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Selective Reduction of Yeast Transfer Ribonucleic Acid with Sodium Borohydride

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A method for the selective reduction of dihydrouridine in transfer RNA from yeast has been developed. The chemistry of the reaction, which uses sodium borohydride as a reducing agent, was studied with dihydrouracil, dihydrouridine and dihydrouridine-2′(3′)-phosphate as starting materials. 3-Ureidopropane-1-ol, *N*-ribosyl-3-ureidopropane-1-ol and *N*-(2′(3′)-phosphorylribosyl)-3-ureidopropane-1-ol, respectively, were proved to be the major reduction products by spectral and chemical evidence. 3-Ureidopropane-1-ol was also synthesized by an independent route. In nucleotide mixtures and in yeast transfer RNA, dihydrouridine was reduced with sodium borohydride with high selectivity. The major nucleotides, pseudouridine and glucosyl-2-thiouridine, were found to be inert towards the reducing agent. N^4-Acetyl-cytidine was reduced to N^1-ribosyl-N^4-acetyl-3,4,5,6-tetrahydrocytidine and 4-thiouridine to *N*-ribosyl-2-oxohexahydropyrimidine under the conditions used for the reduction of RNA.

1. Introduction

Recent progress in nucleic acid chemistry has led to the complete elucidation of the primary structure of five different transfer RNA molecules. The *de novo* chemical synthesis of such molecules, however, seems out of reach for the near future (cf. Khorana, 1966, *152nd Meeting Amer. Chem. Soc.* Abstr. C-2). The selective chemical modification of single nucleotide residues provides an alternative approach for studying the functional significance of the primary, secondary and tertiary structure of transfer RNA. For a number of problems it is especially desirable that a minor nucleotide be the target of such a reaction. This allows the modification of a topographically well-defined region of the polymer for transfer RNA species with known nucleotide sequence.

Dihydrouridine has been detected as a minor component in the sequence of all the tRNA's from yeast which have been investigated in detail (alanine, serine, tyrosine, phenylalanine, valine tRNA). Two or more of these residues were always found in a topographically similar region. The similarity of the dihydrouridine region is especially striking for $tRNA_{ser\ I\ \&\ II}$ and $tRNA_{tyr}$ (Zachau, Dütting, Feldmann, Melchers & Karau, 1966) and for $tRNA_{ala}$ (Holley *et al.*, 1965) and $tRNA_{val}$ (Bayev *et al.*, 1966). It is, however, completely unknown at this time if dihydrouridine and the dihydrouridine region have a special functional role in yeast tRNA.

In the course of our studies of the biological function of dihydrouridine in RNA (Cerutti, Miles & Frazier, 1966; Rottman & Cerutti, 1966) we have developed a method for the selective reduction of these residues in yeast transfer RNA with sodium borohydride.

The heterocyclic portion of dihydrouridine is reductively cleaved and reduced with sodium borohydride to 3-ureidopropane-1-ol (Fig. 1). The chemistry of this novel reaction is described in this paper with dihydrouracil, dihydrouridine and dihydrouridine-2′(3′)-phosphate as respective starting materials. The selectivity of the reaction for dihydrouridine is then demonstrated for model systems containing the major nucleotides 2′(3′)-UMP, 2′(3′)-CMP, 2′(3′)-GMP, 2′(3′)-AMP and dihydrouridine-2′(3′)-phosphate. Finally the reaction is applied to the modification of dihydrouridine in yeast transfer RNA.

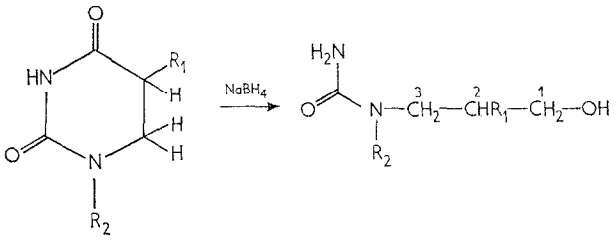

FIG. 1. Reduction of dioxo-dihydropyrimidines with sodium borohydride.

