1978

The acid-soluble nucleotides of encysted embryos of the brine shrimp, Artemia salina.

Sarah Jane. Gilmour
University of Windsor

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

DEDICATION

I would like to dedicate this thesis to Dr. George Lunt who encouraged me to leave my homeland, and to my mother, Joan Rosina Gilmour, who wanted me to stay.

THE ACID-SOLUBLE NUCLEOTIDES OF ENCYSTED EMBRYOS OF THE BRINE SHRIMP, ARTEMIA SALINA

BY

SARAH JANE GILMOUR

A Thesis
Submitted to the Faculty of Graduate Studies
through the Department of Biology in
Partial Fulfillment of the Requirements
for the Degree of Master of Science
at the University of Windsor

Windsor, Ontario, Canada

1978

ABSTRACT

Over thirty acid-soluble UV-absorbing compounds have been isolated from an acid extract of encysted dormant embryos of the brine shrimp, Artemia salina. These compounds have been purified by means of ion-exchange chromatography on columns of Dowex-1-formate and DEAE-cellulose. Several of these compounds have been identified previously as the common mononucleotides; in addition, the diguanosine nucleotides guanosine(5')triphospho(5')guanosine and guanosine(5')tetraphospho(5')guanosine have been shown to be major constituents of the acid-soluble extract. In this thesis six additional UV-absorbing compounds in the acid-soluble fraction of the cysts have been identified. They are uridine 5'-diphosphate, inosine 5'-monophosphate, adenylo-succinic acid, UDP-N-acetylglucosamine, guanosine(5')diphospho (5')guanosine and guanosine(5')triphospho(5')adenosine. The function of these compounds during development of Artemia salina is discussed.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Alden H. Warner, for his invaluable help and encouragement during the course of this study, without which this thesis would not have been possible. I would also like to show my appreciation to the other members of my committee, especially Dr. David Cotter for his critical reading of the manuscript derived from this thesis, and Dr. Keith Taylor who agreed to be a member after this work was in progress. I am very grateful to Dr. Allan Okey of the Biology Department for the use of his Perkin-Elmer Coleman 575 spectrophotometer. Lastly I would like to thank all members of the Biology and Chemistry Departments who have assisted and encouraged me during my stay in Windsor.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Encysted embryos of the brine shrimp, Artemia salina, have been used as a model eukaryotic system for the study of nucleotide metabolism in embryos despite the fact that these embryos (as well as adults of Artemia) are unable to synthesise purines de novo while possessing the de novo pathway for pyrimidine biosynthesis (1). This metabolic deficiency is circumvented in the egg during oogenesis by storage of large quantities of purine-containing compounds in yolk platelets which are used subsequently during development in nucleotide metabolism and nucleic acid synthesis (1,2,3). Thus, the acid-soluble fraction of Artemia cysts is unusually rich in purine-containing compounds.

The major acid-soluble nucleotide in Artemia cysts has been shown to be guanosine(5')tetraphospho(5')guanosine [G(5')p_4(5')G] which comprises about 45% of the total UV-absorbing material in the cold acid extract and 2% of the dry weight of the embryos (2,4). A similar compound, guanosine(5')triphospho(5')guanosine [G(5')p_3(5')G], has also been found in lesser amounts in the cysts (4). In addition to these unusual nucleotide anhydrides, several other nucleotides have been isolated and identified from Artemia cysts (2,4), but many other minor UV-absorbing fractions in extracts from cysts have not yet been characterized. The primary purpose of this study was to re-examine the acid-soluble fraction of encysted embryos of Artemia salina with the purpose of

identifying some of the minor UV-absorbing compounds in the nucleotide fraction. Also, it was hoped that this study might provide further insight into the problem of control of nucleotide metabolism and protein biosynthesis in Artemia embryos.

Since their discovery in Artemia embryos, diguanosine nucleotides have been found in eggs or embryos of the water flea, Daphnia magna (5), the fairy shrimp, Eubranchipus vernalis (6), and in the anostracan, Branchipus stagnalis (7), but in no other living system (8). Thus, these unique nucleotides appear to be restricted to organisms from the subclass Branchiopoda of the Crustacea. By comparison, adenosine(5')tetraphospho(5')adenosine [$A(5')p_4(5')A$] has been found in extracts from several cells of mammalian origin (9). In addition to the naturally occurring dinucleoside polyphosphates described above, hybrid dinucleotides of the type guanosine(5')tetraphospho(5')-adenosine [$G(5')p_4(5')A$] and guanosine(5')triphospho(5')-adenosine [$G(5')p_3(5')A$] have been synthesised in vitro from mononucleotides using an E. coli lysyl-tRNA synthetase system (10,11). However, to the knowledge of this author no other naturally occurring dinucleoside polyphosphates containing guanosine only or adenosine and guanosine as described above have been reported, although Van Denbos and Finamore (12) have suggested that the dinucleoside tetraphosphate, $A(5')p_4(5')G$, may serve as an intermediate in the synthesis of ATP in Artemia nauplii. In this thesis

the presence of two new nucleotide anhydrides in Artemia cysts is described. They are guanosine(5')diphospho(5')-guanosine [G(5')p_2(5')G] and guanosine(5')triphospho(5')-adenosine [G(5')p_3(5')A].

The brine shrimp, as a member of the class Crustacea, possesses an exoskeleton containing chitin which is a high molecular weight homopolymer of N-acetylglucosamine residues (13). During the synthesis of chitin N-acetylglucosamine residues are transferred from UDP-N-acetylglucosamine to β(1→4) linkages in the growing chitin chain (14). Hence, any animal that possesses a chitin-containing exoskeleton would be expected to contain UDP-N-acetylglucosamine in its acid-soluble fraction. It is therefore not surprising that UDP-N-acetylglucosamine has been found to be present in some crustacean tissues (15). In addition, this nucleotide has been found in a variety of bacterial and animal tissues (16). In this study UDP-N-acetylglucosamine has been identified as a prominent component of the acid-soluble fraction of dormant cysts of Artemia salina.

In both eukaryotic and prokaryotic systems the pathway of purine biosynthesis from simple precursors (the de novo pathway) has been well-established (17). In all cases the end-product of de novo purine biosynthesis is the ribonucleotide inosinic acid (IMP) which is an important intermediate in the biosynthesis of AMP and GMP. The conversion of IMP to AMP involves the intermediate adenylosuccinic acid (SAMP) which is an N^6-substituted adenosine monophosphate (17). Generally,

SAMP is not present in cells in large amounts since it is rapidly converted to AMP, although it has been found in measurable quantities in extracts of mammalian liver (18,19). However, it has been synthesised *in vitro* from AMP and fumaric acid using an enzyme preparation from yeast (20), and from IMP and aspartate using an enzyme preparation from *E. coli* (21). In this thesis adenylosuccinic acid is described for the first time in acid extracts of *Artemia* cysts, and its role in *de novo* purine biosynthesis is discussed.

In addition to the above the elution characteristics from a Dowex-1-formate column of several other nucleotides from *Artemia* cysts is described.

EXPERIMENTAL PROCEDURE

METHODS

Extraction of Acid-Soluble Nucleotides

The extraction procedure used was a modification of the method of Clegg et al. (1). One hundred grams of the Utah strain of undeveloped cysts of Artemia salina (Longlife Aquarium Products, St. Thomas, Ontario) were hydrated in 500 ml of 0.5 M NaCl at 0° for 16 h. The hydrated cysts were collected on a sintered glass funnel, washed with about 500 ml of cold 1 N $HClO_4$, and ground for 15 min in 40 g portions in a small amount of 1 N $HClO_4$ using a motorized mortar and pestle. The homogenates were combined, then stirred for an additional 30 min in the cold with about 750 ml of ice-cold 1 N $HClO_4$. The total volume of homogenate at the end of the extraction was about 1000 ml. The extract was centrifuged at 15,000 x g for 20 min and the supernatant fluid was filtered through a sintered glass funnel to remove any floating debris. The 15,000 x g sediment was re-extracted with 600 ml of cold 1 N $HClO_4$ by stirring at low speed for 5 min in a Waring blender, then centrifuged as above to obtain the supernatant fluid. All above steps were carried out at 0-4°. The acid-soluble extracts were pooled and applied to a charcoal column (2 x 20 cm) to deacidify the extracts and remove unwanted embryo constituents as described previously (1). The column was washed with about 1500 ml of distilled water then with approximately 1000 ml of a mixture

of ethanol, concentrated NH_4OH and water (2/1/2 v/v/v). The charcoal eluant, which contained mostly acid-soluble nucleic acid constituents, was concentrated under reduced pressure in a rotary evaporator to remove the ethanol and ammonia, then stored at -20° until needed. The quantity of UV-absorbing material after each step above was determined using a Beckman Spectrophotometer (Model DB).

Fractionation of Acid-Soluble Nucleotides on Anion-Exchange Columns

The charcoal-adsorbable material from the acid-soluble extract was fractionated on a Dowex-1-formate column (2.5 x 38 cm) as described previously (4) using the formate-formic acid procedure of Hurlbert et al. (22). Prior to use the resin (AG 1-X8, Cl^-, Bio-Rad Laboratories, Richmond, Ca.) was washed sequentially with 1 litre of 1 N HCl, water until neutral, 4 N HCOOH containing 1 M $HCOONH_4$ until the wash was chloride-free (approximately 2 litres), and finally with water until neutral. The latter two washes were carried out on the column. The charcoal-adsorbable material was adjusted to approximately 50 ml with water, then applied to the column. The column was washed with about 300 ml of water to remove the non-adsorbable material, then eluted with a series of five gradients beginning with the addition of 1100 ml of 1 N HCOOH dropwise to 1000 ml of distilled water in a mixing chamber. In subsequent steps the following reagents were added dropwise to the mixing flask: 4 N HCOOH, 850 ml; 4 N

HCOOH containing 0.2 M $HCOONH_4$,1450 ml; 4 N HCOOH containing 0.4 M $HCOONH_4$, 800 ml; and 4 N HCOOH containing 1.0 M $HCOONH_4$, 2100 ml. Column fractions of about 15 ml were collected and each was analyzed at 260 and 280 nm using a Beckman Spectrophotometer (Model DB). The contents of each UV-absorbing fraction were pooled and desalted on a charcoal column (1 x 6 cm) using the procedure outlined above. Each column peak which appeared to be heterogeneous by paper or thin layer chromatography was purified further by chromatography on DEAE-cellulose (Whatman DE-11 or DE-23, bicarbonate form) columns using linear gradients of NH_4HCO_3, pH 8.6, according to the procedure of Warner and Finamore (2). The UV-absorbing fractions from the DEAE-cellulose columns were concentrated and desalted by flash evaporation, and in some cases the fraction was percolated through a small Dowex-50W-hydrogen column (1 x 3 cm, AG 50W-X8, Bio-Rad Laboratories, Richmond, Ca.). All purified fractions were stored in aqueous solution at -20° prior to analysis.

Ascending paper chromatography of column fractions was carried out in order to test for homogeneity. Fifty µl of a solution containing 1-2 A_{260} units were applied to Whatman 1 paper sheets (20 x 56 cm) and air dried. The chromatograms were developed in a solvent consisting of 0.1 M sodium phosphate, pH 6.8/$(NH_4)_2SO_4$/n-propanol (100/60/2 v/w/v) (23) for approximately 18 h. The paper was removed from the solvent, air dried, and the UV-absorbing areas detected using a mineral light.

Analytical Procedures

Total phosphate and labile phosphate (where present) were determined by the method of Griswold et al. (24), which is a modification of the procedure of Fiske and Subbarow (25). Total phosphate was determined after digestion of the sample with concentrated H_2SO_4. Labile phosphate, which refers to the phosphate released as orthophosphate after treatment of the phosphorylated compound with 1 N H_2SO_4 at 100° for 17 min, was determined by omitting the acid digestion step. The addition of an acidic solution of ammonium molybdate and a reducing agent (1-amino-2-naphthol-4-sulfonic acid) promoted the development of a phosphomolybdate complex (blue color) which was estimated by absorbance at 820 nm in a Beckman Spectrophotometer (Model DB).

Ribose analyses were performed using the orcinol colorimetric procedure of Mejbaum (26) modified according to the procedure of Volkin and Cohn (27). The sample was treated with an acidic solution of orcinol and $FeNH_4(SO_4)_2$ for 30 min at 100° and the absorbance was read at 660 nm in a Beckman Spectrophotometer (Model DB).

Nitrogen analyses were carried out by direct Nesslerization after digestion with concentrated H_2SO_4 (28). One ml of the sample containing 1-3 A_{260} units was heated with 0.2 ml of 18 N H_2SO_4 for 15 min in a digestion flask. Nessler's reagent was added, and the color was read at 450 nm in a Beckman Spectrophotometer (Model DB).

Periodate oxidations and assays were carried out using the procedure of Khym and Cohn (29). Approximately 10 A_{260} units of the sample were treated with 0.04 N $NaIO_4$ for 15 min at room temperature. The IO_3^- generated in the reaction was estimated by passing the reaction mixture through an AG-1-acetate (1 x 2.5 cm) column to absorb both the oxidized nucleotide as well as the IO_3^-. The IO_3^- was then eluted from the column with 0.1 M NH_4Cl and the amount of IO_3^- eluted determined by absorbance at 232 nm using a molar extinction coefficient of 900 (29).

Colorimetric assays for N-acetylhexosamines were performed using the carbonate method of Aminoff et al. (30) based on the original procedure of Morgan and Elson (31). Approximately 1 μmole of the N-acetylhexosamine was treated with 0.025 M Na_2CO_3 for 5 min at 100°, then an equal volume of a reagent containing 2% p-dimethylaminobenzaldehyde in glacial acetic acid was added. The solution was diluted a further 1 in 5 with glacial acetic acid and the color allowed to develop for 45 min. The absorbance was measured at 550 nm in a Beckman Spectrophotometer (Model DB) and compared to commercially available N-acetylglucosamine.

Ninhydrin oxidation of hexosamines to pentoses was performed using the original procedure of Gardell et al. (32) modified according to the procedure of Pontis (33). A solution of hexosamine was treated with an equal volume of 0.48% ninhydrin in 0.1 M sodium citrate buffer, pH 4.7, at 100° for 30 min. The cooled solution was treated successively with a small amount of AG 50W-X8 (H^+ form) cation exchange

resin and AG 1-X8 (Cl^- form) anion exchange resin (Bio-Rad Laboratories, Richmond, Ca.), and then concentrated in a rotary evaporator. The sample was applied to a sheet of Whatman 1 filter paper (20 x 56 cm) and the chromatogram developed for 42 h in the downward direction with butanol/ethanol/water (4/1/5 v/v/v) (15). The paper was air dried and the spots visualized by spraying with 1% aniline hydrogen phthalate in butanol (34) followed by heating for 5 min at 100° to develop the color. In order to determine $R_{glucose}$ values glucose was run simultaneously and visualized by spraying with 1% p-anisidine in butanol followed by heating for 5 min at 100° (34).

Paper chromatography of hexosamines and N-acetylhexosamines was performed in either an ascending or a descending manner on Whatman 1 filter paper (20 x 56 cm). After sample application the chromatograms were developed in one of the following solvents: butanol/ethanol/water (4/1/5 v/v/v) (15); butanol/pyridine/water (6/4/3 v/v/v) (33); butanol/glacial acetic acid/water (2/1/1 v/v/v) (35), and then allowed to airdry. Ascending chromatograms were developed for about 30 h and descending chromatograms were developed for 40-90 h, allowing the solvent to drip off the lower edge of the paper, to resolve compounds with similar chromatographic mobilities. Hexosamines were visualized by spraying with ninhydrin (Sigma Chemical Company) followed by heating for 5 min at 100°. N-acetylhexosamines were visualized by spraying with a reagent consisting of 1 g of p-dimethylaminobenzaldehyde in 30 ml of

ethanol, 30 ml of concentrated HCl, and 180 ml of butanol (36) followed by heating at 100° for 5 min. $R_{glucose}$ values were obtained as described above.

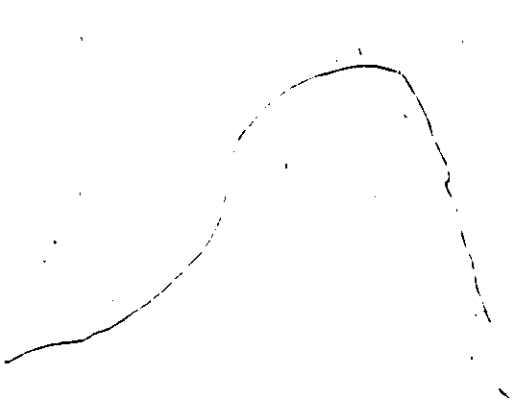
The nitrogenous base constituents were identified by their ultraviolet absorption spectra before and after acid hydrolysis using a Perkin-Elmer Coleman Spectrophotometer (Model 575), and by their chromatographic behaviour on thin layers of PEI-cellulose. Acid hydrolysis of the unknown compounds was carried out with 1 N HCl for 10 min at 100°. The compounds or their base constituents were analyzed by thin layer chromatography on sheets of PEI-cellulose (Brinkmann Instruments, Inc.) that had been washed with water and dried before use. Three different solvent systems were used: (a) 1.4 M LiCl (37); (b) 1.5 M KH_2PO_4 (38); (c) 2.0 N HCOOH/0.5 M LiCl (1/1 v/v) (39). The plates were developed in the solvent for 2-3 h, dried, then analyzed for UV absorbance using a mineral light compared to standard compounds run on the same plate.

The presence of monoesterified phosphate groups in the unknown compounds was determined by measuring the change (if any) in migration rate of the purified compounds on thin layers of PEI-cellulose after treatment with bacterial alkaline phosphatase (BAP). For these analyses, 1-5 units (A_{260}) of the unknown compound were dissolved in a solution containing 29 mM Tris-HCl, pH 8.0, 2.9 mM $MgCl_2$, and 25 µg of BAP in a final volume of 0.2 ml. The reaction mixture was incubated for 1 h at 30° then analyzed by thin layer

chromatography on PEI-cellulose using the solvent systems described above. In an attempt to determine the phosphate linkages in the BAP resistant compounds 2-10 units (A_{260}) were incubated with spleen phosphodiesterase (SPD) (0.05-0.075 units) in 0.2 ml of a buffer containing 25 mM Tris-HCl, pH 7.5, and 2.5 mM $MgCl_2$. After 1 h at 30° the mixture was analyzed by thin layer chromatography on PEI-cellulose as above. In similar experiments 2-13 units (A_{260}) of the unknown compounds were treated with snake venom phosphodiesterase (SVPD) (13-100 µg) in 1.0 ml of a buffer composed of 68 mM Tris-HCl, pH 8.9, and 58 mM $MgCl_2$. The reaction mixtures were incubated for 10 min at 30°, then the reaction terminated by the addition of 0.5 ml of 1 N HCl. The hydrolyzates were neutralized with 1 M NH_4HCO_3, pH 8.6, then chromatographed on columns of DEAE-cellulose (1 x 15 cm) as described above (2). The column fractions were analyzed by spectrophotometry and chromatography on PEI-cellulose compared to standard compounds as described above.

