Tetrahedron. Vol. 29, pp. 2293 to 2296. Pergamon Press 1973. Printed in Great Britain

THE SYNTHESIS OF CARBOXYMETHYL DERIVATIVES OF PURINES AND PYRIMIDINES AND THEIR CONDENSATION WITH NATURALLY OCCURRING MACROMOLECULES

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(*Received in the UK* 15 *February* 1973; *Accepted for publication* 12 *March* 1973)

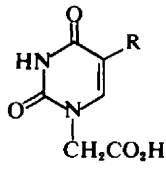
Abstract—1-Carboxymethylthymine, 1-carboxymethylcytosine and 9-carboxymethyladenine were obtained by the direct carboxymethylation of the appropriate bases. In addition a small amount of 3-carboxymethyladenine was obtained. 9-Carboxymethylhypoxanthine was obtained by deamination of 9-carboxymethyladenine. 1-Carboxymethyluracil, 1-carboxymethylthymine and 9-carboxymethylhypoxanthine were each condensed with protamine and with dextran to give water-soluble, base-substituted polymers. The 1-carboxymethylthymine-dextran showed a slow decrease in optical density at 268 nm in 2 × SSC at 20°, of 30%. This did not occur in 7M urea. 1-Carboxymethylthymine-dextran gave an additional hypochromic effect with polyadenylic acid in 2 × SSC at 4° or 14° of 13% and 9% respectively. The ratio of thymine : adenine residues at the point of maximum hypochromicity was 3 : 1. The other dextran derivatives did not show similar hypochromic effects. The 1-carboxymethylthymine-protamine gave a precipitate with polyadenylic acid probably due to electrostatic interaction.

Considerable interest has been shown in introducing the base residues typical of nucleic acids, into proteins and polypeptides. This has been achieved in several ways; the amino groups of polypeptides have been used to build up a pyrimidine ring[1] or to react with the dialdehydes derived from nucleosides or nucleotides by periodate oxidation.[2] The hydroxymethyl group of nucleosides has been converted into a carboxyl group[3,4,5] or substituted with a 5′-O-carboxymethyl group[6] and these derivatives condensed with a polypeptide or protein. These studies have all aimed at the formation of antigens which could be used to investigate the immunology of nucleic acid derivatives. However, if sufficient base residues were present in the molecule, derivatives of this type might interact with natural polynucleotides by means of base-base interactions.

This paper reports an alternative procedure for introducing some nucleic acid base residues into polypeptides, that is by means of carboxymethyl derivatives of the bases. These derivatives have also been condensed with bacterial dextran and when 1-carboxymethylthymine was used, a derivative was formed which interacted with polyadenylic acid.

RESULTS

Synthesis of carboxymethyl derivatives—1-Carboxymethyluracil (**1**, R=H) was synthesised as described by Wheeler and Liddle[7] by the action of sodium chloroacetate on uracil in aqueous alkali. We obtained 1-carboxymethylthymine (**1**, R=Me) in 71% yield by a similar procedure. This is a more direct method than that used by Wang *et al.*[8,9] to prepare this compound. 1-Carboxymethylcytosine **2** was similarly obtained, but in only 20% yield. The rest of the material was largely unchanged cytosine, but attempts to increase the yield of the required product only resulted in the formation of considerable amounts of a disubstituted cytosine compound. 9-Carboxymethyladenine (**3**) was obtained in 22% yield by treatment of adenine with sodium iodoacetate in alkali; less side product was obtained than when sodium chloroacetate was used. The major side product was 3-carboxymethyladenine (**4**) (8% yield). Both these compounds have been synthesised before[10,11] but this is a more direct route. 9-Carboxymethylhypoxanthine (**5**) was obtained by deamination of **3** with nitrous acid. Montgomery and Temple[12] obtained this compound from 6-chloropurine *via* its 9-carboxymethyl derivative. Our attempts to obtain **5** by direct carboxymethylation of hypoxanthine gave complex mixtures.



