0.3*N* formic acid. The eluate was lyophilized to remove formic acid, and the residue was dissolved in distilled water. The sialic acid content was determined by the thiobarbituric acid method (*8*) with crystalline *N*-acetylneuraminic acid as the standard. The chromogen produced by the sialic acid isolated from the cells and that produced by standard *N*-acetylneuraminic acid showed identical spectral characteristics. The sialic acid obtained from both control and induced cells was identified as *N*-acetylneuraminic acid by means of a chromatographic system for the identification and quantitative resolution of mixtures of sialic acids (*9*). Another spot also detected in these chromatograms by the thiobarbituric acid spray reagent (*10*) revealed traces of a material which had an R_F identical to that of standard *N*-glycolylneuraminic acid.

The sialic acid content, when referred to the protein content of the cells, shows a moderate but consistent increase in the preparations treated with hydrocortisone (Table 1). This difference is more striking when the sialic acid content is expressed per cell, in view of the pronounced difference in the cell count between control and hydrocortisone-treated cells. The cell count and protein content reflect the increase in size of the treated cells (*1*). The increase in both the size and the sialic acid content of hydrocortisone-treated cells is consistent with the observations of Kraemer (*4*) who found a direct correlation between cell volume and sialic acid content of synchronized hamster cell lines. The distribution of sialic acid betwen the sedimentable and unsedimentable fractions points out an additional difference between these two types of cells.

The consistent detection of trace amounts of a sialic acid with the chromatographic characteristics of *N*-glycolylneuraminic acid is of considerable interest because this type of sialic acid has never been found in human material (*11*).

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Choline in the Cell Wall of a Bacterium: Novel Type of Polymer-Linked Choline in Pneumococcus

Abstract. *Radioactive choline is incorporated by pneumococcus (strain R36A) into a polymeric substance from which it can be quantitatively recovered as free choline, after hydrolysis by strong acid. The polymeric substance is insoluble in lipid solvents and can be degraded by periodate. Fractionation studies and chemical analyses suggest that choline is linked to a polysaccharide component of pneumococcal cell wall.*

Diplococcus pneumoniae strain R36A has a nutritional requirement for choline (*1*). When pneumococci growing in a chemically defined medium containing choline (*2*) are transferred to choline-free medium, growth continues until the bacterial mass is approximately doubled; after this time protein and ribonucleic acid syntheses come to a halt and growth stops (Fig. 1).

At least a major part, if not all, of choline incorporated by these bacteria appears to be linked to a polymer which is associated with the cell wall fraction and which resembles a polysaccharide. Evidence for this conclusion came from the following experiments.

1) A culture of *Diplococcus pneumoniae* R36A clone R6 was grown at 37°C in a synthetic medium (*2*) supplemented with 0.05 μc (in 2 μg) of radioactive choline per milliliter (*3*). At the end of the logarithmic phase of growth, the cells were harvested by centrifugation, washed three times and suspended in saline at *p*H 8. This cell suspension was used in a number of analyses.

A portion of the washed cells was extracted with a mixture of chloroform and methanol (2 : 1) first at room temperature and then at the boiling temperature of the mixture under reflux. Only about 1 to 5 percent of the incorporated radioactivity became soluble by these treatments. The chloroform-methanol extract was concentrated under nitrogen, and was chromatographed on paper coated with silicic acid (Eastman chromogram sheet, type K301-R-2) with the solvent system consisting of diisobutylketone, acetic acid and water (8 : 5 : 1) (*4*). No more than traces of lecithin could be detected when the chromatograms were developed either by spraying with 2′, 7′-dichlorofluorescein in ethanol or by autoradiography. The major phospholipid component detected by the fluorescein stain had an R_F of 0.77 (lecithin $R_F = 0.41$) and had no radioactivity. Over 99 percent of the radioactivity of the chloroform-methanol extract remained at the origin of the chromatogram. A portion of the cell suspension with radioactivity of 1.02×10^6 count/min was extracted with 5 percent trichloroacetic acid (TCA) at 90°C for 15 minutes. After centrifugation, all of the radioactivity was found in the supernatant. The rest of the cell suspension was lysed by the addition of a few drops of 5 percent deoxycholate (DOC). A portion of this crude lysate was hydrolyzed in 6*N* HCl at 100°C for 10 hours. Chromatography of the hydrolyzate in four solvents which can resolve the methylated derivatives of ethanolamine and serine yielded single radioactive spots with mobilities identical to those of authentic choline (Fig. 2). Another portion of the DOC lysate was passed through a Sephadex G-75 column. The material containing the radioactivity was completely excluded by this gel filter. TCA (in a final concentration of 5 percent) was added to a third portion of the DOC lysate. After 30 minutes extraction at 0°C, the precipitate was removed by centrifugation. A variable but substantial fraction of the total label (between 50 to 90 percent in four separate ex-

