

This article was downloaded by:

On: 25 January 2011

Access details: *Access Details: Free Access*

Publisher *Taylor & Francis*

Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Separation Science and Technology

Publication details, including instructions for authors and subscription information:

<http://www.informaworld.com/smpp/title~content=t713708471>

Purification of Nucleoside-5'-diphosphates: A New Ion-Exchange Method

G. F. Huhn^a; J. H. Jensen^a; M. E. Pierce^a; E. M. Schlabach^a

^a Chambers Works the Du Pont Merck Pharmaceutical Company Deepwater, New Jersey

To cite this Article Huhn, G. F. , Jensen, J. H. , Pierce, M. E. and Schlabach, E. M.(1993) 'Purification of Nucleoside-5'-diphosphates: A New Ion-Exchange Method', *Separation Science and Technology*, 28: 11, 1959 — 1970

To link to this Article: DOI: 10.1080/01496399308016726

URL: <http://dx.doi.org/10.1080/01496399308016726>

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: <http://www.informaworld.com/terms-and-conditions-of-access.pdf>

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

Purification of Nucleoside-5'-diphosphates: A New Ion-Exchange Method

G. F. HUHNS, J. H. JENSEN,* M. E. PIERCE, and
E. M. SCHLABACH

CHAMBERS WORKS
THE DU PONT MERCK PHARMACEUTICAL COMPANY
DEEPWATER, NEW JERSEY 08023-0999

ABSTRACT

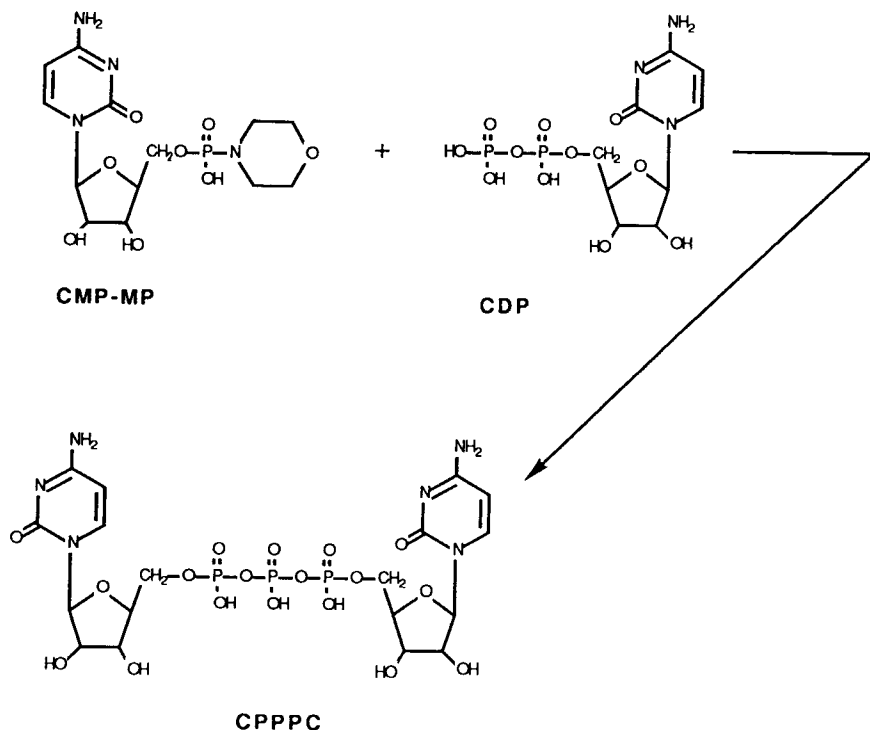
Inosine- and uridine-5'-diphosphates can be purified by ion-exchange chromatography utilizing a new ion-exchange method where Type I ion-exchange resins and the absorbed nucleoside polyphosphates are changed to the basic form by rinsing the column with dilute sodium hydroxide and subsequently eluting with dilute hydrochloric acid and sodium chloride.

INTRODUCTION

Nucleoside-5'-diphosphates (NDPs) (1) containing purine or pyrimidine heterocycles are useful intermediates in the synthesis of biologically active compounds such as oligoribonucleotides. NDPs are also sources of energy in cellular metabolic processes and are important in the biosynthesis of nucleic acids.