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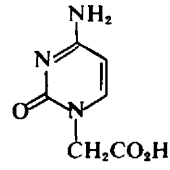

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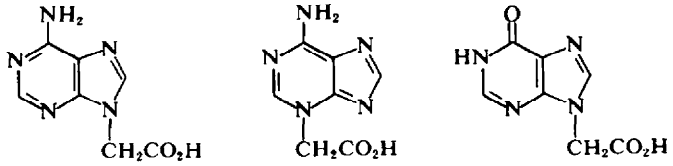
Condensation with protamine. The procedure chosen for the condensation was the mixed anhydride procedure, widely used in peptide chemistry,[13,14] which has been applied to the condensation of "uridine-5′-carboxylic" to bovine serum albumin.[4] 1-Carboxymethyluracil, 1-carboxymethylthymine and 9-carboxymethylhypoxanthine gave highly substituted derivatives when con-



densed with the protamine (see Table 1). 1-Carboxymethylcytosine and 9-carboxymethyladenine did not react satisfactorily, partly because of solubility difficulties.

Condensation with dextran. A number of methods for achieving this condensation were tried including the use of di-imides (dicyclohexylcarbodi-imide and water-soluble di-imides) but they gave very little reaction. The best results were obtained by the use of acid anhydrides. 1-Carboxymethyluracil and 1-carboxymethylthymine in the presence of trifluoroacetic anhydride gave dextran derivatives containing 21% and 33% carboxymethyl-base residues respectively. 9-Carboxymethylhypoxanthine under the same conditions reacted to only a very small extent. Using dichloroacetic anhydride, however, a compound was obtained which contained 3·7% 9-carboxymethylhypoxanthine residues. 9-Carboxymethyladenine and dextran in the presence of trifluoroacetic anhydride gave a product which contained about 5% of substituted adenine residues but the UV absorption spectrum showed in addition to the expected maximum at 258 nm another maximum at 302 nm. This indicated that the adenine residue was probably acylated. The maximum at 302 nm disappeared upon boiling with water, but this treatment removed the carboxymethyladenine residues from the dextran. Under no conditions was there a satisfactory reaction between dextran and either 1-carboxymethylcytosine or acetylated-1-carboxymethylcytosine.

Interactions of 1-Carboxymethylthymine-dextran. Because of the large number of thymine residues in this molecule (one thymine to 2·4 glucose units) it was expected that the compound would act as a polynucleotide analogue and interact with polyadenylic acid. The results shown in Fig 1 indicated, however, that with the 1-carboxymethylthymine-dextran alone in 2× SSC* at 20° there was a slow decrease in UV absorption without a change in λ_{max}. This hypochromic effect was 30%; it did not occur when 7M urea was added to the solution. A mixture of 1-carboxymethylthymine-dextran with polyadenylic acid in 2×SSC after standing at 20° showed no hypochromic effect in addition to that due to the dextran derivative alone. After prolonged standing at 4° or shorter time at 14°, however, hypochromicities of 13% and 9% respectively were obtained (Fig 2).

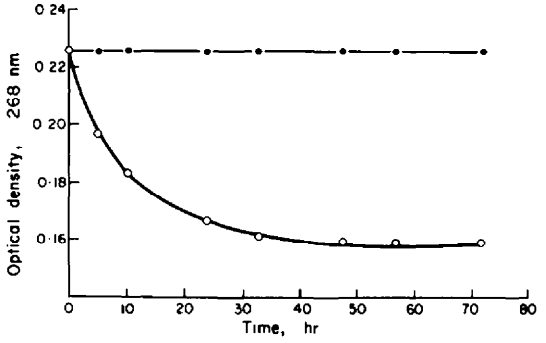


Fig 1. Optical density of 1-carboxymethylthymine-dextran at 20°: ○——○ in 2×SSC (0·03 M Na citrate, 0·3 M NaCl); ●——● in 2×SSC containing 7 M urea.

1-Carboxymethylthymine-dextran was hydrolysed only slowly in aqueous solution; 27% of the UV absorbing material became dialysable after standing at 20° in 10×SSC (pH 7·5) and then dialysing at 20° for 5 days.

