Summary

Uridine-5-carboxaldehyde (I), uridine-5-carboxylic acid (II), and 1-(β-D-ribofuranosyluronic acid)uracil-5-carboxaldehyde (III) were isolated and characterized from the reaction products obtained after catalytic oxidation of uridine-5-methanol (IV). Five aldehyde hydrazone derivatives of I and III were synthesized. Catalytic oxidation of several pyrimidine nucleosides gave the corresponding uronic acid derivatives. The ease of the oxidation of these compounds was found different, depending upon the base moieties.

(Received April 14, 1964)

[Chem. Pharm. Bull. 13(1) 16 ~ 21 (1965)] UDC 612.398.145

3. Yoshiyasu Furukawa, Yoshiko Mizuno, Yasushi Sanno, and Mikio Honjo: Synthesis of Cytidine 5′-Diphosphate- and Deoxycytidine 5′-Diphosphate-L-serine.*1

(*Research Laboratories, Takeda Chemical Industries, Ltd.**2)

CDP-*3 (or dCDP-) choline and CDP- (or dCDP-) ethanolamine participate in the biosythesis of lecithine and phosphatidylethanolamine.[1,2] By analogy, CDP- or dCDP-serine seems to have some bearings on the biosynthesis of phosphatidylserine. However, the occurrence of these compounds in nature has not yet been recorded, and on the contrary, another biosynthetic pathway of phosphatidylserine has been postulated.[3,4] We, therefore, attempted the chemical synthesis of these compounds.

First, a mixture of CMP and O-phosphoryl-L-serine[5] (I) was treated with DCC in aqueous pyridine or a solution of the dicyclohexylguanidinium salt of CMP-NH_2 (II)[6] in *o*-chlorophenol was allowed to react with I. The examination of the reaction mixtures by ion exchange chromatography and paper electrophoresis (PEP), however, showed no formation of the desired CDP-serine. The reason seemed to be due to the interference with the reaction by the free amino group of I. Therefore, O-phosphoryl-N-carbobenzyloxy-L-serine (III) was synthesized and used instead of I. Compound (I) was treated with carbobenzyloxychloride by the usual method in the presence of sodium hydroxide. The crude reaction mixture was applied to the column of Dowex-50 (H^+ form), which on elution with water afforded III and I in succession. Compound (III) gave a positive

*1 Published at the 83rd Annual Meeting of the Pharmaceutical Society of Japan (Nov. 2nd, 1963, Tokyo). Afterwards, Michelson, *et al.* have synthesized CDP-serine by another method, but no detailed description is available (Bull. soc. chim. biol., **45**, 1353 (1963)).

*2 Juso-Nishino-cho, Higashiyodogawa-ku, Osaka (古川純康, 水野芳子, 三野 安, 本庄美喜男).

*3 CDP, cytidine 5′-diphosphate; dCDP, deoxycytidine 5′-diphosphate; CMP, cytidine 5′-phosphate; CMP-NH_2, cytidine 5′-phosphoramidate; DCC, dicyclohexylcarbodiimide; DCPP, P^1,P^2-dicytidine 5′-pyrophosphate; UMP, uridine 5′-phosphate; dCMP-NH_2, deoxycytidine 5′-phosphoramidate; dCMP, deoxycytidine 5′-phosphate.

1) E. P. Kennedy, S. B. Weiss: J. Biol. Chem., **222**, 193 (1956).
2) E. P. Kennedy, L. F. Borkenhagen, S. W. Smith: J. Biol. Chem., **234**, 1998 (1959).
3) G. Hübscher, R. R. Dils, W. F. R. Pover: Biochim. Biophys. Acta, **36**, 518 (1959).
4) J. N. Kanfer, E. P. Kennedy: J. Biol. Chem., **237**, pc 270 (1962).
5) G. Fölsch, O. Mellander: Acta Chem. Scand., **11**, 1234 (1957).
6) R. W. Chambers, P. Shapiro, V. Kurkov: J. Am. Chem. Soc., **82**, 970 (1960).

