THE CHARACTERIZATION AND STEREOCHEMICAL UTILIZATION OF PHOSPHOROTHIOATES PRODUCED BY CHEMICAL AND ENZYMATIC SYNTHESIS

A Thesis

by

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ABSTRACT

The Characterization and Stereochemical Utilization of Phosphorothioates Produced by Chemical and Enzymatic Synthesis

(August 1987)

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Sucrose synthetase (UDP-glucose-fructose glycosyl transferase, E.C. 2.4.1.13) catalyzes the production of uridine diphosphoglucose and fructose from uridine diphosphate and sucrose. An overall stereochemical course for this enzyme has not been established for the β phosphorus of its substrate uridine diphosphoglucose (UDP-glucose). This thesis presents a method for the determination of this stereochemical course through the use of phosphorothioates.

The phosphorothioate analogues of glucose-1-phosphate and uridine diphospho-glucose (UDP-glucose β(S) or UDP-β(S) glucose) were synthesized, purified, and characterized by ^{31}P NMR and by HPLC. In addition, the overall relative rate kinetics of these phosphorothioates are compared to the natural oxygen analogue for each enzyme studied. Uridine diphosphoglucose-β(S) obtained from uridine diphosphoglucose pyrophosphorylase (E.C. 2.4.2.9) is not a kinetically competent substrate for sucrose synthetase. An attempt to synthesize, specify the opposite isomer by using a combination of galactose-1-phosphate uridylyl transferase (E.C. 2.7.12) and phosphoglucomutase (E.C. 2.7.5.1) with glucose-1-phosphorothioate only produced the identical isomer as determined by high field NMR.

Attempts were also made to produce thiocarbamoyl phosphate, carbamoyl phosphorothioate, and thiocitrulline for possible investigation as substrates of enzymes of the urea cycle (carbamoyl phosphate synthetase E.C. 2.7.2.5, ornithine transcarbamoylase E.C. 2.1.3.3, and arginosuccinate synthetase E.C. 6.3.4.5).

ACKNOWLEDGMENT

I wish to express my appreciation to Dr. Frank M. Raushel, Dr. Thomas O. Baldwin and Dr. John Hogg, for their willingness to serve on my committee.

My deepest gratitude goes to all my colleagues and friends for their support and encouragement which has made the task of researching and writing this thesis much easier to bear.

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INTRODUCTION

Phosphate esters occupy a central role in biochemistry. Not only is the genetic blueprint for all living organisms stored in phosphodiester polymer form as DNA, but di- and tri-phosphates of nucleosides are important energy carriers in that groups are activated by phosphorylation. Since phosphate esters are so prevalent, the mechanisms of phosphoryl transfer are important subjects for study.

The mechanism of any enzyme catalyzed reaction is closely related to its stereochemical course. The knowledge of the stereochemical course of a reaction is recognized as one of the most useful kinds of information for distinguishing among possible mechanisms. For a molecule to be able to convey the stereochemistry of a reaction it must contain prochirality at a position essential to the catalytic mechanism of the enzyme. The tetrahedral phosphorus molecule is not chiral and there are only a few examples of naturally occurring phosphodiesters that are chiral. However, phosphates can be made chiral if the tetrahedral phosphorus is labeled with sulfur, ^{16}O, ^{17}O, and ^{18}O atoms. Shown in Scheme I is a phosphorothioate which contains four different groups about the phosphorus molecule and is thus chiral.

In over thirty stereochemical studies of enzymatic substitution at phosphorus the findings have suggested that there is an in-line

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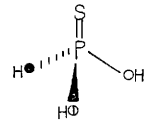
The style and format of the Journal of Biological Chemistry has been used in this thesis.



Scheme I

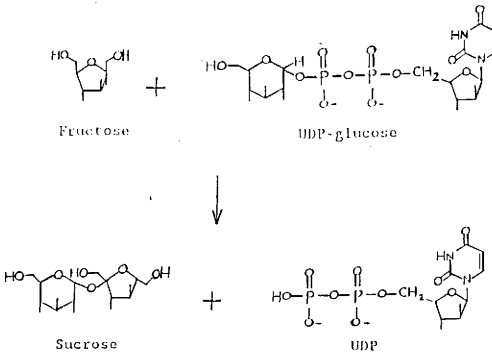
mechanism of reaction which inverts the configuration at the chiral center.[1] The use of chiral phosphorothioates can distinguish between single and double displacement mechanisms at this center. Since all such in-line interactions at chiral phosphorus lead to inversion of configuration, a a single displacement will yield an overall inversion at phosphorus whereas a double displacement will yield retention of the original configuration.

The technique of stereochemical analysis by the use of chiral phosphorothioates was first developed by Eickstein[2] and has been extended and improved upon by a number of researchers.[3,4,5,6,7]

The most pertinent work for our purposes has been that of Knowles et al.[3] and Frey et al.[8] Specifically, Knowles' et al. success with glycolytic enzymes was most important. In proving that pyruvate kinase, glycerol kinase, and hexokinase transfer their phosphorothioate substrates with an identical stereochemical course, Knowles et al. found that some eleven glycolytic enzymes handle phosphorothioates smoothly. Frey et al., were able to chemically and enzymatically produce both the S_p and R_p isomers of uridine diphosphoglucose-α(S). Using these isomers and ^{31}P NMR, they were able to establish an overall stereochemical course for both uridine diphosphoglucose pyrophosphorylase and galactose-1-phosphate uridylyl

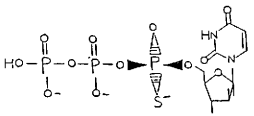
transferase according to Scheme 2. Uridine diphosphoglucose pyrophosphorylase was found to catalyze the reaction with inversion of configuration while galactose-1-phosphate uridylyl transferase proceeded with retention of configuration at the α phosphorus. Thus, two enzymes previously thought to operate by similar mechanisms were shown to follow fundamentally different mechanistic paths.

For our purposes we hoped to employ similar techniques to determine the overall stereochemical course of reactions of sucrose synthetase which catalyzes according to the reaction below.

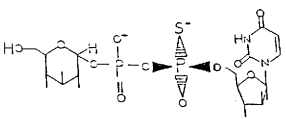


Reaction I

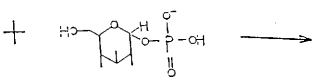
Since almost all glycolytic enzymes handle phosphorothioates well, it is reasonable to assume that a disaccharide phosphorylase like sucrose phosphorylase could handle a phosphorothioate to produce glucose-1-phosphorothioate according to Scheme 4.



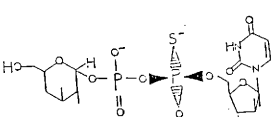
UTPα(S) (R_p)



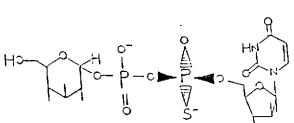
UDPα(S) Glucose (S_p)



Glucose-1-Phosphate

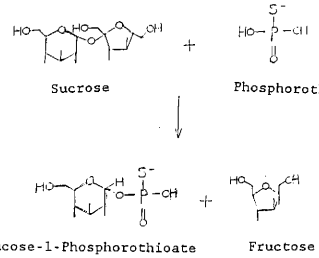


UDPα(S) Galactose (R_p)



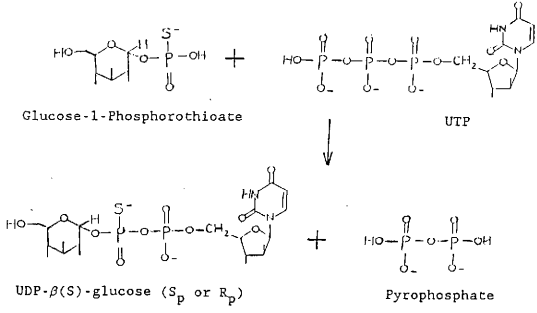
UDPα(S) Glucose (R_p)

Scheme 2.



Scheme 3

Glucose-1-phosphorothioate could then replace glucose-1-phosphate as a substrate in the reaction catalyzed by uridine diphosphoglucose pyrophosphorylase to produce uridine diphosphoglucose-β(S), a molecule that is chiral at the β position. Scheme 4 is shown below.

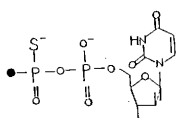


Scheme 4

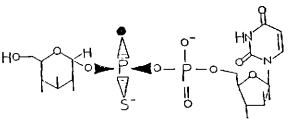
Once uridine diphosphoglucose-β(S) (diastereomer A) is produced, a few questions can be answered with regard to sucrose synthetase. For example, does this enzyme handle phosphorothioates? If sucrose synthetase does catalyze uridine diphosphoglucose-β(S) (diastereomer A) to uridine diphosphate-β(S) then the obvious answer is yes. However, the diastereomer A is not a substrate for sucrose synthetase then there could exist a stereochemical requirement for the opposite diastereomer. To investigate this possibility the synthesis of a mixture of the R_p and S_p diastereomers of uridine diphosphoglucose was undertaken according to modifications of readily available methods.[9] Synthesis of the opposite diastereomer (diastereomer B) could possibly be effected enzymatically by using glucose-1-phosphorothioate as a substrate for galactose-1-phosphate uridylyl transferase, this enzyme operates by a different mechanism than uridine diphosphoglucose pyrophosphorylase.[10]

If neither diastereomer is not a substrate, then sucrose synthetase obviously will not tolerate the substitution of sulfur for oxygen. However, if the diastereomer B is a substrate, then we can investigate this stereochemical requirement by observing the ^{18}O labeling via high field ^{31}P NMR spectroscopy.[7] If uridine diphosphate-β(S), $\beta^{18}O$ can be produced by employing known modifications to synthesize the adenine analogue of this compound[11] then uridine diphosphoglucose-β(S), $\beta^{18}O$ would be produced in the back reaction by sucrose synthetase with either the ^{18}O in a bridging position or in an apical position as in Scheme 5.

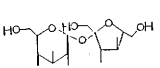
If the ^{18}O occupies a bridging position (S_p isomer) then it will have a bond order of one and we will see a .02 ppm upfield shift for



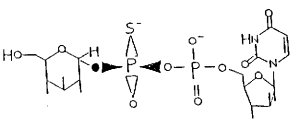
UDPβ(S) R_p and S_p



UDPβ(S) Glucose R_p



Sucrose



UDPβ(S) Glucose S_p

Scheme 5

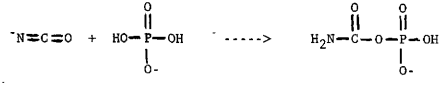
the β phosphorus (relative to no labeling). If the ^{18}O is not bridging and has a bond order of two (as in the R_p isomer) a .04 ppm shift will be observed.[12] Thus careful analysis of the high field NMR spectrum should enable a determination of the stereochemical course of sucrose synthetase.

Other potential studies could include the use of derivatives of carbamoyl phosphate. Carbamoyl phosphate is a key intermediate in both pyrimidine biosynthesis and urea biosynthesis. Carbamoyl phosphate synthetase catalyzes the synthesis of carbamoyl phosphate according to Scheme 6.

$$2\ ATP + HCO_3^- + NH_3 \text{ -----> } 2\ ADP + NH_2COPO_3^= + P_i$$

Reaction 2

Carbamoyl phosphate can also be synthesized chemically by the method of Jones and Lippman according to the scheme.[13]

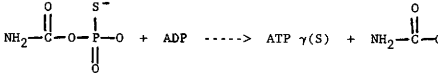


cyanate + orthophosphate — carbamoyl phosphate

Reaction 3

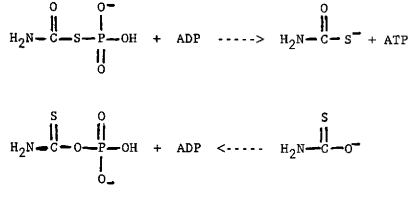
By substituting thio containing reactants for the oxygen containing reactants in this synthesis, it should be possible to synthesize phosphorothioate molecules of interest. The potential significance of these analogues is as follows.

If carbamoyl phosphorothioate is a substrate for carbamoyl phosphate synthetase in the reverse reaction, we could possibly see the production of adenosine triphosphate $\gamma(S)$ as shown in Scheme 6.



Scheme 6

If carbamoyl phosphorothioate is produced with the thio group in the carbonyl position it could prove to be interesting in that a rotational equivalence between the sulfur and oxygen might be seen:

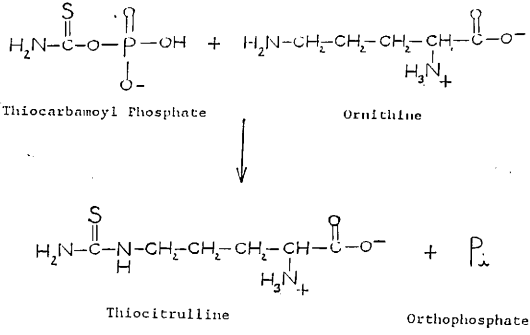


Scheme 7

Previous studies by Raushel and Villafranca[14] have indicated that there is a rotational equivalence of the carboxylate oxygens in carbamoyl phosphate.

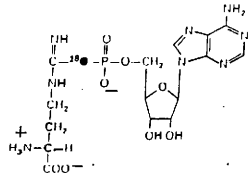
Ornithine transcarbamoylase catalyzes the conversion of carbamoyl phosphate and ornithine to citrulline. If thiocarbamoyl phosphate

can be synthesized, it could prove to be a precursor to thiocitrulline. Thio-carbamoyl phosphate might substitute as a substrate for ornithine transcarbamoylase according to the following scheme.



Scheme 8

Thiocitrulline, which could substitute as a substrate for arginosuccinate synthetase, might possibly provide some insight into the mechanism of this enzyme. Argininosuccinate synthetase has been hypothesized to follow one of three possible mechanisms which are all characterized by the formation of a citrullyl-adenosine monophosphate intermediate[15] shown in Scheme 9.



Scheme 9

Substitution of a sulfur for oxygen in the bridging bond of the citrullyl intermediate will undoubtedly have an effect on this intermediate. If thiocitrulline cannot be synthesized by the above method, it could most likely be synthesized chemically by employing a combination of methods for polypeptide synthesis[16] and those readily available for synthesis of thio ureas.[17,18]

In summary, the objective of this project was to synthesize the aforementioned compounds, purify them through DEAE-cellulose, characterize them through ^{31}P NMR and HPLC, and to investigate the kinetic consequences of their use as enzyme substrates.

EXPERIMENTAL

Decomposition of Carbamoyl Phosphate

Exactly 0.158 g of the dilithium salt of carbamoyl phosphate was weighed out as a solid. The decomposition reaction was begun by the addition of the solid carbamoyl phosphate to 10 mL of 200 mM of PIPES buffer containing 30% D_2O at pH 6.8. Vigorous stirring was applied to ensure that the carbamoyl phosphate dissolved sufficiently. Then 3 mL of this solution was pipetted into a 10 mM NMR tube and the reaction was monitored by ^{31}P NMR spectroscopy at room temperature with the the FT-80 NMR spectrometer at a frequency of 32.203 MHz. Because the peaks for the product orthophosphate and carbamoyl phosphate are separated by only 3 ppm, a small sweep width of 400 Hz was used to view the appearance of the orthophosphate. Other important parameters included an acquisition time of 1.0 second and a seven microsecond pulse width (45° angle). Data were accumulated over a six hundred second interval and were then stored on floppy disk either manually or by the program KINETICS. Kinetic data were acquired by measuring the relative decrease in intensities of the peak associated with the carbamoyl phosphate of the plotted ^{31}P NMR spectrum plotted with respect to time.

The reference orthophosphate peak was established by using 100 mM potassium phosphate in 100 mM PIPES buffer that was 33% in D_2O at pH 6.8. Carbamoyl phosphate was stored over magnesium sulfate in a dessicator below 0 °C when not in use.

Reaction of KOCN with Orthophosphate to Produce Carbamoyl Phosphate

Exactly 0.081 grams (0.001 mole) of solid potassium cyanate was mixed with a 100 mM solution of orthophosphate. This mixture was buffered with 100 mM MES that was 33% in D_2O at pH 5.8 to provide a 100 mM total cyanate solution. Then 3 mL of this 100 mM potassium cyanate reaction mixture was pipetted into a 10 mM NMR tube and the ^{31}P NMR spectrum was then obtained at room temperature by using the FT-80 NMR spectrometer at a frequency of 32.203 MHz. A total of eight frames were obtained over 10 minute intervals (600 transients at 1 second acquisition time) and stored on floppy disk via the KINETICS program. Other NMR parameters were similar to those used for carbamoyl phosphate decomposition experiment except that a sweep width of 2,000 Hz was used and the orthophosphate reference was not centered.

Attempted Synthesis of Thiocarbamoyl Phosphate

Exactly 0.097 g of potassium thiocyanate was added as a solid to 10 mL of a 100 mM MES/33% D_2O solution of 100 mM orthophosphate at pH 5.8. This 100 mM potassium thiocyanate solution was left for 24 hours at room temperature. A 3 mL aliquot of this reaction mixture was then subjected to ^{31}P NMR analysis on the Varian FT-80 NMR. The ^{31}P NMR spectra were obtained using the parameters of the carbamoyl phosphate synthesis experiment.

Reaction of KOCN with Orthophosphorothioate

Exactly 0.203 g (.0025 mole) of potassium cyanate was added to a solution consisting of 250 mM tetra sodium orthophosphorothioate and 250 mM MES buffer that was 33% in D_2O and the pH was adjusted to 5.8.

A 3 mL aliquot of this reaction solution was then pipetted to a 10 mM NMR tube and the reaction followed at room temperature via ^{31}P NMR spectroscopy on the FT-80 NMR spectrometer. ^{31}P NMR parameters were similar to those used to follow the synthesis of carbamoyl phosphate except that an acquisition time of 0.5 seconds was used. Therefore, 1200 transients were accumulated per frame to yield 10 minute time intervals between spectra. Kinetic data were compiled by measuring the relative peak intensity of the emerging orthophosphate peak at 10 minute intervals. Further investigation of this phenomenon was made by monitoring a solution that was only 100 mM in potassium cyanate, but 250 mM in tetra sodium orthophosphorothioate. A similar solution contained the opposite ratio of 250 mM potassium cyanate to 100 mM tetra sodium orthophosphate. The latter reaction was followed for 120 minutes to accommodate the longer reaction time and to confirm the nature of the reaction.

Attempted Removal of Sulfur from AMPS via KOCN

Potassium cyanate was employed as a possible reagent for the removal of sulfur from phosphorothioate nucleotides. Assays were run at 4 different pH levels. Assays at pH 2 and pH 4 were maintained by sodium acetate buffer titrated by HCl to the desired pH. PIPES buffer was used at pH 6.0 and TRIS buffer was employed at pH 8.0. All buffer concentrations were at 100 mM. Assays were also subjected to controlled heating at a constant 50 °C for 24 hours by using a Neslab constant temperature bath. Control samples lacking potassium cyanate were run at each pH level and at room temperature. A typical assay contained a 7 mM concentration of adenosine monophosphorothioate.

The concentrations of potassium cyanate ranged from 7 mM to 200 mM. Any potential progress with this reaction was monitored using a Gilson Model 802B HPLC interfaced with an Apple II computer. UV detection was employed using a detector at 254 nm wavelength. The column employed was a Whatman partisil-10 SAX of the anion exchange type. The elution buffer was orthophosphate at 125 mM in concentration. The pH of the elution buffer was adjusted to 3.5 using phosphoric acid. All other HPLC parameters included a range of 1.0 Aufs, and a flow rate for the elution buffer of 1.0 mL per minute. The program controlled assays were 25 minutes in duration and all injections were 20 μL. A standard of adenosine monophosphate was used to affix the retention times.

Synthesis of n-Butyl Thiourea

The synthesis of n-butyl thiourea was undertaken by a modification of the method of Neville and McGee.[17] Exactly 0.73 grams (0.01 mole, or 1 mL) of dry n-butyl amine were dissolved in dry tetrahydrofuran (THF). Then 0.0025 mole (0.49 grams) of silicon tetra isothiocyanate that was previously dissolved in dry THF was added dropwise with stirring to a three neck 250 mL reaction flask. The reaction flask was sealed under an argon atmosphere and was placed in a water bath to mediate the exothermic reaction. Once the reaction had cooled to room temperature the solution was refluxed under argon for 30 minutes. The THF solvent was removed via rotary evaporation. Dilute isopropyl alcohol (10 mL/2 mL H_2O) was then added to the residue and the whole mixture was refluxed for 30 minutes. Filtration through a course grade sintered glass funnel was

used to remove silica gel. The residue was then washed with 5 mL aliquots of acetone and the resulting liquid was rotary evaporated to a brown oil. Recrystalization was attempted using isopropyl alcohol.

Silicon tetra isothiocyanate was prepared by a modified synthesis of Neville and McGee.[19] Approximately 38 grams (0.5 moles) of dry ammonium thiocyanate was stirred rapidly in a suspension of 200 mL of dry benzene in a sealed 500 ml 3-neck flask filled with argon gas. Exactly 8.5 grams of fresh silicon tetrachloride (0.1 mole) was then added slowly via syringe. Typically, a 5 minute period between 2 mL injections was sufficient to control the reaction. The mixture was then heated under reflux for 3 hours and allowed to cool to 70 °C and then filtered hot through a course glass sintered funnel. Hot THF was then used to wash the compound. The filtrate was distilled to a residual volume of 40 mL and the contents of the reaction flask transferred to a 400 mL beaker. The cold crystals were rapidly filtered, washed with cold THF, pressed and drained, and then weighed (22 grams or a 55% yield). They were stored with in tightly stoppered flask. The crystals of silicon tetra isothio-cyanate melted at 144-146 °C, (Lit. value 144 °C).[17,19] Silicon tetra isothiocyanate was stored in a magnesium sulfate filled dessicator below 0 °C.

An alternative synthesis of the target molecule n-butyl thiourea was explored using a modified method of Nair.[3] A 5 mL portion (0.05 moles) of dry n-butyl amine was slowly added to 6 mL (0.05 mole) of tert-butyl isothiocyanate that was previously dissolved in dry petroleum ether. The reaction vessel was allowed to sit at room temperature for 30 minutes and was then warmed at 30 °C for 2 hours.

