"POLYFUNCTIONAL SUBSTANCES OF IMPORTANCE IN NEUROPHARMACOLOGY AND NEUROCHEMISTRY."

BY

CHEAH, KHAY SENG.

A thesis submitted to the Department of Chemistry, School of General Studies, in conformity with the requirements for the degree of Master of Science.

Australian National University,
Canberra City,
A.C.T.

January, 1965.

G47240

ACKNOWLEDGEMENTS.

The candidate likes to express his sincere thanks and appreciation to Dr. J. C. Watkins for his invaluable guidance, encouragement and understanding throughout the course; to Dr. D. J. Brown who kindly acted as supervisor during Dr. Watkins' absence; Dr. J. E. Fildes for the microanalysis of the synthetic compounds; Dr. J. C. Watkins for the phosphorus analysis and to Professor Sir John Eccles for granting a Research Assistantship in his department.

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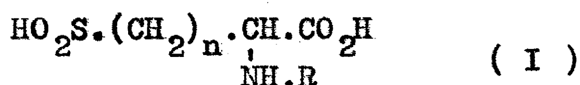
SUMMARY

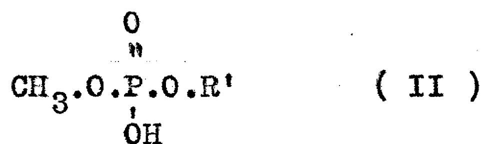
SECTION I : SYNTHESIS OF SOME NEUROPHARMACOLOGICALLY ACTIVE AMINO SULPHINIC ACIDS, PHOSPHORIC ACID DIESTERS, AND QUATERNARY AMMONIUM COMPOUNDS.

Substances belonging to three different structural groups have been synthesized and tested for neuropharmacological activity. The three groups investigated were amino sulphinic acids of general formula



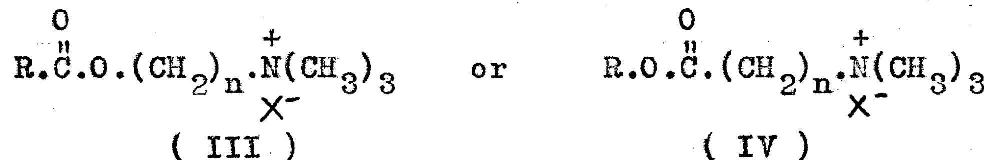
where n = 1 or 2 and R = H or CH_3;

phosphoric acid diesters of general formula



where R' = $-CH_2.CH_2.NH_2$, $-CH_2.CH_2.\overset{+}{N}(CH_3)_3$, or $-CH_2.\underset{CO_2H}{CH}.NH_2$

and quaternary ammonium compounds of general formula



The amino sulphinic acids were of interest in view of the strong excitatory action on neurones within the mammalian central nervous system of compounds related to glutamic and aspartic acids. Four compounds belonging to this group were synthesized : DL-cysteine sulphinic acid (I, n = 1, R = H), N-methyl-DL and L-cysteine sulphinic acid (I, n = 1, R = CH_3), and N-methyl-DL-homocysteine sulphinic acid (I, n = 2, R = CH_3). They were made from the corresponding cystine derivatives by oxidation with perbenzoic acid and alkaline treatment of the resulting disulphoxides. DL-Cysteine sulphinic acid and N-methyl-DL-cysteine sulphinic acid were subsequently shown to be moderately strong excitants of central neurones, with N-methyl-DL-homocysteine sulphinic acid somewhat weaker, and N-methyl-L-cysteine sulphinic acid only a very weak neuronal excitant.

The phosphoric acid diesters were synthesized in view of a theory that the neuropharmacological actions of acetyl choline ($CH_3.CO.O.CH_2.CH_2.\overset{+}{N}(CH_3)_3$), γ-aminobutyric acid ($HO_2C.CH_2.CH_2.CH_2.NH_2$) and glutamic acid ($HO_2C.CH_2.CH_2.\underset{CO_2H}{CH}.NH_2$) are due to the resemblance of their charge distributions to those of the terminal moieties of the phospholipids, lecithin, phosphatidylethanolamine and phosphatidylserine, respectively, which could be involved in the structure of nerve cell membranes. Three compounds were prepared : O-methylphosphorylcholine (II, R' = $-CH_2.CH_2.\overset{+}{N}(CH_3)_3$)

O-methylphosphorylethanolamine (II, $R' = -CH_2.CH_2.NH_2$), O-methylphosphorylserine (II, $R' = -CH_2.CH(CO_2H).NH_2$), all by direct methylation with diazomethane of the corresponding phosphomonoesters of choline, ethanolamine and serine (the latter two phosphomonoesters in the form of their tetramethylammonium salts). Of these three compounds, only O-methylphosphorylserine had any marked pharmacological action, this substance being a moderately strong excitant similar in strength to glutamic acid.

Acetyl choline (III, n = 2, $R = CH_3$) is considered to be the excitatory transmitter normally activating Renshaw cells in the mammalian central nervous system. It was of interest to determine the effects of related quaternary ammonium compounds on Renshaw cells and compare the results with those obtained at other cholinoceptive junctions. Caproyl, lauroyl and palmitoyl esters of choline (III, n = 2, $R = -(CH_2)_4.CH_3$; III, n = 2, $R = -(CH_2)_{10}.CH_3$; III, n = 2, $R = -(CH_2)_{14}.CH_3$, respectively), acetyl homocholine (III, n = 3, $R = CH_3$), β-carbomethoxyethyltrimethylammonium (IV, n = 2, $R = CH_3$), β-carboethoxyethyltrimethylammonium (IV, n = 2, $R = CH_2.CH_3$) and γ-carbomethoxypropyltrimethylammonium (IV, n = 3, $R = CH_3$) were prepared as variants of the basic acetyl choline structure. All the compounds except β-carbomethoxytrimethylammonium and β-carboethoxyethyltrimethylammonium were obtained in the form of their bromides by treatment of the corresponding bromo-esters with dry trimethylamine. The other substances were prepared as their chlorides in an analogous way.

The pharmacological tests showed that the 'reversed esters' β-carbomethoxyethyltrimethylammonium chloride and γ-carbomethoxypropyltrimethylammonium bromide were more active on Renshaw cells than acetyl choline (ACh). Caproyl, lauroyl and palmitoyl choline blocked the action of ACh on Renshaw cells. Comparative results were also obtained on the guinea pig ileum and toad's rectus preparations. β-Carbomethoxyethyltrimethylammonium chloride was more active than ACh on both preparations.

SECTION II : IDENTIFICATION OF SOME ACID-SOLUBLE CYTIDINE NUCLEOTIDES IN OX BRAIN.

Two cytidine-containing substances were isolated from ox brain by a combination of ion-exchange chromatography and paper electrophoresis. It was considered worthwhile to establish completely the structure of these compounds in view of the general involvement of cytidine co-enzymes in lipid biosynthesis and the importance of lipids in cell membrane structure. In particular, the possibility that mono- or dimethyl derivatives of ethanolamine may be involved in the structure of the cytidine derivatives was rigorously investigated. No such evidence was found and the two compounds were identified by a combination of hydrolytic, chromatographic and electrophoretic evidence as cytidine diphosphate choline and cytidine diphosphate ethanolamine.

SECTION I

SYNTHESIS OF SOME NEUROPHARMACOLOGICALLY ACTIVE AMINO SULPHINIC ACIDS, PHOSPHORIC ACID DIESTERS, AND QUATERNARY AMMONIUM COMPOUNDS.

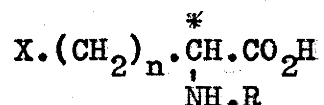
(A). α-AMINO-ALKANE-α-CARBOXYLIC-ω-SULPHINIC ACIDS.

INTRODUCTION & DISCUSSION.

Investigations on the central nervous system are being carried out at the Physiology Department of the John Curtin School of Medical Research, the Australian National University, and part of the research consists of identification and elucidation of the mode of action of transmitter substances. These investigations are conducted by Dr. D. R. Curtis and co-workers on the nervous system of the cat by injecting minute quantities of the chemical substances through glass micro-pipettes into the environment of single cells and the responses of the cells are electronically recorded.

Chemical transmitter substances are of two types, classified according to their mode of action; both are liberated by the nerve endings at the synaptic and neuromuscular junctions. An excitatory transmitter substance causes a reduction of the resting potential across the post-junctional membrane tending to make that cell generate an electrical impulse. In this way nerve impulses are relayed from one nerve fibre to another across the synaptic junction and from a nerve to a muscle fibre at the neuromuscular junction. On the other hand, an inhibitory transmitter substance opposes this effect by raising the resting potential of the post-junctional cell, thus decreasing the effectiveness of simultaneously released excitatory transmitter substances. The balance between these two transmitter effects determines the responses of individual units in the nervous system.

Amino sulphinic acids are considered important in studies of transmitter effects because of the general excitatory action at central synapses by compounds of the type

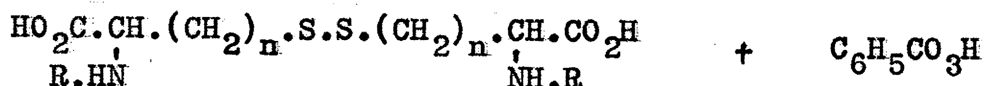


$$X.(CH_2)_n.\overset{*}{C}H(NH.R).CO_2H \qquad (I)$$

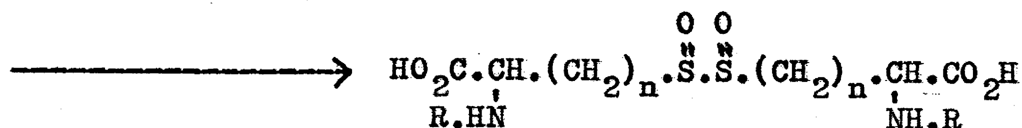
where n = 1 or 2, X = CO_2H, SO_2H or SO_3H, R = H or small alkyl group.

In these compounds, the nature of the N-substituents and the configuration at the asymmetric carbon atom (*) are sometimes of critical importance. N-Methyl-D-aspartic acid (n = 1, X = CO_2H, R = CH_3) and D-homocysteic acid (n = 2, X = SO_3H, R = H) are the two most potent amino acid excitants. The present study involves the synthesis of different optical forms of compounds in which X = SO_2H, n = 1 or 2, and the nitrogen atom carries a methyl substituent.

The compounds prepared in the present work were : DL-cysteine sulphinic acid (IV, $n = 1$, $R = H$), N-methyl-DL and L-cysteine sulphinic acid (IV, $n = 1$, $R = CH_3$), and N-methyl-DL-homocysteine sulphinic acid (IV, $n = 2$, $R = CH_3$). The syntheses were based on the preparation of L-cysteine sulphinic acid by Toennies and Lavine.[1] The appropriate cystine derivative (II) was first prepared and then converted into the disulphoxide (III) with perbenzoic acid. This disulphoxide disproportionated under alkaline conditions into the starting material and the desired sulphinic acid (IV).



(II)



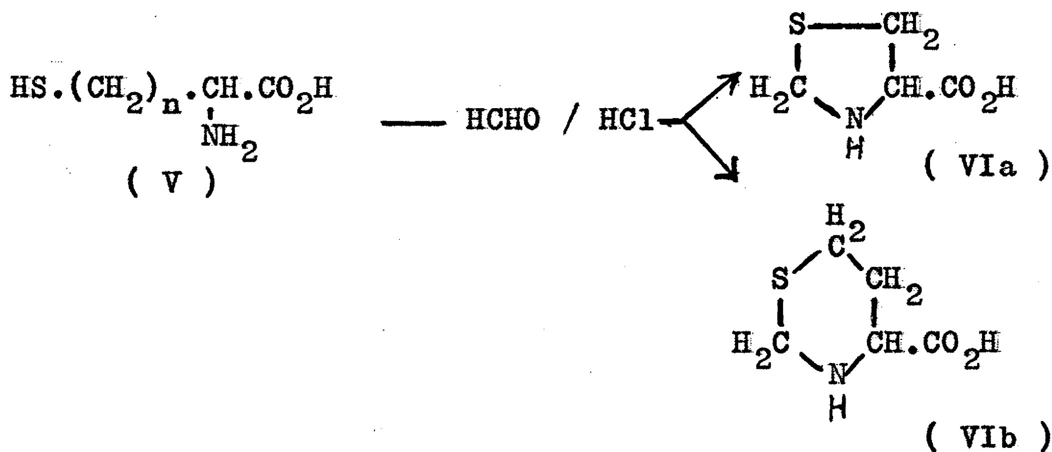
(III)

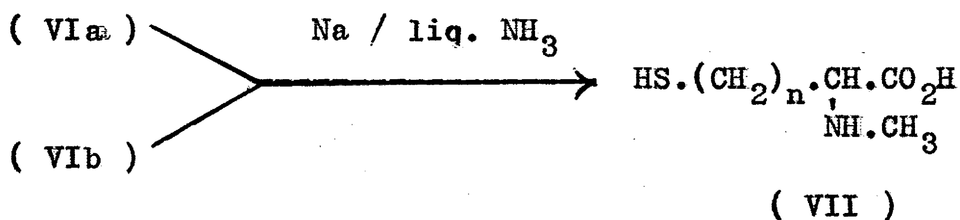
(IV)

+ (II)

The sulphinic acid was then isolated by ion-exchange chromatography on Dowex 1 (acetate) with gradient elution by acetic acid.

DL-Cysteine sulphinic acid (IV, n = 1, R = H) was prepared , according to the above scheme, from DL-cystine (II, n = 1, R = H). For N-methyl-DL and L-cysteine sulphinic acids (IV, n = 1, R = CH_3), the intermediate N-methyl cysteines (VII, n = 1) were first prepared from the appropriate forms of cysteine (V, n = 1) by reaction with formaldehyde under acidic conditions and reduction of the resulting thiazolidine derivative (VIa) with sodium in liquid ammonia. For N-methyl-DL-homocysteine sulphinic acid (IV, n = 2, R = CH_3), the intermediate homocysteine derivative (VII, n = 2) was prepared in an analogous way from DL-homocysteine (V, n = 2). In this case the intermediate product was a six membered ring compound, tetrahydro(1,3-thiazine)-4-carboxylic acid (VIb). The cysteine derivatives were oxidized by air to the corresponding cystine derivatives (II, n = 1 or 2, R = CH_3) for use as the starting materials in the syntheses of the sulphinic acids.





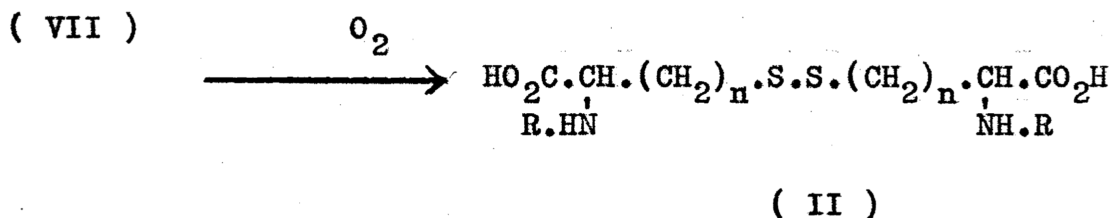


Table I shows the descending order of potency, as excitants of spinal neurones, of the newly prepared amino sulphinic acids (compounds 11, 15, 16 and 30) as compared with the excitatory amino acids previously investigated by Curtis and Watkins.[2] The equally potent DL and L-cysteine sulphinic acids (compounds 11 and 10 respectively) were classified in group G, since both of these substances were about equal to L-glutamic acid (14), the latter being a moderately strong excitant. N-Methyl-DL-cysteine sulphinic acid (15) was somewhat weaker than DL-cysteine sulphinic acid (11), but still of group G strength. Both these acids were very much stronger than N-methyl-L-cysteine sulphinic acid (30) of group I, a group of very weak excitatory amino acids. N-Methyl-DL-homocysteine sulphinic acid (16) was of similar potency to the lower homologue (15).

TABLE I. Order of Potency of Excitatory Amino Acids.

General potency.	Group.	No.	Compound.
Very strong to strong.	A.	1.	N-Methyl-D-aspartic acid.
	B.	2.	D-Homocysteic acid.
	C.	3.	N-Methyl-DL-aspartic acid.
	D.	4.	DL-Homocysteic acid.
	E.	5.	N-Iminomethyl-D-aspartic acid.
		6.	N-Ethyl-D-aspartic acid.
	F.	7.	N-Ethyl-DL-aspartic acid.
		8.	DL-Homocysteine sulphinic acid.
Strong to medium.	G.	9.	L-Cysteic acid.
		10.	L-Cysteine sulphinic acid.
		11.	DL-Cysteine sulphinic acid.
		12.	N-n-Propyl-D-aspartic acid.
		13.	L-Homocysteic acid.
		14.	L-Glutamic acid.
		15.	N-Methyl-DL-cysteine sulphinic acid.
		16.	N-Methyl-DL-homocysteine sulphinic acid.
		17.	L-Aspartic acid.
		18.	N-Methyl-DL-glutamic acid.
		19.	N-Methyl-L-glutamic acid.
		20.	N-Methyl-D-glutamic acid.
		21.	N-Methyl-L-aspartic acid.
Medium to weak.	H.	22.	DL-2-Amino-5-sulpho-n-valeric acid.
		23.	N,N'-Dimethyl-DL-aspartic acid.
		24.	D-Glutamic acid.

TABLE I. (contd.)

