

## Nucleosides and Nucleotides. X. Synthesis of 4-Thiouracil Nucleosides and Nucleotides by the Solvolysis of Cytidine and Its Phosphates with Hydrogen Sulfide<sup>1)</sup>

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Treatment of cytidine with liquid hydrogen sulfide-pyridine afforded 4-thiouridine in an excellent yield. Similar treatment of sodium cytidine 5'-phosphate, cytidine 5'-diphosphate (CDP), cytidine 5'-triphosphate (CTP), and CDP-choline afforded the respective 4-thiouridine phosphates. In the case of CDP and CTP the hydrolytic cleavage of pyrophosphate linkage was noticed.

Several cytidine derivatives such as arabinofuranosylcytosine, 3-methylcytidine, and 2-thiocytidine also underwent sulphydrolysis affording respective 4-thio derivatives. The present method seemed to be the most versatile procedure for the synthesis of 4-thiouracil nucleosides and nucleotides.

4-Thiouridine (Ia), initially synthesized by Fox and co-workers,<sup>3)</sup> has been identified as one of the minor nucleoside constituents of transfer ribonucleic acid (t-RNA).<sup>4)</sup> The structural studies of t-RNA containing Ia revealed that Ia commonly occupies the eighth position from the 5'-terminus. Thereafter a number of studies has been made to clarify the function of this minor component in the structure-activity relationship of t-RNA.<sup>5)</sup> The ultraviolet spectral properties of Ia is unique in that its absorption maximum is located in longer wave-length (330 nm) than that of the major nucleosides. Therefore, it could serve as a reporter molecule if one can introduce Ia into the desired position of nucleic acid molecule. Furthermore, since Ia is known as a useful intermediate in the transformation reaction of the base moiety of pyrimidine nucleosides, the 4-thiouracil moiety in t-RNA becomes a target of chemical modification.

We have recently reported<sup>1,7)</sup> that the solvolytic cleavage of the *O*-cyclo linkage of a *O*<sup>2</sup>,5'-cyclouridine with liquid hydrogen sulfide and pyridine afforded almost exclusively a 2-thiouridine rather than a thiopentofuranosyluracil. In the course of our extended studies of the sulphydrolytic reaction of *O*-cycloctidines we have encountered a facile replacement reaction of amino with thiol group in the cytosine nucleus. This paper describes a versatile method of preparation of Ia and its phosphate esters from cytidine and its phosphates. A preliminary account of the results has appeared.<sup>8)</sup> The application of this method to dinucleoside monophosphates containing cytosine moiety has also been reported in advance.<sup>6)</sup>

Cytidine (II) was treated with a solution of liquid hydrogen sulfide-pyridine-water at 60° for 72 hours in a sealed stainless steel tube. After evaporation of the solvent and the removal of separated elemental sulfur Ia was obtained in high yield as a yellow crystalline mass from ethanol-ethyl acetate (by seeding). The solvent of the reaction can be displaced

1) Part IX: T. Ueda and S. Shibuya, *Chem. Pharm. Bull.* (Tokyo), **22**, 930 (1974).

2) Location: Kita-12, Nishi-6, Kita-ku, Sapporo.

3) J.J. Fox, D.V. Praag, I. Wempen, I.L. Doerr, L. Cheong, J.E. Knoll, M.L. Eidinoff, A. Bendich,<sup>\*</sup> and G.B. Brown, *J. Am. Chem. Soc.*, **81**, 178 (1959).

