Phytochemistry, 1969, Vol. 8, pp. 707 to 723. Pergamon Press. Printed in England

SUGAR NUCLEOTIDES AND NUCLEOTIDE-PEPTIDE COMPLEXES OF *CHLORELLA PYRENOIDOSA*: ISOLATION AND CHARACTERIZATION

GIRDHAR GOPAL SANWAL* and JACK PREISS

Department of Biochemistry and Biophysics, University of California, Davis, California, 95616, U.S.A.

(*Received* 18 *September* 1968)

Abstract—The nucleotides of *Chlorella pyrenoidosa* were extracted with 80% hot ethanol and separated by fractionation on Dowex-1-formate. On the basis of chemical, enzymatic and chromatographic analysis, the following nucleotides were identified: NAD^+, $NADP^+$, 5′-CMP, 5′-UMP, 5′-AMP, 5′-GMP, 5′-IMP, UDP, ADP, ATP and thymidine oligonucleotides. The sugar nucleotides obtained were UDP-D-glucose, UDP-D-galactose, UDP-xylose, UDP-arabinose, UDP-*N*-acetylglucosamine, UDP-glucuronic acid, GDP-D-mannose and ADP-glucose. GDP-D-galactose and ADP-arabinose have not previously been found in green algae. Several nucleotide-peptide complexes, predominantly of adenine nucleotide, were also present. The properties of a derivative of ADP-ribose are discussed. Using ^{35}S-labeled *Chlorella* cells, PAPS and sulfoquinovose were detected. The significance of these findings in relation to the metabolism of *Chlorella* is discussed.

INTRODUCTION

THE NUCLEOSIDE diphosphate sugar compounds have been implicated in a large number of carbohydrate transformations.[1,2] Kauss and Kandler[3] identified ADP-glucose and UDP-glucose in *Chlorella pyrenoidosa*. There is little information available regarding the other sugar nucleotides and various derivatives of nucleotides in green algae. The present communication reports the first isolation of GDP-D-galactose and ADP-arabinose from the green alga, *C. pyrenoidosa*. The isolation and characterization of several adenine nucleotide-peptide complexes, a guanine nucleotide-peptide complex, an unidentified compound, ADP-ribose-X and number of other sugar nucleotides are also described.

RESULTS

The elution pattern of the nucleotides from Dowex 1-X-8-formate column is plotted in Fig. 1. The results of the chemical analysis of the nucleotides are given in Table 1.

Identification of Individual Compounds

NAD^+ (Fractions 1-a, 2-a-II, 3-IV-a and 4-III-a)

The u.v. spectra, chromatographic behaviour in solvents A, B and C and electrophoretic mobility in buffer I were identical with those of authentic NAD^+. A typical absorption peak at 340 nm appeared when ethanol and yeast alcohol dehydrogenase were added together with this component. No $AgNO_3$-reactive material was detected before and after mild acid hydrolysis of the compound.

* Present address: Department of Biochemistry, Lucknow University, Lucknow, U.P., India.

[1] W. J. KELLEHER, *J. Pharm. Sci.* **54**, 1081 (1965).
[2] V. GINSBURG, *Advan. Enzymol.* **26**, 35 (1964).
[3] H. KAUSS and O. KANDLER, *Z. Naturforsch.* **17b**, 858 (1962).

5'-CMP (Fractions 3-IV-b and 4-III-b-A)

The u.v. spectra of these fractions were identical with that of a cytidine derivative. The mobilities of the compound on paper chromatography (solvents A, B, C and E) and electrophoresis (buffers I and II) were identical with those of authentic 5'-CMP. Digestion of this compound with snake venom extract or *E. coli* alkaline phosphatase converted it exclusively to cytidine (solvents A and L).

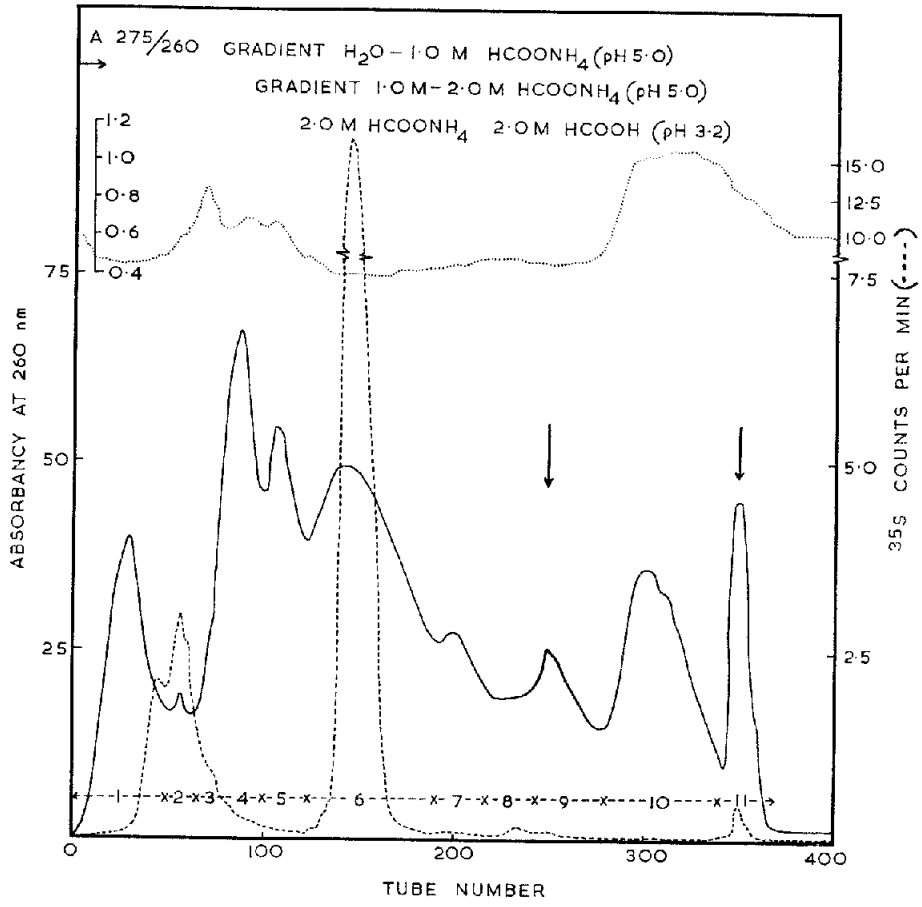


FIG. 1. DOWEX-1-FORMATE COLUMN CHROMATOGRAPHY OF NUCLEOTIDES FROM *Chlorella pyrenoidosa* (670 g).

5'-UMP (Fractions 3-III, 4-II-b-A, 4-III-c-A and 5-III-c)

Spectral analysis showed these components to be uridine derivatives. Paper electrophoretic mobilities in buffers I and II and chromatographic behaviour in solvents A, B, C and E were identical with those of 5'-UMP. No reducing sugar was detected after mild acid hydrolysis. Digestion of the compound with snake venom extract or *E. coli* alkaline phosphatase led to the formation of uridine, which was identified by paper chromatography in solvents A and L.