NDPs are commonly synthesized by reacting an activated 5'-nucleotide, such as NMP-MP, with multiple equivalents of phosphoric acid tertiary amine salt in an anhydrous organic solvent (2–6). Formation of the synthetic by-products, NPPN and NPPPN, usually occurs from the reaction of the activated 5'-nucleotide with NMP and NDP, respectively. For example, CPPPC results from the reaction of the activated morpholidate, CMP-MP, with CDP formed during the desired reaction of morpholidate with phosphoric acid.

* To whom correspondence should be addressed.

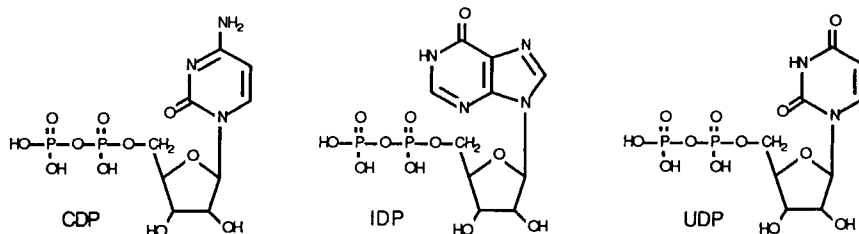


Other impurities present in the solution before purification are the starting NMPs, NTPs, inorganic phosphate, small amounts of morpholine, *N,N'*-dicyclohexyl-4-morpholinocarboxamidine, *N,N'*-dicyclohexyl urea, triethylamine, and the nonaqueous solvent used in the preparation.

NDPs are usually purified by ion-exchange chromatography using strongly basic ion-exchange resins containing quaternary polymer-bound trimethylamine sites (Type 1 resins) (2, 3, 5) or quaternary polymer-bound dimethylmonoethanolamine sites (Type 2 resins) (4, 6). However, it has often been necessary to pretreat the reaction mixtures in various ways to remove solvents and inorganic phosphate before the final ion-exchange purification. Such pretreatments include: vacuum distillation, precipitation of mixtures of various phosphates, adsorption and elution from activated carbon, and extraction with nonpolar solvents such as ether and benzene (1, 3-6).

We wanted to produce many thousands of kilograms of CDP, IDP, and UDP, and we needed to develop purification methods which did not require pretreatment yet gave acceptable yields and purities. These NDPs

were to be used to prepare poly-I and poly-C/U via enzymatic polycondensation for a potential AIDS drug.



After optimizing the reaction sequence as described in the Experimental Section, it was possible to obtain very pure CDP by using a standard ion-exchange procedure followed by crystallization of the hydrochloride salt:

1. Dilute the reaction mass with water and apply to a strongly basic (Type 1) ion-exchange column.
2. Wash the organic material from the column with water.
3. Elute the various phosphate-containing moieties with a combination of aqueous NaCl and HCl.

However, in the case of IDP and UDP we could not separate the NDP from NPPN by using procedures which would be acceptable for commercial-scale production.

Moffatt and Khorana (2) and Smith and Khorana (6) discussed the formation of various NPPNs; however, they purified their materials by using relatively complicated schemes which would be difficult to scale up. Other authors (4, 5, 7) make no mention of NPPN, and we suspect that their analytical techniques were unable to detect these compounds. It is very difficult to determine the concentrations of these compounds even with modern HPLC using ion-exchange columns. These authors used various types of paper chromatography.

EXPERIMENTAL

Raw Materials and Equipment

NMPs were purchased from Yamasa Shoyu Co. and were greater than 98% pure. Inosine-5'-monophosphate (IMP) and uridine-5'-monophosphate (UMP) were purchased as the sodium salts. These compounds had to be converted to the free acids before phosphorylation was used to convert them to IDP and UDP. The free acids were prepared by passing

an aqueous solution of the salt through a cation-exchange resin (Amberlite 200) in the acid form. The eluent was used directly in the subsequent reaction.

Dowex 1×8, a strongly basic (Type 1) ion-exchange resin of 200–400 mesh, was used for all separations after evaluating both Type 1 and Type 2 resins. The resin was conditioned and packed as suggested by Dow Chemical Co.

