STREPTOCOCCAL L FORMS

V. Acid-Soluble Nucleotides of a Group A *Streptococcus* and Derived L Form

JOHN EDWARDS[1] AND CHARLES PANOS[2]

Department of Biological Chemistry, University of Illinois College of Medicine, Chicago, Illinois, and Research Laboratories, Department of Biochemistry, Albert Einstein Medical Center, Philadelphia, Pennsylvania

Received for publication July 14, 1962

ABSTRACT

EDWARDS, JOHN (University of Illinois College of Medicine, Chicago, and Albert Einstein Medical Center, Philadelphia, Pa.) AND CHARLES PANOS. Streptococcal L-forms. V. Acid-soluble nucleotides of a group A *Streptococcus* and derived L form. J. Bacteriol. **84**:1202–1208. 1962.—This report deals with a comparison of the acid-soluble nucleotides from a group A, type 12, β-hemolytic streptococcus and a derived stable L form. This is the first report of the presence of a cell-wall precursor (uridine diphosphate-muramic acid-peptide) in a stable L form. Cells of each organism were obtained during their logarithmic phases of growth, harvested by centrifugation, and washed with osmotically protective NaCl solutions. The analytical procedures were essentially those of Franzen and Binkley. Calculated values indicated that these results could not be accounted for by dry-weight differences due to loss of the streptococcal cell wall. It was found that both organisms contained the same amount of total nucleotide material. The L form contained no uridine monophosphate (UMP), a large concentration of uridine diphosphate (UDP)-muramic acid-peptide, and a significant increase of UDP-*N*-acetylglucosamine. A similar nucleotide containing muramic acid-peptide was not demonstrable in the parent coccus. Instead, UMP and an unidentified uridine nucleotide were resolved in this region. Analyses of extracts from this streptococcal L form indicate the probable presence of two of the three nucleotides originally isolated by Park from penicillin-treated *Staphylococcus aureus*. The presence of the UDP-muramic acid-peptide cell-wall precursor in the L form cultured in the continual absence of penicillin points to an inability of this form to resynthesize the rigid cell wall and indicates that this synthetic mechanism has been permanently impaired.

Park and Johnson (1949) first reported the accumulation of uridine nucleotides in *Staphylococcus aureus* treated with penicillin. Subsequent investigations by Park (1952*a*, *b*, *c*) resulted in the finding of alanine, lysine, and glutamic acids, and a new nine-carbon amino sugar, later called muramic acid, as components of these accumulated nucleotides. Park and Strominger (1957) proposed that penicillin blocked synthesis of the rigid mucopeptide cell wall, thereby causing an accumulation of these uridine muramic acid nucleotide precursors. Penicillin has been employed in the production and isolation of osmotically fragile and reproducing forms lacking a rigid bacterial cell wall, the L form, from many bacterial genera (Dienes and Weinberger, 1951). This antibiotic, in conjunction with a medium of high osmotic pressure, was successfully utilized by Sharp (1954) in the initial production of L forms from the group A streptococci. Lederberg (1957) employed similar conditions for the production of "protoplasts" from *Escherichia coli*.

Very little is known concerning the acid-soluble nucleotide content of L forms of bacteria. This report deals with a comparison of the acid-soluble nucleotides from a group A β-hemolytic streptococcus and a derived stable L form obtained with the aid of penicillin. To our knowledge, this is the first report of the presence of a cell-wall precursor, uridine diphosphate-muramic acid-peptide, in a stable L form.

[1] To be submitted in partial fulfillment for the degree of Doctor of Philosophy.

[2] Senior research fellow (SF 531), 1960. Present address: Research Laboratories, Department of Biochemistry, Albert Einstein Medical Center, Northern Division, Philadelphia, Pa.