TABLE 1. ANALYTICAL DATA OF NUCLEOTIDES ISOLATED FROM *Chlorella pyrenoidosa*

Fraction		Compound identified	Composition*				Yield from 670 g of fresh alga (μmoles)
Major	Minor		Pentose	Acid labile-phosphate	Total phosphate	Reducing sugar after mild acid hydrolysis (moles/mole base)	
1-a	2-a-II 3-IV-a 4-III-a	NAD^+			2·1	—	54·3
3-IV-b	4-III-b-A	5′-CMP	0·92	0·09	1·02	—	5·2
3-III 4-II-b-A	4-III-c-A 5-III-c	5′-UMP	1·09	0·02	0·99	—	40·9
4-II-a-A	5-III-a-A	$NADP^+$			3·10	—	2·4
5-II-A-a 6-I-B-a	4-I-b-A	UDP-sugars	1·06	1·05	2·08	0·98 (glucose)	14·7
4-I-c-A 5-II-B-a	6-I-B-b 6-II-B-a	UDP-*N*-acetyl-glucosamine	1·03	1·08	2·10	1·02 (N-acetylglucosamine)	11·2
5-III-b-A 5-V	6-IV-A-b	5′-IMP				—	0·9
6-V-A 7-VIII-A	8-VII-A 9-VI-A	5′-AMP	0·98	0·05	0·99	—	93·4
6-II-A-a		UDP	0·97	0·95	1·89	—	2·4
6-III-B	7-VI-b-C 8-V-B	Adenine nucleotide-peptide complex I	2·1	0·02	1·90	0·03 (glucose)	5·5
6-V-B-b	7-VIII-B 8-VII-B	ADP-ribose-X	1·98	0·04	2·06	0·50 (glucose)	11·8
7-II-A	8-II	UDP-glucuronic acid		0·95	1·92	1·06 (glucuronic acid)	2·4
8-III		GDP-sugars	1·10	0·98	1·95	0·94 (mannose)	1·4
8-IV	7-V-a 9-IV-B-b	5′-GMP	0·94	0·02	1·10		3·5
9-I-A		Guanine nucleotide-peptide complex	1·02	1·10	2·13	0·05 (glucose)	2·1
8-VI 9-V	7-VII-B-a 10-a-B	ADP	0·96	1·10	2·02		34
8-V-A 9-IV-A		Adenine nucleotide-peptide complex II	1·00	0·02	2·20	0·03 (glucose)	4·5
9-IV-B-a		ADP-sugars		1·02		0·85 (glucose)	0·7
9-IV-C		Adenine nucleotide-peptide complex III	1·98	0·02	2·08	0·01 (glucose)	2·8
10-a-A 11-b-III	10-b-A	ATP	1·01	1·87	2·91		1·6
10-d 10-e 11-d-II	10-f	Thymidine oligomers					51·2

* Based on molar extinction coefficient (λ_{max}) at pH 7·0 of 18×10^3, $15{\cdot}4\times10^3$, $10{\cdot}0\times10^3$, $13{\cdot}7\times10^3$, $9{\cdot}0\times10^3$ and $12{\cdot}2\times10^3$ each for NAD^+ (λ_{max}, 260 nm), adenosine (λ_{max}, 259 nm), uridine (λ_{max}, 262 nm), guanosine (λ_{max}, 252 nm), cytosine (λ_{max}, 271 nm) and inosine (λ_{max}, 249 nm) respectively.

$NADP^+$ (Fractions 4-II-a-A and 5-III-a-A)

Absorption spectra of these fractions, mobilities of the compound on paper chromatography (solvents A, B and C) and electrophoresis (buffer I) resembled those of authentic $NADP^+$. A characteristic absorption peak at 340 nm appeared when D-glucose-6-phosphate and glucose-6-phosphate dehydrogenase were added to this compound.

Uridine diphosphate sugars (Fractions 4-I-b-A, 5-II-A-a and 6-I-B-a)

The u.v. absorption of these fractions at various pH indicated the presence of uridine derivatives. The compound behaved as a single component on paper chromatography in solvents A, B and C and paper electrophoresis in buffer I. The reducing sugar assay on the intact fraction was negative. After mild acid hydrolysis, the fraction showed reducing power (Table 1). Paper chromatography of the hydrolyzate in solvent B showed the disappearance of UDP-sugars and the formation of UDP and a trace of UMP. The nucleotide was subjected to hydrolysis by snake venom phosphodiesterase. The u.v. absorbing material now migrated similarly to UMP (solvent B). Upon mild acid hydrolysis of the nucleotide, glucose, galactose, arabinose and xylose were produced, all of which were identified by cochromatography with authentic sugars in solvents G, H and I.

The combined fractions (4-I-b-A, 5-II-A-a and 6-I-B-a) were separated on Whatman no. 1 paper, using solvent F. The papers were dipped in 0·01 M EDTA, pH 7·0 and dried before use. Three u.v. absorbing bands separated on chromatography for 50 hr. The samples were eluted with water and brought to pH 6·0 with acetic acid, using bromothymol blue as internal indicator[4] and rechromatographed in solvent J to remove borate. The fractions identified were UDP-galactose and UDP-arabinose (Fraction X), UDP-glucose (Fraction Y) and UDP-xylose (Fraction Z). The $R_{glucose\text{-}1\text{-}phospate}$ values of these fractions and authentic samples, are given in Table 2.

TABLE 2. MOBILITIES OF UDP-SUGARS IN SOLVENT F*

	$R_{glucose\text{-}1\text{-}phosphate}$†
Fraction X	0·29
Fraction Y	0·48
Fraction Z	0·58
UDP-galactose	0·30
UDP-glucose	0·49
UDP-xylose	0·58

* Ethanol/methyl ethyl ketone/0·5 M morpholinium tetraborate, pH 8·6 in 0·01 M EDTA (70/20/30).

† Running time 50 hr.

Fraction X. It migrated as a single spot cochromatographing with authentic UDP-D-galactose in solvent F. The molar ratio of uridine:total phosphate:reducing sugar (after mild acid hydrolysis, galactose as standard) was 1:1·9:0·98. However, the analysis of the fractions for D-galactose, using "Galactostat reagent" revealed the ratio of galactose to reducing sugar 0·21:1·0. In order to test whether the sugar sample contained enzyme inhibitors that could lead to decreased value, several μg of D-galactose was added to the assay

[4] H. CARMINATTI, S. PASSERON, M. DANKERT and E. RECONDO, *J. Chromatog.* **18**, 342 (1965).

mixture. Quantitative oxidation of the added sugar was observed. The paper chromatography of the mild acid hydrolyzate of the fraction in solvents H and I gave two spots, cochromatographing with authentic galactose and arabinose on spraying with aniline phthalate reagent. Attempts to resolve UDP-galactose and UDP-arabinose did not meet with success. Analysis of the fraction for pentose, using orcinol reagent with arabinose as standard could account for the sugar other than galactose. The D-configuration of galactose was obvious from its reactivity towards D-galactose oxidase.

Fraction Y. The identification of the fraction as UDP-glucose is based on the following evidence: 1. Mild acid hydrolysis of the material released a reducing sugar which chromatographed with glucose on paper in solvents H and I and had the same mobility on paper electrophoresis in buffer II. The major part of the u.v. containing material cochromatographed with UDP in solvents A and B and on paper electrophoresis at pH 4·0 (buffer I). The rest of the material (about 10 per cent) cochromatographed with UMP in the above solvent systems. 2. The molar ratio of uridine:labile phosphate:total phosphate:glucose was 1:1·1:2·05:0·96. Glucose was determined after mild acid hydrolysis by hexokinase reaction and glucostat reagent (glucose oxidase), using a paper blank. 3. On treatment with snake venom phosphodiesterase, UMP and glucose-1-phosphate were released from UDP-glucose (solvents A and C). 4. UDP-glucose was definitely identified by its quantitative conversion to UDP-glucuronic acid by NAD-linked UDP-glucose dehydrogenase (solvents B and C).

Fraction Z. The fraction was identified as UDP-xylose on the following criteria: 1. The compound cochromatographed with authentic UDP-xylose in solvent F. 2. The molar ratio of uridine:labile phosphate:total phosphate:reducing sugar (after mild acid hydrolysis, xylose as standard) was 1:1:2·2:0·98. Analysis of the fraction for pentose using orcinol reagent with D-xylose as standard, accounted for all the reducing sugar. 3. Mild acid hydrolysis of the compound released UDP (80%) and a small amount of UMP, which were identified by their characteristic mobilities upon chromatography (solvents A and B). Paper chromatography of the hydrolyzate in solvent I revealed a spot which gave the typical red color of pentose and had the mobility of authentic D-xylose. 4. Treatment of the compound with phosphodiesterase followed by *E. coli* alkaline phosphatase released the carbohydrate component which was identified as xylose in solvent G.

Assuming the recovery of UDP sugars in solvent F quantitative, the ratio of UDP-D-galactose:UDP-arabinose:UDP-glucose:UDP-xylose was estimated 1:5:15:4.