2. Materials and Methods

Dihydrouridine and 4-thiouridine disulfide were obtained from Cyclo Chemical Company, Los Angeles, California; dihydrouridine-2′(3′)-phosphate from Calbiochem, Los Angeles, California. The dihydronucleotide was also prepared by the method of Cohn & Doherty (1956) by the reduction of uridylic acid in the presence of a rhodium-on-alumina catalyst. 1-β-D-Glucopyranosyl-2-thiouracil and N^4-acetyl-cytidine were gifts of Dr J. Fox, Sloan-Kettering Institute for Cancer Research. Sodium borotritiide was obtained from New England Nuclear Corporation, Boston, Massachusetts; sodium borodeuteride from Merck & Dohm, Canada. Venom phosphodiesterase (*Crotalus adamanteus*) was a product of Worthington Biochemical Corporation, Freehold, New Jersey. Yeast tRNA, prepared by a modification of Holley's method (Holley, 1963), was obtained from Schwarz Bio-Research, Inc., Orangeburg, New York. The commercial preparation was dialyzed at 4°C for 14 hr against 0·01 M-Tris (pH 7)–0·001 M-sodium EDTA buffer and freed from salts by dialysis against water.

Ultraviolet spectra were measured on a Cary model 15 spectrometer; nuclear magnetic resonance spectra on a Varian A60A spectrometer in D_2O with sodium-3-(trimethylsilyl)-1-propane-sulfonate (in D_2O) as external standard, and mass-spectra on a A.E.I. MS9 spectrometer. Radioactivity on paper chromatograms was determined with a Vanguard automatic chromatogram scanner and, in solutions, with a Packard liquid-scintillation counter series 3000.

Abbreviations and terminology: 2′(3′)-UMP, uridine-2′(3′)-phosphate; 2′(3′)-CMP, cytidine-2′(3′)-phosphate; 2′(3′)-AMP, adenosine-2′(3′)-phosphate; 2′(3′)-GMP, guanosine-2′(3′)-phosphate; *N*-(2′(3′)-phosphorylribosyl)-3-ureidopropane-1-ol, phosphoribosylureidopropanol; [1-^{3}H]*N*-(2′(3′)-phosphorylribosyl)-3-ureidopropane-1-ol, [^{3}H]phosphoribosylureidopropanol; tRNA, transfer ribonucleic acid; *p*-DAB/HCl, *p*-dimethyl-aminobenzaldehyde/HCl (Ehrlich's reagent).

(a) *Reduction of dihydrouracil and its derivatives with sodium borohydride*

(i) *Reduction of dihydrouracil with sodium borohydride to 3-ureidopropane-1-ol*

To a solution of 520 mg dihydrouracil (4·5 m-moles) in 150 ml. of water was added 1 g of $NaBH_4$ (26 m-moles). The solution was stirred for 5 hr. The reaction mixture was then adjusted to pH 4 by addition of Dowex 50W-X8(H^+), filtered and the filtrate lyophilized. The residue was dissolved in 10 ml. methanol and the solution was evaporated to dryness

under reduced pressure. This procedure was repeated three times until all the boric acid was removed as methyl borate. The product was purified by chromatography on a silica gel column (Merck 0·02 to 0·5 mm, 2 cm × 18 cm, propane-1-ol–ammonia (concn) 8 : 1). Fractions of 3 ml. were collected and analyzed on thin-layer chromatography (silica gel G, propane-1-ol–ammonia (conc.) 8 : 1·5). Those fractions (9 to 12) containing material giving a positive color reaction with *p*-DAB/HCl without pretreatment with alkali were pooled and evaporated to dryness. The colorless oil crystallized spontaneously upon standing at room temperature *in vacuo* (yield 82%). Analytical data: m.p. 61 to 62°C. IR(KBr): 1665 cm^{-1}, 1612 cm^{-1}. NMR(D_2O): triplet centered at 3·62 p.p.m., J = 6·5 cyc./sec, (2H): OH—CH_2—; triplet at 3·17 p.p.m., J = 6·5 cyc./sec, (2H): —NH—CH_2—; five-line multiplet with center of gravity at 1·71 p.p.m., (2H): —NH—CH_2—CH_2—CH_2—OH. Mass-spectrum: mass-peak 118. Found: C, 40·57; H, 8·29; N, 23·65. Calc. for $C_4H_{10}N_2O_2$: C, 40·66; H, 8·53; N, 23·71.