Upon returning to room temperature, approximately 7 grams of white crystals precipitated. These crystals were collected and washed with cold petroleum ether, dried, and recrystallized by using a water/ethanol system. The 3-tert-butyl thiocarbamide melted at 91°C (vs 94 °C for the literature value).[18]

A 2.2 g (0.012 moles) sample of the purified 3-tert-butyl thiocarbamide crystals was then subjected to acid hydrolysis using 25 mL of concentrated HCl. The mixture was warmed for 10 minutes at 90 °C.[18] Once cool, the liquid was diluted and neutralized with a sodium bicarbonate solution and the precipitate was washed with benzene to remove the yellow oil from crystals. The remaining solid was recrystallized using a mixture of chloroform and carbon tetrachloride to yield 1.1 gram of colorless crystals that melted at 72 °C. [(lit. value 79°]. TLC was then used to check the purity using 50% ethyl acetate and 50% hexane as solvent. Pre-coated silica gel, 60 F TLC plates from EM Reagents, were employed. Potassium iodide was used to develop the chromatograms.

^{1}H NMR spectra was taken of all starting materials and products using the Varian XL-200 NMR spectrometer operating at a frequency of 200.06 MHz. The acquisition time was 2.5 seconds, the pulse width was 5.0 μseconds, and the sweep width was 3200 Hz. A total of 128 transients were taken and peaks were referenced to zero ppm with TMS. Lock was obtained using $CDCl_3$ solvent.

n-Butyl amine was dried by refluxing under argon atmosphere over calcium hydride for 24 hours. The n-butyl amine was then stored over KOH pellets. Benzene was dried under an argon atmosphere in a

similar way, but was stored over 4° A molecular sieves.

Synthesis of Glucose-1-Phosphorothioate and Kinetic Comparison to its Oxygen Analogue

An assay consisting of 150 μmoles of tetrasodium phosphorothioate and 300 μmoles of sucrose was prepared in 150 mM HEPES buffer that had been titrated to pH 7.5. The deuterium oxide content was 30%. The total volume of the solution was 3 mL. The reaction was initiated by the addition of 6.5 units of sucrose phosphorylase isolated from Leuconostoe mesenteroides. A similar assay was employed using the potassium salt of orthophosphate as a substrate. Exactly 2 units of sucrose phosphorylase were used to initiate this reaction. Both assays were 3 mL in total volume.

The progress of both the glucose-1-phosphorothioate and glucose-1-phosphate reaction assay was monitored on the XL-200 NMR operating at a frequency of 81 MHz. The spectral data were collected in 10 minute frames (500 seconds with 1 second acquisition time). Since the difference in chemical shift for glucose-1-phosphate and orthophosphate is very small, a sweep width of only 300 Hz was employed with the oxygen analogue. However, the difference in chemical shift for glucose-1-phosphorothioate and orthophosphorothioate was much greater. Therefore, a much larger sweep width of 5500 Hz was used. Exactly 18 frames were collected and the data stored for the oxygen analogue experiment. Only 12 frames were collected for the thio analogue because of the large disc space required to store the greater amount of data inherent in a longer sweep width. A five minute delay was imposed between the initial non-enzyme spectrum

and the beginning of accumulations for the first spectrum on the experiment. This was necessary in order to allow time to properly shim the instrument after the addition of sucrose phosphorylase. Other parameters included a transmitter offset of 2400 Hz, a sensitivity enhancement of 0.3, the incorporation of the absolute intensity mode, and a pulse width of 10 μseconds. Data points for the plots for comparison of kinetic relative rates were taken from the integrals of peaks versus time.

Isolation and Purification of Glucose-1-Phosphorothioate

A large scale synthesis of glucose-1-phosphorothioate was used to prepare enough material for purification. This consisted of a 25 mL reaction mixture that was 75 mM in orthophosphorothioate, 100 mM in sucrose, and 200 mM in HEPES buffer at pH 7.5. This assay was also 30% in D_2O. Some 15 units of sucrose phosphorylase were added to initiate the reaction. The reaction was then checked periodically by ^{31}P NMR spectroscopy on the FT-80 using similar parameters as mentioned for earlier experiments. Once the orthophosphorothioate was totally consumed (about 24 hours) the mixture was placed in an Amicon ultrafiltration membrane cone and centrifuged for 30 minutes using a Dupont Sorvall RC-5B refrigerated centrifuge to remove sucrose phosphorylase. The supernatant solution was then sealed in several 8 mL plastic test tubes and stored at -78 °C.

Glucose-1-phosphorothioate was purified using a 50 x 3.0 cm column of DEAE-52 cellulose. A 2 mL solution containing 150 μmoles of glucose-1-phosphorothioate was diluted to 500 mL with dionized water and then titrated to pH 7.8 using dilute KOH. This was then

loaded onto the column at a flow rate of 2 mL/min. The column was eluted with a 3.2 liter gradient of TEA/CO_2 from 50 mM to 350 mM. The TEA/CO_2 buffer was adjusted to pH 7.5 with dry ice. The flow rate of the elution buffer through the column was 2 mL/min. Glucose-1-phosphorothioate eluted behind glucose-1-phosphate at a buffer concentration between 110 and 150 mM.

Since glucose-1-phosphorothioate contains no UV or visible chromophore, detection in the column fractions was accomplished using modifications of 3 methods; the Bochner spray method,[20] the colormetric method of Ames,[21] and the reaction of Ellmans reagent [DTNB,5',5-dithio bis(2-nitrobenzoic acid] with terminal phosphorothioate groups.[22] Both the Bochner and Ames method rely upon free phosphate reaction with molybdate with the reduction of this complex accomplished by ascorbate to produce a blue color detectable at 660 nm. Ellmans reagent will produce a yellow color that can be detected at 412 nm only if a terminal phosphorothioate is present.

The modified Ames method employed the following procedure: 1 drop of concentrated HCl was added to a 0.3 mL aliquot of a fraction collected from the column and this mixture was then boiled for 25 minutes. Then 0.7 mL of a mixture consisting of 1 part 10% ascorbate to 6 parts 0.42% molybdate in 1N H_2SO_4 was then added to the boiled 0.3 mL aliquot. This 1 mL solution was then warmed for 20 minutes at 45 °C and then diluted to 3 mL and the absorbance measured at a 660 nm wavelength. Each sample was checked against a blank consisting of water and mix.

The Bochner spray assay consisted of the following mixture: 2 g of ammonium molybdate were dissolved in 200 mL of methanol with heating and vigorous stirring. The molybdate did not dissolve totally, but when the solution was warm to the touch, 20-30 mL of concentrated nitric acid was added which facilitated the dissolution of the molybdate. After this solution had cooled, approximately 2 g of ascorbate were added and the solution swirled until the ascorbate was totally dissolved. Then approximately 2 g of para-amino benzoic acid was added and allowed to completely dissolve. This produced a harvest gold colored solution that remained stable for 45 minutes. After this time the solution turned to green and then later to red. Once the color of the solution was red, the mix was essentially useless. The chromatograms used were 3.5 inch by 1 inch strips of P.E.I. cellulose plates. The plates were developed in 1.5 M $LiCl/H_2O$ solution. The Bochner spray assay produces a blue color upon reaction with free phosphate. All spots were checked against reference standards. The reference standards had the following R_f values: glucose-1-phosphate; 1.0, glucose-1-phosphorothioate; 0.5, orthophosphate; 0.5, and orthophosphorothioate; 0.2.

The Ellmans reagent assay was considered the easiest to use and the most accurate and so was employed more often than the others. A typical assay consisted of a 100 mL solution that was 2 mM in DTNB. This assay was buffered at pH 7.5 by 50 mM in HEPES. A ratio of 9 parts of this solution was used to 1 part column fraction (usually .9 mL mix to .1 mL fraction) and the resulting mixture was checked at 412 nm on a Gilford 260 spectrophotometer. Fractions identified as

terminal phosphorothioate positive were pooled and rotary evaporated to dryness and then washed with three successive 10 mL aliquots of dry methanol. The resulting residue was taken up in 5 mL of buffer solution and the pH adjusted to 9.0. The glucose-1-phosphorothioate was then analyzed quantitatively via Ellmans reagent to determine the concentration. Decoupled and non-decoupled ^{31}P NMR spectra were taken using the XL-200 NMR spectrometer and parameters already described earlier. The purified material was then stored at -78 °C as a 28 mM solution.

Synthesis of Uridine Diphosphoglucose-β(S) via Uridine Diphosphoglucose Pyrophosphorylase

Glucose-1-phosphorothioate was tested as a substrate for the enzyme uridine diphosphoglucose pyrophosphorylase from Bakers Yeast. A typical 4 mL reaction mixture for the kinetic assays consisted of 4 mM uridine triphosphate (UTP), 4 mM magnesium chloride, 3 mM dithiothreitol (DTE, Cleland's reagent) and 2 mM glucose-1-phosphorothioate. Exactly 15 units of inorganic pyrophosphatase from Bakers Yeast were incorporated into the assay to degrade pyrophosphate to orthophosphate as it was formed. The reaction was initiated by the addition of 2 units of uridine diphosphoglucose pyrophosphorylase. The glucose-1-phosphate assay was similar except that dithiothreitol was not used and the reaction was initiated by only .25 units of uridine diphosphoglucose pyrophosphorylase.

The progress of both reaction assays was followed on a Gilson Model 811 HPLC employing a Whatman 10 SAX partisil anion exchange column. The disappearance of the peak corresponding to uridine

triphosphate was recorded and the area under the curve was then used to determine the rate of the reaction. The HPLC scale used was 0.2 Aufs, the column elution buffer was 450 mM orthophosphate at a pH of 3.5. The injection volume was 20 μL. All runs were at room temperature and followed at a wavelength of 254 nm.

Purification and Characterization of Uridine Diphosphoglucose-β(S)

A large scale reaction mixture for the synthesis of uridine diphosphoglucose-β(S) consisted of a 25 mL stock solution that was 50 mM in glucose-1-phosphorothioate, 60 mM in uridine triphosphate, 50 mM in magnesium chloride, 50 mM in dithiothreitol, and 200 mM in HEPES buffer at pH 7.5. The assay also contained 100 units of inorganic pyrophosphatase. The reaction was initiated by the addition of 20 units of uridine diphosphoglucose pyrophosphorylase. At random time intervals the progress of the reaction was measured by removing 0.1 mL aliquots and diluting them to 2 mL for analysis by HPLC. 20 μL of this solution was then injected onto the column and the disappearance of the uridine triphosphate peak checked. HPLC parameters were the same as those used for the kinetic assays. After the uridine triphosphate had been consumed, the reaction mixture was centrifuged through an Amicon ultrafiltration membrane cone.

Approximately 150 μmoles of uridine diphosphoglucose-β(S) (3 mL of solution) was then diluted to 400 mL with distilled H_2O and the pH adjusted to 7.5. This solution was then loaded onto a 50 x 3.0 cm column of DEAE-52 cellulose anion exchange material and the column was then eluted with a 3.2 liter linear gradient of TEA/CO_2 from 50 mM to 400 mM concentration. The fractions collected were 20 mL in volume.

Those containing uridine diphosphoglucose and uridine diphosphoglucose-β(S) were identified by their recorded UV absorbance at 254 nm. Uridine diphosphoglucose-β(S) eluted well behind uridine diphosphoglucose between buffer concentrations of 220 to 270 mM. Fractions containing uridine diphosphoglucose-β(S) were pooled and rotary evaporated to dryness and then washed 3 times with 10 mL of methanol. The residue was then taken up in 30% D_2O/HEPES buffer at pH 9.0. ^{31}P NMR spectra of the purified compounds were recorded on the XL-200 NMR operating at a frequency of 81 MHz. Other parameters included a sweep width of 7000 Hz, an acquisition time of 2.3 seconds, a pulse width of 15 μseconds, and a transmitter offset of 1,000 Hz. A total of 2,000 transients were completed. Uridine diphosphoglucose-β(S) was then stored at -78 °C as a 25 mM solution in plastic test tubes.

Glucose-1-phosphorothioate was tested as a substrate for the galactose-1-phosphate uridylyl transferase enzyme isolated from adapted yeast. The galactose-1-phosphate uridylyl transferase reaction assay included a phosphoglucomutase/glucose-6-phosphate dehydrogenase coupling system to remove glucose-1-phosphate as it was released from galactose-1-phosphate uridylyl transferase. This assay was 3 mL in total volume and consisted of 3 mM glucose-1-phosphorothioate, 1 mM uridine diphosphoglucose, 5 mM nicotinamide adenine dinucleotide, 4 mM of magnesium chloride, glucose 1,6 diphosphate and 75 mM HEPES buffer pH adjusted to 7.5. The enzymes used were 50 units of phosphoglucomutase from rabbit muscle, and 15 units of glucose-6-phosphate dehydrogenase from Bakers Yeast. Two units of galatose-1-phosphate uridylyltransferase were added last to initiate the

reaction. An alternative assay was utilized that did not incorporate the glucose-6-phosphate dehydrogenase enzyme and its potentially bothersome nicotinamide adenine dinucleotide (NAD^+) which would most likely coelute with uridine-diphosphoglucose-β(S).

Synthesis of Uridine Diphosphoglucose-β(S) from Galatose-1-Phosphate Uridylyl Transferase

The appearance of uridine diphosphoglucose-β(S) was monitored at 254 mM using a Gilson 811 HPLC with a Whatman Partisil 10-SAX Anion exchange column. The elution buffer was 75 mM P_i at a pH of 4.5. The flow rate was 1.0 mL per minute, the range 0.1 Aufs, and a typical run of 20 minutes was long enough to allow the uridine diphosphoglucose-β(S) to elute from the column. All injections were 20 μL in volume.

Purification and Characterization of Uridine Diphosphoglucose-β(S) from Galactose-1-Phosphate Uridylyl Transferase

In order to obtain a sufficient quantity of UDP-β(S)-glucose for further study a large scale synthesis was employed. The large scale assay consisted of 30 μmoles of glucose-1-phosphorothioate, 20 μmoles of uridine diphosphoglucose, 40 μmoles of magnesium chloride, glucose 1,6-diphosphate, and 125 mM HEPES buffer at pH 7.5. Exactly 250 units of phosphoglucomutase were used. The reaction was initiated by the introduction of 40 units of galactose-1-phosphate uridylyl transferase. The total volume of the assay was 40 mL. The reaction was followed for 36 hours or until the assay developed a cloudy tint.

The solution containing 8 μmoles of uridine diphosphoglucose-β(S) was diluted to 400 mL with H_2O and pH adjusted to 7.5. This

solution was then loaded onto a 50 x 3.0 cm column of DEAE-52 cellulose. The column was eluted with a 3.2 liter linear gradient of TEA/CO_2 at a pH of 7.5 from 50 mM to 400 mM. The 20 mL fractions were collected in 160 tubes. Uridine diphosphoglucose-β(S) eluted well behind uridine diphosphoglucose and was collected in tubes 95 through 100. The uridine diphosphoglucose-β(S) was then rotary evaporated to dryness at 20°. The residue was dissolved 3 times with 10 mL of dry methanol and rotary evaporated to dryness. This step was repeated 2 more times. The resulting residue was then dissolved in 3 mL of orthophosphate buffer that was 30% in deuterium oxide. The pH of the buffer solution was 9.0.

The pure uridine diphosphoglucose-β(S) was then examined by ^{31}P NMR spectroscopy. Spectra were taken on the XL-400 NMR spectrometer operating at a frequency of 162 MHz. Other parameters included an acquisition time of 1.2 seconds, a pulse width of 15 μseconds, and a transmitter offset of 2900 Hz. The 300 transients were accumulated with reference to orthophosphate buffer.

To determine the stereochemical make-up of the β phosphorus position, a portion of 23 mM uridine diphosphoglucose-β(S) made from uridine diphosphoglucose pyrophosphorylase was diluted and then mixed with the same compound made via galatose-1-phosphate uridylyl transferase. A ^{31}P NMR spectrum was taken within the region from -39.6 to -40.6 ppm to examine for diastereomers.

Uridine Diphosphoglucose-β(S) Assay with Glycogen Synthetase

Purified uridine diphosphoglucose-β(S) from the uridine diphosphoglucose pyrophosphorylase reaction was tested as a substrate

for glycogen synthetase from rabbit muscle. The reaction assay incorporating uridine diphosphoglucose-β(S) was as follows: 1 mM uridine diphosphoglucose β(S), 6 mg of glycogen and 50 mM HEPES buffer at pH 7.5. A catalytic amount of glucose-6-phosphate was also included for the glucose-6-phosphate dependent form of the enzyme. The reaction was catalyzed by 1 unit of glycogen synthetase. The assay using the natural substrate was similar. Incorporated into this assay were 1 mM uridine diphosphoglucose, 6 mg solid glycogen 50 mM HEPES buffer at pH 7.5, glucose-6-phosphate, and 1 unit of glycogen synthetase. The total volume of both assays was 3.0 mL.

The progress of both reactions assays was followed on a Gilson 811 HPLC. The parameters included an elution buffer of 250 mM phosphate at pH 4.5, a flow rate through the Whatman Partisil 10-SAX anion exchange column of 1.0 mL per minute, and a range of .7 Aufs. The chart speed was 5 mm per minute, the collect time was 15 minutes, and each injection was 20 μL. Upper limits were derived from the uridine diphosphoglucose-β(S) reaction by comparison to the reaction of the oxygen analogue and by estimating the least amount of product, uridine diphosphate (UDP) that could be confidently detected at the range of Aufs used.

Uridine Diphosphoglucose-β(S) Assay with Sucrose Synthetase

Uridine diphosphoglucose was tested as a substrate for sucrose synthetase that was isolated from wheat germ. The enzyme used in this experiment contained 0.76 units per mL. A typical assay involving uridine diphosphoglucose as a substrate consisted of the following: 2 mM uridine diphosphoglucose-β(S), 4 mM dithiothreitol, 50 mM fructose, and 1 unit or 1.3 mL of enzyme solution. The solution was buffered

by 50 mM of HEPES at a pH of 7.3. A control was run for this experiment consisted of the assay listed above except that fructose was omitted. The assay for the reaction of uridine diphosphoglucose with sucrose synthetase consisted of the following: 2 mM UDP-glucose, 50 mM fructose, and 0.7 units of enzyme solution. This assay was also buffered at pH of 7.5 by 50 mM HEPES. Total volume of both assays was 4.0 mL. Each reaction was initiated by addition of fructose. The HPLC parameters for this experiment were exactly as those used for the glycogen synthetase experiment. Upper limits for detection were also calculated in a similar manner as for the glycogen synthetase experiment.

Glucose-1-Phosphorothioate Assay with Phosphoglucomutase

The activity of phosphoglucomutase was checked on a Gilford 260 UV-VIS spectrometer by coupling the product glucose-6-phosphate to a glucose-6-phosphate dehydrogenase enzyme and following the reduction of NAD^+ to NADH at 340 nm. A typical assay consisted of 5 mM glucose-1-phosphate, 2 mM cysteine, 1 mM magnesium chloride, glucose-1,6 diphosphate, and 10 mM NAD^+. The enzymes used were 2 units of phosphoglucomutase and 10 units of glucose-6-phosphate dehydrogenase. The total reaction volume of 3 mL was effectively buffered by 20 mM HEPES at pH 7.5.

The reaction assay using glucose-1-phosphorothioate consisted of the following: a crude sample of 30 mM in concentration of glucose-1-phosphorothioate that was also 20 mM cysteine, 1.5 mM of magnesium chloride, and contained glucose-1,6 diphosphate. Sucrose phosphorylase had been removed by centrifugation using an Amicon

ultrafiltration membrane cone. The pH of this enzyme assay was 7.4 throughout the experiment. The reaction was buffered for a concentration of 100 mM HEPES that contained 25% D_2O. The reaction was initiated by the addition of 15 units of phosphoglucomutase. An assay containing glucose-1-phosphate was also run and consisted of 50 mM glucose-1-phosphate, 20 mM cysteine, 1.5 mM magnesium chloride, and glucose-1,6 diphosphate. This assay was also buffered at 100 mM HEPES and 30% deuterium oxide at pH 7.5. The reaction was initiated by the addition of 5 units of phosphoglucomutase.

The reactions were followed by ^{31}P NMR spectroscopy using the FT-80 multi-nuclear instrument. ^{31}P NMR parameters for the FT-80 included a frequency of 32.203 MHz, a sweep width of 200 Hz, an acquisition time of 0.5 seconds, and a pulse width of 7 μseconds. Exactly 1200 transients were accumulated to allow for 10 minute frames to be collected and stored. Upper limits were established using the best estimate of what could easily be seen in the signal to noise ratio.

Materials Used

The following chemicals were purchased from the Aldrich Chemical Company: deuterium oxide, deuterated chloroform, trimethylsilane, ammonium molybdate, ascorbate, and p-amino benzoic acid. Alpha Chemical Company purchases included: potassium cyanate, potassium thiocyanate, tetra sodium orthophosphorothioate, and silicon tetra isothiocyanate. Purchases from Sigma Chemical Company included: sucrose, DEAE-cellulose, P.E.I. cellulose plates, triethyl amine, Ellmans reagent, NAD^+, NADH, adenosine monophosphate and adenosine triphosphate. The buffers TRIS, PIPES, and HEPES were all purchased

from Sigma Chemical Company as were all the enzymes used except sucrose synthetase which was purified by Dr. A.H. Singh. Adenosine monophosphorothioate was provided by Tim Shull as a 28 mM solution. Dry THF was kindly provided by Dr. Martin E. Newcomb's laboratory. All other chemicals were purchased from Fisher Scientific.

RESULTS

Synthesis of Carbamoyl Phosphate and Related Reactions

The reaction of potassium cyanate and orthophosphate was followed by ^{31}P NMR spectroscopy as shown in Figure 1. The carbamoyl phosphate appears with time at 3.2 ppm upfield from orthophosphate. The reaction was followed for 90 minutes and the time course for the synthesis of carbamoyl phosphate was computed from the intensities of the peaks of carbamoyl phosphate as a percentage of the total phosphate resonance. Figure 2 shows a plot of this time course.

Potassium thiocyanate was then substituted for potassium cyanate in an attempt to produce thiocarbamoyl phosphate. This reaction failed. After 24 hours the ^{31}P NMR spectrum showed in Figure 3 does not differ significantly from the original orthophosphate spectrum. There are no other peaks that could account for thiocarbamoyl phosphate.

When the orthophosphorothioate was substituted for orthophosphate, a reaction was clearly visible by ^{31}P NMR spectroscopy. Figure 4 shows that there is a disappearance of the orthophosphorothioate peak and a corresponding appearance of a peak in the region associated with orthophosphate. Further investigation was undertaken by altering the concentration of reactants. Figure 5 shows how the reactants were varied and how this affected the rate and final product composition. When excess potassium cyanate was used, orthophosphorothioate was totally and rapidly converted to orthophosphate. Shown in Figure 6 is the result of allowing this reaction assay to incubate for 90 minutes. The formation of a peak,

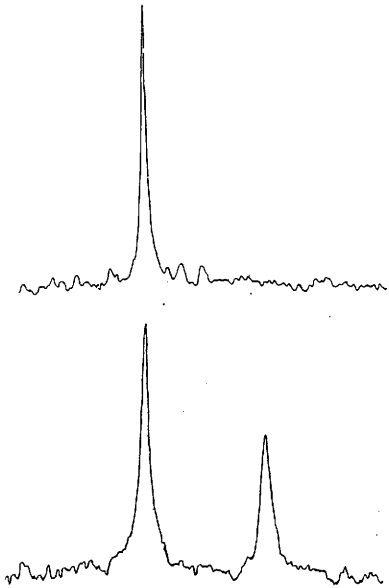


Figure 1: ^{31}P NMR spectra at 32.2 MHz showing the formation with time of carbamoyl phosphate (bottom spectrum) from orthophosphate (top spectrum). The difference between the peaks is 3.2 ppm.