UDP-N-acetylglucosamine (Fractions 4-I-c-A, 5-II-B-a, 6-I-B-b and 6-II-B-a)

These fractions were characterized as UDP-*N*-acetylglucosamine according to the following criteria: 1. The u.v. spectra at various pH indicated the presence of uridine base. 2. The mobilities on paper electrophoresis (buffer I) and paper chromatography (solvents A, B and C) resembled those of UDP-*N*-acetylglucosamine. 3. The treatment of the compound with snake venom phosphodiesterase led to the liberation of UMP (solvent B). 4. Mild hydrolysis of the compound led to the formation of UDP and traces of UMP, as revealed by paper chromatography in solvent B. Paper chromatography of the hydrolyzate in solvent H, showed one spot having the same mobility as *N*-acetylglucosamine. 5. For the further identification of the bound sugar, the fraction was hydrolysed in 6 N HCl at 100° for 6 hr. HCl was removed by evaporation *in vacuo*, and the resulting free amino sugars were degraded with ninhydrin.[5] Paper chromatography in solvent K indicated the presence of

[5] P. J. STOFFYN and R. W. JEANLOZ, *Arch. Biochem. Biophys.* **52**, 373 (1957).

arabinose which would have been derived from glucosamine or mannosamine. The confirmation of the bound sugar as *N*-acetylglucosamine was obtained by chromatography of the mild acid hydrolyzate of the compound in solvent H, using borate buffered Whatman no. 1 paper and spraying with Morgan-Elson reagent.[6] 6. The fraction, after mild acid hydrolysis gave positive test for *N*-acetylaminosugar by the method of Reissig *et al.*[7] The molar ratio of uridine to *N*-acetylglucosamine was 1:1·02.

5′-IMP (Fraction 5-III-b-A)

The u.v. spectra of the fraction indicated that it was an inosine derivative. The electrophoretic mobility in buffer I and chromatographic migration in solvents A, B and C were identical to those of authentic 5′-IMP. No reducing sugar was detected before and after mild acid hydrolysis. The molar ratio of components were those of the authentic IMP. Inosine was formed on hydrolysis with snake venom extract or alkaline phosphatase.

5′-AMP (Fractions 5-V, 6-IV-A-b, 6-V-A, 7-VIII-A, 8-VII-A and 9-VI-A)

These fractions constituted the largest amount of nucleotides isolated from *Chlorella*. The absorption spectra of these fractions indicated the presence of adenine derivative. The mobilities of the compound on paper chromatography (solvents A, B, C, D and E) and electrophoresis (buffers I and II) resembled those of authentic 5′-AMP. No reducing sugar was detected before and after mild acid hydrolysis. The molar ratio of components were those of AMP. Hydrolysis with *E. coli* alkaline phosphatase as well as snake venom extract led to the formation of a compound, which was identified as adenosine on paper chromatography in solvents A and L.

Adenine Nucleotide-Peptide Complex-I (Fractions 6-III-B, 7-VI-b-C and 8-V-B)

These fractions were tentatively identified as Adenine nucleotide-peptide complex on the basis of following criteria: 1. The absorption spectra of these fractions resembled those of adenosine. No new peak of absorption spectrum was detected in the presence of KCN. 2. The electrophoretic mobilities in two buffers (I and II) were close to those of ADP. However, the mobilities of the compound in various solvent systems (A, B and C) were different from those of ADP and of ATP, AMP, NAD^+, $NADP^+$, Desamino NAD^+, Deamino NAD^+ and ADP-ribose (Table 3). 3. No reducing sugar was detected before and after mild acid hydrolysis. The cysteine-sulphuric acid reaction for deoxysugar was negative. The orcinol reaction on the intact nucleotide and on hydrolyzate of the nucleotide obtained after mild acid hydrolysis, gave same pentose values before and after alkaline hydrolysis (0·1 N-NaOH, 100°, 5 min). 4. The molar ratio of adenine:ribose:total phosphate was 1:2·1:1·9 (Table 1). However, the value of labile phosphate was negligible. When the material was heated with 1 N HCl (100°, 10 min) ribose-5-phosphate (solvents B and I) and adenine (solvents A and L) were the only products. No deoxyribose-5-phosphate or other silver-nitrate positive compound detected in the acid hydrolyzate. 5. Exhaustive treatment of the compound with snake venom phosphodiesterase led to the formation of 5′-AMP, which was identified by cochromatography in solvents B, C and E and electrophoresis in buffer II. These solvent and buffer systems distinguish 5′-AMP from 2′-AMP, 3′-AMP and d-AMP. No reducing compound was detected on treatment of the compound with snake venom phosphodiesterase. Treatment of the intact nucleotide with crude snake venom extract led

[6] C. E. CARDINI and L. F. LELOIR, *J. Biol. Chem.* **225**, 317 (1957).
[7] J. L. REISSIG, J. L. STROMINGER and L. F. LELOIR, *J. Biol. Chem.* **217**, 959 (1955).

to the formation of adenosine (solvent L and buffer II). 6. The compound did not react with *E. coli* alkaline phosphatase. 7. The ninhydrin reaction for amino acids was positive on the intact nucleotide. A portion of the nucleotide was hydrolyzed with 6 N HCl (100°, 16 hr). HCl was removed by evaporation *in vacuo* and the resulting residue dissolved in water. Paper chromatography of the residue in solvent M revealed two ninhydrin positive spots which, cochromatographed with authentic glutamic acid and glycine. To test whether glycine was artifact formed during acid hydrolysis[8] with 6 N HCl, another portion of the nucleotide was hydrolyzed with 1 N HCl (100°, 1 hr) and subsequently chromatographed in solvent N. Again two ninhydrin positive spots cochromatographing with glutamic acid and glycine were obtained. Chromatography in solvent N of the nucleotide after alkaline hydrolysis (1 N-NaOH, 100°, 15 min) led to the detection of only one ninhydrin positive spot which did not cochromatograph with any authentic amino acid markers and moved less than glutamic acid and glycine. It may be due to the formation of a peptide of glutamic acid and glycine

TABLE 3. PAPER CHROMATOGRAPHIC AND ELECTROPHORETIC MOBILITIES OF ADENINE NUCLEOTIDE-PEPTIDE COMPLEX I AND ADP-RIBOSE-X

	$R_{5'\text{-AMP}}$ of nucleotide in solvents			$M_{5'\text{-AMP}}$ of nucleotide in buffers	
	A	B	C	I	II
ADP-ribose	0·51	1·30	0·53	2·10	—
ATP	0·49	0·38	0·27	3·10	0·90
ADP	0·74	0·59	0·49	2·30	0·98
NAD^+	0·86	0·85	0·36	0·97	0·78
$NADP^+$	0·50	0·25	0·22	2·20	—
Adenosine	1·46	2·90	—	0·0	0·35
Adenine nucleotide-peptide complex I	0·58	1·81	0·79	2·30	0·98
ADP-ribose-X	1·00	2·80	1·00	2·10	—

which was stable under these conditions. In view of the small quantity of the sample it was not possible to test the linkage between amino acids and nucleotide moiety nor to determine the sequence of amino acids units in the fraction.

ADP-ribose-X (Fractions 6-V-B-b, 7-VIII-B and 8-VII-B)

These fractions yielded a new nucleotide. 1. Analysis of absorption spectrum at various pH indicated it an adenosine derivative. No new peak of absorption obtained in the presence of KCN. 2. Paper chromatographic mobilities in solvents A and C were similar to 5'-AMP (Table 2). The chromatographic mobility in solvent B was faster than 5'-AMP and was similar to adenosine. The electrophoretic migration in buffer I was more than 5'-AMP and was identical to ADP-ribose. 3. Paper chromatography of the nucleotide after mild acid hydrolysis in solvent B revealed three new u.v. absorbing spots which were identified as ADP-ribose (85 per cent), ADP (10 per cent) and 5'-AMP (5 per cent). The nucleotide spot cochromatographing with ADP-ribose was eluted from the paper and re-chromatographed in solvents A and C. In both systems, a single silver nitrate-positive spot was obtained which

[8] H. ISHIHARA, *J. Biochem. (Tokyo)* **47**, 196 (1960).