General potency.	Group.	No.	Compound.
		25.	D-Aspartic acid.
		26.	D-Cysteic acid.
		27.	N-Methyl-DL-cysteic acid.
		28.	N-Methyl-DL-homocysteic acid.
Weak to very weak.	I.	29.	N,N'-Dimethyl-D-aspartic acid.
		30.	N-Methyl-L-cysteine sulphinic acid.
		31.	N-Iminomethyl-L-aspartic acid.
		32.	N-Ethyl-L-aspartic acid.
		33.	N-Methyl-L-cysteic acid.
		34.	DL-α-Aminoadipic acid.
Inactive.	J.	35.	N-n-Propyl-L-aspartic acid.

It can therefore be said that, as with aspartic acid enantiomers, there was little difference between the optical forms of cysteine sulphinic acid. N-Methylation of the latter compounds seemed to have similar effects to N-methylation of the aspartic acid isomers, the L-form being greatly decreased in activity, whilst the D-form would probably be somewhat more active than the unmethylated compound. This probable increase in the case of the D-isomer, however, would be much less marked than in the case of D-aspartic acid. The effect of N-methylation of DL-homocysteine sulphinic acid was similar to that of N-methylation of DL-homocysteic acid, resulting in a marked reduction in the excitatory potency. No information is yet available of the separate forms of these sulphinic acids.

EXPERIMENTAL

Tetrahydro(1,3-thiazine)-4-carboxylic Acid.

24.6 g. of a mixture of DL and meso-homocystine was dissolved in 615 ml. of 4 N hydrochloric acid and 49 g. of small pieces of tin foil was added. The mixture after standing overnight at room temperature was filtered from the undissolved tin foil and diluted with an equal volume of water. This solution was saturated with hydrogen sulphide and treated with 0.5 g. of decolourizing charcoal; the precipitate tin sulphide and charcoal were filtered off through a bed of "hy-flow supercell" using a water-pump. A stream of nitrogen was passed through the clear filtrate to remove the excess of hydrogen sulphide before it was evaporated *in vacuo* at 40° to give a colourless residue, which was then triturated with 300 ml. of a mixture of ether and ethanol (5 : 1, v/v). The crude product obtained on filtration was recrystallized from ethanol yielding 22.3 g. (64 %) of DL-homocysteine hydrochloride monohydrate, m.p. 196°-197°. The product was dissolved in 9.45 ml. of 37 % aqueous formaldehyde, after which the mixture was allowed to stand overnight at room temperature. 9.45 ml. of pyridine was then added, and the powdery precipitate formed after 10 hrs. at room temperature was removed by filtration. To the clear filtrate was added an equal volume of ethanol, and the mixture was kept at 0° overnight, giving 7.24 g. (26.6 %) of colourless tetrahydro(1,3-thiazine)-4-carboxylic acid monohydrate, m.p. 215°-216°.

Anal. Calcd. for $C_5H_9NO_2S.H_2O$: C, 36.36 ; H, 6.71 ; N, 8.48 ; S, 19.41.

Found : C, 36.15 ; H, 6.59 ; N, 8.47 ; S, 19.26.

N,N'-Dimethyl-DL and *meso*-homocystine.

Seven grams of tetrahydro(1,3-thiazine)-4-carboxylic acid monohydrate was dissolved in 220 ml. of liquid ammonia and 0.77 ml. of water added. The solution was then treated with small pieces of sodium, with stirring, until a permanent blue colour was obtained (3.8 g. of sodium required), followed by 6.8 g. of solid ammonium chloride, after which the ammonia was allowed to evaporate overnight at room temperature. After the removal of the final traces of ammonia by standing over concentrated sulphuric acid in a vacuum desiccator for two days at room temperature, the white residue was dissolved in 100 ml. of water and the solution was evaporated to about half its volume at 40° *in vacuo*. The mixture was strongly acidified with 6 N hydrochloric acid, and then evaporated to dryness at 40° *in vacuo*. The mixture was then extracted with 800 ml. of ethanol using 50 ml. for each extraction, and the alcoholic extracts were combined and evaporated at 40° *in vacuo* yielding 13.2 g. of white residue, which was taken up in 400 ml. of water and made strongly alkaline with concentrated ammonia solution (S. G. 0.91). 10 mg. of ferrous sulphate was added to the solution and a stream of air was passed through until the nitroprusside test[3] was negative. The resulting solution was filtered to remove a few mg. of brown suspended material, and the filtrate was acidified with 25 % acetic acid to pH 3.0 and then

treated with 600 ml. of ethanol. The mixture was then allowed to stand at room temperature for two hours and at 0° overnight. The white flocculent precipitate was filtered and recrystallized twice from water yielding 4.3 g. (64 %) of white N,N'-dimethyl-DL and _meso_ homocystine, m.p. 243°-244°. For analysis, a sample was dried at 70° / 0.05 mm. for 27 hr. over phosphorus pentoxide.

Anal. Calcd. for $C_{10}H_{20}N_2O_4S_2$: C, 40.50 ; H, 6.80 ; N, 9.45 ; S, 21.64.

Found : C, 40.31 ; H, 6.88 ; N, 9.48 ; S, 21.43.

N-Methyl-DL-homocysteine Sulphinic Acid.

Three grams of N,N'-dimethyl-DL and _meso_-homocystine ~~was~~ were added to 50 ml. of cold (0°) purified acetonitrile* in 200 ml. volumetric flask. The exactly equivalent quantity of 70 % perchloric acid (1.92 g.) was added with cooling followed by a solution of 5.55 g. of acetic anhydride (sufficient to react with 90 % of the water added with the perchloric acid) in 60 ml. of acetonitrile. A solution of 3.32 g. of perbenzoic acid[4] was added to the reaction mixture which was then made up to the 200 ml. mark with acetonitrile. The solution was allowed to stand for 1 hr. at room temperature, extracted twice with 50 ml-volumes of N hydrochloric acid, and the combined aqueous layers was extracted with five

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* Dried first over K_2CO_3 then refluxed over P_2O_5 and fractionated. [Toennies, G., and Lavine, J. P., _J. Biol. Chem._, 100, 463 (1933)]

10 ml-volumes of chloroform. The aqueous layer was brought to pH 4.0 with 23 ml. of 8 N ammonium hydroxide solution, and allowed to stand overnight at 0°. The solution was allowed to warm to room temperature and was made strongly alkaline with 15 ml. of concentrated ammonia solution (S.G. 0.91). After 1 hr. at room temperature, the solution was evaporated to dryness at 40° in vacuo yielding 19.32 g. of white residue. After the final traces of ammonia were drawn off at the water-pump, 120 ml. of water was added and the insoluble powdery precipitate (370 mg m.p. 243°-244°) was removed by filtration. The clear filtrate was passed through a column of 240 ml. of Dowex 1 (acetate), 200-400 mesh, and the column was carefully washed with water till the washings were neutral, after which it was subjected to gradient elution with 6 N acetic acid. This was done by dropping 6 N acetic acid dropwise into 250 ml. of water in a magnetically stirred mixing flask which was connected by tubing to the top of the ion-exchange chromatographic column. 50 ml. fractions were collected, the seventh to the nineteenth fractions giving a positive ninhydrin test. These fractions were worked up individually yielding 540 mg. of crude N-methyl-DL-homocysteine sulphinic acid on crystallization from a water-alcohol-ether mixture. Three recrystallizations gave 268 mg. of colourless needles, m.p. 158°-159°. A sample of the final product was dried at 62° / 0.05 mm. for 14 hr. over phosphorus pentoxide for analysis.

Anal. Calcd. for $C_5H_{11}NO_4S$: C, 33.14 ; H, 6.12 ; N, 7.73 ; S, 17.69.

Found : C, 33.14 ; H, 6.24 ; N, 7.69 ; S, 17.77.

L-Thiazolidine-4-carboxylic Acid.

This substance was prepared by an adaption of the method used for the preparation of tetrahydro(1,3-thiazine)-4-carboxylic acid. The starting material, L-cysteine hydrochloride monohydrate, was made with slight modification of the method used for DL-homocysteine hydrochloride monohydrate. In this case, 50.0 g. of L-cystine was dissolved in 1 litre of 3 N hydrochloric acid and 35.0 g. of tin added. The mixture was gently refluxed for 2 hr., cooled and then filtered from the undissolved tin and diluted with two volumes of water. This solution was saturated with hydrogen sulphide and the precipitated tin sulphide was mixed with activated charcoal, the mixture filtered, and the filtrate evaporated to dryness *in vacuo* at 40°. The residue was triturated with 150 ml. of a mixture of ether and ethanol (4 : 1, v/v) and filtered to yield crude L-cysteine hydrochloride monohydrate. Yield : 58.6 g. (80 %). From 25.0 g. of this material there was obtained 18.05 g. (95 %) of L-thiazolidine-4-carboxylic acid, m.p. 195°-196°.

N,N'-Dimethyl-L-cystine.

This compound was made from 18.0 g. of L-thiazolidine-4-carboxylic acid in an analogous way to N,N'-dimethyl-DL-homocystine as previously described. Yield : 5.0 g. (21.9 %), m.p. 206°-207° (decomposed).

N-Methyl-L-cysteine Sulphinic Acid.

This substance was prepared in the same way as described for N-methyl-DL-homocysteine sulphinic acid. 2.0 g. of N,N'-dimethyl-L-cystine yielded 2.54 g. of colourless residue after the

evaporation of the ammoniacal solution. This residue was taken up in 9.0 ml. of water and passed through a column of 70 ml. of Dowex 1 (acetate), and eluted with ~~water~~ 6N ACETIC ACID. Ninhydrin-reacting material appeared in the eleventh to seventeenth fractions. These 50 ml. fractions were worked up individually yielding a total 350 mg. of crude N-methyl-L-cysteine sulphinic acid on crystallization from a water-alcohol-ether mixture. A further crop of 125 mg. was obtained from the mother liquors. Three recrystallizations yielded 250 mg. of colourless needles, m.p. 145° (decomposed A sample of the final product was dried at 60° / 0.1 mm. for 8 hr. over phosphorus pentoxide for analysis.

<u>Anal</u>. Calcd. for $C_4H_9NO_4S$: C, 28.74 ; H, 5.43 ; N, 8.38 ; S, 19.18.

Found : C, 28.67 ; H, 5.45 ; N, 8.27 ; S, 19.12.

<u>DL-Thiazolidine-4-carboxylic Acid.</u>

DL-Cysteine hydrochloride monohydrate was made adapting the method used for the preparation of L-cysteine hydrochloride monohydrate. Yield : 72 %.

Ten grams of the product were used to prepare the thiazolidine-4-carboxylic acid by the method described for L-thiazolidine-4-carboxylic acid, giving 7.12 g. of crude product. This was recrystallized from aqueous ethanol yielding 6.0 g. (78 %) of colourless crystals, m.p. 188.5°-189°.

N,N'-Dimethyl-DL-cystine.

This substance was prepared from 5.9 g. of DL-thiazolidine-4-carboxylic acid in an analogous way to the L form previously described. Yield : 3.8 g. (63 %), m.p. 206° (decomposed). The analytical sample was dried at 60° / 0.1 mm. for 6 hr. over phosphorus pentoxide.

Anal. Calcd. for $C_8H_{16}N_2O_4S_2$: C, 35.80 ; H, 6.01 ; N, 10.44 ; S, 23.90.

Found : C, 36.09 ; H, 6.09 ; N, 10.36 ; S, 23.93.

N-Methyl-DL-cysteine Sulphinic Acid.

This compound was made by the same method as that described for N-methyl-L-cysteine sulphinic acid. 2.68 g. of N,N'-dimethyl-DL-cystine yielded 4.66 g. of colourless residue after evaporation of the ammoniacal solution. This was dissolved in 12 ml. of water and the solution passed through a column of 120 ml. of Dowex 1 (acetate), 200-400 mesh, with elution by 6 N acetic acid in the usual way. Ninhydrin-reacting material appeared in the thirteenth to twenty-fourth fractions. The 50 ml. fractions were worked up individually, yielding 615 mg. of crude N-methyl-DL-cysteine sulphinic acid from a water-alcohol-ether mixture. 405 mg. of colourless needles, m.p. 146°, was obtained after three recrystallizations. The analytical sample was dried in an analogous manner to the L form.

Anal. Calcd. for $C_4H_9NO_4S$: C, 28.74 ; H, 5.43 ; N, 8.38 ; S, 19.18.

Found : C, 28.60 ; H, 5.19 ; N, 8.31 ; S, 19.01.

Note : In contrast to the preparation of N-methyl-L-cysteine sulphinic acid, a white precipitate was formed when the aqueous layer was brought to pH 4.0 with 8 N ammonium hydroxide and allowed to stand overnight at 0°. This material was filtered, washed three times with 5 ml-volumes of water, yielding 430 mg. of colourless crystals, m.p. 203°-204°. A few mg. of the by-product was chromatographed using N,N'-dimethyl-DL-cystine as a marker in 70 % aqueous isopropanol (v/v) for 20 hr. and found to have the same R_f as the marker.

DL-Cysteine Sulphinic Acid.

Four grams of DL-cystine hydrochloride was dissolved in about 10 ml. of water. To this solution was added 2.5 ml. of pyridine and the solution kept overnight at 0°. The precipitated DL-cystine was filtered and washed with 5 ml. of water. The crude product was recrystallized from water yielding 3.0 g. of colourless crystals, m.p. 231°-231.5°.

DL-Cystine was converted into DL-cysteine sulphinic acid by the method previously described for the two forms of N-methyl-cysteine sulphinic acid. 2.4 g. of DL-cystine gave 8.6 g. of residue after evaporation of the ammoniacal solution. This was taken up in 100 ml. of water and the solution passed through a column of 200 ml. of Dowex 1 (acetate), 200-400 mesh. The column was washed with water till the washings were neutral and then subjected to gradient elution with 6 N acetic acid. The fourteenth to twenty-sixth fractions gave a positive ninhydrin test. These fractions were worked up individually yielding 435 mg. of crude DL-cysteine sulphinic acid on crystallization from water-alcohol-ether mixture. Three

recrystallizations gave 215 mg. of colourless needles, m.p. 159° (decomposed). The final product was dried at 61° / 0.05 mm. over phosphorus pentoxide for 7½ hr. for analysis.

<u>Anal</u>. Calcd. for $C_3H_7NO_4S.\frac{1}{2}H_2O$: C, 22.22 ; H, 4.97 ; N, 8.64 ; S, 19.77.

Found : C, 22.58 ; H, 4.90 ; N, 8.72 ; S, 19.90.

To support the conclusion that DL-cysteine sulphinic acid crystallized out as the hemi-hydrate, a solution of 11 mg. of the acid in 2.0 ml. of water was titrated against 1.064 N sodium hydroxide solution using methyl orange as indicator.

Calcd. for $C_3H_7NO_4S.\frac{1}{2}H_2O$: Equivalent Weight : 162.2.

Found : Equivalent Weight : 161.4.

Paper chromatography using L-cysteine sulphinic acid and DL-cysteic acid as markers was carried out for 20 hr. in a solvent system made by mixing equal parts by volume of a mixture of pyridine-acetic acid-water (30 : 80 : 110, v/v; pH 4.0) and <u>n</u>-butanol. The DL-cysteine sulphinic acid had the same R_f as the marker, L-cysteine sulphinic acid, which was well separated from the slower running DL-cysteic acid, with no other ninhydrin positive material present. A further sample of the DL-cysteine sulphinic acid was dried at 61° /0.05 mm. for 20½ hr. over phosphorus pentoxide for analysis.

Anal. Calcd. for $C_3H_7NO_4S.\frac{1}{2}H_2O$: C, 22.22 ; H, 4.97 ; N, 8.64 ; S, 19.77.

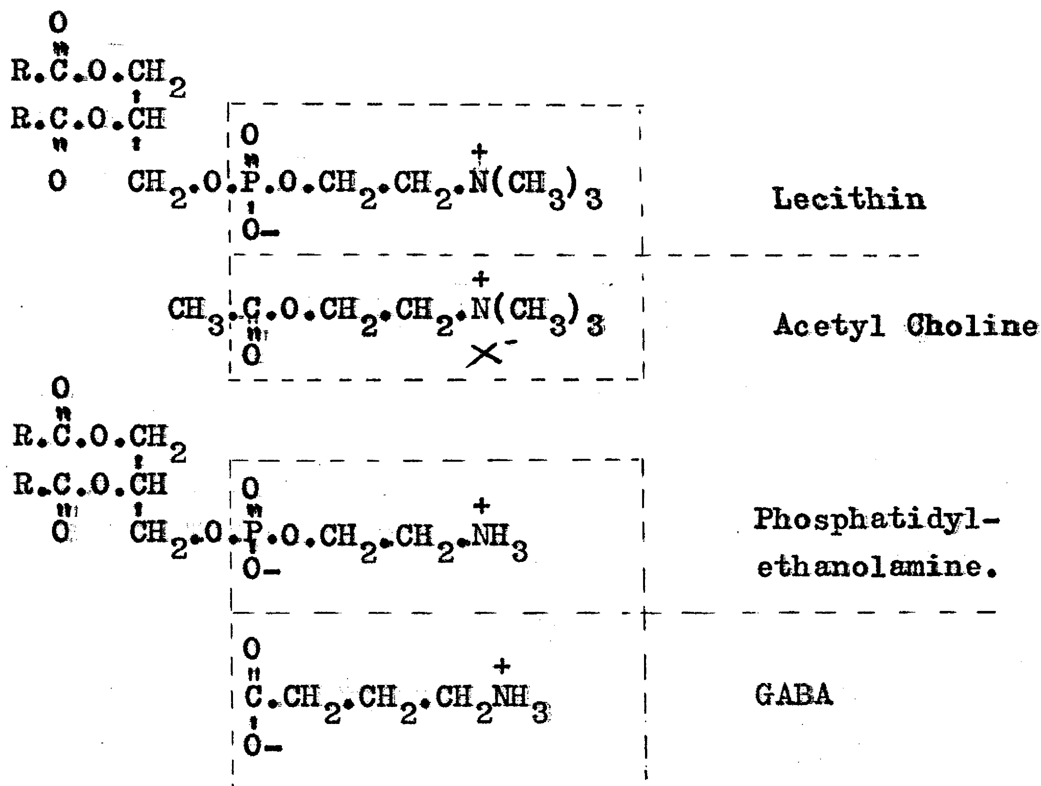
Found : C, 22.42 ; H, 4.90 ; N, 8.95 ; S, 20.39.