test for phosphate with perchloric acid-ammonium molybdate reagent[7] or ferric chloride-sulfosalicylic acid reagent[8] but was negative for a color development with ninhydrin reagent. Compound (Ⅲ) was not obtained in crystalline, however, the following facts would constitute supporting evidence for the assignment of the structure : i) PEP (pH 3.7) gave a single spot at R_{p-ser}*4=1.3, which indicated that the compound was less basic than I; ii) Paper partition chromatography (PPC) in isopropanol-conc. ammonium hydroxide-water (7:1:2) gave a single spot at R_{p-ser}=5.0, which indicated that the compound was more lipophilic than I; iii) Hydrogenolysis of this compound in aqueous methanol with palladium oxide gave I.

Compound (Ⅲ) was allowed to react with the dicyclohexylguanidinium salt of Ⅱ in *o*-chlorophenol at 37° for 10 days. The reaction mixture was chromatographed on Dowex-1 ($HCOO^-$ form) (Fig. 1). The fraction eluted with a mixture of 2*N* formic acid and 0.2*M* ammonium formate contained CDP-N-carbobenzyloxy-L-serine (Ⅳ), which on treatment with snake venom phosphodiesterase*5 gave CMP and Ⅲ. In the case of the large scale preparation of Ⅳ, the step of the isolation of Ⅲ from I was omitted and the mixture was directly allowed to react with Ⅲ, because a large amount of Dowex-50 was required for the isolation of Ⅲ.

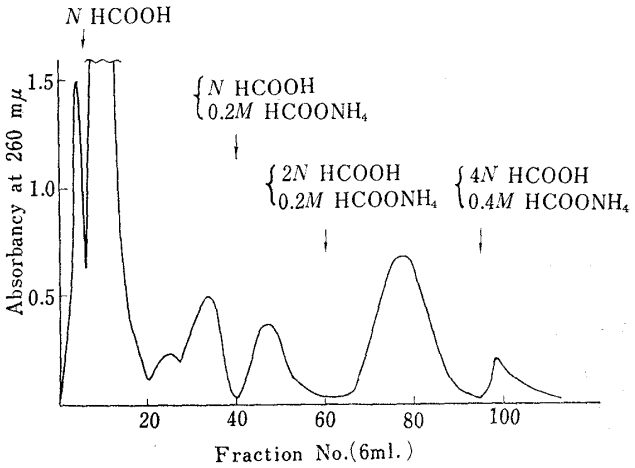

Fig. 1. Fractionation of CDP-N-carbobenzyloxy-L-serine

In order to remove the carbobenzyloxy group, Ⅳ was hydrogenated with palladium oxide in aqueous methanol. The ultraviolet absorption spectrum of the resulting

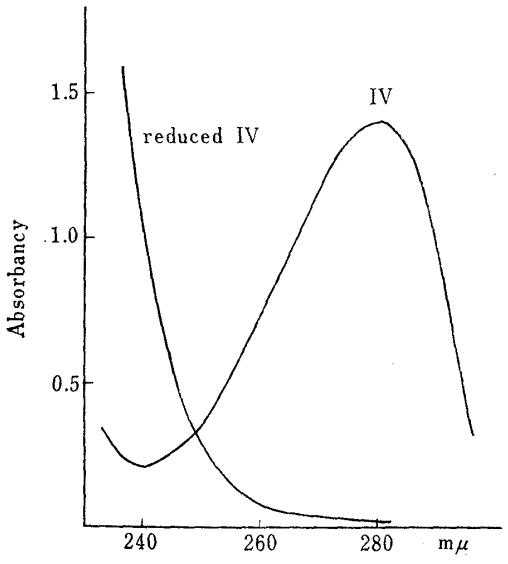

Fig. 2. Ultraviolet Spectra (pH 2)