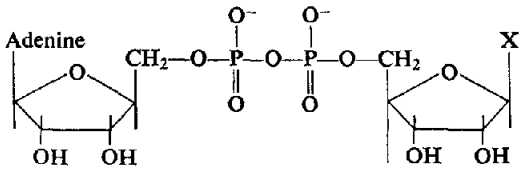
cochromatographed with u.v. absorbing spot and authentic ADP-ribose. Solvent C separates ADP-ribose from ribose-5-phosphate. Additional evidence for ADP-ribose was obtained by the chemical analysis of the eluate. The molar ratio of adenine to ribose to total phosphate in the eluate was 1:2:2. 4. The molar ratio of adenine:ribose:total phosphate for the intact nucleotide fraction was 1:1·98:2·06 (Table 1). No labile phosphate was detected. The pentose value (as ribose) of the nucleotide before or after mild alkaline hydrolysis (0·1 N NaOH, 100°, 5 min) was 2·0. The intact nucleotide when hydrolyzed with mild acid and then subjected to mild alkaline hydrolysis yielded ribose value of 1·0. This indicated that the linkage between ADP-ribose and X was susceptible to mild acid hydrolysis, but was insensitive to mild alkaline hydrolysis. 5. The intact nucleotide did not exhibit any reducing power. Subjecting the intact nucleotide to mild acid hydrolysis gave reducing sugar test equivalent to 0·5 mole (glucose as standard) per mole of base. Under similar conditions, authentic ADP-ribose gave reducing sugar test equivalent to 50 per cent of the reducing power of standard glucose. Tests for uronic acid and TBA assay for 2 deoxyaldoses were negative. 6. Digestion of the nucleotide with snake venom phosphodiesterase released 5'-AMP, identified by paper chromatography in solvents B, C, E and by paper electrophoresis in buffer II and a new phosphate ester (PR-X) with the mobility higher than the intact nucleotide, ribose-5-phosphate, deoxyribose-5-phosphate and ADP-ribose in solvent B (Table 4).

TABLE 4. PAPER CHROMATOGRAPHIC MOBILITIES OF PR-X AND RELATED COMPOUNDS IN SOLVENT B*

	Running distance for 18 hr at 25° cm
Ribose 5-phosphate	9·2
Deoxyribose 5-phosphate	13·8
Ribose	31·6
Deoxyribose	35·0
ADP-ribose	8·2
ADP-ribose-X	17·6
PR-X	19·2
PR-X, after mild acid hydrolysis	9·3
PR-X, after alkaline phosphatase treatment, followed by mild acid hydrolysis	31·2

* 95% ethanol/M ammonium acetate, pH 7·5 (5/2).

The spot of the new phosphate ester PR-X was located by staining with $FeCl_3$-salicylsulfonic acid reagent. The phosphate ester was eluted from the guide paper strip with water. The sample showed the ratio of ribose to total phosphate 1:1. The reducing sugar assay on the phosphate ester was negative. However, mild acid hydrolysis resulted in decomposition of the compound with the liberation of ribose-5-phosphate, which was identified by paper chromatography in solvent B. The phosphate group was easily liberated from PR-X by digestion with *E. coli* alkaline phosphatase. Paper chromatography of phosphatase treated material in solvent B revealed the formation of orthophosphate. Another aliquot of phosphatase treated material was subjected to mild acid hydrolysis and chromatographed in solvent B. An $AgNO_3$-positive spot cochromatographing with authentic ribose was observed. The chemical nature of the compound X could not be ascertained. On the basis of the above evidence, structure (I) is tentatively proposed for this nucleotide.



(I)

UDP (Fraction 6-II-A-a)

Examination of the u.v. spectra of this fraction indicated a uridine derivative. It behaved as UDP on paper chromatography in solvents A, B and C and electrophoresis in buffer I. No reducing sugar could be detected after mild acid hydrolysis. The molar ratio of components were those of UDP.

UDP-glucuronic acid (Fractions 7-II-A and 8-II)

The nucleotide exhibited a u.v. spectrum typical of a uridine derivative. The compound cochromatographed with authentic UDP-glucuronic acid in solvents A, B and C and on paper electrophoresis in buffer I. Mild acid hydrolysis of the material released a reducing sugar which cochromatographed with glucuronic acid on paper (Solvents B and O). The major part of the u.v. containing material (80 per cent) cochromatographed with UDP in solvents B and C and the remaining with 5'-UMP. The uronic acid was eluted from the paper and lactonized by heating it in a sealed tube with glacial acetic acid for 30 min at 80°. Acetic acid was removed by evaporation *in vacuo*. The isolated lactone cochromatographed with glucuronolactone in solvent O, clearly separating it from the other naturally occurring uronic acid lactones. Subjection of the compound to the carbazole and borate carbazole assays[9] indicated the presence of glucuronic acid. The ratio of absorptivity at 530 nm in the absence to the presence of borate was 0·72, indicating the absence of galacturonic acid. Chemical analysis of the material suggested that it contained 1 mole of UDP for every mole of uronic acid. The compound did not exhibit any reducing power. Mild acid hydrolysis of the nucleotide liberated reducing sugar equivalent to one mole of glucuronic acid per mole of uridine.

GDP-sugars (Fraction 8-III)

Paper chromatography of the fraction in solvents A, B and C failed to resolve it into subfractions; it exhibited an identical mobility as that of authentic GDP-D-mannose. Absorption spectra at pH 1 and 7 and reducing sugar tests after mild acid hydrolysis indicated the fraction to be GDP-sugars. The reducing value of the intact nucleotide was negligible. When chromatographed on paper in solvents H and I, the mixture obtained by mild acid hydrolysis of the compound was found to contain mannose as major component and galactose as the minor component. The major part of u.v. absorbing material of mild acid hydrolyzate of the fraction migrated as GDP (80 per cent) in solvent B. When the nucleotide was chromatographed on EDTA-impregnated Whatman no. 1 paper (0·01 M EDTA, pH 7·0) and run for 7 days in solvent F, two u.v. absorbing spots, very close to each other were detected. The fast moving component cochromatographed with authentic GDP-D-mannose and the slow moving component had mobility similar to GDP-D-galactose.[4] As these two were not completely separable, the area of the two spots were cut only by judgement. The characterization of the galactose component of the sugar nucleotide as D-isomer was indicated by its

[9] J. D. GREGORY, *Arch. Biochem. Biophys.* **89**, 157 (1960).

destruction by D-galactose oxidase. However, D-galactose content as assayed by galactose oxidase system of the fraction was only 40 per cent of the total reducing sugar as assayed by Park and Johnson method and calculated as galactose standard. The remaining 60 per cent of the reducing sugar was accounted for mannose, assayed by GDP-mannose dehydrogenase assay.[10]

The confirmation of the fast moving component as GDP-D-mannose is based on the following evidence: It had the same mobility as authentic GDP-D-mannose on paper electrophoresis in buffer I. Paper chromatography of the mild acid hydrolyzate of the fraction in solvent G, revealed a single $AgNO_3$-positive spot, cochromatographing with mannose. The same aliquot of the sugar nucleotide gave 0·03 μmole by the enzymatic technique using the specific GDP-mannose dehydrogenase and 0·034 μmole by the chemical assay of reducing sugar as mannose. The agreement of the results by these two methods indicated that the sugar nucleotide was entirely GDP-mannose.

The ratio of GDP-D-galactose to GDP-mannose was estimated 1:10.

5'-GMP (Fractions 7-V-a, 8-IV and 9-IV-B-b)

Identification of 5'-GMP was made by similar procedures used for characterization of 5'-AMP.

