99. *Investigation of Polymorphism and Isomerism in Cytidine Phosphates.*

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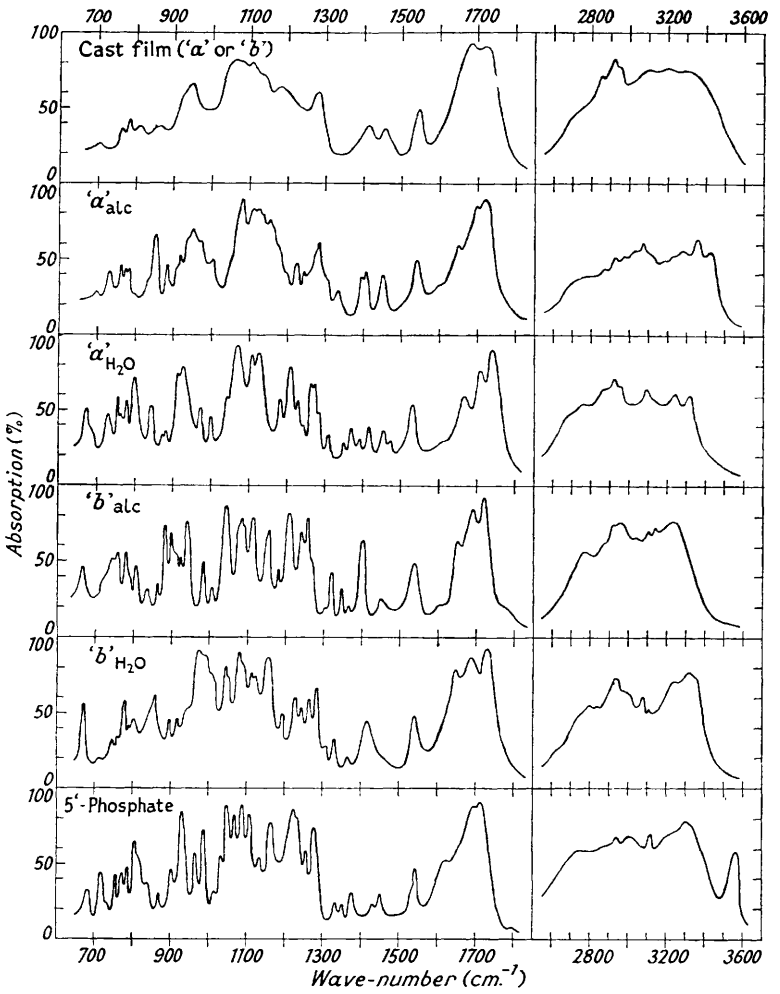
Isolation of two isomeric cytidine phosphates from the products of hydrolysis of yeast nucleic acid is described. Each isomer has been obtained in an amorphous form and in two stable crystalline forms, and these have been characterised by their infra-red spectra. The spectra are discussed in relation to their use in analysis of mixtures of the isomers, and a quantitative method for such analysis in the solid state is presented. Ultra-violet absorption data for the two isomers are presented and the structures of the isomers are discussed.

Acid hydrolysis of yeast pentosenucleic acid, followed by removal of guanine, free acid, and free phosphate, affords a mixture of the pyrimidine nucleotides. The cytosine nucleotides may be separated as pyridine salts, leaving uracil nucleotides in solution. By ion-exchange chromatography, Cohn (*J. Amer. Chem. Soc.*, 1950, **72**, 2811) and Loring *et al.* (*ibid.*, p. 2811; 1951, **73**, 4215; *J. Biol. Chem.*, 1952, **196**, 807) obtained two isomeric

cytidylic acids, designated " a " and " b," from hydrolysates of pentosenucleic acids by various methods. The two cytosine nucleotides, obtained as before from an acid hydrolysate of yeast nucleic acid through their insoluble pyridine salts, have now readily been separated. Cytidylic acid " b " yields an insoluble dibrucine salt, and leaves a brucine-containing mother-liquor from which pure cytidylic acid " a " is obtained. By first separating the cytosine nucleotides from those of uracil, the isomers are separated rather than merely successively isolated by two different procedures, and further the

Plot of percentage absorption against frequency in cm.$^{-1}$ for the isomeric cytidylic acids.



uracil nucleotides are recovered free from either cytidylic acid isomer and may then themselves be obtained pure.