periments) was found in the supernatant.

These findings indicate that the choline incorporated by pneumococci is not extensively converted to other derivatives. The gel-filtration data show that choline is linked to a polymer of rather large molecular size (molecular weight > 50,000). The solubility properties of this material make it unlikely that it is nucleic acid, protein, or lipid.

2) Choline-labeled cells were lysed with DOC, and the lysate was fractionated by a method designed for the preparation of pneumococcal polysaccharide C (*5*). The lysate containing a total of 1.03×10^7 count/min was acidified to *p*H 3 and heated at 100°C for 15 minutes. After centrifugation, 48 percent of the label (4.9×10^6 count/min) was in the precipitate. The rest (5.4×10^6 count/min, 52 percent) remained in the supernatant with the cellular polysaccharides. Upon addition of $CaCl_2$ (1 percent final concentration) and ethanol (20 percent final concentration), a precipitate which contained no radioactivity formed and was discarded. Raising the ethanol concentration to a final concentration of 80 percent precipitated the bulk of the radioactivity in the supernatant (4.0×10^6 count/min or 39 percent of the original radioactivity of the lysate). This crude polysaccharide fraction was dissolved in water (all radioactive material went in solution) and was treated at room temperature for 24 hours with each of the following enzymes (in succession): pancreatic deoxyribonuclease (10 μg/ml, *p*H 8), pancreatic ribonuclease (10 μg/ml, *p*H 8), trypsin (50 μg/ml, *p*H 9), pepsin (50 μg/ml, *p*H 2). The enzyme-treated material was shaken three times with equal volumes of chloroform to remove the protein. The deproteinized material was dialyzed first against water, then against 5*M* urea for 12 hours, and finally against water a second time. During this purification procedure, a maximum of 15 percent of the label became dialyzable, an indication that most of the radioactive choline was covalently bound to a nondialyzable polymer by linkages resistant to the action of the proteolytic and nucleolytic enzymes used.

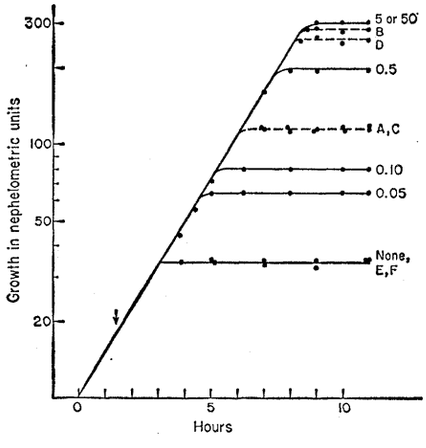

Fig. 1. Pneumococci were grown in the chemically defined medium supplemented with 5 μg of choline per milliliter. The culture was filtered (Millipore), washed, and resuspended in choline-free medium (arrow). This cell suspension was distributed to tubes containing different concentrations of choline, ranging from 0 to 50 μg per milliliter (see numbers on graph). In several additional tubes choline was replaced either by the hydrolyzate of polysaccharide C (10 μl in tube A and 50 μl in tube B), or by chromatographically fractionated hydrolyzate (10 μl and 50 μl "choline fraction" in tubes C and D, respectively). Tubes E and F received 50 μl each of the concentrated fractions either with higher (E) or lower (F) R_F's than choline. The growth of the bacteria was followed in a nepholometer.