MATERIALS AND METHODS

Growth, harvest, and extraction of organisms. The group A, type 12, β-hemolytic *Streptococcus pyogenes* designated AED and its derived L form used in earlier studies (Panos and Barkulis, 1959; Panos, Barkulis, and Hayashi, 1959) were employed. The medium for each of these organisms has been described (Panos and Barkulis, 1959). Another L form cultured in the absence of penicillin for the past 5 years was also investigated. The organisms were grown in three 4-liter Erlenmeyer flasks, each containing 1.5 liters of medium; 159 ml of an 8- to 9-hr culture of the coccus or L form served as an inoculum for each flask. Growth was followed by measuring the optical density (OD) at 660 mμ in a Beckman DU spectrophotometer. The streptococcus and L form were harvested during logarithmic growth at an OD of 0.220 and 0.170, respectively. The maximum attained by cultures of the parent coccus was 0.800, as compared with 0.350 for the L form. The cells were harvested by centrifugation (2 C) at 4,080 × *g* for 15 min. The pellets were resuspended and washed twice with physiological saline for the streptococcus, or 0.5 M NaCl for the osmotically fragile L form.

The combined pellets were extracted with 100 ml of 0.6 N $HClO_4$ at 0 C for 30 min. The suspension was centrifuged at 13,000 × *g* for 10 min at 2 C, and the supernatant neutralized with 10 N KOH. The pellets were extracted again, and the two neutral supernatants combined and lyophilized. The lyophilized powder, which was mainly $KClO_4$, was extracted five times with cold (4 C) water. The combined washings were taken to 50 ml in a volumetric flask with water, and a sample was measured at 260 mμ for determination of total ml OD (Franzen and Binkley, 1961). The sample was then applied to the column.

Column chromatographic procedures. The nucleotides were separated, by the method of Hurlburt et al. (1954), on a Dowex 1 X 4 (formate) column (1.0 by 25 cm) by using a formic acid-ammonium formate gradient elution procedure with a 500-ml mixing flask. The flow rate was 35 to 40 ml per hr. Fractions (6 ml) were collected, and their absorption at 260 and 280 mμ measured. Radioactive adenosine phosphates were added to each preparation as markers. The formic acid and ammonium formate were removed from the nucleotides by reduced-pressure distillation and by sublimation in a modified molecular still, respectively. The recovery of total ultraviolet-absorbing material placed on the column was never below 90%. The data presented are averages of at least three determinations performed at various times and with different cultures of the same organisms. Each fraction was quantitated by integrating the area beneath a peak and dividing by the molar absorbance at 260 mμ at the pH of the eluate. Correction was made for the formate background. The identification of the various fractions was determined as previously described (Franzen and Binkley, 1961).

Descending paper chromatography. Whatman no. 1 paper was employed for all paper chromatograms. The solvent systems used for the nucleotides were isobutyric acid-concd NH_4OH-water (a) 66:1:33 and (b) 57:4:39 (Pabst Brewing Co., 1956); for the amino sugars, butanol-acetic acid-water (c) 4:1:5 (Strange and Dark, 1956); and (d) 57:14:29 and for the amino acids, ethanol-1 N ammonium acetate (e) 5:2 or (f) 7:3, pH 7.5 (Pabst Brewing Co., 1956). The nucleotides were located by the quenching of light from a Mineral Lite (model SL 2537, Arthur H. Thomas Co., Philadelphia, Pa.), the amino sugars by an Elson-Morgan spray (Partridge, 1948), and the amino acids by a ninhydrin spray (0.25% ninhydrin in 95% aqueous acetone). The nucleotides were spotted on paper, chromatographed, eluted with sodium phosphate buffer (pH 7, 0.02 M), and their absorption spectra determined at various pH levels.

Chemical analyses. Partial (1 N HCl for 10 min) and complete (6 N HCl for 3 hr) acid hydrolysis of the isolated nucleotides was performed in sealed tubes at 100 C. Total and labile phosphorus was determined according to Leloir and Cardini (1957), and hexosamine according to the Boas (1953) modification. Diphosphopyridine nucleotide (DPN) was determined enzymatically.