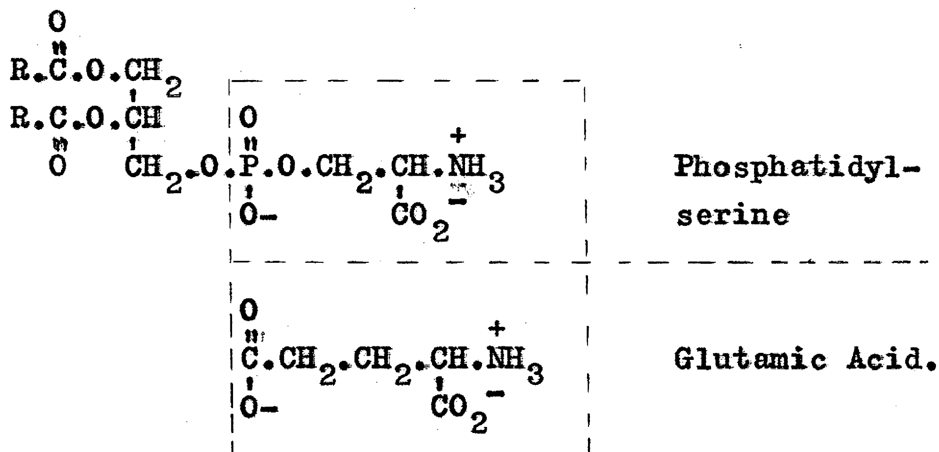
The analysis showed a higher value for nitrogen and sulphur indicating a slight loss of water. Higher drying temperature caused extensive decomposition. A sample of the acid that was heated at 90° over phosphorus pentoxide for 4 hr. at 0.05 mm. pressure showed the presence of other ninhydrin positive materials when chromatographed in the same solvent system as above.

(B). PHOSPHORIC ACID DIESTERS.

INTRODUCTION & DISCUSSION.

It has been suggested by Watkins[5] that the actions of acetyl choline, γ-aminobutyric acid (GABA) and glutamic acid on nervous activity may be due to dissociation of membrane complexes between phospholipids and proteins. The charge distributions of the molecules of acetyl choline, GABA and glutamic acid are very similar to those of the terminal moieties of lecithin, phosphatidylethanolamine and phosphatidylserine as shown below, and it was considered that substances having an even closer structural similarity to these terminal moieties would be of neuropharmacological interest.





Consequently, the O-methyl esters of O-phosphorylcholine, O-phosphorylethanolamine and O-phosphorylserine were synthesized. Direct methylation of the O-phosphoryl-derivatives of choline, ethanolamine and serine was effected with diazomethane. In the case of DL-O-methyl-phosphorylserine and O-methylphosphorylethanolamine, the tetramethylammonium salts of the phosphomonoesters were first prepared by treating DL-O-phosphorylserine and O-phosphorylethanolamine with tetramethylammonium hydroxide. These salts were soluble in methanol, thereby making possible the addition of diazomethane in ether without solubility problems. In the case of O-methyl-phosphorylcholine, a commercial calcium salt of O-phosphorylcholine was first freed of calcium ion by precipitation of calcium oxalate, and the free O-phosphorylcholine was treated in methanol solution with etheral diazomethane. The phosphoric acid diesters were isolated by ion-exchange chromatography using Dowex 50 W (H^+) in all cases.

O-Methylphosphorylserine was found to be a moderately strong excitant of interneurones, being of group G-H potency (Table I). O-Methylphosphorylethanolamine and

and O-methylphosphorylcholine seemed to have a weak depressant action on amino acid-evoked excitation of interneurones, but were very much weaker than GABA. None of the three substances seemed to affect synaptically or ACh-induced firing of Renshaw cells.

EXPERIMENTAL

O-(Methylphosphoryl)-choline.

24 ml. of an aqueous solution of 3.0 g. of a hydrated calcium salt of O-phosphorylcholine chloride ($C_5H_{15}NO_4P.Cl.Ca.3\frac{1}{2}H_2O$) was treated with 8 ml. of a solution containing an equivalent amount (1.18 g.) of hydrated oxalic acid ($C_2H_2O_4.2H_2O$) at room temperature. The resulting suspension was centrifuged at 2,000 r.p.m. for 1 hr. and the clear supernatant was decanted and evaporated to dryness at 40° *in vacuo* giving a gummy residue. This was dried over phosphorus pentoxide for 2 hr. at 0.1 mm. pressure, dissolved in 130 ml. of magnesium dried methanol at room temperature, and the resulting solution was cooled in an ice-bath. A cold (0°) solution of diazomethane (generated from 9.6 g. of nitrosomethylurea[6]) in 80 ml. of ether was added and the yellowish reaction mixture was kept overnight at 0° in a lightly stoppered flask. On evaporation to dryness *in vacuo* at 40°, a colourless gum was obtained, which was dried at 0.1 mm. pressure for 3½ hr. over phosphorus pentoxide at room temperature. The crude product (1.97 g.) was dissolved in 60 ml. of water, centrifuged at 2,000 r.p.m. for 30 min. to remove a few mg. of suspended material (presumably residual calcium oxalate). The clear supernatant solution was then evaporated to dryness at 40° *in vacuo*, the residual colourless gum taken up in 30 ml. of water, the solution passed through a column of 120 ml. of Dowex 50 W (H^+), 200-400 mesh, and the column eluted with water. 10 ml. fractions were collected, the twenty-sixth to forty-fifth fractions giving a positive test with Hanes-Isherwood[7] reagent, indicating the presence of phosphate-containing substance(s). These

fractions were subjected to paper electrophoresis in tris-(hydroxymethyl)-aminomethane—hydrochloric acid buffer[8] (0.05 M), pH 7.2, for 1 hr. at 20 V / cm. using O-phosphorylcholine as marker. The electrophoresis paper was sprayed with Hanes-Isherwood reagent. Only one phosphorus-containing spot was detected, suggesting that fractions 26-45 each contained only a single phosphorus-containing substance, this substance having zero charge at pH 7.2. These fractions were combined, evaporated to dryness at 40° <u>in vacuo</u>, and the colourless gum obtained was dried at 0.2 mm. pressure over phosphorus pentoxide. On standing at 0°, the gum crystallized into a solid mass of O-(methylphosphoryl)-choline monohydrate. Yield : 1.76 g. (87 %).
For analysis, a sample was dried at room temperature over phosphorus pentoxide in a desiccator for 3 days, after which it had a melting point of 112°-114°.

<u>Anal</u>. Calcd. for $C_6H_{16}NO_4P.H_2O$: C, 33.49 ; H, 8.43 ; N, 6.51 ; P, 14.40.

Found : C, 33.55 ; H, 8.35 ; N, 6.41 ; P, 14.27.

Another sample was dried at 56° / 0.1 mm. for 20 hr. over phosphorus pentoxide, m.p. 192°-193°, was also sent for analysis. Weighing of the sample for analysis was carried out under ordinary atmospheric condition and resulted in an obvious uptake of water from the air which led to analytical figures for the monohydrate.

<u>Anal</u>. Calcd. for $C_6H_{16}NO_4P.H_2O$: C, 33.49 ; H, 8.43 ; N, 6.51.

Found : C, 33.56 ; H, 8.36 ; N, 6.67.

The pure product could be recrystallized from ethanol-ether solvent mixture. 200 mg. of the O-(methylphosphoryl)-choline monohydrate yielded 170 mg. of hygroscopic fine white crystals, m.p. 110°-111°. Drying the product at 56° // 0.1 mm. for 20 hr. over phosphorus pentoxide raised the melting point to 193°-194°, probably due to the loss of solvent of crystallization. A sample was sent to be analysed under two conditions of sampling; in one case moisture was rigorously excluded under dry-box conditions, whilst no such precautions were taken in the second case. The analytical figures confirmed that one molecule of water was taken up very rapidly by the anhydrous material.

<u>Anal</u>. Calcd. for $C_6H_{16}NO_4P$: C, 36.55 ; H, 8.18 ; N, 7.10 ; P, 15.71.

Found : C, 36.78 ; H, 8.27 ; N, 7.06 ; P, 15.77.

Calcd. for $C_6H_{16}NO_4P.H_2O$: C, 33.49 ; H, 8.43 ; N, 6.51.

Found : C, 33.73 ; H, 8.53 ; N, 6.77.

<u>O-(Methylphosphoryl)-ethanolamine</u>.

One gram of O-phosphoryl-ethanolamine (7.14 m.mole.) was dissolved in 56 ml. of an aqueous solution containing one equivalent of tetra-methylammonium hydroxide (7.14 m. mole.). The resulting solution was evaporated to dryness at 40° <u>in vacuo</u> yielding a colourless gum, which was then dried at room

temperature for 2 hr. over phosphorus pentoxide at 0.1 mm. pressure. The gum was taken up in 20 ml. of magnesium dried methanol, the methanolic solution was stood in ice for 20 min. and treated with excess of a solution of diazomethane (generated from 2.92 g. of nitrosomethylurea) in 27 ml. of cold (0°) ether. The yellowish reaction mixture was kept at 0° for 20 hr. and then evaporated to dryness at 40° in vacuo. The colourless gum obtained was dried at 0.1 mm. pressure at room temperature over phosphorus pentoxide for 2 hr. yielding 2.07 g. of colourless semi-crystalline solid. This was dissolved in 12 ml. of water, passed through a column of 50 ml. of Dowex 50 W (H^+), 200-400 mesh, and the column was eluted with water at room temperature. 10 ml. fractions were collected, the ninhydrin-reacting material appearing in the seventh to fourteenth fractions. These fractions were subjected to paper electrophoresis in potassium dihydrogen phosphate-sodium hydroxide buffer mixture[9] (0.05 M), pH 7.0, for 1 hr. at 20 V / cm. using O-phosphorylethanolamine and ethanolamine as markers. The dried paper was sprayed with ninhydrin. Only one spot was detected in each case, suggesting that fractions 7-14 contained only a single ninhydrin reacting substance, this substance having a zero net charge. These fractions were combined, evaporated to dryness and then dried at room temperature for 3 hr. at 0.1 mm. pressure over phosphorus pentoxide yielding 1.06 g. of white crystalline solid, m.p. 164° (softening at 130°). The product could be recrystallized from water-ethanol-ether mixture, after which it had m.p. 196°-197°. The analytical sample was dried at 56° / 0.05 mm. for 8 hr. over phosphorus pentoxide.

<u>Anal.</u> Calcd. for $C_3H_{10}NO_4P$: C, 23.25 ; H, 6.49 ;
N, 9.03 ; P, 19.97.

Found : C, 23.45 ; H, 6.57 ; N, 9.03; P, 19.76.

<u>O-(Methylphosphoryl)-serine.</u>

This substance was prepared from 1.0 g. (5.40 m.mole) of O-phosphorylserine[10] in an analogous way to O-methylphosphorylethanolamine except that two equivalents of tetramethylammonium hydroxide were used (86 ml. of 0.126 M tetramethylammonium hydroxide). The colourless gum obtained on evaporation of the aqueous solution of the di-tetramethylammonium salt was dried at 0.1 mm. pressure for 6 hr. at room temperature over phosphorus pentoxide. It was dissolved in 40 ml. of magnesium dried methanol, and the solution was treated with excess of a solution of diazomethane (generated from 2.79 g. of nitrosomethylurea) in 38 ml. of ether in the cold (0°). The reaction mixture was then kept at 0° for 18 hr. and evaporated to dryness at 40° <u>in vacuo</u>. The light yellowish gum so obtained was dried for 4 hr. at 0.1 mm. pressure over phosphorus pentoxide at room temperature giving 1.77 g. of product. This was taken up in 22 ml. of water, passed through a column of 75 ml. of Dowex 50 W (H^+), 200-400 mesh, and the column was eluted with water. 10 ml. fractions were collected, the sixth to fifteenth fractions contained ninhydrin-reacting material. These fractions were then subjected to paper electrophoresis in potassium dihydrogen phosphate-sodium hydroxide buffer mixture (0.05 M), pH 7.0, at 20 V / cm. for 1 hr. with O-phosphorylserine and serine as markers ; spots were detected with ninhydrin.

The electrophoresis results suggested that fractions 6-10 contained only one ninhydrin-reacting substance, this substance being the same in each fraction and having a net -ve charge which was somewhat less than that of the starting material. (Fractions 11-15 contained small amounts of three ninhydrin-reacting substances, all anionic in character, with one behaving like the marker O-phosphorylserine, which had the greatest electrophoretic mobility, another like the substance contained in fractions 6-10, whilst the third had a lower anionic mobility than the other two.) Fractions 6-10 were combined, evaporated to dryness at 40° _in vacuo_ giving a colourless gum that was further dried at room temperature for 2 hr. at 0.1 mm. pressure over phosphorus pentoxide. Yield : 800 mg. Crystallization from a water-ethanol-ether mixture was effected by dissolving the crude product in 3 ml. of water, and adding 27 ml. of ethanol followed by ether till the point of faint turbidity, and allowing the mixture to stand at room temperature for 1½ hr., and at 0° for 18 hr. Yield : 416 mg. of white crystals, decomposed at 120° (the temperature at which the crystals began to froth up). One further recrystallization gave 316 mg. of crystals, decomp. point, 115°, raised to 125° by prolonged drying under vacuum. For analysis, two samples of the final product were dried at 56° / 0.1 mm. over phosphorus pentoxide, one sample for 8 hr. and the other for 30 hr.

<u>Anal.</u> Calcd. for $C_4H_{10}NO_6P$: C, 24.13 ; H, 5.06 ;
N, 7.04 ; P, 15.56.

Found (sample dried for 8 hr.) : C, 26.66 ;
H, 5.48 ; N, 6.56.

Found (sample dried for 30 hr.) : C, 26.57 ;
H, 5.72 ; N, 6.64 ; P, 14.84.

The analytical figures suggested that the product was impure. It was chromatographed in three different solvent systems [A, pyridine-acetic acid-water (30 : 80 : 110) : n-butanol (3 : 2, v/v) ; B, ethanol-water (8 : 1, v/v); C, pyridine-acetic acid-water (30 : 80 : 110)) : n-butanol (2 : 3, v/v)] and subjected to paper electrophoresis in two different buffer systems [A, sodium acetate-acetic acid (0.2 M), pH 4.0 ;

B, tris-(hydroxymethyl)-aminomethane—hydrochloric acid, ('Tris-HCl'), (0.05 M), pH 7.0.]. The papers were sprayed with ninhydrin and Hanes-Isherwood reagent. In all but one of the systems, only one substance was detectable. However, in solvent system A, two strong phosphorus containing spots were demonstrated. The lower R_f spot also showed a strong ninhydrin reaction, but the higher R_f spot reacted only very weakly with ninhydrin. The analytical figures suggested the impurity may have contained more than a single methyl group. However, other methyl groups could not be present as phosphoryl or carboxylic methyl esters as such products would have had different electrophoretic mobilities than O-methyl-phosphorylserine. The only alternative position for extra methyl groups is on the nitrogen. It is difficult

to explain the analytical figures on the basis of contamination by N-methyl-O-methylphosphorylserine, and also such a contaminant would most likely have reacted more strongly with ninhydrin. Watkins (private communication) found that methyl iodide treatment of amino acids gave only a trace of the N-monomethylated derivatives, the main products being more highly methylated compounds. It is suggested that the product obtained is a mixture of O-methylphosphorylserine and the N,N-dimethyl derivative in the approximate proportions of 2 : 1.

Anal. Calcd. for $C_4H_{10}NO_6P$ (67 %) + $C_6H_{14}NO_6P$ (33 %) : C, 26.85 ; H, 5.48 ; N, 6.72 ; P, 14.87.

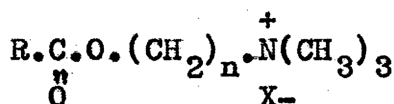
Found (sample dried for 8 hr.) : C, 26.66 ; H, 5.48 ; N, 6.56.

Found (sample dried for 30 hr.) : C, 26.57 ; H, 5.72 ; N, 6.64 ; P, 14.84.

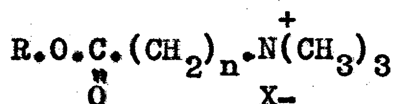
(C). QUATERNARY AMMONIUM COMPOUNDS.

INTRODUCTION & DISCUSSION.

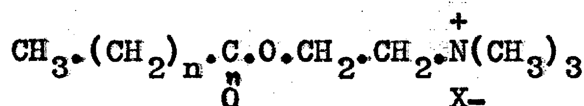
Analogues of acetyl choline are being synthesized and tested for neuropharmacological activity. Substances related to acetyl choline (VIII, n = 2, R = CH_3) were chosen as this compound is the excitatory transmitter substance at the neuromuscular junction and also at synapses in certain restricted regions of the central nervous system.