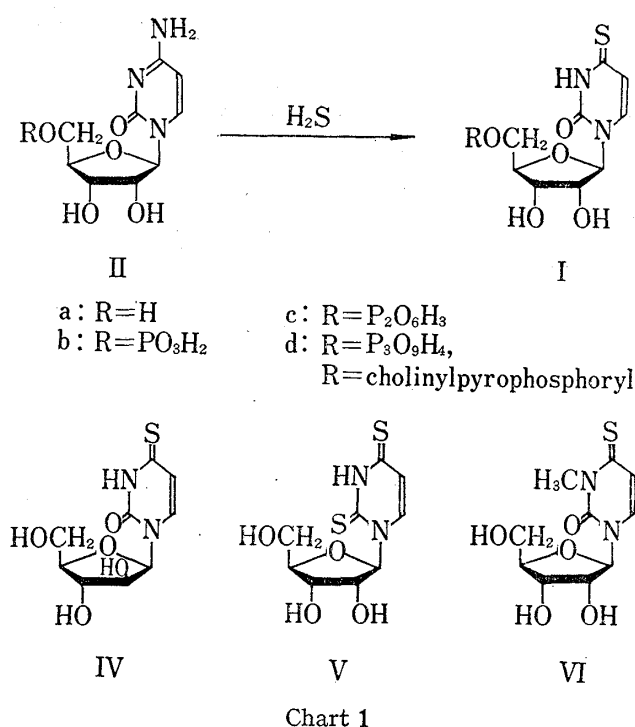
4) M.N. Lipsett, *J. Biol. Chem.*, **240**, 3975 (1965).

5) See references cited in part VI of this series.<sup>6)</sup>

6) K. Miura, M. Shiga, and T. Ueda, *J. Biochem.* (Tokyo), **73**, 1279 (1973).

7) T. Ueda and S. Shibuya, *Chem. Pharm. Bull.* (Tokyo), **18**, 1076 (1970).

8) T. Ueda, M. Imazawa, K. Miura, R. Iwata, and K. Odajima, *Tetrahedron Letters*, **1971**, 2507.



with dimethylformamide, methanol or probably other polar solvents. In a concentrated sodium hydrogen sulfide solution IIa was similarly converted to Ia, although the isolation procedure was rather laborious. The comparison was made of the solvent effect on the rates of formation of Ia from IIa and the results were summarized in Table I along with the solvolysis of 2',3'-O-isopropylidencytidine (III). It can be seen from Table I that; a, aqueous pyridine is the most superior as the solvent; b, 2',3'-O-isopropylidencytidine (III) accelerates the solvolysis. From the accumulated examples<sup>9)</sup> it can be explained that this rate enhancement was brought about by the nucleophilic addition of the 5'-hydroxyl group of III to C-6 position, thus made substitution at C-4' position much easier.

TABLE I. Sulfhydrolysis of Cytidines in Various Solvent Systems

Exp. No.	Compound	Reaction system <sup>a)</sup>	Reaction time at 40°, hr	Yield, %.
1	cytidine (IIa)	NaSH-H <sub>2</sub> O	45	5
2	cytidine (IIa)	H <sub>2</sub> S-DMF	45	18
3	cytidine (IIa)	H <sub>2</sub> S-MeOH-H <sub>2</sub> O	45	45
4	cytidine (IIa)	H <sub>2</sub> S-pyridine-H <sub>2</sub> O	20	42
5	cytidine (IIa)	H <sub>2</sub> S-pyridine-H <sub>2</sub> O	45	67
6	2',3'-O-isopropylidencytidine (III)	H <sub>2</sub> S-pyridine-H <sub>2</sub> O	20	93
7	2',3'-O-isopropylidencytidine (III)	H <sub>2</sub> S-pyridine-H <sub>2</sub> O	45	100

a) For details of the reaction condition see experimental section.