MATERIALS

Standard nucleotides, nucleosides, bases and enzymes were purchased from either P-L Laboratories, Inc. (Milwaukee, Wisconsin), Worthington Biochemical Corporation (Freehold, N.J.) or Sigma Chemical Company (St. Louis, Mo.). Precoated plastic sheets of Polygram Cel 300 PEI were obtained from Brinkmann Instruments, Inc. (Toronto, Ontario). All other chemicals were of reagent grade.



RESULTS

Column Chromatography of Acid-Soluble Extract from Cysts of Artemia salina

Eighteen distinct UV-absorbing fractions from the acid extract of Artemia cysts have been resolved on a column of Dowex-1-formate using a formate-formic acid elution system. The results are shown in Fig. 1. This elution profile is similar to that obtained prevously from the acid-soluble fraction of Artemia salina (4). Several of these fractions have been identified previously, and the identity of some of them has been established (2,4). The identity of the major components is given in the legend to Fig. 1.

Many of the column fractions from Dowex-1-formate were found to be heterogeneous by paper or thin layer chromatography. Therefore, these fractions were purified further by chromatography on columns of DEAE-cellulose (bicarbonate form). In some cases further purification on a small column of AG-50 (H^+ form) was considered necessary to remove traces of heavy metals before the enzyme studies, but in general fractions homogeneous by chromatographic criteria were not subjected to further purification after chromatography on DEAE-cellulose.

Identification of Fraction 10 from Dowex-1-formate

The results shown in Fig. 2 were obtained when fraction 10 from the Dowex-1-formate column was chromatographed on a column of DEAE-cellulose (bicarbonate form).

FIGURE 1

Fractionation of UV-absorbing components in the acid-soluble extract from dormant embryos of Artemia salina on Dowex-1-formate.

The acid-soluble fraction from 100 g of dry cysts was chromatographed on a 2.5 x 38 cm column of Dowex-1-formate as described under Experimental Procedure. The following eluents were added at points A to E as indicated: A, 1 N HCOOH; B, 4 N HCOOH; C, 4 N HCOOH containing 0.2 M $HCOONH_4$; D, 4 N HCOOH containing 0.4 M $HCOONH_4$; E, 4 N HCOOH containing 1.0 M $HCOONH_4$. Column fractions of about 15 ml were collected and analyzed for absorbance at 260 and 280 nm. The identity of some of the fractions has been determined previously (4). The primary components in these fractions are as follows: 1, guanosine; 2, CMP; 4, AMP; 8, GMP; 10, UMP; 11, ADP; 15, GDP; 16, ATP and G(5')p_3(5')G; 17, GTP and G(5')p_4(5')G. The identity of fractions 3,5,6,7,9,12, 13 and 14 has not been previously established.

Figure 1

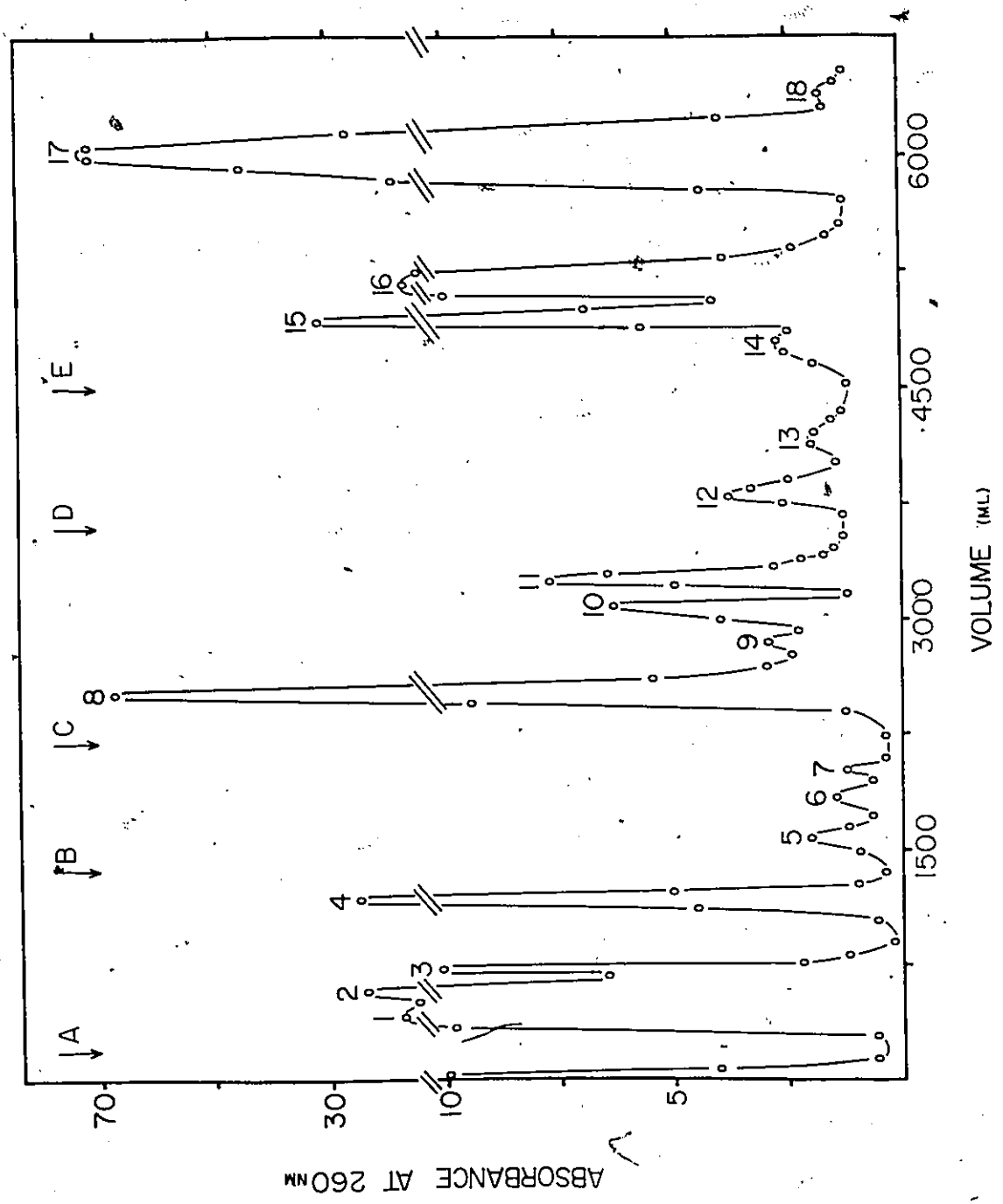
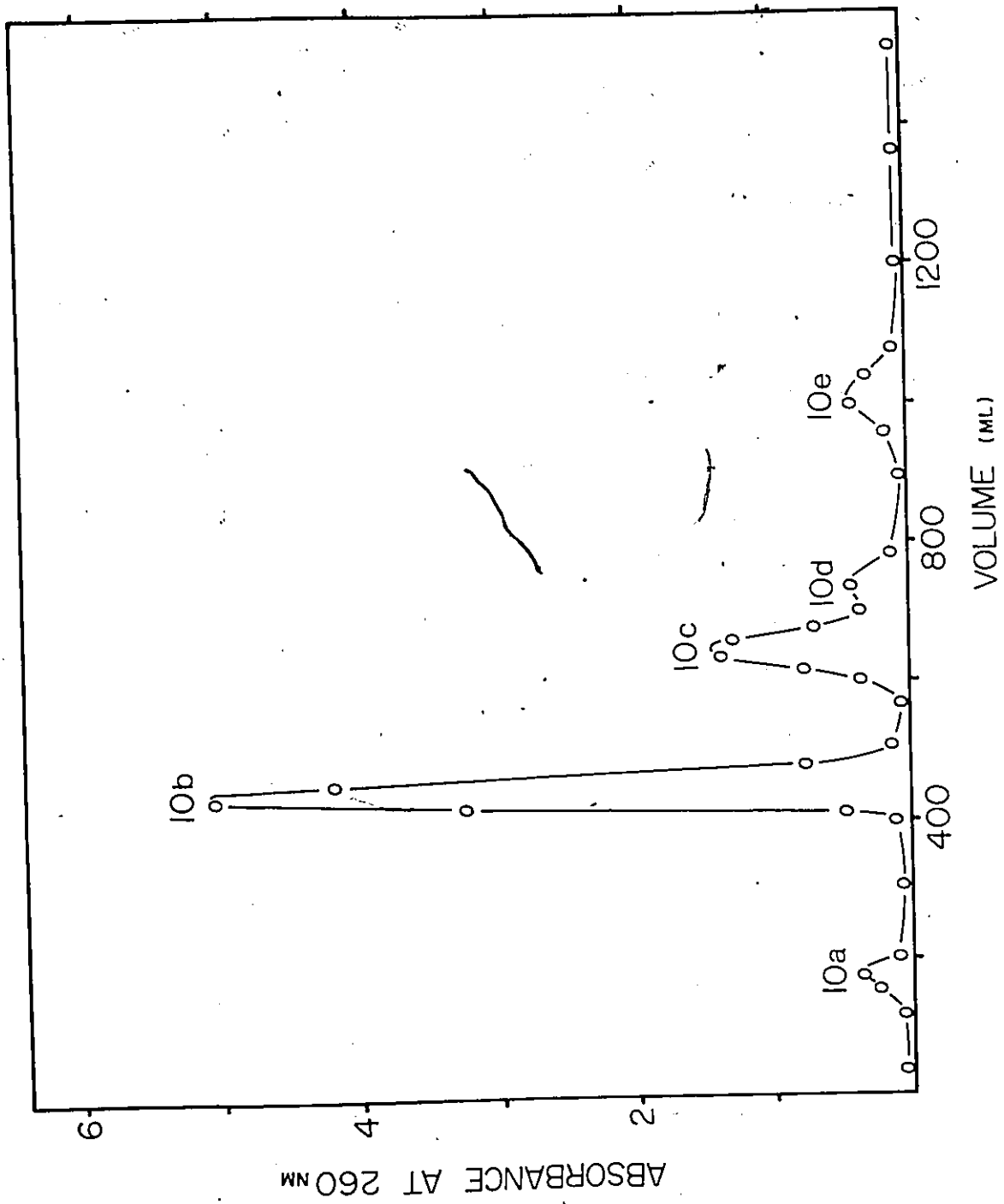

ABSORBANCE AT 260NM
70
30
10
5
VOLUME (ML)
1500
3000
4500
6000
A
B
C
D
E
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

FIGURE 2

Fractionation of peak 10 from Dowex-1-formate on DEAE-cellulose.

Peak 10 from the Dowex-1-formate column shown in Fig.1 was chromatographed on a 1.5 x 26 cm column of DEAE-cellulose using a 1500 ml linear gradient of NH_4HCO_3, pH 8.6 from 0.002 M to 0.20 M. Each column fraction was analyzed for absorbance at 260 nm. Fractions 10b and 10c were saved for additional study, but fractions 10a, 10d and 10e were not identified in this study.

Figure 2


ABSORBANCE AT 260 NM
6
4
2
400
800
1200
VOLUME (ML)
10a
10b
10c
10d
10e

The major peak (10b), which was found to contain one phosphate group per base, was identified by means of its spectral properties (see Table 1) and chromatographic mobility on Whatman 1 paper to be identical to UMP. This finding confirmed results obtained previously (2,4).

The second largest UV-absorbing peak from the DEAE-cellulose column shown in Fig. 2 was peak 10c. This fraction was found to have the ultraviolet absorption properties presented in Table 2. The spectral ratios of 10c correspond very closely to those of IMP, and when absorption spectra of both compounds were compared they were found to be superimposible. Furthermore, phosphate and ribose analyses indicated that 10c contained one phosphate group and one ribose per base (data not shown). The supposition that 10c is IMP was confirmed by paper and thin layer chromatography as shown in Table 3.

Identification of Fraction 11 from Dowex-1-formate

Chromatography of fraction 11 from Dowex-1-formate on DEAE-cellulose (bicarbonate form) yielded five distinct UV-absorbing peaks as shown in Fig. 3. The identity of fractions 11c and 11d are described in this section. Initially, the ultraviolet absorption characteristics of fraction 11c were examined and the data are presented in Table 4. These data suggest that 11c is an adenosine-containing compound, and the results shown in Table 5 for ribose and phosphate analyses are consistent with the view that fraction 11c is

Table 1. Ultraviolet absorption characteristics of fraction 10b compared to UMP*.

pH	Absorbancy Ratio			Wavelength (nm)	
	$A_{250}:A_{260}$	$A_{280}:A_{260}$	$A_{290}:A_{260}$	Maximum	Minimum
Acid (adjusted to 0.01 N HCl)	0.77 (0.76)	0.38 (0.40)	0.07 (0.06)	262 (262-3)	230 (233)
Neutral	0.75 (0.70)	0.40 (0.39)	0.08 (0.03)	262 (263)	230 (232-3)
Basic (adjusted to 0.01 N NaOH)	0.80 (0.73)	0.35 (0.41)	0.08 (0.05)	262 (263)	238 (235)

* Values for UMP are shown in parentheses.

Table 2. Ultraviolet absorption characteristics of fraction 10c compared to IMP*.

pH	Absorbancy Ratio			Wavelength (nm)	
	$A_{250}:A_{260}$	$A_{280}:A_{260}$	$A_{290}:A_{260}$	Maximum	Minimum
Acid (adjusted to 0.01 N HCl)	1.44 (1.39)	0.28 (0.26)	0.10 (0.10)	250 (250)	226 (225)
Neutral	1.57 (1.57)	0.30 (0.28)	0.08 (0.07)	250 (250)	225 (225)
Basic (adjusted to 0.01 N NaOH)	1.07 (1.13)	0.23 (0.23)	0.05 (0.05)	259 (258)	228 (229)

* Values for IMP are shown in parentheses.

Table 3. Chromatography of fraction 10c on Whatman 1 paper and thin layers of PEI-cellulose.

Compound	R_f Value*		
	PEI-cellulose		Whatman 1
	Solvent 1	Solvent 2	Solvent 3
Hypoxanthine	0.49	0.50	-
IMP	0.70	0.85	0.54
10c	0.70	0.85	0.52
10c after Acid Hydrolysis†	0.49	0.50	-

* Solvent 1, 1.4 M LiCl; solvent 2, 1.5 M KH_2PO_4; solvent 3, 0.1 M sodium phosphate, pH 6.8/$(NH_4)_2SO_4$/n-propanol (100/60/2 v/w/v).

† Acid hydrolysis was carried out by treatment with 1.0 N HCl for 10 min at 100°.

FIGURE 3

Fractionation of peak 11 from Dowex-1-formate on DEAE-cellulose.

Peak 11 from the Dowex-1-formate column shown in Fig. 1 was chromatographed on a 1.5 x 23 cm column of DEAE-cellulose using a 1500 ml linear gradient of NH_4HCO_3, pH 8.6 from 0.002 M to 0.20 M. Each column fraction was analyzed for absorbance at 260 nm. Fractions 11c and 11d were saved for further examination, but fractions 11a, 11b and 11e were not identified in this study.

Figure 3

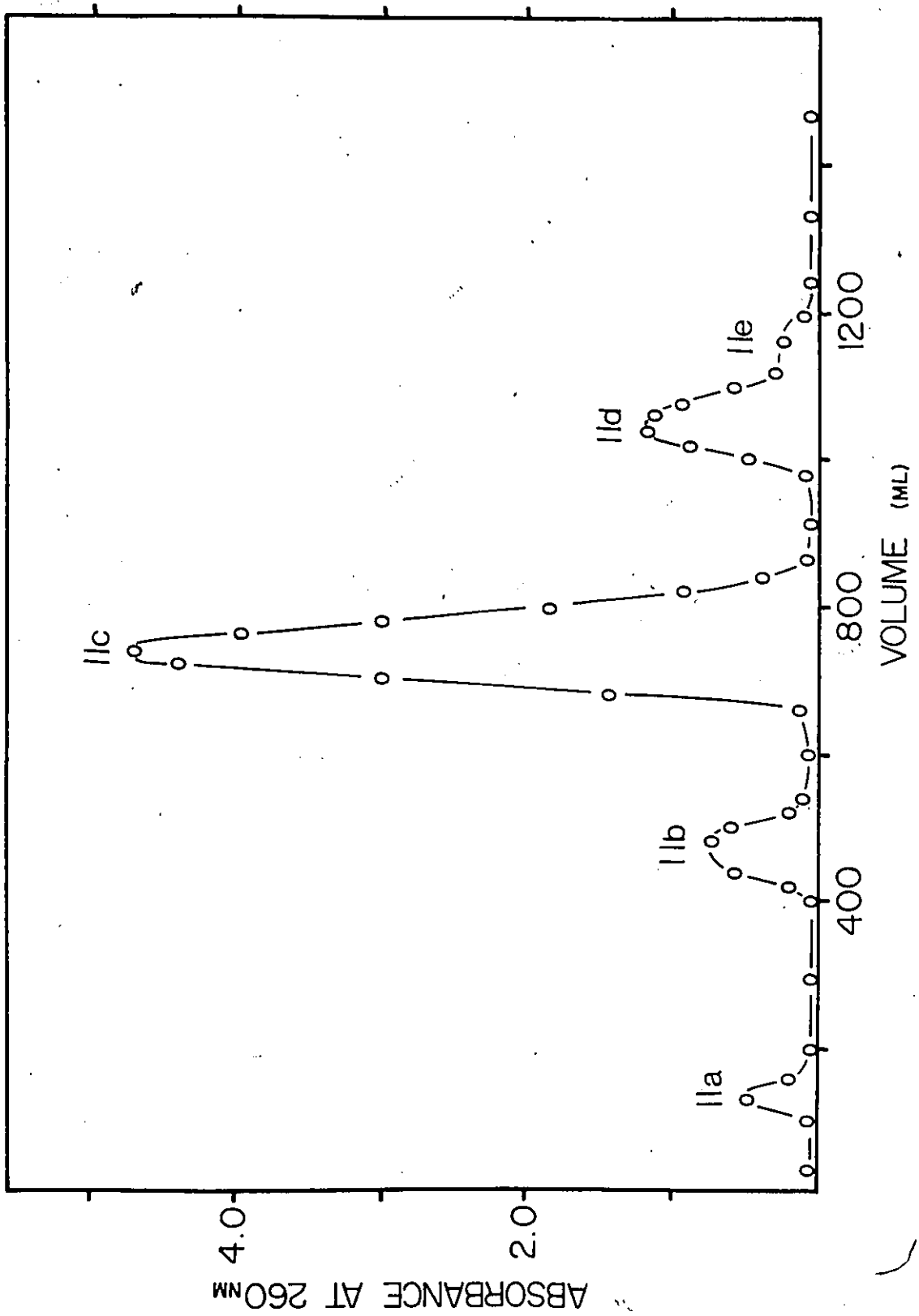

ABSORBANCE AT 260NM
4.0
2.0
400
800
1200
VOLUME (ML)
IIa
IIb
IIc
IId
IIe

Table 4. Ultraviolet absorption characteristics of fraction 11c.

pH	Absorbancy Ratio			Wavelength (nm)	
	$A_{250}:A_{260}$	$A_{280}:A_{260}$	$A_{290}:A_{260}$	Maximum	Minimum
Acid (adjusted to 0.01 N HCl)	0.85	0.25	0.07	257	228
Neutral	0.80	0.17	0.02	259	226
Basic (adjusted to 0.01 N NaOH)	0.80	0.18	0.04	259	229

Table 5. Partial chemical analysis of fraction 11c.

