

67. The Constitution of Yeast Ribonucleic Acid. Part XI. Synthesis of Uridine-2' Phosphate.

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Uridine-2' phosphate has been synthesised by procedures which define clearly the position of the phosphoryl group. It follows, therefore, that uridylic and cytidylic acids are 3'-phospho-derivatives of the nucleosides, as postulated hitherto without proof. The stabilities of uridine-2' phosphate and uridylic acid towards alkali are closely similar, and thus give rise to the view that the facile alkaline fission of yeast ribonucleic acid is not due to a marked and inherent lability of the ester linkage in position 2', but is a property of the polynucleotide. The significance of these observations is discussed.

It was postulated by Levene and Tipson (*J. Biol. Chem.*, 1935, **109**, 623) that phospho-ester groups at C2' of the ribonucleosides would be labile, and that the presence of these linkages in ribonucleic acid would account for the ease with which the polynucleotide suffers hydrolysis in cold dilute alkaline solution. This postulate has been accepted by others (Makino, *Z. physiol. Chem.*, 1935, **236**, 201; Bredereck, *Fortschr. Chem. Org. Naturstoffe*, 1938, **1**, 152). No nucleoside-2' phosphate however has, as yet, either been identified among the fission products of yeast ribonucleic acid or been prepared synthetically; the behaviour of such compounds towards hydrolytic agents has therefore been a matter of conjecture, although Gulland and Walsh (*J.*, 1945, 172) have pointed out that there is no record of substituents in the hydroxyls of carbohydrates differing so markedly in stability as to explain the complete fission of an ester at C2' whilst a similar linkage at C3' remains unattacked.

The sugar of uridine was shown to be in furanose form by Levene and Tipson (*J. Biol. Chem.*, 1933, **101**, 529), and the same authors prepared and characterised uridine-5' phosphate by phosphorylating its 2' : 3'-isopropylidene derivative (*ibid.*, 1934, **106**, 113).

Uridylic acid, obtained by alkaline fission of yeast ribonucleic acid, is different from uridine-5' phosphate and is therefore uridine-2' or -3' phosphate. By analogy with the purine nucleotides, in which the phosphoryl group was proved to esterify the hydroxyl at C3' (Levene and Harris, *ibid.*, 1932, **98**, 9; Levene, Harris, and Stiller, *ibid.*, 1934, **105**, 153), it has been generally assumed without proof that uridylic acid, and hence cytidylic acid, which can be converted into uridylic acid by deamination (Bredereck, *Z. physiol. Chem.*, 1934, **224**, 79), are 3'-phospho-esters of the corresponding nucleosides. Uridylic acid was synthesised by phosphorylation of uridine in barium hydroxide solution (Gulland and Hobday, *J.*, 1940, 746) and of 5'-trityl uridine (Bredereck and Berger, *Ber.*, 1940, **73**, 1124), but these syntheses shed no light on the choice between positions 2' and 3'. Through the synthesis of uridine-2' phosphate now described, and the demonstration that this nucleotide is different from uridylic acid, it is proved that uridylic and cytidylic acids are 3'-phospho-esters of the nucleosides.

3' : 5'-Benzylidene uridine (I, R = H) was prepared in good yield by condensation of uridine with benzaldehyde in the presence of zinc chloride. The allocation of the benzylidene group to these positions is deduced as follows. It is known (Haworth and Hirst, *Ann. Rev. Biochem.*, 1936, **5**, 83) that benzaldehyde condenses readily with alternate, but rarely with adjacent, hydroxyl groups of a sugar, and on stereochemical considerations condensation will not occur with hydroxyl groups at C2' and C5' of the *d*-ribose residue. The remote possibility that the sugar might have changed from the furanose to the pyranose form during the condensation in presence of zinc chloride was ruled out by recovery of uridine from benzylidene uridine after hydrolysis with dilute acid; the presence of the furanose ring in the recovered and the original uridine was confirmed by periodate titrations (Lythgoe and Todd, *J.*, 1944, 592). If, after phosphorylation of benzylidene uridine and removal of the benzylidene radical, a nucleotide were obtained differing from uridine-5' phosphate and from uridylic acid, that nucleotide would be a 2'-phospho-ester, benzylidene uridine would be the 3' : 5'-derivative, and uridylic acid would be a 3'-phospho-ester. The differences expected were realised in fact, and these conclusions are therefore valid.