During the solvolysis the hydrolytic deamination was also observed in some case (Exp. no. 1 in Table I) which can be practically excluded by the use of a large excess of hydrogen sulfide, or the deletion of water if the solubility of the substrate allows. By the similar procedure cytosine, arabinofuranosylcytosine, 2-thiocytidine and 3-methylcytidine were also converted to 4-thiouracil, arabinofuranosyl-4-thiouracil (IV), 2,4-dithiouridine (V),<sup>10)</sup> and 3-methyl-4-thiouridine (VI),<sup>11)</sup> respectively. Furthermore this method was successfully extended to the synthesis of various phosphoric esters of I. Treatment of sodium cytidine 5'-phosphate (CMP, IIb) with hydrogen sulfide-pyridine-water system at 60° for 41 hours afforded disodium 4-thiouridine 5'-phosphate (Ib) as the crystalline form from aqueous ethanol in a yield of 70%. Cytidine 5'-diphosphate (CDP, IIc) and 5'-triphosphate (CTP, IId) were likewise converted to their 4-thiouracil counterparts (Ic, Id). During the solvolysis of IId

9) For example see: a) D.V. Santi and C.F. Brewer, *J. Am. Chem. Soc.*, **90**, 6236 (1968); b) R.W. Chambers and V. Kurkov, *J. Am. Chem. Soc.*, **85**, 2160 (1963); c) H. Hayatsu, Y. Wataya, K. Kai, and S. Iida, *Biochem.*, **9**, 2858 (1970).

10) T. Ueda, Y. Iida, K. Ikeda, and Y. Mizuno, *Chem. Pharm. Bull. (Tokyo)*, **16**, 1788 (1968).

11) K.H. Scheit, *Tetrahedron Letters*, **1967**, 113.

(and IIc), however, extensive hydrolysis and dismutation of pyrophosphate linkages<sup>12)</sup> were observed, and the products from IIc were 4-thioUMP (Ib), -UDP(Ic), -UTP(Id), and 4-thiouridine 5'-tetrakisphosphate along with inorganic phosphate and pyrophosphate (see Fig. 1). If this dismutation and degradation were promoted by the sulfhydrolysis of the pyrophosphate linkages the phosphorothioate analogs of these nucleotides or phosphorothioic acids should be detected in the reaction products. To test this possibility uridine 5'-triphosphate (UTP) was kept under similar reaction conditions and the degradates were separated on a DEAE-cellulose column chromatography into five fractions according to the charge number. Each fraction was checked of the identity with the uridine phosphates on thin-layer chromatogram (PEI-cellulose). As shown in Table II neither fraction contained any phosphorothioates detectable with the SH-spray reagent.<sup>13)</sup> Therefore the dismutation of UTP to  $UP_{1-5}$  must be catalyzed by pyridine and not by sulfhydryl ion. The use of 2,6-lutidine in place of pyridine<sup>12b)</sup> did not prevent this dismutation. Cytidine diphosphate choline (CDP-choline, IIe) was quite stable even under forced reaction conditions and was converted almost quantitatively to 4-thio-UDP-choline (Ie).

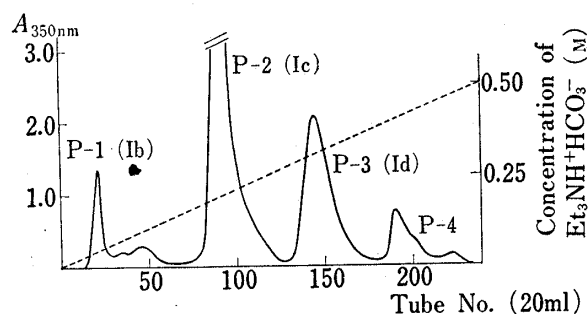


Fig. 1. Ion-exchange Chromatography of Sulfhydrolytic Products of Cytidine 5'-triphosphate (IIc)<sup>a)</sup>

a) For the details see experimental section.