3-Ureidopropane-1-ol was also obtained in small yields if a suspension of β-ureidopropionic acid in tetrahydrofuran was reduced with $LiAlH_4$. The reductive cleavage of dioxo-dihydropyrimidines is accompanied by the loss of the ultraviolet absorption in the region of 230 to 240 mμ (0·1 N-NaOH) and can therefore easily be followed spectrophotometrically. The formation of the reduction product on the other hand can be measured with the Archibald assay (Archibald, 1944). The ring-opened product yields a positive assay without pretreatment with alkali, due to its free ureido-function (Fig. 2). Ureido alcohols are also readily identified and distinguished from dihydropyrimidines on paper chromatograms and on thin-layer chromatography. In contrast to dioxo-dihydropyrimidines they yield an intense yellow color with *p*-DAB/HCl without pretreatment with alkali.

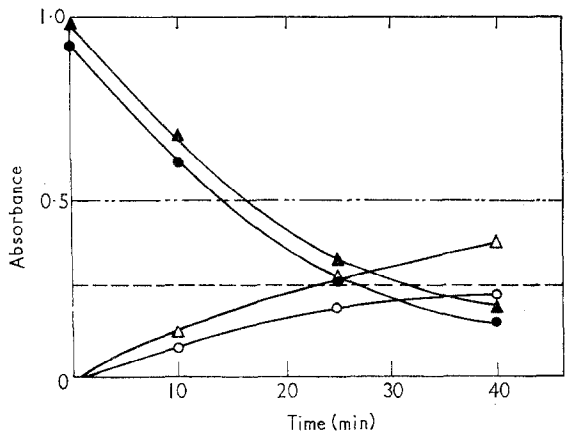

FIG. 2. The reduction of dihydrouracil and dihydrouridine with $NaBH_4$.

—▲—▲—, Disappearance of dihydrouracil, A_{228} (0·1 N-NaOH); —△—△—, formation of 3-ureidopropane-1-ol, Archibald assay (A_{490}); — — —, 100% 3-ureidopropane-1-ol; —●—●—, disappearance of dihydrouridine, A_{234} (0·1 N-NaOH); —○—○—, formation of *N*-ribosyl-3-ureidopropane-1-ol, Archibald assay (A_{490}); — — — —, 100% *N*-ribosyl-3-ureidopropane-1-ol.

(ii) *Reduction of dihydrouridine with sodium borohydride to* N-*ribosyl-3-ureidopropane-1-ol*

Dihydrouridine (2 m-moles) in 250 ml. water was exposed in the dark to 20 m-moles of $NaBH_4$ for 5 hr. The reaction was stopped by the addition of Dowex 50W-X8(H^+) with vigorous stirring until the pH dropped to 4. Boric acid was removed as before. The reduction product was not purified further. It was characterized by thin-layer chromatography (microcrystalline cellulose, formic acid–*tert.*-amylalcohol–water, 3 : 3 : 1) and paper chromatography (Whatman 3 MM, propane-1-ol–ammonia (conc.)–water, 55 : 10 : 35).

The reduction products developed an intense yellow color with *p*-DAB/HCl on the chromatograms. A minor contaminant with a smaller R_F-value in both systems gave a reddish color reaction with the same reagent after heating for 10 min at 60°C. This side product was not investigated further. Analytical data: IR(KBr): 1660 cm^{-1}; NMR(D_2O): triplet at 3·60 p.p.m., J = 6·5 c/s: OH—CH_2—; triplet at 3·30 p.p.m., J = 6·5 c/s, (2H): —NR—CH_2—; five-line multiplet with center at 1·82 p.p.m. (2H): —NR—CH_2—CH_2—CH_2—OH.

(iii) *Reduction of dihydrouridine-2′(3′)-phosphate with sodium borohydride to* N-(2′(3′)-*phosphorylribosyl)-3-ureidopropane-1-ol*

Dihydrouridine-2′(3′)-phosphate (1 m-mole) was reduced with $NaBH_4$ (10 m-moles) in 150 ml. water under the conditions described in section (ii) above. After removal of boric acid the reaction mixture was applied to Dowex 1-X8($HCOO^-$) (200 to 400 mesh, 0·9 cm × 20 cm). The column was first washed with 50 ml. of water. Small amounts of 3-ureidopropane-1-ol were detected in the water washings after evaporation to dryness. The elution was continued with 0·2 N-formic acid (2 to 3 ml./min, 10-ml. fractions). Portions of the fractions were analyzed with the orcinol assay for ribose and the Archibald assay (Archibald, 1944), without pretreatment with alkali, for free ureido functions. Fractions 65 to 90, which contained orcinol and Archibald-positive material, were pooled and evaporated to dryness. The material contained in these fractions was further analyzed by paper chromatography and thin-layer chromatography under the conditions described in section (ii) above and by paper electrophoresis (Whatman no. 41, 0·05 M-$NaHCO_3$–Na_2CO_3, 36 v/cm, 45 min). Again a minor contaminant with a reddish color reaction with *p*-DAB/HCl was detected after heating.

