a small amount

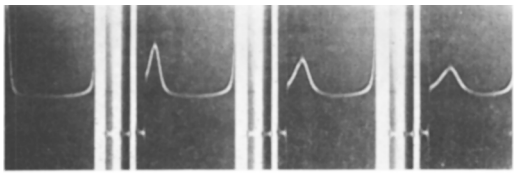

FIG. 1. Ultracentrifugation of 1% solution of C substance at 59,780 rpm; pictures taken at 32 min intervals using Schleiren optical system.

TABLE I. Analyses of C Substance from Pneumococci.

Analysis	%
Polypeptide (biuret)	9.0–11.0
Nitrogen	5.8
*Reducing sugar	36
*Hexosamine	27
*Muramic acid	3.5
Methyl pentose	<.1
Uridine	5–19
Phosphorus	4.0

* Calculated on sample hydrolyzed in 2 N HCl for 2 hr at 105°.

of inactive polysaccharide is not completely ruled out. When 0.1 mg amounts of pneumococcal nucleic acid, yeast DNA or thymus DNA were added to 1 ml aliquots of CRP positive serum, no precipitate occurred, nor did the presence of these nucleic acids inhibit the C-CRP reaction. Incubation with DNA-ase and RNA-ase followed by dialysis did not alter the U-V absorption by the C substance (Fig. 2) or its serological titer. No RNA nucleotides were recovered when 100 mg of C substance was subjected to mild alkaline hydrolysis and examined by the Astrachan-Volkin method(4). The silver-nitrate, bromphenol blue test for purines and cytidine was negative. Following acid hydrolysis, paper chromatography and U-V absorption spectra indicated that the absorbing material was uridine monophosphate. The serologically active C substance was completely precipitated by streptomycin and redissolved in 1 M NaCl. The chemical analyses are shown in Table I. Tests for lipids, uronic acid and sialic acid were negative. Paper chromatography indicated the presence of glucosamine and galactosamine, muramic acid, and glucose. Quantitative analysis of sugars separated by paper chromatography indicated that glucosamine and galactosamine constituted 27% of the recoverable material and the galactosamine/glucosamine ratio was approximately 2:1. Muramic acid was estimated at 3.5%. The amino acids were identified as alanine, glycine, glutamic and diamino-pimelic with molar ratios and percentages as given in Table II. These values were obtained by use of the amino acid analyzer (Fig. 3). The material also contained a trace amount of serine. Since di-

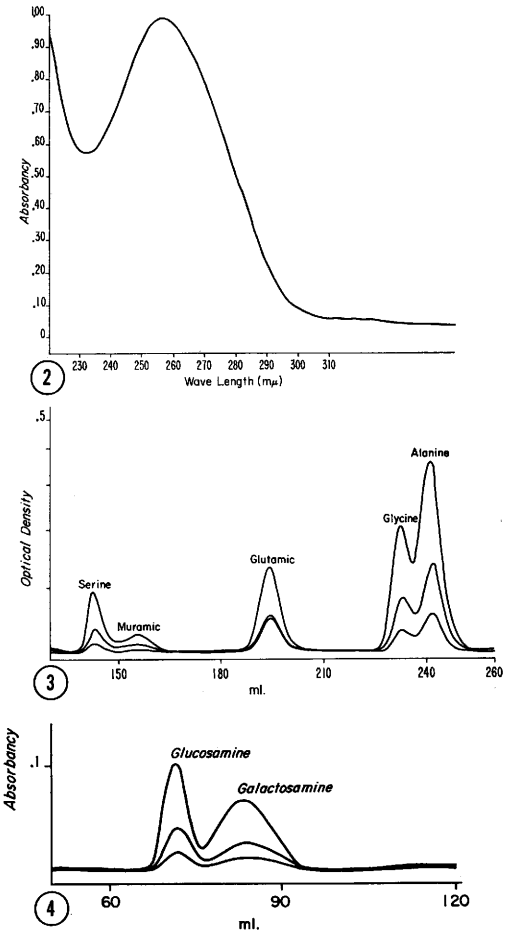

FIG. 2. U-V absorption by C substance of pneumococci (1 mg C/ml at pH 7).

FIG. 3. Amino acid analysis of C substance of pneumococci using Beckman/Spinco model 120 Amino Acid Analyzer. 2 mg sample, long column, buffer pH 3.25 at 30° to pH 4.25 at 50°. Color developed with ninhydrin.

FIG. 4. Amino sugar analysis of C substance of pneumococci using Beckman/Spinco model 120 Amino Acid Analyzer. 2 mg sample, medium column, buffer pH 4.20 at 30° to pH 4.26 at 50°. Color developed with ninhydrin.

TABLE II. Amino Acid Composition of C Substances. Results obtained by amino acid analyzer using 2 mg C.

	μmoles	Molar ratio	μg	% of C
Alanine	.86	3	76.6	3.8
Glutamic	.29	1	41.7	2.1
Glycine	.58	2	43.5	2.2
Diaminopimelic	.29	1	58.0	2.9
Total			219.8	11

aminopimelic acid comes off the column at the same place as methionine, it was further identified by means of 2-dimensional paper chromatography. Glucosamine, galactosamine and muramic acid could also be detected in the amino acid analyzer and the galactosamine-glucosamine ratio confirmed (Fig. 4). Calculations based on the foregoing data suggest that the serologically active C substance probably has a molecular weight which is a multiple of 8000 and is composed of a uridine nucleotide (UDP-muramic acid-peptide) joined to a polysaccharide fraction which consists chiefly of glucose, glucosamine and galactosamine, one or more of the sugars being phosphorylated.

