513. *Nucleotides. Part XIV.* A Method Suitable for the Large-scale Preparation of Deoxyribonucleosides from Deoxyribonucleic Acids.*

By W. Andersen, C. A. Dekker, and A. R. Todd.

The deoxyribosides of guanine, adenine, cytosine, thymine, and uracil have been isolated from an enzymic digest of commercial herring-sperm deoxyribonucleic acid. Ion-exchange chromatography has been employed in the separation procedure which is suitable for the preparation of these deoxyribonucleosides on the large scale.

Work in progress in this laboratory on the synthesis of nucleotides, which forms the subject of this series of papers, requires for its prosecution substantial quantities of ribonucleosides and deoxyribonucleosides. Whereas well-defined and successful methods are available for the isolation of the former, we found that even recently published methods for the preparation of the latter (Schindler, *Helv. Chim. Acta*, 1949, **32**, 979; Reichard and Estborn, *Acta Chem. Scand.*, 1950, **4**, 1047) were generally unsatisfactory when applied on the large scale. Moreover the authors of these methods omitted to inhibit adenine deoxyriboside deaminase by adding silver salts (Klein, *Z. physiol. Chem.*, 1934, **224**, 244; *ibid.*, 1938, **255**, 82) and so isolated hypoxanthine deoxyriboside instead of the adenine derivative which was important to us. The present paper describes a new procedure which we have found satisfactory for relatively large-scale preparation of deoxyribonucleosides from herring-sperm deoxyribonucleic acid.

The stability of the phosphodiester linkage in deoxyribonucleic acids (cf. Brown and Todd, *J.*, 1952, 52) and the instability of the glycosidic linkage in the purine deoxyribonucleotides to acids preclude the use of either alkali or acid for the satisfactory cleavage of the deoxyribonucleic acids either to their constituent nucleotides or nucleosides. It is, therefore, necessary to resort to enzymic hydrolysis and this involves the addition to the nucleic acid solution of protein (enzymes) and salts (activating ions and buffering system) which must subsequently be removed before isolation of the nucleosides. Thus, the use of an ammonia—ammonium sulphate buffer has in the past necessitated subsequent quantitative removal of sulphate ions as barium sulphate and extensive washing of the precipitate to avoid loss by adsorption. We have circumvented these steps by extracting the concentrated enzymic digest with ethanol. The ethanol extract so obtained was shown by paper chromatography to contain the deoxyribosides of guanine, adenine, cytosine, thymine, and uracil. The detection of uracil deoxyriboside in enzymic hydrolysates of deoxyribonucleic acids from various sources has been reported recently from this laboratory (Dekker and Todd, *Nature*, 1950, **166**, 557; Dekker and Elmore, *J.*, 1951, 2864). It could be produced from cytosine deoxyriboside either by partial degradation during the manufacture of the commercial nucleic acid, used as starting material, or by the action of a bacterial deaminase during hydrolysis. A formic acid hydrolysate of the nucleic acid used in our experiments was shown by paper chromatography to contain uracil (Vischer and Chargaff, *J. Biol. Chem.*, 1948, **176**, 715). Furthermore the ratio uracil : thymine in the formic acid hydrolysate corresponded fairly closely to the ratio of their deoxyribosides in the enzymic

* Part XIII, preceding paper.

digest. In view of these facts it is probable that the uracil deoxyriboside isolated was present in the original deoxyribonucleic acid and was not produced during the hydrolysis. The presence of uracil deoxyriboside in the hydrolysate caused, of course, a corresponding drop in the yield of cytosine deoxyriboside.

The mixture obtained from the ethanol extract of the enzymic digest was resolved into its component nucleosides by ion-exchange chromatography in two stages. Conditions similar to those which Cohn (*J. Amer. Chem. Soc.*, 1950, **72**, 1471) found successful in the separation of the ribonucleosides were equally applicable to the first stage in the separation of the deoxyribonucleosides. The thymine, uracil, and guanine derivatives, which by virtue of their weakly acidic hydroxyl groups are capable of forming anions above pH 10 were adsorbed on a quaternary ammonium type anion-exchange resin (Dowex-2 in the formate form) while the deoxyribosides of adenine and cytosine, which do not contain acidic functional groups, were not retained on the column. The adsorbed nucleosides were separated by elution with an ammonium formate buffer at pH $\not>$ 8. The separation of the thymine and uracil deoxyribosides from one another was not complete. As a rule paper chromatography of the uracil deoxyriboside fractions revealed a slight contamination with thymidine. Pure uracil deoxyriboside was obtained from them by recycling through a smaller Dowex-2 column under the same conditions as above.