Source of chemicals. The nucleotides were purchased from the Pabst Laboratories (Milwaukee, Wis.). AMP-8-C^{14}, ADP-8-C^{14}, and ATP-8-C^{14} with specific activities of 0.75, 1.45, and 1.25 μc per mg, respectively, were obtained from the Schwarz Laboratories, Inc. (Mt. Vernon, N.Y.). Analytical grade Dowex 1 X 4 (200 to 400 mesh) anion-exchange resin was purchased from Bio-Rad Laboratories (Richmond, Calif.).

RESULTS

Total amount of extractable acid-soluble ultraviolet-absorbing material. As mentioned previously, the streptococcus and its L form were harvested during the early part of their logarithmic phases of growth. Attempts to use the KSB-R Servall Szent-Gyorgyi and Blum continuous-flow system for the collection of the L form were unsuccessful. It was found that considerable breakage of this fragile L form occurred, with loss of intracellular components. The extractable nucleotide content of the L form harvested in this manner was nil. Similar results were not obtained with the parent coccus. To evaluate the nucleotide content of the various extracts from the coccus and L form, their total absorbance was determined and compared (Table 1). No difference was noted with the L form cultured in the presence and absence of penicillin. Barkulis and Jones (1957) reported that the cell walls of group A streptococci comprise 25%, by wt, of the intact coccus. For comparison, the calculated values in Tables 1 and 2 are those determined on the assumption that the streptococcal protoplasm was increased by an amount equivalent to the weight of the cell wall lost upon conversion to the L form. This increase is closely comparable to the protoplasm of the L form on a dry weight basis. There was no significant change in the total amount of acid-soluble materials obtained from the streptococcus and L form on an equivalent weight basis (Table 1). The differences noted between the L form and calculated figures were not considered to be significant, and were similar to those experienced during replicate determinations.

Characteristics of the individual nucleotide peaks. The position of elution (Fig. 1) and the concentration of each of the nucleotides obtained (Table 2) from the coccus and L form are presented. As is apparent, the major differences are in peaks VI, VII, and IX, which are uridine-containing compounds. No differences were noted upon comparing elution diagrams of the L form grown in the absence or presence of penicillin. Peak I was composed of nonpolar substances which were eluted from the column with water. Their total concentration varied during repeated determinations and they are, in part, contaminants from the growth medium. Peak II did not contain phosphorus, was ninhydrin-negative, and did not migrate as a possible nucleoside or nucleotide on paper chromatography. Peak III did not reveal the presence of cytidine diphosphate (CDP)-glycerol or CDP-ribitol. Cytidine monophosphate (CMP) was the only compound isolated. The amount of adenosine monophosphate (AMP) depicted in the elution diagrams is exaggerated. After correcting for the AMP-8-C^{14} added, the coccus and

TABLE 1. *Extractable acid-soluble ultraviolet-absorbing material**

Organism	Total	Nonpolar material†	Nucleotide content
Streptococcus	469	58	411
L form	594	13	581
Calculated‡	587	73	514

* (OD_{260} of perchloric acid extract) × (volume of extract in ml) per g (dry wt) of cells (Franzen and Binkley, 1961). Average of six determinations.

† Water fraction from column chromatographic analyses data (see text).

‡ Theoretical: streptococcal protoplasm increased by 25% to compare with the L form (see text).

TABLE 2. *Nucleotide composition** of the AED Streptococcus and stable L form*

Peak no.	Probable identity	Streptococcus	L form	Calculated†
I	Nonpolar	—	—	—
II	Calculated as uridine	2.91	1.84	3.64
III	CMP	2.14	2.13	2.68
IV	DPN	1.75	2.61	2.19
V	AMP	0.55	0.87	0.69
VI_S	UMP	3.66	—	4.58
VI_L	UDP-mur-peptide	—	8.81	—
VII	Uridine nucleotide	2.07	—	2.59
VIII	ADP	0.46	0.48	0.58
IX	UDP-N-AG	2.03	4.21	2.54
X	UDP-hexosamine	0.88	1.12	1.10
XI	GDP	1.27	1.79	1.59
XII	UDP	1.45	1.61	1.81
XIII	ATP	1.12	1.90	1.40

* Average of three determinations (μmoles/g dry wt of cells).