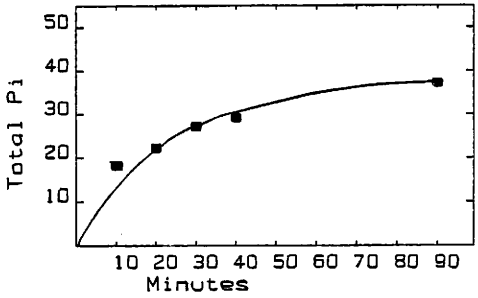


Figure 2: Time course of carbamoyl phosphate synthesis followed by ^{31}P NMR.

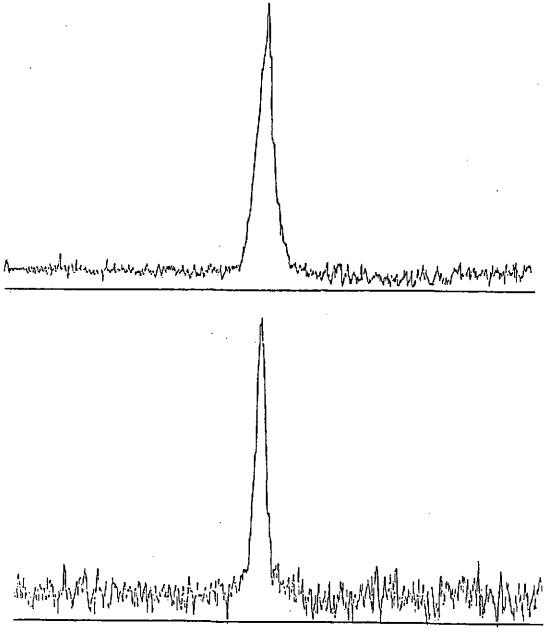


Figure 3: ^{31}P NMR spectra at 32.2 MHz showing orthophosphate at time zero (top spectrum) and again after 24 hours (bottom spectrum). The initial spectrum is essentially unchanged.

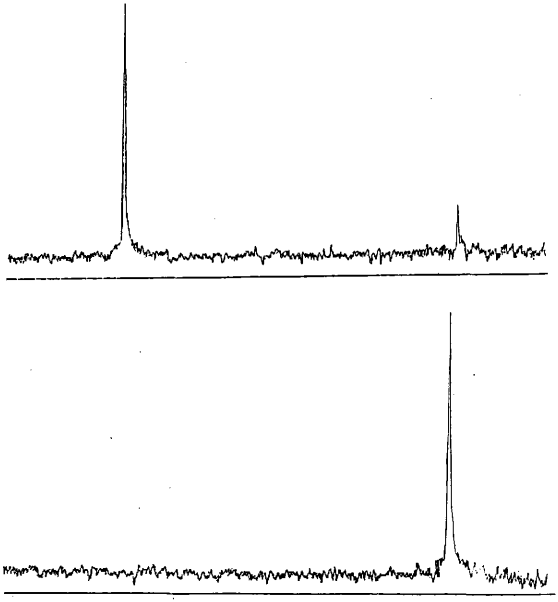


Figure 4: ^{31}P NMR spectra of orthophosphorothioate at time zero (note contaminating orthophosphate) is shown in the top spectrum. The bottom spectrum shows the results after 10 minutes had passed since the addition of potassium cyanate in a 2:1 ratio (200 mM to 100 mM of orthophosphorothioate).

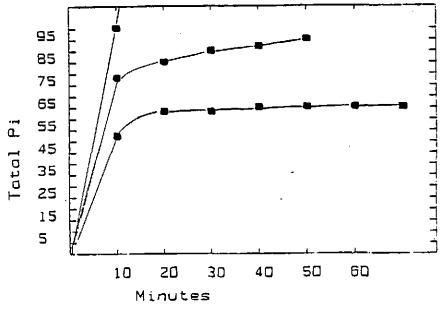


Figure 5: The time course of the reaction of orthophosphorothioate with cyanate at different ratios of potassium cyanate to orthophosphorothioate. Top: 2:1, middle: 1:1, and bottom: 1:2. Points were acquired by a comparison of the ^{31}P NMR peak intensities vs time.

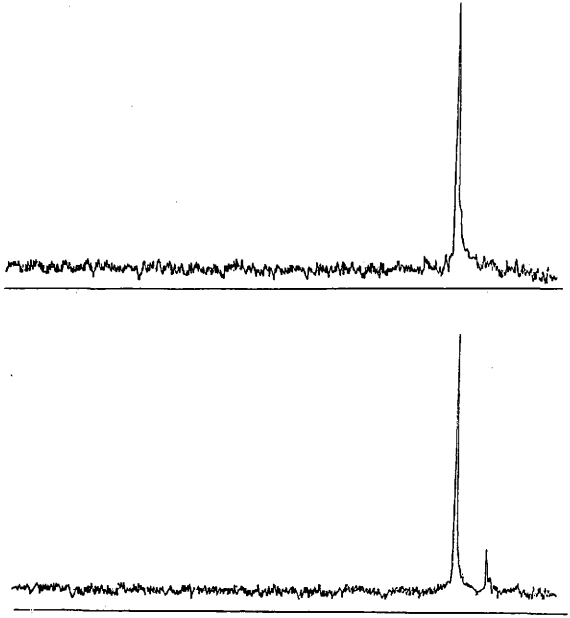


Figure 6: The ^{31}P spectrum at 32.2 MHz shows the peak resulting from the reaction of potassium cyanate and thiophosphate (top). After 90 minutes the formation of a peak 3 ppm from the peak in the top spectrum is seen (bottom). A comparison to Figure 1 suggests that the more downfield peak is orthophosphate while that peak forming at 3 ppm upfield is carbamoyl phosphate.

at 3.2 ppm from orthophosphate, confirms that orthophosphate has been produced.

Several attempts were made to try to utilize potassium cyanate in a desulfurization reaction as a method for the possible introduction of O^{17} or O^{18} isotopes of oxygen into phosphate esters as according to Scheme 10.

$$O = C = N \quad + \quad AMP(S) \quad \text{-----}> \quad AMP(O) \quad + \quad NH_3 \quad + \quad O = C = S$$

Scheme 10

The pH as well as the potassium cyanate concentration and assay temperature were all varied in different combinations. No conditions were found that resulted in the loss of sulfur from AMP(s).

Attempted Synthesis of Thiocitrulline

After the attempt to produce thiocarbamoyl phosphate failed, we attempted the chemical synthesis of thiocitrulline. It was hoped that thiocitrulline could be easily synthesized by modifications to the method of Nevell and McGee shown in the Scheme 11.

$$Si \quad (N = C = S)_4 \quad + \quad R{-}NH_2 \quad \text{-----}> \quad R{-}\underset{H}{N}{-}\overset{\overset{S}{\|}}{C}{-}NH_2$$

Scheme 11

However, crystals of n-butyl thiourea were very difficult to obtain when this method was employed. An efficient method of recrystallizing the brown oil that was isolated was not devised. However, more favorable results were obtained by using the modification of the method of G.V. Nair as shown in Scheme 12.

1) $N=C=S + R\text{-}NH_2$ ----------> substituted, tert butyl thiocarbamide

2) $R'\text{-}N(H)\text{-}C(=S)\text{-}N(H)\text{-}R$ ----------> hydrolysis $H_2N\text{-}C(=S)\text{-}N(H)\text{-}R$ + t-butylchloride

Scheme 12

The tert-butyl thiocarbamide was easily obtained and recrystallized in excellent yield. However, the hydrolysis of this thiocarbamide produced a messy product that had to be washed and recrystallized several times in order to obtain a compound that melted within a reasonably close range to the accepted literature value for the target compound n-butyl thiourea. The proton NMR of the recrystallized compound did not yield encouraging results. The pertinent spectra are shown in Figures 7 and 8. Although the spectrum of the final isolated compound does contain resonances one would expect for an alkyl thiourea, it also contains other nitrogen containing species that are very difficult to attribute to a pure compound. The method of Nair was deemed an insufficient method for the synthesis of thiocitrulline.

Synthesis and Characterization of Glucose-1-Phosphorothioate

Orthophosphorothioate was submitted to the action of sucrose phosphorylase in the presence of excess sucrose. Orthophosphate was likewise used as a substrate to yield a comparison to the enzymes natural substrate. Since the reaction assay did not contain an easily identifiable chromophore the rate of the reaction was monitored by following the ^{31}P NMR spectrum and recording the

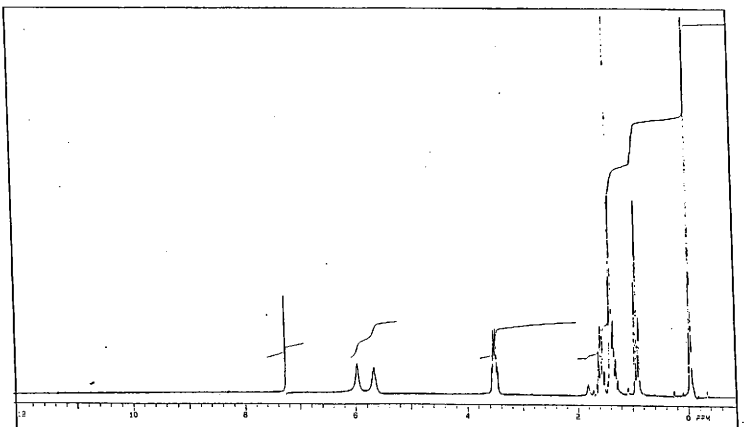


Figure 7: ^{1}H NMR at 200 MHz of n-butyl, t-butyl thio carbamide.

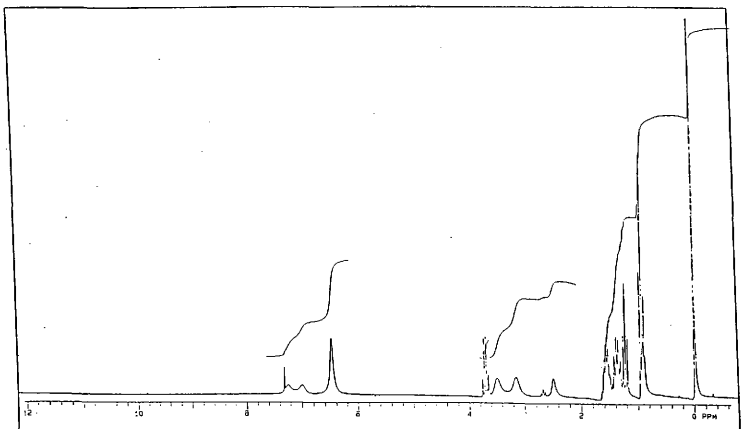


Figure 8: ^{1}H NMR at 200 MHz of the compound hydrolyzed from n-butyl, t-butyl thio carbamide. "Humps" at 7.0, 6.5, and 3.3 could be attributed to nitrogen containing compounds.

integrals of the peaks over time. In Figure 9, the ^{31}P NMR spectrum does show that orthothiophosphate is well utilized as a substrate, but the rate of the reaction of the thio analogue is much slower than that of the oxygen analogue (Figure 10). The overall rate of reaction for the thio analogue was found to be 0.13 μmoles per minute whereas that for the oxygen analogue was 0.54 μmoles per minute. Therefore, the thio analogue was utilized at only 24% of the reaction rate that was seen with orthophosphate as a substrate.

The purification of glucose-1-phosphorothioate was achieved by DEAE cellulose chromatography and was confirmed by comparing the ^{31}P NMR spectrum of pre-column assay material to post-column material as shown in Figure 11. The purified sample is essentially free of phosphates except for the peak at -45.9 ppm that represents glucose-1-phosphorothioate. The undecoupled ^{31}P NMR spectra yielded a single doublet with a coupling constant of 9.72 Hz versus 6.16 Hz for the oxygen analogue. Approximately 150 μmoles of crude assay material were loaded onto the column and 125 μmoles were isolated for a 83% yield.

Glucose-1-Phosphorothioate as a Substrate for Phosphoglucomutase

Glucose-1-phosphorothioate was tested as a substrate for phosphoglucomutase according to Scheme 13.

glucose-1-phosphorothioate glucose-6-phosphorothioate

Scheme 13

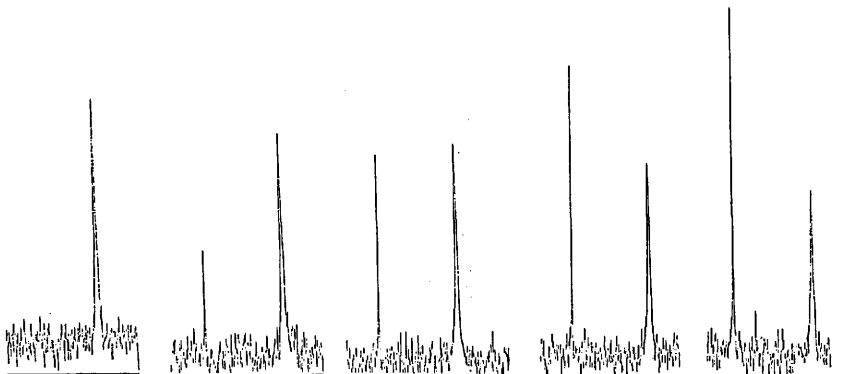


Figure 9: Shows the ^{31}P NMR at 81 MHz of the reaction of orthophosphorothioate and sucrose catalyzed by sucrose phosphorylase. Glucose-1-phosphorothioate is easily seen approximately 10 ppm downfield of orthophosphorothioate after 10 minutes (second frame from left). The glucose-1-phosphorothioate peak continues to grow with time as seen in the successive frames.

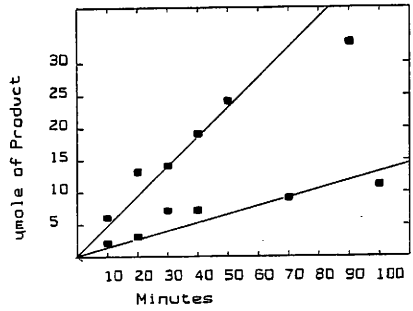


Figure 10: A comparison of the observed rate of the orthophosphate (top line) and orthophosphorothioate (bottom line) as μmoles of product produced per unit per minute. The points were taken from the integrals of the peaks obtained from the ^{31}P spectra.

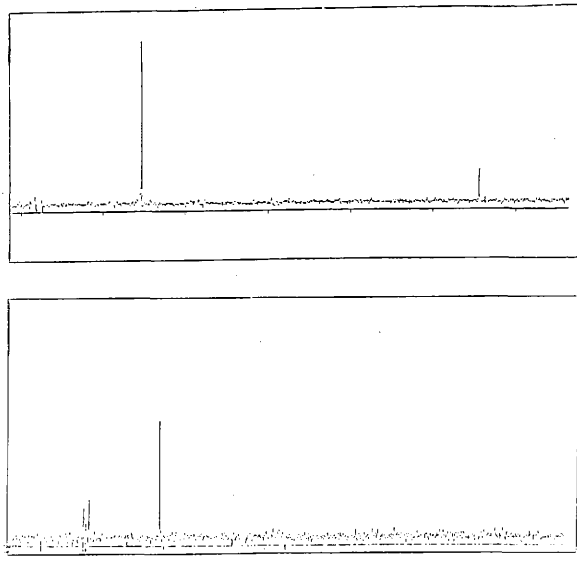


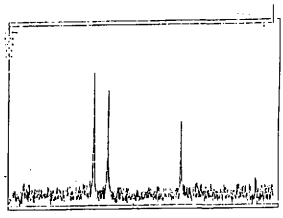
Figure 11: The ^{31}P NMR spectrum (top) of glucose-1-phosphorothioate (at -45.9 ppm) before undergoing column purification. The ^{31}P NMR spectrum (bottom) after column purification does not contain glucose-1-phosphate (at -5 ppm).

This would have enabled us to monitor the conversion of orthophosphorothioate to glucose-1-phosphorothioate on a smaller and perhaps more accurate scale by coupling glucose-1-phosphorothioate to glucose-6-phosphate dehydrogenase enzyme and consequently following the reduction of NAD^+ to NADH. However, phosphoglucomutase does not accept glucose-1-phosphorothioate as a substrate. The ^{31}P NMR spectrum shown in Figure 12 remains essentially unchanged in the glucose-1-phosphorothioate region at room temperature with time even after the addition of the dithiothreitol. However, it is easily noticed that there has been a reaction between the glucose-1-phosphate and the enzyme to produce glucose-6-phosphate. The upper limit for the reaction of glucose-1-phosphorothioate with phosphoglucomutase was calculated to be less than 1.25% of the rate of the oxygen analogue.

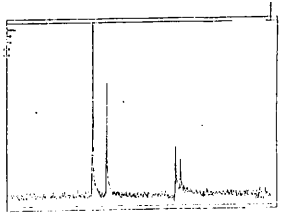
Synthesis and Characterization of Uridine Diphosphoglucose-β(S) Glucose from Uridine Diphosphoglucose Pyrophosphorylase

Glucose-1-phosphorothioate was subjected to the action of yeast uridine diphosphoglucose pyrophosphorylase in the presence of excess uridine triphosphate. The inclusion of inorganic pyrophosphatase insured that the pyrophosphate produced would be hydrolyzed to orthophosphate. Dithiothreitol was imperative to the success of all assays that contained glucose-1-phosphorothioate. Dithiothreitol was not needed for assays that included glucose-1-phosphate. According to Figure 13, glucose-1-phosphorothioate was utilized at 12.5% of the rate of the natural substrate glucose-1-phosphate. Glucose-1-phosphorothioate had a rate of 0.02 μmoles per minute vs 0.16 μmoles

A. glucose-1-phosphorothioate, orthophosphorthioate, and glucose-1-phosphate before addition of phosphoglucomutase.



B. 1 hour after the addition of phosphoglucomutase: glucose-6-phosphate has formed slightly downfield from glucose-1-phosphate.



C. After 24 hours only glucose-1-phosphorothioate is seen. Spectrum resembles A except that glucose-1-phosphate has been converted to glucose-6-phosphate.

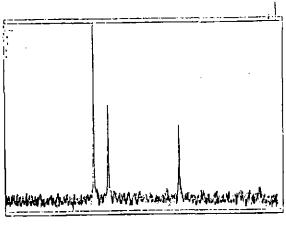


Figure 12: ^{31}P NMR spectra at 32 MHz.

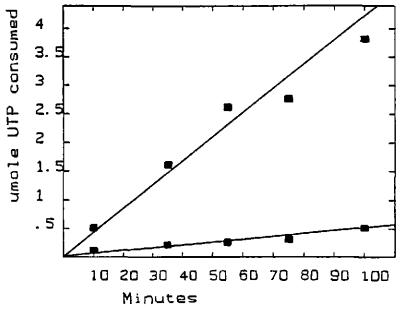


Figure 13: A comparison of the observed rate of the glucose-1-phosphorothioate and glucose-1-phosphate as μmoles of UTP consumed per unit per minute. These points were taken from the integrals of the HPLC peaks corresponding to UTP.

per minute for the oxygen analogue for a given amount of enzyme.

Purification of uridine diphosphoglucose-β(S) is shown in Figure 14, and was achieved by DEAE cellulose chromatography with the elution profiles recorded at 254 nm showing clear separation between uridine diphosphoglucose and uridine diphosphoglucose-β(S). 270 μmoles of uridine diphosphoglucose-β(S) were loaded onto the column and 190 μmoles were isolated for a 75% yield. The ^{31}P NMR spectra are shown before and after column purification in Figure 15. The spectrum shows that uridine diphosphoglucose-β(S) has been purified from orthophosphate, uridine diphosphoglucose-β(S), and any unreacted uridine triphosphate. Doublets are seen at -45.9 and 11.0 ppm for the thio analogue and 8.6 and 10.4 for the oxygen analogue. The ^{31}P NMR also yielded coupling constants of 27.81 for Jα, β(S) and Jα, β vs 20.25 Jα, β, respectively. (Literature value 20.75).[4]

Uridine Diphosphoglucose-β(S) as a Substrate for Glycogen Synthetase and Sucrose Synthetase

Uridine diphosphoglucose-β(S) was tested as a substrate for both sucrose synthetase and glycogen synthetase (E.C. 2.4.1.11) enzymes. The reaction for glycogen synthetase is shown below.

$$\text{Uridine diphosphoglucose} + (\text{glycogen})_n \text{ -----> } \text{UDP} + (\text{glycogen})_{n+1}$$

The reactions were followed by HPLC and monitored for the appearance of any peak with a retention time similar to uridine diphosphate (approximately 11.5 minutes). Both sucrose synthetase and glycogen synthetase catalyzed the natural uridine diphosphoglucose substrate

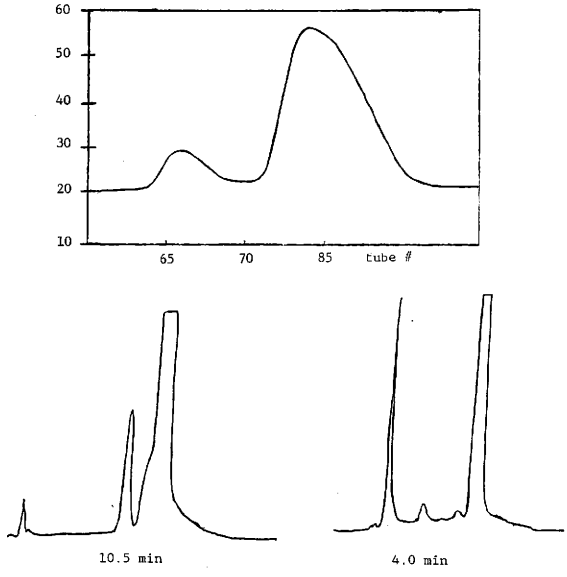


Figure 14: The purification of uridine diphosphoglucose-β(S) on DEAE-cellulose. The HPLC spectrum on the left shows uridine diphosphoglucose-β(S) before purification (peak at 10.5 minutes is UDP-glucose). The HPLC trace on the right shows uridine diphosphoglucose-β(S) after purification.