From the fractions representing the first peak, in the Dowex-2 ion-exchange chromatogram (*i.e.*, the adenine-cytosine deoxyriboside mixture) it was possible to crystallise about 90% of the adenine deoxyriboside directly. Cytosine deoxyriboside and the remainder of the adenine deoxyriboside were most efficiently separated by recycling their neutral solution through a carboxylic type cation-exchange resin (IR C-50) buffered at pH 3·5. Elution with water sufficed to resolve these two weakly basic nucleosides; this separation appears to be more dependent on adsorption than on ion-exchange characteristics since the nucleosides are eluted in the reverse order to that which would be predicted from their basic dissociation constants. A similar phenomenon has been observed in certain instances by Cohn (*loc. cit.*) in his extensive work on the separation of the ribonucleotides.

The preparative procedure described in the Experimental section has several advantages over existing methods. It is, for example, unnecessary to remove the last traces of protein from the nucleic acid before hydrolysis, thus permitting the direct use of fairly crude commercial samples of deoxyribonucleic acid. The laborious chloroform extraction procedure which Schindler (*loc. cit.*) described for partially purifying the guanine deoxyriboside is eliminated. The latter author's use of adsorption chromatography on alumina for the separation of the pyrimidine nucleosides from the other components is also avoided. Apart from minor losses incurred in the ethanol extraction and in the final crystallisation, the new method gives almost quantitative recovery of the deoxyribonucleosides.

In preliminary experiments in which we attempted to separate the nucleosides on a sulphonic acid type cation-exchange resin (Dowex-50) (Reichard and Estborn, *loc. cit.*), extensive hydrolysis of the purine nucleosides was consistently encountered. This occurred even when the resin was in the ammonium salt form and when buffered solutions were used for elution. Adenine deoxyriboside, which remains on the column for the longest period of time, was particularly affected.

Experimental

M. p.s are uncorrected. Evaporation of aqueous solutions was carried out in all cases under reduced pressure at a bath-temperature not exceeding 45°.

Purification of Intestinal Phosphatase.—A glycerol extract of calf intestinal mucosa was prepared according to Klein (*Z. physiol. Chem.*, 1932, **207**, 125). To 250 c.c. of the glycerol extract ethanol (500 c.c.) was added with thorough mixing. The precipitate was separated by centrifugation (3000 r.p.m. for 20 minutes) and washed once with ethanol (400 c.c.) and twice with water (200 c.c. and 150 c.c.). After the final centrifugation the residue was suspended in water (200 c.c.).

Enzymic Hydrolysis of Herring-sperm Deoxyribonucleic Acid.—Herring-sperm deoxyribonucleic acid (180 g.; 7·3% of P) of commercial origin (Isaac Spencer and Co., Ltd., Aberdeen) was dissolved in warm water (1400 c.c.). Magnesium sulphate (54 g. of the heptahydrate) and

deoxyribonuclease (250 mg.; supplied by Gea Ltd., Copenhagen) were added and the pH was adjusted to 6·5—7·0 with ammonia (4N). The volume was made up to a total of 1650 c.c. and the solution incubated at 37° for 44 hours.

For convenience the above solution was divided into two equal parts for further hydrolysis. To each portion (825 c.c.) was added silver sulphate (2·15 g., dissolved in water by dropwise addition of concentrated ammonia), N-ammonia–N-ammonium sulphate buffer (720 c.c.; 1 : 1), and calf intestinal phosphatase (200 c.c. of the suspension described above). The volume was made up to 1900 c.c. and the solution was incubated at 37° for 24 hours. More phosphatase suspension (200 c.c.) was added, the pH readjusted to 9 with ammonia, and the incubation continued for a further 24 hours. By this means 80% of the original organically bound phosphorus was liberated as inorganic phosphate. Magnesium ammonium phosphate and insoluble protein were removed by centrifugation, and the clear supernatant liquid was concentrated to a thick slurry. This slurry was extracted once with 99% ethanol (1000 c.c.) and twice with 85% ethanol (500 c.c. and 250 c.c.). The extractions were carried out by mechanical shaking for 4 hours in each case. The combined extracts were concentrated to a thick syrup *in vacuo*, diluted to 400 c.c. with water, and filtered through a thin layer of activated charcoal. The filtrate was made up to 550 c.c. with distilled water.