† Theoretical: increasing streptococcal protoplasm by 25% to compare with the L form (see text).

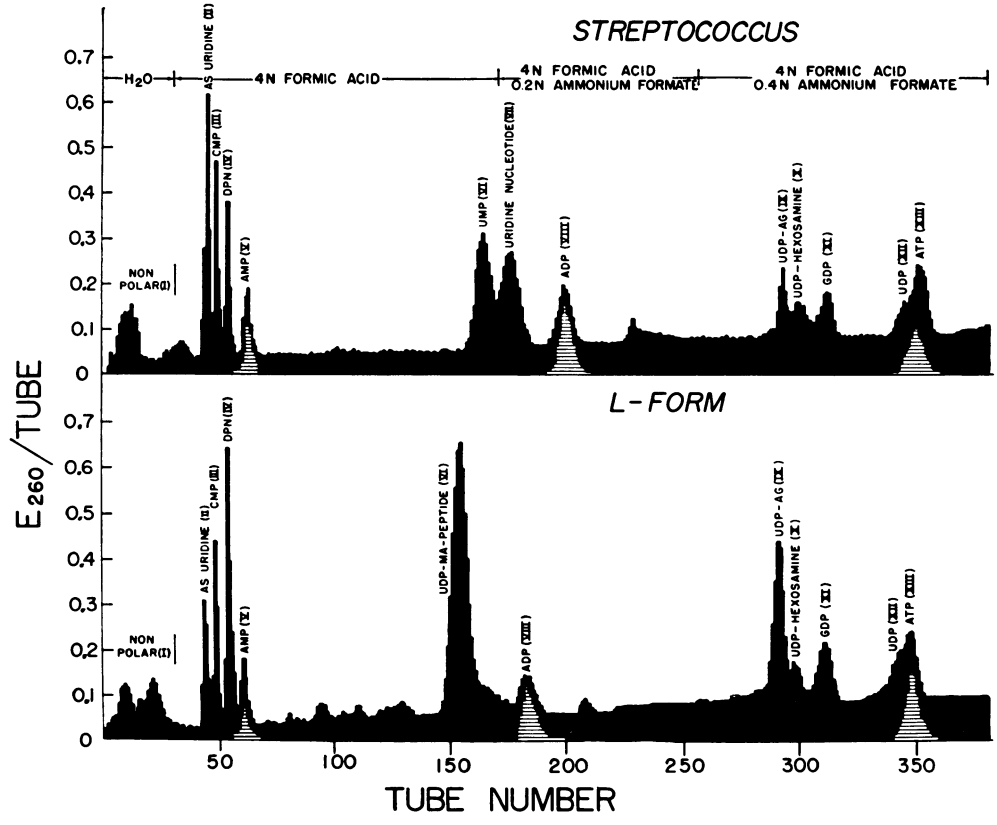

FIG. 1. *Typical nucleotide elution diagrams of the parent streptococcus and derived L form during logarithmic growth. Amount of material on column: 127 and 133 ml OD for the streptococcus and L form, respectively. Lined areas indicate relative amounts of radioactivity eluted from resins where markers were added along with the acid extracts.*

derived L form were found to contain similar concentrations of this nucleotide.