All other reagents and solvents were reagent grade.

Eluent fractions were collected using a Gilson Model 12 UV/Vis Detector at 254 μm (with a millivolt recorder) and a Gilson Model 202 Fraction Collector. A parallel loop was used to bypass part of the stream around the UV cell to reduce the back pressure.

An in-line pH probe was used to monitor the eluent pH just after the UV detector.

Calibrated FMI piston pumps were used to transfer solutions. Pressure gauges were used in various parts of the system to assure that the working pressure of the glass column was not exceeded. In general, the flow had to be reduced when loading the reaction solution because of high back pressure. The flow could be increased after eluting the organic solvent and salts.

HPLC/ion-exchange analysis was done with Hewlett-Packard Series 1090 HPLC instruments using a DuPont Zorbax® Bio Series SAX column (6.2 mm i.d. \times 8 cm). The compounds were eluted with 0.1 M K_2HPO_4 buffer adjusted to pH 6.5 ± 0.2 with 85% H_3PO_4 .

Chemical Procedures

All previously unknown compounds such as NPPN and NPPPN were prepared as described, and their structures were confirmed by carbon, hydrogen, and phosphorus NMR and mass spectroscopy.

NDPs

The nucleoside-5'-diphosphates were prepared from the phosphomorpholidates by reaction with phosphoric acid. For example: Dimethylacetamide (DMAc) (640 mL) and deionized water (220 mL) were charged to a 2-L round-bottomed flask followed by cytidine-5'-monophosphate (104 g) and a water rinse (20 mL). Morpholine (88 mL) was added and the resulting slurry heated to 80°C, giving a clear solution. *N,N'*-Dicyclohexylcarbodiimide (DCC) (127 g) dissolved in DMAc (135 mL) was added over 17 minutes. Analysis by HPLC indicated 99% conversion to the cytidine-5'-phosphomorpholidate.

The mixture was cooled to 6°C and vacuum filtered to remove most of the dicyclohexylurea. The filter cake was washed with 308 mL DMAc. The combined filtrates were vacuum distilled at 50–60 mmHg up to 85°C until 400 mL distillate solvent was removed. Karl Fischer titration of the residue indicated 0.3% water was present. DMAc (100 mL) was added to the residue, giving 893 g of a solution containing 0.320 mol cytidine-5'-phosphomorpholidate.

An aliquot of the morpholidate solution (222 g, 0.8 mol) was charged to a 500-mL flask. Triethylamine (39 mL), followed by 85% phosphoric acid (27.5 mL), were added. The mixture was heated to 55–65°C, held for 4 hours, cooled to 50°C, and diluted with water (52 mL). The overall yield, determined by HPLC, was 86.6% cytidine-5'-diphosphate based on the starting cytidine-5'-monophosphate.

NPPNs and NPPPNs

The dinucleotide monophosphates were prepared by reacting the nucleotide-5'-mono- or diphosphate with the appropriate nucleotide-5'-phosphomorpholidate to produce NPPN and NPPPN, respectively. An example follows.

CPPPC

CMP (32.5 g), morpholine (27.5 mL), water (75 mL), and DMAc (200 mL) were charged into a 1-L round-bottomed flask. The mixture was heated to 80°C and a solution of DCC (40 g) in DMAc (40 mL) was added over 10 minutes. The reaction was held at 80–85°C for 2 hours, then cooled to room temperature. The dicyclohexylurea by-product was removed by vacuum filtration and washed with 65 mL DMAc. To the combined filtrate was added cytidine-5'-diphosphate (23 g). The mixture was heated to 60–65°C for 24 hours. The mixture was cooled to room temperature, filtered, and diluted to 1.0 L with water.

The dilute dinucleotide solution was loaded onto a 2.5 × 50 cm ion-exchange column containing Dowex Type 1, 200–400 mesh resin. After washing with water (1.0 L), the product was eluted with 0.1 N NaCl and a step gradient of 0.005, 0.01, 0.015, and 0.02 N HCl. Product-rich fractions totaling 1.3 L were adjusted to pH 11 with 1 N NaOH (20 mL). This solution was loaded onto the same ion-exchange column and washed with water (1.0 L). The product was then eluted as the free acid with 0.20 N HCl. Product-rich fractions were combined (655 mL) and slowly added with stirring to ethanol (650 mL). The product was isolated by vacuum filtration, washed with ethanol (100 mL), and dried *in vacuo* at 45°C overnight. Yield: 5.5 g. Purity (HPLC): 95%.