(1) Preparative acid hydrolysis of the sodium borohydride reduction product(s) of dihydrouridine-2′(3′)-phosphate: A solution of 310 mg reduction product in 3 ml. N-H_2SO_4 or 3 ml. 2N-HCl was kept at 65°C for 5 hr. The reaction was stopped by the addition of 30 ml. 0·1 N-$Ba(OH)_2$ if dilute sulfuric acid was used for the hydrolysis. The $BaSO_4$ was separated by centrifugation and the supernatant lyophilized. If dilute HCl was used the reaction mixture was evaporated to dryness under reduced pressure. The residue was taken up in a small amount of water and a mixture of propane-1-ol–ammonia (concn) 10 : 1 and applied to a silica gel column (silica gel, Merck, 0·05 to 0·2 mm, 2 cm × 19 cm). The column was eluted with propane-1-ol–ammonia (concn) 10 : 1 and fractions of 3 ml. were taken. Fractions 14 to 22 contained two products with a positive reaction with *p*-DAB/HCl on thin-layer chromatography (silica gel G, propane-1-ol–ammonia (conc.) 8 : 1) and R_F-values identical and slightly smaller than 3-ureidopropane-1-ol. After prolonged elution (approx. 500 ml.), fractions containing D-ribose and D-ribose-2′(3′)-phosphate were collected. The identity of these products was assessed by comparison with authentic materials by thin-layer chromatography under the above conditions (development of the spots with anisaldehyde–H_2SO_4 (Stahl & Kaltenbach, 1961)). The two *p*-DAB/HCl-positive products were separated on a second silica gel column (0·05 to 0·2 mm, 1·5 cm × 16 cm; elution with propane-1-ol–ammonia (concn) 10 : 1; 2-ml. fractions). Fractions 25 to 35 contained 65 mg of a product which was identical in all respects to authentic 3-ureidopropane-1-ol (cf. section (i) above). In fractions 37 to 42 a product with a dark yellow color reaction with *p*-DAB/HCl and a smaller R_F-value than 3-ureidopropane-1-ol on thin-layer chromatography (for conditions see above) was detected. The structure of this minor product, which crystallized spontaneously after evaporation of the solvents, is now being elucidated.

(2) The reduction of dihydrouridine-2′(3′)-phosphate with sodium borotritiide: Dihydrouridine-2′(3′)-phosphate (40 μmoles) was taken up in 0·5 ml. water and the pH of the solution adjusted to 7 with 0·1 N-NaOH. After exposure to [^{3}H]$NaBH_4$ (threefold excess, 2 mc/m-mole) for 2 hr in the dark, the reaction mixture was acidified (pH 4) with N-formic acid and lyophilized. The residue was taken up in methanol and the solution evaporated to dryness on the rotatory evaporator. This procedure was repeated 5 times.

(3) Rate of hydrolysis of [1-^{3}H]*N*-(2′(3′)-phosphorylribosyl)-3-ureidopropane-1-ol: Samples (0·8 μmole) of the tritiated reduction product from (2) above were treated with 0·5 N-H_2SO_4 for 0 min and 1, 2, 3, 4 hr at 65°C. The reaction was stopped by the addition

of an equivalent amount of 0·1 N-$Ba(OH)_2$ and the $BaSO_4$ separated by centrifugation. A portion of the supernatant was lyophilized and the residue taken up in a small amount of water and quantitatively applied to Whatman no. 41 paper for electrophoresis at pH 10·3 (0·05 N-Na_2CO_3–$NaHCO_3$ buffer, 36 v/cm, 45 min). In all experiments authentic samples of 3-ureidopropane-1-ol, *N*-(2′(3′)-phosphorylribosyl)-3-ureidopropane-1-ol, dihydrouridine-2′(3′)-phosphate and *N*-(2′(3′)-phosphorylribosyl)-β-ureidopropionic acid were applied to the paper and their mobilities compared to those of the radioactive components of the hydrolysis mixture. The electropherograms were first analyzed with a Vanguard automatic chromatogram scanner and then cut into 2 cm × 4 cm strips. The strips were eluted with 1 ml. water for 12 hr. The radioactivity was determined by taking samples of the eluates from each strip in a Packard liquid-scintillation counter using Bray's solution (Bray, 1960).