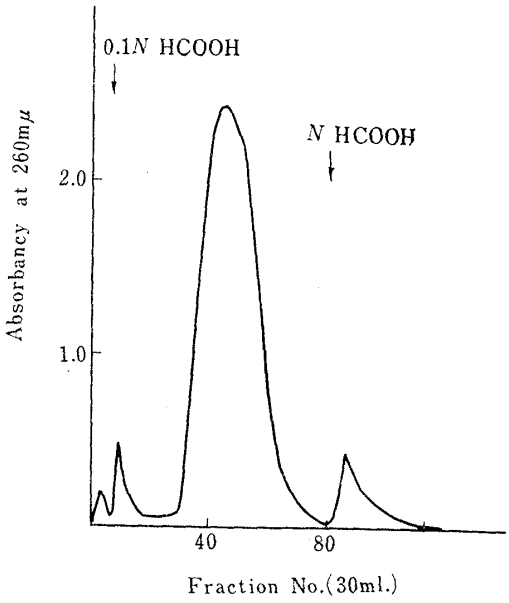

Fig. 3. Fractionation of CDP-L-serine

*4 R_{p-ser} : Ratio of the migration distance of a sample to that of O-phosphoryl-L-serine.
*5 Kindly supplied by Dr. S. Iwanaga, Institute for Protein Research, Osaka University.

7) C. S. Hanes, F. A. Isherwood : Nature, **164**, 1107 (1949).
8) H. E. Wade, D. E. Morgan : *Ibid.*, **171**, 529 (1953).

compound differed from that of Ⅳ and showed practically no absorption around 280 mμ (Fig. 2). PEP (pH 3.7) of the reaction mixture gave a single ninhydrin-positive fluorescent spot at $R_{CMP}=10$. These results were reminiscent of not only the splitting of the carbobenzyloxy group, but also some unknown side reaction at the cytosine moiety. As a model experiment, CMP was hydrogenated under the same conditions to give a compound, which was eventually shown to be 5,6-dihydro-UMP from the following evidences : i) PEP (pH 3.7 and pH 9.2) gave a single fluorescent spot corresponding to that of UMP. ii) It showed two dissociation constants at 9.4 and 6.2, indicating that the one was the pKa[9] of the hydroxyl group at C_4 of uracil and the other that of the secondary phosphate group.[9] iii) The elemental analysis of the hydrogenation product was in agreement with the theoretical value. Batt, *et al.*[10] has isolated 5,6-dihydrouracil by the hydrogenation of uracil with Adams catalyst in glacial acetic acid. By analogy, the hydrogenation product of Ⅳ was presumed to be 5,6-dihydro-UDP-L-serine. In alternative approach, Ⅳ was hydrogenated in aqueous methanol using 10% palladised charcoal to yield, after purification by chromatography on Dowex-1 ($HCOO^-$ form) (Fig. 3), the barium salt of CDP-L-serine as a crystalline and analytically pure powder. Ultraviolet absorbance ratios of this compound as well as their λ_{max}, λ_{min} were identical with those of CMP. Cytosine : inorganic phosphorus : total phosphorus[11] = 1:0:1.9 (calculated value, 1:0:2.0). Degradation of this compound with snake venom phosphodiesterase gave CMP and I.

In a manner similar to the synthesis of CDP-L-serine we have also synthesized the barium salt of dCDP-L-serine as a crystalline white powder. The present synthesis of