Resin Affinity

Three grams of resin conditioned to the chloride form were added to 38 g of solution containing IMP, IDP, and IPPI. The pH was adjusted as shown in Table 1, and the solution was placed in a rocker for 6 hours. The resin was removed by filtration, and the solutions were analyzed by HPLC.

Ion-Exchange Chromotography

A 60×2.5 cm glass column was packed with 50 cm of Dowex 1×8 strongly basic (Type 1) ion-exchange resin, 200–400 mesh. The resin was conditioned by alternating 2 N HCl and 1.5 N NaOH with appropriate water washings between the exchanges. The resin was equilibrated to the Cl^- form with 2 N HCl and washed free of Cl^- until a negative test for Cl^- was obtained.

A solution of IDP (168.6 g) containing IDP (34.6 mM), IMP (3.3 mM), IPPI (0.69 mM), IPPPI (1.4 mM), ITP (0.44 mM), IMP-MP (0.48 mM), and inorganic phosphate (~ 125 mM) in DMAc with small amounts of morpholine and *N,N'*-dicyclohexyl-4-morpholinocarboxamidine was diluted with water to 1020 g, clarified, and adjusted to pH 6 with 2.4 mL of 50% NaOH. The solution was passed through the ion-exchange column at 23 mL/min. The eluent was monitored by an in-line UV detector and an in-line pH meter. The results are shown in Fig. 2. The resin was washed with water until the unretained materials had eluted and the eluent UV absorbance had become sufficiently low. The flow rate was then increased to 29 to 30 mL/min, and a solution of NaCl (0.12 M) was passed through the column to elute inorganic phosphate and IMP. Aqueous sodium hydroxide (0.06 M) was then passed through the column as the main IMP peak began to elute, and it was continued until IMP ceased to elute and the pH of the eluent increased sharply to greater than 12. The column was washed

TABLE I

| pH | % IMP | % IPPI | % IDP |
|---------|--------|--------|-------|
| Initial | 0.0040 | 0.0024 | 0.74 |
| 4.92 | 0.0041 | 0.0020 | 0.64 |
| 5.96 | 0.0064 | 0.0022 | 0.65 |
| 6.95 | 0.0090 | 0.0023 | 0.67 |
| 8.21 | 0.0079 | 0.0020 | 0.66 |
| 9.53 | 0.0088 | 0.0011 | 0.61 |
| 10.69 | 0.0056 | 0.0000 | 0.60 |
| 11.80 | 0.0048 | 0.0000 | 0.61 |

with water until the pH decreased to 9.65. A solution of sodium chloride (0.2 M) and HCl (0.01 M) was passed through the column, and fractions containing purified IDP were collected when the eluent UV absorbance began to rise; collection was continued until the pH decreased to 8.5. A total of 1903 g of solution was collected and combined, and HPLC analysis of this solution assayed 26 mM IDP of 99.2 HPLC area % purity. The recovery of purified IDP was 75%. The remaining fractions collected after the pH dropped below 8.5 contained between 4.8 and 29.1 HPLC area % IPPI. The column was then washed with HCl (2.0 M) until the absorbance had become sufficiently low. HPLC analysis of the 2.0 M HCl wash showed IPPPI, ITP, and residual IMP, IDP, and IPPI.

RESULTS AND DISCUSSION

Although standard ion-exchange purification yielded fairly good purities for IDP and UDP, usually greater than 98%, the dinucleotides IPPI and UPPU always eluted with IDP and UDP, respectively (Fig. 1).