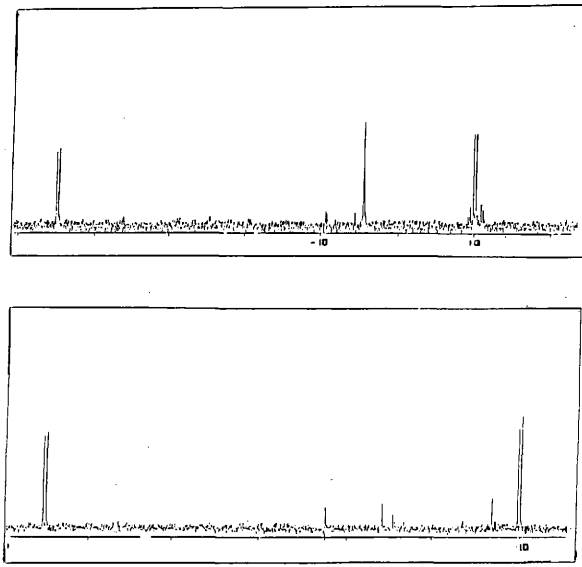


Figure 15: ^{31}P NMR at 81 MHz of uridine diphosphoglucose-β(S). The figure at the top is unpurified material. The figure at the bottom shows the result of the column purification: uridine diphosphoglucose and orthophosphate have been removed.

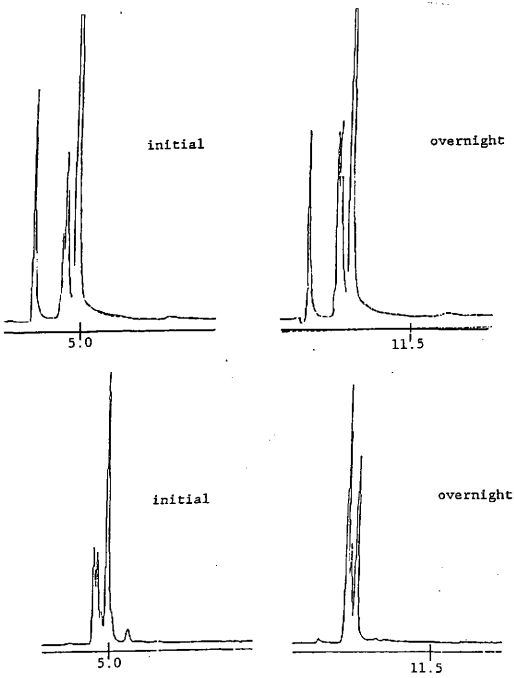


Figure 16: Reaction of uridine diphosphoglucose-β(S) with sucrose synthetase (top) and fructose shows no appearence of uridine diphosphoglucose-β(S). The control without fructose is shown at bottom. Uridine diphosphoglucose and uridine diphosphate-β(S) eluted at 5.0 minutes.

very efficiently with sucrose synthetase producing uridine diphosphate at .149 μmole per minute and with glycogen synthetase producing uridine diphosphate at .054 μmole per minute. No uridine diphosphate-β(S) could be detected by HPLC (Figures 16 and 17). It would be safe to conclude from the data that at best uridine diphosphoglucose-β(S) is an extremely poor substrate for sucrose synthetase and glycogen synthetase. The upper limits for the reaction were calculated at less than 0.01% of the rate of the natural substrate for sucrose synthetase and less than 0.2% of the rate of the natural substrate for glycogen synthetase.

Glucose-1-Phosphorothioate as a Substrate for Galactose-1-Phosphate Uridylyl Transferase

Glucose-1-phosphorothioate was submitted as a substrate for galactose-1-phosphate uridylyl transferase. The process of the reaction is outlined in Figures 18, 19, and 20. This reaction was followed by HPLC and the results are shown in Figure 21. The reaction proceeded to 75% completion with a rate of .0021 μmoles per minute. It was determined that the glucose-6-phosphate dehydrogenase enzyme was not essential in order to produce uridine diphosphoglucose-β(S). This enabled us to scale up the reaction assay for characterization of this substrate.

The uridine diphosphoglucose-β(S) from galactose-1-phosphate uridylyl transferase was purified on a column of DEAE cellulose according to methods used to purify the uridine diphosphoglucose-β(S) produced from uridine diphosphoglucose pyrophosphorylase. Eight μmoles were loaded onto the column and six μmoles were isolated for

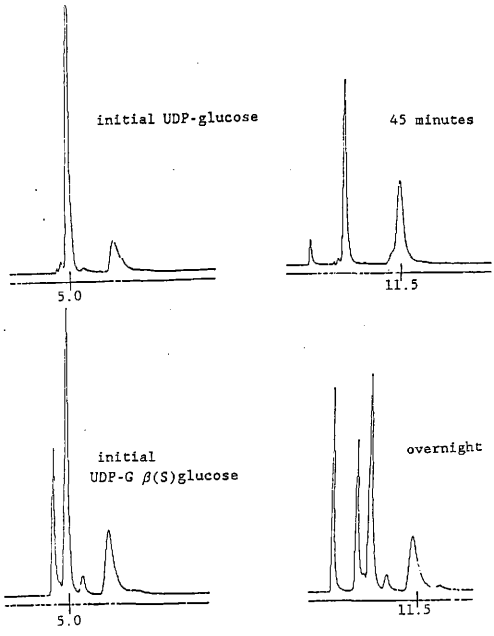


Figure 17: HPLC comparison of UDP-glucose (top) and UDP-G β(S) glucose (bottom) with glycogen synthetase. UDP is quite visable after 45 minutes (second frame at top at 11.5 minutes retention time) but there is not any UDP like peak with UDP-β(S) glucose as substrate (second frame at bottom) even after 24 hours.

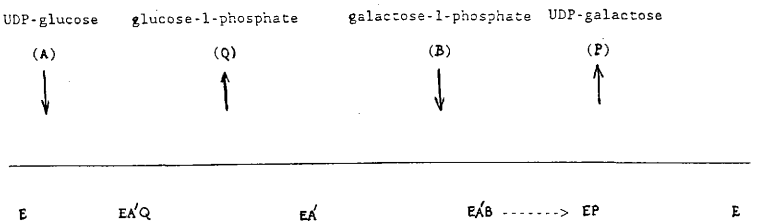


Figure 18: Bi-Bi order for binding of uridyl transferase enzyme showing binding of substrates and release of products.

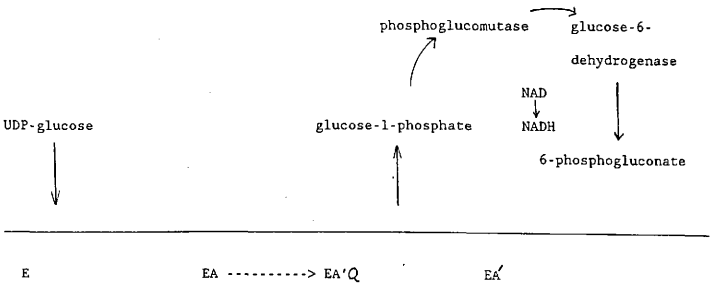


Figure 19: Full enzyme substrate complex is not allowed to form since galactose-1-phosphate was not included in the assay. UDP-glucose is bound, E ----> EA, and glucose-1-phosphate is released as UMP remains bound as EA'. Glucose-1-phosphate is then coupled with phosphoglucomutase and glucose-1-phosphate dehydrogenase to formed non-substrate 6-phosphogluconate.

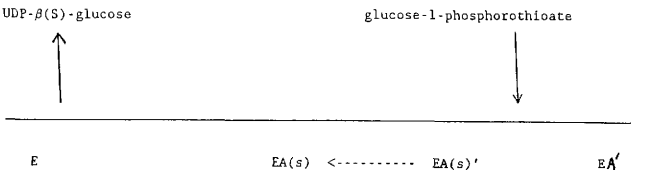
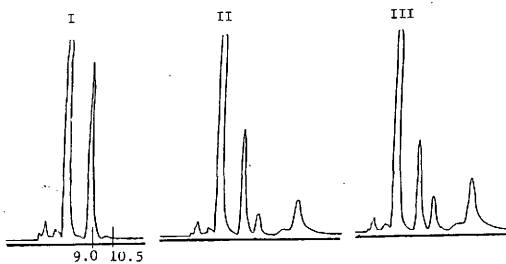


Figure 20: Excess glucose-1-phosphorothioate is then bound by the EA-like complex (EA(s)' enzyme and reforms the complex (sulfur is now substituted at the β phosphorus position). The EA'(s) complex is then converted to the EA(s) complex and UDP-β(S) glucose is released.



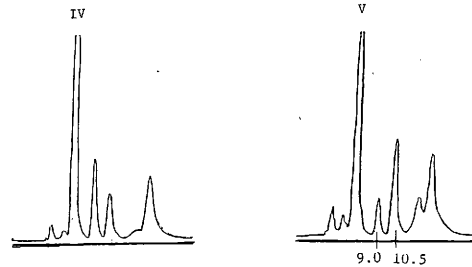


Figure 21: Shown are five frames from the HPLC. I is at time 0. II is at 100 minutes after the addition of the enzyme. III is at 170 minutes and IV is at 240 minutes since the addition of enzyme. V shows the HPLC after 24 hours. Retention times were as follows: NAD^+, 6.5 minutes; NADH, 15 minutes; uridine diphosphoglucose, 9.0 minutes; and uridine diphosphoglucose-β(S) at 10.5 minutes.

a 75% yield. The ^{31}P NMR spectrum showed that uridine diphosphoglucose-β(S) contained no other phosphorus containing compounds except for the orthophosphate buffer.

In order to best test for diastereomerism, an authentic sample of uridine diphosphoglucose-β(S) from the uridine diphosphoglucose pyrophosphorylase reaction (Figure 22) was mixed with that produced by galactose-1-phosphate uridylyl transferase (Figure 23) and the ^{31}P NMR spectrum of the mixture was recorded. The resulting ^{31}P NMR spectra shown in Figure 24 yielded a single doublet between -38 ppm to -43 ppm instead of the doublet of doublets that one would expect for a mixture of two diastereomers. Therefore, the uridine diphosphoglucose-β(S) produced from uridine diphosphoglucose pyrophosphorylase and that produced by uridylyl transferase are the same isomer unless the ^{31}P resonances are coincidentally identical.

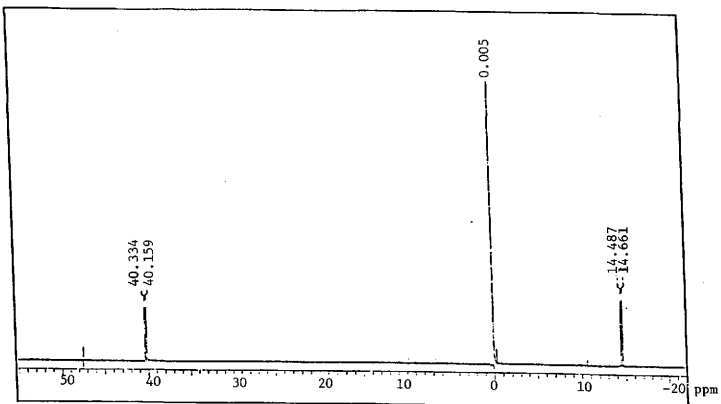


Figure 22: ^{31}P NMR from uridine diphosphoglucose pyrophosphorylase. Doublets appear at -40.2 ppm and 14.5 ppm when referenced to orthophosphate buffer.

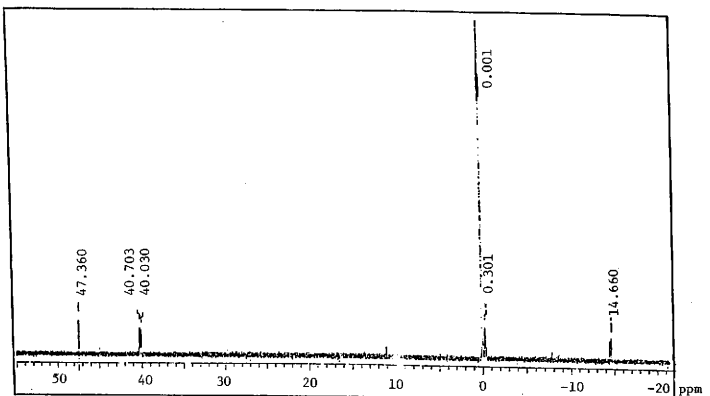


Figure 23: ^{31}P NMR from galactose-1-phosphate uridylyl transferase. Doublets appear at -40 ppm and 14.6 ppm when referenced to orthophosphate buffer.

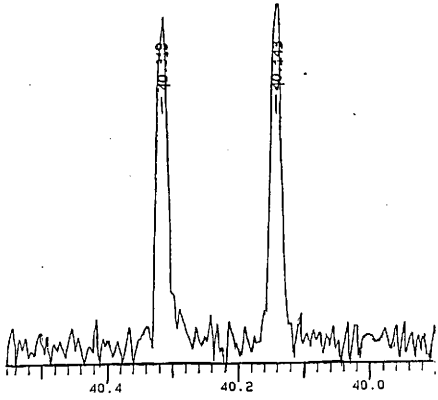


Figure 24: The ^{31}P NMR spectrum of the region from -39.9 to -40.5.

DISCUSSION

The Synthesis of Carbamoyl Phosphate Derivatives

The synthesis and decomposition of carbamoyl phosphate have been well studied. In our investigation we found that the synthesis of carbamoyl phosphate could be followed on the FT-80 accurately. Any differences from the literature rate of synthesis most likely would arise from differences in method. Jones et al., employed the Fiske-Subbarrow method for analysis of free phosphate.[13] However, carbamoyl phosphate reacts with molybdate to produce free phosphate. This could lead to an artificially high concentration of orthophosphate and thus a net slower appearance of carbamoyl phosphate. Once the reliability of the ^{31}P NMR method was established, our attention turned toward the synthesis of target compounds thio carbamoyl phosphate and carbamoyl phosphorothioate.

Thiocarbamoyl phosphate failed to form from thiocyanate and orthophosphate at the optimum synthesis pH of 5.8. However, in the attempt to form carbamoyl phosphorothioate from cyanate and orthophosphorothioate, we encountered surprising results. It was clear from the ^{31}P NMR that the phosphorothioate monoanion was being catalyzed to orthophosphate since the contaminating orthophosphate peak grew in direct proportion to the decrease in the phosphorothioate peak. This suspicion was confirmed when an excess of cyanate produced a new resonance at 3 ppm upfield from orthophosphate. This could only have happened had the excess cyanate reacted with orthophosphate to produce carbamoyl phosphate. Obviously the simple mechanistic

scheme outline envisioned earlier does not apply to this reaction. The mechanistic pathway of Figure 25 is a more likely approach.

It was conceivable that the highly electropositive carbonyl carbon of cyanate is attacked by the nucleophilic sulfur of the phosphorothioate to form an intermediate compound highly susceptible to P-S bond cleavage which could then lead to the formation of carbon oxysulfide or carbon dioxide (depending on the rate sulfur is "washed out" during the reaction) and ammonia. Catalyzed desulfurizations are known in the literature.[23] This mechanism is also consistent with gas build up that was observed in the reaction vessel.

Since the above reaction undoubtedly involves the substitution of an oxygen from a water molecule for sulfur on the phosphorothioate, we attempted to repeat this experiment using adenosine monophosphorothioate. This could have proven to be an effective method for the introduction of O^{18} or O^{17} labeling. However, with adenosine monophosphorothioate the loss of sulfur was not catalyzed. The cause of this failure was not investigated.

Attempted Synthesis of Thiocitrulline

We were unsuccessful in our attempts to synthesize a n-butyl thiourea precursor to thiocitrulline. The failure of the synthesis of n-butyl thiourea according to the method of Nevel and McGee[17] could be attributed to the inability to produce totally anhydrous conditions in the reaction mixture. Since the starting material of silicon tetraisothiocyanate reacts readily with H_2O to produce SiO_2, the conditions must be strictly anhydrous for success. Our experience with silicon tetraisothiocyanate showed it to be

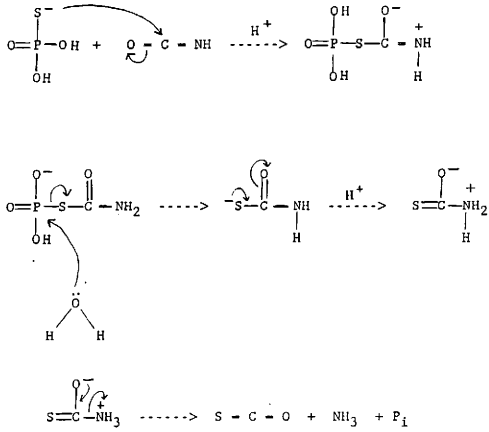


Figure 25: Mechanistic scheme showing the formation of thiocarbamoyl phosphorothioate with the sulfur in the bridging position. This molecule then is possibly decomposed to carbonyl oxysulfide, ammonia, and orthophosphate.

extremely sensitive to atmospheric moisture. Silicon tetra-isothiocyanate will decompose from an off-white color to bright orange when left to the open air even after a few seconds. This extreme sensitivity to moisture made the method of Nevel and McGee[17] unsuitable for work with the milligram quantities which we expected to use with the blocked ornithine.

The method of G.V. Nair[18] displayed some initial success. The intermediate disubstituted n-butyl, t-butyl thiocarbamate was successfully synthesized in high yield and purity. However, the hydrolysis of this compound yielded a sample that was difficult to purify by recrystalization. Since the Nair method involves the formation of an alkyl cation it is reasonable to assume that there must exist some degree of carbocation rearrangement that could lead to a mix of similar alkyl monosubstituted thioureas. The disappointing results with the hydrolysis of the thiocarbamide and the potential for unspecific hydrolysis with the blocked ornithine led us to conclude that the method of Nair was also unsuitable for producing thiocitrulline.

Phosphorothioates as Substrates for Sucrose Phosphorylase, Phosphoglucomutase, Uridine Diphosphoglucose Pyrophosphorylase, Galactose-1-Phosphorothioate Uridylyl Transferase, Sucrose Synthetase, and Glycogen Synthetase

Sucrose phosphorylase was able to catalyze the reaction of sucrose and orthophosphorothioate to glucose-1-phosphorothioate although this production was quite sluggish in comparison to the orthophosphate substrate. The slower overall rate is a direct

consequence of the subtle chemical differences between sulfur and oxygen. Phosphorothioates are almost always poorer substrates than their oxygen analogues although there are some exceptions.[24,25] Glucose-1-phosphorothioate also exhibits the characteristic downfield chemical shift that is typical of phosphorothioates. In addition, glucose-1-phosphorothioate also exhibits a greater affinity for ion exchange material than does its oxygen relative glucose-1-phosphate.

Glucose-1-phosphorothioate was also a typical phosphorothioate in that it was not catalyzed to glucose-6-phosphorothioate by phosphoglucomutase. Once again, this is not surprising when one considers that another well studied phosphosugar mutase, phosphoglycerate mutase, does not accept the phosphorothioate analogues of 3' or 2' phosphoglycerate.[24]

However, glucose-1-phosphorothioate was accepted by both uridine diphosphoglucose pyrophosphorylase and galactose-1-phosphate uridylyl transferase. Once again, a reduced rate was seen with each enzyme. The inclusion of a thiol reducing agent such as dithiothreitol was essential to the catalytic integrity of uridine diphosphoglucose pyrophosphorylase. This suggests that glucose-1-phosphorothioate is acting in an inhibitory manner by forming disulfide bonds either directly in the active site or in close proximity to it. Glucose-1-phosphorothioate did not necessitate the use of thiol reducing agents with galactose-1-phosphate uridylyl transferase. Both uridine diphosphoglucose-β(S) products from galactose-1-phosphate uridylyl transferase and uridine diphosphoglucose pyrophosphorylase showed the downfield ^{31}P NMR chemical shifts characteristics of phosphorothioate

analogues of uridine diphosphoglucose. They also had a greater affinity for ion exchange since they eluted on the HPLC and DEAE-cellulose at longer times than the oxygen analogue. However, the HPLC of purified uridine diphosphoglucose-β(S) did show that this compound was unstable at room temperature.

Since uridine diphosphoglucose-β(S) is a chiral molecule with respect to the β phosphorus, the possibility of isomerism was investigated using high field ^{31}P NMR. It was hoped that the different enzymes would produce different isomers. Unfortunately, the only conclusion that could be reached from the ^{31}P NMR results is that uridine diphosphoglucose-β(S) from uridine diphosphoglucose pyrophosphorylase and galactose-1-phosphate contained the same stereochemistry at the β phosphorus position since the highfield ^{31}P NMR resonances were identical. It is possible that the existence of diastereomers could not be detected by high field NMR (i.e. the ^{31}P NMR resonances could be identical for both the S_p and R_p diastereomers). However, Frey was able to clearly account for the existence of R_p and S_p diastereomers of uridine diphosphoglucose-α(S) by their easily distinguishable ^{31}P NMR spectra. Nucleotide phosphorothioate compounds that could not be distinguishable by their ^{31}P NMR were shown to be the same isomer. Therefore, it would be highly unlikely for the beta-sulfur substituted diastereomers of uridine diphosphoglucose to exhibit strikingly dissimilar ^{31}P NMR resonance behavior.

Uridine diphosphoglucose-β(S) was tested as a substrate for both sucrose synthetase and glycogen synthetase with the same negative

result. Since uridine diphosphoglucose-β(S) is undoubtedly a single isomer, it is likely that the opposite isomer may prove to be a substrate for one or both of these enzymes.

CONCLUSION

The modifications of the method of Jones[13] for the synthesis of carbamoyl phosphate failed to produce the desired thio-derivatives of carbamoyl phosphate; thiocarbamoyl phosphate and carbamoyl phosphorothioate. The investigation of the ^{18}O labeling possibilities of cyanate with phosphorothioates proved to be negative in that the monophosphorothioate, adenosine monophosphorothioate showed no signs of sulfur cleavage.

The chemical synthesis of thiocitrulline was not undertaken since all attempts to produce a precursor compound, n-butylthiourea, were unfruitful.

The strategy for producing uridine diphosphoglucose-β(S) was successful. Unfortunately, the isomer produced by uridine diphosphoglucose pyrophosphorylase and that produced by galactose-1-phosphate uridylyl transferase were the same. This diastereomer was not a kinetically competent substrate for sucrose synthetase nor was it a substrate for a similar enzyme, glycogen synthetase. Further investigation of sucrose synthetase must await the synthesis of the opposite diastereomer of uridine diphosphoglucose-β(S) for the back reaction catalyzed by this enzyme.