The progress of this separation can be followed by determination of optical rotation (Loring, *loc. cit.*) or of ultra-violet absorption spectra, especially the ratio of the optical density at 280 mμ to that at 260 mμ (Loring, Cohn, *locc. cit.*). Infra-red spectral measurements have been used in the present work; these had to be made on the solid material and were complicated by differences of physical form in the same chemical entity. Each isomer has been obtained (see Experimental section) in an amorphous form and in two crystalline forms (from aqueous alcohol or water); the latter are referred to as " a "$_{alc}$ or " a "$_{H_2O}$, etc. None of the forms contains water of crystallisation, and the two crystalline forms of each isomer are not, in general, interconvertible except through recrystallisation.

On one occasion, however, the " a " isomer was obtained from water in a metastable crystalline form, which changed into the usual " a "$_{H_2O}$ form in a few days; its spectrum is not reproduced in view of its transient nature and production on only one occasion.

The spectra are presented in the Figure; that of cytidine-5′ phosphate is of a sample kindly supplied by Dr. W. E. Cohn, who also supplied samples of his " a " and " b " isomers for comparison. Only in the case of the adenosine phosphates have the infra-red spectra been published (Brown and Todd, *J.*, 1952, 44) of nucleotides definitely known to be separated isomers. The spectrum of cytidylic acid has been reported by Blout and Fields (*J. Biol. Chem.*, 1949, **178**, 335) and by Clark (Thesis, Columbia, 1950) although in both cases the resolution was small: both spectra were obtained on samples from the Schwartz laboratories, yet are markedly different; comparison with our spectra has shown the difference to be due to crystal form, both materials being predominantly the " b " isomer.

In spite of the polymorphism, there is no difficulty in recognising the spectra or in obtaining an estimate of the amount of each isomer present in a mixture. For recognition of the forms present, the detail available in the infra-red spectrum is more characteristic than any other single property, and it suffices to examine only a small region of the spectrum, that from 1200 to 1350 cm.$^{-1}$ being the most sensitive. Furthermore, as each isomer is easily obtained in a single crystal form by crystallisation under constant conditions, the analysis reduces to that of one of four possible two-component mixtures, and is easily carried out as described below. Finally, extraneous impurities can easily be detected and allowed for, so that even in their presence the isomeric composition can be estimated.

In the analysis of solid mixtures, two difficulties appear. First, the films scatter as well as absorb the incident energy and, secondly, it is difficult to measure the amount of material absorbing. The former was overcome by measuring differences in optical density at an absorption maximum and a neighbouring minimum (Pirlot, *Bull. Soc. chim. Belg.*, 1949, **58**, 28); the amount of scatter will not affect this difference, as the percentage scatter will be approximately equal at the two positions, owing to their proximity. The second difficulty was overcome by measuring the ratio of two such differences, chosen so that the value is large for one component of the mixture and small for the other. The relation between the value of this ratio and the composition of the mixture may be obtained empirically, but is easily calculated from the value of the ratio for three different samples; the calculation and the measurements are simplified by taking for these the two pure components and a 1 : 1 mixture.

Let the extinctions of the two components, a and b, for unit thickness, be a_1 and b_1 at one frequency and a_2 and b_2 at a second. If r_a, r_b, and $r_{\frac{1}{2}}$ are the values of the ratio of optical density at the first position to that at the second, then $r_a = a_1/a_2$, $r_b = b_1/b_2$, and $r_{\frac{1}{2}} = (a_1 + b_1)/(a_2 + b_2)$, irrespective of thickness, provided that Beer's law holds.