Phosphate/Base*		Ribose/Base*
Total	Labile	
2.07	1.00	1.16

* Based on the spectral properties of fraction 11c the molar extinction coefficient at 260 nm of 15400 for adenosine was used in determining these ratios (23).

adenosine diphosphate. The chromatographic data presented in Table 6 support this proposition.

When the ultraviolet absorption characteristics of peak 11d were determined at acid, basic and neutral pH values and compared to those for G(5')p_2(5')G the results shown in Table 7 were obtained. These data, together with UV absorbance spectra of acid hydrolyzed 11d (1.0 N HCl, 10 min at 100°, data not shown) indicate that guanine is the only nitrogenous base in this fraction. When acid hydrolyzed fraction 11d was chromatographed on a thin layer sheet of PEI-cellulose using a LiCl/HCOOH solvent system, only one UV-absorbing spot was detected and its mobility is shown in Table 8. These data indicate that guanine is the sole UV-absorbing product obtained from the acid hydrolysis of column fraction 11d. Column fraction 11d was also analyzed for phosphate and ribose, and the results of these analyses are shown in Table 9. These results are consistent with those expected for either GMP, cGMP or G(5')p_2(5')G.

In an effort to elucidate the chemical structure of column fraction 11d samples of this fraction were treated with either BAP, SPD or SVPD then chromatographed on a thin layer sheet of PEI-cellulose using a LiCl/HCOOH solvent system. The results shown in Table 10 indicate that the chromatographic mobility of fraction 11d is not altered by BAP compared to non-treated samples, and suggest that the phosphate group(s) in this compound is(are) diesterified as in diguanosine polyphosphates (4,41). Similarly, the

Table 6. Chromatography of fraction 11c compared to ADP*.

Compound	R_f Value		
	PEI-cellulose		Whatman 1
	Solvent 1	Solvent 2	Solvent 3
ADP	0.57	0.71	0.30
11c	0.57	0.70	0.29

* Thin layer chromatography on PEI-cellulose and ascending paper chromatography on Whatman 1 paper sheets was performed as described in Experimental Procedure.
Solvent 1, 1.4 M LiCl; solvent 2, 1.5 M KH_2PO_4; solvent 3, 0.1 M sodium phosphate, pH 6.8/$(NH_4)_2SO_4$/n-propanol (100/60/2 v/w/v).

Table 7. Ultraviolet absorption characteristics of fraction 11d compared to G(5')p_2(5')G*.

pH	Absorbancy Ratio			Wavelength (nm)	
	$A_{250}:A_{260}$	$A_{280}:A_{260}$	$A_{290}:A_{260}$	Maximum	Minimum
Acid (adjusted to 0.01 N HCl)	0.95 (0.94)	0.61 (0.64)	0.42 (0.44)	256 (256)	228-9(230)
Neutral	1.08 (1.07)	0.65 (0.65)	0.36 (0.33)	252 (252-3)	225-6(226)
Basic (adjusted to 0.01 N NaOH)	1.02 (1.07)	0.63 (0.65)	0.26 (0.32)	256 (254)	230 (228)

* Values for G(5')p_2(5')G are shown in parentheses.

Table 8. Chromatography of acid hydrolyzed fraction 11d on PEI-cellulose*.

Compound	R_f Value
Guanine	0.69
7-Methyl guanine	0.65
11d	0.27
11d after acid hydrolysis	0.70

* About 2 A_{260} units of fraction 11d were treated with 1 N HCl for 10 min at 100°. After cooling, 25 µl of the sample were applied to a plastic coated sheet of PEI-cellulose that had been washed with water prior to use. Standard guanine and 7-methyl guanine as well as untreated fraction 11d were applied to the PEI-cellulose sheet and air-dried. The plate was developed for 2 h using a solvent consisting of 0.5 M LiCl/2.0 N HCOOH (1/1 v/v).

Table 9. Partial chemical analysis of fraction 11d.

Phosphate/Base*		Ribose/Base*
Total	Labile	
1.10	0	1.10

* Based on the spectral properties of fraction 11d, a molar extinction coefficient of 23400 at 260 nm was used in determining these ratios based on the molar extinction coefficient of 11700 for GMP (23). However Michelson has observed a hypochromic effect of 12-25% for $A(5')p_2(5')A$ (40); therefore the above extinction coefficient may be a conservative estimation.

Table 10. Thin layer chromatography on PEI-cellulose of fraction 11d after incubation with BAP, SPD and SVPD.

Compound	R_f Value*		
	Solvent 1	Solvent 2	Solvent 3
GMP	0.43	0.41	0.56
cGMP	0.39	0.51	0.39
11d	0.27	0.44	0.0
11d, after treatment with BAP†	0.27	-	-
11d, after treatment with SPD†	0.26	-	-
11d, after treatment with SVPD†	0.43	0.43	0.56

* Solvent 1, 0.5 M LiCl/2.0 N HCOOH (1/1 v/v); solvent 2, 1.4 M LiCl; solvent 3, 1.5 M KH_2PO_4.

† Treatment with BAP, SPD and SVPD was performed as described under Experimental Procedure. The products of SVPD hydrolysis were passed through a DEAE-cellulose column as described under Experimental Procedure prior to analysis on thin layers of PEI-cellulose.

chromatographic mobility of fraction 11d was not altered by SPD treatment. Since SPD will only hydrolyze dinucleoside phosphates containing a free 5' hydroxyl group, it was concluded that 11d contains a substituted 5' hydroxyl group.

In contrast to the above results, fraction 11d was found to be sensitive to hydrolysis by SVPD. When the product(s) of SVPD treatment were chromatographed on a column of DEAE-cellulose the results shown in Fig. 4 were obtained. Except for a slight shoulder only one UV-absorbing product was obtained upon hydrolysis of 11d by SVPD, and this product has spectral (data not shown) and chromatographic properties identical to GMP (see Table 10). Although 11d is identical chemically to GMP and cGMP, the enzyme and chromatographic data are not consistent with that expected for either GMP or cGMP (see Table 10). However, these results are consistent with the hypothesis that 11d is G(5')p_2(5')G of the structure illustrated in Fig. 5. Finally, the identity of 11d as G(5')p_2(5')G was confirmed by chromatography of 11d compared to commercially available G(5')p_2(5')G on thin layers of PEI-cellulose using three different solvent systems. The results of these experiments are presented in Table 11 and support the conclusion that fraction 11d is identical to G(5')p_2(5')G.

Identification of Fraction 12 from Dowex-1-formate

When fraction 12 from Dowex-1-formate was chromatographed on a column of DEAE-cellulose (bicarbonate

FIGURE 4

Release of GMP from G(5')p_2(5')G (DEAE-cellulose fraction 11d) by SVPD.

Thirteen units (A_{260}) of column fraction 11d, proposed to be G(5')p_2(5')G, were treated with 50 µg of SVPD for 10 min at 30° as described under Experimental Procedure. The reaction mixture was chromatographed on a 1 x 15 cm column of DEAE-cellulose using a 400 ml linear gradient of NH_4HCO_3, pH 8.6, from 0.002 M to 0.20 M. Under these conditions only one UV-absorbing fraction eluted from the column (o——o), and this material was identified as GMP (see Table 10). The elution position of unaltered G(5')p_2(5')G (peak 11d) is shown here for comparison (x——x).

Figure 4

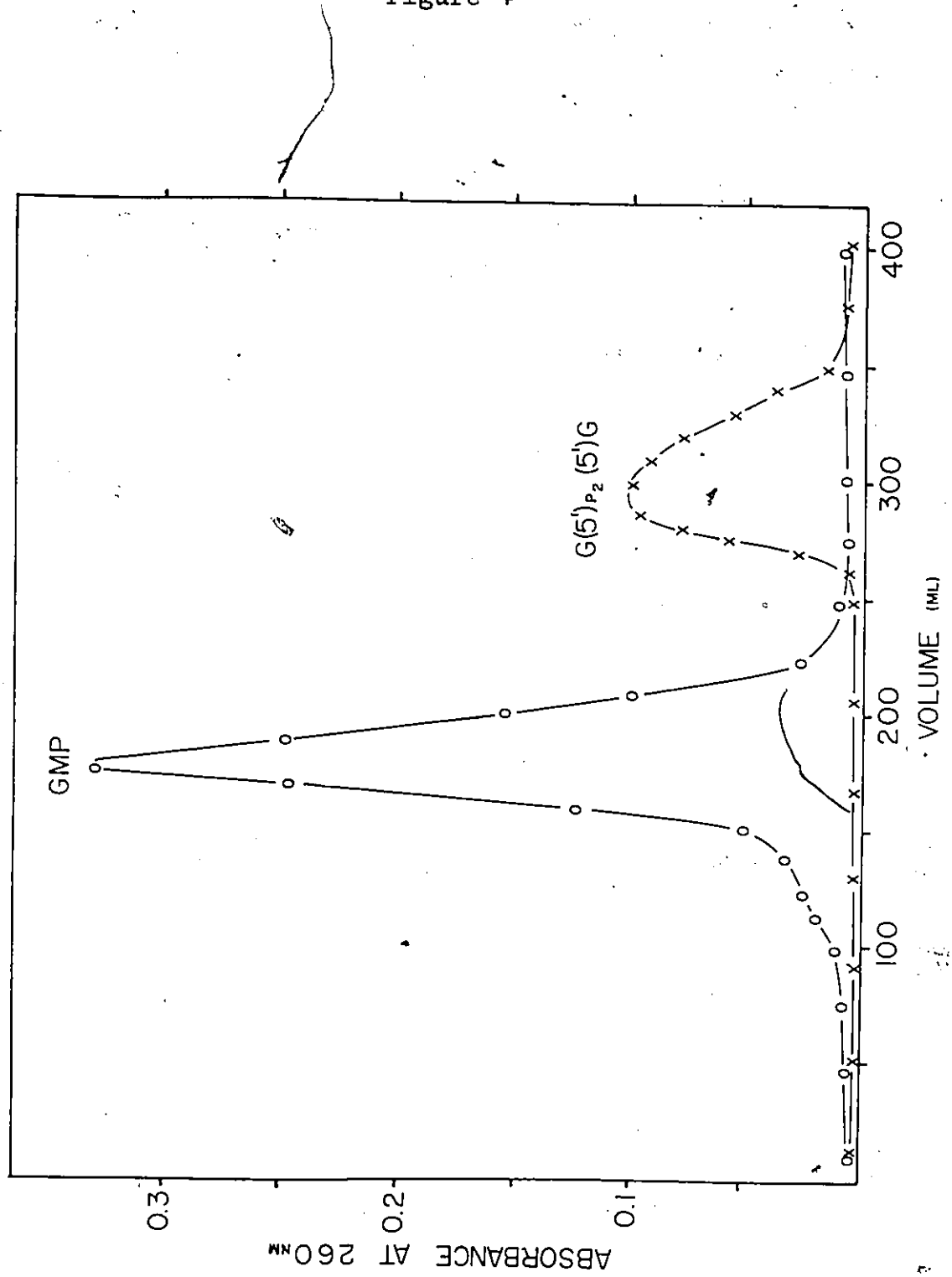

ABSORBANCE AT 260NM
0.3
0.2
0.1
100
200
300
400
VOLUME (ML)
GMP
G(5')P2 (5')G

FIGURE 5

Proposed structure of guanosine(5')diphospho(5')guanosine (DEAE-cellulose fraction 11d).

The arrow indicates the position of hydrolysis by SVPD.

Figure 5

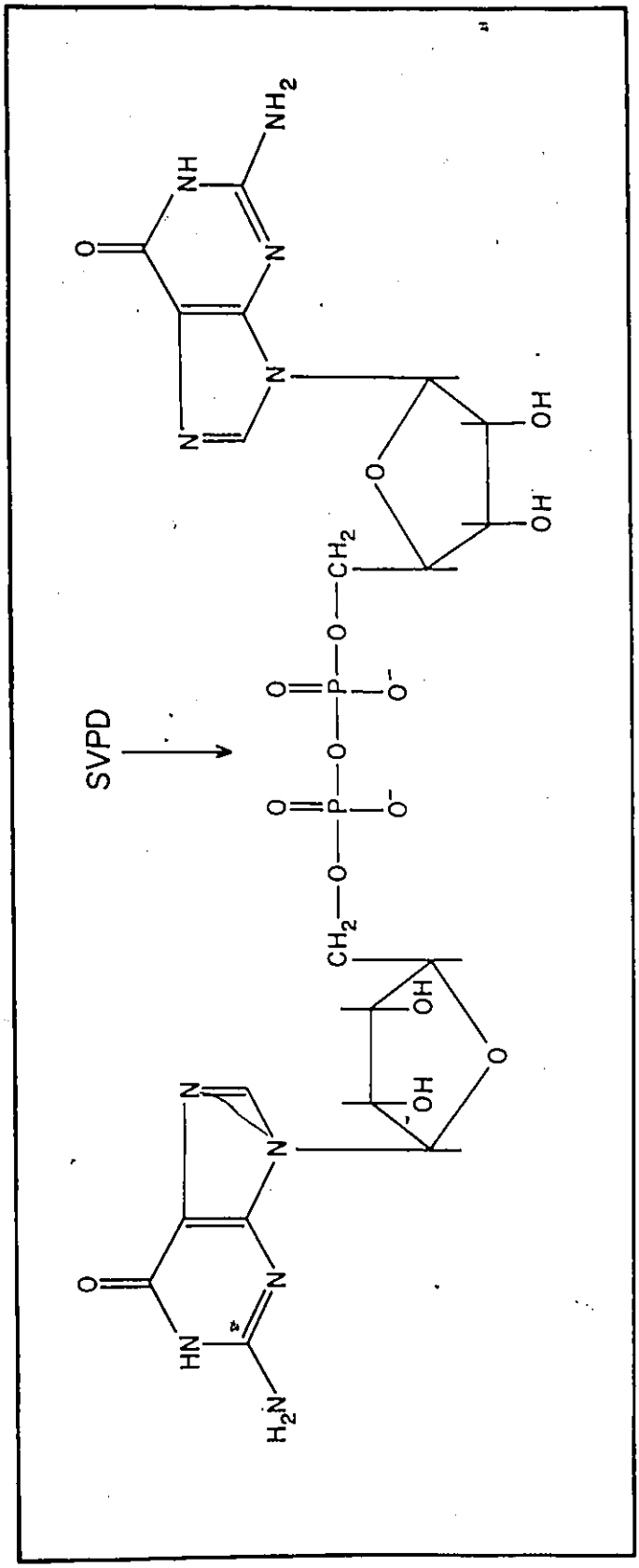


Table 11. Thin layer chromatography on PEI-cellulose of fraction 11d compared to G(5')p_2(5')G.

Compound	R_f Value*		
	Solvent 1	Solvent 2	Solvent 3
11d	0.22	0.46†	0.0
G(5')p_2(5')G	0.23	0.46†	0.0

* Solvent 1, 0.5 M LiCl/2.0 N HCOOH (1/1 v/v); solvent 2, 1.4 M LiCl; solvent 3, 1.5 M KH_2PO_4.

† Considerable trailing was observed using this solvent.

form) the results shown in Fig. 6 were obtained. Two large and three small peaks were obtained from fraction 12, and the two major peaks were analyzed further. Fraction 12b was found to have ultraviolet absorption properties identical to those for uridine-containing compounds, and the ultraviolet absorption spectra of fraction 12b and UMP were found to be superimposible. Selected UV absorption data are shown in Table 12. Phosphate analysis indicated that 12b is a diphosphate with one of the phosphate groups labile to hydrolysis by dilute acid (see Table 13). However, column fraction 12b was found to be insensitive to hydrolysis by BAP as determined by its mobility on PEI-cellulose (see Table 14). Thus it was concluded that 12b consists of uridine diphosphate with a diesterified terminal phosphate. Since periodate oxidation of 12b consumed 2 moles of periodate per mole of uridine (see Table 13), it appeared likely that 12b was UDP esterified to a carbohydrate component. This hypothesis was strengthened by the findings presented in Table 15 which show that treatment of 12b with 0.01 N HCl for 5 min at 100° produced a UV-absorbing product with a chromatographic mobility similar to UDP, whereas treatment with 0.1 N HCl for 30 min at 100° produced a UV-absorbing compound which chromatographed like UMP on PEI-cellulose. Thus it appears that UDP is associated with a non UV-absorbing component in fraction 12b that can be easily removed by mild acid hydrolysis. Therefore an attempt was made to characterize this component.

FIGURE 6

Fractionation of peak 12 from Dowex-1-formate on DEAE-cellulose.

Peak 12 from the Dowex-1-formate column shown in Fig. 1 was chromatographed on a 1 x 30 cm column of DEAE-cellulose using a 1000 ml linear gradient of NH_4HCO_3, pH 8.6 and 0.002 M to 0.20 M. Each column fraction was analyzed for absorbance at 260 nm. Fractions 12b and 12e were saved for further analysis, but fractions 12a, 12c and 12d were not examined further in this study.

