Fig. 1. O, Sedimentation coefficients plotted against molecular weight for samples of glycogen investigated by Bell *et al.* (1948). The full curve was drawn freehand and used to estimate the approximate molecular weights of the present samples of liver glycogen (●) and kidney glycogen (■).

prepared and measured under the same conditions. The approximate average molecular weights of the latter samples (determined by sedimentation and

diffusion, at 1 g./100 ml.) are plotted in Fig. 1 against the sedimentation coefficients. Extrapolation of this curve indicates, for the present samples, average molecular weights of approximately 7.1×10^6 (kidney glycogen) and 6.6×10^6 (liver glycogen).

Orrell & Bueding (1958) have reported much higher values for sedimentation coefficients of glycogens, and Stetten, Katzen & Stetten (1958) have found much higher values of molecular weights by light-scattering. It is not clear how far these differences may be due to the use of different methods of extraction or to measurement under different conditions.

REFERENCES

- Baldwin, R. L. (1953). *Brit. J. exp. Path.* **34**, 217.
 Bell, D. J., Gutfreund, H., Cecil, R. & Ogston, A. G. (1948). *Biochem. J.* **42**, 405.
 Cecil, R. & Ogston, A. G. (1948). *Biochem. J.* **43**, 592.
 Orrell, S. A. & Bueding, E. (1958). *J. Amer. chem. Soc.* **80**, 3800.
 Stetten, M. R., Katzen, H. M. & Stetten, D., jun. (1958). *J. biol. Chem.* **232**, 475.

The Preparation and Purification of Cytidine 2':3'-Phosphate

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In the degradation of ribonucleic acid by pancreatic ribonuclease, uridine and cytidine 2':3'-phosphates are formed as intermediates and subsequently hydrolysed to the corresponding 3'-phosphates (Markham & Smith, 1952; Brown, Dekker & Todd, 1952). The kinetics of the action of ribonuclease on ribonucleic acid are complex (Vandendriessche, 1953) and interpretation awaits detailed investigations with substrates of low molecular weight. A first step in the logical approach to this problem must involve studies of the hydrolysis of the cyclic substrates. Thus it is necessary that these should be available in reasonable quantity and a high degree of purity. Both chemical and enzymic methods are available for the preparation of these substances. The chemical methods are to be preferred since the isolation of the product is difficult when the enzymic method is employed. The chemical methods involve intramolecular esterification of the adjacent hydroxyl group of the nucleoside 2'- or 3'-phosphate; both trifluoroacetic anhydride (Brown, Magrath & Todd, 1952) and dicyclohexyl carbodi-imide (Dekker & Khorana, 1954) have been used as condensing agents. The

trifluoroacetic anhydride method appeared to be the easier route and we have investigated it in some detail.

EXPERIMENTAL AND RESULTS

Cytidylic acid. This (a mixture of cytidine 2'- and 3'-phosphates) was prepared by the method of Cohn & Carter (1950), De-Acidite FF (formate form) being used in place of Amberlite IRA 400.

Trifluoroacetic acid. This was obtained from Eastman Kodak Co., Kirby Industrial Estate, Liverpool.

Trifluoroacetic anhydride. This was prepared by the method of Swarts (1922), with the modification that the proportion of trifluoroacetic acid to phosphorus pentoxide was 40:65 (w/w).

Ribonuclease. This (lot no. 21630) was obtained from Armour and Co. Ltd., Hampden Park, Eastbourne, Sussex.

Paper chromatography. Whatman no. 4 paper, previously washed with 2 N-acetic acid, then with water by descending chromatography, was employed. The chromatograms were developed by the ascending method for 14 hr., with propan-2-ol-water-aq. NH_3 soln. (sp.gr. 0.88) (70:25:5, by vol.) as solvent (Brown, Magrath & Todd, 1952). The papers were dried in a stream of air and the nucleotides detected by examination in u.v. light. In some instances the

phosphate spray of Hanes & Isherwood (1949) was also employed.