Phosphorylation of benzylidene uridine with diphenyl chlorophosphonate* in anhydrous pyridine gave a good yield of 3' : 5'-benzylidene uridine-2' diphenyl phosphate [I, R = (PhO)₂PO]. Hydrolysis of this substance with *N*/2-sodium hydroxide solution at 100° for 30 minutes yielded benzylidene uridine phenyl phosphate, which was converted directly by hydrolysis with hot

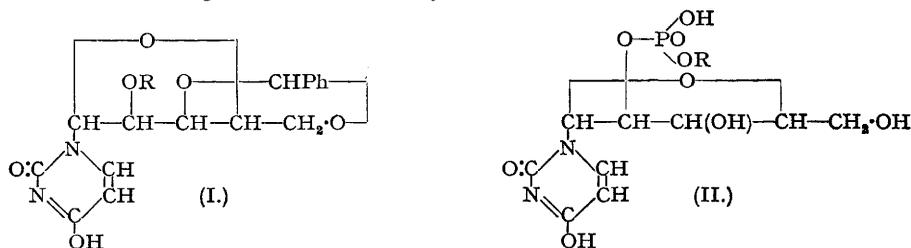
* The term diphenyl chlorophosphonate is here used in place of diphenylphosphoryl chloride used previously (*J.*, 1940, 746) in view of a recent decision that PO(OH)₂Cl should be called chlorophosphonic acid by analogy with corresponding compounds of sulphur and other elements.

$\text{N}/4$ -sulphuric acid into *uridine-2' phenyl phosphate* (II, R = Ph). This was characterised as the *brucine* salt and was further proved to be a di-ester by electrometric titration and estimation of one molecular equivalent of phenol after fission with hot 10N -sodium hydroxide. This graded hydrolysis of $3' : 5'$ -benzylidene uridine-2' diphenyl phosphate is in contrast with the complete dephenylation of diphenylphosphoryl derivatives of uridine, cytidine, and adenosine through the action of $\text{N}/2$ -sodium hydroxide for 30 minutes as recorded by Bredereck, Berger, and Ehrenberg (*Ber.*, 1940, **73**, 269) and Bredereck and Berger (*ibid.*, p. 1124).

Determinations of free phenol and inorganic phosphate during hydrolysis of $3' : 5'$ -benzylidene uridine-2' diphenyl phosphate with N -sodium hydroxide at 100° showed that the second phenyl group was liberated at the same rate as dephosphorylation and formation of inorganic phosphate. Hydrogenolysis with platinum oxide and hydrogen at room temperature and pressure readily removed the residual phenyl radical from uridine-2' phenyl phosphate; in these mild conditions reduction of the uracil radical did not occur (cf. Levene and La Forge, *Ber.*, 1912, **45**, 608; Levene and Jorpes, *J. Biol. Chem.*, 1929, **81**, 579; Levene and Tipson, *ibid.*, 1934, **106**, 113; Brown and Johnson, *J. Amer. Chem. Soc.*, 1923, **45**, 2702).

Uridine-2' phosphate (II, R = H) was thus obtained in good yield and characterised as the *barium* and *brucine* salts. It was shown to be different from uridylic acid and from uridine-5' phosphate by comparisons of the acid hydrolysis curves of the three nucleotides, of the conditions of precipitation of their lead salts, of the solubilities of their barium salts, and of the crystalline forms, solubilities, melting points, and rotations of their brucine salts.