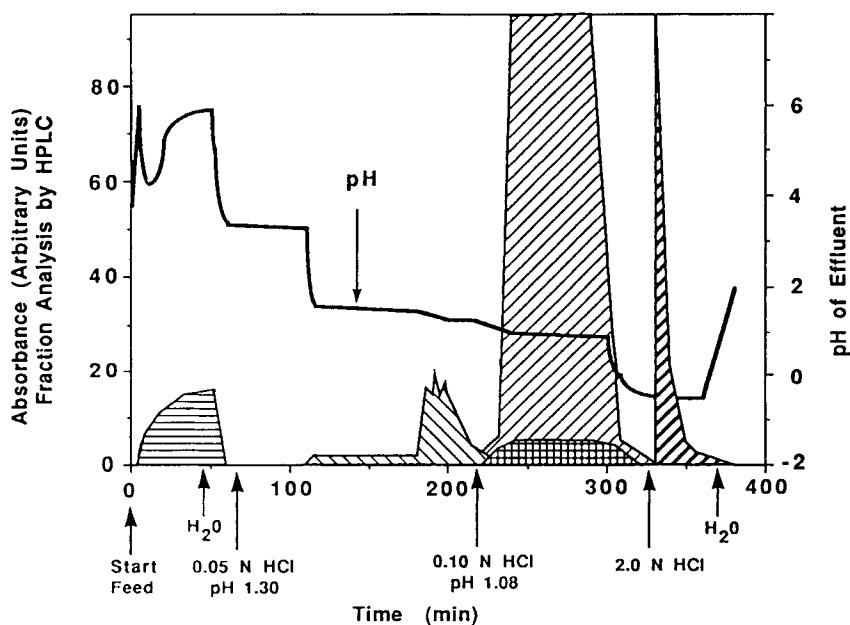


FIG. 1 Elution of (unretained), (IMP, IMP-MP, phosphates), (IDP), (IPPI), and (IPPPI, IPPPI, ITP).

At the time of this work, there was some evidence that IPPI might inhibit the enzyme, PNPase, used to polymerize the NDPs to poly-I and poly-C/U, particularly when polymer-bound enzyme was used for many cycles. However, after the present work was finished, it was shown that this inhibition does not occur (8).

Resin affinity experiments showed that IPPI was selectively adsorbed by Type 1 resin at pHs greater than 9.5 (Table 1). Therefore, we examined various elution schemes to determine if elution at higher pHs would give a good separation of IDP and IPPI (and UDP/UPPU).

Loading the solution at pH 4.8 and eluting with 0.05 to 1.0 N NaOH failed. This was not entirely surprising since OH^- has a poor affinity for Type 1 resins. In addition, at high pH the phosphate groups will be more ionized and thus less likely to be displaced.

Loading the solutions at low pHs and then eluting with 0.05 to 0.1 N NaCl at pH 11.5 gave IDP free of IPPI and IMP; however, the yield was only 55%. It appears that the mono- and disodium salts of IDP eluted, but the trisodium salt remained on the column.

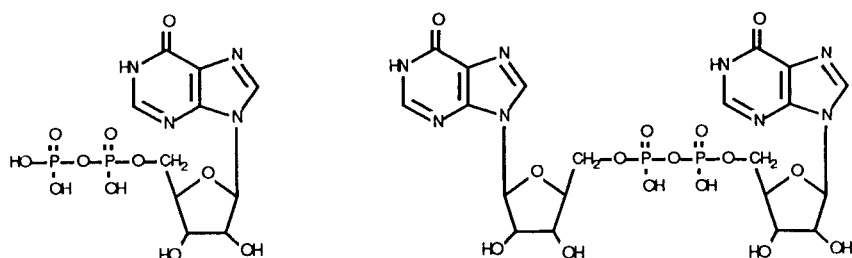
Loading the solution at pH 11.5 and then eluting with neutral NaCl solutions gave mixtures of IMP and IDP with long tailing. Loading the solution at pH 10 and eluting with NaCl solution at pH 4.5 to 3.8 gave similar results.

These results showed that IDP could be separated from IPPI by manipulating the pH of the column. The problem now became one of separating IDP from IMP. The rest of the impurities could be separated easily.

We found that an acceptable separation could be obtained by loading the solution at a slightly acidic pH, eluting IMP and other fast eluting compounds such as DMAc and other organics at low pH and NaCl concentration, converting the column to the hydroxide form, and then eluting the IDP with a weakly acidic NaCl solution. As expected, IPPI did not begin to elute until the end of the IDP peak when the pH had dropped to below 9.0. The difference in the two methods is shown in Figs. 1 and 2.