Guanosine Nucleotide-peptide Complex (Fraction 9-I-A)

The u.v. absorption at different pH indicated it a guanosine derivative. Paper chromatographic mobilities in solvents A, B and C were identical to authentic GDP-glucose. The mobility on paper electrophoresis in buffer I was slightly less than GDP-glucose. No reducing sugar was detected before and after mild acid hydrolysis. GMP was produced on treatment of the compound with snake venom phosphodiesterase (solvent B). There was one mole of labile phosphate per mole of base. The molar ratio of guanosine:ribose:total phosphate was 1:1·02:2·13 (Table 1). The nucleotide gave a positive ninhydrin test. Hydrolysis of the nucleotide with 6 N HCl (100°, 16 hr) and subsequent paper chromatography in solvent M led to the identification of glycine and leucine (or isoleucine). Hydrolysis with 1 N HCl (100°, 2 hr) led to the detection of 3 ninhydrin positive spots (solvent N). Two of the spots cochromatographed with glycine and leucine (isoleucine). The third spot had mobility less than known amino acids. The bond between peptide and nucleotide could not be decided with data now available. On the basis of the above evidences the complex appears to be a guanosine diphosphate-peptide.

ADP (Fractions 7-VII-B-a, 8-VI, 9-V and 10-a-B)

Identification of ADP was based on the data obtained from analyses similar to those previously described for characterization of UDP.

Adenine Nucleotide-peptide Complex II (Fractions 8-V-A and 9-IV-A)

The u.v. spectra of these fractions indicated an adenosine derivative. The mobilities on paper chromatogram in solvents A, B and C and on paper electrophoresis in buffer I was similar to authentic ATP. In all these solvent systems ^{35}S radioactivity was associated with the intact nucleotide. Hydrolysis of the nucleotide with either 0·01 N HCl or 0·1 N HCl (100°, 10 min) did not release any new ^{35}S compound (solvents B and C). No labile phosphate

[10] J. PREISS, *J. Biol. Chem.* **239**, 3127 (1964).

was detected. The reducing sugar test on the nucleotide before and after mild acid hydrolysis was negative. Digestion of the compound with crude snake venom led to the formation of adenosine (solvent L). Treatment with snake venom phosphodiesterase and subsequent chromatography in solvents A and E revealed the liberation of 5′-AMP. The fraction gave a positive ninhydrin test. The ninhydrin positive spot and u.v. absorbing spot of the intact nucleotide cochromatographed in solvent N; the migration of the compound was less than known amino acids. Hydrolysis with 6 N NCl (100°, 16 hr) and subsequent chromatography in solvents M and N led to the identification of three ninhydrin positive spots with identical mobilities of cysteine, glycine and serine. No attempts were made to determine the amino acid sequence. The fraction appears to be a peptide of adenine nucleotide.

ADP-sugars (*Fraction* 9-*IV-B-a*)

The nucleotide had u.v. spectra identical to that of adenosine. Paper chromatography in solvents A, B and C and electrophoresis in buffer I revealed a single spot with the mobility of ADP-glucose. Upon mild acid hydrolysis, it gave two u.v. absorbing spots on papergram (solvent B), which were identified as ADP (80 per cent) and 5′-AMP (20 per cent). Paper chromatography of the mild acid hydrolyzate in solvents H and I revealed the production of glucose and a small proportion of arabinose. Upon treatment with snake venom phosphodiesterase and subsequent chromatography in solvent A revealed the formation of 5′-AMP. The molar ratio of base to reducing sugar (glucose) was 0·85.

Adenine Nucleotide-peptide Complex III (*Fraction* 9-*IV-C*)

Analysis of the u.v. spectrum at different pHs indicated an adenosine derivative. The mobilities of the intact nucleotide on paper chromatograms (solvents A and C) and electrophoretogram (buffer I) was similar to ADP-glucose. However, the mobility of the nucleotide was slightly more than authentic ADP-glucose in solvent B. No reducing sugar was found before and after mild acid hydrolysis. Analysis showed that the compound had one mole of adenine, two moles of ribose and two moles of phosphate (Table 1). The value for labile phosphate was negligible. Treatment with snake venom phosphodiesterase and subsequent paper chromatography in solvents A and E and electrophoresis in buffer II revealed the formation of 5′-AMP. The compound was insensitive to *E. coli* alkaline phosphatase. It gave a positive ninhydrin and u.v. absorbing spot at the same position of the chromatogram (solvent B). Hydrolysis of the compound in 6 N HCl (100°, 16 hr) and subsequent chromatography in solvents M and N led to the identification of glutamic acid, glycine and cysteine. The chemical nature of this fraction appears to be similar to adenine nucleotide peptide complex I but containing one more amino acid viz. cysteine (cystine).

ATP (*Fractions* 10-*a-A*, 10-*b-A and* 11-*b-III*)

These fractions had characteristic u.v. spectrum of adenosine derivatives. Electrophoretic mobility in buffer I and migration on papergrams in solvents A, B and C were identical to authentic ATP. No reducing sugar was detected before and after mild acid hydrolysis. The molar ratio of components were those of ATP (Table 1). Treatment of the nucleotide with snake venom phosphodiesterase led to the formation of 5′-AMP (solvent A).

Thymidine-oligonucleotides (*Fractions* 10-*d*, 10-*e*, 10-*f and* 11-*d-II*)

The u.v. spectrum of these fractions at various pH values were those of thymidine derivatives. The mobilities of these fractions on paper chromatogram in solvent C were slightly more

than TMP. These compounds did not cochromatograph with 5′-TMP or thymidine in solvents A and B. In both the solvent systems the mobilities of these fractions were more than TMP but less than thymidine. No reducing sugar was detected before and after acid hydrolysis (0·01 N HCl, 100°, 15 min and 0·1 N HCl, 100°, 10 min). Treatment of these compounds with either snake venom phosphodiesterase or crude snake venom extract and subsequent paper chromatography in solvents A and L and paper electrophoresis in buffer I, did not reveal the formation of any new u.v. absorbing compound. It is likely that the fractions contain either thymine dimers or oligonucleotides containing 3′-monophosphate.

^{35}S *Compounds*

6-Sulfoquinovose-^{35}S (*fractions* 1-*b and* 2-*b*). Identification of sulfoquinovose was based on identical migration with those of authentic 6-sulfoquinovose on paper chromatography in solvents A, B, C, G and H and paper electrophoresis in buffers I and II. Only one radioactive and $AgNO_3$ reactive material was detected before and after mild acid hydrolysis of the compound. The compound gave positive reaction for deoxysugar with cysteine-sulfuric acid reagent. Its concentration in the extract, based on deoxysugar test, was 2·6 μmoles (approximately 0·5 per cent of the ^{35}S activity in the extract).

PAPS-^{35}S (*fraction* 1 1-*b-I*). The u.v. spectrum of the fraction revealed its adenine derivatives. The mobility of the ^{35}S labeled compound was identical to authentic PAPS on paper electrophoresis in buffer I and on papergrams in solvents A, B and C. Hydrolysis of the compound with 0·1 N HCl at 37° for 60 min and subsequent paper electrophoresis in buffer I and paper chromatography in solvent C showed the formation of a new ^{35}S compound with identical mobility of inorganic sulfate (Fig. 2). The concentration of the compound was 0·3 μmole (approximating 0·1 per cent of the ^{35}S activity in the extract).

Nucleoside diphsopho-6-sulfoquinovose-^{35}S. Each fraction was tested for release of any ^{35}S-sulfoquinovose on acid hydrolysis (0·01 N HCl and 0·1 N HCl, 100°, 15 min). No compound was detected which liberated sulfoquinovose on acid hydrolysis.