The most pronounced nucleotide is peak VI (Table 2 and Fig. 1) from the acid-soluble nucleotides of the L form and is probably identical with the uridine diphosphate (UDP)-*N*-acetylmuramic acid-peptide (UDP-MA-peptide) isolated by Park (1952*c*) from *S. aureus* treated with penicillin and from certain streptococci by McCluer, van Eys, and Touster (1955). The properties of this peak (VI-L form) are given in Table 3. The concentrations of the various amino acids of the peptide were not quantitated. However, paper chromatograms of hydrolysates always revealed considerably more alanine than lysine or glutamic acid. Although uridine monophosphate (UMP) was present in the greatest concentrations of all of the other nucleotides in extracts from the coccus (Fig. 1 and Table 2), a similar discrete peak was not observed with the L form. Small amounts of UMP were detected, however, in the area between peaks VI and VII of the L form (Table 2 and Fig. 1). Peaks VI and VII from the parent streptococcus did not migrate on paper as the UDP-MA-peptide of the L form. These nucleotides were Elson-Morgan and ninhydrin-negative after hydrolysis and did not possess an acid-labile phosphate. Peak VI from the coccus was identified as UMP by paper chromatography with an authentic sample. In contrast, peak VII did not display the characteristics of the other nucleotides from either organism. It was found to contain uridine and to migrate slower than UMP by paper chromatography (solvent systems a and b, R_F values of peak VII, 0.085 and 0.24; UMP, 0.13 and 0.34, respectively). This peak is probably an unidentified uridine nucleotide, and no further characterization was attempted.

TABLE 3. *Properties of peak VI of the L form leading to its identity as the UDP-muramic acid-peptide*

I. *Spectrophotometric identification of base as uridine**

	Minimum (mμ)	Maximum (mμ)	Ratios at 250, 260, 280 mμ as 250/260	as 280/260
pH 7	231 (230)	261 (262)	0.75 (0.75)	0.31 (0.36)
pH 11	242 (242)	260 (261)	0.80 (0.83)	0.33 (0.29)

II. *Chromatographic results of peak VI hydrolysates for:*

A. Amino sugar-

Solvent system	Peak VI‡	R_F values Muramic acid	Glucosamine
c	0.27	(0.28)	(0.15)
d	0.31	(0.33)	(0.13)
f	0.70	(0.69)	—

B. Amino acids†

Solvent system	Glutamic acid	Lysine	Alanine
b	0.35 (0.35)	0.52 (0.54)	0.60 (0.59)
e	0.35 (0.34)	0.50 (0.51)	0.58 (0.57)
f	0.37 (0.37)	0.54 (0.54)	0.62 (0.60)

III. *Analysis of phosphate content of peak VI*

	μMP:μM Uridine
Labile (10 min, 100 C, 1 N H_2SO_4)	1.1:1
Total	2.1:1

IV. *Chromatographic comparison with UMP*

Solvent system	R_F values Peak VI	UMP
a	0.04	0.19
b	0.24	0.34

* Figures in parentheses are literature values for uridine.

† Values in parentheses are chromatographic standards.

‡ Also, characteristic Elson-Morgan chromogen (orange) and spectrophotometric maximum (505 mμ) agreed with authentic sample of muramic acid.

Peak VIII is adenine diphosphate (ADP), as determined by position of elution and the addition of ADP-8-C^{14} as a marker.

The amount of UDP-*N*-acetylglucosamine (UDP-*N*-AG) found in the L form was almost twice as much as that from the coccus on an equivalent dry weight basis. This compound was identified as stated earlier (Materials and Methods), and revealed a pink chromogen (maximum, 530 mμ) after the Elson-Morgan reaction. Peak X was found in small amounts and appears to be an unknown uridine nucleotide containing an amino sugar. Hydrolysis of this nucleotide followed by the Elson-Morgan test resulted in the appearance of an orange chromogen with an absorption maximum at 505 mμ. This agreed closely with an authentic sample of muramic acid kindly supplied by J. A. Hayashi. Paper chromatography indicated that this nucleotide (peak X) was not UDP-glucose. Insufficient amounts of this compound prevented further characterization. Peaks XI, XII, and XIII were identified in the usual manner and by an isotope marker (ATP-8-C^{14}) and found to be guanosine diphosphate (GDP), UDP, and adenine triphosphate (ATP), respectively. The values for ATP (Table 2) are probably conservative, since it has been shown (Binkley and Franzen, 1961) that the values determined by the luciferase method for this nucleotide are always 10 to 25% higher

than that calculated from elution diagram curves. Further elution of 100 fractions beyond ATP (Fig. 1) with 4 N formic acid-0.8 N ammonium formate did not reveal the presence of other nucleotide components.