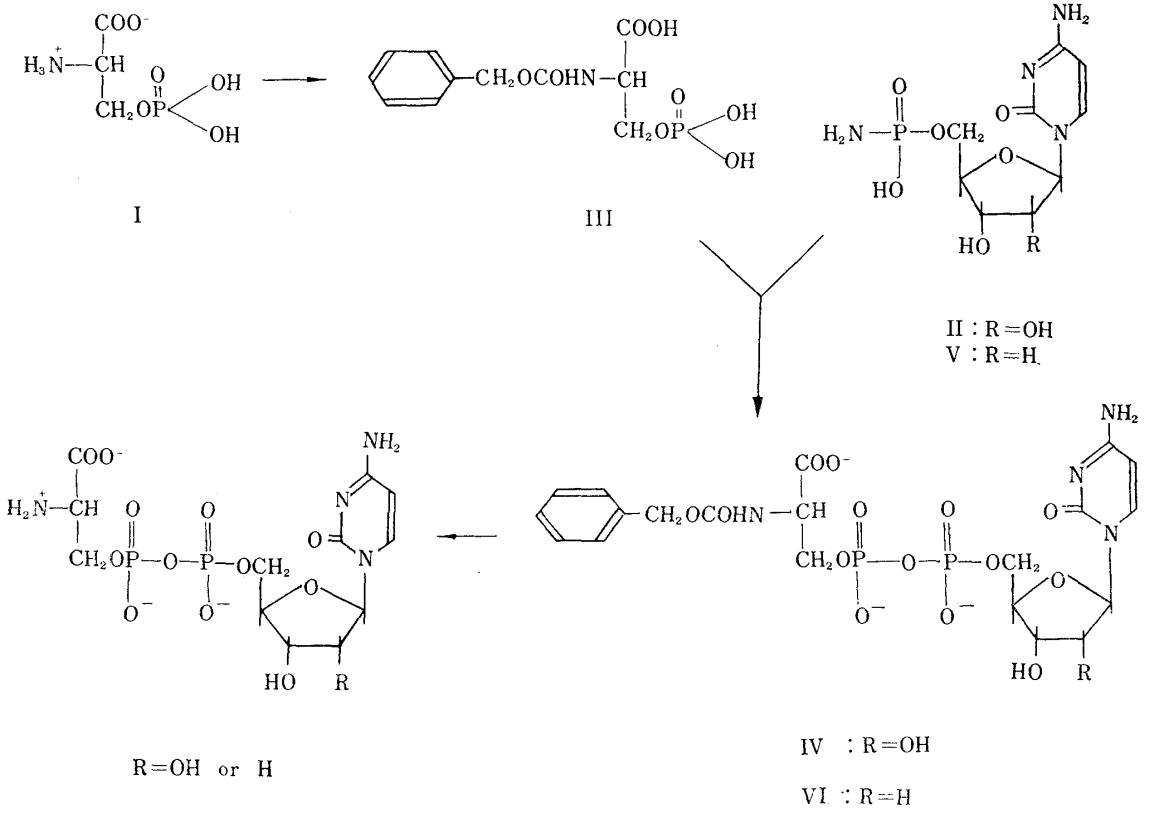

Chart 1.

9) E. Chargaff, J. N. Davidson : "The Nucleic Acids," Vol. I, 269 (1955), Academic Press Inc., New York.
10) R. D. Batt, J. K. Martin, J. M. Ploeser, J. Murray : J. Am. Chem. Soc., **76**, 3663 (1954).
11) R. J. L. Allen : Biochem. J., **34**, 858 (1940).

CDP- and dCDP-L-serine is the first success for the synthesis of this type of nucleoside diphosphate amino acids,*1 which might be of interest as potential intermediates in the biosynthesis of phosphatidylserine.

Experimental

Paper Electrophoresis (PEP) and Paper Partition Chromatography (PPC)——PEP was carried out on Whatman No. 1 filter paper at 22 v./cm. for 1.5 hr. using the following buffers: 1, 0.05 *M* citrate buffer (pH 3.7); 2, 0.1*M* acetate buffer (pH 5.0); 3, 0.05*M* borate buffer (pH 9.2). PPC was carried out on Whatman No. 1 filter paper by the ascending method using the following solvents: A, *iso*-PrOH-conc. NH_4OH-H_2O=7:1:2; B, *iso*-butyric acid-½*N* NH_4OH=10:6; C, satd. $(NH_4)_2SO_4$-1*M* AcONa-*iso*-PrOH=79:19:2.