(VIII)



(XII)

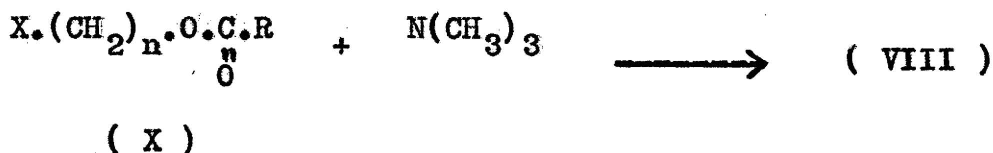


(IX)

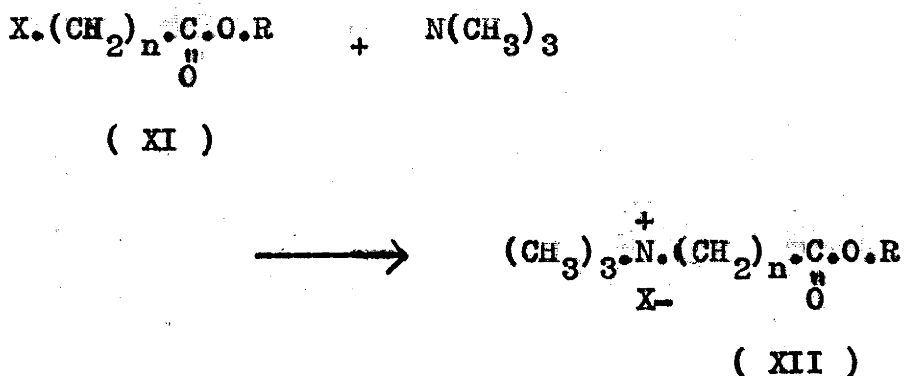
In other regions of the central nervous system acetyl choline has no action but the possibility exists that a structurally similar compound may have transmitter function in these parts.

Acetyl homocholine (VIII, n = 3, R = CH_3) was prepared as the first variation of the basic structure of acetyl choline. Other modifications included the variation of the alkyl group (VIII), and reversal of the carbonyl and ether oxygen atoms of the acyl group (XII). The method employed for the preparation of

these quaternary ammonium esters was by direct treatment of the respective halo-esters with dry trimethylamine according to the reactions



and



Acetyl homocholine (VIII, n = 3, R = CH_3) has a longer carbon chain than acetyl choline (VIII, n = 2, R = CH_3) and this increases the distance between the ester and the quaternary ammonium groupings by which the active compounds are thought to become associated with the structural components of the junctional membrane and thus to manifest their characteristic actions. Acetyl homocholine has been previously prepared as the iodide by Zu and Wilson[11] by treating γ-dimethylamino-propyl acetate with methyl iodide. In the present preparation, trimethylamine and γ- bromopropyl acetate were reacted together in alcohol solution at room temperature giving the required acetyl homocholine in

the form of its bromide. The intermediate γ-bromopropyl acetate was prepared by the action of acetyl bromide on trimethylene glycol.[12]

Caproyl choline (IX, n = 4), lauroyl choline (IX, n = 10) and palmitoyl choline (IX, n = 14) have hydrocarbon 'tails' of increasing length which render the substances more lipid soluble. This feature was thought to be of interest because of the postulated nature of biological membranes, in which a bimolecular lipid layer is thought to be an essential feature. The 2-bromoethyl esters of the appropriate fatty acids were prepared by refluxing 2-bromoethanol and the fatty acid in toluene containing sulphuric acid, with azeotropic removal of water. The esters were then reacted with dry trimethylamine which gave the quaternary ammonium compounds as the bromides. Each of these three choline derivatives had previously been prepared as their iodides by the action of methyl iodide on the respective 2-dimethylaminoethyl esters of the fatty acids.[13]

A series of 'reversed' analogues of choline and homocholine was prepared to investigate the effect of this particular structural modification on the neuropharmacological activity. These substances are represented by formula (XII).

β-carbomethoxyethyltrimethylammonium chloride (XII, n = 2, R = CH_3, X = Cl) is an isomer of acetyl choline chloride (VIII, n = 2, R = CH_3, X = Cl) but has the carbonyl and ether oxygen atoms in the reverse order. This was prepared by treating methyl-β-chloropropionate (XI, n = 2, R = CH_3, X = Cl) with a solution of dry trimethylamine in methanol at reflux

temperature. The intermediate methyl-β-chloropropionate was obtained by esterification of β-chloropropionic acid with methanol in the presence of dry hydrogen chloride.

β-carboethoxyethyltrimethylammonium chloride (XII, $n = 2$, $R = CH_2.CH_3$, $X = Cl$) is the 'reversed' analogue of propionyl choline (VIII, $n = 2$, $R = CH_2.CH_3$, $X = Cl$) which has an action similar to acetyl choline at most cholinoceptive junctions. The compound was prepared by the same method as for β-carbomethoxyethyltrimethylammonium chloride. The intermediate, ethyl-β-chloropropionate (XI, $n = 2$, $R = CH_2.CH_3$, $X = Cl$) was made using ethanol instead of methanol. No record of previous preparations of either of these two quaternary ammonium esters could be found in the chemical literature.

γ-carbomethoxypropyltrimethylammonium bromide (XII, $n = 3$, $R = CH_3$, $X = Br.$) has one methylene group more than β-carbomethoxyethyltrimethylammonium chloride and is thus the 'reversed' analogue of acetyl homocholine bromide. The starting material, methyl-γ-bromobutyrate (XI, $n = 3$, $R = CH_3$, $X = Br$) was obtained by ring opening of γ-butyrolactone with dry methanol and hydrogen bromide at 0°. On reaction with trimethylamine in the usual way, methyl-γ-bromobutyrate was converted into the quaternary ammonium bromide which has not been previously synthesized. Unlike the two other 'reversed' esters, this substance is not hygroscopic.

Table II illustrates the pharmacological actions of the quaternary ammonium compounds compared with that of acetyl choline (ACh) which was used as the reference substance and was given an arbitary rating of 100. On

Renshaw cells, the equally potent β-carbomethoxyethyl-trimethylammonium chloride and γ-carbomethoxypropyltri-methylammonium bromide had a greater activity than ACh, with acetyl homocholine bromide less potent than β-carbo-ethoxyethyltrimethylammonium chloride, the latter having the same activity as ACh. Caproyl, lauroyl and palmitoyl choline (all as their bromides), unlike the rest of the quaternary ammonium compounds, functioned by blocking the activity of ACh. Caproyl choline bromide was two and a half times as active as ACh on the toad's rectus, and β-carbomethoxyethyltrimethylammonium chloride six and a half times as potent. The bromides of acetyl homocholine and γ-carbomethoxypropyltrimethylammonium were about four-five times less active than ACh. On guinea pig ileum, only β-carbomethoxyethyltrimethylammonium chloride was slightly more active than ACh, while γ-carbomethoxypro-pyltrimethylammonium bromide, acetyl homocholine bromide and caproyl choline bromide were much weaker than ACh.

TABLE II. Pharmacological Actions of Quaternary Ammonium Compounds.

Quaternary Ammonium Compds.	*Rectus (toad)	*Ileum (guinea pig)	Renshaw cell.
Acetyl Choline.	100.	100.	+++
Acetyl Homocholine Bromide.	21.	1.6.	+
Caproyl Choline Bromide.	250.	0.5.	⌿
Lauroyl Choline Bromide.	-	-	⌿
Palmitoyl Choline Bromide.	-	-	⌿

TABLE II. (contd.)

'Reversed' Choline Esters.	*Rectus (toad)	*Ileum (guinea pig.)	Renshaw cell. (cat.)
β-Carbomethoxyethyltri-methylammonium Chloride.	650.	130	+++++
β-Carboethoxyethyltri-ammonium Chloride.	-	-	+++
γ-Carbomethoxypropyltri-ammonium Bromide.	26.	6.4.	+++++

Note : - : Not tested.
* : Potency of acetyl choline taken as 100.
+++ : Activity of acetyl choline.
+ : Activity less than that of acetyl choline.
+++++ : Activity greater than that of acetyl choline.
/ : Blocks activity of acetyl choline.

Results obtained from experiments conducted by Dr. D. R. Curtis and R. W. Ryall.

EXPERIMENTAL

γ-Bromopropyl acetate.

One hundred grams of acetyl bromide was added to an equivalent quantity of trimethylene gylcol (147.2 g.) and the mixture kept at 0° overnight. The reaction mixture was warmed at 100° for 1 hr. and distilled under reduced pressure, yielding crude γ-bromopropyl acetate which was contaminated with trimethylene bromohydrin. The crude product was digested under reflux for 5 hr. with acetyl bromide and the unreacted acetyl bromide together with hydrogen bromide was removed by vacuum distillation. The remaining mixture was fractionated under reduced pressure and the fraction boiling at 102°-103° / 30 mm. was collected. Yield : 105.4 g. (71.6 %)

Acetyl homocholine bromide.

Ten grams of γ-bromopropyl acetate was treated with 12 ml. of 33 % ethanolic trimethylene and the mixture kept overnight at room temperature. Ether was added to the mixture to the point of incipient turbidity after which it was allowed to stand at 0° for 24 hr. The supernatant solution was decanted from the oily precipitate, which was recrystallized three times from hot ethanol and petroleum ether (b.p. 80°-100°) yielding 4.2 g. (31.7 %) of very hygroscopic crystals of acetyl homocholine bromide, m.p. 125°. For analysis, a sample was dried at 50° over phosphorus pentoxide for 10 hr. at 0.1 mm. pressure.

Anal. Calcd. for $C_8H_{18}BrNO_2$: C, 40.00 ; H, 7.53 ; Br, 33.27 ; N, 5.88.

Found : C, 39.97 ; H, 7.25 ; Br, 33.33 ; N, 5.87.

Methyl-β-chloropropionate.

Dry hydrogen chloride was passed continuously through a solution of 21.0 g. of β-chloropropionic acid in excess of methanol while the mixture was heated under reflux for 2½ hr. The reaction mixture was concentrated in vacuo until all the hydrogen chloride had been removed, and the ester was distilled under reduced pressure yielding 14.5 g. (61.1 %) of colourless liquid.

β-Carbomethoxyethyltrimethylammonium chloride.

Methyl-β-chloropropionate (10.3 g.) was refluxed with an equal amount of methanol while excess dry trimethylamine was passed through the solution. The latter was prepared by refluxing a solution (34 ml.) of 33 % of trimethylamine in ethanol over poatssium hydroxide pellets and the evolved gas was dried by passage through a soda-lime tube. The reaction mixture was kept overnight at room temperature and the crude product was filtered yielding 11.4 g. (75 %) of white crystals. This material was recrystallized from a mixture of methanol and ether yielding hygroscopic white crystals, m.p. 173.5°-174°. A sample for analysis was dried at 70° // 0.2 mm. over phosphorus pentoxide.

Anal. Calcd. for $C_7H_{16}ClNO_2$: C, 46.28 ; H, 8.87 ; Cl, 19.52 ; N, 7.71.

Found : C, 46.58 ; H, 8.77 ; Cl, 19.70 ; N, 7.75.

<u>Ethyl-β-chloropropionate.</u>

This substance was prepared exactly as the methyl ester from 29.0 g. of β-chloropropionic acid, except that ethanol was used instead of methanol. Yield : 19.3 g. (53 %), b.p. 61.5° / 50 mm.

<u>β-Carboethoxyethyltrimethylammonium chloride.</u>

This compound was prepared in an analogous way to the methyl ester from a solution of 4.0 g. of ethyl-β-chloropropionate in equal amount of dry ethanol. The trimethylamine gas was generated from 30 ml. of 33 % alcoholic trimethylamine in the usual way. M.p. 170°-170.5°. The analytical sample was dried at 65° / 0.2 mm. over phosphorus pentoxide for 24 hr.

<u>Anal.</u> Calcd. for $C_8H_{18}ClNO_2$: C, 49.10 ; H, 9.26 ; Cl, 18.11 ; N, 7.16.

Found : C, 49.25 ; H, 9.07 ; Cl, 18.06 ; N, 7.17.

<u>Methyl-γ-bromobutyrate.</u>

A mixture of thirty grams of γ-butyrolactone and 100 ml. of dry methanol was saturated with dry hydrogen bromide gas at 0°. The resulting solution was kept at 0° for two days and at room temperature for one day. The reacted solution was then subjected to fractionation <u>in vacuo</u> and the fraction boiling at 50°-52° / 1.3 mm. was collected. Yield : 30.3 g. (48 %).

γ-Carbomethoxypropyltrimethylammonium bromide.

Six grams of the methyl-γ-bromobutyrate was refluxed with 10 ml. of dry methanol and dry trimethylamine was continuously passed through the mixture. The trimethylamine gas was generated from 50 ml. of 22.87 % (w / v) alcoholic trimethylamine and the evolved gas dried by a soda-lime tube. The reaction mixture was kept overnight at room temperature before ether was added to the point of incipient turbidity and then allowed to stand at 0° for 24 hr. The white crystalline deposit was filtered and recrystallized three times from methanol-ether mixture. Yield : 2.2 g., m.p. 76°-77°. For analysis, a sample of the product was vacuum dried at room temperature over phosphorus pentoxide for 24 hr.

Anal. Calcd. for $C_8H_{18}BrNO_2$: C, 40.00 ; H, 7.55 ; Br, 33.27 ; N, 5.83.

Found : C, 39.48 ; H, 7.51 ; Br, 32.88 ; N, 5.88.

2-Bromoethyl caproate.

A solution of 15 g. of n-caproic acid, 48 g. of 2-bromoethanol, 2.3 g. of concentrated sulphuric acid in 100 ml. of toluene was refluxed for 3½ hr. with azeotropic removal of water. The dark brown solution was cooled, washed three times with 60 ml-volumes of water, dried with anhydrous sodium sulphate for 24 hr. and subjected to fractional distillation under reduced pressure. The fraction boiling at 84°-85° / 2.5 mm. was collected. Yield : 28.8 g. (65 %).

Caproyl choline.

Excess of dry trimethylamine gas, prepared from 30 ml. of a 23 % (w / v) solution of trimethylamine in alcohol in the usual way, was passed through 5.0 g. of warm 2-bromoethyl caproate. A white deposit formed after the reaction was allowed to stand over-night at room temperature. The excess trimethylamine was removed in a vacuum desiccator over concentrated sulphuric acid, yielding 5.05 g. of crude product, which was washed eight times with 8 ml-volumes of dry ether. Because of the hygroscopic nature of the product, the ether washings were carried out by allowing the suspension to settle, and carefully pipetting off the supernatant ethereal extract; residual ether from the last washing operation was allowed to evaporate at room temperature through a calcium chloride tube. For analysis, a sample was dried at 60° over phosphorus pentoxide for 7 hr. at 0.05 mm. pressure. M.p. 108°-109°.

Anal. Calcd. for $C_{11}H_{24}BrNO_2$: C, 46.81 ; H, 8.57 ; Br, 28.32 ; N, 4.96.

Found : C, 46.20 ; H, 8.43 ; Br, 29.28 ; N, 5.12.

2-Bromoethyl laurate.

This substance was prepared by the method described for 2-bromoethyl caproate from 50 g. of lauric acid, 40 g. of 2-bromoethanol, 1 g. of concentrated sulphuric acid in 100 ml. of toluene. Yield : 13 g. (56.5 %). The colourless ester was refractionated and the fraction boiling at 125°-127° / 0.1 mm. was analysed.

Anal. Calcd. for $C_{14}H_{27}BrO_2$: C, 54.74 ; H, 8.86 ; Br, 26.01.

Found : C, 54.81 ; H, 8.95 ; Br, 26.24.

Lauroyl choline.

This compound was synthesized in an analogous way to caproyl choline from 1.7 g. of 2-bromoethyl laurate. The trimethylamine gas was generated from 70 ml. of 22.3 % (w/v) alcoholic trimethylamine in the usual way. The analytical sample was dried at 58° / 0.05 mm. over phosphorus pentoxide for 7 hr. M.p. 150°.

Anal. Calcd. for $C_{17}H_{36}BrNO_2$: C, 55.72 ; H, 9.90 ; Br, 21.81 ; N, 3.82.

Found : C, 55.34 ; H, 10.16 ; Br, 22.31 ; N, 3.94.

2-Bromoethyl palmitate.

This was prepared in the same way as the previous fatty acid esters. 25 g. of palmitic acid gave 10.4 g. of product, m.p. 44°-45.5°. A further crop of 6.34 g. was obtained from the filtrate giving a total yield of 16.74 g. (46.4 %). The crude product was recrystallized three times from minimal amounts of hot acetone which raised the melting point to 45°-46°. The analytical sample was dried in air at room temperature for 20 hr.

Anal. Calcd. for $C_{18}H_{35}BrO_2$: C, 59.50 ; H, 9.71 ; Br, 21.99.

Found : C, 59.74 ; H, 9.47 ; Br, 22.14.

Palmitoyl choline.

Excess dry trimethylamine gas prepared in the usual way was passed through a warm solution of 3 g. of 2-bromoethyl palmitate, The trimethylamine gas was generated from 100 ml. of 23 % (w/v) alcoholic trimethylamine. The white solid formed was kept in a vacuum desiccator over concentrated sulphuric acid overnight to remove the excess trimethylamine, and further dried over phosphorus pentoxide, giving a crude yield of 3.18 g. (90 %).
The product was recrystallized three times from hot acetone, yielding white crystals, m.p. 176°-178°. The analytical sample was dried at 59° / 0.05 mm. over phosphorus pentoxide for 6 hr.