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THE CHARACTERIZATION AND STEREOCHEMICAL UTILIZATION OF PHOSPHOROTHIOATES PRODUCED BY CHEMICAL AND ENZYMATIC SYNTHESIS

A Thesis

by

JOEY SCOTT NEWBORN

Submitted to the Graduate College of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 1987

Major Subject: Chemistry

THE CHARACTERIZATION AND STEREOCHEMICAL UTILIZATION OF PHOSPHOROTHIOATES PRODUCED BY CHEMICAL AND ENZYMATIC SYNTHESIS

A Thesis

by

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ABSTRACT

The Characterization and Stereochemical Utilization of Phosphorothioates Produced by Chemical and Enzymatic Synthesis

(August 1987)

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Sucrose synthetase (UDP-glucose-fructose glycosyl transferase, E.C. 2.4.1.13) catalyzes the production of uridine diphosphoglucose and fructose from uridine diphosphate and sucrose. An overall stereochemical course for this enzyme has not been established for the β phosphorus of its substrate uridine diphosphoglucose (UDP-glucose). This thesis presents a method for the determination of this stereochemical course through the use of phosphorothioates.

The phosphorothioate analogues of glucose-1-phosphate and uridine diphospho-glucose (UDP-glucose β(S) or UDP-β(S) glucose) were synthesized, purified, and characterized by ^{31}P NMR and by HPLC. In addition, the overall relative rate kinetics of these phosphorothioates are compared to the natural oxygen analogue for each enzyme studied. Uridine diphosphoglucose-β(S) obtained from uridine diphosphoglucose pyrophosphorylase (E.C. 2.4.2.9) is not a kinetically competent substrate for sucrose synthetase. An attempt to synthesize, specify the opposite isomer by using a combination of galactose-1-phosphate uridylyl transferase (E.C. 2.7.12) and phosphoglucomutase (E.C. 2.7.5.1) with glucose-1-phosphorothioate only produced the identical isomer as determined by high field NMR.

Attempts were also made to produce thiocarbamoyl phosphate, carbamoyl phosphorothioate, and thiocitrulline for possible investigation as substrates of enzymes of the urea cycle (carbamoyl phosphate synthetase E.C. 2.7.2.5, ornithine transcarbamoylase E.C. 2.1.3.3, and arginosuccinate synthetase E.C. 6.3.4.5).

ACKNOWLEDGMENT

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INTRODUCTION

Phosphate esters occupy a central role in biochemistry. Not only is the genetic blueprint for all living organisms stored in phosphodiester polymer form as DNA, but di- and tri-phosphates of nucleosides are important energy carriers in that groups are activated by phosphorylation. Since phosphate esters are so prevalent, the mechanisms of phosphoryl transfer are important subjects for study.

The mechanism of any enzyme catalyzed reaction is closely related to its stereochemical course. The knowledge of the stereochemical course of a reaction is recognized as one of the most useful kinds of information for distinguishing among possible mechanisms. For a molecule to be able to convey the stereochemistry of a reaction it must contain prochirality at a position essential to the catalytic mechanism of the enzyme. The tetrahedral phosphorus molecule is not chiral and there are only a few examples of naturally occurring phosphodiesters that are chiral. However, phosphates can be made chiral if the tetrahedral phosphorus is labeled with sulfur, ^{16}O, ^{17}O, and ^{18}O atoms. Shown in Scheme I is a phosphorothioate which contains four different groups about the phosphorus molecule and is thus chiral.

In over thirty stereochemical studies of enzymatic substitution at phosphorus the findings have suggested that there is an in-line

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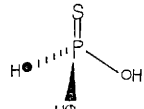
The style and format of the Journal of Biological Chemistry has been used in this thesis.



Scheme I

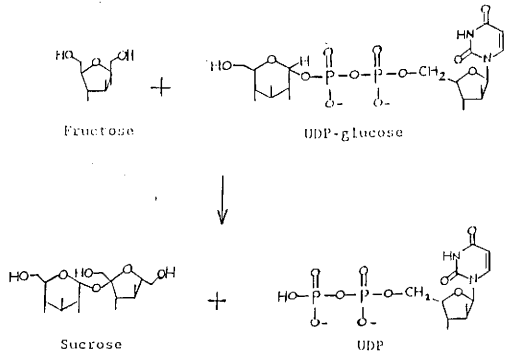
mechanism of reaction which inverts the configuration at the chiral center.[1] The use of chiral phosphorothioates can distinguish between single and double displacement mechanisms at this center. Since all such in-line interactions at chiral phosphorus lead to inversion of configuration, a a single displacement will yield an overall inversion at phosphorus whereas a double displacement will yield retention of the original configuration.

The technique of stereochemical analysis by the use of chiral phosphorothioates was first developed by Eickstein[2] and has been extended and improved upon by a number of researchers.[3,4,5,6,7]

The most pertinent work for our purposes has been that of Knowles et al.[3] and Frey et al.[8] Specifically, Knowles' et al. success with glycolytic enzymes was most important. In proving that pyruvate kinase, glycerol kinase, and hexokinase transfer their phosphorothioate substrates with an identical stereochemical course, Knowles et al. found that some eleven glycolytic enzymes handle phosphorothioates smoothly. Frey et al., were able to chemically and enzymatically produce both the S_p and R_p isomers of uridine diphosphoglucose-α(S). Using these isomers and ^{31}P NMR, they were able to establish an overall stereochemical course for both uridine diphosphoglucose pyrophosphorylase and galactose-1-phosphate uridylyl

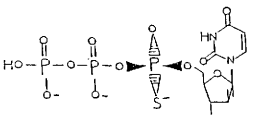
transferase according to Scheme 2. Uridine diphosphoglucose pyrophosphorylase was found to catalyze the reaction with inversion of configuration while galactose-1-phosphate uridylyl transferase proceeded with retention of configuration at the α phosphorus. Thus, two enzymes previously thought to operate by similar mechanisms were shown to follow fundamentally different mechanistic paths.

For our purposes we hoped to employ similar techniques to determine the overall stereochemical course of reactions of sucrose synthetase which catalyzes according to the reaction below.

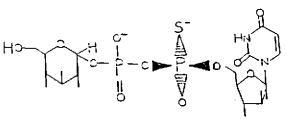


Reaction I

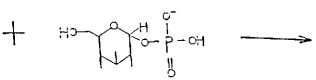
Since almost all glycolytic enzymes handle phosphorothioates well, it is reasonable to assume that a disaccharide phosphorylase like sucrose phosphorylase could handle a phosphorothioate to produce glucose-1-phosphorothioate according to Scheme 4.



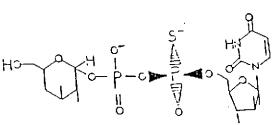
UTPα(S) (R_p)



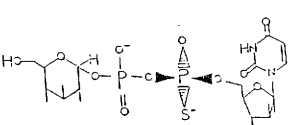
UDPα(S) Glucose (S_p)



Glucose-1-Phosphate

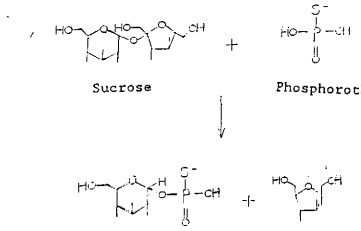


UDPα(S) Galactose (R_p)



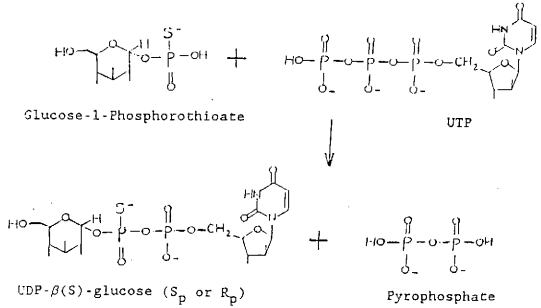
UDPα(S) Glucose (R_p)

Scheme 2.



Scheme 3

Glucose-1-phosphorothioate could then replace glucose-1-phosphate as a substrate in the reaction catalyzed by uridine diphosphoglucose pyrophosphorylase to produce uridine diphosphoglucose-β(S), a molecule that is chiral at the β position. Scheme 4 is shown below.

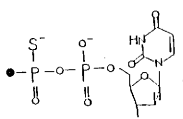


Scheme 4

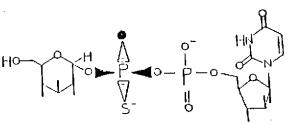
Once uridine diphosphoglucose-β(S) (diastereomer A) is produced, a few questions can be answered with regard to sucrose synthetase. For example, does this enzyme handle phosphorothioates? If sucrose synthetase does catalyze uridine diphosphoglucose-β(S) (diastereomer A) to uridine diphosphate-β(S) then the obvious answer is yes. However, the diastereomer A is not a substrate for sucrose synthetase then there could exist a stereochemical requirement for the opposite diastereomer. To investigate this possibility the synthesis of a mixture of the R_p and S_p diastereomers of uridine diphosphoglucose was undertaken according to modifications of readily available methods.[9] Synthesis of the opposite diastereomer (diastereomer B) could possibly be effected enzymatically by using glucose-1-phosphorothioate as a substrate for galactose-1-phosphate uridylyl transferase, this enzyme operates by a different mechanism than uridine diphosphoglucose pyrophosphorylase.[10]

If neither diastereomer is not a substrate, then sucrose synthetase obviously will not tolerate the substitution of sulfur for oxygen. However, if the diastereomer B is a substrate, then we can investigate this stereochemical requirement by observing the ^{18}O labeling via high field ^{31}P NMR spectroscopy.[7] If uridine diphosphate-β(S), $\beta^{18}O$ can be produced by employing known modifications to synthesize the adenine analogue of this compound[11] then uridine diphosphoglucose-β(S), $\beta^{18}O$ would be produced in the back reaction by sucrose synthetase with either the ^{18}O in a bridging position or in an apical position as in Scheme 5.

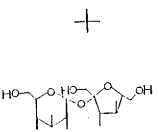
If the ^{18}O occupies a bridging position (S_p isomer) then it will have a bond order of one and we will see a .02 ppm upfield shift for



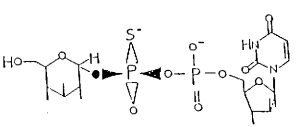
UDPβ(S) R_p and S_p



UDPβ(S) Glucose R_p



Sucrose



UDPβ(S) Glucose S_p

Scheme 5

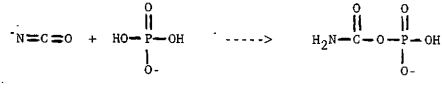
the β phosphorus (relative to no labeling). If the ^{18}O is not bridging and has a bond order of two (as in the R_p isomer) a .04 ppm shift will be observed.[12] Thus careful analysis of the high field NMR spectrum should enable a determination of the stereochemical course of sucrose synthetase.

Other potential studies could include the use of derivatives of carbamoyl phosphate. Carbamoyl phosphate is a key intermediate in both pyrimidine biosynthesis and urea biosynthesis. Carbamoyl phosphate synthetase catalyzes the synthesis of carbamoyl phosphate according to Scheme 6.

$$2\ ATP + HCO_3^- + NH_3 \text{ -----> } 2\ ADP + NH_2COPO_3^{=} + P_i$$

Reaction 2

Carbamoyl phosphate can also be synthesized chemically by the method of Jones and Lippman according to the scheme.[13]

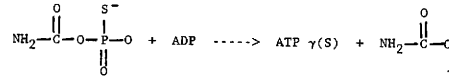


cyanate + orthophosphate — carbamoyl phosphate

Reaction 3

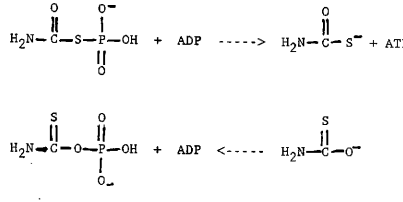
By substituting thio containing reactants for the oxygen containing reactants in this synthesis, it should be possible to synthesize phosphorothioate molecules of interest. The potential significance of these analogues is as follows.

If carbamoyl phosphorothioate is a substrate for carbamoyl phosphate synthetase in the reverse reaction, we could possibly see the production of adenosine triphosphate $\gamma(S)$ as shown in Scheme 6.



Scheme 6

If carbamoyl phosphorothioate is produced with the thio group in the carbonyl position it could prove to be interesting in that a rotational equivalence between the sulfur and oxygen might be seen:

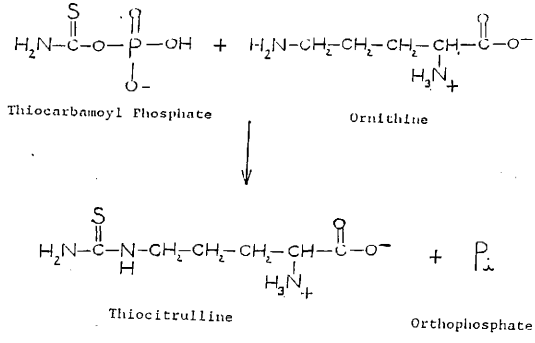


Scheme 7

Previous studies by Raushel and Villafranca[14] have indicated that there is a rotational equivalence of the carboxylate oxygens in carbamoyl phosphate.

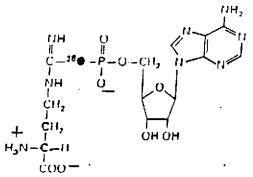
Ornithine transcarbamoylase catalyzes the conversion of carbamoyl phosphate and ornithine to citrulline. If thiocarbamoyl phosphate

can be synthesized, it could prove to be a precursor to thiocitrulline. Thio-carbamoyl phosphate might substitute as a substrate for ornithine transcarbamoylase according to the following scheme.



Scheme 8

Thiocitrulline, which could substitute as a substrate for arginosuccinate synthetase, might possibly provide some insight into the mechanism of this enzyme. Argininosuccinate synthetase has been hypothesized to follow one of three possible mechanisms which are all characterized by the formation of a citrullyl-adenosine monophosphate intermediate[15] shown in Scheme 9.



Scheme 9

Substitution of a sulfur for oxygen in the bridging bond of the citrullyl intermediate will undoubtedly have an effect on this intermediate. If thiocitrulline cannot be synthesized by the above method, it could most likely be synthesized chemically by employing a combination of methods for polypeptide synthesis[16] and those readily available for synthesis of thio ureas.[17,18]

In summary, the objective of this project was to synthesize the aforementioned compounds, purify them through DEAE-cellulose, characterize them through ^{31}P NMR and HPLC, and to investigate the kinetic consequences of their use as enzyme substrates.

EXPERIMENTAL

Decomposition of Carbamoyl Phosphate

Exactly 0.158 g of the dilithium salt of carbamoyl phosphate was weighed out as a solid. The decomposition reaction was begun by the addition of the solid carbamoyl phosphate to 10 mL of 200 mM of PIPES buffer containing 30% D_2O at pH 6.8. Vigorous stirring was applied to ensure that the carbamoyl phosphate dissolved sufficiently. Then 3 mL of this solution was pipetted into a 10 mM NMR tube and the reaction was monitored by ^{31}P NMR spectroscopy at room temperature with the the FT-80 NMR spectrometer at a frequency of 32.203 MHz. Because the peaks for the product orthophosphate and carbamoyl phosphate are separated by only 3 ppm, a small sweep width of 400 Hz was used to view the appearance of the orthophosphate. Other important parameters included an acquisition time of 1.0 second and a seven microsecond pulse width (45° angle). Data were accumulated over a six hundred second interval and were then stored on floppy disk either manually or by the program KINETICS. Kinetic data were acquired by measuring the relative decrease in intensities of the peak associated with the carbamoyl phosphate of the plotted ^{31}P NMR spectrum plotted with respect to time.

The reference orthophosphate peak was established by using 100 mM potassium phosphate in 100 mM PIPES buffer that was 33% in D_2O at pH 6.8. Carbamoyl phosphate was stored over magnesium sulfate in a dessicator below 0 °C when not in use.

Reaction of KOCN with Orthophosphate to Produce Carbamoyl Phosphate

Exactly 0.081 grams (0.001 mole) of solid potassium cyanate was mixed with a 100 mM solution of orthophosphate. This mixture was buffered with 100 mM MES that was 33% in D_2O at pH 5.8 to provide a 100 mM total cyanate solution. Then 3 mL of this 100 mM potassium cyanate reaction mixture was pipetted into a 10 mM NMR tube and the ^{31}P NMR spectrum was then obtained at room temperature by using the FT-80 NMR spectrometer at a frequency of 32.203 MHz. A total of eight frames were obtained over 10 minute intervals (600 transients at 1 second acquisition time) and stored on floppy disk via the KINETICS program. Other NMR parameters were similar to those used for carbamoyl phosphate decomposition experiment except that a sweep width of 2,000 Hz was used and the orthophosphate reference was not centered.

Attempted Synthesis of Thiocarbamoyl Phosphate

Exactly 0.097 g of potassium thiocyanate was added as a solid to 10 mL of a 100 mM MES/33% D_2O solution of 100 mM orthophosphate at pH 5.8. This 100 mM potassium thiocyanate solution was left for 24 hours at room temperature. A 3 mL aliquot of this reaction mixture was then subjected to ^{31}P NMR analysis on the Varian FT-80 NMR. The ^{31}P NMR spectra were obtained using the parameters of the carbamoyl phosphate synthesis experiment.

Reaction of KOCN with Orthophosphorothioate

Exactly 0.203 g (.0025 mole) of potassium cyanate was added to a solution consisting of 250 mM tetra sodium orthophosphorothioate and 250 mM MES buffer that was 33% in D_2O and the pH was adjusted to 5.8.

A 3 mL aliquot of this reaction solution was then pipetted to a 10 mM NMR tube and the reaction followed at room temperature via ^{31}P NMR spectroscopy on the FT-80 NMR spectrometer. ^{31}P NMR parameters were similar to those used to follow the synthesis of carbamoyl phosphate except that an acquisition time of 0.5 seconds was used. Therefore, 1200 transients were accumulated per frame to yield 10 minute time intervals between spectra. Kinetic data were compiled by measuring the relative peak intensity of the emerging orthophosphate peak at 10 minute intervals. Further investigation of this phenomenon was made by monitoring a solution that was only 100 mM in potassium cyanate, but 250 mM in tetra sodium orthophosphorothioate. A similar solution contained the opposite ratio of 250 mM potassium cyanate to 100 mM tetra sodium orthophosphate. The latter reaction was followed for 120 minutes to accommodate the longer reaction time and to confirm the nature of the reaction.

Attempted Removal of Sulfur from AMPS via KOCN

Potassium cyanate was employed as a possible reagent for the removal of sulfur from phosphorothioate nucleotides. Assays were run at 4 different pH levels. Assays at pH 2 and pH 4 were maintained by sodium acetate buffer titrated by HCl to the desired pH. PIPES buffer was used at pH 6.0 and TRIS buffer was employed at pH 8.0. All buffer concentrations were at 100 mM. Assays were also subjected to controlled heating at a constant 50 °C for 24 hours by using a Neslab constant temperature bath. Control samples lacking potassium cyanate were run at each pH level and at room temperature. A typical assay contained a 7 mM concentration of adenosine monophosphorothioate.

The concentrations of potassium cyanate ranged from 7 mM to 200 mM. Any potential progress with this reaction was monitored using a Gilson Model 802B HPLC interfaced with an Apple II computer. UV detection was employed using a detector at 254 nm wavelength. The column employed was a Whatman partisil-10 SAX of the anion exchange type. The elution buffer was orthophosphate at 125 mM in concentration. The pH of the elution buffer was adjusted to 3.5 using phosphoric acid. All other HPLC parameters included a range of 1.0 Aufs, and a flow rate for the elution buffer of 1.0 mL per minute. The program controlled assays were 25 minutes in duration and all injections were 20 μL. A standard of adenosine monophosphate was used to affix the retention times.

Synthesis of n-Butyl Thiourea

The synthesis of n-butyl thiourea was undertaken by a modification of the method of Neville and McGee.[17] Exactly 0.73 grams (0.01 mole, or 1 mL) of dry n-butyl amine were dissolved in dry tetrahydrofuran (THF). Then 0.0025 mole (0.49 grams) of silicon tetra isothiocyanate that was previously dissolved in dry THF was added dropwise with stirring to a three neck 250 mL reaction flask. The reaction flask was sealed under an argon atmosphere and was placed in a water bath to mediate the exothermic reaction. Once the reaction had cooled to room temperature the solution was refluxed under argon for 30 minutes. The THF solvent was removed via rotary evaporation. Dilute isopropyl alcohol (10 mL/2 mL H_2O) was then added to the residue and the whole mixture was refluxed for 30 minutes. Filtration through a course grade sintered glass funnel was

used to remove silica gel. The residue was then washed with 5 mL aliquots of acetone and the resulting liquid was rotary evaporated to a brown oil. Recrystalization was attempted using isopropyl alcohol.

Silicon tetra isothiocyanate was prepared by a modified synthesis of Neville and McGee.[19] Approximately 38 grams (0.5 moles) of dry ammonium thiocyanate was stirred rapidly in a suspension of 200 mL of dry benzene in a sealed 500 ml 3-neck flask filled with argon gas. Exactly 8.5 grams of fresh silicon tetrachloride (0.1 mole) was then added slowly via syringe. Typically, a 5 minute period between 2 mL injections was sufficient to control the reaction. The mixture was then heated under reflux for 3 hours and allowed to cool to 70 °C and then filtered hot through a course glass sintered funnel. Hot THF was then used to wash the compound. The filtrate was distilled to a residual volume of 40 mL and the contents of the reaction flask transferred to a 400 mL beaker. The cold crystals were rapidly filtered, washed with cold THF, pressed and drained, and then weighed (22 grams or a 55% yield). They were stored with in tightly stoppered flask. The crystals of silicon tetra isothio-cyanate melted at 144-146 °C, (Lit. value 144 °C).[17,19] Silicon tetra isothiocyanate was stored in a magnesium sulfate filled dessicator below 0 °C.

An alternative synthesis of the target molecule n-butyl thiourea was explored using a modified method of Nair.[3] A 5 mL portion (0.05 moles) of dry n-butyl amine was slowly added to 6 mL (0.05 mole) of tert-butyl isothiocyanate that was previously dissolved in dry petroleum ether. The reaction vessel was allowed to sit at room temperature for 30 minutes and was then warmed at 30 °C for 2 hours.