Similarly, for a mixture of x parts of a to $(1 - x)$ parts b

$$r_x = \frac{xa_1 + (1 - x)b_1}{xa_2 + (1 - x)b_2}, \text{ whence } x = \frac{p(r_b - r_x)}{p(r_b - r_x) + (r_x - r_a)}$$

where $p = b_2/a_2 = (r_a - r_{\frac{1}{2}})/(r_{\frac{1}{2}} - r_b)$. The composition is therefore determined by the values of r_x, r_a, r_b, and $r_{\frac{1}{2}}$. Provided all the densities measured are below about 0·7 the assumption of Beer's law is valid, as the band-width of the band used is large compared with the resolution (Philpotts, Thain, and Smith, *Analyt. Chem.*, 1951, **23**, 268). Instead of optical densities at a single frequency, differences in optical densities, used for the reasons given above, can be used.

From their infra-red spectra, Dr. Cohn's samples of cytidylic acid " a " and " b " were found to be in the form " a "$_{H_2O}$ and " b "$_{alc}$ respectively. In order to correlate the nature of our samples with those of Loring, optical rotation figures were obtained. The " a " isomer gave $[\alpha]_D^{20}$ +19·8° (c, 0·95 in H_2O) and the " b " isomer $[\alpha]_D^{20}$ +47·2° (c, 0·97 in H_2O), with a probable error in each case of ±1·0°. These figures agree well with those of Loring (1951, *loc. cit.*), who also reported some crystal data; both isomers were quoted as having oblique extinction, being therefore biaxial, but only two refractive indices were given for

each, and these were noted to be " for crystals obtained from water or aqueous alcohol." We have not attempted to carry out an exhaustive determination of the crystal data but have measured extinction angles and found straight extinction in all cases, with the possible exception of the " a "$_{H_2O}$ form, which shows a value not greater than 2° (the error of measurement being about $\pm 1°$). The alcohol forms crystallised in needles and the water forms in plates—the " b "$_{H_2O}$ form has readily given flat crystals about 2 × 4 mm.

The ultra-violet absorption spectra of cytidylic acid isomers and mixtures have been measured at various stages during chemical separation in 0·01N-HCl, 0·01N-NaOH, and in 0·05M-phosphate buffer of pH 7·0. The complete data for the " a " and " b " isomers are given in the Table.

Loring *et al.* (1952, *loc. cit.*, Fig. 1) reported the first maximum for each isomer, in acid solution, and noted its higher extinction value in the spectrum of the " b " isomer. Our repeated measurements have confirmed that this difference, together with the small wave-length differences at maxima and minima, are significant. The additional data (see Table) for alkaline and pH 7·0 buffer solutions, including that for the hitherto unreported band at low wave-lengths, further distinguish the two sets of spectra. The short-wave-length band is broader and differences between the isomers are less clearly defined in alkali and phosphate than in acid solutions, and the data for alkali and phosphate are in parentheses in the Table. Measurement of the spectrum of Cohn's cytidine-5′ phosphate shows its resemblance to that of the " b " rather than that of the " a " isomer, with a further 5—10 Å shift to longer wave-lengths for the first maximum in the 5′-phosphate, at each pH value. Its $\varepsilon_{280}/\varepsilon_{260}$ value (0·01N-HCl) is 2·1, compared with 1·99 and 1·83 for the " b " and " a " isomer respectively.

Ultra-violet absorption data.

		Cytidylic acid " a "			Cytidylic acid " b "		
		0·01N-HCl	0·01N-NaOH	Buffer pH 7·00	0·01N-HCl	0·01N-NaOH	Buffer pH 7·00
1st Max.	mμ	278	270	270	279·5	270·5	271
	ε	12,740	8,890	8,860	13,400	9,350	9,400
Min.	mμ	240·5	251·5	250·5	241	250	250
	ε	2,030	6,980	6,910	1,750	6,750	6,840
2nd Max.	mμ	211·5	(232)	(232)	212·5	(231)	(230)
	ε	10,520	(8,270)	(8,140)	10,150	(8,300)	(8,330)

The hydrolysis of the " a " and " b " cytidylic acids to cytidine (Loring *et al.*, *J. Biol. Chem.*, 1952, **196**, 821) is consistent with their formulation as cytidine phosphates; both resist oxidation by periodate and therefore cannot have the phosphate in the 5′-position. This leaves the 2′- and 3′-positions as possibilities, and Loring *et al.* (1952, *loc. cit.*, p. 827) have suggested tentatively a 2′-phosphate structure for the " a " isomer on the basis of its lower solubility, acidity, and ultra-violet absorption maximum. They suggest that these physico-chemical differences would result from a greater tendency to zwitter-ion formation, deduced from examination of an atom model of the 2′-isomer.