The C-CRP reaction was not inhibited by n-acetyl glucosamine, n-acetyl galactosamine or muramic acid in the amounts tested (up to 2 mg/ml). Uridine monophosphate (1 mg/ml) completely inhibited the C-CRP reaction. Mild hydrolysis of the C substance (5 minutes at pH 2 and 100°) followed by dialysis resulted in complete inactivation of the non-dialyzable fraction. The dialyzable fraction completely inhibited the C-CRP reaction when tested as described above. Paper chromatography and spectrophotometric analysis showed that it contained UMP, phosphate, muramic acid and a second ninhydrin positive substance in amount too small for identification. Tests for glucosamine, galactosamine and glucose were negative.

Discussion. The results reported here appear to confirm the view of McCarty(2) that the serologically active C polysaccharide consists chiefly of cell wall material, since two major constituents are glucosamine and galactosamine. They also support the view of Goebel(1) that an U-V absorbing substance is an integral part of the molecule and does not represent contamination with nucleic acid, since the active material which was homogeneous in the ultracentrifuge and in paper electrophoresis continued to absorb in the U-V. Moreover treatment with DNA-ase and RNA-ase failed to affect the U-V absorption. Arguing also against the presence of contaminating RNA are the negative tests for purines and cytidine and the failure to isolate RNA nucleotides by the method of Astrachan and Volkin. The failure of mild alkaline hydrolysis in the latter method to remove UMP from the amino sugars reflects the comparative stability of such amino sugar nucleotides under these conditions. The fact that the C substance forms a precipitate with streptomycin suggests that it may be an anionic polymer and raises the question whether the same mechanism determines the C-CRP reaction. The C-CRP complex, as well as the C-streptomycin complex, can be redissolved in 1 M NaCl. However, CRP does not precipitate DNA and the C-CRP reaction requires Ca^{++} whereas the precipitation of anionic polymers with streptomycin is partially inhibited by Ca^{++}. With respect to the C-CRP reaction, it would seem that the protein combines with the nucleotide portion of the molecule, since removal of the nucleotide alone rendered the remaining sugar component inactive and since the uridine nucleotide which was removed was capable of inhibiting completely the C-CRP reaction, as was uridine monophosphate (1 mg/ml).

It was noted that when heat (100°) and acid (pH 4.5) were used to remove protein, the polysaccharide recovered failed to remove all CRP from human CRP positive serum, irrespective of concentration employed. This suggests that the heating at acid pH released an inhibitor which partially blocked the reaction. Uridine nucleotides are readily split off from sugars by this process and thus it may be that the less active polysaccharide preparation represents the result of the combined activity of the parent compound and the released uridine nucleotide. On the other hand, the mechanism operative in the rabbit system may differ from that in human serum and may involve a heat and acid labile component other than the uridine nucleotide.

Summary. The serologically active C polysaccharide of pneumococcus was isolated as a substance fairly homogeneous in ultracentrifuge ($S_{20w} = 3$) and on paper electrophoresis consisting of glucosamine, galactosamine, and glucose, and a uridine nucleotide composed of UDP, muramic acid and a peptide consisting chiefly of alanine, glycine, glutamic and diaminopimelic acids with a trace of serine. It could be precipitated by streptomycin

and redissolved in 1 M NaCl. Removal of the uridine nucleotide by mild acid hydrolysis destroyed the serological activity and the uridine nucleotide removed was capable of inhibiting the C-CRP reaction, as was uridine monophosphate in a concentration of 1 mg/ml. It is suggested that the uridine nucleotide portion of the C substance is responsible for its complexing with human CRP.

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Production of Specific Neutralizing Antibody with Soluble Antigens of Type 5 Adenovirus.* (28579)

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Biosynthesis of 3 well-defined soluble antigens and infectious virus follows adenovirus infection of a number of mammalian cells *in vitro*(1,2). The soluble antigens, which are readily separable from mature virus particles as well as from each other(1,2,3), are predominantly protein in nature(4) and have been shown to represent virus structural subunits produced in excess(4,5,6,7). To obtain additional evidence on the relationship of the soluble antigens to the virus proteins, advantage was taken of the fact that injection of the purified soluble antigens into rabbits leads to the production of specific antibodies directed against the type-specific (E) or group-specific (L) soluble antigens present in type 5 virus-infected cells(2). The investigation described here had as its objective to characterize further the nature and role of the E and L soluble antigens by immunological means, employing antigen-specific antisera in neutralization, complement-fixation, and cross-adsorption tests for this purpose. The data thus obtained strengthen the evidence that the soluble antigens represent virus subunits which have been made in excess but have not been incorporated into virus particles.

Materials and methods. Tissue culture. HeLa cells and, on occasion, KB cells(8) propagated in continuous serial culture as previously described(9) were employed. For infectivity and neutralization titrations cul-

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