Anal. Calcd. for $C_{21}H_{44}BrNO_2$: C, 59.69 ; H, 10.50 ; Br, 18.91 ; N, 3.32.

Found : C, 58.88 ; H, 10.63 ; Br, 19.28 ; N, 3.26.

The analysis showed the carbon figure to be slightly low and another sample of the product was dried at 59° / 0.05 mm. for 14 hr. over phosphorus pentoxide.

Anal. Calcd. for $C_{21}H_{44}BrNO_2$: C, 59.69 ; H, 10.50 ; Br, 18.91 ; N, 3.32.

Found : C, 59.24 ; H, 10.57 ; Br, 19.89 ; N, 3.32.

The second analysis gave a higher bromine value than expected though the carbon content was closer to the theoretical value.

SECTION II

IDENTIFICATION OF SOME ACID-SOLUBLE NUCLEOTIDE DERIVATIVES IN OX BRAIN.

INTRODUCTION & DISCUSSION.

Watkins had shown that when a perchloric acid extract of brain was subjected to ion-exchange chromatography on Dowex 50 W (H^+), 200-400 mesh, many U.V-absorbing substances were weakly adsorbed and could be eluted by water. Some of the eluted substances were readily identified by electrophoresis, chromatography and U.V-absorption characteristics, but others remained unidentified at that time. The latter seemed to contain cytidine and adenosine chromophores. The order of elution found by Watkins was as follows :-

1. Guanosine-5'-phosphate, GMP(5').
2. Adenylylsuccinic acid.
3. An unidentified cytidine derivative.
4. An unidentified adenosine derivative.
5. An unidentified cytidine derivative (not identical with 3.)
6. Cytidine-5'-phosphate, CMP(5').
7. Nicotinamide-adenine dinucleotide, NAD.
8. Adenosine-5'-phosphate, AMP(5').

It was therefore of interest to investigate the unidentified substances detected in these eluates and the cytidine-containing substances were initially chosen in view of the importance of cytidine co-enzymes in phospholipid synthesis, and the role of the latter substances in nervous processes. Cytidine diphosphate choline (CDP-C) and cytidine diphosphate ethanolamine (CDP-E) had both previously been detected in brain,[14] but there remained the possibility that related substances such as cytidine diphosphate N-methylethanolamine and cytidine diphosphate

N,N-dimethylethanolamine might also occur and be involved in similar metabolic pathways.

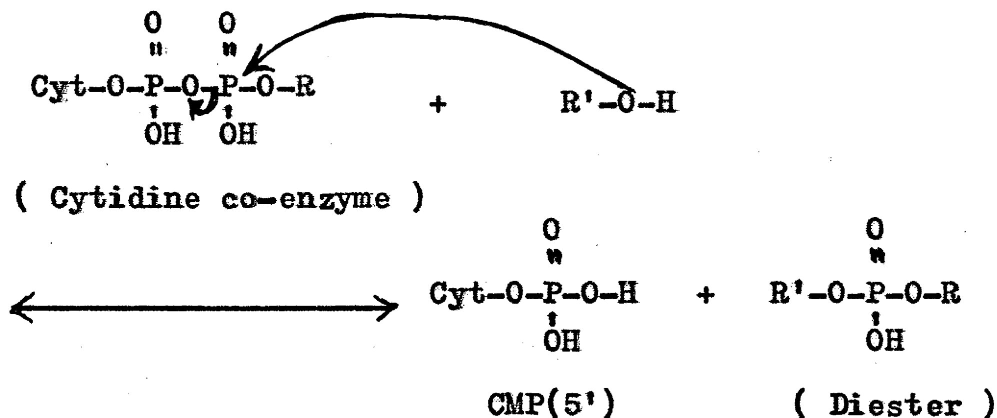
Cytidine Co-Enzymes and Phospholipid Biosynthesis.

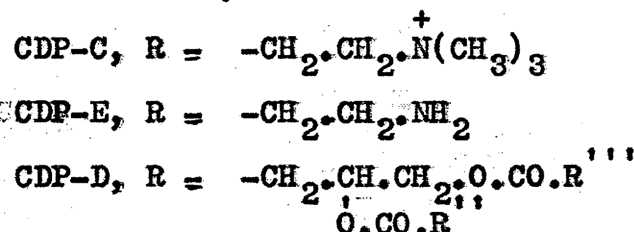
Kennedy and his co-workers had demonstrated that phosphatidylcholine[15] and sphingomyelin[16] could be formed by the reaction of CDP-C with a D-$\alpha\beta$-diglyceride and N-acylsphingosine respectively, and phosphatidylethanolamine[15] from CDP-E and D-$\alpha\beta$-diglyceride. They also postulated that phosphatidylserine was most likely to be formed in nature in an analogous way, with serine first converted into phosphorylserine, then to cytidine diphosphate serine (CDP-S) and finally to phosphatidylserine by reaction of CDP-S with a D-$\alpha\beta$-diglyceride. If this was the case, the identification and isolation of CDP-S would form a step towards proving the existence of this metabolic pathway for the biosynthesis of phosphatidylserine in nature. The demonstrations of the existence of either cytidine diphosphate N-methylethanolamine or cytidine diphosphate N,N-dimethylethanolamine of both of these compounds in brain would be of great interest since one would expect that these cytidine derivatives, like CDP-C and CDP-E, would react with a D-$\alpha\beta$-diglyceride to form the respective phosphatidyl derivatives. If this were the case, the possibility of another biosynthetic pathway could not be ruled out. One might expect, for example, the cytidine diphosphate N-methylethanolamine to react with a D-$\alpha\beta$-diglyceride to form a phosphatidyl N-methylethanolamine, which by direct methylation, probably involving methyl-S-adenosylmethionine, could be converted to phosphatidylcholine via phosphatidyl N,N-dimethylethanolamine. The existence of both phosphatidyl N-methyl-

ethanolamine and phosphatidyl N,N-dimethylethanolamine, and the process of direct methylation with methyl-S-adenosylmethionine were reported by Bremer and Greenberg.[17] Using liver tissues *in vitro*, they demonstrated that labelled phosphatidylcholine was formed from methyl-labelled S-adenosylmethionine and offered convincing evidence that the water-soluble forms of choline such as phosphorylcholine and CDP-C were not directly produced. Instead, they succeeded in detecting the formation of labelled phosphatidyl N-methylethanolamine and phosphatidyl N,N-dimethyethanolamine, suggesting that methylation of ethanolamine to choline actually took place with the phospholipids acting as successive intermediates. They, thus supported the early work of Crowder and Artom,[18] who illustrated that phospholipids could be formed *in vitro* from dimethylaminoethanol while Nyc and his collaborators[19,20] had shown that a mutant strain of *Neurospora crassa* (47904) which normally required choline for optimal growth, accumulated phospholipids containing mono- and dimethylaminoethanol when grown in the absence of choline. The early results of Nyc and Artom were consistent with the hypothesis that methylation of ethanolamine to choline was possible via phosphatidylethanolamine with phosphatidyl N-methylethanolamine and phosphatidyl N,N-dimethylethanolamine acting as successive intermediates.

After having isolated CDP-C, CDP-E, CDP-D (cytidine diphosphate diglyceride), CDP-G (cytidine diphosphate glycerol) and CDP-R (cytidine diphosphate ribitol) from bacteria, Baddiley[21,22] showed that the first three cytidine containing co-enzymes were essential in the formation of the phospho-diester linkages of phospholipids in living cells. In cytidine co-enzymes reactions, it is

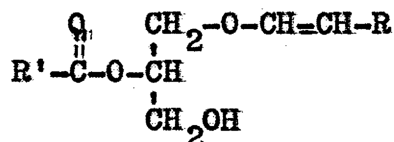
generally believed that an enzyme-catalysed nucleophilic attack of the oxygen of a hydroxyl group occurs with the displacement of CMP(5'), and the formation of a mono-phosphorus diester linkage as illustrated thus :-





Baddiley also illustrated that CDP-D was involved in phosphatidylinositol and phosphatidylglycerophosphate formation by reaction with myo-inositol and L-α-glycerophosphate respectively. The biosynthesis of the plasmalogenic phosphatides were still obscure as no direct pieces of evidence were yet available. Like other conventional phosphatides e.g. phosphatidylcholine and phosphatidylethanolamine, they were widely distributed in nature, and have structures closely similar to them.

Generally in the plasmalogenic series the fatty acid ester linkage in the α-position of the glycerophosphate is replaced by an aldehydogenic bond, in the form of an αβ-unsaturated ether. Compounds having the general structure XIII, which is closely similar to αβ-diglycerides,



(XIII)

can react with CDP-C or CDP-E in the presence of a particular enzyme to form the plasmalogenic phospholipids of phosphatidylcholine and phosphatidylethanolamine respectively.[23]

Phospholipids and Membrane Structure.

Because of the role played by the cytidine nucleotides in phospholipid biosynthesis, they are of great importance in the synthesis and maintenance of cellular structure. Cell membranes are believed to consist of an orientated bi-molecular layer of phospholipids coated externally and internally by a monolayer of protein as illustrated diagrammatically in Fig. I.

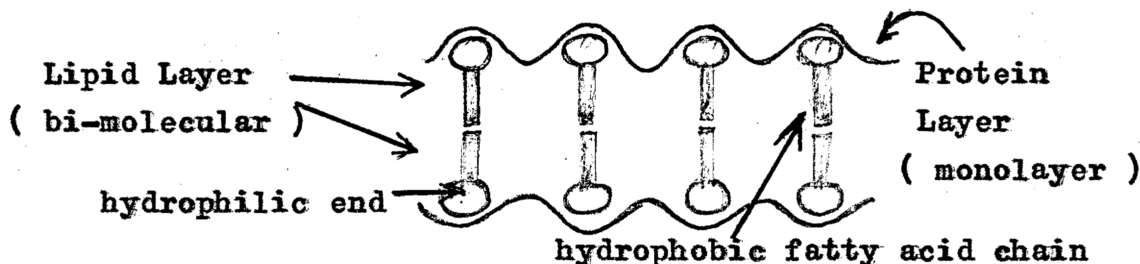


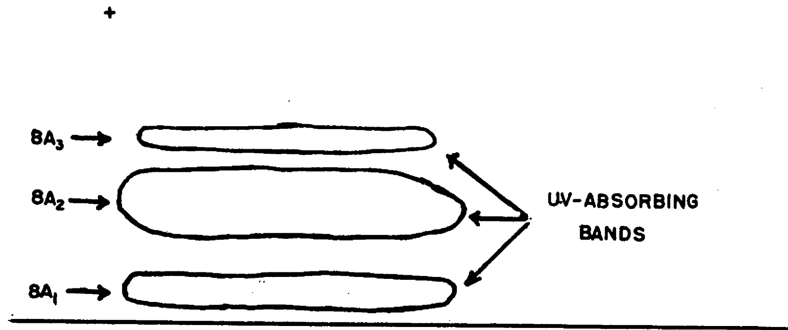
Fig. I. Postulated Membrane Structure.

The hydrophobic fatty acid chains are held together by Van der Waals' forces, and constitute a hydrocarbon interior which is responsible for the high electrical resistance of the membrane. The polar groups of both lipid and protein are probably involved in transport phenomena including the specific and controlled movements of sodium and potassium ions across the membrane during the conduction of impulses along nerve fibres. Thus by their association with the membrane structure, the phospholipids, and hence also the cytidine containing substances, are linked with the neurophysiology of nervous conduction and transmission.

In model systems,[24,25] lipid bi-layers seem to be impermeable to ions, and the mechanism by which ions across cellular membranes are not known. A variety of proposals have been made, one of the more important being the "Lipoid-Pore Hypothesis". The 'pore' in the membrane is suggested to be formed by a protein helix or by a group of such helices with charged groups which have appreciable freedom of movement. Under these conditions, the protein or other substances facilitating transport surround the ion transported and avoid the need to introduce it as a hydrated ion to the hydrocarbon chains of the lipid, which also carry charged groupings. Pharmacological agents are thought to act by forming complexes with the membrane molecules and changing the membrane permeability. For example, acetyl choline is believed to be bound to a membrane molecule by virtue of an interaction between the quaternary ammonium and ester groupings and complementary sites on the receptor molecule. This results in conformational changes and gives rise to the increased permeability to sodium and potassium ions responsible for the induced nervous and muscular activity.

Identification of Two Cytidine-containing Substances from Ox Brain.

The present work describes the extraction and identification of two cytidine derivatives from ox brain. Extraction was carried out with perchloric acid, adopting the same proceedure as that used by Watkins. Different methods of extraction were recorded in the literature by various workers e.g. Ansell and Bayliss[26] used 10 % ice-cold trichloroacetic acid, an ice-cold mixture of ethanol-water (2 : 1, v/v) by Kennedy and Weiss[27] and hot aqueous extract by Lunt and Kent.[28] Perchloric acid was most convienent as the perchlorate ion could be removed as the insoluble potassium perchlorate at pH 4.0 with potassium hydroxide. The cytidine nucleotides were then partially separated from the other U.V-absorbing substances by passing the perchloric acid extract through a column of Dowex 50 W (H^+), 200-400 mesh, and the U.V-absorbing compounds which were loosely held by the resin were eluted with water. The U.V-absorbing substances in each fraction were initially examined by paper electrophoresis in sodium acetate-acetic acid buffer mixture (0.2 M), pH 4.0.[29] The patterns so obtained were compared with those obtained by Watkins in order to establish correspondence between fractions. At pH 4.0, the cytidine-containing substances had the lowest electrophoretic mobility in an anionic direction, and for convienence such substances were designated by the suffix A_1 (A refering to anion and 1 to the fact that the U.V-absorbing region was closest to the line of application of the fraction on the paper.) This is illustrated in page 48 of a paper electrophoresis pattern obtained using fraction eight as an example.



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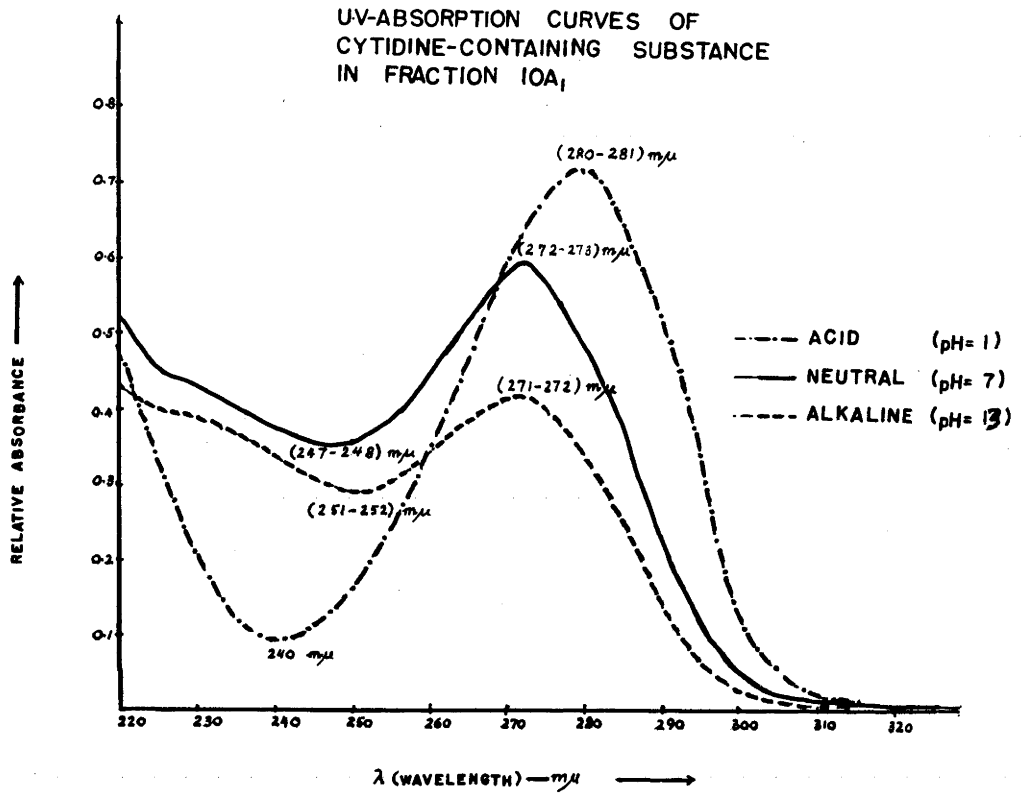
PAPER ELECTROPHORESIS
OF FRACTION 8

BUFFER: $NaCO_2CH_3$- CH_3COOH
0.2M, pH4·0

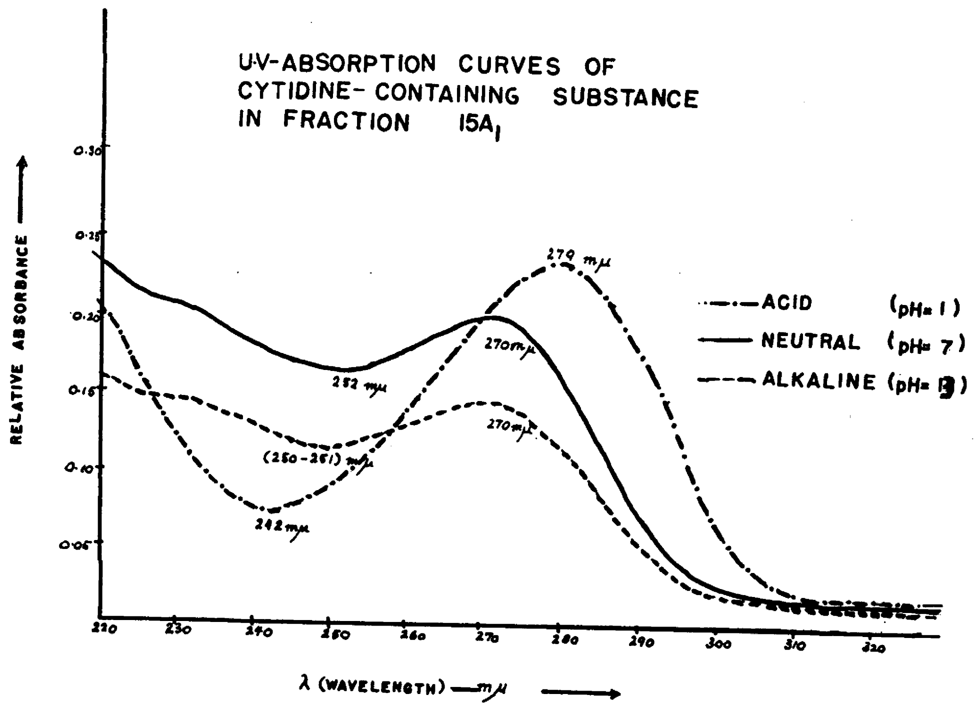
VOLTAGE: 400V (=10V/cm)
TIME: 1·5 hr

Preliminary identification of substances thus separated was made by cutting out the U.V-absorbing regions, eluting them with water, and examination of their U.V. spectra at three different pHs. Those cytidine-containing substances which remained unidentified by Watkins were present in the eighth to eleventh and the thirteenth to sixteenth fractions. The first group reacted with ninhydrin but the second group did not. Each of the fractions contained a substance with a characteristic U.V-absorption of a cytidine derivative with a maximium absorption at about 280 mμ in acid medium (pH 1.0) and this is illustrated by the U.V-absorption curves of fraction 10 and fraction 15 at three different pHs. Fraction 10 is taken to represent the ninhydrin reacting group, and fraction 15 the group which did not react with ninhydrin. The U.V-absorption curves are illustrated in page 50-51.