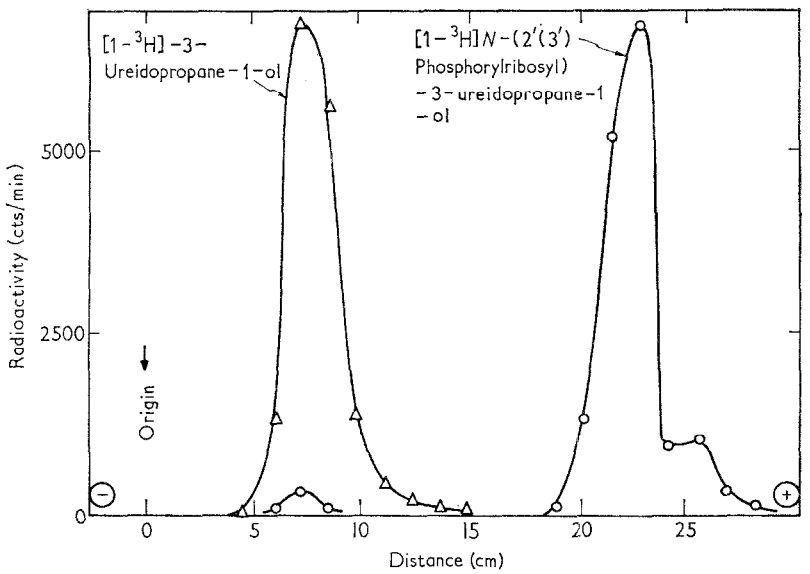

FIG. 3. Distribution of the radioactivity before —○—○— and after —△—△— acid hydrolysis of [1-³H]*N*-(2′(3′)-phosphorylribosyl)-3-ureidopropane-1-ol as measured in the eluates of the electropherogram. (For experimental details see Materials and Methods, section (a)(iii)(3).)

The pseudo first-order rate of hydrolysis of the *N*-glycosidic bond in *N*-(2′(3′)-phosphorylribosyl)-3-ureidopropane-1-ol was determined with radioactive starting material ([1-³H]*N*-(2′(3′)-phosphorylribosyl)-3-ureidopropane-1-ol)†, obtained from the reduction of dihydrouridine-2′(3′)-phosphate with [³H]$NaBH_4$. It was found to be approximately 2×10^{-2} min^{-1} at 65°C in 0·5 N-H_2SO_4. In these experiments the hydrolysis mixture was separated by paper electrophoresis and the radioactivity measured in the eluates of the electropherogram. After 4 hr hydrolysis more than 90% of the radioactivity had shifted from the location of *N*-(2′(3′)-phosphorylribosyl)-3-ureidopropane-1-ol on the electropherogram to the location of 3-ureidopropane-1-ol. At each stage of the hydrolysis the loss of counts at the location of *N*-(2′(3′)-phosphorylribosyl)-3-ureidopropane-1-ol could be completely accounted for by the gain of counts in 3-ureidopropane-1-ol (Fig. 3). No cleavage of the *N*-glycosidic bond in *N*-(2′(3′)-phosphorylribosyl)-3-ureidopropane-1-ol was observed upon treatment with 0·3 N-KOH for 18 hr at 37°C.

† The position of the isotope follows from our studies of the reduction of dihydrouracil with $NaBD_4$: the NMR-signal for the two methylene protons at C_1 (at 3·62 p.p.m.) disappeared if the reduction was carried out with $NaBD_4$ instead of $NaBH_4$ (cf. also Ballé, Cerutti & Witkop, 1966).

(b) *Selective reduction of dihydrouridylic acid with sodium borotritiide in nucleotide mixtures*