Upon returning to room temperature, approximately 7 grams of white crystals precipitated. These crystals were collected and washed with cold petroleum ether, dried, and recrystallized by using a water/ethanol system. The 3-tert-butyl thiocarbamide melted at 91°C (vs 94 °C for the literature value).[18]

A 2.2 g (0.012 moles) sample of the purified 3-tert-butyl thiocarbamide crystals was then subjected to acid hydrolysis using 25 mL of concentrated HCl. The mixture was warmed for 10 minutes at 90 °C.[18] Once cool, the liquid was diluted and neutralized with a sodium bicarbonate solution and the precipitate was washed with benzene to remove the yellow oil from crystals. The remaining solid was recrystallized using a mixture of chloroform and carbon tetrachloride to yield 1.1 gram of colorless crystals that melted at 72 °C. [(lit. value 79°]. TLC was then used to check the purity using 50% ethyl acetate and 50% hexane as solvent. Pre-coated silica gel, 60 F TLC plates from EM Reagents, were employed. Potassium iodide was used to develop the chromatograms.

^{1}H NMR spectra was taken of all starting materials and products using the Varian XL-200 NMR spectrometer operating at a frequency of 200.06 MHz. The acquisition time was 2.5 seconds, the pulse width was 5.0 μseconds, and the sweep width was 3200 Hz. A total of 128 transients were taken and peaks were referenced to zero ppm with TMS. Lock was obtained using $CDCl_3$ solvent.

n-Butyl amine was dried by refluxing under argon atmosphere over calcium hydride for 24 hours. The n-butyl amine was then stored over KOH pellets. Benzene was dried under an argon atmosphere in a

similar way, but was stored over 4° A molecular sieves.

Synthesis of Glucose-1-Phosphorothioate and Kinetic Comparison to its Oxygen Analogue

An assay consisting of 150 μmoles of tetrasodium phosphorothioate and 300 μmoles of sucrose was prepared in 150 mM HEPES buffer that had been titrated to pH 7.5. The deuterium oxide content was 30%. The total volume of the solution was 3 mL. The reaction was initiated by the addition of 6.5 units of sucrose phosphorylase isolated from Leuconostoe mesenteroides. A similar assay was employed using the potassium salt of orthophosphate as a substrate. Exactly 2 units of sucrose phosphorylase were used to initiate this reaction. Both assays were 3 mL in total volume.

The progress of both the glucose-1-phosphorothioate and glucose-1-phosphate reaction assay was monitored on the XL-200 NMR operating at a frequency of 81 MHz. The spectral data were collected in 10 minute frames (500 seconds with 1 second acquisition time). Since the difference in chemical shift for glucose-1-phosphate and orthophosphate is very small, a sweep width of only 300 Hz was employed with the oxygen analogue. However, the difference in chemical shift for glucose-1-phosphorothioate and orthophosphorothioate was much greater. Therefore, a much larger sweep width of 5500 Hz was used. Exactly 18 frames were collected and the data stored for the oxygen analogue experiment. Only 12 frames were collected for the thio analogue because of the large disc space required to store the greater amount of data inherent in a longer sweep width. A five minute delay was imposed between the initial non-enzyme spectrum

and the beginning of accumulations for the first spectrum on the experiment. This was necessary in order to allow time to properly shim the instrument after the addition of sucrose phosphorylase. Other parameters included a transmitter offset of 2400 Hz, a sensitivity enhancement of 0.3, the incorporation of the absolute intensity mode, and a pulse width of 10 μseconds. Data points for the plots for comparison of kinetic relative rates were taken from the integrals of peaks versus time.

Isolation and Purification of Glucose-1-Phosphorothioate

A large scale synthesis of glucose-1-phosphorothioate was used to prepare enough material for purification. This consisted of a 25 mL reaction mixture that was 75 mM in orthophosphorothioate, 100 mM in sucrose, and 200 mM in HEPES buffer at pH 7.5. This assay was also 30% in D_2O. Some 15 units of sucrose phosphorylase were added to initiate the reaction. The reaction was then checked periodically by ^{31}P NMR spectroscopy on the FT-80 using similar parameters as mentioned for earlier experiments. Once the orthophosphorothioate was totally consumed (about 24 hours) the mixture was placed in an Amicon ultrafiltration membrane cone and centrifuged for 30 minutes using a Dupont Sorvall RC-5B refrigerated centrifuge to remove sucrose phosphorylase. The supernatant solution was then sealed in several 8 mL plastic test tubes and stored at -78 °C.

Glucose-1-phosphorothioate was purified using a 50 x 3.0 cm column of DEAE-52 cellulose. A 2 mL solution containing 150 μmoles of glucose-1-phosphorothioate was diluted to 500 mL with dionized water and then titrated to pH 7.8 using dilute KOH. This was then

loaded onto the column at a flow rate of 2 mL/min. The column was eluted with a 3.2 liter gradient of TEA/CO_2 from 50 mM to 350 mM. The TEA/CO_2 buffer was adjusted to pH 7.5 with dry ice. The flow rate of the elution buffer through the column was 2 mL/min. Glucose-1-phosphorothioate eluted behind glucose-1-phosphate at a buffer concentration between 110 and 150 mM.

Since glucose-1-phosphorothioate contains no UV or visible chromophore, detection in the column fractions was accomplished using modifications of 3 methods; the Bochner spray method,[20] the colormetric method of Ames,[21] and the reaction of Ellmans reagent [DTNB,5',5-dithio bis(2-nitrobenzoic acid] with terminal phosphorothioate groups.[22] Both the Bochner and Ames method rely upon free phosphate reaction with molybdate with the reduction of this complex accomplished by ascorbate to produce a blue color detectable at 660 nm. Ellmans reagent will produce a yellow color that can be detected at 412 nm only if a terminal phosphorothioate is present.

The modified Ames method employed the following procedure: 1 drop of concentrated HCl was added to a 0.3 mL aliquot of a fraction collected from the column and this mixture was then boiled for 25 minutes. Then 0.7 mL of a mixture consisting of 1 part 10% ascorbate to 6 parts 0.42% molybdate in 1N H_2SO_4 was then added to the boiled 0.3 mL aliquot. This 1 mL solution was then warmed for 20 minutes at 45 °C and then diluted to 3 mL and the absorbance measured at a 660 nm wavelength. Each sample was checked against a blank consisting of water and mix.

The Bochner spray assay consisted of the following mixture: 2 g of ammonium molybdate were dissolved in 200 mL of methanol with heating and vigorous stirring. The molybdate did not dissolve totally, but when the solution was warm to the touch, 20-30 mL of concentrated nitric acid was added which facilitated the dissolution of the molybdate. After this solution had cooled, approximately 2 g of ascorbate were added and the solution swirled until the ascorbate was totally dissolved. Then approximately 2 g of para-amino benzoic acid was added and allowed to completely dissolve. This produced a harvest gold colored solution that remained stable for 45 minutes. After this time the solution turned to green and then later to red. Once the color of the solution was red, the mix was essentially useless. The chromatograms used were 3.5 inch by 1 inch strips of P.E.I. cellulose plates. The plates were developed in 1.5 M $LiCl/H_2O$ solution. The Bochner spray assay produces a blue color upon reaction with free phosphate. All spots were checked against reference standards. The reference standards had the following R_f values: glucose-1-phosphate; 1.0, glucose-1-phosphorothioate; 0.5, orthophosphate; 0.5, and orthophosphorothioate; 0.2.

The Ellmans reagent assay was considered the easiest to use and the most accurate and so was employed more often than the others. A typical assay consisted of a 100 mL solution that was 2 mM in DTNB. This assay was buffered at pH 7.5 by 50 mM in HEPES. A ratio of 9 parts of this solution was used to 1 part column fraction (usually .9 mL mix to .1 mL fraction) and the resulting mixture was checked at 412 nm on a Gilford 260 spectrophotometer. Fractions identified as

terminal phosphorothioate positive were pooled and rotary evaporated to dryness and then washed with three successive 10 mL aliquots of dry methanol. The resulting residue was taken up in 5 mL of buffer solution and the pH adjusted to 9.0. The glucose-1-phosphorothioate was then analyzed quantitatively via Ellmans reagent to determine the concentration. Decoupled and non-decoupled ^{31}P NMR spectra were taken using the XL-200 NMR spectrometer and parameters already described earlier. The purified material was then stored at -78 °C as a 28 mM solution.

Synthesis of Uridine Diphosphoglucose-β(S) via Uridine Diphosphoglucose Pyrophosphorylase

Glucose-1-phosphorothioate was tested as a substrate for the enzyme uridine diphosphoglucose pyrophosphorylase from Bakers Yeast. A typical 4 mL reaction mixture for the kinetic assays consisted of 4 mM uridine triphosphate (UTP), 4 mM magnesium chloride, 3 mM dithiothreitol (DTE, Cleland's reagent) and 2 mM glucose-1-phosphorothioate. Exactly 15 units of inorganic pyrophosphatase from Bakers Yeast were incorporated into the assay to degrade pyrophosphate to orthophosphate as it was formed. The reaction was initiated by the addition of 2 units of uridine diphosphoglucose pyrophosphorylase. The glucose-1-phosphate assay was similar except that dithiothreitol was not used and the reaction was initiated by only .25 units of uridine diphosphoglucose pyrophosphorylase.

The progress of both reaction assays was followed on a Gilson Model 811 HPLC employing a Whatman 10 SAX partisil anion exchange column. The disappearance of the peak corresponding to uridine

triphosphate was recorded and the area under the curve was then used to determine the rate of the reaction. The HPLC scale used was 0.2 Aufs, the column elution buffer was 450 mM orthophosphate at a pH of 3.5. The injection volume was 20 μL. All runs were at room temperature and followed at a wavelength of 254 nm.

Purification and Characterization of Uridine Diphosphoglucose-β(S)

A large scale reaction mixture for the synthesis of uridine diphosphoglucose-β(S) consisted of a 25 mL stock solution that was 50 mM in glucose-1-phosphorothioate, 60 mM in uridine triphosphate, 50 mM in magnesium chloride, 50 mM in dithiothreitol, and 200 mM in HEPES buffer at pH 7.5. The assay also contained 100 units of inorganic pyrophosphatase. The reaction was initiated by the addition of 20 units of uridine diphosphoglucose pyrophosphorylase. At random time intervals the progress of the reaction was measured by removing 0.1 mL aliquots and diluting them to 2 mL for analysis by HPLC. 20 μL of this solution was then injected onto the column and the disappearance of the uridine triphosphate peak checked. HPLC parameters were the same as those used for the kinetic assays. After the uridine triphosphate had been consumed, the reaction mixture was centrifuged through an Amicon ultrafiltration membrane cone.

Approximately 150 μmoles of uridine diphosphoglucose-β(S) (3 mL of solution) was then diluted to 400 mL with distilled H_2O and the pH adjusted to 7.5. This solution was then loaded onto a 50 x 3.0 cm column of DEAE-52 cellulose anion exchange material and the column was then eluted with a 3.2 liter linear gradient of TEA/CO_2 from 50 mM to 400 mM concentration. The fractions collected were 20 mL in volume.

Those containing uridine diphosphoglucose and uridine diphosphoglucose-β(S) were identified by their recorded UV absorbance at 254 nm. Uridine diphosphoglucose-β(S) eluted well behind uridine diphosphoglucose between buffer concentrations of 220 to 270 mM. Fractions containing uridine diphosphoglucose-β(S) were pooled and rotary evaporated to dryness and then washed 3 times with 10 mL of methanol. The residue was then taken up in 30% D_2O/HEPES buffer at pH 9.0. ^{31}P NMR spectra of the purified compounds were recorded on the XL-200 NMR operating at a frequency of 81 MHz. Other parameters included a sweep width of 7000 Hz, an acquisition time of 2.3 seconds, a pulse width of 15 μseconds, and a transmitter offset of 1,000 Hz. A total of 2,000 transients were completed. Uridine diphosphoglucose-β(S) was then stored at -78 °C as a 25 mM solution in plastic test tubes.

Glucose-1-phosphorothioate was tested as a substrate for the galactose-1-phosphate uridylyl transferase enzyme isolated from adapted yeast. The galactose-1-phosphate uridylyl transferase reaction assay included a phosphoglucomutase/glucose-6-phosphate dehydrogenase coupling system to remove glucose-1-phosphate as it was released from galactose-1-phosphate uridylyl transferase. This assay was 3 mL in total volume and consisted of 3 mM glucose-1-phosphorothioate, 1 mM uridine diphosphoglucose, 5 mM nicotinamide adenine dinucleotide, 4 mM of magnesium chloride, glucose 1,6 diphosphate and 75 mM HEPES buffer pH adjusted to 7.5. The enzymes used were 50 units of phosphoglucomutase from rabbit muscle, and 15 units of glucose-6-phosphate dehydrogenase from Bakers Yeast. Two units of galatose-1-phosphate uridylyltransferase were added last to initiate the

reaction. An alternative assay was utilized that did not incorporate the glucose-6-phosphate dehydrogenase enzyme and its potentially bothersome nicotinamide adenine dinucleotide (NAD^+) which would most likely coelute with uridine-diphosphoglucose-β(S).

Synthesis of Uridine Diphosphoglucose-β(S) from Galatose-1-Phosphate Uridylyl Transferase

The appearance of uridine diphosphoglucose-β(S) was monitored at 254 mM using a Gilson 811 HPLC with a Whatman Partisil 10-SAX Anion exchange column. The elution buffer was 75 mM P_i at a pH of 4.5. The flow rate was 1.0 mL per minute, the range 0.1 Aufs, and a typical run of 20 minutes was long enough to allow the uridine diphosphoglucose-β(S) to elute from the column. All injections were 20 μL in volume.

Purification and Characterization of Uridine Diphosphoglucose-β(S) from Galactose-1-Phosphate Uridylyl Transferase

In order to obtain a sufficient quantity of UDP-β(S)-glucose for further study a large scale synthesis was employed. The large scale assay consisted of 30 μmoles of glucose-1-phosphorothioate, 20 μmoles of uridine diphosphoglucose, 40 μmoles of magnesium chloride, glucose 1,6-diphosphate, and 125 mM HEPES buffer at pH 7.5. Exactly 250 units of phosphoglucomutase were used. The reaction was initiated by the introduction of 40 units of galactose-1-phosphate uridylyl transferase. The total volume of the assay was 40 mL. The reaction was followed for 36 hours or until the assay developed a cloudy tint.

The solution containing 8 μmoles of uridine diphosphoglucose-β(S) was diluted to 400 mL with H_2O and pH adjusted to 7.5. This

solution was then loaded onto a 50 x 3.0 cm column of DEAE-52 cellulose. The column was eluted with a 3.2 liter linear gradient of TEA/CO_2 at a pH of 7.5 from 50 mM to 400 mM. The 20 mL fractions were collected in 160 tubes. Uridine diphosphoglucose-β(S) eluted well behind uridine diphosphoglucose and was collected in tubes 95 through 100. The uridine diphosphoglucose-β(S) was then rotary evaporated to dryness at 20°. The residue was dissolved 3 times with 10 mL of dry methanol and rotary evaporated to dryness. This step was repeated 2 more times. The resulting residue was then dissolved in 3 mL of orthophosphate buffer that was 30% in deuterium oxide. The pH of the buffer solution was 9.0.

The pure uridine diphosphoglucose-β(S) was then examined by ^{31}P NMR spectroscopy. Spectra were taken on the XL-400 NMR spectrometer operating at a frequency of 162 MHz. Other parameters included an acquisition time of 1.2 seconds, a pulse width of 15 μseconds, and a transmitter offset of 2900 Hz. The 300 transients were accumulated with reference to orthophosphate buffer.

To determine the stereochemical make-up of the β phosphorus position, a portion of 23 mM uridine diphosphoglucose-β(S) made from uridine diphosphoglucose pyrophosphorylase was diluted and then mixed with the same compound made via galatose-1-phosphate uridylyl transferase. A ^{31}P NMR spectrum was taken within the region from -39.6 to -40.6 ppm to examine for diastereomers.

Uridine Diphosphoglucose-β(S) Assay with Glycogen Synthetase

Purified uridine diphosphoglucose-β(S) from the uridine diphosphoglucose pyrophosphorylase reaction was tested as a substrate

for glycogen synthetase from rabbit muscle. The reaction assay incorporating uridine diphosphoglucose-β(S) was as follows: 1 mM uridine diphosphoglucose β(S), 6 mg of glycogen and 50 mM HEPES buffer at pH 7.5. A catalytic amount of glucose-6-phosphate was also included for the glucose-6-phosphate dependent form of the enzyme. The reaction was catalyzed by 1 unit of glycogen synthetase. The assay using the natural substrate was similar. Incorporated into this assay were 1 mM uridine diphosphoglucose, 6 mg solid glycogen 50 mM HEPES buffer at pH 7.5, glucose-6-phosphate, and 1 unit of glycogen synthetase. The total volume of both assays was 3.0 mL.

The progress of both reactions assays was followed on a Gilson 811 HPLC. The parameters included an elution buffer of 250 mM phosphate at pH 4.5, a flow rate through the Whatman Partisil 10-SAX anion exchange column of 1.0 mL per minute, and a range of .7 Aufs. The chart speed was 5 mm per minute, the collect time was 15 minutes, and each injection was 20 μL. Upper limits were derived from the uridine diphosphoglucose-β(S) reaction by comparison to the reaction of the oxygen analogue and by estimating the least amount of product, uridine diphosphate (UDP) that could be confidently detected at the range of Aufs used.

Uridine Diphosphoglucose-β(S) Assay with Sucrose Synthetase

Uridine diphosphoglucose was tested as a substrate for sucrose synthetase that was isolated from wheat germ. The enzyme used in this experiment contained 0.76 units per mL. A typical assay involving uridine diphosphoglucose as a substrate consisted of the following: 2 mM uridine diphosphoglucose-β(S), 4 mM dithiothreitol, 50 mM fructose, and 1 unit or 1.3 mL of enzyme solution. The solution was buffered

by 50 mM of HEPES at a pH of 7.3. A control was run for this experiment consisted of the assay listed above except that fructose was omitted. The assay for the reaction of uridine diphosphoglucose with sucrose synthetase consisted of the following: 2 mM UDP-glucose, 50 mM fructose, and 0.7 units of enzyme solution. This assay was also buffered at pH of 7.5 by 50 mM HEPES. Total volume of both assays was 4.0 mL. Each reaction was initiated by addition of fructose. The HPLC parameters for this experiment were exactly as those used for the glycogen synthetase experiment. Upper limits for detection were also calculated in a similar manner as for the glycogen synthetase experiment.

Glucose-1-Phosphorothioate Assay with Phosphoglucomutase

The activity of phosphoglucomutase was checked on a Gilford 260 UV-VIS spectrometer by coupling the product glucose-6-phosphate to a glucose-6-phosphate dehydrogenase enzyme and following the reduction of NAD^+ to NADH at 340 nm. A typical assay consisted of 5 mM glucose-1-phosphate, 2 mM cysteine, 1 mM magnesium chloride, glucose-1,6 diphosphate, and 10 mM NAD^+. The enzymes used were 2 units of phosphoglucomutase and 10 units of glucose-6-phosphate dehydrogenase. The total reaction volume of 3 mL was effectively buffered by 20 mM HEPES at pH 7.5.

The reaction assay using glucose-1-phosphorothioate consisted of the following: a crude sample of 30 mM in concentration of glucose-1-phosphorothioate that was also 20 mM cysteine, 1.5 mM of magnesium chloride, and contained glucose-1,6 diphosphate. Sucrose phosphorylase had been removed by centrifugation using an Amicon

ultrafiltration membrane cone. The pH of this enzyme assay was 7.4 throughout the experiment. The reaction was buffered for a concentration of 100 mM HEPES that contained 25% D_2O. The reaction was initiated by the addition of 15 units of phosphoglucomutase. An assay containing glucose-1-phosphate was also run and consisted of 50 mM glucose-1-phosphate, 20 mM cysteine, 1.5 mM magnesium chloride, and glucose-1,6 diphosphate. This assay was also buffered at 100 mM HEPES and 30% deuterium oxide at pH 7.5. The reaction was initiated by the addition of 5 units of phosphoglucomutase.

The reactions were followed by ^{31}P NMR spectroscopy using the FT-80 multi-nuclear instrument. ^{31}P NMR parameters for the FT-80 included a frequency of 32.203 MHz, a sweep width of 200 Hz, an acquisition time of 0.5 seconds, and a pulse width of 7 μseconds. Exactly 1200 transients were accumulated to allow for 10 minute frames to be collected and stored. Upper limits were established using the best estimate of what could easily be seen in the signal to noise ratio.

Materials Used

The following chemicals were purchased from the Aldrich Chemical Company: deuterium oxide, deuterated chloroform, trimethylsilane, ammonium molybdate, ascorbate, and p-amino benzoic acid. Alpha Chemical Company purchases included: potassium cyanate, potassium thiocyanate, tetra sodium orthophosphorothioate, and silicon tetra isothiocyanate. Purchases from Sigma Chemical Company included: sucrose, DEAE-cellulose, P.E.I. cellulose plates, triethyl amine, Ellmans reagent, NAD^+, NADH, adenosine monophosphate and adenosine triphosphate. The buffers TRIS, PIPES, and HEPES were all purchased

from Sigma Chemical Company as were all the enzymes used except sucrose synthetase which was purified by Dr. A.H. Singh. Adenosine monophosphorothioate was provided by Tim Shull as a 28 mM solution. Dry THF was kindly provided by Dr. Martin E. Newcomb's laboratory. All other chemicals were purchased from Fisher Scientific.

RESULTS

Synthesis of Carbamoyl Phosphate and Related Reactions

The reaction of potassium cyanate and orthophosphate was followed by ^{31}P NMR spectroscopy as shown in Figure 1. The carbamoyl phosphate appears with time at 3.2 ppm upfield from orthophosphate. The reaction was followed for 90 minutes and the time course for the synthesis of carbamoyl phosphate was computed from the intensities of the peaks of carbamoyl phosphate as a percentage of the total phosphate resonance. Figure 2 shows a plot of this time course.

Potassium thiocyanate was then substituted for potassium cyanate in an attempt to produce thiocarbamoyl phosphate. This reaction failed. After 24 hours the ^{31}P NMR spectrum showed in Figure 3 does not differ significantly from the original orthophosphate spectrum. There are no other peaks that could account for thiocarbamoyl phosphate.