Uridine-2' phosphate was stable for 3 days in 1% sodium hydroxide solution at room temperature, and comparison of its alkaline hydrolysis curve with that of uridylic acid showed that the phosphoryl group of the synthetic acid was only slightly more labile than that of the natural nucleotide. The difference, as such, was totally inadequate to account for the supposed complete fission of the ester linkage at C2' and stability of that at C3'.



In the light of this communication, the instability of the internucleotide linkage is a characteristic of the polynucleotide structure, and not an inherent property of the ribose-2' phospho-ester group as postulated by Levene and Tipson. Two possibilities must be considered. Either the alkaline fission of yeast ribonucleic acid leads solely, as hitherto postulated, to the formation of 3'-phospho-nucleosides, or it produces a mixture of nucleosides phosphorylated at C3' and also at C2' (or C5').

As regards the former alternative, some factor must operate to promote the instability of the phosphoryl group which is not at C3'. An unorthodox speculation as to the nature of this factor is that the sugar radicals of the polynucleotide are not in the furanose form as found in the nucleosides, but are 1 : 2-glycosides doubly esterified by phosphoryl groups at C3' and C4', the lability of this ring as compared with the stability of the furanose form providing a driving force which results in the extrusion of the phosphoryl at C4'.

In the case of the latter alternative, it may be recalled that yields of 3'-phospho-nucleosides isolated from the fission of yeast ribonucleic acid have always fallen short of the theoretical. The triply esterified phosphoryl groups of the polynucleotide (Fletcher, Gulland, and Jordan, *J.*, 1944, **33**) could be expected to exhibit increased alkali lability as compared with the phosphoryl groups of mononucleotides by analogy with other phosphoryl tri-esters (Lossen and Kohler, *Annalen*, 1891, **262**, 209; Drushel, *Amer. J. Sci.*, 1915, **40**, 643; Drushel and Felty, *ibid.*, 1917, **43**, 57; Autenrieth, *Ber.*, 1897, **30**, 2369; Cavalier, *Compt. rend.*, 1898, **127**, 114; Plimmer and Burch, *J.*, 1929, 279); the behaviour of $3' : 5'$ -benzylidene uridine-2' diphenyl phosphate on hydrolysis conforms to the general pattern. Phosphoryl di-esters, however, are more stable towards alkaline hydrolysis, but it is relevant to refer to the quantitative conversion of $\alpha\beta$ -diglycerol monophosphate into α - and β -monoglycerol phosphoric acids, without liberation of inorganic phosphate, as a result of hydrolysis by one molar proportion of sodium hydroxide

in dilute solution (Bailly and Gaumé, *Bull. Soc. chim.*, 1926, **39**, 1420; *Compt. rend.*, 1926, **183**, 67). It is possible that the lability of a phosphoryl ester towards alkali might be increased by the presence of a second phosphoryl ester at the adjacent carbon atom.

The syntheses of other 2'-phospho-nucleosides will be reported shortly, and the alternatives outlined above are being investigated.

EXPERIMENTAL.

Uridine, colourless needles, m. p. 164—165°, $[\alpha]_D^{20} + 4.8^\circ$, was prepared from yeast ribonucleic acid by combining the procedures of Bredereck (*Ber.*, 1941, **74**, 694) and of Gulland and Hobday (*J.*, 1940, 746).

3': 5'-Benzylidene Uridine.—A mixture of uridine (10 g.), dried at 100° over phosphoric oxide in a vacuum for 4 hours, zinc chloride (15 g.), and freshly distilled benzaldehyde (50 c.c.) was shaken at room temperature for 12 hours. After standing for 48 hours, the clear brown solution was poured into ether (500 c.c.) in a separating funnel and zinc chloride was extracted by successive small quantities of water. After the ethereal suspension had remained at 0° for 3 hours, crude *benzylidene uridine* (11.3 g.) was collected, washed with ether, and crystallised repeatedly from water (65 parts), from which it (8.4 g.) separated in colourless needles, m. p. 189—190° (Found, in anhydrous material: C, 57.8; H, 4.9; N, 8.5. $C_{16}H_{18}O_6N_2$ requires C, 57.8; H, 4.9; N, 8.4%).