DISCUSSION

Various nucleotide-peptide compounds have been described, ranging from protein-bound nucleotides[11] to nucleic acid-bound peptides.[8,12] Harris and associates[13–16] obtained a mixture of nucleotide-peptide compounds in aqueous alcoholic extracts of brewers yeast. Several nucleotide-peptide compounds have been isolated from TCA extracts of green alga, *Chlorella ellipsoidea* by Hase *et al.*[17–20] They observed that cysteine (cystine) was a common component of all the compounds and that glutamic acid, aspartic acid, serine, glycine and alanine were also associated with the peptide moiety, while adenine either free or combined

[11] W. NIEMIERKO, M. DYDNSKA, W. DRABIKOWSKI, I. KAKOL and H. ZALUSKA, *Acta Biol. Exptl* **17**, 373 (1957).
[12] V. HABERMANN, *Biochim. Biophys. Acta* **32**, 297 (1959).
[13] G. HARRIS and G. E. NEAL, *Biochim. Biophys. Acta* **47**, 122 (1961).
[14] G. HARRIS and A. WISEMAN, *Biochim. Biophys. Acta* **55**, 374 (1962).
[15] G. HARRIS and A. WISEMAN, *Biochim. Biophys. Acta* **55**, 775 (1962).
[16] A. H. COOPER, G. HARRIS and G. E. NEAL, *Biochim. Biophys. Acta* **61**, 573 (1962).
[17] E. HASE, S. MIHARA, H. OTSUKA and H. TAMIYA, *Arch. Biochem. Biophys.* **83**, 170 (1959).
[18] E. HASE, S. MIHARA, H. OTSUKA and H. TAMIYA, *Biochim. Biophys. Acta* **32**, 298 (1959).
[19] E. HASE and S. MIHARA, *J. Gen. Appl. Microbiol.* **5**, 221 (1960).
[20] E. HASE, S. MIHARA and H. TAMIYA, *Biochim. Biophys. Acta* **39**, 381 (1960).

to uracil, to uracil and guanine or to an unknown substance (x) were detected together with ribose as a part of the nucleotide-peptide. No attempts were made to determine the chemical structure of these nucleotide-peptides. The nucleotide-peptide complexes found by the present authors in ethanolic extracts of *C. pyrenoidosa* differ from those of *C. ellipsoidea* in that the base is either adenine or guanine and not a mixture of bases and that the composition of the peptides is different.

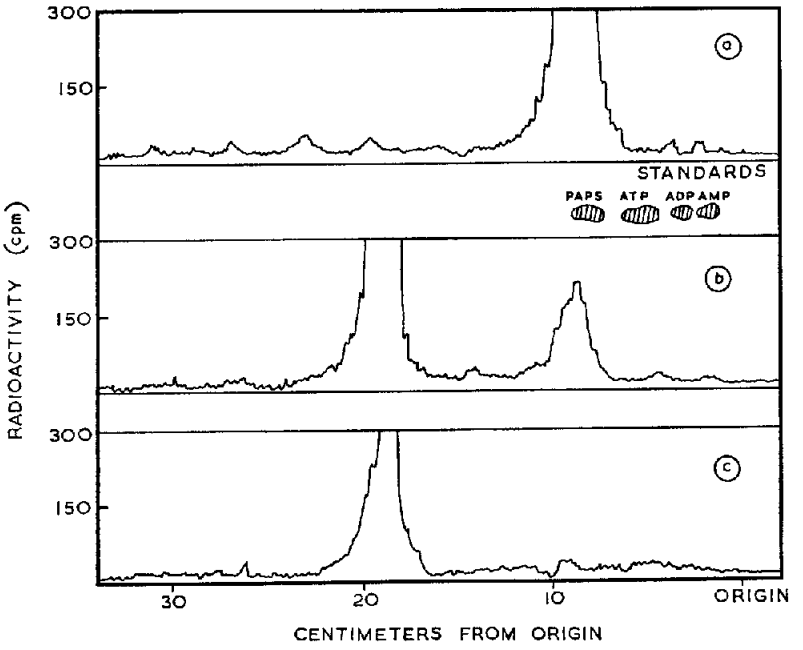


FIG. 2. THE RADIOACTIVE PROFILE OF COMPOUND 11-b-I ON PAPER ELECTROPHORESIS IN BUFFER I. 4,000 V, 30 min (a) INTACT NUCLEOTIDE (b) AFTER HYDROLYSIS WITH 0·1 N HCl (37°, 60 min) (c) STANDARD INORGANIC SULFATE ^{35}S.

Adenine nucleotide-peptide complexes I and III are, to our knowledge, the first known nucleotide-peptide complexes having the ratio of base to ribose to total phosphate as 1:2:2. Complex I is probably derived from complex III by loss of a cysteine (cystine) residue. In the past few years a large number of gutamyl-peptides have been isolated from plant sources.[21–24] These peptides may be synthesized from adenine nucleotide-peptide complex I and complex III by transpeptidation reactions. A number of nucleotide derivatives containing sugar-peptides, that are precursors of peptides present in bacterial cell walls have been isolated in recent years.[25,26] However, since algal cell walls are composed of carbohydrate and are supposedly free of amino acids and peptides,[27] it is unlikely that such compounds would in this case be associated with the cell wall development.

[21] A. I. VIRTANEN, *Angew. Chem.*, Intern. Ed. Engl. **1**, 299 (1962).
[22] E. J. MATIKKALA and E. I. VIRTANEN, *Acta Chem. Scand.* **16**, 2461 (1962).
[23] R. M. ZACHARIUS, C. J. MORRIS and J. F. THOMSON, *Arch. Biochem. Biophys.* **80**, 199 (1959).
[24] C. J. MORRIS, J. F. THOMSON and R. M. ZACHARIUS, *J. Biol. Chem.* **238**, 650 (1963).
[25] H. R. PERKINS, *Bacteriol. Rev.* **27**, 18 (1963).
[26] H. H. MARTIN, *Ann. Rev. Biochem.* **35**, 457 (1966).
[27] T. K. VIRUPAKSHA and A. SHRIFT, *Biochim. Biophys. Acta* **80**, 587 (1964).

The guanine nucleotide-peptide complex appears to be a mixed anhydride of guanosine-diphosphate with peptides. The biological significance of the natural occurrence of GDP-peptide complex is not apparent. The adenine nucleotide-peptide complex II is similar to GDP-peptide complex in having similar ratio of base to ribose to total phosphate. However, in contrast to GDP-peptide complex, none of the two phosphate groups are acid labile. Cysteine (cystine) is one of the components of the peptide chain of complex II. Cysteine (cystine) is also common to all the nucleoside-peptides isolated from *C. vulgaris*[27] and of nucleotide-peptides isolated from *C. ellipsoidea*[18] and *Chlamydomonas moewusu*.[28] It has been pointed out that cysteine plays an essential role in the process of cell division.

The compound ADP-ribose-X appears to represent a new class of adenine sugar nucleotides. The linkage between terminal ribose unit of the compound to X is very acid labile, like the glycosidic linkage of sugar nucleotides. Chambon *et al.*[29] and Doly *et al.*[30] obtained a polymer of ADP-ribose by the action of liver nuclear extracts on NAD. More recently, Hasegawa *et al.*[31] and Reeder *et al.*[32] made detailed studies on the polymer of ADP-ribose. Digestion of the polymer with venom phosphodiesterase produced an acid-soluble product with a similar structure of ADP-ribose. This product was also isolated from yeast grown in medium containing ^{32}P-orthophosphate.[31] ADP-ribose-X, obtained in the present experiment differs from the acid-soluble product of ADP-ribose polymer in being insensitive to alkaline phosphatase hydrolysis. At the present time, little can be said concerning the possible role of this compound in biosynthetic processes. It may be that the compound is initially hydrolyzed to ADP-ribose which then is cleaved to ribose-5-phosphate and ADP, the two easily utilizable substrates. Alternatively, the compound may function as activated molecule in transfer reactions involving X. Definite assignment of the role of this compound in the metabolism of *Chlorella* must await elucidation of the chemical nature of X.