Mixtures of the major nucleotides (0·4 μmole of 2′(3′)-UMP, 2′(3′)-CMP, 2′(3′)-GMP and 2′(3′)-AMP) in the presence or absence of an equimolar amount of dihydrouridine-2′(3′)-phosphate in water were incubated with a sixfold excess of [^{3}H]$NaBH_4$ (4 mc/m-mole) for 0, 0·5, 2, 6 and 9 hr. Before addition of the reducing agent the pH of the solution was adjusted to 10 with 0·1 N-NaOH. The reactions were stopped by acidification with 0·5 N-HCl (pH 4) and the components of the reaction mixture quantitatively separated by ion-exchange chromatography on Dowex 50W-X4(H^+) and Dowex 1-X8($HCOO^-$) following the procedure of Katz & Comb (1963). After measuring the ultraviolet spectra, the fractions were lyophilized. The residues were taken up in 1 ml. of water and the radioactivity determined in 0·25-ml. portions as outlined in (a)(iii)(3) above. The remaining solutions were relyophilized, the residues dissolved in 50 μl. of water and the samples quantitatively applied to Whatman no. 41 paper. The contents of the fractions were compared to authentic samples of 2′(3′)-UMP, 2′(3′)-GMP, 2′(3′)-CMP, 2′(3′)-AMP and *N*-(2′(3′)-phosphorylribosyl)-3-ureidopropane-1-ol by electrophoresis at pH 10·3 (for conditions see section (a)(iii)(3)). The electropherogram of the fractions containing [1-^{3}H]*N*-(2′(3′)-phosphorylribosyl)-3-ureidopropane-1-ol were cut into 2 cm × 4 cm strips, the cuts eluted with 1 ml. water for 12 hr and the ultraviolet spectra and the radioactivity measured in the eluates.

In a number of experiments the fractions containing 2′(3′)-UMP and [1-^{3}H]*N*-(2′(3′)-phosphorylribosyl)-3-ureidopropane-1-ol were taken up in 0·5 N-H_2SO_4 or N-HCl and heated for 5 hr at 65°C. The samples were neutralized with 5 ml. 0·1 N-$Ba(OH)_2$, centrifuged and the supernatant lyophilized. If dilute HCl was used in the hydrolysis, the reaction mixture was evaporated to dryness under reduced pressure. The residues were taken up in a small amount of water and applied to Whatman no. 41 for electrophoresis at pH 10·3 (for conditions see section (a)(iii)(3)). Authentic samples of 3-ureidopropane-1-ol, *N*-(2′(3′)-phosphorylribosyl)-3-ureidopropane-1-ol and 2′(3′)-UMP were always included on the electropherogram for comparison. The electropherograms were cut into strips, eluted and analyzed as described above.

(c) *Selective reduction of dihydrouridine in yeast tRNA*

(i) *Incorporation of tritium into yeast tRNA upon treatment with sodium borotritiide*

Samples of 0·5 mg yeast tRNA in 0·3 ml. of water (0·14 M in Na^+ from [^{3}H]$NaBH_4$), in 0·3 ml. 0·02 M-Mg^{2+} (+ 0·14 M in Na^+ from [^{3}H]$NaBH_4$) or in 0·3 ml. 0·1 M-boric acid–KCl–NaOH buffer, pH 9·0 (0·24 M in Na^+ and 0·1 M in K^+) were exposed to 17 μmoles [^{3}H]$NaBH_4$ (10 mc/m-mole) in the dark. In experiments where no buffer was used the pH of the solutions rose to 9·8 to 10·1 due to the decomposition of $NaBH_4$. In the buffered experiments a shift of the pH from 9·0 to 9·4 was observed. [^{3}H]$NaBH_4$ was added again at 6-hr intervals in experiments with prolonged exposure to the reducing agent. The reaction was stopped by acidification (pH 4) with 0·1 N-HCl and the samples were allowed to stand for 10 min at room temperature. After addition of 50 μl. 20% potassium acetate (pH 5) and 0·5 mg RNA as carrier, the RNA was precipitated with 3 vol. ethanol (95%, −15°C). The samples were kept at −15°C for 10 min, centrifuged at 0°C and the supernatant solution discarded. The residues were taken up in 0·5 ml. water and the ethanol precipitation repeated 3 times as outlined above. Finally the RNA was precipitated with 4 vol. 10% trichloroacetic acid (0°C). The samples were collected on Millipore filters and the precipitates washed with 10 ml. cold 10% trichloroacetic acid. The filters were dried and added to 10 ml. toluene–PPO–POPOP counting solution and counted in a Packard liquid-scintillation counter.

In some experiments the treated tRNA samples were desalted after acidification by a passage through a 1 cm × 8 cm column of Sephadex G25 (10^{-3} M-Tris, pH 7·0) and the ultraviolet absorbing fractions (260 mμ) were collected and lyophilized. The residues were taken up in 0·5 ml. water and further purified by repeated ethanol precipitation as outlined above.