Uridine was regenerated by hydrolysis of benzylidene uridine (1 g.) at 100° for $\frac{3}{4}$ hour with *N*/4-sulphuric acid (100 c.c.). After removal of the benzaldehyde by extraction with ether, and of the sulphuric acid by means of baryta, the solution was evaporated to a syrup which was dissolved in hot absolute alcohol (10 c.c.). On cooling, uridine crystallised in colourless needles, m. p. 164—165°, $[\alpha]_D^{20} + 4.6^\circ$; a mixed m. p. with authentic uridine was not depressed. This material absorbed 0.98 mol., and the original uridine 0.99 mol., of sodium metaperiodate per mol. No formic acid was liberated in either case.

3': 5'-Benzylidene Uridine-2' Diphenyl Phosphate.—A solution of freshly distilled diphenyl chlorophosphate (2.75 c.c.) (Brigl and Müller, *Ber.*, 1939, **72**, 2121) in dry pyridine (14 c.c.) was added during 15 minutes to a mechanically stirred solution of dry benzylidene uridine (2.75 g.) in dry pyridine (33 c.c.), cooled in an ice-salt mixture and protected from moisture. Stirring was continued for 2 hours in the freezing mixture and for 1 hour at room temperature; the solution was again cooled, water (8 c.c.) added during 15 minutes, and stirring continued for 1 hour. The yellow oil which separated when the mixture was poured into ice-water (300 c.c.) was extracted with chloroform, and the solution washed successively with dilute sulphuric acid, water, sodium bicarbonate, water, and then evaporated completely under reduced pressure. The residual gum was dissolved in ice cold *N*/10-sodium hydroxide, and the solution filtered and neutralised exactly with *N*/10-sulphuric acid. **3': 5'-Benzylidene uridine-2' diphenyl phosphate** separated as an amorphous powder, m. p. 56—60° (4.0 g.), which was washed by decantation, collected, and dried in a vacuum desiccator over phosphoric oxide (Found, on anhydrous material: C, 59.6; H, 4.6; N, 4.9; P, 5.4. $C_{28}H_{25}O_9N_2P$ requires C, 59.6; H, 4.5; N, 5.0; P, 5.5%). It formed an intensely yellow solution in cold alkali which faded on removal of the first phenyl radical (see below). It dissolved readily in the usual organic solvents, but was sparingly soluble in carbon tetrachloride and insoluble in light petroleum. Attempts to crystallise it have so far failed.

Hydrolysis of 3': 5'-Benzylidene Uridine-2' Diphenyl Phosphate with *N*-Sodium Hydroxide.—A flask containing the compound (48 mg.) and *N*-sodium hydroxide (2 c.c.) was heated to 100°, closed, and left in a boiling water-bath. At intervals, small quantities of the hot solution were removed and cooled rapidly to room temperature, and determinations of inorganic phosphate (Briggs, *J. Biol. Chem.*, 1922, **53**, 13) and of free phenol (Folin and Ciocalteu, *ibid.*, 1927, **73**, 627) were made on samples (0.1 c.c.). For the determination of free phenol, the sample was acidified with tartaric acid and steam-distilled. The following table records the molar equivalents of phosphate and phenol liberated per mol. of 3': 5'-benzylidene uridine-2' diphenyl phosphate:

Time, hours	0.5	1.0	1.5	2.0	4.0
Inorganic phosphate, mols.	0	0.06	0.09	0.13	0.25
Free phenol, mols.	1.00	1.09	1.11	1.12	1.25