Identification of these cytidine derivatives was done principally by acid hydrolysis. The hydrolytic products were identified by paper chromatography, paper electrophoresis and U.V-absorption. In all cases, known markers were used. The "ninhydrin -ve" cytidine derivatives of fraction thirteen to sixteen, on N hydrochloric acid hydrolysis at 100° for 30 mins. in sealed tubes, liberated two compounds having the same R_fs as CMP(5') and phosphorylcholine. The latter was detected on chromatographic papers by Hanes-Isherwood reagent[30] and the former by means of U.V-absorption and also by Hanes-Isherwood phosphate spray. With 6 N hydrochloric acid hydrolysis at 100° for 60 hr. in a sealed tube, three U.V-absorbing spots were detected, these substances having R_fs equivalent to cytosine, cytidine and CMP(5'). Furthermore, free choline

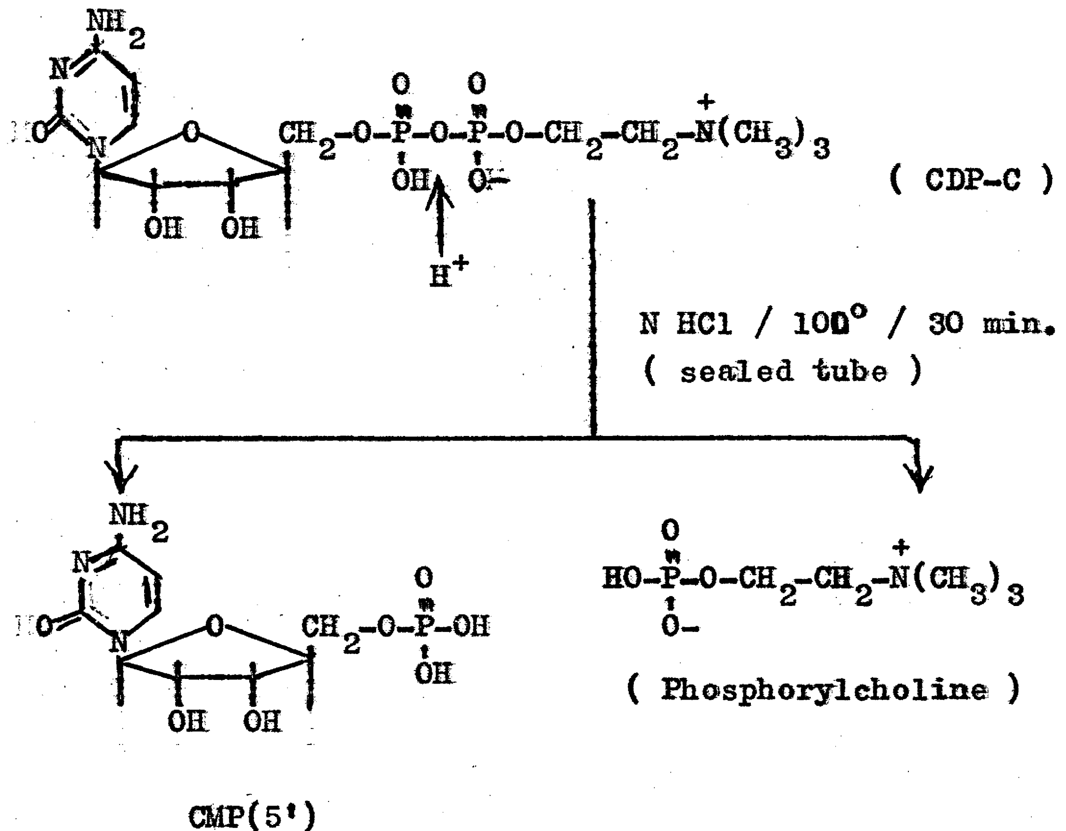


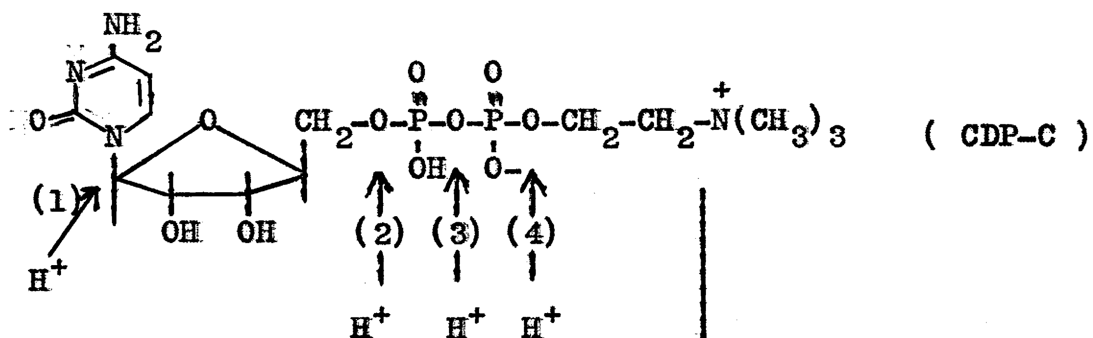
UV-ABSORPTION CURVES OF
CYTIDINE-CONTAINING SUBSTANCE
IN FRACTION 10A1
(280-281) mμ
(272-273) mμ
(271-272) mμ
(247-248) mμ
(251-252) mμ
240 mμ
ACID (pH= 1)
NEUTRAL (pH= 7)
ALKALINE (pH= 13)
RELATIVE ABSORBANCE
λ (WAVELENGTH) — mμ



U.V-ABSORPTION CURVES OF
CYTIDINE- CONTAINING SUBSTANCE
IN FRACTION 15A1
RELATIVE ABSORBANCE
0.30
0.25
0.20
0.15
0.10
0.05
279 mμ
270 mμ
252 mμ
270 mμ
(250-251) mμ
242 mμ
ACID (pH= 1)
NEUTRAL (pH= 7)
ALKALINE (pH= 13)
220
230
240
250
260
270
280
290
300
310
320
λ (WAVELENGTH) — mμ

and inorganic phosphate were present among the products of the 6 N hydrochloric acid hydrolysis of fraction 15 A_1, which was taken to represent the "ninhydrin -ve" group. Free choline was detected by using 0.2 % dipicrylamine in acetone.[31] It thus appeared that the cytidine derivative of this group might be CDP-C, since an authentic sample of CDP-C when similarly treated gave identical results. The positions of the bonds hydrolyzed by the two strengths of hydrochloric acid in order to give the products detected are illustrated below.

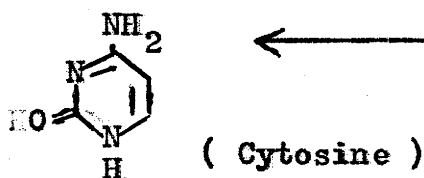




(CDP-C)

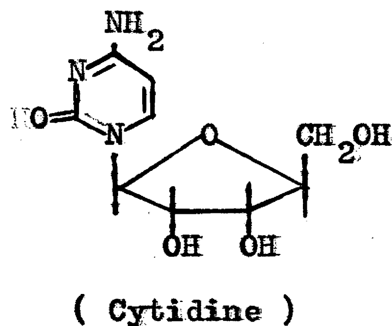
6 N HCl // 100° // 60 hr.
(sealed tube)

(1)



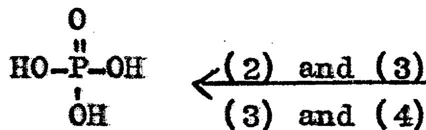
(Cytosine)

(2)



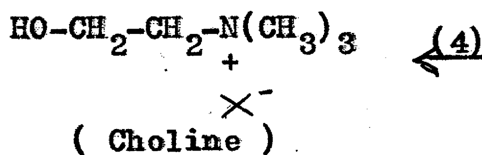
(Cytidine)

(3) → CMP(5')



(2) and (3)
(3) and (4)

(Inorganic Phosphate)



(4)

(Choline)

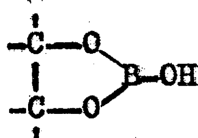
Practically identical results were obtained with the "ninhydrin +ve" substances of fraction nine to eleven when hydrolyzed with N and 6 N hydrochloric acid under the same conditions as the "ninhydrin -ve" group. With N hydrochloric acid hydrolysis at 100° for 30 min., besides CMP(5'), phosphorylethanolamine was detected instead of phosphorylcholine. Both phosphorylethanolamine and ethanolamine, instead of choline, were present among the 6 N hydrochloric acid hydrolysis products, together with the same three U.V-absorbing substances (i.e. cytosine, cytidine, CMP(5').) as obtained before. Ethanolamine and phosphorylethanolamine were detected with ninhydrin reagent. At this stage, it appeared that the "ninhydrin +ve" compound was most likely to be CDP-E. The only difference between the "ninhydrin -ve" and the "ninhydrin +ve" groups was in the nature of the terminal moiety, this being ethanolamine in the latter case, and choline in the former.

The "ninhydrin +ve" and "ninhydrin -ve" groups behaved chromatographically and electrophoretically exactly like the authentic markers, CDP-E and CDP-C respectively. Paper electrophoresis was carried out in three different solvent systems having different pH values (i.e. 4.0, 7.0 and 10.7).

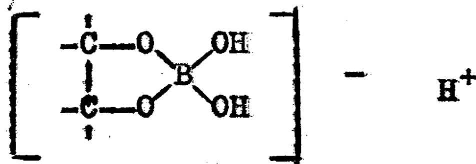
As further confirmation that the substance of fraction 15 A_1 was CDP-C, the pyrimidine base of this fraction was liberated by treating an electrophoretically pure sample with 70 % perchloric acid at 100° for 30 min. and identified as cytosine by U.V-absorption measurement followed by paper chromatography and electrophoresis against an authentic marker. Perchloric acid was used

for two reasons. Firstly, it gives rise to less deamination than sulphuric or hydrochloric acid,[32] and secondly, it causes no appreciable destruction of either the pyrimidine or purine bases under the conditions used.

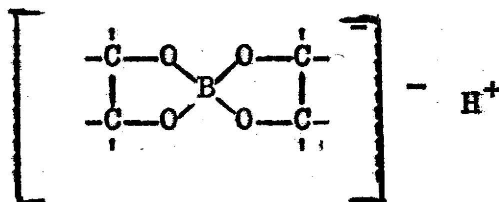
The sugar residue of the cytidine derivatives was determined using borate electrophoresis at pH 9.1. Under these conditions, the sugar ring would form a complex with the boric acid generally of the types illustrated thus :-



(I)



(II)



(III)

Types (II) and (III) are formed when adjacent <u>cis</u>-hydroxyls are present in the sugar residue, and type (II) predominates when the relative proportions of borate to sugar are high in dilute solutions.[33] On the other hand, no such complexes can be formed with the deoxy-ribose ring and its derivatives. Thus by means of the borate complex formation, a new dissociating acid group is introduced into a sugar molecule possessing two <u>cis</u>-hydroxyl groups. The pK of the first dissociation of the boric acid is 9.0, and by using borate buffer at pH 9.1 the acidic group of the complex is partially dissociated

giving the complex a net negative charge thereby causing it to migrate towards the anode during electrophoresis. The rate of movement is determined by the negative charges due to the borate complex and those on the enol hydroxyl groups of the bases. Thus, the deoxy-ribose and its derivatives, carrying charges only on the partially dissociated enol hydroxyl groups, would be expected to move much slower than the ribose compounds, and this constitutes a good method of identifying the sugar component in the cytidine derivatives. Electrophoretically pure samples of the two cytidine-containing substances from ox brain were hydrolyzed with 6 N hydrochloric acid at 100° for 60 hr. in a sealed tube and the hydrolysis products were subjected to paper electrophoresis in borate buffer,[34] (0.05 M), pH 9.1 at 20 V / cm for 1 hr. with appropriate markers. The results clearly indicated that the sugar residue in both the cytidine nucleotides was ribose. Although the completely liberated sugar itself was destroyed in this process, the cytidine and CMP(5') obtained by incomplete hydrolysis had the same R_fs as authentic cytidine markers and were well separated from deoxy-cytidine markers. That some deamination had occurred was idicated by the presence of uracil, which was formed from cytosine through loss of ammonia.

The content of phosphorus was determined by subjecting an electrophoretically pure sample of Fraction 15 A_1 (taken to represent the "ninhydrin -ve" group) to acid digestion in order to liberate inorganic phosphate which was then estimated by the King's colorimetric method.[35] The amount of phosphorus was then compared with the theoretical value calculated from the U.V-absorption

curve of a duplicate sample of Fraction 15 A_1 based on the assumption that the substance was CDP-C. The results were completely consistent with the identification of the extracted substance as CDP-C.

In summary, the accummulated evidence showed that the cytidine nucleotide in fractions thirteen to sixteen was CDP-C, and that in fractions eight to eleven was CDP-E since :

1. The substances behaved like authentic CDP-C and CDP-E electrophoretically and chromatographically.

2. The pyrimidine nucleoside was cytidine in both cases.

3. One of the substances contained choline, the other ethanolamine.

4. Identical products were obtained by the hydrolysis of Fraction 15 A_1 and an authentic sample of CDP-C.

5. The estimated phosphorus content of Fraction 15 A_1 was identical with that calculated on the basis of spectral data, assuming the compound to be CDP-C.

EXPERIMENTAL

Perchloric Acid Extraction of Ox Brain.

2.4 kg. of brain was obtained from six freshly slaughtered cattle, freed as much as possible from blood vessels, and cut into small pieces. They were frozen in liquid nitrogen, roughly crushed and then immediately homogenized portionwise with an approximately equal amount of cold (0°) 10 % perchloric acid. The combined homogenate after rehomogenization was centrifuged (2,000 r.p.m. / 20 min.) at 0°. The supernatant (I) was set aside at 0° and the residue re-extracted twice with 300 ml. of cold (0°) 5 % perchloric acid with centrifuging at 0°, (3,000 r.p.m. / 20 min.), giving supernatant II. The combined supernatant (I + II) was carefully brought to pH 4.0 with 50 % cold (0°) potassium hydroxide and cooled overnight at 0°. The clear supernatant was decanted from the precipitated potassium perchlorate and evaporated to dryness <u>in vacuo</u> at 40° giving 28.7 g. of light yellow residue. This was taken up in 145 ml. of water and passed through a column of 294 g. of Dowex 50 W (H^{+}), 200-400 mesh. The column was washed with water till the washings reached pH 4.0 and was then eluted with M pyridine solution until ninhydrin-reacting material no longer appeared in the pyridine eluate. The pH 4.0 eluate (but not the more acidic earlier fractions) and the pyridine eluate were combined and evaporated to dryness at 40° yielding 8.7 g. of yellow gum. This was dissolved in 113 ml. of water and subjected to ion-exchange chromatography using 124 ml. of Dowex 50 W (H^{+}), 200-400 mesh. 50 ml. fractions were collected and freeze-dried overnight.

They were then taken up in 5 ml. of water and stored in the frozen state. The scheme of the acid extraction is illustrated thus :-

Scheme of Perchloric Acid Extraction of Ox Brain.

Brain Pieces (2.4 kg.)

↓ Liquid Nitrogen

Frozen Brain Pieces

↓
1. Crushed.
2. Homogenized (2 min.) with equal amount of 10 % cold (0°) perchloric acid.

Homogenate (0°)

↓
1. Rehomogenized. (30 min.)
2. Centrifuged (2,000 r.p.m. / 20 min. 0°.)

Residue. → Washed (twice) with 300 ml. of 5 % cold (0°) perchloric acid followed by centrifuged (3,000 r.p.m. / 20 min. / 0°.) → Precipitate (950 ml.) and Supernatant II

Supernatant I → Combined with supernatant II → Combined Supernatant (I + II)

Scheme of Perchloric Acid Extraction of Ox Brain. (contd.)