TABLE II. *R<sub>f</sub>* Values of the Thin-Layer Chromatography (TLC) of the Degradates of Uridine 5'-Triphosphate under Sulfhydrolytic Conditions

Compound(peak) <sup>a)</sup>	Tube No.	ODU <sub>260nm</sub>	<i>R<sub>f</sub></i> <sup>b)</sup>
UMP	—	—	0.92
UDP	—	—	0.76
UTP	—	—	0.41
Peak-1	69—77	90	0.92
Peak-2	116—132	1050	0.76
Peak-3	135—149	3000	0.40
Peak-4	160—172	155	0.15
Peak-5	201—220	45	0.03
			(0.15, 0.40) <sup>c)</sup>

a) Peaks and tube no. are those of the eluents of the column chromatography as described in the text. One tube contains 20 ml of the eluent.

b) TLC was performed on polyethyleneimine-cellulose (Cel 300PEI, Machery, Nagl, West Germany) sheets with following solvent system: IpOH-0.75M  $KH_2PO_4$ , pH 3.5 (5:20). The sheets were previously developed twice with the solvent, dried, and used.

c) Partially degraded during the work-up.

The presently described method of preparation of I is unique when compared with the prevalent procedures. The thiation of uridine to Ia requires prior protection of the sugar hydroxyl groups.<sup>1)</sup> The phosphorylation of Ia to Ib requires several steps and choice of the phosphorylating agents adaptable to this particular case is rather limited because of the

12) a) D.L.M. Verheyden, W.E. Wehrli, and J.G. Moffatt, *J. Am. Chem. Soc.*, **87**, 2257 (1965); b) W.E. Wehrli and J.G. Moffatt, *J. Am. Chem. Soc.*, **87**, 3760 (1965).

13) R.S. Goody and F. Eckstein, *J. Am. Chem. Soc.*, **93**, 6252 (1971).

lability of the 4-thio function toward acid or alkaline hydrolytic conditions.<sup>14,15)</sup> Providing that the proper cytosine derivatives are readily accessible, the present method is superior, since no protection (hence deblocking) of sugar and phosphate portion is necessary. In fact, several dinucleoside monophosphates containing 4-thiouridine have been prepared from those of cytidine.<sup>6)</sup>

Reports on the conversion of an amino group to a thiol group in nitrogen-containing heteroaromatic compounds are relatively rare although the reverse reactions are quite general. To our knowledge the conversion of 6-amino-5,5-dialkyluracils to 5,5-dialkyl-4-thiobarbituric acids by alcoholic hydrogen sulfide under elevated temperature is the sole previous report related to the present procedures in pyrimidines.<sup>16)</sup> In general, this type of substitution together with the present procedure can be correlated to the method of preparation of thiono esters and thioamides by the sulfhydrolysis of imino esters<sup>17)</sup> and amidines,<sup>18)</sup> respectively. The applicability of the present method to other aminopyrimidines and heteroaromatic amines are currently undertaken in our laboratory.<sup>19)</sup>

### Experimental

**4-Thiouridine (Ia) from Cytidine**—Cytidine (1.0 g) was dissolved in 15 ml of H<sub>2</sub>O and to this was added H<sub>2</sub>S-pyridine solution (prepared by condensing H<sub>2</sub>S gas in 15 ml of pyridine under dry-ice acetone cooling to a volume of 40 ml) in a refrigerated stainless steel tube. The sealed tube was heated at 60° for 72 hr in an oil bath. After vaporization of the most of H<sub>2</sub>S gas the solvent was removed *in vacuo* and the residue was taken up in H<sub>2</sub>O. This procedure was repeated several times and the final residue (TOD<sub>330nm</sub>, 90000) was dissolved in EtOH-EtOAc and set aside at room temperature with seeding. Separated yellow crystals of Ia, 640 mg, were collected by filtration. The identification was made by the direct comparison of ultraviolet (UV) and mp (135–138°) with the authentic material.<sup>20)</sup> From the mother liquor more crystals (130 mg, total yield 69%) were obtained. Treatment of 1.5 g of cytidine in 50 ml of DMF with H<sub>2</sub>S-pyridine (1:1, 50 ml) at 50° for 3 days afforded 800 mg of Ia.