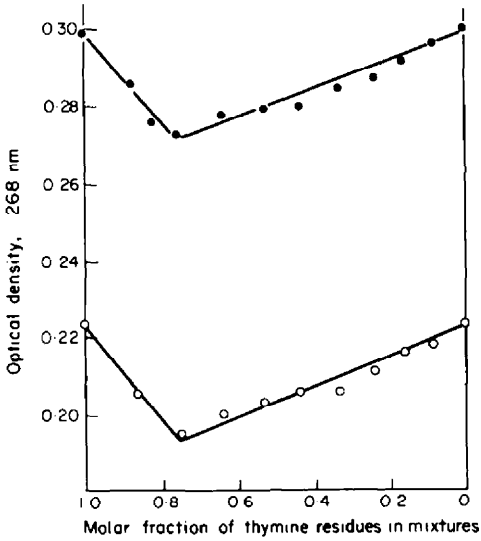


Fig 2. Optical density of mixtures of 1-carboxymethylthymine-dextran and polyadenylic acid: ○——○ after 116 hours at 4°, ●——● after 48 hours at 14°. In both cases the OD of the solution containing 1-carboxymethylthymine-dextran alone had decreased by 30% of the original.

*SSC = 0·015 M sodium citrate, 0·15 M sodium chloride.

DISCUSSION

The methods described here for the condensation of nucleic acid base residues with proteins and polysaccharides have given satisfactory results in the case of the 1-carboxymethyl derivatives of thymine and uracil. The products obtained have as high or higher degrees of substitution than examples quoted in the literature for products that have immunological activity.[1-6] 1-Carboxymethylhypoxanthine did not react so readily with dextran, but the product formed was nevertheless probably sufficiently substituted to be of use in immunological experiments. The 1-carboxymethylthymine-dextran was sufficiently highly-substituted with thymine residues to act as a polynucleotide analogue in that it interacted with polyadenylic acid in solution. In addition the analogue showed evidence of intramolecular interaction or aggregation. The fact that there was a decrease in UV absorption at high ionic strength and that this decrease did not occur in 7M urea indicated that hydrogen bonding of the type occurring in polynucleotides might be involved and that base stacking was taking place to give some type of ordered structure. In the interaction with polyadenylic acid the maximum hypochromicity was 13%. This was in addition to the self hypochromism of the thymine-containing polymer so that the hypochromism shown by the interaction of "disorganised" 1-carboxymethylthymine–dextran with polyadenylic acid would be about 43%. The rate of interaction of the two polymers at 4° was very low and this might have been due to a slow break down of intramolecular and formation of intermolecular interactions. At the point of maximum hypochromicity there was considerable excess of thymine residue compared to adenine residues (3 : 1). With the natural polynucleotides under these conditions the ratio of thymine or uracil residues to adenine residues is usually 2 : 1, and it appears that a triple stranded complex is formed. In the present case this higher ratio most probably indicates that many thymine residues are not interacting with adenine residues because of the random nature of the distribution of the thymine residues on the dextran backbone.

The 1-carboxymethyluracil and 9-carboxymethylhypoxanthine derivatives of dextran showed no evidence of interaction with polynucleotides. This was attributed to their relatively low degree of substitution. 1-Carboxymethylthymine-protamine gave a precipitate with polyadenylic acid which made it difficult to determine whether base–base interaction was taking place.

EXPERIMENTAL

Dextran (mol wt 2–2·75 × 10^5) and protamine (salmine sulphate) were obtained from BDH Ltd. The compounds obtained below were characterised by their UV absorption spectra, and elemental analysis. Paper chromatography, paper electrophoresis and tlc were used to check homogeneity.

1-Carboxymethyluracil. This was obtained from uracil (5 g) by essentially the same procedure as described by Wheeler and Liddle.[7] The product was obtained in 83% yield after being crystallised twice from water m.p. 287°, λ_{max} 263 nm, ϵ_{max} 9·7 × 10^3 (pH 2); λ_{max} 266 nm, ϵ_{max} 7·4 × 10^3 (pH 12) (Found: C, 42·5; H, 3·8; N, 16·7. Calc. for $C_6H_6N_2O_4$, C, 42·4; H, 3·5; N, 16·5%).