O-Phosphoryl-N-carbobenzyloxy-L-serine (III)——Compound (I)[5] (370 mg., 2 mmoles) was dissolved in 2*N* NaOH (2 ml.) and to this were added dropwise (10 min.) 4*N* NaOH (0.5 ml.) and a 30% carbobenzyloxy-chloride solution in toluene (1.15 g., 2 mmoles) with stirring at 0°. The mixture was continued to stir at 0° for 20 min. and then at room temperature for 30 min. PPC (solvent A) of the aqueous layer gave two phosphate-positive spots at Rf 0.04 and 0.2. The former spot was ninhydrin-positive and corresponding to that of the starting material (I) and the latter was presumed to be that of III. The reaction mixture was freed of toluene and 3/4 volume of the aqueous layer was applied to a Dowex-50 (H^+ form) column (1 L.), which was eluted with H_2O. Fraction 1: tube No. 5~11, 350 ml. (0.75 mmole, 50% yield based on the quantitative analysis of total phosphorus)[11]; Fraction 2: tube No. 13~25, 650 ml. Fraction 1 was concentrated to a colorless sirup. This sirup (3 mg.) was dissolved in 50% MeOH (10 ml) and hydrogenated with PdO (5 mg.) for 8 hr. The catalyst was filtered off and the filtrate was concentrated and submitted to PEP (buffer 1) and PPC (solvent A) which showed a single ninhydrin-positive spot corresponding to that of I.

CDP-N-carbobenzyloxy-L-serine (IV)——The dicyclohexylguanidinium salt of II (52 mg., 0.1 mmole) and III (15 mg., 0.05 mmole) were dissolved in *o*-chlorophenol (5 ml.) and the solution was kept at 37° for 10 days. To the reaction mixture was added ether (20 ml.) and the mixture was extracted with three portions of H_2O (10 ml.). The combined aqueous layer was washed with ether (20 ml.), freed of ether *in vacuo* and then fractionated with a column of Dowex-1, X-8 ($HCOO^-$ form, 200~400 mesh) (Fig. 1).

Table I.

Fraction	Solvent	Volume (ml.)	TOD*6
1	H_2O	30	25
2	*N* HCOOH	90	480
		110	32
3	*N* HCOOH+0.2*M* $HCOONH_4$	120	24
4	2*N* HCOOH+0.2*M* $HCOONH_4$	200	60
5	4*N* HCOOH+0.4*M* $HCOONH_4$	200	20

Fraction 4 was adsorbed on a charcoal column (50 mg.) which was washed with H_2O and eluted with conc. NH_4OH-H_2O-EtOH (0.5:50:50, 10 ml.), and the eluate was concentrated. PEP (buffer 1 and 2) of the concentrated solution gave a single UV absorption spot at R_{CMP}=1.8 and R_{CMP}=8.0 in two different systems. An aliquot (TOD 35) of the concentrated solution was evaporated to dryness *in vacuo* and the residue was dissolved in 0.1*M* glycine-NaOH-NaCl buffer (pH 9.2) (1 ml.). To this were added 0.3*M* $MgCl_2$ (0.01 ml.) and snake venom phosphodiesterase*5 (0.1 ml., 20 γ protein) and the mixture was incubated at 37° overnight. PEP (buffer 2) of the reaction mixture gave a single UV absorbing spot corresponding to that of CMP. PPC (solvent B) gave phosphate-positive spots corresponding to CMP (Rf 0.43) and III (0.64). The yield of IV was 19% based on III.

Hydrogenation of CDP-N-carbobenzyloxy-L-serine (IV) with Palladium Monoxide——Compound (IV) (TOD 240, 32 μmoles) was hydrogenated in 30% MeOH (30 ml.) with PdO (40 mg.) for 15 hr. The optical density at 260 mμ (OD_{260}) of the reaction mixture was reduced to 6.4% that of the starting material.