Microanalysis. This was carried out by Weiler and Strauss, Oxford.

Effect of trifluoroacetic acid on the cyclization of cytidylic acid in the presence of trifluoroacetic anhydride

When possible mechanisms of the cyclization with trifluoroacetic anhydride are considered, it is apparent that there are a number of species which might act catalytically. For instance, trifluoroacetic acid, produced by trifluoroacetylation of sugar hydroxyl groups, would be present during the reaction. The effect of adding varying quantities of trifluoroacetic acid to the reaction mixture was therefore investigated.

Yeast cytidylic acid (0.19 g.) was dissolved in the trifluoroacetic acid and trifluoroacetic anhydride added. The reaction flask was stoppered and the mixture kept at room temperature for 18 hr. The solvents were evaporated at room temperature under reduced pressure and the residue was dissolved in liquid NH_3 (10 ml.). The flask was closed with a drying tube containing powdered KOH in order to exclude moisture. After 10 min. the NH_3 was removed by evaporation under reduced pressure at room temperature. A sample was chromatographed, the paper examined under u.v. light and the absorbing spots were marked with pencil. Rectangles of paper containing the nucleotides were cut and eluted with 0.05M-sodium acetate buffer, pH 5.0 (5 ml.). The extinctions of the eluates were determined at 268 $\text{m}\mu$, which is the isosbestic point of cytidine 2':3'-phosphate and cytidine 3'-phosphate under these conditions. A blank was prepared from a piece of the chromatogram of the same area and R_F .

Three spots were visible on the chromatograms: the fastest (R_F 0.41) was cytidine 2':3'-phosphate; that of R_F 0.18 was unreacted cytidylic acid; the slowest (R_F 0.08) was presumably cytidylecytidylic acid and was formed in approx. 6% yield. The results are shown in Table 1. The percentage conversion into cytidine 2':3'-phosphate was calculated assuming that the slowest-running material is isosbestic at 268 $\text{m}\mu$ with cytidine 3'-phosphate on a pyrimidine basis.

Large-scale preparation of cytidine 2':3'-phosphate

Cytidylic acid (3 g.) was added to a mixture of trifluoroacetic acid (7.5 ml.) and trifluoroacetic anhydride (15 ml.) which had previously been cooled in an ice bath. The flask was stoppered, removed from the ice bath and shaken gently under a stream of water from the cold tap until a homogeneous solution was obtained. The reaction mixture was allowed to stand for 14 hr. at room temperature and then evaporated under reduced pressure. The last traces of volatile materials were removed in a desiccator over powdered KOH at a pressure less than 0.1 mm. The residual gum was dissolved in anhydrous liquid NH_3 (50 ml.), and the reaction mixture thus obtained was protected from atmospheric moisture by means of a KOH guard tube. After 15 min. most of the NH_3 was distilled off under reduced pressure and the final traces were removed in a desiccator over P_2O_5 . Paper chromatography as described above showed that 91% conversion into the cyclic phosphate had been achieved.

Purification of cytidine 2':3'-phosphate

Preliminary solvent fractionation. This is suitable for preparations of cytidine 2':3'-phosphate which are relatively heavily contaminated with cytidylic acid. The crude samples used in these experiments were obtained in the preliminary experiments to find the optimum conditions for cyclization.

The product of a cyclization run (2 g.) assaying 69% of cytidine 2':3'-phosphate, in which cytidylic acid was the major impurity, was extracted with anhydrous ethanol (4 x 100 ml.); the insoluble material after each extraction was separated by centrifuging. Excess of anhydrous ether (dried over sodium metal) was added with vigorous stirring to each of the ethanol extracts, the precipitates were removed by centrifuging, washed with anhydrous ether and dried *in vacuo* over P_2O_5 . The four fractions were bulked (1.1 g.); the material obtained assayed 89% of cytidine 2':3'-phosphate, demonstrating that this is preferentially extracted by ethanol. A portion (1 g.) of the product was extracted once with 100 ml. of ethanol and then twice with 50 ml. portions of ethanol. The nucleotides were precipitated with ether as described above. The results are shown in Table 2. It can be seen that, by using this relatively simple technique, preparations of cytidine 2':3'-phosphate of 95% purity can be obtained. Other organic solvents were tried; of these only methanol, which gave results similar to ethanol, was found to be of any value.