1-Carboxymethylthymine. Thymine (5 g), potassium hydroxide (11 g) and chloroacetic acid (7·5 g) were dissolved in water (100 ml) and the solution boiled for 45 min. The solution was cooled, adjusted to pH 3 and the resulting precipitate filtered off and recrystallised twice from water to give 1-carboxymethylthymine (5·2 g; 71% yield) m.p. 270°, λ_{max} 268 nm, ϵ_{max} 9·3 × 10^3 (pH 2); λ_{max} 271 nm, ϵ_{max} 7·3 × 10^3 (pH 12) (Found: C, 45·5; H, 4·4; N, 15·2. Calc. for $C_7H_8N_2O_4$, C, 45·7; H, 4·4; N, 15·2%).

1-Carboxymethylcytosine. Cytosine (2 g), chloroacetic acid (1·7 g) and sodium hydroxide (1·4 g) were dissolved in water (40 ml) and the solution boiled for 1 hr. The pH of the solution was then adjusted to pH 3·5 and the resulting precipitate, which contained cytosine and the required product, filtered off. It was then fractionated on a column of Dowex-1 (Cl^- form). The cytosine was removed with water and the required product with dilute acetic acid. It was obtained as a powder upon evaporation of the eluate. The powder was crystallised from water to give *1-carboxymethylcytosine* (0·61 g, 20% yield) m.p. 280°, λ_{max} 280 nm, ϵ_{max} 12·1 × 10^3 (pH 2); λ_{max} 274 nm, ϵ_{max} 8·6 × 10^3 (pH 12) (Found: C, 42·4; H, 4·0; N, 25·0. $C_6H_7N_3O_3$ requires C, 42·6; H, 4·2; N, 24·8%).

9-Carboxymethyladenine. Adenine (5·0 g), iodoacetic acid (6·9 g) and potassium hydroxide (4·1 g) were dissolved in water (100 ml) and the solution boiled for 45 min. More potassium hydroxide was added as necessary to maintain the pH above 10. The solution was cooled, adjusted to pH 2·5 the resulting precipitate filtered off and the solid dissolved in ammonia (pH 9) and the products fractionated on Dowex-1 (Cl^- form). The column was eluted with water (which removed adenine) and then acetic acid, pH 3·7 (which removed 3-carboxymethyladenine) and finally with 0·1 M sodium chloride to remove the required product. The fractions containing this were evaporated to a small volume, adjusted to pH 2·5, the resulting precipitate filtered off and crystallised from water to give 9-carboxymethyladenine (1·6 g, 22% yield), decomp 275°, λ_{max} 258 nm, ϵ_{max} 14·0 × 10^3 (pH 2); λ_{max} 261 nm, ϵ_{max} 13·7 × 10^3 (pH 12) (Found: C, 43·6; H, 3·9; N, 36·1. Calc. for $C_7H_7N_5O_2$, C, 43·5; H, 3·7; N, 36·3%).

3-Carboxymethyladenine. The acetic acid eluate obtained in the foregoing experiment was concentrated and adjusted to pH 3·3. The resulting precipitate was filtered off and crystallised from water to give 3-carboxymethyladenine (0·6 g, 8·4% yield), m.p. 314° (d), λ_{max} 276 nm, ϵ_{max} 17·2 × 10^3 (pH 2); λ_{max} 274 nm, ϵ_{max} 12·3 × 10^3 (pH 12). (Found: C, 43·2; H, 3·7; N, 36·4. Calc. for $C_7H_7N_5O_2$, C, 43·5; H, 3·6; N, 36·3%).