The identification of GDP-D-galactose in *C. pyrenoidosa* is the first report of its occurrence in green algae. GDP-D-galactose has been detected in animal tissues.[33, 34] Goudsmit and Neufeld,[35] isolated GDP-L-galactose from the albumin gland of *Helix pomatia*. Su and Hassid[36] reported GDP-L-galactose and GDP-D-mannose in red alga *Porphyra perforata* and postulated GDP-L-galactose to be an intermediate in the biosynthesis of L-galactose residues of galactan. The present authors, however, found GDP-D-galactose, instead of GDP-L-galactose, together with GDP-D-mannose and UDP-D-galactose in the green algae. It appears that the galactan synthesis in green algae is different from that in red algae. The transfer of mannose residues from GDP-D-mannose to yeast mannan has been accomplished.[37]

The formation of UDP-glucuronic acid in green algae probably takes place through enzymic dehydrogenation of UDP-D-glucose. Ankel *et al.*[38] presented evidence of UDP-glucuronatecarboxylase in *E. gracilis*, *S. quadricauda* and *A. flos-aquae* catalysing the decarboxylation of UDP-glucuronic acid to UDP-D-xylose. The presence of UDP-xylose in

[28] R. F. JONES and R. A. LEWIN, *Exptl Cell. Res.* **22**, 86 (1961).
[29] P. CHAMBON, J. D. WEILL, M. T. STROSSER and P. MANDEL, *Biochem. Biophys. Res. Commun.* **25**, 638 (1966).
[30] J. DOLY and P. MANDEL, *Compt. Rend.* **264**, 2683 (1967).
[31] S. HASEGAWA, S. FUJIMURA, Y. SHIMIZU and T. SUGIMURA, *Biochim. Biophys. Acta* **149**, 369 (1967).
[32] R. H. REEDER, K. UEDA, T. HONJO, Y. NISHIZUKA and O. HAYAISHI, *J. Biol. Chem.* **242**, 3172 (1967).
[33] R. DENAMUR, G. FAUCONNEAU and G. J. GUNTZ, *Ann. Biol. Anim. Biochem. Biophys.* **1**, 74 (1961).
[34] E. KEMPF and P. MANDEL, *Compt. Rend.* **253**, 2155 (1961).
[35] E. M. GOUDSMIT and E. F. NEUFELD, *Biochim. Biophys. Acta* **121**, 192 (1966).
[36] J. C. SU and W. J. HASSID, *Biochemistry* **1**, 474 (1962).
[37] I. D. ALGRANATI, H. CARMINATTI and E. CABIB, *Biochem. Biophys. Acta* **136**, 172 (1967); *Res. Commun.* **12**, 504 (1963).
[38] H. ANKEL, E. ANKEL, D. S. FEINGOLD and J. S. SCHUTZBACH, *Biochem. Biophys. Acta* **136**, 172 (1967).

Chlorella is indicative of the role of the sugar nucleotide as xylosyl donor in the synthesis of pentosans. D-Xylose is a common constituent of algal polysaccharides. It occurs in mucilage of many green algae.[39] UDP-D-xylose has been demonstrated in higher plants[40] and *Cryptococcus laurentii*.[41]

UDP-arabinose detected in *Chlorella pyrenoidosa* may be formed from UDP-D-xylose by UDP-arabinose 4-epimerase. The enzyme has been demonstrated in mung bean.[42] The role of UDP-arabinose and ADP-arabinose is obscure. These nucleoside diphosphate arabinoses could be the precursors of arabofuranosyl residue of algal polysaccharide chain. UDP-L-arabinose has been detected in the brown alga, *Fucus gardneri*[43] and higher plants.[40] It may be of interest to note that ADP-arabinose has been observed to occur in green algae for the first time. Lin and Hassid[43] found a compound similar but not identical to ADP-arabinose in *F. gardneri*.

The amino sugar, UDP-*N*-acetylglucosamine may be involved in the synthesis of algal cell wall substances. UDP-*N*-acetylglucosamine has also been demonstrated in *C. ellipsoidea*.[44]

Barber and Chang[45] reported the synthesis of UDP-L-rhamnose by enzymes of *C. pyrenoidosa*. Kauss[46] isolated the compound itself from the golden brown alga, *Ochromonas malhamensis*. The failure to detect UDP-L-rhamnose in *C. pyrenoidosa* by the present authors may be due to the synthesis of UDP-L-rhamnose occurring during only a portion of the cell's life. Murakami *et al.*[47] found that the formation of the cell walls in *Chlorella* occurred during autospore generation and over a relatively short period of time.

Shibuya *et al.*[48] reported a nucleoside diphosphosulfoquinovose in *Chlorella* and proposed that it might be a key intermediate in the biosynthetic pathways of various sulfoquinovosides including sulfolipid. Attempts to demonstrate the presence of this compound in *C. pyrenoidosa* were unsuccessful. It may be that the compound was present only during the short life of algal cell. Wedding and Black[49] found PAPS and APS in perchloric acid extracts of *C. pyrenoidosa* exposed to radioactive sulfate. These compounds were not detected when the cells were extracted with 80% hot alcohol. The present authors, however, could demonstrate PAPS in the ethanolic extracts of the ^{35}S-labeled algal cells. The experiment of Davies *et al.*[50] suggested that PAPS may take part in sulfolipid biosynthesis in *E. gracilis*.

EXPERIMENTAL

Growth of Algae

Chlorella pyrenoidosa was grown in glucose-beef extract media as described earlier.[51] The cells were harvested at the end of six days. In one experiment the cells were uniformly labeled with ^{35}S by being grown on

[39] R. A. LEWIN, *Can. J. Microbiol.* **2**, 665 (1956).
[40] V. GINSBURG, P. K. STUMPF and W. Z. HASSID, *J. Biol. Chem.* **223**, 977 (1956).
[41] H. ANKEL, D. G. FARRELL and D. S. FEINGOLD, *Biochim. Biophys. Acta* **90**, 397 (1964).
[42] E. F. NEUFELD, *Arch. Biochem. Biophys.* **69**, 602 (1957).
[43] T. Y. LIN and W. J. HASSID, *J. Biol. Chem.* **241**, 3282 (1966).
[44] T. IWAMURA, T. KANAZAWA and K. KANAZAWA, in *Studies on Microalgae and Photosynthetic Bacteria*, p. 577, Special Issue of *Plant Cell Physiol.*, Tokyo (1963).
[45] G. A. BARBER and M. T. Y. CHANG, *Arch. Biochem. Biophys.* **118**, 659 (1967).
[46] H. KAUSS, *Biochem. Biophys. Res. Commun.* **18**, 170 (1965).
[47] S. MURAKAMI, Y. MORIMURA and A. TAKAMIYA, in *Studies on Microalgae and Photosynthetic Bacteria*, p. 65, Special Issue of Plant Cell Physiol, Tokyo (1963).
[48] I. SHIBUYA, T. V. YAGI and A. A. BENSON, in *Studies on Microalgae and Photosynthetic Bacteria*, p. 627, Special Issue of *Plant Cell Physiol.*, Tokyo (1963).
[49] R. T. WEDDING and M. K. BLACK, *Plant Physiol.* **35**, 72 (1960).
[50] W. H. DAVIES, E. I. MERCER and T. W. GOODWIN, *Biochem. J.* **98**, 369 (1966).
[51] J. PREISS and E. GREENBERG, *Arch. Biochem. Biophys.* **118**, 702 (1967).

glucose-beef extract media in the presence of ^{35}S-sulfate (10 mC, specific activity after dilution with Na_2SO_4 488 mC/m mole).

Isolation of Nucleotides

In a typical experiment, 50 g of freshly harvested *Chlorella* cells were immediately dropped into 500 ml of boiling 80% ethanol. The boiling was continued for 10 min with vigorous stirring. The resulting suspension was cooled and centrifuged at 16,000 × *g* for 15 min. The insoluble residue was re-extracted with an additional 250 ml of 80% boiling ethanol as before. The extracts were combined, concentrated under reduced pressure at 30° to about 100 ml and extracted 4 times with equal volume of $CHCl_3$ to remove neutral lipids and pigments. The glycolipids were removed by extraction (× 4) with $CHCl_3$-methanol (2:1); a precipitate which formed at the interphase during extraction was removed by passing the solution through a sintered funnel. The aqueous phase, pale yellow in color, was concentrated to about 20 ml under reduced pressure and stored frozen.

The aqueous extract obtained in 12 different batches and the batch in which uniformly labeled ^{35}S-*Chlorella* cells used, were combined. The total volume was 260 ml with the total absorptivity at 260 nm of 13,700. The pH of the combined extract was 6·8.