Figure 6

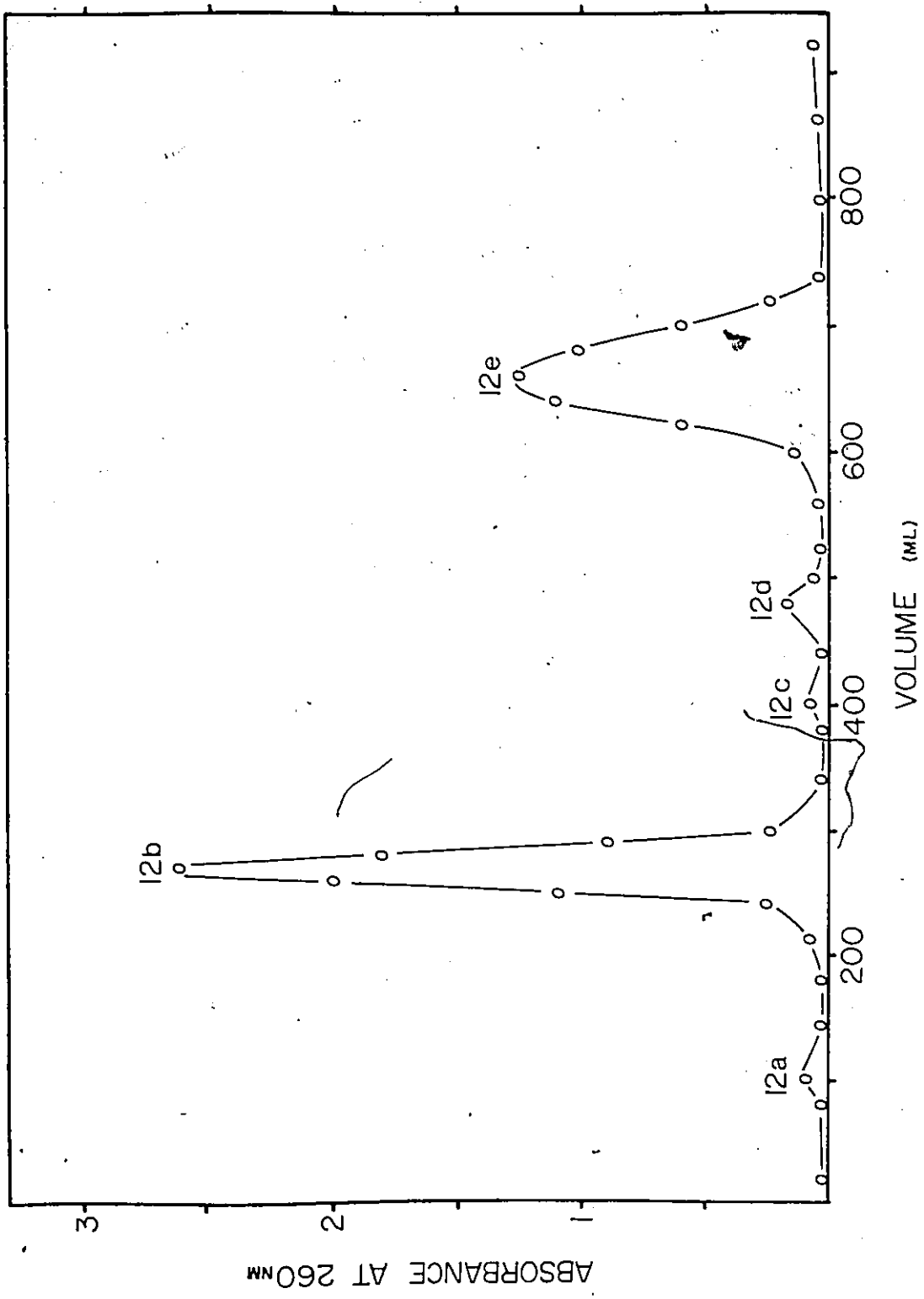

ABSORBANCE AT 260NM
3
2
1
200
400
600
800
VOLUME (ML)
12a
12b
12c
12d
12e

Table 12. Ultraviolet absorption characteristics of fraction 12b compared to UMP*.

pH	Absorbancy Ratio			Wavelength (nm)	
	$A_{250}:A_{260}$	$A_{280}:A_{260}$	$A_{290}:A_{260}$	Maximum	Minimum
Acid (adjusted to 0.01 N HCl)	0.76 (0.76)	0.35 (0.40)	0.04 (0.06)	262 (262-3)	230 (233)
Neutral	0.76 (0.70)	0.36 (0.39)	0.05 (0.03)	262 (263)	229 (232-3)
Basic (adjusted to 0.01 N NaOH)	0.79 (0.73)	0.33 (0.31)	0.05 (0.05)	262 (263)	234 (235)

* Values for UMP are shown in parentheses.

Table 13. Partial chemical analysis of fraction 12b.

Phosphate/Base*		Periodate Consumed /Base*	Nitrogen/Base*
Total	Labile		
1.96	1.01	2.11	3.07

* Based on the spectral properties of fraction 12b the molar extinction coefficient of 9900 at 260 nm for uridine was used in determining these ratios (23).

Table 14. Thin layer chromatography on PEI-cellulose of fraction 12b before and after treatment with BAP*.

Compound	R_f Value†
Uridine	0.91
UMP	0.61
UDP	0.11
12b (Control)	0.38
12b (Treated)	0.40

* Treatment with BAP was carried out as outlined under Experimental Procedure.

† The solvent used was 0.5 M LiCl/2.0 N HCOOH (1/1 v/v).

Table 15. Thin layer chromatography of fraction 12b on PEI-cellulose before and after acid hydrolysis.

Compound	R_f Value*
UMP	0.67
UDP	0.16
12b (Control)	0.33
12b (Treatment 1)	0.17
12b (Treatment 2)	0.67

* The solvent was 0.5 M LiCl/2.0 N HCOOH (1/1 v/v). Treatment 1, 5 min at 100° in 0.01 N HCl; treatment 2, 30 min at 100° in 0.1 N HCl.

After mild hydrolysis (0.01 N HCl for 5 min at 100°), 12b was found to give a positive Morgan and Elson reaction (carbonate method) (30) for N-acetylhexosamines in the ratio of one mole of sugar per mole of uridine. The chromogen produced in this reaction was found to have a spectrum identical to that produced by N-acetylglucosamine in the same reaction. It was concluded, therefore, that 12b consists of a UDP esterified to an N-acetylhexosamine. This hypothesis was strengthened by the results shown in Table 16 which show that mild acid hydrolysis of 12b produces a component that migrates on paper chromatograms with N-acetylglucosamine. The observation that 12b contains 3 nitrogens per uridine (see Table 13) is consistent with the conclusion that N-acetylglucosamine is present in the molecule. When the N-acetylhexosamine from 12b was isolated and subjected to acid hydrolysis under conditions that cause deacetylation of an N-acetyl sugar (2.0 N HCl for 2-3 h at 100°), a compound was produced which chromatographed with glucosamine and gave a positive ninhydrin reaction (see Table 17). The final proof that this substance is an amino sugar was obtained when it was found to be oxidized by ninhydrin to give a compound with chromatographic properties identical to arabinose (see Table 18). The results presented here indicate that 12b possesses an N-acetylglucosamine which is linked to UDP, and support the thesis that 12b is UDP-N-acetylglucosamine as shown in Fig. 7. This view was confirmed by chromatography of 12b with authentic UDP-N-

Table 16. Ascending paper chromatography of the carbohydrate component of fraction 12b*.

Compound	R_f Value†	
	Solvent 1	Solvent 2
N-acetylglucosamine	0.29	0.31
12b (Carbohydrate component)	0.29	0.31

* Approximately 3 μmoles of fraction 12b were treated with 0.01 N HCl for 5 min at 100°. The solution was applied to a column of DEAE-cellulose (1 x 4 cm) and washed with water. The first 20 ml of the water wash (containing the carbohydrate component) was collected and concentrated to 250 μl in a rotary evaporator. Approximately 20 μl of the sample were applied to Whatman 1 paper which had been pretreated with 0.2 M sodium tetraborate and air-dried (42). After development in the appropriate solvent for about 30 h the chromatograms were dried and the spots visualized by spraying them with the Morgan and Elson spray reagent (36) for N-acetylhexosamines as described under Experimental Procedure.

† Solvent 1, butanol/ethanol/water (4/1/5 v/v/v); solvent 2, butanol/pyridine/water (6/4/3 v/v/v).

Table 17. Paper chromatography of the deacetylated carbohydrate component from fraction 12b*.

Compound	R_f Value†			$R_{glucose}$‡
	Solvent 1	Solvent 2	Solvent 3	Solvent 1
Mannosamine	0.14	0.35	-	0.91
Galactosamine	0.13	0.28	-	0.76
Glucosamine	0.16	0.29	0.30	0.85
12b (Deacetylated carbohydrate component)	0.15	0.30	0.29	0.83

* The carbohydrate component from fraction 12b obtained as described in the legend to Table 16 was treated with 2.0 N HCl for 2-3 h at 100° in a sealed tube. These conditions cause N-acetylhexosamines to undergo a deacetylation reaction to produce hexosamines.

† Approximately 20 µl of the sample were applied to Whatman 1 paper and air-dried. After development in the appropriate solvent for about 30 h the chromatograms were dried and the spots visualized by spraying with ninhydrin spray (Sigma Chemical Company). Development was in the ascending manner.

‡ Approximately 20 µl of the sample were applied to Whatman 1 paper and air-dried. The chromatogram was developed in the descending manner for 42 h. Glucose was run simultaneously

and visualized by spraying with a p-anisidine reagent as described under Experimental Procedure.

Solvent 1, butanol/ethanol/water (4/1/5 v/v/v); solvent 2, butanol/pyridine/water (6/4/3 v/v/v); solvent 3, butanol/glacial acetic acid/water (2/1/1 v/v/v).

Table 18. Descending paper chromatography of the carbohydrate component from fraction 12b after deacetylation and ninhydrin oxidation*.

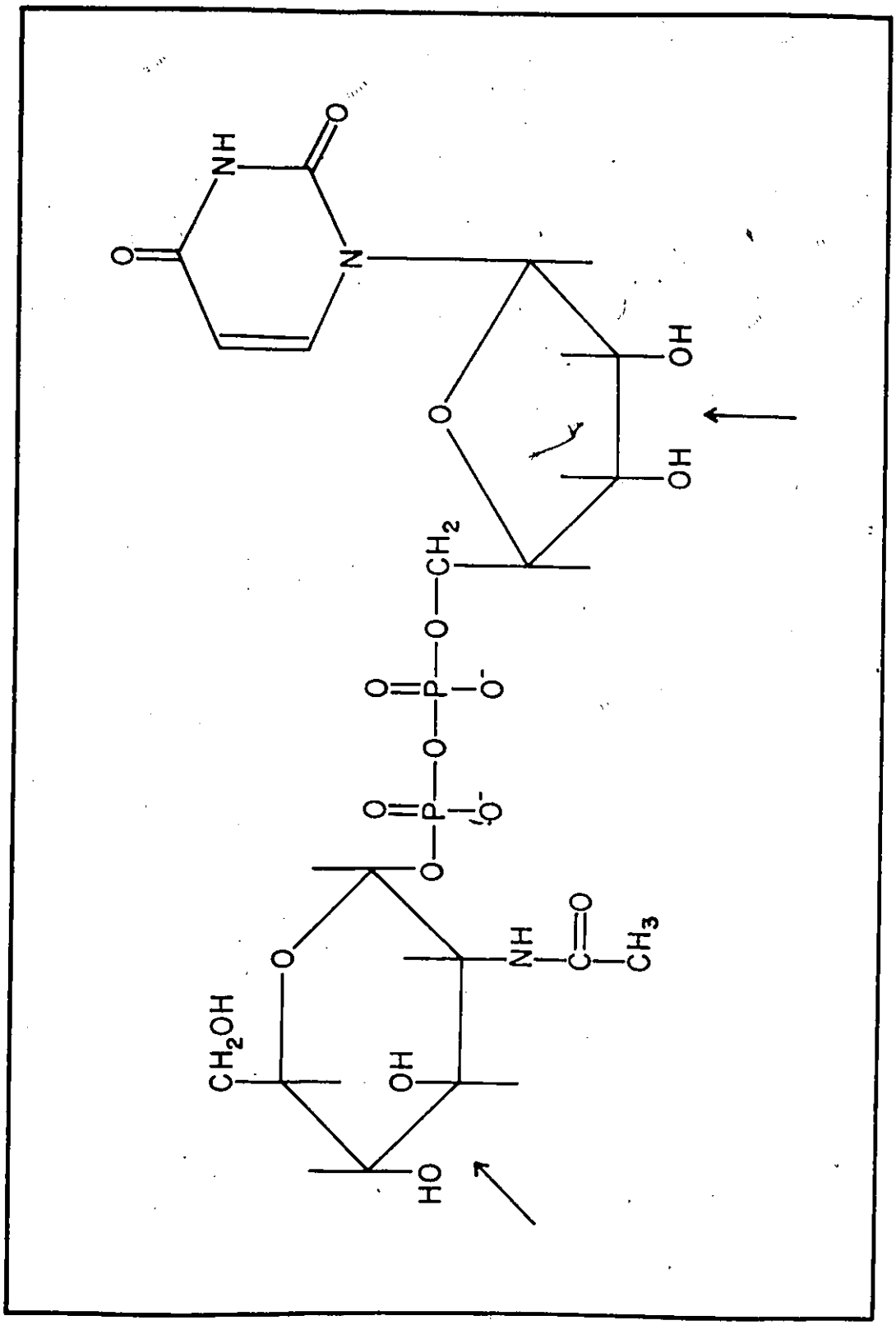
Compound	$R_{glucose}$
Ribose	1.62
Arabinose	1.24
Xylose	1.39
Lyxose	1.50
12b (Deacetylated and ninhydrin oxidized carbohydrate component)	1.23

* The deacetylated carbohydrate component from fraction 12b (obtained as described in the legend to Table 17) was oxidized to a pentose by ninhydrin as described under Experimental Procedure. Approximately 20 µl of the sample were applied to Whatman 1 paper and air-dried. The chromatogram was developed in the downward direction for 42 h, and the spots were visualized by spraying with an aniline phthalate reagent as described in Experimental Procedure. Glucose was run simultaneously and visualized as described in the legend to Table 17. The solvent was butanol/ethanol/water (4/1/5 v/v/v).

FIGURE 7

Structure of UDP-N-acetylglucosamine (DEAE-cellulose fraction 12b).

The arrows indicate positions of oxidation by periodate.


NH
O
O
N
OH
O
OH
CH2
O
P
O-
O
P
O-
O
O
NH
C
O
CH3
CH2OH
O
OH
HO

acetylglucosamine on thin layers of PEI-cellulose (see Table 19).

The second major peak (12e) obtained as a result of chromatography of fraction 12 from Dowex-1-formate on a column of DEAE-cellulose (see Fig. 6) was found to have ultraviolet absorption characteristics similar to those of an N^6 substituted adenosine compound. The ultraviolet absorption characteristics of fraction 12e before and after acid hydrolysis are shown in Table 20. Phosphate and ribose analyses indicate that 12e contains one phosphate group and one ribose per base (see Table 21). Incubation of 12e with BAP produced a change in the mobility of the compound (Table 22), which indicates that the phosphate group is monoesterified and thus susceptible to removal by BAP. In order to detect the presence of a substituent at the N^6 position of the adenine ring, 12e was subjected to a 5 h hydrolysis in 6 N HCl at 100° and the hydrolyzate chromatographed on Whatman 1 paper. The results shown in Table 23 indicate that a ninhydrin-positive compound was produced which chromatographed with aspartic acid. Thus, it appears that fraction 12e is AMP containing a succinate moiety at the N^6 position of the adenine ring, and therefore identical to adenylosuccinate of the structure shown in Fig. 8. This hypothesis was confirmed when the mobilities of 12e and authentic adenylosuccinate and their acid hydrolysis products were compared on thin layers of PEI-cellulose (Table 22).

Table 19. Thin layer chromatography of fraction 12b on PEI-cellulose compared to UDP-N-acetylglucosamine.

Compound	R_f Value*		
	Solvent 1	Solvent 2	Solvent 3
UDP-N-acetylglucosamine	0.34	with solvent front	0.40
12b	0.33	with solvent front	0.40

* Solvent 1, 0.5 M LiCl/2.0 N HCOOH (1/1 v/v); solvent 2, 1.4 M LiCl; solvent 3, 0.25 M LiCl.

Table 20. Ultraviolet absorption characteristics of fraction 12e compared to adenylosuccinic acid before and after acid hydrolysis*

pH	Absorbancy Ratio			Wavelength (nm)	
	$A_{250}:A_{260}$	$A_{280}:A_{260}$	$A_{290}:A_{260}$	Maximum	Minimum
Acid (adjusted to 0.01 N HCl)	0.61 (0.59)	0.76 (0.73)	0.26 (0.24)	235 (235)	267 (267)
Neutral	0.55 (0.54)	0.90 (0.81)	0.32 (0.26)	233 (234)	268 (268)
Basic (adjusted to 0.01 N NaOH)	0.57 (0.53)	0.90 (0.89)	0.32 (0.31)	234 (234)	270 (269)
After Acid Hydrolysis†					
Acid (adjusted to 0.01 N HCl)	0.60 (0.56)	1.56 (1.60)	0.91 (0.95)	236 (237)	277 (277)
Neutral	0.58 (0.54)	0.96 (0.97)	0.27 (0.27)	233 (233)	270 (270)
Basic (adjusted to 0.01 N NaOH)	0.59 (0.54)	0.97 (0.99)	0.27 (0.27)	234 (234)	269 (270)

* Values for adenylosuccinic acid are shown in parentheses.

† Acid hydrolysis was carried out by treatment in 1.0 N HCl for 10 min at 100°.

Table 21. Partial chemical analysis of fraction 12e.

Phosphate/Base*		Ribose/Base*
Total	Labile	
1.10	0.0	1.15

* Data were calculated using an extinction coefficient at 268 nm (the maximum for fraction 12e) of 19300 (43).

Table 22. Thin layer chromatography on PEI-cellulose of fraction 12e after acid hydrolysis and treatment with BAP*.

Compound	R_f Value†		
	Solvent 1	Solvent 2	Solvent 3
Adenylosuccinic acid	0.44	0.51	0.78
N^6-succinyladenine	0.83	0.67	0.72
N^6-succinyladenosine	0.79	0.81	-
12e (untreated)	0.44	0.53	0.80
12e (acid hydrolyzed)	0.84	0.66	0.70
12e (BAP treated)	0.80	0.82	-

* Acid hydrolysis was carried out by treatment with 1.0 N HCl for 10 min at 100°. Incubation with BAP was performed as described in Experimental Procedure.

† Solvent 1, 0.5 M LiCl/2.0 N HCOOH (1/1 v/v); solvent 2, 1.4 M LiCl; solvent 3, 1.5 M KH_2PO_4.