The purified polysaccharide fraction was lyophilized, and the white asbestos-like material was analyzed. Treatment of a few milligrams of this material with a mixture of chloroform and methanol (2 : 1) at room temperature or refluxing it for 3 hours with this mixture did not extract any radioactive material. After the material was refluxed with a mixture of absolute methanol and 6*N* HCl (1 : 1) for 90 minutes, none of the radioactivity could be partitioned into petroleum ether. Hydrolysis with 6*N* HCl at 100°C for 10 hours liberated a single radioactive substance which chromatographed in solvents 1, 2, 3, and 4 (Fig. 2) as free choline. To a portion of the hydrolyzate 1 mg of nonradioactive (carrier) choline and reinecke acid were added, and the red-colored reineckate was recrystallized three times from a mixture of absolute ethanol and *n*-propanol. The crystalline material showed a constant specific radioactivity (100,000 count/min ± 15 percent per optical density unit measured at 526 mμ) after each of the recrystallizations (*6*).

The "polysaccharide" material was dissolved in water and passed through a Sephadex G-200 column; part of the radioactivity was excluded, part was retained and showed considerable heterogeneity in size. Sephadex G-75 and G-25 gel filters completely excluded the radioactivity. After treatment with $5 \times 10^{-2}M$ KIO_4 at *p*H 7 for 3 to 4 hours in the dark, all radioactivity could be recovered as small fragments which were retained by both Sephadex G-75 and G-25 and which were somewhat larger in molecular size than free choline. Acid hydrolysis (6*N* HCl, 100°C for 10 hours) of the periodate fragments liberated a single radioactive component which was identified as choline on the basis of mobility in chromatographic solvents 1 to 4 (see legend to Fig. 2).

The purified polysaccharide (1.8 mg) was hydrolyzed in sealed, evacuated tubes with 6*N* HCl at 108°C for 22 hours and analyzed in the Technicon automatic amino acid analyzer (*7*). Table 1 indicates that the preparation contained amino acids and amino sugars typical of the composition of pneumococcal polysaccharide C characterized recently by Gotschlich and Liu (*5*, *8*). A number of additional amino acids not typical to the polysaccharide C were also present in smaller quantities, probably representing contamination by protein (maximum 2 percent). No attempt was made to measure the degree of destruction of the amino sug-

	R_F (relative to choline) in solvents			
	1	2	3	4
Ethanolamine	0.65	1.2		0.17
Monomethyl ethanolamine				0.49
Dimethyl ethanolamine				0.84
Choline	1	1	1	1
D, L Serine		0.35	0.40	
6N Hydrolyzate of pneumococcal lysate	1	0.98	1	1

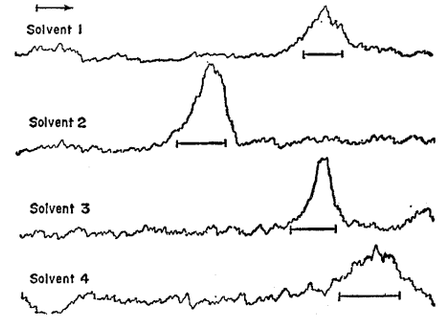

Fig. 2. Chromatographic identification of choline liberated by hydrolysis with strong acid from the crude DOC-lysate of pneumococci. Descending chromatography was carried out, and the radioactive spots were located on the chromatogram in a Packard radiochromatogram scanner (Model 7200). *Solvent 1*: *n*-butanol, ethanol, acetic acid, and water (8 : 2 : 1 : 3); *Solvent 2*: ethanol, ammonia, and water (90 : 5 : 5); *Solvent 3*: ethanol, acetic acid, and water (90 : 5 : 5); *Solvent 4*: phenol, *n*-butanol, 80 percent formic acid, and water (50 : 50 : 3 : 10) (*15*). The location of choline standard is indicated by the horizontal bars. Nonradioactive choline was located on the paper strips by the phosphomolybdate stain method (*9*).