Uridine-2' Phenyl Phosphate.—3': 5'-Benzylidene uridine-2' diphenyl phosphate (1.4 g.) and *N*/2-sodium hydroxide (50 c.c.) were heated at 100° for 30 minutes, *N*-sulphuric acid (50 c.c.) was added, and the heating continued for 45 minutes. After the phenol and benzaldehyde had been extracted with ether, the mixture was neutralised with sodium hydroxide, mixed with a slight excess of 25% barium acetate to precipitate sulphate ions, diluted to 300 c.c., and mixed with 25% lead acetate solution and sufficient ammonia to adjust the reaction to pH 9; the lead salt was not precipitated at pH 6.8—7.0. The lead salt was centrifuged, washed, and decomposed with hydrogen sulphide, and the solution, after being freed from lead sulphide by filtration and from hydrogen sulphide by aeration, was made alkaline to litmus with a 10% solution of brucine in alcohol, and evaporated to a syrup. This was dissolved in hot water (5 c.c.), filtered, and left in a refrigerator until a small quantity of brucine had separated and been removed. The solution was evaporated to a syrup, mixed with alcohol, and poured into ether. The resulting precipitate of *brucine* salt separated in micro-crystalline form from 97% alcohol. When dried for 48 hours at 15 mm. over phosphoric oxide, placed in a bath at 213°, and heated at 4° per min., slight shrinkage occurred at 218°, and at 222—225° the salt melted to a transparent liquid (Found, in material dried at 60° and 0.25 mm.: C, 57.5; H, 5.7; N, 6.9; P, 3.9; brucine, 51.2. $C_{28}H_{26}O_4N_2, C_{15}H_{17}O_9N_2P$ requires C, 57.4; H, 5.5; N, 7.0; P, 3.9; brucine, 49.6%. Loss, 2.4% of dry weight; H_2O requires 2.3%). Material dried as for analysis had $[\alpha]_D^{24} - 31.5^\circ$ in dry pyridine (*c*, 0.99). Potentiometric titration of, and estimation of total phenol (liberated by 10*N*-sodium hydroxide at 100° in 4 hours) in a solution of

the acid, prepared by decomposition of the lead salt with hydrogen sulphide, showed that one phosphoryl dissociation and one phenol radical per mol. were present.

Uridine-2' Phosphate.—3':5'-Benzylidene uridine-2' diphenyl phosphate (3.7 g.) and $n/2$ -sodium hydroxide (140 c.c.) were heated at 100° for 30 minutes, n -sulphuric acid (140 c.c.) was added, and the heating continued for 45 minutes. After extraction of the phenol and benzaldehyde with ether, n -sodium hydroxide (69 c.c.) was added, and the solution evaporated under reduced pressure to about 35 c.c., and mixed with industrial methylated spirit (350 c.c.) to precipitate sodium sulphate, which was collected. The filtrate and washings were evaporated to a small volume under reduced pressure, diluted with water, and evaporated to 30 c.c. When this solution was shaken in 1 atm. of hydrogen at room temperature for 4½ hours with Adams's platinum oxide (0.5 g., added in three batches), absorption took place of 95% of the volume of hydrogen theoretically required for hydrogenolysis of the phenyl ester. The solution was filtered from the catalyst, mixed with a little barium acetate to remove sulphate ions, and then with lead acetate solution and ammonia to adjust the reaction to pH 6.8–7.0. The lead salt was separated (centrifuged), washed, and decomposed with hydrogen sulphide, and the solution filtered, aerated, concentrated under reduced pressure to 30 c.c., and mixed with saturated barium hydroxide solution till alkaline to phenolphthalein. Excess of barium hydroxide was removed from the solution by passage of carbon dioxide, boiling, cooling, and filtering, and the resulting solution was concentrated to 25 c.c. and mixed with absolute alcohol (50 c.c.). The solid was collected, washed with 66% alcohol, 95% alcohol, and ether, and dried in a vacuum. It was purified by solution in water, filtration and reprecipitation. The barium salt (1.6 g.) formed a white amorphous powder, readily soluble in water (Found, in two batches of anhydrous material: P, 6.7, 6.8; Ba, 30.9, 31.6. $C_9H_{11}O_9N_2P$ requires P, 6.8; Ba, 29.9%). The salt contained no inorganic phosphate, *e.g.*, barium phosphate, and all attempts failed to bring the barium analytical figures closer to those theoretically required.