Table 23. Descending paper chromatography of fraction 12e after hydrolysis in 6 N HCl*

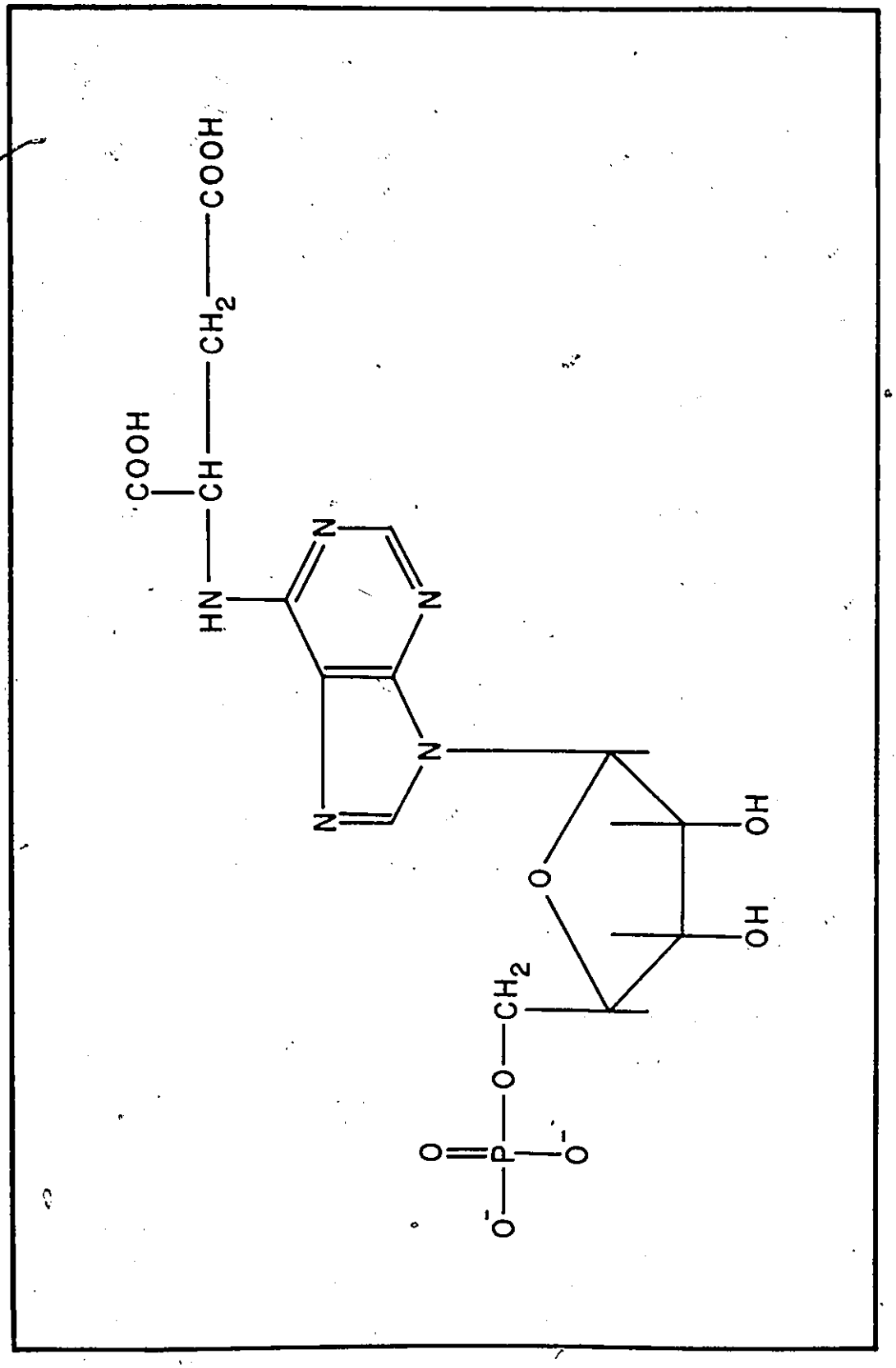
Compound	R_f Value
Aspartic acid	(a) 0.15
AMP (Acid hydrolyzed)	(a) - (b) 0.21
Adenylosuccinic acid (Acid hydrolyzed)	(a) 0.16 (b) 0.23
12e (Acid hydrolyzed)	(a) 0.15 (b) 0.21

* Acid hydrolysis was carried out by treatment with 6 N HCl for 5 h at 100° in a sealed tube. Approximately 20 µl of the hydrolyzates were applied to a sheet of Whatman paper, and the chromatogram was developed in the downward direction for 27 h using phenol saturated with water as a solvent. After air-drying the chromatogram the spots were visualized by spraying with ninhydrin reagent (Sigma Chemical Company).

FIGURE 8

Structure of adenylosuccinic acid (DEAE-cellulose fraction 12e).

Figure 8



Identification of fraction 13 from Dowex-1-formate

When fraction 13 from the Dowex-1-formate column was chromatographed on DEAE-cellulose (bicarbonate form) the results illustrated in Fig. 9 were obtained. Three major peaks were obtained from this fraction, and the first of these (13b) was found to be resistant to hydrolysis by BAP, and therefore similar to a dinucleoside compound. Column fractions 13c and 13d were both modified by BAP and not analyzed further in this study.

The results presented in Table 24 show some of the ultraviolet absorption characteristics of peak 13b at acid, basic and neutral pH values. These data are consistent with the hypothesis that fraction 13b contains equimolar amounts of both adenine and guanine, and since this fraction was found to be homogeneous by several chromatographic criteria, both purine bases must be present in the same molecule. When fraction 13b was treated with 1.0 N HCl for 10 min at 100° and the products chromatographed on a thin layer of PEI-cellulose the results shown in Table 25 were obtained. These data support the view that both adenine and guanine are present in the same molecule. The data shown in Table 26 were obtained when column fraction 13b was subjected to total and labile phosphate and ribose analyses. The observed ratios were obtained using a calculated molar extinction coefficient of 25800 as described in Table 26, and the values are consistent with the hypothesis that 13b is a dinucleoside polyphosphate similar to $G(5')p_3(5')A$.

FIGURE 9

Fractionation of peak 13 from Dowex-1-formate on DEAE-cellulose.

Peak 13 from the Dowex-1-formate column shown in Fig. 1 was chromatographed on a 1.5 x 26 cm column of DEAE-cellulose using a 1700 ml gradient of NH_4HCO_3, pH 8.6 from 0.002 M to 0.25 M. Each column fraction was analyzed at 260 nm. Fraction 13b was saved for further analysis, but fractions 13a, 13c and 13d were not identified in this study.

Figure 9

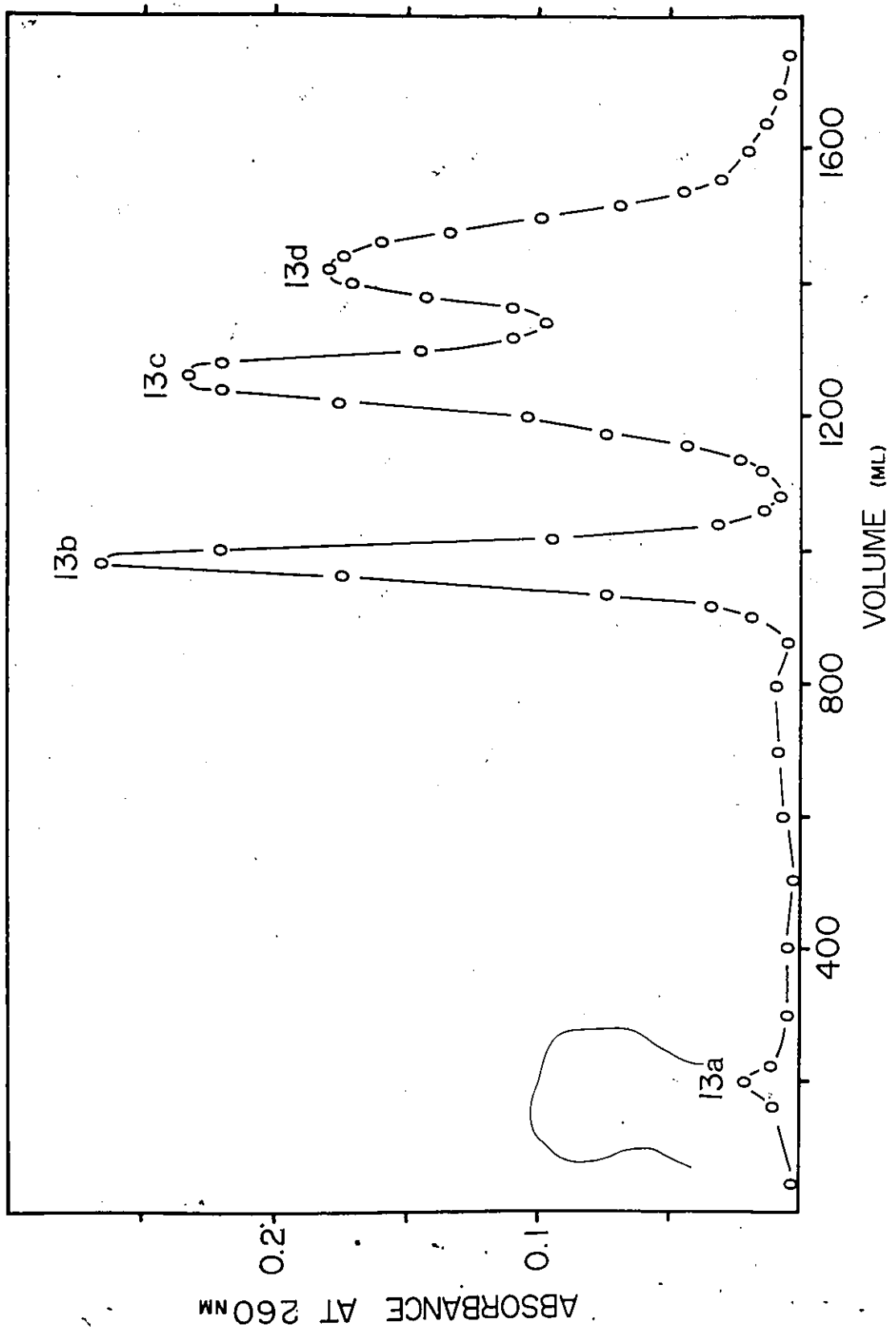

ABSORBANCE AT 260NM
0.2
0.1
VOLUME (ML)
400
800
1200
1600
13a
13b
13c
13d

Table 24. Ultraviolet absorption characteristics of fraction 13b compared to $G(5')p_3(5')A$*.

pH	Absorbancy Ratio			Wavelength (nm)	
	$A_{250}:A_{260}$	$A_{280}:A_{260}$	$A_{290}:A_{260}$	Maximum	Minimum
Acid (adjusted to 0.01 N HCl)	0.95 (0.93)	0.46 (0.42)	0.26 (0.21)	255 (256)	230 (229)
Neutral	0.99 (0.98)	0.42 (0.39)	0.18 (0.15)	255 (255-6)	227 (227)
Basic (adjusted to 0.01 N NaOH)	0.95 (0.96)	0.44 (0.40)	0.19 (0.15)	255 (256)	229 (228)

* Values for $G(5')p_3(5')A$ are shown in parentheses.

Table 25. Thin layer chromatography of acid hydrolyzed fraction 13b on PEI-cellulose*.

Compound	R_f Value
Adenine	0.83
Guanine	0.69
7-Methyl guanine	0.65
13b	0.04
13b after acid hydrolysis	(a) 0.70 (b) 0.83

* About 2 A_{260} units of fraction 13b were treated with 1.0 N HCl for 10 min at 100°. After cooling, 25 µl of the sample were applied to a plastic coated sheet of PEI-cellulose that had been washed with water prior to use. Standard compounds were also applied to the plates and air-dried. The plates were developed for 2 h using a solvent consisting of 0.5 M LiCl/2.0 N HCOOH (1/1 v/v).

Table 26. Partial chemical analysis of fraction 13b.

Phosphate/Base*		Ribose/Base*
Total	Labile	
1.56	0.57	0.94

* For the determination of base content of compound 13b a molar extinction coefficient at 260 nm of 25800 was used based on the summation of those for AMP and GMP (23), and assuming a 5% hypochromic effect for a dinucleotide containing AMP and GMP. However, Michelson has observed a hypochromic effect of 12-25% for $A(5')p_2(5')A$ (40); therefore the above extinction coefficient may be a conservative estimate.

In an attempt to elucidate the structure of fraction 13b samples of this fraction were treated with either BAP, SPD or SVPD then chromatographed on a thin layer of PEI-cellulose using a LiCl/HCOOH solvent system. Treatment with BAP and SPD did not alter the chromatographic mobility of fraction 13b compared to a non-treated sample, and these findings suggested that the phosphate groups in this compound are diesterified as in the diguanosine polyphosphates (4,41). Since SPD only hydrolyzes dinucleoside phosphates containing a free 5' hydroxyl group it was concluded that column fraction 13b contains a substituted 5' hydroxyl group as described for fraction 11d [$G(5')p_2(5')G$].

In contrast to the above experiments, 13b was found to be sensitive to hydrolysis by SVPD, and several UV-absorbing spots were detected after chromatography on PEI-cellulose (data not shown). When the products of SVPD treatment of fraction 13b were chromatographed on columns of DEAE-cellulose the results shown in Fig. 10 and 11 were obtained. In these experiments four UV-absorbing products were found after hydrolysis by SVPD. The identity of these fractions was determined by their elution positions from the column, by their spectral characteristics at acid, neutral and basic pH values (data not shown), and by their chromatographic behaviour on thin layers of PEI-cellulose compared to standard compounds (see Table 27). The products of SVPD hydrolysis of fraction 13b were identified as AMP, GMP, ADP and GDP. In addition, the results presented in Table 28

FIGURE 10

Partial hydrolysis of G(5')p_3(5')A (DEAE-cellulose fraction 13b) by SVPD.

Nine units (A_{260}) of column fraction 13b shown in Fig. 9 were treated with 13 μg of SVPD for 10 min at 30° as described under Experimental Procedure. The reaction mixture was chromatographed on DEAE-cellulose as described in the legend to Fig. 4 and each column fraction was analyzed for UV absorbance at 260 nm. Four distinct fractions eluted from the column ahead of the unhydrolyzed substrate [G(5')p_3(5')A] and these were identified by their UV spectra and chromatographic properties to be AMP, GMP, ADP and GDP (see Table 27).

Figure 10

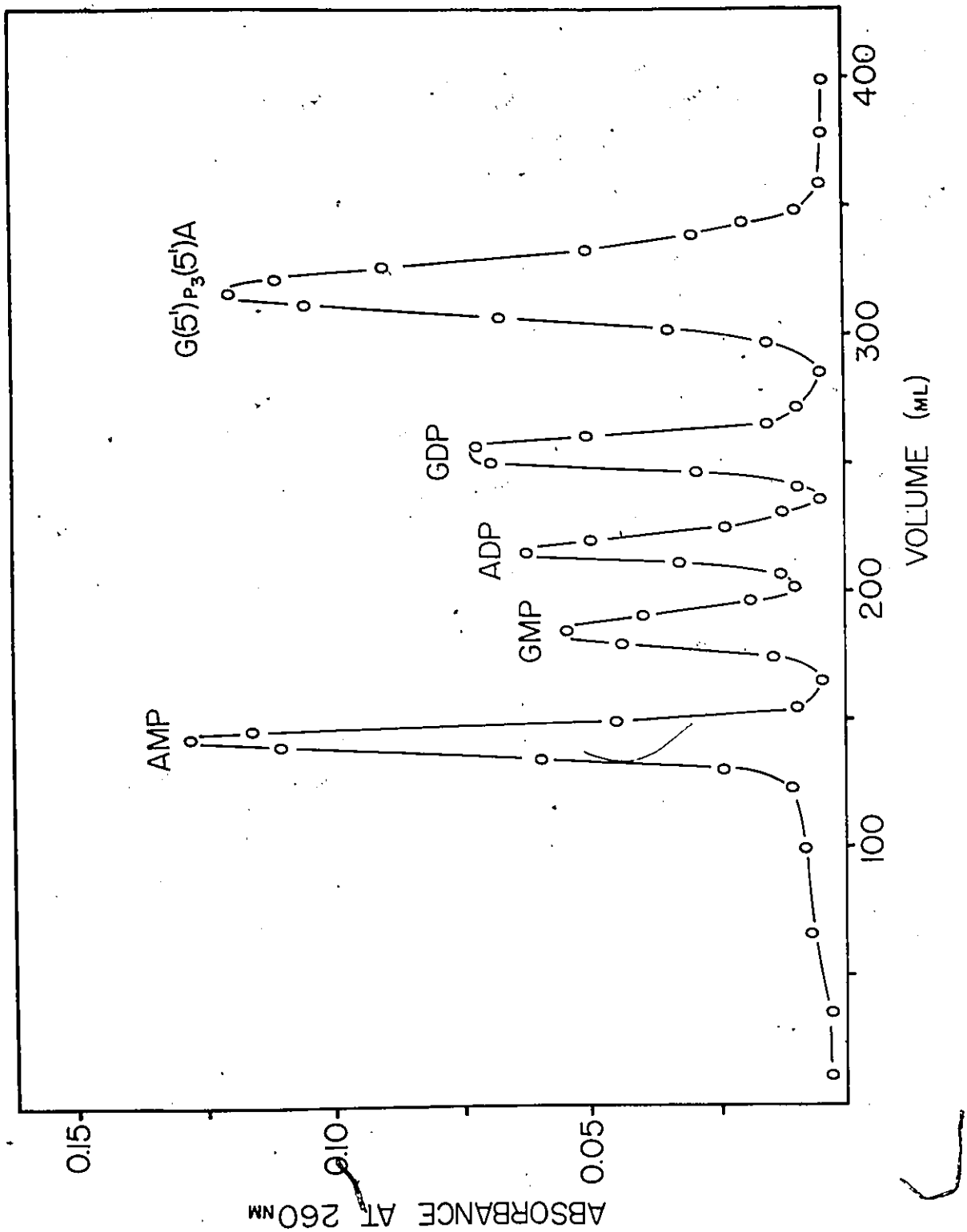

G(5')P3(5')A
GDP
ADP
GMP
AMP
0.15
0.10
0.05
ABSORBANCE AT 260NM
100
200
300
400
VOLUME (ML)

FIGURE 11

Complete hydrolysis of G(5')p_3(5')A (DEAE-cellulose fraction 13b) by SVPD.

Nine units (A_{260}) of column fraction 13b were treated with 100 µg of SVPD and incubated for 10 min at 30° as described under Experimental Procedure. The reaction mixture was chromatographed on DEAE-cellulose and the products analyzed as described in the legend to Fig. 10.