When the orthophosphorothioate was substituted for orthophosphate, a reaction was clearly visible by ^{31}P NMR spectroscopy. Figure 4 shows that there is a disappearance of the orthophosphorothioate peak and a corresponding appearance of a peak in the region associated with orthophosphate. Further investigation was undertaken by altering the concentration of reactants. Figure 5 shows how the reactants were varied and how this affected the rate and final product composition. When excess potassium cyanate was used, orthophosphorothioate was totally and rapidly converted to orthophosphate. Shown in Figure 6 is the result of allowing this reaction assay to incubate for 90 minutes. The formation of a peak,

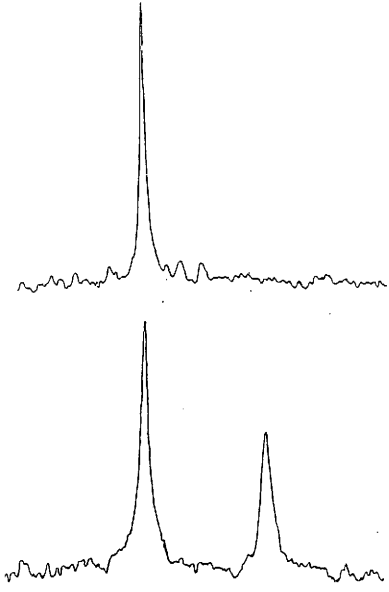


Figure 1: ^{31}P NMR spectra at 32.2 MHz showing the formation with time of carbamoyl phosphate (bottom spectrum) from orthophosphate (top spectrum). The difference between the peaks is 3.2 ppm.

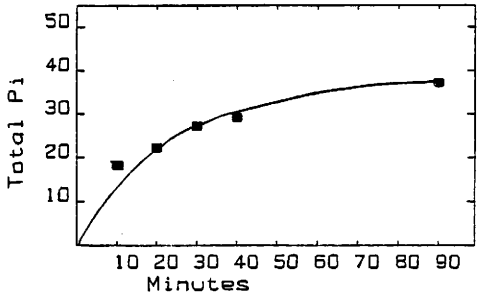


Figure 2: Time course of carbamoyl phosphate synthesis followed by ^{31}P NMR.

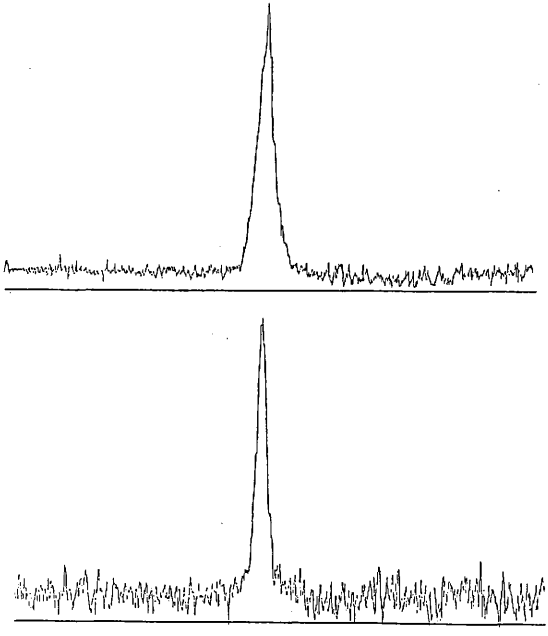


Figure 3: ^{31}P NMR spectra at 32.2 MHz showing orthophosphate at time zero (top spectrum) and again after 24 hours (bottom spectrum). The initial spectrum is essentially unchanged.

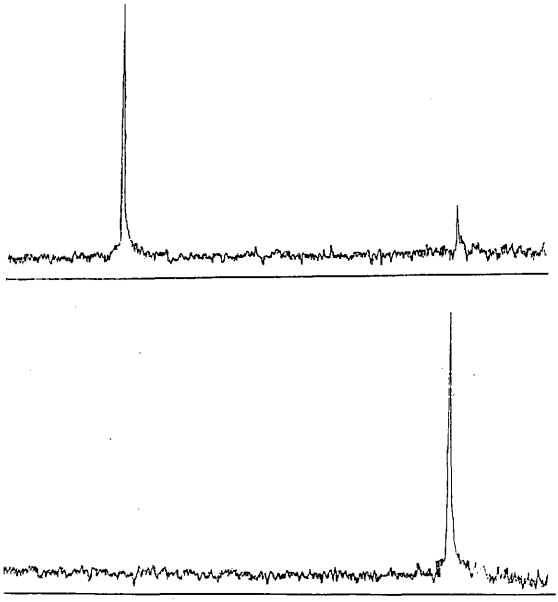


Figure 4: ^{31}P NMR spectra of orthophosphorothioate at time zero (note contaminating orthophosphate) is shown in the top spectrum. The bottom spectrum shows the results after 10 minutes had passed since the addition of potassium cyanate in a 2:1 ratio (200 mM to 100 mM of orthophosphorothioate).

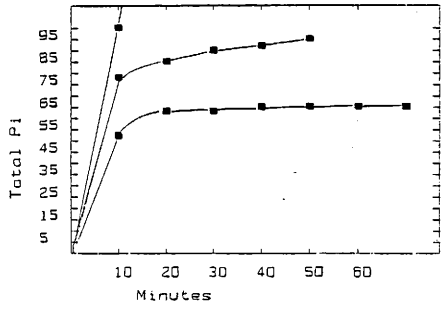


Figure 5: The time course of the reaction of orthophosphorothioate with cyanate at different ratios of potassium cyanate to orthophosphorothioate. Top: 2:1, middle: 1:1, and bottom: 1:2. Points were acquired by a comparison of the ^{31}P NMR peak intensities vs time.

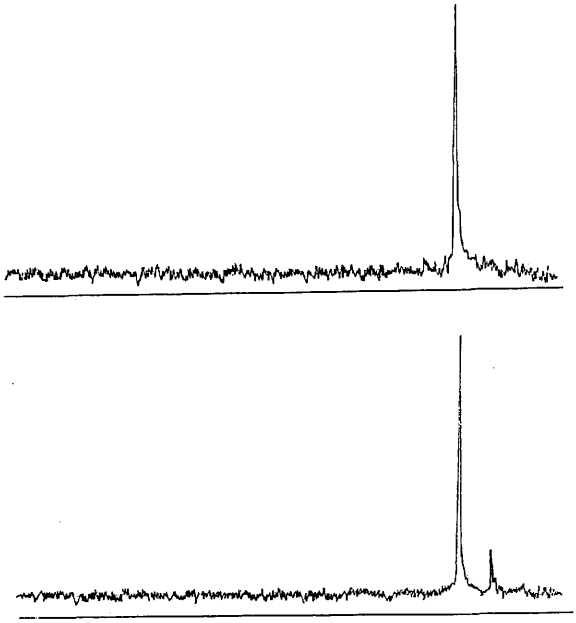


Figure 6: The ^{31}P spectrum at 32.2 MHz shows the peak resulting from the reaction of potassium cyanate and thiophosphate (top). After 90 minutes the formation of a peak 3 ppm from the peak in the top spectrum is seen (bottom). A comparison to Figure 1 suggests that the more downfield peak is orthophosphate while that peak forming at 3 ppm upfield is carbamoyl phosphate.

at 3.2 ppm from orthophosphate, confirms that orthophosphate has been produced.

Several attempts were made to try to utilize potassium cyanate in a desulfurization reaction as a method for the possible introduction of O^{17} or O^{18} isotopes of oxygen into phosphate esters as according to Scheme 10.

$$O = C = N \quad + \quad AMP(S) \quad \text{-----}> \quad AMP(O) \quad + \quad NH_3 \quad + \quad O = C = S$$

Scheme 10

The pH as well as the potassium cyanate concentration and assay temperature were all varied in different combinations. No conditions were found that resulted in the loss of sulfur from AMP(s).

Attempted Synthesis of Thiocitrulline

After the attempt to produce thiocarbamoyl phosphate failed, we attempted the chemical synthesis of thiocitrulline. It was hoped that thiocitrulline could be easily synthesized by modifications to the method of Nevell and McGee shown in the Scheme 11.

$$Si\ (N = C = S)_4 \quad + \quad R{-}NH_2 \quad \text{-----}> \quad R{-}\underset{H}{N}{-}\overset{\overset{S}{\|}}{C}{-}NH_2$$

Scheme 11

However, crystals of n-butyl thiourea were very difficult to obtain when this method was employed. An efficient method of recrystallizing the brown oil that was isolated was not devised. However, more favorable results were obtained by using the modification of the method of G.V. Nair as shown in Scheme 12.

1) $N=C=S + R\text{-}NH_2$ ----------> substituted, tert butyl thiocarbamide

2) $R'\text{-}N(H)\text{-}C(=S)\text{-}N(H)\text{-}R$ ----------> hydrolysis $H_2N\text{-}C(=S)\text{-}N(H)\text{-}R$ + t-butylchloride

Scheme 12

The tert-butyl thiocarbamide was easily obtained and recrystallized in excellent yield. However, the hydrolysis of this thiocarbamide produced a messy product that had to be washed and recrystallized several times in order to obtain a compound that melted within a reasonably close range to the accepted literature value for the target compound n-butyl thiourea. The proton NMR of the recrystallized compound did not yield encouraging results. The pertinent spectra are shown in Figures 7 and 8. Although the spectrum of the final isolated compound does contain resonances one would expect for an alkyl thiourea, it also contains other nitrogen containing species that are very difficult to attribute to a pure compound. The method of Nair was deemed an insufficient method for the synthesis of thiocitrulline.

Synthesis and Characterization of Glucose-1-Phosphorothioate

Orthophosphorothioate was submitted to the action of sucrose phosphorylase in the presence of excess sucrose. Orthophosphate was likewise used as a substrate to yield a comparison to the enzymes natural substrate. Since the reaction assay did not contain an easily identifiable chromophore the rate of the reaction was monitored by following the ^{31}P NMR spectrum and recording the

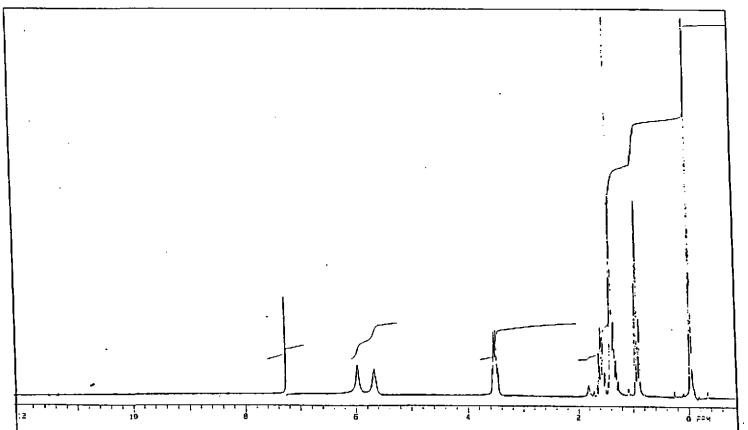


Figure 7: ^{1}H NMR at 200 MHz of n-butyl, t-butyl thio carbamide.

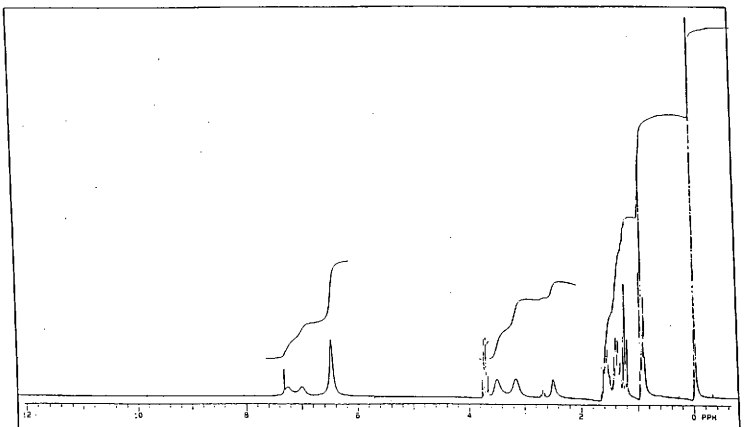


Figure 8: ^{1}H NMR at 200 MHz of the compound hydrolyzed from n-butyl, t-butyl thio carbamide. "Humps" at 7.0, 6.5, and 3.3 could be attributed to nitrogen containing compounds.

integrals of the peaks over time. In Figure 9, the ^{31}P NMR spectrum does show that orthothiophosphate is well utilized as a substrate, but the rate of the reaction of the thio analogue is much slower than that of the oxygen analogue (Figure 10). The overall rate of reaction for the thio analogue was found to be 0.13 μmoles per minute whereas that for the oxygen analogue was 0.54 μmoles per minute. Therefore, the thio analogue was utilized at only 24% of the reaction rate that was seen with orthophosphate as a substrate.

The purification of glucose-1-phosphorothioate was achieved by DEAE cellulose chromatography and was confirmed by comparing the ^{31}P NMR spectrum of pre-column assay material to post-column material as shown in Figure 11. The purified sample is essentially free of phosphates except for the peak at -45.9 ppm that represents glucose-1-phosphorothioate. The undecoupled ^{31}P NMR spectra yielded a single doublet with a coupling constant of 9.72 Hz versus 6.16 Hz for the oxygen analogue. Approximately 150 μmoles of crude assay material were loaded onto the column and 125 μmoles were isolated for a 83% yield.

Glucose-1-Phosphorothioate as a Substrate for Phosphoglucomutase

Glucose-1-phosphorothioate was tested as a substrate for phosphoglucomutase according to Scheme 13.

glucose-1-phosphorothioate glucose-6-phosphorothioate

Scheme 13

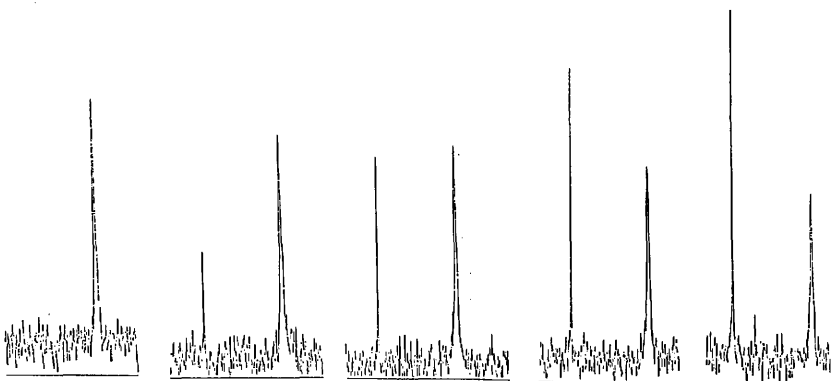


Figure 9: Shows the ^{31}P NMR at 81 MHz of the reaction of orthophosphorothioate and sucrose catalyzed by sucrose phosphorylase. Glucose-1-phosphorothioate is easily seen approximately 10 ppm downfield of orthophosphorothioate after 10 minutes (second frame from left). The glucose-1-phosphorothioate peak continues to grow with time as seen in the successive frames.

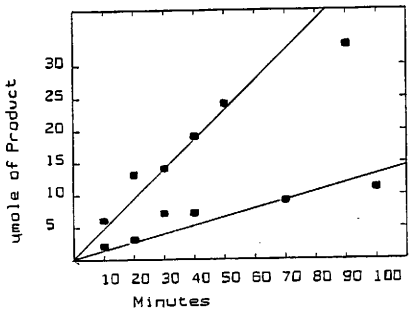


Figure 10: A comparison of the observed rate of the orthophosphate (top line) and orthophosphorothioate (bottom line) as μmoles of product produced per unit per minute. The points were taken from the integrals of the peaks obtained from the ^{31}P spectra.

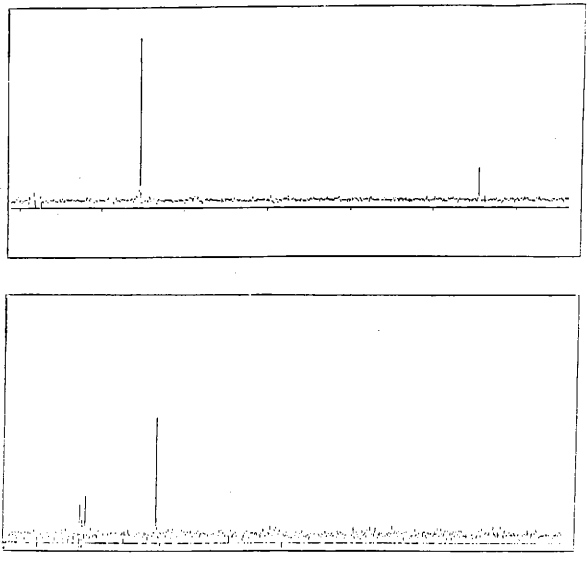


Figure 11: The ^{31}P NMR spectrum (top) of glucose-1-phosphorothioate (at -45.9 ppm) before undergoing column purification. The ^{31}P NMR spectrum (bottom) after column purification does not contain glucose-1-phosphate (at -5 ppm).

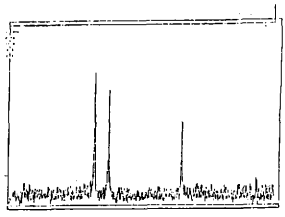
This would have enabled us to monitor the conversion of orthophosphorothioate to glucose-1-phosphorothioate on a smaller and perhaps more accurate scale by coupling glucose-1-phosphorothioate to glucose-6-phosphate dehydrogenase enzyme and consequently following the reduction of NAD^+ to NADH. However, phosphoglucomutase does not accept glucose-1-phosphorothioate as a substrate. The ^{31}P NMR spectrum shown in Figure 12 remains essentially unchanged in the glucose-1-phosphorothioate region at room temperature with time even after the addition of the dithiothreitol. However, it is easily noticed that there has been a reaction between the glucose-1-phosphate and the enzyme to produce glucose-6-phosphate. The upper limit for the reaction of glucose-1-phosphorothioate with phosphoglucomutase was calculated to be less than 1.25% of the rate of the oxygen analogue.

Synthesis and Characterization of Uridine Diphosphoglucose-β(S)

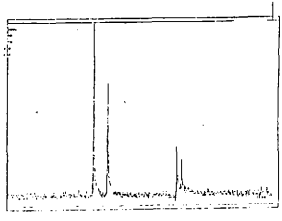
Glucose from Uridine Diphosphoglucose Pyrophosphorylase

Glucose-1-phosphorothioate was subjected to the action of yeast uridine diphosphoglucose pyrophosphorylase in the presence of excess uridine triphosphate. The inclusion of inorganic pyrophosphatase insured that the pyrophosphate produced would be hydrolyzed to orthophosphate. Dithiothreitol was imperative to the success of all assays that contained glucose-1-phosphorothioate. Dithiothreitol was not needed for assays that included glucose-1-phosphate. According to Figure 13, glucose-1-phosphorothioate was utilized at 12.5% of the rate of the natural substrate glucose-1-phosphate. Glucose-1-phosphorothioate had a rate of 0.02 μmoles per minute vs 0.16 μmoles

A. glucose-1-phosphorothioate, orthophosphorthioate, and glucose-1-phosphate before addition of phosphoglucomutase.



B. 1 hour after the addition of phosphoglucomutase: glucose-6-phosphate has formed slightly downfield from glucose-1-phosphate.



C. After 24 hours only glucose-1-phosphorothioate is seen. Spectrum resembles A except that glucose-1-phosphate has been converted to glucose-6-phosphate.

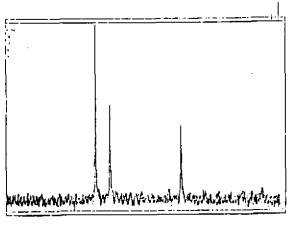


Figure 12: ^{31}P NMR spectra at 32 MHz.

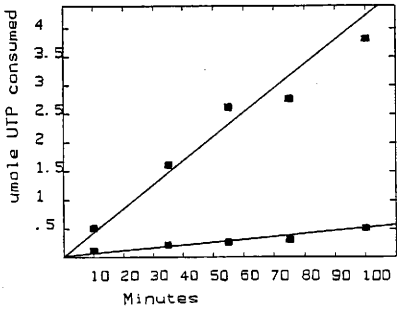


Figure 13: A comparison of the observed rate of the glucose-1-phosphorothioate and glucose-1-phosphate as μmoles of UTP consumed per unit per minute. These points were taken from the integrals of the HPLC peaks corresponding to UTP.

per minute for the oxygen analogue for a given amount of enzyme.

Purification of uridine diphosphoglucose-β(S) is shown in Figure 14, and was achieved by DEAE cellulose chromatography with the elution profiles recorded at 254 nm showing clear separation between uridine diphosphoglucose and uridine diphosphoglucose-β(S). 270 μmoles of uridine diphosphoglucose-β(S) were loaded onto the column and 190 μmoles were isolated for a 75% yield. The ^{31}P NMR spectra are shown before and after column purification in Figure 15. The spectrum shows that uridine diphosphoglucose-β(S) has been purified from orthophosphate, uridine diphosphoglucose-β(S), and any unreacted uridine triphosphate. Doublets are seen at -45.9 and 11.0 ppm for the thio analogue and 8.6 and 10.4 for the oxygen analogue. The ^{31}P NMR also yielded coupling constants of 27.81 for Jα, β(S) and Jα, β vs 20.25 Jα, β, respectively. (Literature value 20.75).[4]

Uridine Diphosphoglucose-β(S) as a Substrate for Glycogen Synthetase and Sucrose Synthetase

Uridine diphosphoglucose-β(S) was tested as a substrate for both sucrose synthetase and glycogen synthetase (E.C. 2.4.1.11) enzymes. The reaction for glycogen synthetase is shown below.

$$\text{Uridine diphosphoglucose} + (\text{glycogen})_n \text{ -----> } \text{UDP} + (\text{glycogen})_{n+1}$$

The reactions were followed by HPLC and monitored for the appearance of any peak with a retention time similar to uridine diphosphate (approximately 11.5 minutes). Both sucrose synthetase and glycogen synthetase catalyzed the natural uridine diphosphoglucose substrate

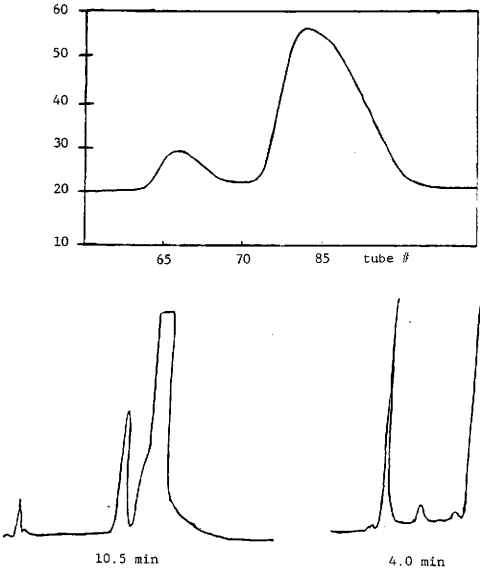


Figure 14: The purification of uridine diphosphoglucose-β(S) on DEAE-cellulose. The HPLC spectrum on the left shows uridine diphosphoglucose-β(S) before purification (peak at 10.5 minutes is UDP-glucose). The HPLC trace on the right shows uridine diphosphoglucose-β(S) after purification.