The *dibrucine* salt was prepared from the barium salt (1.4 g.) by removing the barium quantitatively from an aqueous solution by means of $n/10$ -sulphuric acid, adding an alcoholic solution of brucine (2.1 mols.), and evaporating the mixture to dryness under reduced pressure. The salt was crystallised twice from 35% alcohol (8 parts) and twice from water (18 parts), from which it separated in irregular, coarse prisms (2.3 g.); the m. p. (see below) was not changed by three more recrystallisations from water (Found, in material dried at 60° and 0.25 mm.: C, 59.5; H, 6.1; N, 7.5; P, 2.8; brucine, 73.2. $C_9H_{13}O_9N_2P \cdot 2C_{23}H_{26}O_4N_2$ requires C, 59.3; H, 5.9; N, 7.6; P, 2.8; brucine, 70.8%. Loss 7.5% of dry weight; $4.5H_2O$ requires 7.3%).

Dibrucine Uridylate and Uridine-5' Phosphate.—These salts were required for comparison. The former salt was prepared from yeast ribonucleic acid by a modification of the method of Bredereck and Richter (*Ber.*, 1938, **71**, 718) to be published shortly, and crystallised four times from 33% alcohol (Found, in material dried at 60° and 0.25 mm.: C, 59.1; H, 6.1; N, 7.6; P, 2.8. Calc. for $C_9H_{13}O_9N_2P \cdot 2C_{23}H_{26}O_4N_2$, C, 59.3; H, 5.9; N, 7.6; P, 2.8%). The latter salt was a sample prepared by Gulland and Hobday (*J.*, 1940, 746).

Comparisons of Uridylic Acid, Uridine-5' Phosphate, and Uridine-2' Phosphate.—(i) *Hydrolysis in n/10-sulphuric acid*. The percentage dephosphorylations given for uridylic acid and uridine-5' phosphate are the means of those recorded by Levene and Tipson (*J. Biol. Chem.*, 1934, **106**, 113) and Gulland and Hobday (*loc. cit.*). The dephosphorylation of uridine-2' phosphate was determined as follows. A solution of the dibrucine salt (equivalent to about 0.18 g. of anhydrous material) in hot water (20 c.c.) was cooled, and before crystallisation began barium hydroxide solution (5 c.c.; equivalent to 2.20 c.c. of 1.020N-sulphuric acid) was added. Brucine was extracted by five successive portions (each 15 c.c.) of chloroform, 1.020N-sulphuric acid (2.40 c.c.) was added, and the precipitated barium sulphate centrifuged off and washed. The combined centrifugate and washings, together with 1.020N-sulphuric acid (9.61 c.c.), were diluted with water to 100 c.c. The total phosphorus in 5 c.c. of this solution was 0.238 mg. (average of 4 concordant estimations). Samples (5.0 c.c.) were sealed in tubes and placed in a boiling water-bath. At intervals two tubes were removed and cooled rapidly, and the inorganic phosphate of the contents determined. The variation between these duplicates never exceeded 3% and the mean figure is quoted.

	P, mg./10 c.c. N/10-acid.	Time, hrs.	2.	4.	6.	8.	20.	24.	28.	30.
2'-Phosphate	0.48	Inorg. P,	0.012	0.019	0.025	0.032	0.067	0.076	0.092	0.097
		mg./5 c.c. Dephosph.,	5.0	8.0	10.5	13.4	28.2	32.0	38.6	40.7
5'-Phosphate	0.50, 0.60	Dephosph.,	3.0	5.5	7.5	9.5	19.5	23.0	26.0	28.0
3'-Phosphate	0.35, 0.40, 0.62	Dephosph.,	15.0	25.0	33.5	40.0	68.0	74.0	79.0	81.0
		%								