Combined Supernatant (I + II)

1. Brought to pH 4.0 with 50 % Potassium Hydroxide.
2. Allowed to stand overnight at 0°.

Supernatant

Precipitate (Potassium Perchlorate)

Supernatant → Evaporated (20°-25° / 0.7 mm.)

Residue (28.7 g.)

1. Dried. (2 hr. / 0.1 mm.)
2. Dissolved in water. (145 ml.)
3. Subjected to Ion-exchange Chromatography. (294 g. of Dowex 50 W (H^+), 200-400 mesh.)

Chromatographic Column

1. Eluted with water until eluate was pH 4.0 (540 ml.)
2. Eluted with M pyridine solution.
3. Combined pH 4.0 water eluate and pyridine eluate evaporated.

Yellow Gum (8.7 g.)

(continued next page)

Scheme of Perchloric Acid Extraction of Ox Brain. (contd.)

Yellow Gum (contd.)

1. Dissolved in water. (113 ml.)
2. Adsorbed on Dowex 50 W (H^+), 200-400 mesh. (124 ml.)
3. Eluted with water.

↓ Filtrate (113 ml.)

↓ Fractions (50 ml.)

Freeze-dried. (0° // 0.4 mm.)

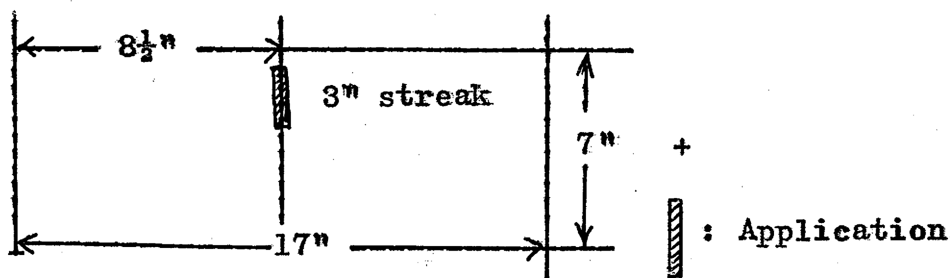
↓ Dried Fractions (32)

Analysis of Ion-Exchange Fractions. (1-32.)

This was done by paper chromatography, electrophoresis and U.V-absorption measurements to determine which fractions contained the cytidine derivatives. Individual fractions, dissolved in 5 ml. of water, were tested for the presence of U.V-absorbing and phosphate-containing substance(s) by examination of spots of the solutions applied to paper under U.V. light and after treatment with Hanes-Isherwood reagent. If these tests were positive, the solutions were subjected to paper chromatography and electrophoresis using cytidine-5'-diphosphate choline, (CDP-C) and cytidine-5'-diphosphate ethanolamine, (CDP-E), as markers in the following solvent systems :-

Paper Chromatography.	1. 70 % aqueous isopropanol (v/v). 2. isobutyric acid-ammonium hydroxide (0.5 N), 10 : 6 (v/v). 3. isopropanol-concentrated hydrochloric acid-water (170 : 41 : 39, v/v).
Paper Electrophoresis.	4. sodium acetate-acetic acid buffer mixture (0.2 M), pH 4.0 5. potassium dihydrogen phosphate-sodium hydroxide buffer (0.05 M), pH 7.0. 6. glycine-sodium hydroxide buffer (0.05 M), pH 10.7.

Paper chromatography was carried out at room temperature using the descending technique in glass tanks with Whatman No. 1 paper. For paper electrophoresis, Carl Schleicher and Schull No. 2043b Mgl paper was used. Authentic markers were always employed where possible. Phosphates were detected by the Hanes-Isherwood reagent, and amino compounds with 0.25 % ninhydrin in water-saturated *n*-butanol and then heating the paper for 5-10 min. at 105°. The U.V-absorption was measured with a Shimadzu Spectrophotometer (Model QR-50) at three different pH values. For spectrophotometric analysis, 0.15 ml. of each fraction was applied along a 3 inch line on a 7X17 inch paper as shown :-



Electrophoreses were run for 1½ hr. at 400 volts in sodium acetate-acetic acid buffer mixture (0.2 M), pH 4.0, and the U.V-absorbing region was carefully marked, cut into small pieces and transferred into a 30 ml. serum bottle. The paper cuttings were eluted with 5 ml. of water, filtered and the absorption was measured at 220-350 mμ against a blank solution. The latter was prepared by cutting a band region equal in area and electrophoretic mobility to the test band and treated identically. The absorption was also read in acid and alkaline media by adding 0.5 ml. of N hydrochloric acid and subsequently 1 ml. of N sodium hydroxide to both the fractions and blanks. The chromatographic, electrophoretic and spectrophotometric results indicated that there were two types of cytidine compounds present, the "ninhydrin +ve" substances, contained in fractions 8-11 and the "ninhydrin -ve" substances, contained in fractions 13-16. These substances corresponded to those detected by Watkins but not identified. CMP(5'), identified by Watkins, appeared first in fraction 17 of the present work. All these fractions showed characteristic U.V-absorption curves of a cytidine derivative with the maximium absorption within the wave-length range 279-281 mμ in acid (pH 1.0), 270-275 mμ in neutral (pH 7.0) and 270-273 mμ in alkaline (pH 13) as illustrated in Table I. The cytidine-containing substances of fractions 13-16 (represented by Fraction 15 A_1) and that of fractions 8-11 (represented by Fraction 8 A_1) behaved like CDP-C and CDP-E in all chromatographic and electrophoretic systems employed (Table II). The amount of the cytidine-containing substance in each fraction was estimated

either on the basis of CDP-C or CDP-E as appropriate. These estimates are shown in Table III. Methods by which the substances were fully identified as CDP-C and CDP-E are described in the pages following Table III.

Table I

U.V-Absorption of Electrophoretic Fractions.

	Acid (pH 1)		Neutral (pH 7)		Alkaline (pH 13)	
Fractions.	λ Max.	λ Min.	λ Max.	λ Min.	λ Max.	λ Min.
@ 8 (A_1).	281.	241.	271.	250.	272.	250.
@ 9 (A_1).	279-280.	240.	271.	250.	271-272.	250-251.
@ 10 (A_1).	279.	242.	270.	252.	270.	250-251.
@ 11 (A_1).	279-280.	241-242.	270-271.	249-250.	271.	249.
& 13 (A_1).	279-280.	240-241.	272.	249-250.	272-273.	251.
& 14 (A_1).	280.	241.	271.	250.	272.	250.
& 15 (A_1).	280-281.	240	272-273.	247-248.	271.	251-252.
& 16 (A_1).	280-281.	240.	272.	249.	271-272.	249.
CDP-C (Sigma)	279-280.	240.	275.	245.	270-271.	249-250.
≠ Cytidine.	280.	241.	271.	250.5.	273.	251.5.

Note : @ :- Ninhydrin +ve fractions.

& :- Ninhydrin -ve fractions.

≠ :- Recorded by J. J. Fox and D. Shugar in Biochem. et Biophys. Acta., 9, 369 (1952)

λ :- Wavelength (mu).

Table II.

(A). Paper Chromatography.

Solvent System.	Compound.	R_f ∅	
70 % Aqueous isopropanol (v/v).	CDP-E.	0.34 (a,b)	
	Fraction 8 A_1.	0.35 (a,b)	
	CDP-C.		0.49 (a).
	Fraction 15 A_1.		0.49 (a).
Isobutyric acid-ammonium hydro-xide (0.5 N), 10 : 6 (v/v).	CDP-E.	0.34 (a,b)	
	Fraction 8 A_1.	0.34 (a,b)	
	CDP-C.		0.45 (a).
	Fraction 15 A_1.		0.45 (a).
Isopropanol-conc. hydrochloric-water. (170 : 41 : 39, v/v.)	CDP-E.	0.29 (a,b)	
	Fraction 8 A_1.	0.29 (a,b)	
	CDP-C.		0.65 (a).
	Fraction 15 A_1.		0.65 (a).

Note :

∅ Spot detection as follows :-

(a) U.V. Absorption.

(b) Ninhydrin Spray.

CDP-E : Cytidine diphosphate ethanolamine.

CDP-C : Cytidine diphosphate choline.

Table II (contd.)

(B). Paper Electrophoresis.

Solvent System.	Compound.	ø Distance (cm) moved towards anode :-	
Sodium acetate-acetic acid buffer mixture (0.2 M), pH 4.0.	CDP-E.	0.75 (a,b).	
	Fraction 8 A_1.	0.75 (a,b).	
	CDP-C.	0.75 (a).	
	Fraction 15 A_1	0.75 (a).	
Potassium dihydrogen phosphate-sodium hydroxide buffer (0.05 M) pH 7.0.	CDP-E.	1.5 (a,b).	
	Fraction 8 A_1.	1.45 (a,b).	
	CDP-C.	1.50 (a).	
	Fraction 15 A_1	1.50 (a).	
Glycine-sodium hydroxide buffer (0.05 M), pH 10.7.	CDP-E.		2.7 (a).
	Fraction 8 A_1		2.7 (a).
	CDP-C.	1.50 (a).	
	Fraction 15 A_1	1.50 (a).	

Note :

ø Spot detection as follows :-

(a) U.V. Absorption.

(b) Ninhydrin Spray.

Table III.

Amount of Cytidine Derivatives.

Fraction.	Estimated Weight of Cytidine Derivative. (mg.)	Total Amount of Cytidine Derivative.
@ 8 A_1.	6.41.	15.47 mg. of CDP-E.
@ 9 A_1.	7.14.	
@10 A_1.	1.05	
@11 A_1.	0.87	
&13 A_1.	1.96.	14.15 mg. of CDP-C.
&14 A_1.	4.37.	
&15 A_1.	4.70.	
&16 A_1.	3.12.	

Note :

CDP-C :- Cytidine diphosphate choline.
CDP-E :- Cytidine diphosphate ethanolamine.
@ :- Ninhydrin +ve fractions.
& :- Ninhydrin -ve fractions.

Estimates based on U.V.-absorption measurements assuming Fractions $8A_1$-$11A_1$ are CDP-E and Fractions 13-16 (A_1) are CDP-C.

(A). "Ninhydrin -ve" Cytidine Derivatives.

(Fractions $13A_1$-$16A_1$.)

Specific Investigation of Fraction $15A_1$.

This fraction was chosen since it contained the highest concentration of the cytidine derivative in this particular group. The substance was subjected to

1. Acid Hydrolysis.

The sample was prepared by streaking 0.32 ml. of a solution of Fraction 15 containing 0.3 mg. of cytidine derivative along a 5 inch. line on a 7"X17" Schleicher and Schull No. 2043b Mgl paper. Electrophoresis was run for 2 hr. at 400 volts in sodium acetate-acetic acid buffer mixture (0.2 M), pH 4.0. The paper was then dried in air at room temperature, the U.V.-absorbing region marked, cut into small pieces, transferred into a 30 ml. serum bottle, eluted with 7 ml. of water and the eluate was filtered into a 25 ml. round bottomed flask. The paper cuttings were washed three times with 1 ml. of water. The combined eluate and washings were evaporated to dryness *in vacuo* at 40° giving a colourless residue. This was taken up in 0.5 ml. of N hydrochloric acid and hydrolyzed in a sealed tube at 100° for 30 min. The hydrolyzate was then pipetted into a 25 ml. round bottomed flask, the reaction tube washed three times with 2 ml-volumes of water and the combined hydrolyzate and washings evaporated to dryness at 40° *in vacuo* yielding a colourless residue. The residual

hydrochloric acid was removed by standing the sample over potassium hydroxide under vacuum for 10 hr., after which the residue was taken up in 0.25 ml. of water and kept at 0°. An authentic sample of 2 mg. of CDP-C was similarly treated. For 6 N hydrochloric acid hydrolysis, the sample was prepared in the same way as for the N hydrochloric acid hydrolysis from 0.4 ml. of starting material, and eluted with 10 ml. of water. The hydrolysis was carried out at 100° with 0.8 ml. of 6 N hydrochloric acid for 60 hr., giving a colourless residue on evaporation in vacuo at 40°. After removal of residual hydrogen chloride over potassium hydroxide in vacuo for 10 hr. at room temperature, the reaction product was taken up in 0.3 ml. of water and kept at 0°. 2 mg. of an authentic sample of CDP-C was similarly treated.

2. Paper Chromatography.

The hydrolyzates of Fraction 15 A_1 and of the samples of authentic CDP-C were chromatographed using CDP-C, CMP(5'), phosphorylcholine, cytidine, cytosine, and inorganic phosphate as markers in the following solvent systems :-

1. isopropanol-concentrated hydrochloric acid-water (170 : 41 : 39.), v/v/
2. isobutyric acid-sodium hydroxide-water. (69 ml. : 0.85 g. : 31 ml.)
3. isobutyric acid-ammonium hydroxide (0.5 N), (10 : 6), v/v.
4. methanol-concentrated ammonium hydroxide (S.G. 0.91)-water. (60 : 10 : 30.), v/v.
5. ethanol-water (8 : 1.), v/v.

The last two solvent systems were used only for detecting free choline among the products of 6 N hydrolysis. The U.V.-absorbing spots were marked before the papers were sprayed with appropriate reagents. Ninhydrin and Hanes-Isherwood spray were used for amino group and phosphorus-containing compounds respectively, and 0.20 % dipicrylamine in 50 % acetone was used for the detection of choline.

Table IV. N Hydrochloric Acid Hydrolysis.

Solvent System.	Compound.	ϕ R_f		
Isobutyric acid-Sodium hydroxide-Water. (69 ml. : 0.85 g. : 31 ml.)	CDP-C.	0.31. (a)		
	CMP(5').		0.26. (a,b)	
	P-Choline.			0.46 (b).
	CDP-C. Hydrolyzate		0.26. (a,b)	0.46 (b).
	Fraction $15A_1$ Hydrolyzate		0.27. (a,b)	0.46 (b).

Note :

ϕ Spot detection as follows :-

(a) U.V.-Absorption.

(b) Hanes-Isherwood Spray.

P-Choline :- Phosphorylcholine.

CDP-C :- Authentic sample of cytidine diphosphate choline.

Table IV. (contd.)

Solvent System.	Compound.	ø R_f		
Isopropanol-concentrated hydrochloric acid-Water. (170 : 41 : 39, v/v.)	CDP-C.			0.42 (a).
	CMP(5').		0.56. (a,b)	
	P-Choline.	0.85. (b)		
	CDP-C. Hydrolyzate.	0.84. (b)	0.56. (a,b)	
	Fract. 15 A_1 Hydrolyzate	0.84. (b)	0.57. (a,b)	
Isobutyric acid -Ammonium hydroxide (0.5 N), 10 : 6, v/v.	CDP-C.		0.25. (a).	
	CMP(5').			0.21 (a,b).
	P-Choline.	0.35. (b).		
	CDP-C. Hydrolyzate	0.34. (b).		0.21 (a,b).
	Fract. 15 A_1 Hydrolyzate	0.34. (b)		0.21 (a,b).

Note :

ø Spot detected as follows :-

(a) U.V-Absorption.

(b) Hanes-Isherwood Spray.

Table V. 6 N Hydrochloric Acid Hydrolysis.

Solvent System.	Compound.	∅ R_f			
Isobutyric acid-Ammonium hydroxide (0.5 N), 10 : 6, v/v.	Choline (chloride)	0.71. (c).			
	Cytosine.		0.68. (a).		
	Cytidine.			0.53. (a).	
	CMP(5').				0.30. (a,b).
	Fract. 15 A_1 Hydrolyzate[1]	0.71. (c).	0.68. (a).	0.53. (a).	0.30. (a,b).
Isobutyric acid-Sodium hydroxide-Water. (69 ml : 0.85 g. : 31 ml.)	Choline. (chloride)	0.75. (c).			
	Cytosine.		0.75. (a)		
	Cytidine.			0.56. (a)	
	CMP(5').				0.29. (a,b)
	Fract. 15 A_1 Hydrolyzate[1]	0.74. (c).	0.75. (a).	0.54. (a).	0.28. (a,b).

Note :

∅ Spot detection as follows :-

(a) U.V.-Absorption.

(b) Hanes-Isherwood Spray.

(c) 0.2 % Dipicrylamine in 50 % acetone.

Table V. (contd.)

Solvent System.	Compound.	ø R_f.			
Isobutyric acid-Sodium hydroxide-Water (69 ml. : 0.85 g. : 31 ml.)	Choline. (chloride)	0.68. (c)			
	Cytosine.		0.66. (a)		
	Cytidine.			0.50. (a)	
	CMP(5').				0.22 (a,b)
	CDP-C. Hydrolyzate	0.68. (c)	0.64. (a)	0.48. (a)	0.23. (a,b)
Isobutyric acid-Ammonium hydroxide. (0.5 N), 10 : 6, v/v.	Choline. (chloride)		0.60. (c)		
	Cytosine.	0.62. (a)			
	Cytidine.			0.49. (a).	
	CMP(5').				0.14. (a,b)
	CDP-C. Hydrolyzate	0.62. (a)	0.60. (c)	0.48. (a)	0.14. (a,b)
Methanol-conc. Ammonium hydroxide-Water. (60 : 10 : 30), v/v.	Choline. (chloride)	0.84. (c)			
	Fract. 15 A_1 Hydrolyzate	0.84. (c)			
Ethanol -Water. (8 : 1), v/v.	Choline. (chloride)	0.71. (c)			
	Fract. 15 A_1 Hydrolyzate	0.71. (c)			

3. Paper Electrophoresis.

The 6 N hydrochloric acid hydrolyzate was subjected to paper electrophoresis in borate buffer, (0.05 M), pH 9.1 at 15 volts / cm. for 1 hr. using ribose, deoxy-ribose, cytosine, cytidine, CMP(5'), deoxy-cytidine, uracil, and uridine as markers. The paper was dried in air at room temperature, the U.V.-absorbing spots were marked and the paper was then sprayed with aniline hydrogen phthalate reagent. The migrations observed were illustrated in Table VI (page 76).