Figure 11

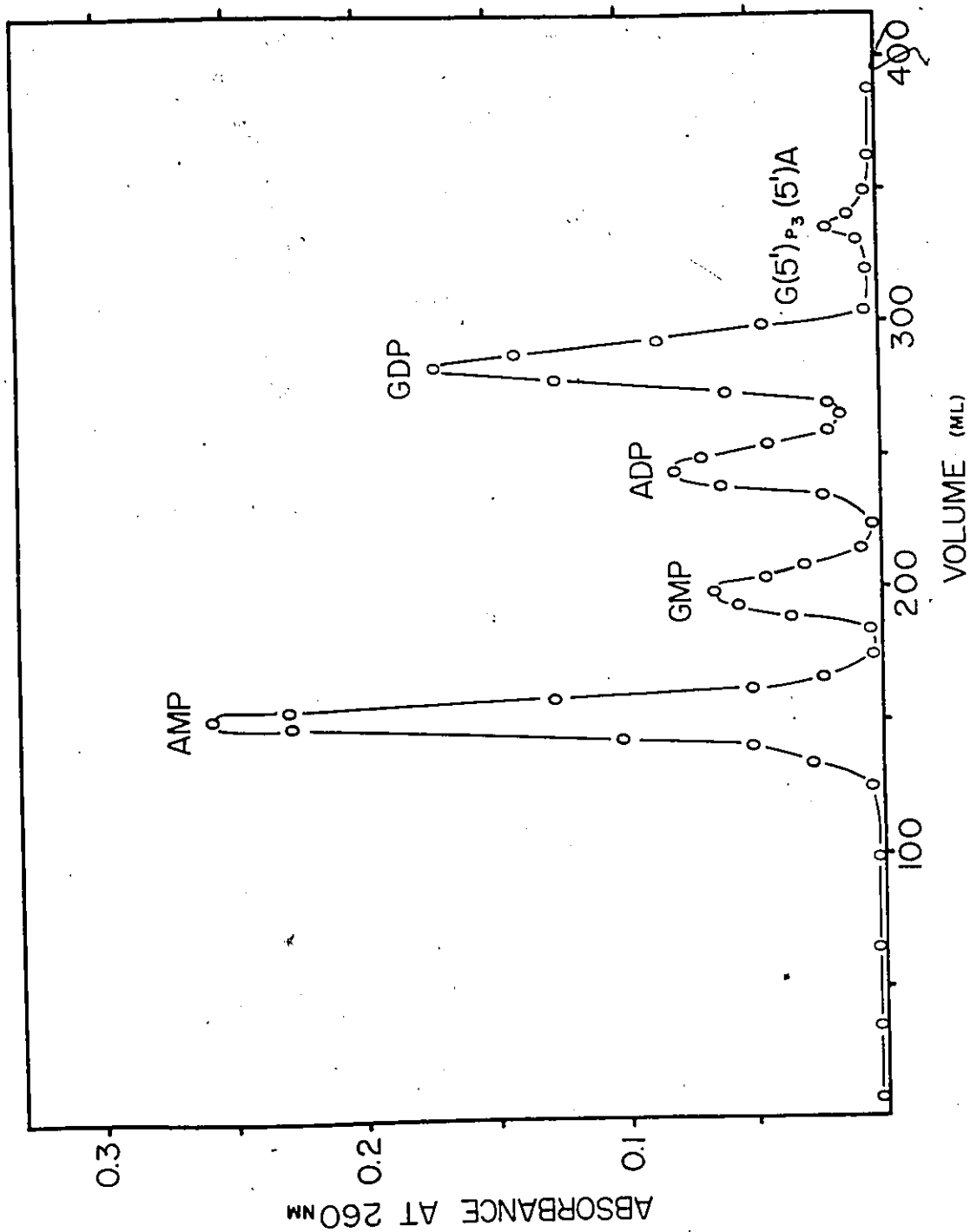

AMP
GMP
ADP
GDP
G(5')P3(5')A
100
200
300
400
VOLUME (ML)
0.1
0.2
0.3
ABSORBANCE AT 260NM

Table 27. Thin layer chromatography of SVPD hydrolysis products of fraction 13b on PEI-cellulose*.

	Compound	R_f Value		
		Solvent 1	Solvent 2	Solvent 3
Standard Compounds	AMP	0.82	0.54	0.71
	GMP	0.43	0.37	0.56
	ADP	0.20	0.39	0.69
	GDP	0.05	0.25	0.57
Column Fractions	Peak 1	0.80	0.56	0.67
	Peak 2	0.42	0.45	0.63
	Peak 3	0.22	0.34	0.66
	Peak 4	0.10	0.21	0.57

* Nine units (A_{260}) of fraction 13b were treated with 100 µg of SVPD for 10 min at 30° as described under Experimental Procedure. The hydrolysis products were chromatographed on a DEAE-cellulose column as shown in Fig. 11, and the contents of each peak were pooled and concentrated in a rotary evaporator. About 50 µl of each sample were applied to PEI-cellulose sheets which had been washed with water prior to use, and the chromatograms were run for 2-3 h in the appropriate solvent.

Solvent 1, 0.5 M LiCl/2.0 N HCOOH (1/1 v/v); solvent 2, 1.4 M LiCl; solvent 3, 1.5 M KH_2PO_4.

Table 28. Products of SVPD hydrolysis of fraction 13b*.

Column Fractions	Partial Hydrolysis	Complete Hydrolysis
AMP	0.114	0.273
GMP	0.078	0.102
ADP	0.066	0.101
GDP	0.106	0.266
13b	0.250	-

* The data are shown in μmoles and were calculated from the results shown in Fig. 10 and 11.

show that hydrolysis of fraction 13b by SVPD results in the production of nearly equimolar quantities of AMP and GDP, and less but nearly equimolar quantities of GMP and ADP. These results are consistent with the hypothesis that column fraction 13b is G(5')p_3(5')A of the structure shown in Fig. 12. Finally, the identity of fraction 13b as G(5')p_3(5')A was confirmed by chromatography along with commercially available G(5')p_3(5')A on thin layers of PEI-cellulose using three different solvent systems. The results of these experiments are presented in Table 29, and they support the conclusion that fraction 13b is G(5')p_3(5')A.

Identification of fraction 16 from Dowex-1-formate

When fraction 16 from Dowex-1-formate was chromatographed on DEAE-cellulose (bicarbonate form) six UV-absorbing fractions were resolved. The results of this experiment are illustrated in Fig. 13. The major peak (16f) has been identified previously (4) to be G(5')p_3(5')G. In a previous study (A.H. Warner, unpublished observations) ATP was found to be present in the same column fraction as G(5')p_3(5')G from Dowex-1-formate. Paper chromatography and UV-absorption analysis of column fraction 16d compared to authentic ATP confirmed the identity of fraction 16d in Fig. 13 as ATP (data not shown).

By comparison peak 16b from DEAE-cellulose was found to have ultraviolet absorption properties similar to those for uridine-containing compounds. These UV-absorption characteristics are shown in Table 30. When a sample of

FIGURE 12

Proposed structure of guanosine(5')triphospho(5')adenosine (DEAE-cellulose fraction 13b).

The arrows indicate the positions of hydrolysis by SVPD.

Figure 12

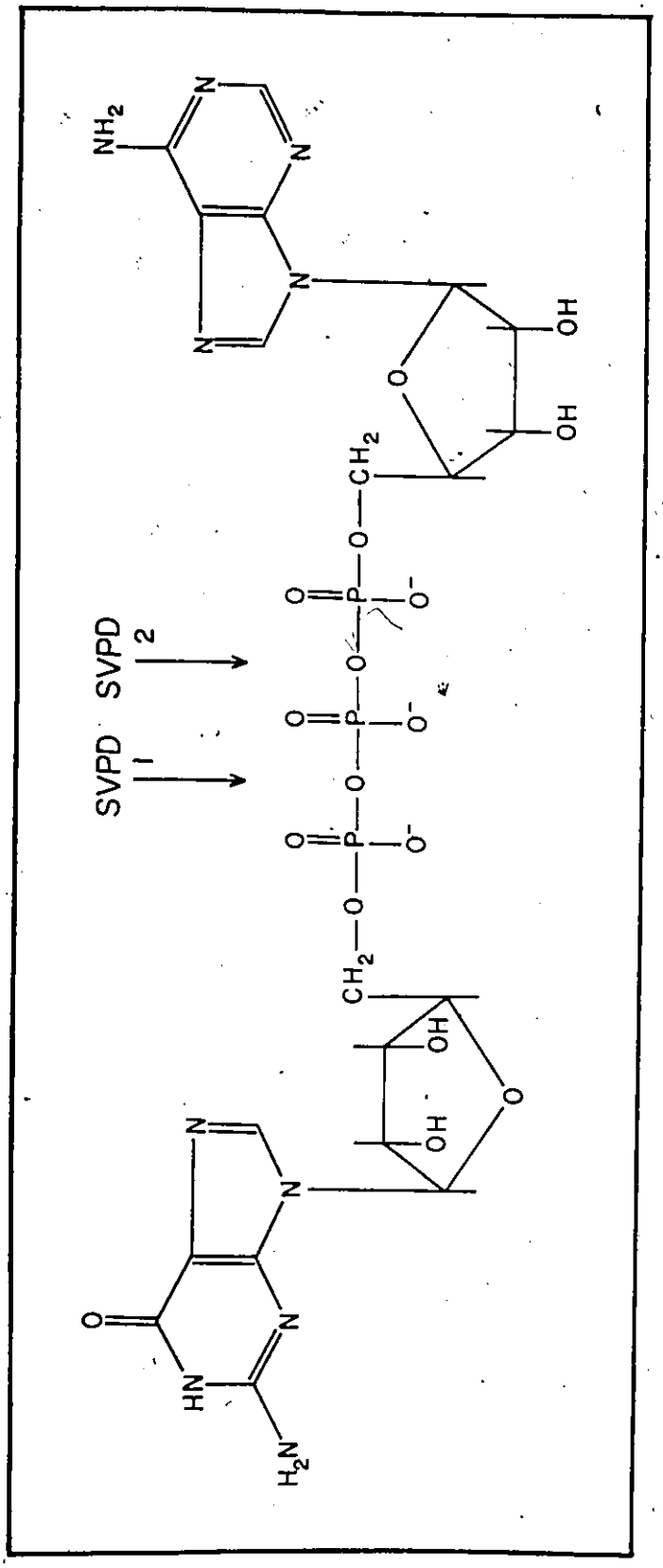


Table 29. Thin layer chromatography of fraction 13b compared to authentic dinucleoside compounds on PEI-cellulose.

Compound	R_f Value*		
	Solvent 1	Solvent 2	Solvent 3
$G(5')p_2(5')G$	0.23	0.46†	0.0
$G(5')p_3(5')G$	0.01	0.25	0.0
$G(5')p_3(5')A$	0.04	0.46	0.57
$G(5')p_4(5')G$	0.0	0.07†	0.0
13b	0.04	0.47	0.56

* Solvent 1, 0.5 M LiCl/2.0 N HCOOH (1/1 v/v); solvent 2, 1.4 M LiCl, solvent 3, 1.5 M KH_2PO_4.

† These compounds were found to trail considerably in this solvent.

FIGURE 13

Fractionation of peak 16 from Dowex-1-formate on DEAE-cellulose.

Peak 16 from the Dowex-1-formate column shown in Fig. 1 was chromatographed on a 2.5 x 50 cm column of DEAE-cellulose using a 2300 ml gradient of NH_4HCO_3, pH 8.6 from 0.002 M to 0.25 M. Each column fraction was analyzed at 260 nm and the identity of the major peak (16f) was confirmed by its spectral and chromatographic properties to be G(5')p_3(5')G as described previously (4). The identity of peak 16d was also confirmed by means of its spectral and chromatographic properties to be ATP. Fraction 16b was saved for further analysis, but fractions 16a, 16c and 16e were not identified in this study.

Figure 13

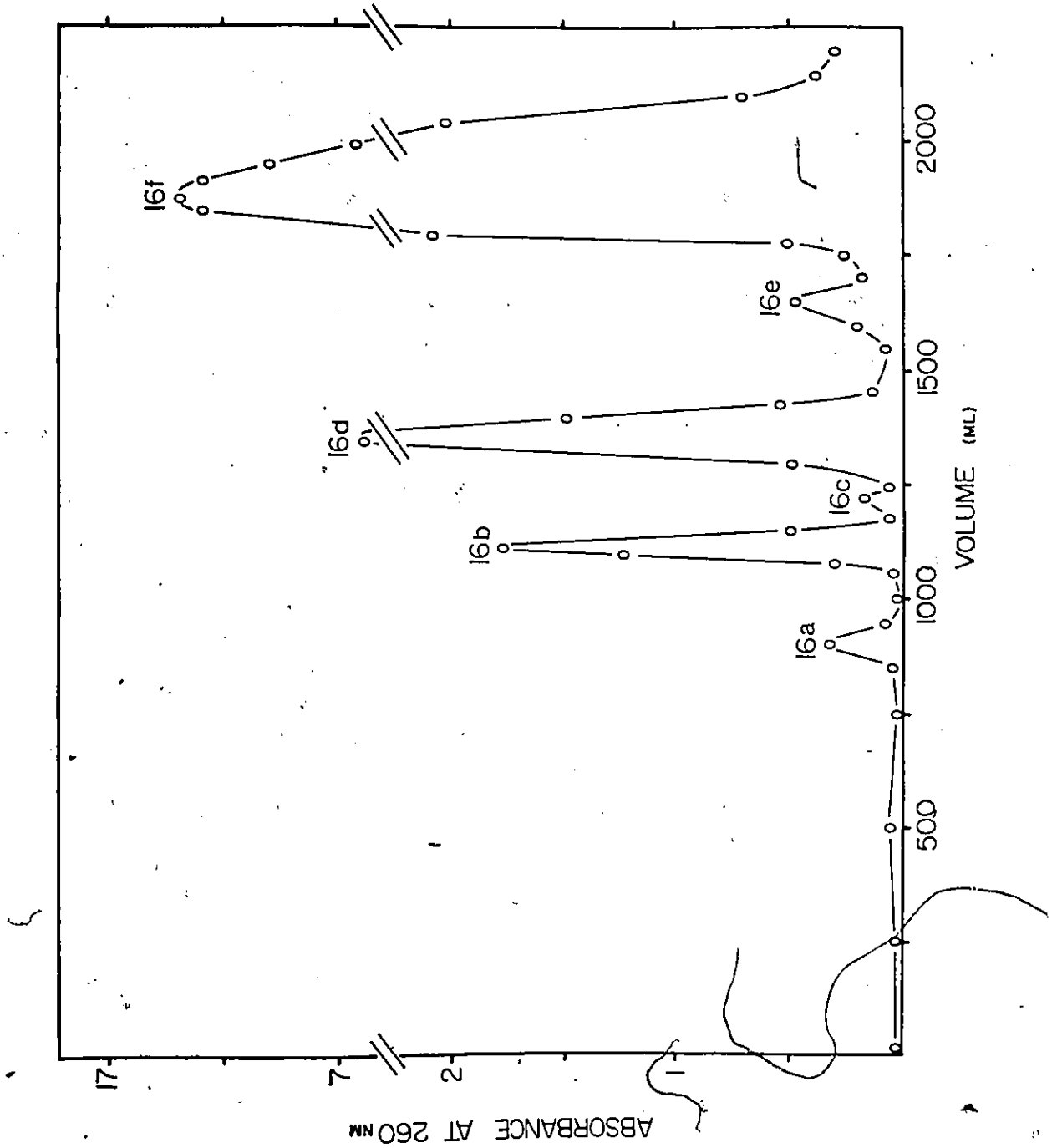

ABSORBANCE AT 260 NM
17
7
2
1
500
1000
1500
2000
VOLUME (ML)
16a
16b
16c
16d
16e
16f

Table 30. Ultraviolet absorption characteristics of fraction 16b compared to UMP*.

pH	Absorbancy Ratio			Wavelength (nm)	
	$A_{250}:A_{260}$	$A_{280}:A_{260}$ †	$A_{290}:A_{260}$	Maximum	Minimum
Acid (adjusted to 0.01 N HCl)	0.75 (0.76)	0.45 (0.40)	0.11 (0.06)	262 (262-3)	231 (233)
Neutral	0.75 (0.70)	0.46 (0.39)	0.09 (0.03)	262 (263)	232 (232-3)
Basic (adjusted to 0.01 N NaOH)	0.75 (0.73)	0.47 (0.31)	0.08 (0.05)	262 (263)	233 (235)

* Values for UMP are shown in parentheses

† Values for $A_{280}:A_{260}$ are higher than expected for a uridine-containing compound. This is due to the fact that 16b was not completely pure as shown by thin layer chromatography.

fraction 16b was treated with either 1.0 N HCl (100° for 10 min) or BAP (30° for 1 h) its chromatographic mobility on PEI-cellulose was altered as shown in Table 31. The hydrolytic products which resulted from acid or BAP treatment had chromatographic mobilities identical to UMP and uridine, respectively. These data together with the spectral analyses suggest that fraction 16b is identical to UDP.

Identification of fraction 17 from Dowex-1-formate

In some chromatographic experiments of the acid extract from Artemia, fraction 17 which contains G(5')p_4(5')G (4) was found to separate into two peaks when the concentration of G(5')p_4(5')G was very low. In the experiment shown in Fig. 1 no fractionation of the components of peak 17 was apparent. However, when the trailing edge of peak 17 was pooled and chromatographed on a column of DEAE-cellulose (bicarbonate form) the results shown in Fig. 14 were obtained. Spectral analyses of fraction 17a and 17b indicate that both peaks contain only guanosine as the major UV-absorbing moiety (data not shown), and chemical and chromatographic analyses indicate that peak 17a is identical to GTP (see Tables 32 and 33). Peak 17b was found to be G(5')p_4(5')G as described previously (4). Thus, the major component of fraction 17 from Dowex-1-formate (shown in Fig. 1) is G(5')p_4(5')G, and the trailing edge is rich in GTP which usually is not resolved from G(5')p_4(5')G in this chromatographic system unless the concentration of G(5')p_4(5')G is low.

Table 31. Chromatography of acid hydrolyzed and BAP treated fraction 16b*.

Compound	R_f Value†	
	PEI-cellulose Solvent 1	Whatman 1 Solvent 2
Uridine	0.84	-
UMP	0.60	-
UDP	0.10	0.74
16b	0.08	0.75
16b (Acid hydrolyzed)	0.60	-
16b (BAP treated)	0.86	-

* Acid hydrolysis was carried out by treatment with 1.0 N HCl for 10 min at 100° under conditions which hydrolyze pyrimidine-containing nucleoside diphosphates to nucleoside monophosphates. Treatment with BAP was as described under Experimental Procedure.

† Solvent 1, 0.5 M LiCl/2.0 N HCOOH (1/1 v/v); solvent 2, 0.1 M sodium phosphate, pH 6.8/$(NH_4)_2SO_4$/n-propanol (100/60/2 v/w/v).

FIGURE 14

Fractionation of peak 17 from Dowex-1-formate on DEAE-cellulose.

The trailing edge of peak 17 from the Dowex-1-formate column shown in Fig. 1 was chromatographed on a 1 x 30 cm column of DEAE-cellulose using a 1000 ml linear gradient of NH_4HCO_3, pH 8.6 from 0.002 M to 0.25 M. Each column fraction was analyzed for absorbance at 260 nm and the identity of peaks 17b as $G(5')p_4(5')G$ (4) was confirmed by its spectral and chromatographic properties. Fraction 17a was saved for further analysis.