Separation on a charcoal column. The charcoal was obtained from Sutcliffe, Speakman and Co. Ltd., Leigh, Lancs (no. 130, mesh 80-200). It was washed successively with 2N-HCl, 2N-acetic acid and water until the washings were neutral, then with a solution of ethylenediaminetetra-acetic acid (0.1M, adjusted to pH 9.0 with NH_3); this was followed by acetic acid and finally water until the washings were neutral.

A slurry of the washed charcoal in aqueous 80% (v/v) ethanol was used to prepare a column (30 cm. x 5.5 cm.),

Table 1. *Effect of trifluoroacetic acid on the cyclization of cytidylic acid in the presence of trifluoroacetic anhydride*

Vol. of trifluoroacetic anhydride (ml.)	Vol. of trifluoroacetic acid (ml.)	Percentage cyclization
1.2	0.0	63
1.0	0.2	84
0.6	0.6	80
0.3	0.9	62
0.0	1.2	3

Table 2. *Purification of cytidine 2':3'-phosphate by successive extractions with ethanol*

	Vol. of ethanol used (ml.)	Wt. of fraction (g.)	Percentage of cytidine 2':3'-phos- phate
Original material	—	1.0	89
First extraction	100	0.57	95
Second extraction	50	0.19	94
Third extraction	50	0.08	85
Residue	—	0.14	55

which was washed thoroughly with 80% ethanol. The preparation of cytidine 2':3'-phosphate (1 g., 88% purity), dissolved in 80% ethanol (150 ml.), was run on the column, which was washed with 80% ethanol (200 ml.). Elution was effected by the use of a linear gradient of pyridine in 80% ethanol: this was achieved by connecting together two vessels of identical dimensions by means of a siphon tube. The mixing vessel, which was connected to the column, contained initially 80% ethanol (3260 ml.); the reservoir vessel contained a mixture of pyridine and 80% ethanol [3260 ml.; 20% (v/v) pyridine]. The contents of the mixing vessel were stirred with a magnetic stirrer.

The eluate was collected in 100 ml. fractions at a flow rate of 400 ml./hr. Pyridine was first detected in fraction 16. A sample from each fraction was spotted on paper and chromatographed: fractions 17–20 contained cytidine 2':3'-phosphate; fractions 21–22 were slightly contaminated with cytidylic acid; fractions 23–25 were substantially cytidylic acid.

Fractions 17–20 were bulked and evaporated under reduced pressure to a small volume; anhydrous ethanol (50 ml.) was added and the mixture evaporated to dryness under reduced pressure. The residue was dissolved in ethanol (100 ml.) and cytidine 2':3'-phosphate precipitated by the addition of a large excess of anhydrous ether. The product was collected by centrifuging, washed with anhydrous ether and dried *in vacuo* over P_2O_5 . Yield, 500 mg. A further small amount was obtained from the mother liquors.

The purity of the material was checked by potentiometric titration with KOH. There was no buffering in the region pH 6.0–7.0, demonstrating that cytidylic acid and pyridinium ions were absent. Buffering was observed in the region pH 4.0–5.0, showing that the cytosine ring is partly in the protonated form. The product is therefore a mixture of the ammonium salt and the dipolar ion of cytidine 2':3'-phosphate. Chromatography confirmed the absence of any u.v.-absorbing or phosphorus-containing contaminants.