Table VI.

COMPOUND.	Distance (cm.) moved towards :					
	CATHODE		ANODE			
Fract. 15 A_1 Hydrolyzate.		1.25. (a)	1.40. (a)	2.70. (a)		7.80. (a)
Cytosine.		1.20. (a).				
D-Cytidine.	1.50. (a)					
D-Ribose.	0.80. (b)					
Ribose.					0.70. (b).	
Uracil.			1.40. (a)			
Cytidine.				2.70. (a)		
CMP(5')						7.70. (a)

Note :

Spot detection as follows :-

(a) U.V-Absorption.

(b) Aniline hydrogen phthalate Spray.

D : Deoxy e.g. D-Cytidine - deoxy-cytidine.

The results indicated that there were four U.V.-absorbing compounds in the 6 N hydrochloric acid hydrolyzate. One of these travelled towards the cathode end of the paper and had the same electrophoretic mobility as the marker, cytosine. The three anionic components possessed the same electrophoretic mobility as uracil, cytidine and CMP(5') in respective order of distance from the origin. The uracil U.V.-absorbing spot was comparatively much weaker in intensity than the rest, and was formed from the deamination of cytosine in the acid medium. Ribose was not detected as it would have been destroyed under the vigorous hydrolytic condition.

4. Phosphorus Estimation.

The sample of Fraction $15A_1$ was prepared in the same way as for the acid hydrolysis from 0.4 ml. of Fraction 15, and electrophoresis was run for 2½ hr. at 10 volts / cm. in sodium acetate-acetic acid buffer mixture (0.2 M), pH 4.0. The U.V.-absorbing band (A_1) was eluted with 7 ml. of water, and aliquots of the eluate was used for the phosphorus estimation and for the determination of its U.V.-absorption. For phosphorus estimation, 2 ml. of Fraction $15A_1$ eluate was added to a mixture of 1 ml. of micro-analytical grade nitric acid and 1 ml. of 10 N sulphuric acid in a micro-Kjeldahl digestion flask. A glass bead was then added to the mixture which was heated on a Kjeldahl rack until all the water had evaporated. When the fuming had ceased, the contents were further heated strongly for another 10 mins. After the flask had cooled to room temperature, 5 ml. of water was added and the contents were hydrolyzed on a boiling water-bath for

45-60 mins. and then made up to a known volume with water for phosphorus determination by King's colorimetric method. The U.V.-absorption of another aliquot of the $15A_1$ eluate was measured at pH 1.0. From the spectrographic data of pure CDP-C, an estimate of the amount in a 2 ml-aliquot of $15A_1$ was made. From this value the theoretical amount of phosphorus was calculated and compared with that obtained by the direct determination.

Calcd. (spectrographic data) : 13.41 µg.

Found (direct determination) : 13.45 µg.

5. Perchloric Acid Hydrolysis.

A sample of Fraction $15A_1$ was prepared in the same way as before from 0.4 ml. of Fraction 15. The colourless residue obtained on evaporation of the $15A_1$ eluate at 40° *in vacuo* was dissolved in 0.4 ml. of water and then hydrolyzed in a boiling water-bath with an equal volume of 70 % perchloric acid. The hydrolyzate, after cooling to room temperature, was brought to pH 4.0 with 0.1 N potassium hydroxide and the resulting solution kept overnight at 0°. The supernatant was carefully pipetted, evaporated, and a sample subjected to paper chromatography in isopropanol : concentrated hydrochloric acid : water (130 : 24 : 46, v/v). After drying the paper in air at room temperature, the U.V.-absorbing spot was marked, cut into small pieces into a 10 ml. serum bottle and eluted with 5 ml. of water. The residue obtained on evaporation of the eluate *in vacuo* at 40° was taken up in 0.3 ml. of water

and then subjected to paper electrophoresis for 1½ hr. at 10 volts / cm. in sodium acetate-acetic acid buffer mixture (0.2 M), pH 4.0, with cytosine as the marker. The perchloric acid hydrolysis was repeated with 2 mg. of an authentic sample of CDP-C.

The free base of Fraction $15A_1$ and that of the authentic sample of CDP-C had the same R_f and electrophoretic mobility as the marker, cytosine.

6. <u>Acid Hydrolysis of other "Ninhydrin -ve" Cytidine Derivatives.</u>

The cytidine-containing components of fractions 13, 14 and 16 (Fractions $13A_1$, $14A_1$ and $16A_1$) were prepared from 1 ml., 0.45 ml., and 0.6 ml. respectively of fractions 13, 14 and 16, and were then hydrolyzed at 100° for 30 mins. in a sealed tube with 0.5 ml. of N hydrochloric acid in each case. The hydrolyzates were chromatographed in identical solvent systems to those employed for the N hydrochloric acid hydrolyzate of Fraction $15A_1$, using CDP-C, CMP(5') and phosphorylcholine as markers.

Table VII. N Hydrochloric Acid Hydrolysis of other "Ninhydrin -ve" Cytidine Derivatives.

Solvent System.	Compound.	ø R_f		
Isopropanol-conc-centrated Hydro-chloric-Water. (170 : 41 : 39, v/v.)	P-Choline.	0.92. (b)		
	CMP(5').		0.71. (a,b)	
	CDP-C.			0.68 (a)
	Fract. 13A_1. Hydrolyzate	0.92. (b)	0.70. (a,b)	
	Fract. 14A_1. Hydrolyzate	0.92. (b)	0.70. (a,b)	
	Fract. 16A_1. Hydrolyzate	0.92. (b)	0.71. (a,b)	

Note :

ø Spot detection as follows :-

(a) U.V.-Absorption.

(b) Hanes-Isherwood Spray.

P-Choline :- Phosphorylcholine.

Table VII. (contd.)

Solvent System.	Compound.	∅ R_f		
Isobutyric acid-Ammonium hydroxi-ide. (0.5 N), 10 : 6, v/v.	P-Choline.	0.45. (b)		
	CDP-C.		0.37. (a)	
	CMP(5').			0.27 (a,b)
	Fract. $13A_1$. Hydrolyzate	0.45. (b)		0.27 (a,b)
	Fract. $14A_1$. Hydrolyzate	0.45. (b)		0.27 (a,b)
	Fract. $16A_1$. Hydrolyzate	0.45. (b)		0.28 (a,b)
Isobutyric acid-Sodium hydroxide-Water. (69 ml. : 0.85 g. : 31 ml.)	P-Choline.	0.49. (b)		
	CDP-C.		0.30. (a)	
	CMP(5').			0.28.(a,b)
	Fract. $13A_1$ Hydrolyzate	0.48. (b)		0.28 (a,b)
	Fract. $14A_1$. Hydrolyzate	0.49. (b)		0.28 (a,b)
	Fract. $16A_1$. Hydrolyzate	0.48. (b)		0.27 (a,b)

(B). "Ninhydrin +ve" Cytidine Derivatives.

(Fractions $8A_1$-$11A_1$)

Specific Investigation of Fraction $8A_1$.

This fraction was taken as representative of the "ninhydrin +ve" cytidine-containing electrophoretic fractions.

1. Acid Hydrolysis.

A sample of Fraction $8A_1$ was prepared from 0.3 ml. of Fraction 8 by the same proceedure as that previously described for Fraction $15A_1$, and was then hydrolyzed at 100° for 30 min. with 0.5 ml. of N hydrochloric acid in a sealed tube. The experiment was repeated using 0.45 ml. of Fraction 8 with the modification that 6 N hydrochloric acid (0.8 ml.) was used for the hydrolysis, which was carried out for 60 hr. at 100°.

2. Paper Chromatography.

The N hydrochloric acid hydrolyzate was chromatographed in three of the same solvent systems as those used for the N hydrochloric acid hydrolyzate of Fraction $15A_1$, viz.

1. isobutyric acid-sodium hydroxide-water. (69 ml. : 0.85 g. : 31 ml.)

2. isobutyric acid-ammonium hydroxide (0.5 N), 10 : 6 (v/v).

3. isopropanol-conc. hydrochloric acid-water. (170 : 41 : 39, v/v).

using CMP(5'), O-phosphorylethanolamine and O-phosphorylserine as markers. For the 6 N hydrochloric acid hydrolyzate, CMP(5'), cytidine, cytosine, O-phosphorylethanolamine, ethanolamine and serine were used as markers in the above solvent systems (1) and (2), and O-phosphorylethanolamine and ethanolamine in the following solvent systems :-

4. ethanol (95 %)-conc. ammonium hydroxide (95 : 5, v/v).
5. ethanol-water. (8 : 1, v/v)
6. 70 % aqueous isopropanol. (v/v)

These last three solvent systems were satisfactory for distinguishing between the ninhydrin reacting markers.

Table VIII. N Hydrochloric Acid Hydrolysis.

Solvent System.	Compound.	ø R_f.		
Isopropanol-conc. hydrochloric acid -water. (170 : 41 : 39, v/v)	O-phosphorylethanolamine.	0.65. (c)		
	O-phosphorylserine.		0.56. (c)	
	CMP(5').			0.56 (a,b)
	Fraction $8A_1$. Hydrolyzate	0.65. (c)		0.56 (a,b)

Note :

ø Spot detection as follows :-

(a) U.V.-Absorption.

(b) Hanes-Isherwood

Table VIII. (contd.)

Note :

ϕ Spot detection as follows :-

(c) Ninhydrin.

Solvent System.	Compound.	ϕ R_f.		
Isobutyric acid-Ammonium hydroxide. (0.5 N), 10 : 6, v/v.	O-phosphoryl-ethanolamine.	0.31. (c)		
	O-phosphoryl-serine.			0.14 (c)
	CMP(5').		0.30. (a,b)	
	Fraction $8A_1$. Hydrolyzate	0.30. (c)	0.30. (a,b)	
Isobutyric acid-Sodium hydroxide-Water. (69 ml. : 0.85 g. : 31 ml.)	O-phosphoryl-ethanolamine.	0.39. (c)		
	O-phosphoryl-serine.			0.18 (c)
	CMP(5').		0.35. (a,b)	
	Fraction $8A_1$. Hydrolyzate.	0.39. (c)	0.34. (a,b)	

<u>Table IX.</u> 6 N Hydrochloric Acid Hydrolysis.

Solvent System.	Compound.	ϕ R_f.				
Isobutyric acid-Ammonium hydroxide (0.5 N), 10 : 6, v/v.	Cytosine.	0.71. (a)				
	Cytidine.		0.57. (a)			
	CMP(5').			0.31. (a)		
	O-phosphoryl-ethanolamine.					0.31 (b)
	Ethanolamine.				0.72. (b)	
	Serine.					0.39 (b)
	Fraction $8A_1$. Hydrolyzate[1]	0.71. (a)	0.57. (a)	0.31. (a)	0.72. (b)	0.31 (b)

Note :

ϕ Spot detection as follows :-

(a) U.V-Absorption.

(b) Ninhydrin Spray.

Table IX. (contd.)

Solvent System.	Compound.	∅ R_f.				
Isobutyric acid-Sodium hydroxide-Water. (69 ml. : 0.85 g. : 31 ml.)	Cytosine.	0.75. (a)				
	Cytidine.		0.61. (a)			
	CMP(5').			0.35. (a)		
	O-phosphoryl-ethanolamine					0.39 (b)
	Ethanolamine.				0.74. (b)	
	Serine.					0.46 (b)
	Fraction $8A_1$. Hydrolyzate.	0.76. (a)	0.61. (a)	0.36. (a)	0.74. (b)	0.39 (b)
Ethanol ; Water. (8 : 1, v/v)	O-phosphoryl-ethanolamine				0.59 (b)	
	Ethanolamine.					0.12 (b)
	Fraction $8A_1$. Hydrolyzate.				0.59 (b)	0.12 (b)
70 % aqueous isopropanol (v/v)	O-phosphoryl-ethanolamine.					0.42 (b)
	Ethanolamine.				0.69. (b)	
	Fraction $8A_1$. Hydrolyzate.				0.69. (b)	0.43 (b)

Note :

∅ Spot detection as follows :-

(A) U.V.-Absorption.

(b) Ninhydrin Spray.

Table IX. (contd.)

Solvent System.	Compound	ϕ R_f.	
Ethanol (95 %) : conc. Ammonium hydroxide. (95 : 5, v/v.)	O-phosphoryl-ethanolamine	0.00 (b).	
	Ethanolamine.		0.55 (b).
	Fraction $8A_1$. Hydrolyzate[1]	0.00 (b)	0.55 (b).

3. Paper Electrophoresis.

The 6 N hydrochloric acid hydrolyzate was subjected to paper electrophoresis in borax-sodium hydroxide buffer, (0.05 M), pH 10.4 for 2 hr. at 7.5 volts / cm. using CMP(5'), cytidine, cytosine, ribose and deoxy-ribose as markers.

The results indicated that there were three U.V-absorbing compounds in the 6 N hydrochloric acid hydrolyzate. One of these travelled towards the cathode end of the paper and had the same electrophoretic mobility as the marker, cytosine. The two anionic components possessed the same electrophoretic mobilities as cytidine and CMP(5') in respective order of distance from the origin. This illustrated that the sugar component of the cytidine derivative was ribose.

REFERENCES.

1. Toennies, G., and Lavine, J. F., J. Biol. Chem., 113, 571 (1936).

2. Curtis, D. R., and Watkins, J. C., J. Physiol., 166, 1 (1963).

3. Toennies, G., and Kolb, J. J., Anal. Chem., 23, 823 (1951).

4. "Organic Synthesis", Coll. Vol. I, 2nd. Edition, Gilman, H., and Blatt, A. H., Eds., John Wiley and Sons. New York, N. Y., 1948., p. 431.

5. Watkins, J. C., (to be published in 1965).

6. Arndt, F., "Organic Synthesis", Coll. Vol. II, Edited by Blatt, A. H., John Wiley and Sons, New York, 1944., p. 165.

7. Hanes, C. S., and Isherwood, F. A., Nature, 164, 1107 (1949).

8. Sigma Chemical Co. Bulletin, 106.

9. Clark, W. M., J. Biol. Chem., 25, 479 (1916).

10. Neuhaus, F. C., and Korkes, S., Biochem. Prep., 6, 75 (1958).

11. Zu, Y. K., and Wilson, W., Brit. J. Pharmacol. and Chemotherapy, 12, 38 (1957).

12. Bogert, M. T., and Slocum, E. M., J. Am. Chem. Soc., 46, 766 (1924).

13. Zu, Y. K., and Wilson, W., Brit. J. Pharmacol. and Chemotherapy, 12, 38 (1957).

14. Miani, N., Atti Soc. med.-chir., Padova, 34, 3 (1956-1957).

15. Kennedy, E. P., and Weiss, S. B., J. Biol. Chem., 222, 193 (1956).

16. Kennedy, E. P., and Sribeny, J. Biol. Chem., 233, 1315 (1958).

17. Bremer, J., and Greenberg, D. M., Biochim. Biophys. Acta., 35, 287 (1959).

18. Crowder, M., and Artom, C., Fed. Proc., 11, 199 (1952).

19. Nyc, J. F., and Hall, M. O., J. Am. Chem. Soc., 81, 2275 (1959).

20. Nyc, J. F., and Wolf, B., J. Biol. Chem., 234, 1068 (1959).

21. Baddiley, J., Buchanan, J. G., Mathias, A. P., and Sanderson, A. R., Biochem. J., 64, 599 (1956).

22. Baddiley, J., Buchanan, J. G., Carss, B., and Mathias, A. P., Biochim. Biophys. Acta., 21, 191 (1956).

23. Kiysau, T. Y., and Kennedy, E. P., J. Biol. Chem., 235, 2590 (1960).

24. Huang, C., Wheeldon, L., and Thompson, T. E., J. Molec. Biol., 8, 148 (1964).

25. Mueller, P., Rudin, D.O., Ti Tien, H., and Wiescolt, W. C., Circulation., 26, 1167 (1962).

26. Ansell, G. B., and Bayliss, B. J., Biochem. J., 78, 209 (1961).

27. Kennedy, E. P., and Weiss, S. B., J. Biol. Chem., 222, 193 (1956).
(cf Biochem. J., 78, 210 (1961).)

28. Lunt, M. R., and Kent, P. W., Biochem. J., 78, 128 (1961).

29. Walpole, G. S., J. Chem. Soc., 105, 2501 (1914).

30. Hanes, C. S., and Isherwood, F. A., Nature, 164, 1107 (1949).

31. Malyoth, G., and Stein, H. W., Biochemische Zeitschrift., 322, 165 (1951).

32. Levene, P. A., and Bass, L. W., "Nucleic Acids", ACS Monograph Series. The Chemical Catalogue Co., New York, p. 57 (1931).

33. Isbell, H. S., Brewster, J. F., Holt, N. B., and Frush, H. L., J. Research. Natl. Bur. Standards, 40, 129 (1948).

34. "Methods in Enzymology", Vol. I., Academic Press. Inc., New York (1955), p. 146., after Clark, W. M., and Lubs, H. A., J. Bacteriol., 2, 1 (1917).

35. King, E. J., Biochem. J., 26, 292 (1932).