Figure 1 is a "normal" ion-exchange purification. The solution was loaded at pH 6.0, the column washed with water, and the nucleotides eluted with increasing concentrations of HCl. There was virtually no separation of IDP and IPPI.

In contrast, very pure IDP was obtained by converting the column and the compounds adsorbed on the column to the multicharged form with NaOH and eluting with NaCl and a very low concentration of HCl, as shown in Fig. 2. The recoveries were 75 to 81% of pure IDP in solution. We believe that the mechanism of the separation is due to a subtle difference in the ionization constants of the nucleosides.



At higher pH, multiple negative charges on the nucleosides will give very strong bonding to the column. As the pH is reduced, the nucleosides will be protonated and elute according to the pK_a of each site. The mechanism is not a displacement by hydroxide ion. Neither compound is eluted with hydroxide, and as can be seen from Fig. 2, IDP does not elute until

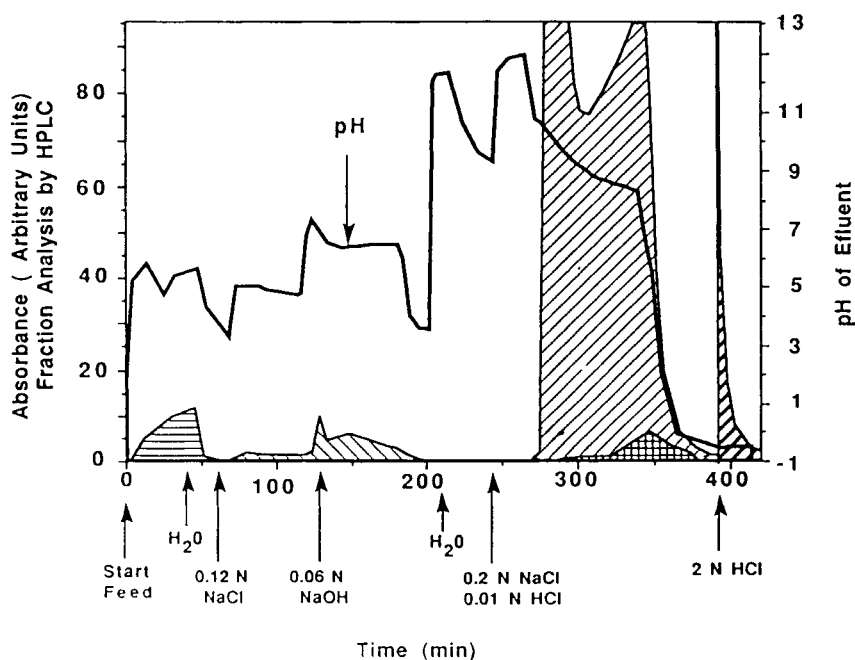
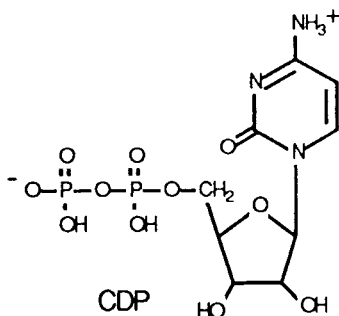


FIG. 2 Elution of (unretained), (IMP, IMP-MP, phosphates), (IDP), (IPPI), and (IPPI, IPPPI, ITP).

the pH has dropped to about 10, and IPPI does not elute until the pH drops to about 8.5. Although not shown, UDP gives exactly the same behavior.

We did not demonstrate this purification scheme for CDP. The standard ion-exchange elution scheme gave pure CDP. We believe that the NH_2 group exists as the zwitterion at the low pHs of the elution and gives the necessary separation.



The purified NDPs were concentrated and desalted by reverse osmosis and then spray-dried from the concentrated solutions. CDP was also crystallized by addition of the concentrated solution to acetone. UDP was crystallized by addition of the concentrated solution to methanol. The concentrated solutions and all of the solid NDPs were polymerized successfully with PNPase to form either poly-I or poly-C/U.

This new ion-exchange method has a second advantage. The NDPs are not very stable in acidic solution, but they are quite stable at higher pHs. Figure 3 shows the rate of decomposition of IDP to IMP as a function of time and pH. IDP is the least stable; however, CDP and UDP have very similar stabilities as a function of pH.