(ii) *Isolation and characterization of [1-^3H]N-(2′(3′)-phosphorylribosyl)-3-ureidopropane-1-ol from yeast tRNA exposed to sodium borotritiide*

Yeast tRNA was treated with [^{3}H]$NaBH_4$, the reaction stopped and the samples desalted on Sephadex G25 as described in the preceding section (i). The RNA was then digested with 0·5 ml. 0·3 N-KOH at 37°C for 18 hr or by treatment with venom phosphodiesterase (*Crotalus adamanteus*) for 24 hr at 37°C in 0·1 M-Tris (pH 8·9) and 0·02 M-$CaCl_2$ (Nihei & Cantoni, 1963). If alkaline digestion was used the samples were neutralized with 25 μl. of cold 6 N-$HClO_4$ and the $KClO_4$ separated by centrifugation. The supernatant solution was then brought to 0·05 N in HCl by the addition of 0·5 ml. 0·1 N-HCl and quantitatively applied to a Dowex 50W-X4(H^+) column. In experiments using enzymic digestion, dilute HCl was added to bring the solution to 0·05 N in HCl and the samples were applied directly to the ion exchange column. The separation of the digests was then carried out as described by Katz & Comb (1963). The further analysis of the fractions followed the procedures outlined in section (b) for the model systems.

3. Results

(a) *Selective reduction of dihydrouridylic acid with sodium borotritiide in the presence of the major nucleotides*

After studying the reduction of dihydrouracil, dihydrouridine and dihydrouridine-2′(3′)-phosphate with sodium borohydride (see section (a) of Materials and Methods) it was considered a necessary prerequisite to investigate the selectivity of the reaction for dihydrouridine in the presence of the major nucleotides before the method was applied to yeast transfer RNA.

Dihydrouridine-2′(3′)-phosphate was reduced with highest selectivity in the presence of the major nucleotides at pH 10. The method of Katz & Comb (1963) was used for the separation of the nucleotides on Dowex 50W-X4(H^+) and Dowex 1-X8($HCOO^-$) after exposure to [^{3}H]$NaBH_4$. The ultraviolet spectra of the fractions obtained from experiments with or without dihydrouridine-2′(3′)-phosphate and with 0 to 9 hours exposure to [^{3}H]$NaBH_4$, were found to be qualitatively and quantitatively identical. Comparison of the contents of each fraction with authentic samples by paper electrophoresis further proved the complete inertness of the major nucleotides towards [^{3}H]$NaBH_4$ under conditions which lead to extensive reduction of dihydrouridine-2′(3′)-phosphate. Only a small percentage of the total radioactivity was detected in the fractions containing 2′(3′)-GMP (2·5%), 2′(3′)-CMP (0·5%) and 2′(3′)-AMP (0·5%), whereas 96·5% was found in the fraction containing 2′(3′)-UMP and [^{3}H]phosphoribosylureidopropanol. Fast initial incorporation of tritium into the fraction containing 2′(3′)-UMP and [^{3}H]phosphoribosylureidopropanol was observed during the first half hour of exposure to the reducing agent. The tritium uptake then went on at a rather small rate but did not come to a complete stop. The localization of the radioactive label in phosphoribosylureidopropanol rather than 2′(3′)-UMP was demonstrated after electrophoretic separation of the two components at pH 10·3. Distinct peaks for the absorption at 260 mμ and for the radioactivity were found in the eluates of the electropherogram. The mobilities of the peaks aligned with authentic 2′(3′)-UMP and phosphoribosylureidopropanol, respectively. Selective acidic hydrolysis of phosphoribosylureidopropanol in the presence of 2′(3′)-UMP followed by separation of the products by paper electrophoresis led to a complete shift of the radioactivity from the location of phosphoribosylureidopropanol to the location 3-ureidopropane-1-ol.

(b) *Behavior of the minor nucleotides towards sodium borohydride*

In a number of cases the minor nucleotides of tRNA have been found to be more susceptible towards an organic reagent than their major nucleotide precursors (e.g. pseudouridine towards acrylonitrile, Chambers, 1965; Ofengand, 1965; Yoshida & Ukita, 1965; photolysis of pseudouridine, Tomasz & Chambers, 1964; oxidation of 4-thiouridine with iodine, Carbon, Hung & Jones, 1965). It was therefore considered to be important to include a study of the behavior of the minor nucleotides towards sodium borohydride.

There is no reason to believe that the methylated bases of tRNA should be susceptible to $NaBH_4$ and no experiments with these compounds have been carried out. Pseudouridine and glucosyl-2-thiouracil were found to be resistant under the conditions used for the reduction of dihydrouridine in RNA.