ars which is known to occur to a considerable extent during acid hydrolysis (*5*). Thus, an estimate of the total recovery could not be made. The molar ratios of the stable amino acids alanine, glutamic acid, and lysine were 2.2 : 1.0 : 0.9. These three components made up 1.6 (alanine), 1.22 (glutamic acid), and 1.1 (lysine) percent of the weight of the preparation. In the polysaccharide C preparation of Liu and Gotschlich (*5*) alanine, glutamic acid, and lysine (molar ratios 1.84 : 1.0 : 0.81) represented 1.6, 1.6, and 1.3 percent respectively of the weight of the preparation. These authors could account for about 80 percent of the weight of their preparations of polysaccharide C in terms of the peptidoglycan components, galactosamine phosphate, acetyl groups, and glucose (*5*).

All radioactivity in the hydrolyzed sample was associated with choline, and there was no loss in the radioactivity during hydrolysis. If the specific radioactivity of the choline did not change during incorporation, the 1.8-mg polysaccharide preparation contained 64 μg of choline.

3) Choline was identified as a component of polysaccharide C prepared from R36A-strain pneumococci which were grown in beef-heart infusion broth, that is, in a complex medium very different from the one used in the previous experiments. (This preparation was supplied by Dr. T .Y. Liu of this university.) The preparation was free of protein and nucleic acid. Chromatography of acid-hydrolyzed polysaccharide C (6*N* HCl, 100°C, 10 hours) in solvents 1, 2, and 3 (Fig. 2) yielded a single phosphomolybdate-staining component (*9*) with an R_f identical to that of the choline standard and portions of the hydrolyzate added to choline-free growth medium could support growth of pneumococci (Fig. 1).

The hydrolyzate was chromatographed in solvent 2 and various sections of the chromatogram were eluted individually. The growth-supporting activity of the hydrolyzate could be quantitatively recovered from a single area on the chromatogram, and this area coincided with the location of choline (Fig. 1).

4) Cell walls were prepared from choline-labeled pneumococci. The washed-cell suspension in saline was heated for 10 minutes at 65°C to inactivate autolytic enzymes. The suspension was then shaken with glass beads in the Mickle disintegrator for 15 minutes. Observation in the phase-contrast microscope indicated disruption of more than 99 percent of the cocci. After purification by eleven successive washings with cold distilled water and overnight treatments with proteolytic enzymes [first trypsin (50 μg/ml, *p*H 8); then pepsin (50 μg/ml, *p*H 2)], followed by nine additional washings with water, 40 percent of the incorporated radioactive choline (2.5×10^5 count/min) remained associated with the protein-free cell walls (that is, material sedimenting at 5000*g* in 20 minutes).

Table 1. Amino acid and amino sugar components (μmole per 1.8 mg of sample) of the polysaccharide preparation. Results are:

Component	Amount (μmole)	Component	Amount (μmole)
Glutamic acid	0.148	Galactosamine	0.711
Lysine	.134	Aspartic acid	.026
Alanine	.322	Isoleucine	.009
Serine	.075	Leucine	.02
Glycine	.048	Tyrosine	.004
Muramic acid	.224	Phenylalanine	.008
Glucosamine	.313	Arginine	.005

5) In a further experiment cells were labeled with radioactive choline, harvested, and washed as described. To half of the washed cell suspension KIO_4 was added (final concentration of $5 \times 10^{-2}M$); the other half of the suspension served as control. After various periods of incubation at 37°C in the dark, portions were centrifuged, and the radioactivity in the supernatant was determined. The periodate treatment quantitatively released the radioactivity from the cells within 60 minutes after the addition of the reagent (Fig. 3). After treatment for 30 minutes wih KIO_4, the cells, as judged by phase contrast microscopy, still had the normal coccoid shape and refractivity.