Separation on ion-exchange resins. The resin used was Dowex 1 (4% cross-linked, 200–400 mesh). It was converted into the acetate form and packed into a column 60 cm. $\times$ 3 cm. The column was equilibrated with triethylamine acetate (0.2M, pH 7.4). A solution of cytidine 2':3'-phosphate (1 g., 80–90% purity, dissolved in 20 ml. of water and adjusted to pH 8.0 with NH_3) was run on to the column, which was then washed with water (20 ml.). Triethylamine acetate solution (0.2M, pH 7.4) was run through the column at 150 ml./hr. and the eluate collected in 50 ml. fractions. The extinctions of samples of the fractions suitably diluted were read at 268 $m\mu$. Cytidine 2':3'-phosphate emerged in fractions 81–96; these were bulked and freeze-dried. The product was dissolved in anhydrous ethanol (20 ml.), and anhydrous ether (200 ml.) was added with stirring. The suspension was kept at 0° for 2 hr. the product collected by centrifuging, washed with anhydrous ether and dried *in vacuo* over P_2O_5 . Yields, 0.7 g. Chromatography showed a single spot with R_F 0.41. A small sample was incubated with ribonuclease (50 $\mu g./ml.$) in acetate buffer (0.05M, pH 5.0) at room temperature for 3 hr. Paper chromatography of the incubation mixture showed complete conversion into cytidylic acid. Electro-metric titration of the material isolated from the column showed it to be a mixture of the triethylamine salt and the

dipolar ion in the approximate molar proportions 7:1. No buffering in the region pH 6.0–7.0 was observed, confirming the absence of cytidylic acid.

Conversion of the triethylamine into the potassium salt. Salts of volatile bases are unsatisfactory and it was considered advisable to attempt the conversion of the material into the salt of an alkali metal. A column (10 cm. $\times$ 1.5 cm.) of Amberlite IRC50 (XE64) was converted into the potassium form with carbonate-free KOH. The mixture of triethylamine salt and dipolar ion (1.5 g.) was dissolved in boiled-out glass-distilled water (10 ml.) and the solution was adjusted to pH 8.0 with carbonate-free KOH. It is essential to use carbonate-free alkali to avoid contamination of the product with $KHCO_3$. The solution was run through the column and followed by CO_2 -free water. The eluate was collected in 5 ml. fractions; those containing u.v.-absorbing material were bulked and freeze-dried. The residue was dissolved in anhydrous methanol (25 ml.), filtered through sintered glass and the potassium salt of cytidine 2':3'-phosphate precipitated by the addition of anhydrous ether. The product was collected by centrifuging, washed with anhydrous ether and dried *in vacuo* over P_2O_5 . The recovery was nearly quantitative. The titration curve showed no buffering in the region pH 6.0–7.0, demonstrating the absence of cytidylic acid. Furthermore, the cytosine ring is essentially all in the unprotonated form (Found: C, 30.1; H, 4.2; N, 11.6; P, 8.8. $C_9H_{11}O_7N_3PK \cdot H_2O$ requires C, 29.9; H, 3.6; N, 11.6; P, 8.7%). The material is considered to be the monopotassium salt of cytidine 2':3'-phosphate monohydrate.

DISCUSSION

The method of Brown, Magrath & Todd (1952) has been improved in two important respects. The addition of trifluoroacetic acid to the trifluoroacetic anhydride is the major improvement as this enables at least 90% cyclization to be achieved, thus simplifying the purification.

Elucidation of the role of trifluoroacetic acid would require detailed kinetic studies to determine whether the effect is primarily catalytic or due to the displacement of equilibria. Cyclization occurs to a small extent in trifluoroacetic acid alone; this reaction is presumably an acid-catalysed esterification. It is consistent with the known interconversion of the 2' and 3' nucleotides in acid media which probably involves the formation of a cyclic intermediate (Brown, Magrath & Todd, 1952).