Hydrogenation of CMP with Palladium Monoxide——CMP (free, 500 mg.) was hydrogenated in 30% MeOH (300 ml.) with PdO (400 mg.) for 16 hr. The OD_{260} of the reaction mixture was reduced to 0.5% that of the starting material. The catalyst was filtered off and to the filtrate was added $Ba(OAc)_2 \cdot H_2O$ (420 mg.). The solution was concentrated and MeOH was added to yield a precipitate, which was reprecipitated from H_2O and MeOH to give a white powder of 5,6-dihydro-UMP·Ba. *Anal.* Calcd. for $C_9H_{13}O_9N_2PBa \cdot CH_3OH \cdot 2H_2O$: C, 21.72; H, 3.44; N, 5.63; P, 6.22. Found: C, 22.50; H, 3.86; N, 5.29; P, 5.93.

*6 TOD=Optical density at 260 mμ × ml.

Hydrogenation of CDP-N-carbobenzyloxy-L-serine (IV) with Palladised Charcoal——Compound (IV) (TOD 3600) was hydrogenated in 30% MeOH (300 ml.) with 10% Pd-C (600 mg.) for 8 hr. The catalyst was filtered and eluted with conc. NH_4OH-H_2O-EtOH (0.5:50:50, 50 ml.). The filtrate and eluate were combined and concentrated. PEP (buffer 1) of the concentrated solution gave two ninhydrin-positive spots, one of which was a UV absorbing at $R_{CMP}=6$ and the other a fluorescent spot at $R_{CMP}=10$.

CDP-L-serine——The hydrogenation product of IV with Pd-C was fractionated with a column (15 ml.) of Dowex-1, X-8 ($HCOO^-$ form, 200~400 mesh) (Fig. 3).

Table II.

Fraction	Solvent	Volume (ml.)	TOD
1	H_2O	300	20
2	0.1*N* HCOOH	600	40
3	0.1*N* HCOOH	1200	2930
4	*N* HCOOH	900	—

Fraction 3 was adsorbed on a charcoal column (6 g.), which was washed with H_2O and eluted with conc. NH_4OH-H_2O-EtOH (0.5:50:50, 200 ml.). PEP (buffer 1) and PPC (solvent B) of the concentrated eluate showed a single UV absorbing ninhydrin-positive spot at $R_{CMP}=6$ and 0.62, respectively.
UV absorbance data :

Table III.

	λ_{max}	λ_{min}	$A_{250\,m\mu}/A_{260\,m\mu}$	$A_{280\,m\mu}/A_{260\,m\mu}$	$A_{290\,m\mu}/A_{260\,m\mu}$
pH 2	280 mμ	241 mμ	0.46	2.07	1.54
pH 7.2	270 mμ	250 mμ	0.85	0.93	0.30

$Ba(OAc)_2 \cdot H_2O$ (180 mg.) and EtOH were added to the concentrated solution to yield a white precipitate, which was recrystallized from aq. EtOH to give a white crystalline powder (300 mg.) of CDP-L-serine·Ba. *Anal.* Calcd. for $C_{12}H_{18}O_{13}N_4P_2Ba \cdot C_2H_5OH \cdot H_2O$: C, 24.42; H, 3.81; N, 8.13; P, 8.99; EtO, 6.53. Found : C, 24.99; H, 4.25; N, 7.86; P, 8.71; EtO, 6.04. $[\alpha]_D^{22}$ +7° (c=1.0, H_2O).

dCDP-N-carbobenzyloxy-L-serine (VI)——Compound (I) (400 mg., 2.16 mmoles) was treated with carbobenzyloxychloride as described before to give a 68% yield of III which was determined by PPC (solvent A). The solution was passed through a column (15 ml.) of IR-120 (H^+ form) and the column was washed with H_2O. The combined solution was evaporated to dryness. The resultant sirup (a mixture of III (450 mg., 1.5 mmoles) and I (120 mg)) and the dicyclohexylguanidinium salt of V[6] (1.5 g., 3 mmoles) were dissolved in *o*-chlorophenol (60 ml.) and the solution was kept at 37° for 10 days. The reaction mixture was worked up as described previously for IV, followed by ion exchange chromatography with a column (100 ml.) of Dowex-1, X-8($HCOO^-$ form, 200~400 mesh).

Table IV.