9-Carboxymethylhypoxanthine. 9-Carboxymethyladenine (0·42 g) was dissolved with warming in N-hydrochloric acid (250 ml), the solution cooled, sodium nitrite (1·53 g) added and the mixture kept at 20° for 18 hr. It was then evaporated to dryness and the residue extracted three times with portions (30 ml) of dimethylformamide. The dimethylformamide extract was evapor-

Table 1. Condensation of carboxymethyl derivatives with protamine

Carboxymethyl derivative	Triethylamine (mmole)	ClCOOEt (mmole)	Yield of product (mg)	Degree of substitution Base residue/arginine residue
1-Carboxymethyl-thymine (0·27 mmol)	0·27	0·30	27	1:4·1
1-Carboxymethyl-uracil (0·27 mmole)	0·82	1·21	27	1:2·2
9-Carboxymethyl-hypoxanthine (0·29 mmole)	0·95	1·4	21	1:3·6

ated to dryness and the residue crystallised from water to give 9-carboxymethylhypoxanthine m.p. 300°, λ_{max} 250 nm, ϵ_{max} $11{\cdot}0 \times 10^3$ (pH 1); λ_{max} 252 nm ϵ_{max} $12{\cdot}8 \times 10^3$ (pH 13). (Found: C, 43·3; H, 3·1; N, 28·8. Calc. for $C_7H_6N_4O_3$, C, 43·3; H, 3·3; N, 28·8%).

Condensation of carboxymethyl derivatives with protamine. The carboxymethyl compound was dissolved in dry dimethylformamide (4 ml), triethylamine in dry dimethylformamide (0·5 ml) was added and the solution cooled to 0°. A solution of ethylchloroformate in dry dimethylformamide (0·5 ml) was then added and the solution kept at 0° for 10 min. To the mixture was then added a solution of salmine sulphate (50 mg, 0·2 mmole arginine residues) dissolved in 3N-sodium hydroxide (15 ml) cooled to 0° and the mixture kept at 0° for 10 min. It was then neutralised with acetic acid, diluted with water and the solution dialysed exhaustively against distilled water. In the case of the 9-carboxymethylhypoxanthine derivative there was insoluble material remaining inside the dialysis tube and this was centrifuged off. The clear solutions of non-diffusible material were freeze-dried to give the required products. In each case they gave the characteristic UV absorption spectrum of the purine or pyrimidine derivative and from these the extent of reaction was determined. The results and conditions used are summarised in Table 1.

Condensation of carboxymethyl derivatives with dextran. (a) *1-Carboxymethylthymine.* 1-Carboxymethylthymine (50 mg) was allowed to stand with trifluoroacetic anhydride (0·5 ml) for 30 min at 20°. Freeze-dried dextran (50 mg, moisture content, 10%) and trifluoroacetic anhydride (1·5 ml) was added and the mixture boiled under reflux for 24 hr. The resulting clear, viscous liquid was cooled and mixed with an excess of sodium hydrogen carbonate solution and the resulting solution exhaustively dialysed against distilled water at 0°. The non-diffusible material was freeze-dried to give a 1-carboxymethyl derivative of dextran (67 mg, water content, 10%). It had a UV absorption spectrum typical of a 1-carboxymethylthymine derivative and it contained 33% of 1-carboxymethylthymine residues (based on dry weight).

(*b*) *1-Carboxymethyluracil.* 1-Carboxymethyluracil (200 mg), dextran (190 mg) and trifluoroacetic anhydride (1·5 ml) were heated at 55° for 4 hr and then at 70° for 2 hr in a sealed vessel. The reaction mixture was worked up as described above to give a product (118 mg, water content, 10%) which had a UV absorption spectrum typical of a 1-carboxymethyluracil derivative. It contained 21% 1-carboxymethyluracil residues (based on dry weight).

(*c*) *9-Carboxymethylhypoxanthine.* 9-Carboxymethylhypoxanthine (26 mg), dextran (24 mg), and dichloroacetic anhydride (1 ml) were heated at 60° for 22 hr. The product was worked up as described above (yield, 17 mg, water content, 10%).* The product contained 3·7% 9-carboxymethylhypoxanthine residues. No dichloroacetate groups were present.

*Some water-insoluble material was centrifuged off before freeze-drying.

Acknowledgements—The authors thank the Medical Research Council and the Cancer Research Campaign for financial assistance and the Science Research Council for research studentships (to P. L. and S. F. W.).

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