Figure 14

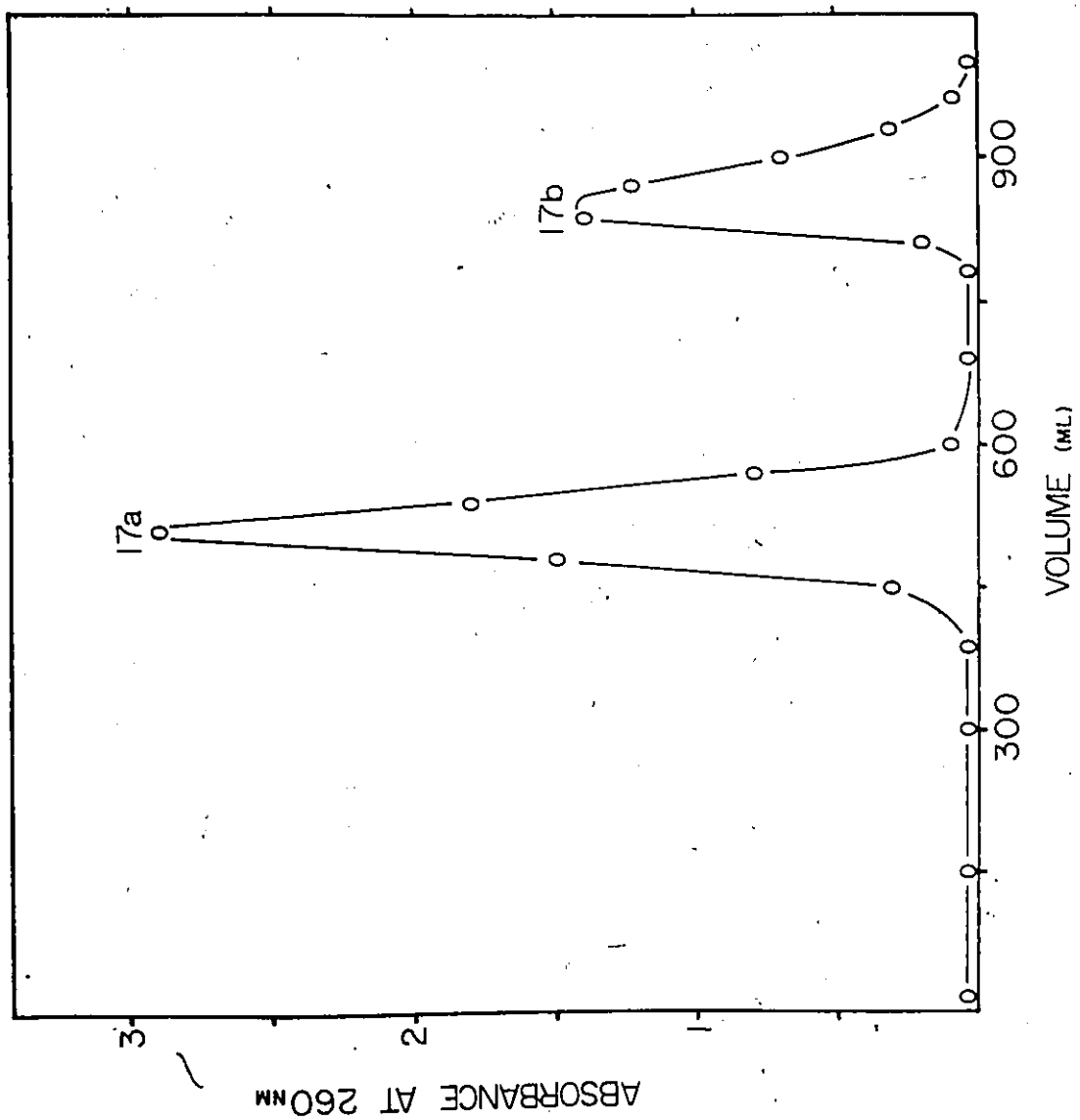


Table 32. Partial chemical analysis of fraction 17a*.

Phosphate/Base		Ribose/Base	Nitrogen/Base
Total	Labile		
2.84	1.94	0.90	5.00

* Determinations of base content were made using a molar extinction coefficient of 11700 at 260 nm for guanosine (23).

Table 33. Chromatography of fraction 17a compared to GTP.

Compound	R_f Value*		
	PEI-cellulose		Whatman 1
	Solvent 1	Solvent 2	Solvent 3
GTP	0.23	0.40	0.56
17a	0.21	0.40	0.56

* Solvent 1, 1.4 M LiCl; solvent 2, 1.5 M KH_2PO_4; solvent 3, 0.1 M sodium phosphate, pH 6.8/$(NH_4)_2SO_4$/n-propanol (100/60/2 v/w/v).

DISCUSSION

By the use of ion-exchange chromatography the acid-soluble nucleotide fraction from dormant embryos of the brine shrimp, Artemia salina, has been resolved into more than 25 nucleotide components. Most of the common nucleotides were shown previously to be present in dormant encysted embryos of Artemia in addition to large amounts of diguanosine tri- and tetraphosphate [$G(5')p_3(5')G$ and $G(5')p_4(5')G$] (2,4). However, several UV-absorbing acid-soluble compounds derived from Artemia cysts remained to be identified.

Dormant embryos of Artemia salina are encased in a hard shell consisting of two regions. The outer region which contains a dark pigment is of maternal origin, whereas the less pigmented inner region is of embryonic origin (44). The shell has been shown by Dutrieu to contain 50% protein, 10% chitin, and a number of other components (45). During development of dormant embryos of the brine shrimp the larvae first "emerge" from the outer shell enclosed in a hatching membrane, and then after several hours this membrane ruptures and the larvae "hatch" (44). During this process the larval exoskeleton appears, although its origin in the undeveloped cyst remains undetermined. The exoskeleton of Crustacea consists basically of a hardened cuticle composed of a chitin-protein complex, stiffened and hardened by the deposition of calcium salts or organic materials such as quinones (13). Chitin, which is located in the inner thick layer of the

cuticle (the endocuticle) is a major constituent of the exoskeleton. It consists of a high molecular weight homopolymer of β(1→4) linked N-acetyl-D-glucosamine with no branching in the carbohydrate chain (13). It is synthesised from UDP-N-acetylglucosamine in a reaction catalyzed by chitin synthetase as shown below (14).

$$\text{UDP-N-acetylglucosamine} + (\text{N-acetylglucosamine})_n \xrightarrow{\text{chitin synthetase}} \text{UDP} + \underset{\text{lengthened chitin chain}}{(\text{N-acetylglucosamine})_{n+1}}$$

Therefore, if N-acetylglucosamine is a soluble precursor of chitin it should be present in a system such as Artemia embryos in which exoskeleton formation is occurring. Thus, of the several compounds identified in this study, one was found to be UDP-N-acetylglucosamine (UDP-NAG) (column fraction 12b) as shown in Fig. 7. This compound is probably identical to UDP-X which was described by Warner and Finamore (2), but which was not identified by them. Although UDP-NAG has not been described previously in the brine shrimp, it has been described in other crustacea (15) and in a variety of other animal tissues and bacteria (16). Chitin biosynthesis has not been studied in Artemia but it appears that the UDP-NAG found in acid extracts of Artemia cysts is the primary source of N-acetylglucosamine for chitin biosynthesis during exoskeleton formation in the developing embryo.

Previous studies of nucleotide metabolism in adults and embryos of the brine shrimp have indicated that Artemia possesses a unique mechanism among eukaryotic organisms for

the synthesis of purine-containing compounds. First of all, both adults and embryos are unable to synthesise purines de novo (1), and secondly the embryos appear to utilize part of the large store of G(5')p_4(5')G as the primary source of ATP during development (1). In this study two compounds important in purine biosynthesis have been found in acid extracts of dormant embryos. They have been purified by ion-exchange chromatography and identified by their spectral, chemical and chromatographic properties. They are inosine 5'-monophosphate (IMP) (column fraction 10c) and adenylosuccinic acid (SAMP) (column fraction 12e). The chemical structure of the latter is shown in Fig. 8, and both compounds are of considerable interest in the regulation of purine metabolism in Artemia embryos. It is well known that IMP is a key intermediate in de novo purine biosynthesis and thus it is a precursor for both adenine and guanine-containing nucleotides. In addition, SAMP is an important intermediate in the biosynthesis of AMP. The relationship between IMP and SAMP in purine biosynthesis is shown in Fig. 15.

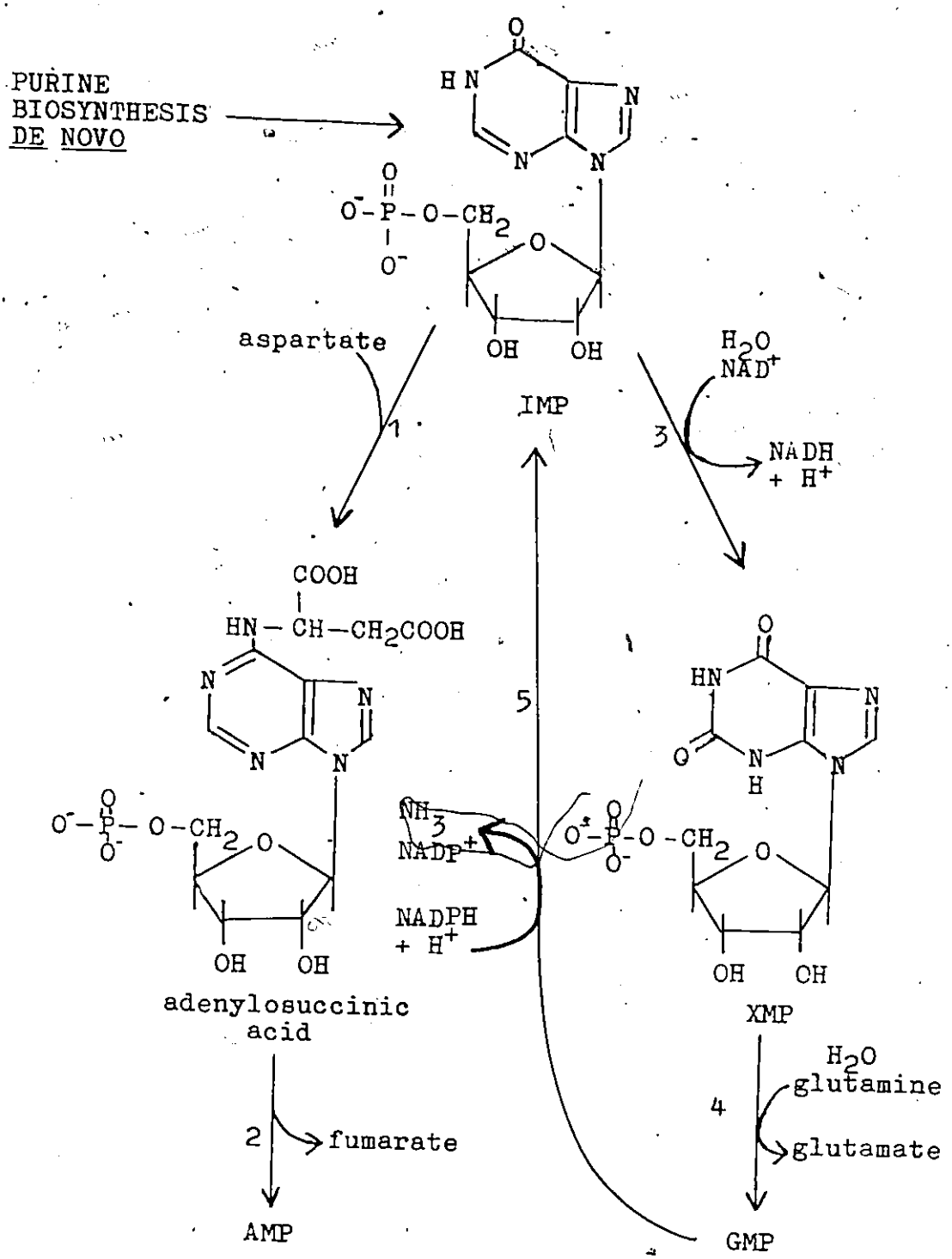
In an earlier study Clegg et al. (1) showed that nauplii of Artemia salina are incapable of de novo purine biosynthesis, but convert guanine compounds to adenine compounds as required by the organism for the synthesis of adenosine nucleotides. The reason(s) for this inability of Artemia nauplii to synthesise purines de novo is (are) unknown at the present time, but it is possible that one or all of the enzymes in the de novo pathway of purine biosynthesis are inhibited or

FIGURE 15

Pathway for the production of AMP and GMP from IMP.

Enzymes are: 1, adenylosuccinate synthetase; 2, adenylosuccinate lyase; 3, inosine monophosphate dehydrogenase; 4, guanosine monophosphate synthetase; 5, guanosine monophosphate reductase.

Figure 15


PURINE
BIOSYNTHESIS
DE NOVO
aspartate
1
IMP
3
H_2O
NAD^+
NADH
+ H^+
COOH
HN – CH – CH_2COOH
5
NH_3
$NADP^+$
NADPH
+ H^+
adenylosuccinic
acid
2
fumarate
AMP
XMP
4
H_2O
glutamine
glutamate
GMP

lacking. This same metabolic deficiency is also shared by Artemia adults (6).

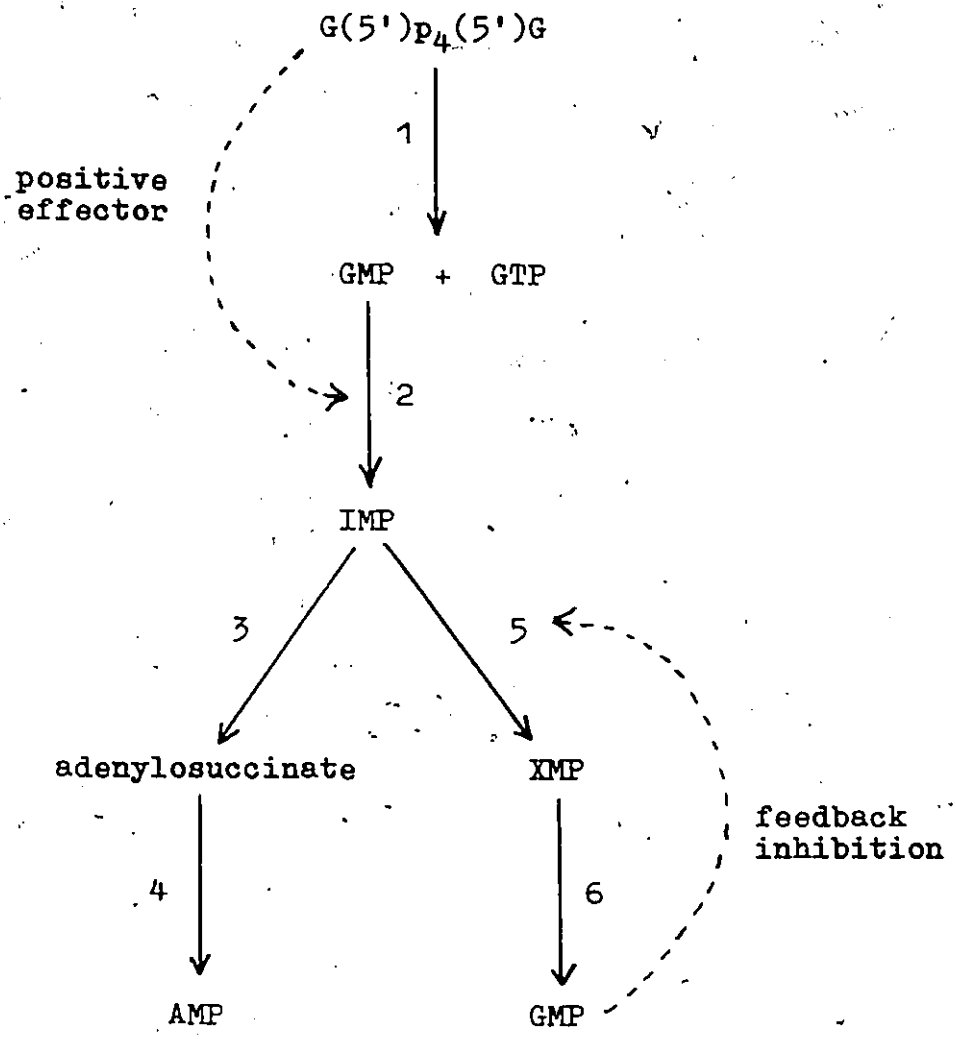
Purine biosynthesis in micro-organisms is known to be regulated at two levels (46,47). The first of these involves regulation of the de novo pathway leading to IMP, and therefore provides common control over the biosynthesis of all purine nucleotides. The second level of control involves regulation of the branch pathways leading from IMP to AMP or GMP, and serves to regulate the relative amounts of these two nucleotides. Perhaps purine biosynthesis in the brine shrimp is under similar control to that described above, and therefore high levels of purine nucleotides as observed in Artemia embryos might inhibit de novo purine biosynthesis. In particular, the extremely high levels of diguanosine nucleotides present in undeveloped embryos could have a profound effect on the quantities of purine nucleotides synthesised. In this respect, it is noteworthy that guanine derivatives inhibit the enzyme IMP dehydrogenase (46) and thus inhibit the formation of XMP. Of interest is the fact that no XMP could be detected in brine shrimp cysts in this study. Thus, it appears that IMP dehydrogenase is inhibited by the high level of GMP found in extracts of cysts resulting in the accumulation of IMP. GMP is indeed present in a high concentration in the cysts (see Fig. 1, peak 8), and most of this nucleotide is present in the cytosol (3). Since GMP is an effective feedback inhibitor of its own biosynthesis from IMP (see Fig. 16) it is unlikely that this compound arises

FIGURE 16

Proposed pathway for the production of AMP from $G(5')p_4(5')G$.

Enzymes are: 1, diguanosinetetraphosphate guanylohydrolase; 2, guanosine monophosphate reductase; 3, adenylosuccinate synthetase; 4, adenylosuccinate lyase; 5, inosine monophosphate dehydrogenase; 6, guanosine monophosphate synthetase.

Figure 16


G(5')p4(5')G
1
positive effector
GMP + GTP
2
IMP
3
5
adenylosuccinate
XMP
feedback inhibition
4
6
AMP
GMP

from IMP in the cysts but from some other source. Most likely GMP is derived during early development from the hydrolysis of $G(5')p_4(5')G$ by the enzyme diguanosine tetraphosphate guanylohydrolase which is present in the cytosol of encysted embryos (48,49). Part of the GMP produced in this way is probably converted to IMP by GMP reductase as suggested by Renart et al. (50) (see Fig. 15 and 16). Furthermore, the formation of IMP from GMP is probably enhanced by $G(5')p_4(5')G$ which has been shown to be a positive effector of GMP reductase (50). Therefore, both $G(5')p_4(5')G$ and guanosine tetraphosphate guanylohydrolase appear to be important as stimulators of AMP synthesis (see Fig. 16). The existence of this pathway in Artemia is supported by the finding of relatively large quantities of SAMP in Artemia cysts. In Artemia embryos SAMP may accumulate as a result of the absence or inhibition of the enzyme adenylosuccinate lyase which converts SAMP to AMP. It is interesting to note that this enzyme is also required in de novo purine biosynthesis at a step prior to IMP (51); thus if it is inhibited or deficient in brine shrimp cysts purine biosynthesis would be inhibited during development. This view is consistent with the observations of Clegg et al. (1) who demonstrated that purine biosynthesis de novo does not occur in the nauplius larva of Artemia.

In certain mutants of micro-organisms (52) Gots and Gollub have observed that the lack of the enzyme adenylo-succinate lyase causes a block in two positions of the purine biosynthetic pathway. First there is a block in the conversion

of SAMP to AMP, and second there is a block in the conversion of 5-amino-4-imidazole-N-succinocarboxamide ribonucleotide (SAICAR) to 5-amino-4-imidazolecarboxamide ribonucleotide (AICAR) in which case SAICAR accumulates. Perhaps in Artemia there is an analogous situation to account for the accumulation of SAMP (and maybe SAICAR also), and consequently de novo purine biosynthesis cannot occur. An examination of the levels of SAICAR and of the enzyme adenylosuccinate lyase would be useful in testing this hypothesis.