Column Chromatography

Chromatographic procedures were carried out in a cold room at 0–3°. The aqueous extract was carefully layered on to a column of Dowex-1-X-8 (formate form, 100 to 200 mesh, 100 ml bed volume). The column was washed with 900 ml of cold deionized water until the absorptivity of the eluate at 260 nm fell to less than 0·1. Nucleotides were then eluted from the column by ammonium formate gradient, which was performed in three ranges. The first range was began with 1750 ml water in the mixing chamber and 1750 ml of 1 M ammonium formate, pH 5·0 in the reservoir chamber. The second range was obtained by 750 ml of 1 M ammonium formate, pH 5·0 in the mixer and 750 ml of 2 M ammonium formate, pH 5·0 in the reservoir. The third range was obtained by stepwise elution with 2 M ammonium formate containing 2 M formic acid, pH 3·2. The flow rate was adjusted to 3 ml per min. Fractions of 14·5 ml were collected and the appearance of nucleotides in the eluate followed by the absorbance at 260 nm and 275 nm. An aliquot was also assayed for ^{35}S counts by gas flow G.M. counter. The material eluted from the column was combined into 11 fractions (Fraction 1 to Fraction 11, Fig. 1). To each of the pooled fractions sufficient amounts (1 mg per absorptivity unit at 260 nm) of acid-washed Norit was added to remove at least 90 per cent of the 260 nm absorbing material. The Norit suspension was stirred vigorously and after standing in cold for 30 min was centrifuged at 16,000 × *g* for 30 min. The Norit was washed thoroughly with cold distilled water and the nucleotides eluted with 50% ethanol containing 0·1% NH_4OH. The eluates were then concentrated *in vacuo* at 30–32°. The average recovery was 80–90 per cent.

Paper Chromatography

Since analysis by paper chromatography showed that each fraction contained more than one compound, the nucleotides were further separated by descending chromatography on Whatman No. 3 MM paper in Solvent A: isobutyric acid/1 M ammonia/0·1 M EDTA, pH 7·2 (10/6·0/0·16); Solvent B: 95% ethanol/1 M ammonium acetate, pH 7·5 (5/2); and Solvent C: 95% ethanol/1 M ammonium acetate, pH 3·8 (5/2).

The u.v. absorbing components were located on paper under a Mineralight, cut from the paper and the solvents removed by keeping the paper strips in contact with absolute ethanol for a few hours and drying at room temperature. The nucleotides were eluted from the paper with deionized water and subjected to further analysis. For quantitative analysis, a blank strip was cut from the same chromatogram and treated in the same manner. If analysis by paper chromatography and paper electrophoresis showed that isolated fraction contained more than one component the nucleotides were further separated by descending chromatography. The separation of the various fractions in solvent A has been designated by Roman numerals, in solvent B, by capital and in solvent C by small letters according to increasing R_f (Table 1). The following solvent systems were also used in descending paper chromatography on Whatman No. 1 filter paper—Solvent D: 600 g $(NH_4)_2SO_4$ in 1 l. of 0·1 M sodium phosphate, pH 6·8 and 20 ml of *n*-propanol; Solvent E: 0·8 sat. $(NH_4)_2SO_4$ containing 2% propanol; Solvent F: 95% ethanol/methyl ethyl ketone/0·5 M morpholinium tetraborate pH 8·6, in 0·01 M EDTA (70/20/30); Solvent G: ethyl acetate/pyridine/water (3·6/1/1·15); Solvent H: *n*-butanol/pyridine/water (6/4/3); Solvent I: phenol/water (80/20 v/v); Solvent J: methanol/1 M ammonium acetate, pH 3·8 (70/35); Solvent K: *n*-butanol/ethanol/water (4/1/1); Solvent L: *n*-butanol saturated with 10% urea; Solvent M: 77% ethanol/1% diethylamine; Solvent N: *tert*-butanol/butanone/diethylamine/water (40/40/4/20); and Solvent O: ethyl acetate/pyridine/water (40/11/6). Paper electrophoresis was carried out in a GME high voltage electrophorator with 0·05 M citrate buffer, pH 4·0 and 0·05 M borate buffer, pH 9·2.

Alkaline $AgNO_3$ [52] and aniline phthalate [53] were used for the detection of reducing sugars, and Morgan-Elson reaction [54] for *N*-acetylaminosugars. To locate phosphate esters, chromatograms sprayed with 0·1%

[52] W. E. TREVELYAN, D. P. PROCTER and J. S. HARRISON, *Nature* **166**, 444 (1950).
[53] S. M. PARTRIDGE, *Nature* **164**, 443 (1949).
[54] S. M. PARTRIDGE, *Biochem. J.* **42**, 238 (1948).

$FeCl_3$, $6H_2O$ in 80% ethanol, dried in air at room temperature and sprayed with 10% salicylsulfonic acid in 80% ethanol.[55] Amino acids were detected by dipping the dried chromatograms in 0·02% ninhydrin in acetone, drying in air and heating them for 2 min at 100°. For the detection of ^{35}S radioactivity on paper chromatograms or electrophoretograms, the strips were scanned for radioactivity in a 4π scanner (Nuclear Chicago Corp. U.S.A.).

Analytical Procedures

Total, acid-labile and inorganic phosphate were determined by the method of Fiske and Subbarow[56] and Ames and Dubin.[57] The pentose content of the nucleotides was determined by the orcinol method.[58] The 5′-adenylic acid and 5′-uridylic acid served as the standard for the determinations of pentose in the purine and pyrimidine nucleotides, respectively. Reducing values of the sugars liberated from the sugar nucleotides after mild acid hydrolysis (0·01 N HCl, 100°, 10 min) were determined according to Park and Johnson.[59] The procedure for measurements of uronic acids,[9, 60] deoxysugar,[61] pentose,[58] reducing sugar,[59] *N*-acetylamino-sugar[7] and ketodeoxygluconate[62] were modified to a micro scale so that the amount of the sample were in the range 0·01 to 0·1 μmole. The base moiety of the nucleotides were identified by spectral measurements in the region of 360–210 nm at pH 1, 7 and 12.

Conditions for Hydrolysis with Phosphodiesterase and Alkaline Phosphatase

Hydrolysis of nucleotides or oligonucleotides with snake venom phosphodiesterase were carried out in the reaction mixture containing 0·1 μmole of nucleotide, 5 μl of 0·1 M Tris-HCl buffer, pH 8·5, 5 μl of 0·1 M $MgCl_2$ and sufficient amounts of water and the enzyme (10 μl) in a total volume of 25 μl at 37° for 60 min.

Hydrolysis of nucleoside monophosphates or sugar phosphates with *E. coli* alkaline phosphatase was carried out in the reaction mixture containing 0·1 μmole of substrate, 5 μl of 0·05 M glycyl-glycine buffer, pH 8·0, 5 μl of 0·02 M $MgCl_2$ and sufficient amounts of water and enzyme (10 μl) in a total volume of 25 μl at 37° for 45 min.

Materials

6-sulfoquinovose was a gift from Dr. Benson. Snake venom was a lyophilized sample from *Crotalus adamanteus*. GDP-mannose dehydrogenase was purified from *Arthrobacter* by the procedure of Preiss[10] and assayed accordingly.

Acknowledgements—This work was supported by a financial grant from the U.S. Public Health Service under contract number AI 05520. One of us (G.G.S.) thanks Fulbright Foundation for the travel award.

[55] H. E. Wade and D. M. Morgan, *Nature* **171**, 529 (1953).
[56] C. H. Fiske and Y. Subbarow, *J. Biol. Chem.* **66**, 375 (1925).
[57] B. N. Ames and D. T. Dubin, *J. Biol. Chem.* **235**, 769 (1960).
[58] Z. Dische, *J. Biol. Chem.* **204**, 983 (1953).
[59] J. T. Park and M. J. Johnson, *J. Biol. Chem.* **181**, 149 (1949).
[60] T. Bitter and H. M. Muir, *Anal. Biochem.* **4**, 330 (1962).
[61] Z. Dische and L. B. Shettles, *J. Biol. Chem.* **175**, 595 (1948).
[62] A. Weissbach and J. Hurwitz, *J. Biol. Chem.* **234**, 705 (1959).