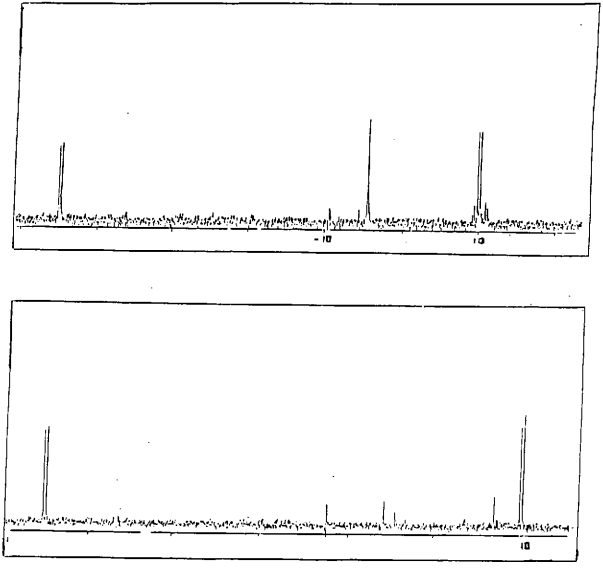


Figure 15: ^{31}P NMR at 81 MHz of uridine diphosphoglucose-β(S). The figure at the top is unpurified material. The figure at the bottom shows the result of the column purification: uridine diphosphoglucose and orthophosphate have been removed.

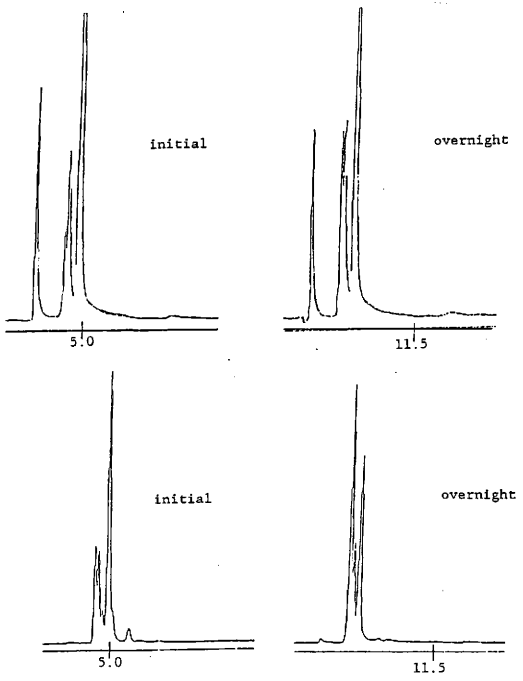


Figure 16: Reaction of uridine diphosphoglucose-β(S) with sucrose synthetase (top) and fructose shows no appearence of uridine diphosphoglucose-β(S). The control without fructose is shown at bottom. Uridine diphosphoglucose and uridine diphosphate-β(S) eluted at 5.0 minutes.

very efficiently with sucrose synthetase producing uridine diphosphate at .149 μmole per minute and with glycogen synthetase producing uridine diphosphate at .054 μmole per minute. No uridine diphosphate-β(S) could be detected by HPLC (Figures 16 and 17). It would be safe to conclude from the data that at best uridine diphosphoglucose-β(S) is an extremely poor substrate for sucrose synthetase and glycogen synthetase. The upper limits for the reaction were calculated at less than 0.01% of the rate of the natural substrate for sucrose synthetase and less than 0.2% of the rate of the natural substrate for glycogen synthetase.

Glucose-1-Phosphorothioate as a Substrate for Galactose-1-Phosphate Uridylyl Transferase

Glucose-1-phosphorothioate was submitted as a substrate for galactose-1-phosphate uridylyl transferase. The process of the reaction is outlined in Figures 18, 19, and 20. This reaction was followed by HPLC and the results are shown in Figure 21. The reaction proceeded to 75% completion with a rate of .0021 μmoles per minute. It was determined that the glucose-6-phosphate dehydrogenase enzyme was not essential in order to produce uridine diphosphoglucose-β(S). This enabled us to scale up the reaction assay for characterization of this substrate.

The uridine diphosphoglucose-β(S) from galactose-1-phosphate uridylyl transferase was purified on a column of DEAE cellulose according to methods used to purify the uridine diphosphoglucose-β(S) produced from uridine diphosphoglucose pyrophosphorylase. Eight μmoles were loaded onto the column and six μmoles were isolated for

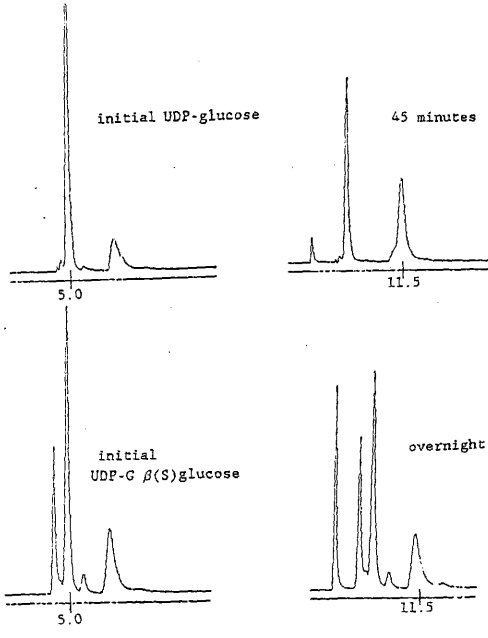


Figure 17: HPLC comparison of UDP-glucose (top) and UDP-G β(S) glucose (bottom) with glycogen synthetase. UDP is quite visable after 45 minutes (second frame at top at 11.5 minutes retention time) but there is not any UDP like peak with UDP-β(S) glucose as substrate (second frame at bottom) even after 24 hours.

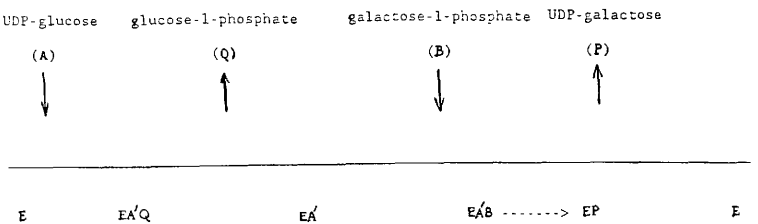


Figure 18: Bi-Bi order for binding of uridyl transferase enzyme showing binding of substrates and release of products.

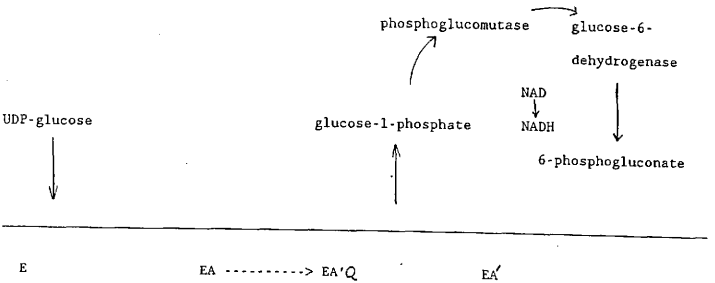


Figure 19: Full enzyme substrate complex is not allowed to form since galactose-1-phosphate was not included in the assay. UDP-glucose is bound, E ----> EA, and glucose-1-phosphate is released as UMP remains bound as EA'. Glucose-1-phosphate is then coupled with phosphoglucomutase and glucose-1-phosphate dehydrogenase to formed non-substrate 6-phosphogluconate.

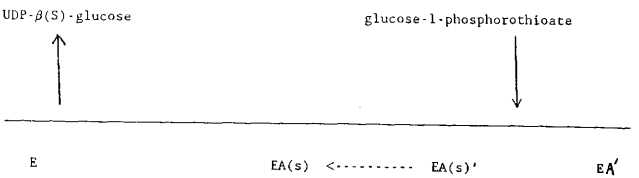


Figure 20: Excess glucose-1-phosphorothioate is then bound by the EA-like complex (EA(s)' enzyme and reforms the complex (sulfur is now substituted at the β phosphorus position). The EA'(s) complex is then converted to the EA(s) complex and UDP-β(S) glucose is released.

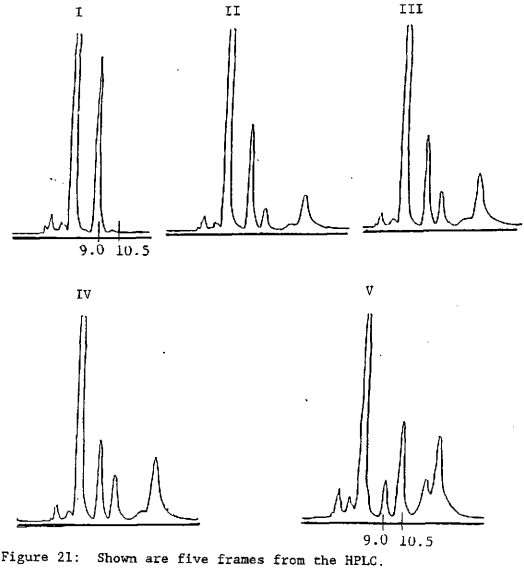


Figure 21: Shown are five frames from the HPLC. I is at time 0. II is at 100 minutes after the addition of the enzyme. III is at 170 minutes and IV is at 240 minutes since the addition of enzyme. V shows the HPLC after 24 hours. Retention times were as follows: NAD^+, 6.5 minutes; NADH, 15 minutes; uridine diphosphoglucose, 9.0 minutes; and uridine diphosphoglucose-β(S) at 10.5 minutes.

a 75% yield. The ^{31}P NMR spectrum showed that uridine diphosphoglucose-β(S) contained no other phosphorus containing compounds except for the orthophosphate buffer.

In order to best test for diastereomerism, an authentic sample of uridine diphosphoglucose-β(S) from the uridine diphosphoglucose pyrophosphorylase reaction (Figure 22) was mixed with that produced by galactose-1-phosphate uridylyl transferase (Figure 23) and the ^{31}P NMR spectrum of the mixture was recorded. The resulting ^{31}P NMR spectra shown in Figure 24 yielded a single doublet between -38 ppm to -43 ppm instead of the doublet of doublets that one would expect for a mixture of two diastereomers. Therefore, the uridine diphosphoglucose-β(S) produced from uridine diphosphoglucose pyrophosphorylase and that produced by uridylyl transferase are the same isomer unless the ^{31}P resonances are coincidentally identical.

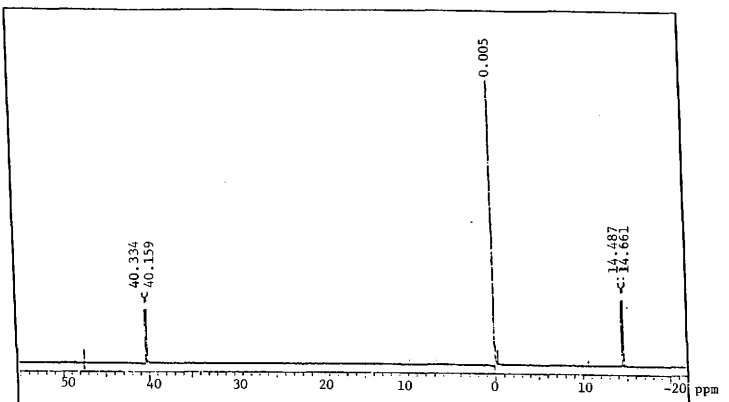


Figure 22: ^{31}P NMR from uridine diphosphoglucose pyrophosphorylase. Doublets appear at -40.2 ppm and 14.5 ppm when referenced to orthophosphate buffer.

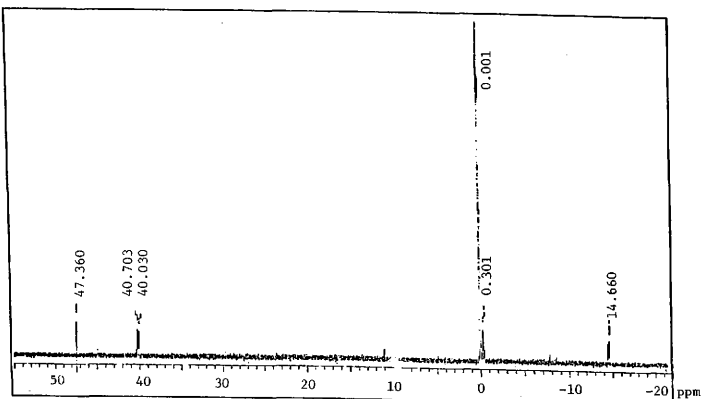


Figure 23: ^{31}P NMR from galactose-1-phosphate uridylyl transferase. Doublets appear at -40 ppm and 14.6 ppm when referenced to orthophosphate buffer.

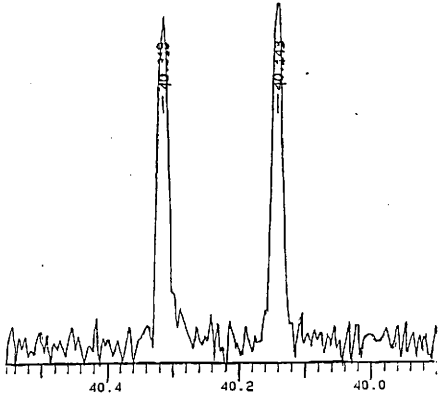


Figure 24: The ^{31}P NMR spectrum of the region from -39.9 to -40.5.

DISCUSSION

The Synthesis of Carbamoyl Phosphate Derivatives

The synthesis and decomposition of carbamoyl phosphate have been well studied. In our investigation we found that the synthesis of carbamoyl phosphate could be followed on the FT-80 accurately. Any differences from the literature rate of synthesis most likely would arise from differences in method. Jones et al., employed the Fiske-Subbarrow method for analysis of free phosphate.[13] However, carbamoyl phosphate reacts with molybdate to produce free phosphate. This could lead to an artificially high concentration of orthophosphate and thus a net slower appearance of carbamoyl phosphate. Once the reliability of the ^{31}P NMR method was established, our attention turned toward the synthesis of target compounds thio carbamoyl phosphate and carbamoyl phosphorothioate.

Thiocarbamoyl phosphate failed to form from thiocyanate and orthophosphate at the optimum synthesis pH of 5.8. However, in the attempt to form carbamoyl phosphorothioate from cyanate and orthophosphorothioate, we encountered surprising results. It was clear from the ^{31}P NMR that the phosphorothioate monoanion was being catalyzed to orthophosphate since the contaminating orthophosphate peak grew in direct proportion to the decrease in the phosphorothioate peak. This suspicion was confirmed when an excess of cyanate produced a new resonance at 3 ppm upfield from orthophosphate. This could only have happened had the excess cyanate reacted with orthophosphate to produce carbamoyl phosphate. Obviously the simple mechanistic

scheme outline envisioned earlier does not apply to this reaction. The mechanistic pathway of Figure 25 is a more likely approach.

It was conceivable that the highly electropositive carbonyl carbon of cyanate is attacked by the nucleophilic sulfur of the phosphorothioate to form an intermediate compound highly susceptible to P-S bond cleavage which could then lead to the formation of carbon oxysulfide or carbon dioxide (depending on the rate sulfur is "washed out" during the reaction) and ammonia. Catalyzed desulfurizations are known in the literature.[23] This mechanism is also consistent with gas build up that was observed in the reaction vessel.

Since the above reaction undoubtedly involves the substitution of an oxygen from a water molecule for sulfur on the phosphorothioate, we attempted to repeat this experiment using adenosine monophosphorothioate. This could have proven to be an effective method for the introduction of O^{18} or O^{17} labeling. However, with adenosine monophosphorothioate the loss of sulfur was not catalyzed. The cause of this failure was not investigated.

Attempted Synthesis of Thiocitrulline

We were unsuccessful in our attempts to synthesize a n-butyl thiourea precursor to thiocitrulline. The failure of the synthesis of n-butyl thiourea according to the method of Nevel and McGee[17] could be attributed to the inability to produce totally anhydrous conditions in the reaction mixture. Since the starting material of silicon tetraisothiocyanate reacts readily with H_2O to produce SiO_2, the conditions must be strictly anhydrous for success. Our experience with silicon tetraisothiocyanate showed it to be

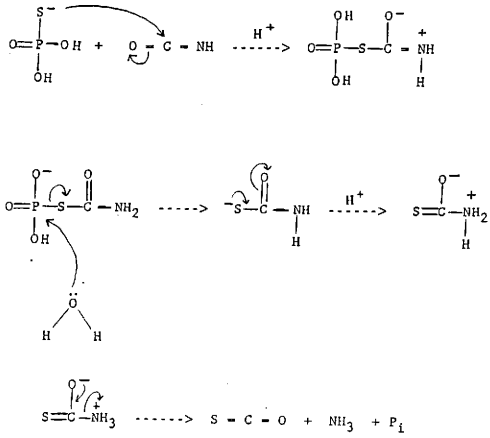


Figure 25: Mechanistic scheme showing the formation of thiocarbamoyl phosphorothioate with the sulfur in the bridging position. This molecule then is possibly decomposed to carbonyl oxysulfide, ammonia, and orthophosphate.

extremely sensitive to atmospheric moisture. Silicon tetra-isothiocyanate will decompose from an off-white color to bright orange when left to the open air even after a few seconds. This extreme sensitivity to moisture made the method of Nevel and McGee[17] unsuitable for work with the milligram quantities which we expected to use with the blocked ornithine.

The method of G.V. Nair[18] displayed some initial success. The intermediate disubstituted n-butyl, t-butyl thiocarbamate was successfully synthesized in high yield and purity. However, the hydrolysis of this compound yielded a sample that was difficult to purify by recrystalization. Since the Nair method involves the formation of an alkyl cation it is reasonable to assume that there must exist some degree of carbocation rearrangement that could lead to a mix of similar alkyl monosubstituted thioureas. The disappointing results with the hydrolysis of the thiocarbamide and the potential for unspecific hydrolysis with the blocked ornithine led us to conclude that the method of Nair was also unsuitable for producing thiocitrulline.

Phosphorothioates as Substrates for Sucrose Phosphorylase, Phosphoglucomutase, Uridine Diphosphoglucose Pyrophosphorylase, Galactose-1-Phosphorothioate Uridylyl Transferase, Sucrose Synthetase, and Glycogen Synthetase

Sucrose phosphorylase was able to catalyze the reaction of sucrose and orthophosphorothioate to glucose-1-phosphorothioate although this production was quite sluggish in comparison to the orthophosphate substrate. The slower overall rate is a direct

consequence of the subtle chemical differences between sulfur and oxygen. Phosphorothioates are almost always poorer substrates than their oxygen analogues although there are some exceptions.[24,25] Glucose-1-phosphorothioate also exhibits the characteristic downfield chemical shift that is typical of phosphorothioates. In addition, glucose-1-phosphorothioate also exhibits a greater affinity for ion exchange material than does its oxygen relative glucose-1-phosphate.

Glucose-1-phosphorothioate was also a typical phosphorothioate in that it was not catalyzed to glucose-6-phosphorothioate by phosphoglucomutase. Once again, this is not surprising when one considers that another well studied phosphosugar mutase, phosphoglycerate mutase, does not accept the phosphorothioate analogues of 3' or 2' phosphoglycerate.[24]

However, glucose-1-phosphorothioate was accepted by both uridine diphosphoglucose pyrophosphorylase and galactose-1-phosphate uridylyl transferase. Once again, a reduced rate was seen with each enzyme. The inclusion of a thiol reducing agent such as dithiothreitol was essential to the catalytic integrity of uridine diphosphoglucose pyrophosphorylase. This suggests that glucose-1-phosphorothioate is acting in an inhibitory manner by forming disulfide bonds either directly in the active site or in close proximity to it. Glucose-1-phosphorothioate did not necessitate the use of thiol reducing agents with galactose-1-phosphate uridylyl transferase. Both uridine diphosphoglucose-β(S) products from galactose-1-phosphate uridylyl transferase and uridine diphosphoglucose pyrophosphorylase showed the downfield ^{31}P NMR chemical shifts characteristics of phosphorothioate

analogues of uridine diphosphoglucose. They also had a greater affinity for ion exchange since they eluted on the HPLC and DEAE-cellulose at longer times than the oxygen analogue. However, the HPLC of purified uridine diphosphoglucose-β(S) did show that this compound was unstable at room temperature.

Since uridine diphosphoglucose-β(S) is a chiral molecule with respect to the β phosphorus, the possibility of isomerism was investigated using high field ^{31}P NMR. It was hoped that the different enzymes would produce different isomers. Unfortunately, the only conclusion that could be reached from the ^{31}P NMR results is that uridine diphosphoglucose-β(S) from uridine diphosphoglucose pyrophosphorylase and galactose-1-phosphate contained the same stereochemistry at the β phosphorus position since the highfield ^{31}P NMR resonances were identical. It is possible that the existence of diastereomers could not be detected by high field NMR (i.e. the ^{31}P NMR resonances could be identical for both the S_p and R_p diastereomers). However, Frey was able to clearly account for the existence of R_p and S_p diastereomers of uridine diphosphoglucose-α(S) by their easily distinguishable ^{31}P NMR spectra. Nucleotide phosphorothioate compounds that could not be distinguishable by their ^{31}P NMR were shown to be the same isomer. Therefore, it would be highly unlikely for the beta-sulfur substituted diastereomers of uridine diphosphoglucose to exhibit strikingly dissimilar ^{31}P NMR resonance behavior.

Uridine diphosphoglucose-β(S) was tested as a substrate for both sucrose synthetase and glycogen synthetase with the same negative

result. Since uridine diphosphoglucose-β(S) is undoubtedly a single isomer, it is likely that the opposite isomer may prove to be a substrate for one or both of these enzymes.

CONCLUSION

The modifications of the method of Jones[13] for the synthesis of carbamoyl phosphate failed to produce the desired thio-derivatives of carbamoyl phosphate; thiocarbamoyl phosphate and carbamoyl phosphorothioate. The investigation of the ^{18}O labeling possibilities of cyanate with phosphorothioates proved to be negative in that the monophosphorothioate, adenosine monophosphorothioate showed no signs of sulfur cleavage.

The chemical synthesis of thiocitrulline was not undertaken since all attempts to produce a precursor compound, n-butylthiourea, were unfruitful.

The strategy for producing uridine diphosphoglucose-β(S) was successful. Unfortunately, the isomer produced by uridine diphosphoglucose pyrophosphorylase and that produced by galactose-1-phosphate uridylyl transferase were the same. This diastereomer was not a kinetically competent substrate for sucrose synthetase nor was it a substrate for a similar enzyme, glycogen synthetase. Further investigation of sucrose synthetase must await the synthesis of the opposite diastereomer of uridine diphosphoglucose-β(S) for the back reaction catalyzed by this enzyme.

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