**Comparison of the Rate of Reaction of Cytidine with H<sub>2</sub>S in Various Solvent Systems**—Cytidine (300 mg) or 2',3'-O-isopropylidencytidine (III, 350 mg) in a solvent was mixed with the following solvent system in a sealed tube and treated under the reaction conditions described in Table I: Exp. No. 1, in 10 ml of H<sub>2</sub>O with 10 ml of 40% NaSH solution previously saturated with H<sub>2</sub>S; No. 2, in 5 ml of DMF with 20 ml of H<sub>2</sub>S containing 5 ml of DMF; No. 3, in 5 ml of H<sub>2</sub>O with 20 ml of H<sub>2</sub>S containing 5 ml of MeOH; No. 4–7, in 5 ml of H<sub>2</sub>O with 20 ml of H<sub>2</sub>S containing 5 ml of pyridine. After the removal of the solvent the residue was dissolved in H<sub>2</sub>O and UV spectra were taken. The yield was calculated from the absorption ratio of  $A_{260nm}$  to  $A_{330nm}$ , taking molar absorptivity of cytidine as 7500 (260 nm) and 4-thiouridine as 21000 (330 nm) and 2850 (260 nm).

**1-β-D-Arabinofuranosyl-4-thiouracil (IV)**—In a steel tube 10 g of arabinofuranosylcytosine was dissolved in H<sub>2</sub>O (10 ml) and pyridine (2 ml), followed by the addition of H<sub>2</sub>S-pyridine solution (35 ml: 15 ml) under dry-ice-acetone cooling. The tube was sealed and heated at 60° for 113 hr. After vaporization of H<sub>2</sub>S, the solvent was evaporated *in vacuo* and the residue was taken in H<sub>2</sub>O. After removal of the insoluble material the solvent was removed, residue was taken in H<sub>2</sub>O, and this process was repeated until no precipitation occurred. The final residue was taken in EtOH and again concentrated to leave a syrup which was dissolved in a least amount of acetone. EtOAc was added slowly to obtain a turbid solution which was set aside overnight. The separated crystals were collected and recrystallized from acetone to

- 14) N.K. Kochetkov, E.I. Budowski, N.N. Shibaev, G.I. Yeliseeva, M.A. Grachev, and V.P. Demushikin, *Tetrahedron*, **19**, 1207 (1963).
- 15) Thiation of uridine phosphates has been reported: M. Saneyoshi, *Chem. Pharm. Bull.* (Tokyo), **19**, 493 (1971).
- 16) J.H. Boothe and C.O. Wilson, *J. Am. Chem. Soc.*, **68**, 448 (1946).
- 17) M.J. Janssen in "The chemistry of carboxylic acids and esters (The chemistry of functional groups)", S. Patai ed., Interscience-Publishers, London, 1969, p. 741.
- 18) W. Walter and J. Voss in "The chemistry of amides (The chemistry of functional groups)", J. Zabicky ed., Interscience-Publishers, London, 1970, p. 418.
- 19) Amino group in certain purine nucleosides underwent sulfhydrolysis. See ref. 8.
- 20) V.N. Shibaev, M.A. Grachev, and S.M. Spiridonova, in "Synthetic Procedures in Nucleic Acid Chemistry," vol. 1 ed. by W. Werner Zorbach and R.S. Tipson 1968, p. 503.

yield 8.4 g, 51%, of IV, mp 165—166°. *Anal.* Calcd. for  $C_9H_{12}O_5N_2S$ : C, 41.53; H, 4.64; N, 10.74; S, 12.29. Found: C, 41.49; H, 4.63; N, 10.93; S, 12.12. UV  $\lambda_{\max}^{H_2O}$  (nm); 331, 244.