Fraction	Solvent	Volume (L.)	TOD
1	H_2O	1	3700
2	*N* HCOOH	8	14000
3	*N* HCOOH + 0.2*M* $HCOONH_4$	5	440
4	2*N* HCOOH + 0.2*M* $HCOONH_4$	6	1500
5	2*N* HCOOH + 0.4*M* $HCOONH_4$	5	500

Fraction 4 was treated with charcoal as described previously for IV, and the resulting solution showed a single UV absorbing spot at $R_{CMP}=8.0$ on PEP (buffer 1). The yield was 16% based on III.

dCDP-L-serine——The above mentioned solution (TOD 1500) was hydrogenated in 30% MeOH (150 ml.) with 10% Pd-C (300 mg.) for 7 hr. The catalyst was filtered and eluted with conc. NH_4OH-H_2O-EtOH (0.5:50:50, 30 ml.). The combined filtrate and eluate was concentrated *in vacuo* to show a UV absorbing spot at $R_{CMP}=6.0$ and a fluorescent spot at $R_{CMP}=10$ on PEP (buffer 1). The solution was fractionated with a column (7 ml.) of Dowex-1, X-8 ($HCOO^-$ form, 200~400 mesh).

Table V.

Fraction	Solvent	Volume (ml.)	TOD
1	H_2O	300	6
2	0.1*N* HCOOH	400	14
3	0.1*N* HCOOH	700	1100

Fraction 3 was worked up as mentioned above, and the barium salt of dCDP-L-serine (90 mg.) was isolated as a white crystalline powder, homogeneous on PEP (buffer 1). *Anal.* Calcd. for $C_{12}H_{18}O_{12}N_4P_2Ba \cdot \frac{1}{2}C_2H_5OH \cdot 5H_2O$: N, 7.76; P, 8.57; EtO, 3.11. Found: N, 7.87; P, 8.47; EtO, 2.91. $[\alpha]_D^{23}$ +9° (c=1.0, H_2O).

Enzymatic Degradation of CDP- and dCDP-L-serine——CDP-L-serine·$(NH_4)_2$ (2 mg.) and dCDP-L-serine·Ba (2 mg.) were dissolved in 0.1*M* glycine-NaCl-NaOH buffer (pH 9.2, 1 ml.), respectively. To these solutions were added 0.3*M* $MgCl_2$ (0.05 ml.) and snake venom phosphodiesterase*5 (0.05 ml., 10 γ protein) and the mixture were incubated at 37° for 1 hr. PEP (buffer 1) of the reaction mixtures gave both a UV absorbing spot at $R_{CMP}=1$ and a ninhydrin-positive spot at $R_{CMP}=9$. PPC (solvent B) gave a single UV absorbing spot at Rf 0.37 (=CMP) or at Rf 0.47 (dCMP) and a ninhydrin-positive spot at Rf 0.2 (=I), respectively. PPC (solvent C) gave a single UV absorbing spot at 0.74 (=CMP) or at Rf 0.65 (dCMP) and a ninhydrin-positive spot at Rf 0.95 (=I), respectively.

The authors are grateful to Prof. O. Hayaishi of the Department of Medical Chemistry, Faculty of Medicine, Kyoto University and Dr. K. Tanaka of this laboratories for their encouragement during the present work. Thanks are also due to Mr. M. Kan, *et al.* for elementary analysis, to Miss T. Hiratsuka for physico-chemical measurements.

Summary

O-Phosphoryl-L-serine (I) was treated with carbobenzyloxychloride to give O-phosphoryl-N-carbobenzyloxy-L-serine (III). Condensation of III with dicyclohexylguanidinium cytidine 5′-phosphoramidate (II) gave cytidine 5′-diphosphate-N-carbobenzyloxy-L-serine (IV), which was hydrogenated with palladised charcoal to obtain cytidine 5′-diphosphate-L-serine. Likewise, deoxycytidine 5′-diphosphate-L-serine was synthesized from III and dicyclohexylguanidinium deoxycytidine 5′-phosphoramidate (V).

(Received August 10, 1964)