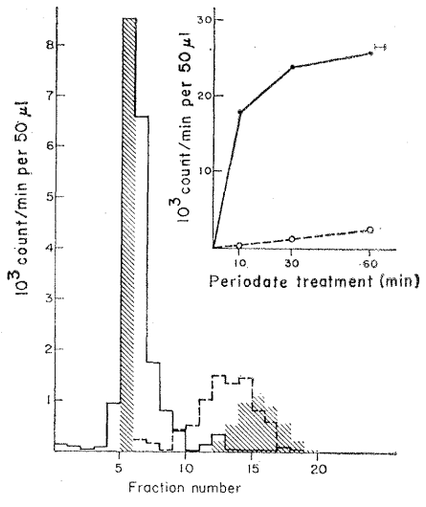

Fig. 3. Chromatography by the purified choline-labeled "polysaccharide" fraction on Sephadex G-75 before (solid line) and after (dashed line) treatment with periodate. Cross-hatched bars represent the elution volumes of blue dextran and choline standard. The inset depicts the release of choline by periodate treatment of choline-labeled cells. Radioactivity in the supernatant in KIO_4-treated cells (solid line) and in the untreated control (dashed line). The horizontal bar indicates the total radioactivity in the cells.

The control suspension was allowed to autolyze at 37°C for 12 hours, and examination under the phase-contrast microscope indicated complete conversion of the cocci to "autolytic spheres." These structures are essentially cell membrane ghosts, practically free of cell wall material, and they sediment only at somewhat higher centrifugal fields than the intact cells do (*10*). After centrifugation at 10,000*g* for 30 minutes, no radioactivity was found associated with the sedimented cell membrane ghosts (all radioactivity remained in the supernatant).

The quantitative release of choline label by KIO_4 treatment of whole labeled cells suggests that most of the choline may be linked to a single type of macromolecule. The loss of radioactivity to the precipitate during heating of the crude DOC lysate and during successive dialyses may be due to heterogeneity introduced into the choline-containing polymer (or polymers) by heating at low *p*H.

Even as a component of phospholipids, choline is relatively rare in bacteria (*11*). The results of the experiments described suggest that in pneumococcus choline is present in a hitherto unknown form, apparently linked to a polysaccharide component of the bacterial cell wall. This suggestion is consistent with the solubility properties of the incorporated choline, its fractionation with the polysaccharide C and with the cell walls of pneumococcus. The absence of choline from cell-membrane preparations and the absence of lecithin in the pneumococcal phospholipids also supports this suggestion. Whether choline is linked to the peptido-glycan-poly(*N*-acetyl)galactosamine phosphate complex of the pneumococcal walls (*8*) or to some other yet unidentified polymer (*12*) remains to be determined. In the structural polysaccharides of the pneumococcal cell wall, choline may occupy a position analogous to that of ethanolamine in the lipopolysaccharides of *Salmonella* (*13*).

It may be of interest to note that

choline was required for the conversion of pneumococci to the competent physiological state, in which these bacteria can absorb DNA molecules and undergo genetic transformation (*14*).

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Insulin Biosynthesis: Evidence for a Precursor

Abstract. *Human islet cell tumor tissue and isolated islets of Langerhans from rats incorporated radioactive amino acids in vitro into insulin and a larger acid-alcohol soluble protein which could be separated from insulin by gel filtration. The amino acids were incorporated into the larger protein earlier than into insulin; only after incubation of islets for approximately 30 minutes did radioactivity begin to appear in insulin. The transfer of about 70 percent of the radioactivity of the larger protein to insulin was demonstrated in the absence of new peptide bond synthesis (cycloheximide), or during incubation with unlabeled amino acid (chase). The results indicate that the larger protein is a precursor in the biosynthesis of insulin. The name "proinsulin" is suggested for this protein.*

Slices from a human, insulin-producing β-cell tumor incorporated labeled amino acids into insulin and another acid-alcohol extractable protein (designated component *b*) which is larger than insulin (*1*). The latter is related immunologically to insulin, and insulin apparently is released from it upon incubation with small amounts of trypsin. It was postulated that the larger protein is a precursor of insulin which had accumulated in small amounts in the tumor. The studies to be presented here on the time course of insulin biosynthesis were carried out with another human β-cell tumor, and also with isolated islets of Langerhans from rat pancreas. The results support the earlier interpretation.