**2,4-Dithiouridine (V) from 2-Thiocytidine**—2-Thiocytidine (27.7 mg) in 1 ml of  $H_2O$  was added  $H_2S$ -pyridine (3 ml: 1 ml) and heated at 50° for 66 hr in a sealed tube. After removal of the solvent the residue was crystallized from  $H_2O$  to furnish greenish yellow plates (mp 170.5—171.5°, 20 mg). The spectral properties are identical with those prepared by us.<sup>10</sup> The mp was 4° higher than that prepared by the thiation method.<sup>10</sup>

**3-Methyl-4-thiouridine (VI)**—3-Methylcytidine methosulfate<sup>21</sup> (500 mg) in 5 ml of  $H_2O$  was added  $H_2S$ -pyridine (10 ml: 5 ml) and heated at 55° for 27 hr in a sealed tube. After the removal of the solvent the residue was taken in  $H_2O$  and concentrated. The residue was again taken in  $H_2O$ , the precipitates removed and the filtrate was concentrated to leave a mass. TLC of the product with silica gel (AcOEt-EtOH, 10: 1) showed single spot, *Rf* 0.45 (*Rf* of Ia, 0.33). This was applied on a column of silica gel and eluted with EtOAc-EtOH (9: 1). Fractions containing VI were collected and evaporated to leave a mass which was crystallized from AcOEt to furnish 294 mg of VI, mp 147—148°. UV  $\lambda_{\max}^{H_2O}$  nm( $\epsilon$ ): 327 (23700), 260sh (3320), 246 (3520).  $\lambda_{\min}^{H_2O}$  nm( $\epsilon$ ): 278 (2500), 225 (2460). In 0.1N NaOH the maxima changed slowly to 318 nm (17700) and 271 nm (15300), respectively, in 3 hr.

**4-Thiouracil from Cytosine**—Cytosine (25 mg) was suspended in 4 ml of DMF to which was added  $H_2S$ -pyridine (1: 1, 20 ml) and heated at 50° for 2 days in a sealed tube. After removal of the solvent the residue was taken in MeOH and insoluble material (containing unreacted cytosine) was filtered and the filtrate was kept at room temperature. The yellow crystals of 4-thiouracil, 18 mg, were obtained and identified by the direct comparison of UV and paper chromatography with the authentic sample.

**4-Thiouridine 5'-Phosphate (Ib)**—Disodium cytidine 5'-phosphate (IIb, 400 mg) was taken in 5 ml of  $H_2O$  and was added 15 ml of  $H_2S$  containing 5 ml of pyridine under dry-ice cooling, and sealed. After treatment at 60° for 41 hr the solvent was removed *in vacuo*. The residue was dissolved in  $H_2O$ , the insoluble materials filtered off, and concentrated to a small volume to which was added EtOH slowly. The separated crystals were collected by centrifugation, washed with EtOH, ether and dried. The yield of Ib was 300 mg, 70%. UV  $\lambda_{\max}^{0.05N HCl}$  nm( $\epsilon$ ): 333 (23500), 246 (4300);  $\lambda_{\max}^{0.05N NaOH}$ : 318 (19500). *Anal.* Calcd. for  $C_9H_{11}O_8N_2Na_2PS \cdot 1.5H_2O$ : C, 26.27; H, 3.41; N, 6.81. Found: C, 26.31; H, 3.90; N, 6.82.