(ii) *Hydrolysis in n/10-sodium hydroxide*. A solution of brucine salt (equivalent to about 0.18 g. of anhydrous material) in hot water (50 c.c.) was cooled, and before crystallisation began 1.033N-sodium hydroxide (3.0 c.c.) was added. Brucine was extracted by five successive portions of chloroform (15 c.c. each), the solution was filtered to remove chloroform drops and, with the addition of 1.033N-sodium hydroxide (7.0 c.c.), diluted to 100 c.c. with water. Total phosphorus was determined in three samples (2 c.c.), and the absence of inorganic phosphate was shown by analysis of two samples (5 c.c.). The remainder of the solution was transferred to a flask fitted with an efficient condenser, heated rapidly to boiling and immersed in a boiling water-bath. At intervals samples of the hot liquid were removed and cooled rapidly, and the inorganic phosphate determined in duplicate (Briggs, *loc. cit.*). A slight greenish tint in the colour developed decreased the accuracy of the determinations, and the duplicates had a maximum variation of 8%; mean figures are quoted.

	P, mg./5 c.c. N/10-NaOH.	Time, hrs.								
			1.	2.	3.	4.	5.	6.	7.	8.
3'-Phosphate	0.259	Inorg. P,	0.007	0.012	0.018	0.024	0.028	0.035	0.040	0.046
		Dephosph., mg./5 c.c. %	2.7	4.6	6.9	9.3	10.8	13.5	15.5	17.8
2'-Phosphate	0.248	Inorg. P,	0.013	0.020	0.026	0.034	0.040	0.047	0.051	0.059
		Dephosph., mg./5 c.c. %	5.2	8.1	10.5	13.7	16.1	19.0	20.6	23.8

(iii) *Characteristics of salts.* This table is based on our observations and information taken from Levene and Tipson, Gulland and Hobday (*loc. cit.*), and Levene (*J. Biol. Chem.*, 1919, **40**, 395).

Characteristic.	3'-Phosphate.	5'-Phosphate.	2'-Phosphate.
(a) Pptn. of Pb salt	pH 6.8—7.0	Dil. ammonia	pH 6.8—7.0
(b) Solubility of Ba salt in cold water	Sparingly	Easily	Easily
(c) Brucine salt :			
(i) Form	Needles; 7H ₂ O	Needles	Prisms; 4.5H ₂ O
(ii) [α] _D in dry pyridine	−55.0° (c, 1.00), −55.9° (c, 1.06)	−69.7° (c, 0.97), −68.8° (c, 1.05)	−76.1° (c, 0.96), t = 17°
(iii) Solubility :			
(a) Water	Sparingly sol., cold and hot	Mod. sol. cold, easily sol. hot	Sparingly sol. cold, 1 : 18 pts. hot
(β) 33% EtOH	Sparingly sol. cold, 1 : ca. 80 pts. hot	Mod. sol. cold, easily sol. hot	Mod. sol. cold, 1 : 8 pts. hot
(γ) Abs. EtOH	Sparingly sol. cold, and hot	Sparingly sol. cold, easily sol. hot	Sparingly sol. cold, mod. sol. hot
(iv) M. p. :	These are unsharp and conditions must be standardised. Salt was dried for 48 hours over P ₂ O ₅ at 15 mm.; m. p. tube 1 mm. diam.; temp. rise 4°/min.		
(a) Placed in bath at 153°	Gradually swelled to opaque mass from 175° to 185°; had become transparent drops by 188—190°	Shrank at 159°; formed a transparent mass, 162—165°	Shrank to opaque mass from 161° to 174°; had become trans- parent drops by 177— 180°
(β) Placed in bath at 12° below top of range	In at 183°. Shrank, effervesced, and swelled to opaque mass 185—187°; had become transparent drops by 195°	In at 153°; as above.	In at 168°. Shrank 171°, swelled to opaque mass 172— 175°; had become transparent drops by 180°

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