Brown, Magrath & Todd (1952) have proposed that in trifluoroacetic anhydride an unsymmetrical anhydride of cytidylic acid and trifluoroacetic acid is formed as an intermediate. It has been shown that the reactions with nucleophilic reagents of unsymmetrical anhydrides of carboxylic and trifluoroacetic acids differ in both rate and product composition from those of a solution of the carboxylic acid in trifluoroacetic anhydride (Bourne, Stacey, Tatlow & Worrall, 1954; Tedder, 1955). The unsymmetrical anhydrides yield mixed products on treatment with alcohols, whereas solutions of a carboxylic acid in trifluoroacetic anhydride

yield primarily carboxylate esters. The only difference between these two reacting systems is the presence of trifluoroacetic acid in the latter. The acid probably functions by donating a hydrogen ion to the most basic oxygen of the mixed anhydride, which would be the carbonyl oxygen atom of the carboxylic acid. Thus nucleophilic attack is favoured at the carboxylic rather than at the trifluoroacetic carbonyl carbon atom: this explains the preponderance of the carboxylate ester. In the absence of trifluoroacetic acid the major product should be the ester of the stronger acid component of the mixed anhydride, if the reaction is of the S_N2 type and steric factors do not become important.

It can be assumed that the first step in the cyclization in the absence of added acid is the formation of a mixed anhydride of cytidylic acid and trifluoroacetic acid, which might well be catalysed by strong acids.

In the presence of trifluoroacetic acid the ionization of a proton from the hydroxyl group attached to the phosphorus atom would be suppressed. This should favour the attack of the adjacent hydroxyl group at the phosphorus rather than the carbonyl carbon atom, and would explain the increased yield of cyclic phosphate in the presence of trifluoroacetic acid. Thus the reaction would be similar to that of other mixed anhydrides in the presence of trifluoroacetic acid.

The second improvement is the use of liquid ammonia in place of propanol-2-ammonia for the removal of trifluoroacetyl groups (J. G. Buchanan, personal communication). It eliminates the formation of esters as by-products, and this simplifies the purification procedure.

The purification by chromatography on charcoal columns was found in general to be satisfactory, but with some batches of charcoal a poor separation was obtained. Careful purification of the charcoal is essential.

It has been reported (Brown, Magrath & Todd, 1952) that cytidine 2':3'-phosphate and cytidylic acid cannot be separated by ion-exchange chromatography with formic acid as the eluent. This is

not surprising, since resolution can only be expected over the pH range where ionization of the secondary phosphate of cytidylic acid occurs. For this reason the eluting solution should have a pH greater than 5.0. Experiments with ammonium formate were abandoned owing to the difficulty of removing the electrolyte completely by freeze-drying. Triethylamine acetate was satisfactory because solutions of sufficiently high pH can be obtained and it can readily be removed by freeze-drying.

SUMMARY

1. An improved method of cyclizing 2'- and 3'-cytidylic acid to the 2':3'-cyclic phosphate with trifluoroacetic anhydride has been devised. Yields are approximately 90% of cyclic ester, of purity over 95%.

2. Fractionation on Dowex 1, in the presence of a triethylamine-acetate buffer (pH 7.4), raises the purity to over 98%.

3. Methods involving simple ethanol fractionation or fractional elution from charcoal columns are described for the purification of crude cyclic ester. By their use, purity can be increased from 60–80% to 90–95%.

4. The mechanism of the cyclization reaction is discussed.

REFERENCES

- Bourne, E. J., Stacey, M., Tatlow, J. C. & Worrall, R. (1954). *J. chem. Soc.* p. 2006.
Brown, D. M., Dekker, C. A. & Todd, A. R. (1952). *J. chem. Soc.* p. 2715.
Brown, D. M., Magrath, D. I. & Todd, A. R. (1952). *J. chem. Soc.* p. 2708.
Cohn, W. & Carter, C. (1950). *J. Amer. chem. Soc.* **72**, 2606.
Dekker, C. & Khorana, H. (1954). *J. Amer. chem. Soc.* **76**, 3522.
Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
Markham, R. & Smith, J. D. (1952). *Biochem. J.* **52**, 552.
Swarts, F. (1922). *Acad. Belg. Cl. Sci.* **8**, 343.
Tedder, J. M. (1955). *Chem. Rev.* **55**, 787.
Vandendriessche, L. (1953). *Acta chem. scand.* **7**, 699.