**4-Thiouridine 5'-Triphosphate (Id)**—Trisodium cytidine 5'-triphosphate (IIId, 275 mg, contaminated with 5.5% of CDP) was treated as described above for 67 hr at 50°. After removal of the solvent *in vacuo* the residue was taken up in  $H_2O$ , applied on a column of DEAE-cellulose (bicarbonate form,  $2.3 \times 45$  cm), and eluted with a linear gradient of triethylammonium bicarbonate ( $H_2O$  and 0.5M  $Et_3NH^+HCO_3^-$ , pH 8.5, 2.5 l each). The elutes were separated in four main peaks as shown in Fig. 1. Fractions corresponding to each peak were collected and concentrated to a dryness. Peak 1 (tube No. 18—25, 875ODU<sub>330</sub>), peak 2 (tube No. 83—111, 3775ODU<sub>330</sub>), peak 3 (tube No. 136—167, 2210ODU<sub>330</sub>), peak 4 (tube No. 186—197, 382ODU<sub>330</sub>) were identified as that of Ib, Ic, Id, and 4-thiouridine 5'-tetrakisphosphate, respectively, by paper chromatography and ionophoresis (solvent system; EtOH-1M  $NH_4OAc$  (pH 7.0, 5: 2, descending) and 0.05M triethylammonium bicarbonate, pH 8, 700 volts, 80 min). A similar reaction from 275 mg of IID was performed using 2,6-lutidine in place of pyridine. Elution from the column was performed with a linear gradient of LiCl (0—2M, 2.5 l each). Fractions containing Id (peak 3) were collected and diluted with 4 fold volume of  $H_2O$  and applied to a column of DEAE-cellulose (bicarbonate form,  $2.3 \times 35$  cm) and eluted with a linear gradient of triethylammonium bicarbonate (0.05—0.5M, pH 8, 2 l each). Fractions composed of peak 2 were collected and concentrated to a small mass which was taken up in  $H_2O$ . This was concentrated again and the process was repeated several times. Final concentrate contained Id, 3500ODU<sub>330</sub>, 35%.  $\epsilon_{(p)330nm} = 7400$ ; *Rf* = 0.21 (isobutyric acid-2N  $NH_4OH$ -0.2M EDTA, 120: 12: 3).

**4-Thiouridine 5'-Diphosphate (Ic)**—Trisodium cytidine 5'-diphosphate (IIc, 244 mg) was treated with  $H_2S$  as described in the synthesis of Id, for 90 hr at 40°. By the purification through a column of DEAE-cellulose with LiCl solution as the eluent, then with triethylammonium bicarbonate as described in the previous section, triethylammonium salt of Ic was obtained (5750ODU<sub>330</sub>, 55%);  $\epsilon_{(p)} = 10800$ .

**Treatment of Uridine 5'-Triphosphate with  $H_2S$** —Trisodium uridine 5'-triphosphate (300 mg) was dissolved in 5 ml of  $H_2O$  and to which was added 20 ml of  $H_2S$ -pyridine (1: 1) and kept for 96 hr at 40° in a sealed tube. After removal of the solvent the residue was applied to a column of DEAE-cellulose (bicarbonate form, 2.8 60 cm). Elution was performed as described in the reaction with IID. Five peaks were obtained which were collected separately and applied to TLC. The results were shown in Table II. Phosphoric acid and pyrophosphoric acid were detected in the fraction No. 57—66 and 134—146, respectively. Neither spots on TLC was positive for  $NaN_3-I_2$  spray test.<sup>13</sup>

**4-Thiouridine Diphosphate Choline (Ie)**—Monosodium salt of CDP-choline (IIe, 225 mg) was taken in 6 ml of aqueous pyridine (5: 1) to which was added  $H_2S$ -pyridine (20 ml, containing 5 ml of pyridine) and sealed. The tube was heated at 70° for 48 hr. After the usual work-up EtOH was added to the aqueous solution of Ie slowly and kept in a refrigerator overnight. The precipitate was collected by centrifugation,

21) P. Brookes and P.D. Lawley, *J. Chem. Soc.*, 1348 (1962).

washed with EtOH, ether, and dried *in vacuo* at 50° to afford 254 mg, 96%, of Ie. UV  $\lambda_{\text{max}}^{\text{H}_2\text{O}}$  nm: 245, 330:  $\lambda_{\text{max}}^{\text{NaOH}}$  315.  $\epsilon_{(p)330\text{nm}}=11300$ . NMR ( $\delta$ , ppm in D<sub>2</sub>O): 3.30 (s, Me<sub>3</sub>N<sup>+</sup>). *Rf* (EtOH-1M NH<sub>4</sub>OAc, pH 7.0, 5: 2)=0.25 (*Rf* of IIe, 0.15).

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