Chromatographic Examinations.—An aliquot of this solution was placed on paper (Whatman No. 1) and run on a two-dimensional chromatogram with *n*-butanol saturated with water in one direction and 5% disodium hydrogen phosphate–*iso*amyl alcohol in the other. Examination of the paper chromatogram by means of a " Mineralite " ultra-violet lamp revealed five spots with R_F values corresponding to those of the deoxyribosides of guanine, adenine, cytosine, uracil, and thymine (Buchanan, *Nature*, 1951, **168**, 1091). The spots were cut out and eluted separately with 0·1M-sodium phosphate buffer (5·0 c.c.; pH 7·0), and the optical density of the eluates at 260 mμ was determined with a Beckman spectrophotometer. From the known ultra-violet absorption characteristics of the compounds, amounts of individual nucleosides present in the original solution were calculated. The results are expressed in g. per 36 g. of nucleic acid, corresponding to 110 c.c. of the above solution, batches of this size being used in ion-exchange separations described below: Guanine deoxyriboside, 2·7 g.; adenine deoxyriboside, 3·4 g.; cytosine deoxyriboside, 1·8 g.; uracil deoxyriboside, 1·0 g.; thymidine, 4·9 g. The ratio uracil deoxyriboside : thymidine was 1 : 5.

A sample of the deoxyribonucleic acid used as starting material (30 mg.) was hydrolysed with formic acid (2 c.c.) at 175° for 40 minutes. The hydrolysate was examined by paper chromatography in *iso*propyl alcohol–6N-hydrochloric acid (65 : 35). The thymine and uracil spots (R_F—0·76 and 0·66 respectively) were cut out and eluted as above. Determination of optical densities of the eluates at 260 mμ gave a ratio uracil : thymine = 1 : 4.

Separation of the Nucleosides.—A column (32 × 3·3 cm.) of Dowex-2 resin (250—500 mesh; chloride form) was set up and the resin converted into the formate form before applying a portion of the above nucleoside solution (110 c.c., equiv. to 36 g. of deoxyribonucleic acid) previously adjusted to pH 10·5 with ammonia (190 c.c. of 4N). The column was adjusted to a flow rate of 270 c.c. per hour and 200-c.c. fractions were collected. Initially elution was carried out with ammonium formate buffer of pH 9·8 (0·01N with respect to formate), cytosine and adenine deoxyribosides passing through together and being collected in fractions 1—8. At this point the eluant was changed to neutral ammonium formate buffer of pH 7·4 (0·1N with respect to formate) and washing was continued until a total of 28 fractions had been collected from the column and all nucleosidic material had been eluted. The course of the separation was followed by determining the optical densities of individual fractions (after suitable dilution) at 280 and 260 mμ with a Beckman spectrophotometer. The chromatogram details are summarised below:

Deoxyriboside of	Optical density ratio (280/260 mμ)	Range of fractions	No. of fractions to peak
Adenine	0·13	1—8	2
Cytosine	0·95		
Thymine	0·74	12—17	16
Uracil	0·38	18—19	
Guanine	0·61	20—28	25

Thymine deoxyriboside (*thymidine*). Fractions 9, 10, and 11 were discarded. Fractions 12—17 inclusive (1200 c.c.) were evaporated to 50 c.c. and set aside in the refrigerator. Thymine deoxyriboside (2·7 g.) separated. The mother-liquors were evaporated to dryness and ammonium formate was removed by sublimation at 45°/0·5 mm. The residue was dissolved in methanol

(50 c.c.) and set aside, giving a further crop of nucleoside (total yield, 4·2 g.). Recrystallised from methanol the nucleoside had m. p. 182—183° (Found : C, 49·8; H, 5·7; N, 11·8. Calc. for $C_{10}H_{14}O_5N_2$: C, 49·6; H, 5·8; N, 11·6%).