The progressive long-wave-length shift from the " a " to the " b " to the 5′-phosphate spectrum observed in the present investigation, taken together with evidence of the spectroscopic effects of hindrance to resonance interaction between the phenyl and pyrimidine rings in phenylpyrimidine derivatives (Maggiolo and Russell, *J.*, 1951, 3297), provides some support for these conclusions.

Experimental

A mixture of pyrimidine nucleotides prepared by acid hydrolysis of yeast ribonucleic acid (50 g.) (Pharmaco-Chemical Products Ltd.) by the method of Barker *et al.* (*J.*, 1949, 904) was separated into insoluble pyridine cytidylates and soluble pyridine uridylates by extraction with ice-cold pyridine.

Cytidylic Acid Fraction.—The pyridine cytidylate was dissolved in water (300 ml.) and evaporated to a syrup (100 ml.) under reduced pressure. Brucine (30 g.) in hot ethanol (30 ml.)

was then added. Separation of crystalline dibrucine cytidylate " b " began in a few minutes and was complete after 60 hours at 0°. The salt was washed with a little water, dried *in vacuo* (28 g.), and recrystallised from 35% aqueous alcohol (225 ml.). 24 G. were obtained.

Cytidylic Acid " b."—Dibrucine cytidylate " b," suspended in water (20 pts. by wt.), was heated to 85°. Ammonia solution (*d* 0·880) was then added (1 ml. per g. of salt). The solution was cooled to room temperature and free brucine removed by filtration. This precipitate was re-extracted with ammonia in the same way. The combined solutions were extracted with chloroform until free from brucine and evaporated *in vacuo* to low volume (*ca.* 2 ml. per g. of original salt). Diammonium cytidylate " b " was not isolated and the solution was used for the next stage.

To the solution of diammonium salt, sufficient 25% lead acetate solution was added to ensure complete precipitation. The lead cytidylate was collected by centrifugation and the deposit washed twice with water. The salt was resuspended in water and decomposed with hydrogen sulphide. The lead sulphide was removed and washed. The combined filtrates, freed from hydrogen sulphide by filtration, were concentrated *in vacuo*. Crystalline cytidylic acid " b " separated. 450 Mg. of this was recrystallised by dissolving it in 19 ml. of boiling water and adding 20 ml. of boiling ethanol: this gave 330 mg. of cytidylic acid " b," decomp. 232—233° (determined with an electrically heated m. p. apparatus, 1-mm. sealed tube put in the block at 230°; heating of 2° per minute). The nucleotide showed $[\alpha]_D^{20}$ +47·2° (*c*, 0·97 in H_2O) (Found, in anhyd. material: N, 13·15; P, 9·8. Calc. for $C_9H_{14}O_8N_3P$: N, 13·0; P, 9·6%).

Cytidylic Acid " a."—The filtrate (150 ml.) from dibrucine cytidylate " b " was concentrated *in vacuo* to 50 ml. The brucine was removed by addition of ammonia, followed by extraction with chloroform, and diammonium cytidylate " a " converted into the free nucleotide as described above. 250 Mg. of this cytidylic acid " a " gave 160 mg. after recrystallisation from 33% ethanol. The nucleotide melted at 235—236° (decomp.) (conditions described above), and showed $[\alpha]_D^{20}$ +19·8° (*c*, 0·95 in H_2O) (Found, in anhyd. material: N, 12·8; P, 9·95%).