In embryos of Artemia salina the mode of biosynthesis of adenosine nucleotides is unusual, and the synthesis of AMP and ATP appears to occur by different routes (12). In fact, Van Denbos and Finamore have proposed that ATP is synthesised from $G(5')p_4(5')G$ through the intermediate $A(5')p_4(5')G$ (12). However, $A(5')p_4(5')G$ was not detected in acid extracts of Artemia cysts in this study; therefore proof of this pathway must await further experimentation.

In this study two new dinucleotide compounds of potential interest in nucleic acid metabolism were identified. They are guanosine(5')diphospho(5')guanosine [$G(5')p_2(5')G$] (column fraction 11d) and guanosine(5')triphospho(5')adenosine [$G(5')p_3(5')A$] (column fraction 13b). To the knowledge of the author this is the first time these nucleotide anhydrides have been described in a biological system, although $G(5')p_2(5')G$ has been synthesised chemically (53) and $A(5')p_3(5')G$ synthesised enzymatically in vitro using E. coli lysyl tRNA synthetase (11). (A report describing the

identification of these two compounds in dormant embryos of the brine shrimp has been submitted for publication (54)).

The identity of column fractions 11d and 13b as G(5')p_2(5')G and G(5')p_3(5')A, respectively was based on the results of several chemical, physical and enzymatic analyses of these compounds compared to commercially available standards. Chemically, fraction 11d is identical to GMP and cGMP, but it is resistant to hydrolysis by BAP and has chromatographic properties on thin layers of PEI-cellulose different from either of these nucleotides. Similarly, fraction 13b was found to be resistant to hydrolysis by BAP. These data suggest that the phosphate groups in both compounds are diesterified as in diguanosine tri- and tetraphosphates described previously (4,41). In addition, the enzyme studies indicate that the phosphate groups in both molecules are esterified through the 5' position on the ribose. Thus, both compounds are resistant to hydrolysis by spleen phosphodiesterase which requires a free 5' hydroxyl group for hydrolysis, and sensitive to snake venom phosphodiesterase which requires a free 3' hydroxyl group for hydrolysis. The chemical and enzymatic analyses are consistent with the structures shown in Fig. 5 and 12 for column fractions 11d and 13b, respectively. Furthermore the results also show that SVPD hydrolyzes G(5')p_3(5')A preferentially at position 2 shown in Fig. 12, rather than at position 1. Thus, GDP and AMP are produced in equimolar quantities at a faster rate than GMP and ADP, the products of hydrolysis at position 1.

In dormant embryos of Artemia salina the most abundant nucleotide is G(5')p_4(5')G which comprises about 45% of the UV-absorbing material in the extract and about 2%* of the embryo dry weight (2,4). By comparison, the two new dinucleoside compounds described here [G(5')p_2(5')G and G(5')p_3(5')A] comprise only about 0.5% each of the UV-absorbing material in the acid-soluble fraction from cysts or about 0.03% each of the cyst dry weight. The significance and function of G(5')p_2(5')G and G(5')p_3(5')A in Artemia embryos as well as their mode of biosynthesis is unknown at the present time.

It is noteworthy, however, that about 75% of Artemia cyst mRNA has the 5' terminal "cap" structure of m^7G(5')p_3(5')AmpGp, whereas the remainder of the "capped" mRNA has the structure m^7G(5')p_3(5')GmpGp (55). Since translation of Artemia mRNA in a cell-free wheat germ system is inhibited by synthetic "caps" such as m^7G(5')p_3(5')Gm and m^7G(5')p_3(5')Am (55), it is tempting to speculate that G(5')p_3(5')G and G(5')p_3(5')A play a role in the regulation of protein synthesis during the development of Artemia. This could only be the case, however, if the embryos contain an enzyme system that specifically methylates the guanosine moiety at the 7 position.

---

* This value was calculated from the data in Ref. 2, and on the basis that there are 250 cysts per mg dry weight.

The origin of the dinucleotide compounds described in this study in undeveloped embryos of the brine shrimp is still obscure. It is possible that G(5')p_3(5')A arises due to mRNA hydrolysis, although no methylated dinucleotides were detected in acid extracts of Artemia cysts in this study. Thus, if G(5')p_3(5')A [and G(5')p_3(5')G] is a "cap" metabolite demethylation of the "cap" must have occurred before "cap" hydrolysis.

Another possible source of G(5')p_3(5')A may be from the amino acid activation reaction. Several years ago Zamecnik and his colleagues reported that various dinucleoside polyphosphates are synthesised in vitro from mononucleotides in the back reaction of amino acid activation using lysyl-tRNA synthetase from E. coli (10,11). In their studies these investigators observed that hybrid dinucleoside polyphosphates such as A(5')p_4(5')G and A(5')p_3(5')G are also synthesised in vitro when GTP is added to their lysine activating system. In contrast, partially purified lysyl-tRNA synthetase from Artemia cyst cytosol is inactive in the synthesis of G(5')p_4(5')G (A.H. Warner, unpublished observations) but it is not known whether this enzyme will catalyze the synthesis of dinucleoside polyphosphates or hybrids such as G(5')p_3(5')A. Another possibility is that G(5')p_3(5')A is stored in yolk platelets in Artemia and its synthesis catalyzed by GTP:GTP guanylyltransferase from Artemia yolk platelets in the presence of an adenosine nucleotide (56,57). Since G(5')p_3(5')G has been shown to be derived from G(5')p_4(5')G

in yolk platelets by the nucleophilic attack of GDP, perhaps ADP can also act as a nucleophile in a similar reaction with $G(5')p_4(5')G$ to yield $G(5')p_3(5')A$ and GTP. Similarly, $G(5')p_2(5')G$ could be formed by a nucleophilic attack by GMP on $G(5')p_3(5')G$ or $G(5')p_4(5')G$. An understanding of the mode of biosynthesis of $G(5')p_2(5')G$ and $G(5')p_3(5')A$ will contribute greatly to our overall knowledge of dinucleotide metabolism in Crustacea.

In addition to those compounds described above several other compounds have been identified in the acid extract of brine shrimp cysts. They are UMP (10b), ADP (11c), UDP (16b) and GTP (17a). These compounds (with the exception of UDP) have been demonstrated previously in acid extracts of Artemia embryos (2) and consequently their presence has not been elaborated upon in this thesis.

BIBLIOGRAPHY

1. Clegg, J.S., Warner, A.H. and Finamore, F.J. (1967). Evidence for the function of P^1,P^4-diguanosine 5'-tetraphosphate in the development of Artemia salina. J. Biol. Chem. 242, 1938-1943.

2. Warner, A.H. and Finamore, F.J. (1967). Nucleotide metabolism during brine shrimp embryogenesis. J. Biol. Chem. 242, 1933-1937.

3. Warner, A.H., Puodziukas, J.G. and Finamore, F.J. (1972). Yolk platelets in brine shrimp embryos. Site of biosynthesis and storage of the diguanosine nucleotides. Exptl. Cell Res. 70, 365-375.

4. Warner, A.H. and Finamore, F.J. (1965). Isolation, purification, and characterization of P^1,P^3-diguanosine 5'-triphosphate from brine shrimp eggs. Biochim. Biophys. Acta 108, 525-530.

5. Oikawa, T.G. and Smith, M. (1966). Nucleotides in the encysted embryos of Daphnia magna. Biochemistry 5, 1517-1521.

6. Warner, A.H. and McClean, D.K. (1968). Studies on the biosynthesis and role of diguanosine tetraphosphate during growth and development of Artemia salina. Dev. Biol. 18, 278-293.

7. Zagalsky, P.F. and Gilchrist, B.M. (1976). Isolation of a blue canthaxanthin-lipovitellin from the yolk platelets of Branchipus stagnalis (L) (Crustacea: Anostraca). Comp. Biochem. Physiol. 55B, 195-200.

8. Warner, A.H., Thomas, Dides S., Shridhar, V. and McCurdy, H.D. (1977). The diguanosine nucleotides: do they exist in aquatic fungi? Can. J. Biochem. 55, 841-846.

9. Rapaport, E. and Zamecnik, P.C. (1976). Presence of diadenosine 5',5'''-P^1,P^4-tetraphosphate (Ap_4A) in mammalian cells in levels varying widely with proliferative activity of the tissue: A possible positive "pleiotypic activator". Proc. Natl. Acad. Sci. U.S. 73, 3984-3988.

10. Zamecnik, P.C., Stephenson, M.L. and Janeway, C.M. (1966). Enzymatic synthesis of diadenosine tetraphosphate and diadenosine triphosphate with a purified lysyl-sRNA synthetase. Biochem. Biophys. Res. Commun. 24, 91-97.

11. Randerath, K., Janeway, C.M., Stephenson, M.L. and Zamecnik, P.C. (1966). Isolation and characterization of dinucleoside tetra- and triphosphates formed in the presence of lysyl-sRNA synthetase. Biochem. Biophys. Res. Commun. 24, 98-105.

12. Van Denbos, G. and Finamore, F.J. (1974). An unusual pathway for the synthesis of adenosine triphosphate by the purine-requiring organism Artemia salina. J. Biol. Chem. 249, 2816-2818.

13. Dennell, R. (1960). Integument and exoskeleton. In The Physiology of Crustacea (Waterman, T.H., ed.), vol. 1, pp. 449-472. Academic Press, New York and London.

14. Glaser, L. and Brown, D.H. (1957). The synthesis of chitin in cell-free extracts of Neurospora crassa. J. Biol. Chem. 228, 729-742.

15. Lunt, M.R. and Kent, P.W. (1961). Evidence for the occurrence of uridine diphosphate N-acetylglucosamine in crustacean tissues. Biochem. J. 78, 128-134.

16. Kochetkov, N.K. and Shibaev, V.N. (1973). Glycosyl esters of nucleoside pyrophosphates. In Advances in Carbohydrate Chemistry and Biochemistry (Tipson, R.S. and Horton, D., eds.), vol. 28, pp. 307-399. Academic Press, New York and London.

17. Buchanan, J.M. (1960). Biosynthesis of purine nucleotides. In The Nucleic Acids (Chargaff, E. and Davidson, J.N., eds.), vol. 3, pp. 303-322. Academic Press, New York and London.

18. Joklik, W.K. (1956). The occurrence of adenine- and adenyl-succinic acid in mammalian liver. Biochim. Biophys. Acta 22, 211-212.

19. Okuhara, E. and Hansen, R.G. (1964). Reinvestigation of bovine liver nucleotide-peptide and separation of adenylosuccinic acid (6-succinoaminopurine-9-ribosyl-5'-phosphate). J. Biochem. 55, 287-292.

20. Carter, C.E. and Cohen, L.H. (1956). The preparation and properties of adenylosuccinase and adenylosuccinic acid. J. Biol. Chem. 222, 17-30.

21. Lieberman, I. (1956). Involvement of guanosine triphosphate in the synthesis of adenylosuccinate from inosine-5'-phosphate. J. Amer. Chem. Soc. 78, 251.

22. Hurlbert, R.B., Schmitz, H., Brumm, A.F. and Potter, V.R. (1954). Nucleotide metabolism II. Chromatographic separation of acid-soluble nucleotides. J. Biol. Chem. 209, 23-39.

23. P-L Biochemicals, Inc. (1967). Ultraviolet absorption spectra of 5'-ribonucleotides. Circular OR-10.

24. Griswold, B.L., Humoller, F.L. and McIntyre, A.R. (1951). Inorganic phosphates and phosphate esters in tissue extracts. Anal. Chem. 23, 192-194.

25. Fiske, C.H. and Subbarow, Y. (1925). The colorimetric determination of phosphorus. J. Biol. Chem. 66, 375-400.

26. Mejbaum, W. (1939). Estimation of small amounts of pentose especially in derivatives of adenylic acid. Z. Physiol. Chem. 258, 117-120.

27. Volkin, E. and Cohn, W.E. (1954). Estimation of nucleic acids. In Methods of Biochemical Analysis (Glick, D., ed.), vol. 1, pp. 287-305. Interscience Publishers, Inc., New York and London.

28. Koch, F.C. and McMeekin, T.L. (1924). A new direct nesslerization micro-Kjeldahl method and a modification of the Nessler-Folin reagent for ammonia. J. Amer. Chem. Soc. 46, 2066-2069.

29. Khym, J.X. and Cohn, W.E. (1960). Characterizations and some chemical reactions of periodate-oxidized nucleosides. J. Amer. Chem. Soc. 82, 6380-6386.

30. Aminoff, D., Morgan, W.T.J. and Watkins, W.M. (1952). Studies in immunochemistry. II. The action of dilute alkali on the N-acetylhexosamines and the specific blood-group mucoids. Biochem. J. 51, 379-389.

31. Morgan, W.T.J. and Elson, L.A. (1934). A colorimetric method for the determination of N-acetylglucosamine and N-acetylchondrosamine. Biochem. J. 28, 988-995.

32. Gardell, S., Heijkenskjöld, F. and Rochnorlund, A. (1950). Oxidation of glucosamine and galactosamine with ninhydrin to arabinose and lyxose and their identification with paper chromatography. Acta Chem. Scand. 4, 970.

33. Pontis, H.G. (1955). Uridine diphosphate acetylgalactosamine in liver. J. Biol. Chem. 216, 195-202.

34. Putman, E.W. (1957). Paper chromatography of sugars. In Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), vol. 3, pp. 62-72. Academic Press, New York and London.

35. Carter, C.E. (1956). Synthesis of 6-succinoaminopurine. J. Biol. Chem. 223, 139-146.

36. Partridge, S.M. (1948). Filter paper partition chromatography of sugars. Biochem. J. 42, 238-248.

37. Manandhar, M.S.P. and Van Dyke, K. (1974). Guanosine triphosphate analysis: PEI-thin layer purification and luciferin-luciferase liquid scintillation quantitation. Anal. Biochem. 60, 122-129.

38. Cashel, M., Lazzarini, R.A. and Kalbacher, B. (1969). An improved method for thin layer chromatography of nucleotide mixtures containing ^{32}P-labeled orthophosphate. J. Chromatog. 40, 103-109.

39. Randerath, K. and Randerath, E. (1967). Thin-layer separation methods for nucleic acid derivatives. In Methods in Enzymology (Grossman, L. and Moldave, K., eds.), vol. 12A, pp. 323-347. Academic Press, New York and London.

40. Michelson, A.M. (1962). Hypochromicity of oligo- and polynucleotides. Biochim. Biophys. Acta 55, 841-848.

41. Finamore, F.J. and Warner, A.H. (1963). The occurrence of P^1,P^4-diguanosine 5'-tetraphosphate in brine shrimp eggs. J. Biol. Chem. 238, 344-348.

42. Cabib, E., Leloir, L.F. and Cardini, C.E. (1953). Uridine diphosphate acetylglucosamine. J. Biol. Chem. 203, 1055-1070.

43. Tornheim, K. and Lowenstein, J.M. (1972). The purine nucleotide cycle. The production of ammonia from aspartate by extracts of rat skeletal muscle. J. Biol. Chem. 247, 162-169.

44. Morris, J.E. and Afzelius, B.A. (1967). The structure of the shell and outer membranes in encysted Artemia salina embryos during cryptobiosis and development. J. Ultrastruct. Res. 20, 244-259.

45. Dutrieu, J. (1960). Observations biochimiques et physiologiques sur le developpement d'Artemia salina leach. Arch. de Zool. Exp. et Gen. 99, 1-134.

46. Nierlich, D.P. and Magasanik, B. (1971). Control by feedback repression of the enzymes of purine biosynthesis in Aerobacter aerogenes. Biochim. Biophys. Acta 230, 349-361.

47. Lehninger, A.L. (1975). Biochemistry, 2nd ed. Worth Publishers, Inc., New York.

48. Warner, A.H. and Finamore, F.J. (1965). Isolation, purification, and characterization of P^1,P^4-diguanosine 5'-tetraphosphate asymmetrical-pyrophosphohydrolase from brine shrimp eggs. Biochemistry 4, 1568-1575.

49. Vallejo, C.G., Sillero, M.A.G. and Sillero, A. (1974). Diguanosinetetraphosphate guanylohydrolase in Artemia salina. Biochim. Biophys. Acta 358, 117-125.

50. Renart, M.F., Renart, J., Sillero, M.A.G. and Sillero, A. (1976). Guanosine monophosphate reductase from Artemia salina: Inhibition by xanthosine monophosphate and activation by diguanosine tetraphosphate. Biochemistry 15, 4962-4966.

51. Miller, R.W., Lukens, L.N. and Buchanan, J.M. (1957). The enzymatic cleavage of 5-amino-4-imidazole-N-succinocarboxamide ribotide. J. Amer. Chem. Soc. 79, 1513-1514.

52. Gots, J.S. and Gollub, E.G. (1957). Sequential blockade in adenine biosynthesis by genetic loss of an apparent bifunctional deacylase. Proc. Natl. Acad. Sci. U.S. 43, 826-834.

53. Adam, A. and Moffat, J.G. (1966). Dismutation reactions of nucleoside polyphosphates. V. Syntheses of P^1,P^4-di(guanosine-5') tetraphosphate and P^1,P^3-di (guanosine-5') triphosphate. J. Amer. Chem. Soc. 88, 838-842.

54. Gilmour, S.J. and Warner, A.H. (1978). The presence of guanosine(5')diphospho(5')guanosine and guanosine (5')triphospho(5')adenosine in brine shrimp embryos. J. Biol. Chem. (submitted).

55. Groner, Y., Grosfeld, H. and Littauer, U.Z. (1976). 5'-capping structures of Artemia salina mRNA and the translational inhibition by cap analogs. Eur. J. Biochem. 71, 281-293.

56. Warner, A.H., Beers, P.C. and Huang, F.L. (1974). Biosynthesis of the diguanosine nucleotides. I. Purification and properties of an enzyme from yolk platelets of brine shrimp embryos. Can. J. Biochem. 52, 231-240.

57. Warner, A.H. and Huang, F.L. (1974). Biosynthesis of the diguanosine nucleotides. II. Mechanism of action of GTP:GTP guanylyltransferase on nucleotide metabolism in brine shrimp embryos. Can. J. Biochem. 52, 241-251.

VITA AUCTORIS

1950: Born in Bath, England, March 29, 1950.

1955-57: Attended Weston St. John's Infant School, Bath, England.

1957-61: Attended Newbridge Junior School, Bath, England.

1961-68: Attended the City of Bath Girls' School, Bath, England.

1968-69: Employed as a laboratory technician in the Botany Department of Bath University, Bath, England.

1969-73: Attended Bath University, Bath, England. Graduated in 1973 with the degree of Bachelor of Science (Honours) in Applied Biochemistry.

1971: Employed at the Fruit and Vegetable Preservation Research Association, Chipping Campden, England to study the pectic enzymes of the potato.

1972: Employed at the Grassland Research Institute, Hurley, England to study lactic acid levels during silage fermentation.

1973: Accepted into the Faculty of Graduate Studies at the University of Windsor, Windsor, Ontario.