The NDPs elute at a very low pH, approximately 1.0, when the standard elution scheme is used, and they decompose to some extent during processing. This is not a serious concern at the laboratory scale; however, this is a potential problem at the manufacturing scale. This new scheme eliminates this problem because the solutions elute at a pH of 8.5 to 10.0 where the NDPs are very stable.

In summary, we have shown that this new scheme of ion exchange gives very high purity IDP and UDP with the advantage that these NDPs are stable in solution.

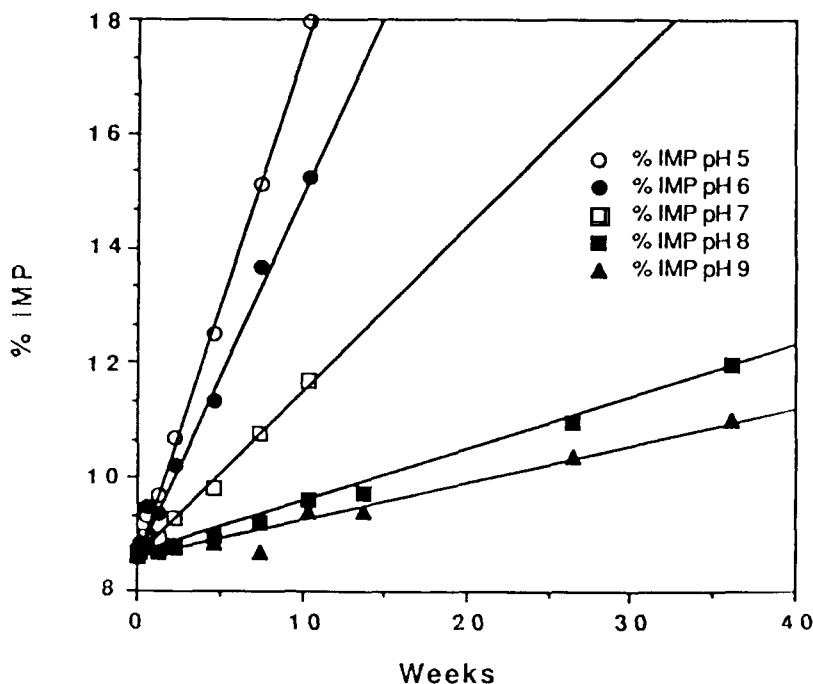


FIG. 3 Stability of IMP as a function of pH and time.

We believe that it may be possible to apply this new technology to the separation of other multivalent compounds, such as other phosphates and perhaps sulfates.

ACKNOWLEDGMENTS

We thank J. R. Matos for polymerizing the NDPs to poly-I and poly-I/C, K. W. Sigvardson for help in confirming the structure by mass spectroscopy and NMR, and Richard Pease for helpful suggestions for the ion-exchange processes.

REFERENCES AND NOTES

1. Abbreviations used in this paper are: nucleoside-5'-monophosphate (NMP); nucleoside-5'-diphosphates (NDP); nucleoside-5'-triphosphates (NTP); 1,2-di-(5'-nucleoside-diphosphate) (NPPN); 1,2-di-(5'-nucleoside-triphosphate) (NPPPN); nucleoside-5'-mono-

phosphomorpholidate (NMP-MP); cytidine-5'-monophosphate (CMP); inosine-5'-monophosphate (IMP); uridine-5'-monophosphate (UMP); cytidine-5'-diphosphate (CDP); inosine-5'-diphosphate (IDP); uridine-5'-diphosphate (UDP); inosine-5'-triphosphate (ITP); *N,N'*-dicyclohexylcarbodiimide (DCC); dimethylacetamide (DMAc).

2. J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 649 (1961).
3. A. M. Michelson, *Biochim. Biophys. Acta*, **91**, (1964).
4. Y. Fujimoto and M. Teranishi, U.S. Patent 3,534,017.
5. H. U. Bergmeyer, U.S. Patent 3,803,125.
6. M. Smith and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 1141 (1958).
7. Y. Fujimota and M. Teranishi, U.S. Patent 3,509,128.
8. J. R. Matos, Private Communication.

Received by editor August 25, 1992