When material from a second insulin-producing tumor became available, a study was undertaken to examine the time course of incorporation of radioactive amino acids into component *b* and insulin (*2*). It was assumed that if component *b* is a precursor of insulin, radioactivity would appear first in it, and that upon subsequent incubation radioactivity would be transferred into the fraction containing insulin.

Slices from the tumor (10 to 20 mg) were incubated at 37°C for various intervals in small plastic tubes with the medium containing glucose (3 mg/ml) and L-leucine-H^3 (5 c/mmole) or L-phenylalanine-H^3 (3 c/mmole) described previously (*1*). In order to terminate labeling and initiate a chase, unlabeled leucine or phenylalanine was added to the medium at various times during incubation to give a final concentration of 30 μg/ml. After incubation each mixture was frozen at −79°C and transported on dry ice from Iowa City to Chicago, where the study was completed.

Data on the incorporation of amino acids into the acid-alcohol insoluble protein of the slices (Fig. 1) indicated that protein synthesis proceeded at a nearly linear rate. When unlabeled amino acid was added to dilute the labeled amino acid about 100 fold, the rate of incorporation of radioactivity decreased markedly. The acid-alcohol soluble fraction was separated and partially purified, and then it was passed through columns of Sephadex G-50 equilibrated with 1*M* acetic acid as described previously (*1*). The results are shown in Fig. 2. After incubation for 40 minutes label was present in a peak which eluted at a position corresponding to that of component *b* derived from the original tumor, but no label eluted in a position corresponding to that of added authentic porcine insulin. After incubation for 80 minutes peak *b* was higher and a small shoulder of radioactivity had appeared in the region of the insulin marker which, when separated and rerun (Fig. 2, inset), indicated the presence of a small amount of labeled insulin. Essentially similar results were obtained in experiments where a chase was carried out over the period from 40 to 80 minutes. After incubation of slices for 210 minutes a definite peak of radioactivity eluted in a position corresponding to that for insulin. The tubes containing peaks *b* or *c* were combined, the acetic acid was evaporated, and the protein was dissolved in 0.01*N* HCl for further analysis.

Material from both peaks *b* and *c* was bound by antibody to porcine insulin as noted previously (*1*). At least 50 percent of the radioactivity from peak *b* from this tumor was converted

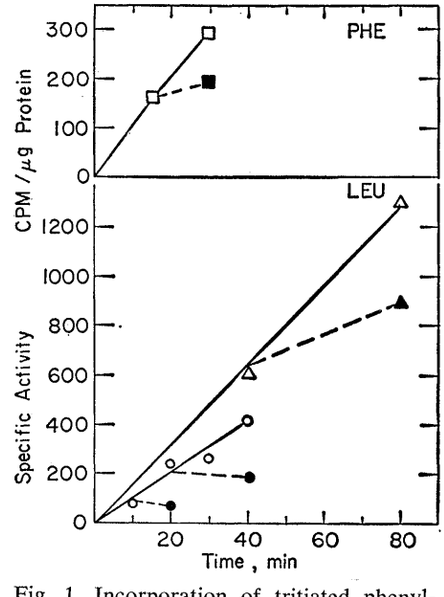

Fig. 1. Incorporation of tritiated phenylalanine (Phe) or leucine (Leu) into the acid-insoluble protein fraction of islet tumor slices. The residues obtained after acid-alcohol extraction and centrifugation were heated to 100°C in 5 percent trichloroacetic acid, then washed three times with 5 percent trichloroacetic acid and three times with 95 percent alcohol. The precipitates were dissolved in 0.3*N* KOH, their radioactivity was measured in Bray's solution (*15*) in a liquid-scintillation counter, and the protein concentration was measured by the method of Lowry (*16*). Solid symbols indicate samples in which excess unlabeled amino acid was added at the time indicated by the dashed line.