Production of the Polymorphic Forms.—The forms described above are respectively " b "$_{alc}$ and " a "$_{alc}$. The amorphous forms were prepared by drying aqueous solutions rapidly over P_2O_5 *in vacuo*. To obtain the $_{H_2O}$-forms, saturated solutions of the respective isomers were prepared in distilled water, *viz.*, about 1% for the " a " and about 3% for the " b " isomer. The solution was then filtered and set aside on a watch-glass; a small amount of crust was formed round the edges and simultaneously crystals began to form in the solution. When the volume of solution had been reduced to about a quarter, the liquid was poured off and filtered and the resulting crystals were washed with aqueous alcohol, then absolute alcohol. Examination of the crust showed it to be in the $_{alc}$-form; two processes were therefore taking place simultaneously, true crystallisation depositng the $_{H_2O}$-forms from the body of the solution and evaporation of water leaving the $_{alc}$-forms at its edge. This suggests that the commencement of crystallisation is easier for the $_{alc}$-forms, the $_{H_2O}$-forms only being produced if sufficient time is allowed for crystallisation centres to arise.

The forms obtained from mixtures, by crystallisation from aqueous alcohol, depend partly on the composition. The less soluble " a " isomer always crystallises in the " a "$_{alc}$ form, but the more soluble " b " isomer can crystallise in either form, the " b "$_{alc}$ form being favoured by a preponderance of the " b " isomer in the mixture or by the addition of excess of alcohol; the two " b " forms have not been found together in a mixture.

Optical-rotation Determinations.—These were made on a Schmidt and Haensch polarimeter, fitted with a three-prism polariser and with a vernier scale reading directly to 0·01°. The half shadow angle was adjusted to 5° and the standard deviation for one position was 0·007°. For each determination the blank and the solution were each measured ten times to give a probable error of the mean of 0·01°. In each case, the cell was 1-cm. long.

Infra-red Data.—These were obtained on a Perkin–Elmer 12C spectrometer fitted with 13-cycle amplifier. The spectra from 3600 to 2500 cm.$^{-1}$ were obtained with a lithium fluoride prism on mulls in a fully fluorinated oil; from 1900 to 650 cm.$^{-1}$, a rock salt prism was used on mulls in liquid paraffin, except from 1500 to 1330 cm.$^{-1}$ where the " fluorocarbon " mull was used. Scatter in the films was generally small and was allowed for by placing a treated plate of rock salt in the radiation path when recording the incident energy; this plate was ground with emery paper to give the same transmission as the film at 2000 cm.$^{-1}$, where no absorption bands are found. The accuracy of band position, expressed as wave-length deviation, remains approximately constant over the entire region at about $\pm$0·01 μ.

Infra-red Analytical Data.—In the application of the method described above, the frequencies at which the optical densities are measured are chosen so that the value of the

optical-density ratio function is as different as possible for the two compounds. The best ratios to use are :

$$\text{"a"}_{alc} + \text{"b"}_{alc}: \frac{d \text{ at } 854 - d \text{ at } 820}{d \text{ at } 806 - d \text{ at } 820} \text{ or } \frac{d \text{ at } 854 - d \text{ at } 820}{d \text{ at } 1043 - d \text{ at } 1015}$$

$$\text{"a"}_{alc} + \text{"b"}_{H_2O}: \frac{d \text{ at } 854 - d \text{ at } 805}{d \text{ at } 1044 - d \text{ at } 1028}$$

$$\text{"a"}_{H_2O} + \text{"b"}_{alc}: \frac{d \text{ at } 929 - d \text{ at } 891}{d \text{ at } 1155 - d \text{ at } 1172}$$

$$\text{"a"}_{H_2O} + \text{"b"}_{H_2O}: \frac{d \text{ at } 929 - d \text{ at } 897}{d \text{ at } 1156 - d \text{ at } 1182}$$

In each case the first frequency refers to the position of a strong band of one component where the second component absorbs only weakly; that in the numerator is for the " a " component and that in the denominator for " b," so that the ratio is large for " a," and small for " b." The second frequency refers to the position of a minimum of the strongly absorbing material, the exact position being chosen to reduce the interference of the other isomer.

Ultra-violet Absorption Data.—Hilger Uvispek and Beckman DU instruments were used. The wave-length scales had been adjusted and calibrated by means of a mercury arc and gave deviations of 0—2 Å over the region of measurement. Concentrations and cell-lengths were adjusted to give optical densities within the range 0·2—0·8, for maximum accuracy of determination of extinction coefficients.

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