DISCUSSION

On an equivalent weight basis, no significant difference in the total extractable acid-soluble ultraviolet-absorbing material was observed from the parent streptococcus and derived L form (Table 1) during logarithmic growth. However, major differences were observed in the concentrations and types of nucleotides in these extracts (Table 2 and Fig. 1). It is evident from these data that the change in the nucleotide content of the L form cannot be accounted for by dry weight differences due to loss of the streptococcal cell wall (Table 2, calculated values), and the data are indicative of a concomitant metabolic alteration with loss of the coccal cell wall.

One of the L forms employed in this investigation had been cultured in the absence of penicillin for the past 5 years without a reversion to the parent streptococcus and was, therefore, considered to be stable, i.e., nonreverting. The finding of the cell-wall precursor, UDP-muramic acid-peptide (peak VI), in extracts from the L form cultured in the absence, as well as the presence, of penicillin indicates that cell-wall mucopeptide synthesis remains blocked. Strominger (1962) proposed that penicillin somehow blocks the transfer of the muramic acid-peptide from the nucleotide to the cell wall. The inability of the stable L form to resynthesize a rigid cell wall in the absence of penicillin indicates that this synthetic mechanism has been permanently impaired. Other differences have been noted between the L form and parent cocci. Recently, Panos (1962) documented an alteration in carbohydrate metabolism in this stable L form, derived with the aid of penicillin, as compared with the parent coccus.

The presence of glucosamine as well as muramic acid in group A streptococcal cell-wall preparations and their possible position as components of this structure have been reported and reviewed (Barkulis, 1960). The increase of UDP-*N* acetylglucosamine (peak IX) in the L form, therefore, was expected and must be a reflection of the inhibition of new cell-wall material. The inability to detect UMP and the uridine nucleotide in the L form is probably the result of the accumulation of the cell-wall intermediates, UDP-MA-peptide and UDP-*N*-AG. This would seem plausible since, based upon an equivalent total percentage recovery from each organism of the components listed in Table 2, UMP and uridine nucleotide can account for 86% of the total UDP-muramic acid-peptide precursor found within the L form.

The finding of an orange chromogen with an absorption maximum of 505 mμ (*see* Results) indicates that muramic acid may be a component of the UDP-hexosamine found in both organisms. This would be the second nucleotide found within the L form which contained muramic acid. Park (1952*a*, *b*, *c*) originally isolated three such compounds from *S. aureus* that had been treated with penicillin. His third nucleotide, which contained the amino acid L-alanine attached to the muramic acid moiety of the nucleotide, was not detected in these experiments. The concentration of UDP-hexosamine (peak X) did not change markedly, as would have been expected from the earlier findings of Park (1952*a*, *b*, *c*) if it were involved in cell-wall synthesis, indicating that penicillin had no effect on the biosynthesis of this nucleotide.

Various methods have been employed to illustrate that L forms lack a rigid bacterial cell wall. However, chemical analyses of a stable streptococcal L form have repeatedly indicated minute amounts of a cell-wall component, glucosamine, to be present, suggesting that complete removal of the cell wall had not been attained. The finding of various amino sugars as components of the nucleotides of this L form probably explains this discrepancy. Panos et al. (1959) reported that muramic acid was not found as a structural component of this streptococcal L form. The finding of muramic acid as a component of a nucleotide of an L form during this investigation, even when cultured in the absence of penicillin, confirms this earlier finding and represents the first report of the presence of this component in acid extracts from a stable L form.

ACKNOWLEDGMENTS

We are grateful to M. J. Firszt for her technical assistance during this investigation. We are also indebted to S. B. Binkley, Department of Biochemistry, University of Illinois College of Medicine, for his helpful suggestions during the course of this investigation, and to Gerald D. Shockman,

Temple University Medical School, for his helpful criticisms during the preparation of this manuscript.

This study was made possible by grants E-4543 and E-4495 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service.

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