Uracil deoxyriboside. Fractions 18 and 19 (400 c.c.), which contained the uracil deoxyriboside contaminated with a small amount of the thymine derivative, were evaporated and ammonium formate was sublimed off. The residue was dissolved in water (35 c.c.), and the solution adjusted to pH 10—11 with a few c.c. of ammonia (4N) and run on to a smaller column (9 × 2·4 cm.) of Dowex-2 in the formate form, elution being effected with ammonium formate buffer of pH 7·4 (0·1N with respect to formate). The eluate was collected in fractions (60 c.c.); aliquots of each fraction were diluted and the optical densities at 280 and 260 mμ determined. Fractions 7 and 8 which had an optical density ratio (D_{280} : D_{260}) of 0·35—0·38, were evaporated to dryness and freed from ammonium formate by sublimation. The residue, dissolved in methanol (10 c.c.) and kept in the refrigerator, yielded uracil deoxyriboside (0·42 g.). Recrystallised from methanol the compound had m. p. 161—162° (Found : C, 47·4; H, 5·3; N, 12·3. Calc. for $C_9H_{12}O_5N_2$: C, 47·4; H, 5·3; N, 12·3%).

Guanine deoxyriboside. Fractions 20—28 inclusive (1800 c.c.) were concentrated to small bulk (300 c.c.). Guanine deoxyriboside (1·90 g.) crystallised when the solution was kept in the refrigerator. If the evaporation was carried too far the nucleoside separated in an amorphous form. The mother-liquors were evaporated to dryness, ammonium formate sublimed off, and the residue recrystallised from water (35 c.c.). A further amount of guanine deoxyriboside (0·38 g.) was thus obtained, giving a total yield of 2·28 g. The nucleoside, recrystallised from water, had no definite m. p. (Found, in material dried at 135°/1 mm. for 20 hours : C, 44·8; H, 4·6; N, 26·4. Calc. for $C_{10}H_{13}O_4N_5$: C, 45·0; H, 4·9; N, 26·2%).

Separation of the deoxyribosides of adenine and cytosine. The first eight fractions (1600 c.c.) containing the mixed adenine and cytosine deoxyribosides were combined, concentrated to small bulk (50 c.c.), and set aside overnight in the refrigerator. Adenine deoxyriboside monohydrate (3·16 g.) separated and was recrystallised from water (Found : C, 44·9; H, 5·9; N, 26·3. Calc. for $C_{10}H_{13}O_3N_5,H_2O$: C, 44·6; H, 5·6; N, 26·0%). Dried overnight at 80°/0·5 mm. the nucleoside had m. p. 189—190°.

A column (10 × 3·3 cm.) of IR C–50 resin (particle size less than 150 mesh) was washed successively with N-sodium hydroxide, water, ammonium formate buffer of pH 3·4 (N with respect to formate), and finally with water until the eluate was neutral. The mother-liquors from the above crystallisation of adenine deoxyriboside were taken to dryness and freed from ammonium formate by sublimation, and the residue was dissolved in water (50 c.c.). The solution was run on to the column, eluted with water (flow rate 250 c.c. per hour), and collected in fractions (120 c.c.) which were examined in the usual manner by optical-density determinations. Fractions 3—10 inclusive (960 c.c.), containing cytosine deoxyriboside, were combined and evaporated to dryness. Methanol (10 c.c.) was added and the solution was again evaporated under reduced pressure. The residue was dissolved in methanol (30 c.c.) and after its filtration a solution of dry hydrogen chloride in methanol (5 c.c. of 3N) was added and the whole set aside in the refrigerator. Cytosine deoxyriboside separated as its crystalline hydrochloride (1·59 g.); when heated the hydrochloride melted and decomposed at 161—164° (Found : C, 41·0; H, 5·0; N, 15·6. Calc. for $C_9H_{13}O_4N_3$,HCl : C, 41·0; H, 5·4; N, 15·9%). Fractions 12—20 inclusive (1080 c.c.) were combined and concentrated to small bulk (15 c.c.). Adenine deoxyriboside (0·11 g.) separated, making the total yield of this nucleoside 3·27 g.

Grateful acknowledgment is made to the Teknisk Videnskabelige Forskningsraad (Denmark) and the American Cancer Society for Fellowships (to W. A. and C. A. D. respectively), and to Messrs. Gea Ltd., Copenhagen, for a generous gift of deoxyribonuclease.

UNIVERSITY CHEMICAL LABORATORY, CAMBRIDGE. [*Received, March 19th,* 1952.]