4-Thiouridine reacts quite readily with $NaBH_4$ with concomitant loss of the absorption at 331 mμ (pH 2) to *N*-ribosyl-2-oxohexahydropyrimidine (cf. Fox & Van Praag, 1960, *N*-(2′,3′,5′-tri-O-benzoyl-β-D-ribosyl)-2-oxohexahydropyrimidine was obtained by reduction of benzoylated 4-thiouridine with Raney nickel). The implications of the reduction of 4-thiouridine for the modification of *Escherichia coli* tRNA are under investigation.

N^4-Acetyl-cytidine is partially deacetylated to cytidine and partially reduced to N^1-ribosyl-N^4-acetyl-3,4,5,6-tetrahydro-cytidine in the presence of $NaBH_4$ (pH 10). N^4-Acetyl-cytidine is also readily deacetylated at pH 9 or 10 in the absence of $NaBH_4$ (half completion at pH 9, 37°C in 5·5 hours; half completion at pH 10, room temperature in 1 hour).

Details of the chemistry of these novel reactions of 4-thiouridine and N^4-acetyl-cytidine with $NaBH_4$ and their implications for the modification of these residues in RNA will be discussed elsewhere.

(c) *Selective reduction of dihydrouridine in yeast transfer RNA*

Incorporation of tritium into yeast tRNA upon treatment with sodium borotritiide and isolation of [1-^{3}H]*N*-(2′(3′)-phosphorylribosyl)-3-ureidopropane-1-ol from the treated RNA:

Extensive incorporation of tritium into yeast tRNA was observed upon treatment with excess [^{3}H]$NaBH_4$ at pH 9·8 to 10·1 (0·14 M-Na^+) or at pH 9·0 to 9·4 (0·24 M-Na^+ and 0·1 M-K^+). RNA which had been exposed to [^{3}H]$NaBH_4$ up to 24 hours to the reducing agent could be purified to constant specific activity (counts/O.D., min) by chromatography on Sephadex G25 and repeated ethanol precipitation.

In a number of experiments the RNA was digested with alkali or venom phosphodiesterase after exposure to [^{3}H]$NaBH_4$ for 0 to 12 hours and the nucleotides separated by ion exchange chromatography on Dowex 50W-X4(H^+) and Dowex 1-X8($HCOO^-$) according to Katz & Comb (1963). Qualitatively and quantitatively identical ultraviolet spectra in corresponding fractions were obtained from tRNA which had been exposed or had not been exposed to [^{3}H]$NaBH_4$. The following distribution of the radioactivity was observed in the fractions from experiments with two-hour exposure to the reducing agent: 84 to 86% in the fractions containing 2′(3′)-UMP and [^{3}H]phosphoribosylureidopropanol, 10 to 12% in the fractions containing 2′(3′)-GMP and residual [^{3}H]phosphoribosylureidopropanol, 3% in each of the fractions contain-

ing 2′(3′)-AMP and 2′(3′)-CMP, respectively. The components of the fractions containing [^{3}H]phosphoribosylureidopropanol and 2′(3′)-UMP or 2′(3′)-GMP were separated by paper electrophoresis and it was demonstrated that the radioactive label was contained in phosphoribosylureidopropanol rather than in 2′ (3′)-UMP or 2′ (3′)-GMP. In all experiments distinctive peaks for the radioactivity and for the absorption at 260 mμ were obtained in the eluates from the electropherograms (Fig. 4). The electrophoretic mobility of the peak for the radioactivity was found to be equal to the mobility of authentic phosphoribosylureidopropanol and the peak for the absorption at 260 mμ with 2′(3′)-UMP or 2′(3′)-GMP, respectively. Acid treatment of the fractions containing 2′(3′)-UMP and [^{3}H]phosphoribosylureidopropanol (i.e. selective hydrolysis of the *N*-glycosidic bond in [^{3}H]phosphoribosylureidopropanol) lead to the shift of only 70 to 80% of the radioactivity from the location of phosphoribosylureidopropanol to the location of 3-ureidopropane-1-ol on the electropherogram. This is in contrast to the experiments with nucleotide mixtures. The possibility that the modification product of a second, minor nucleotide could be present in these fractions has to be considered. Such a product would be characterized by the lack of ultraviolet absorption in the 260 mμ region, similar mobility to that of phosphoribosylureidopropanol on paper electrophoresis at pH 10·3 and resistance to mild acid hydrolysis.

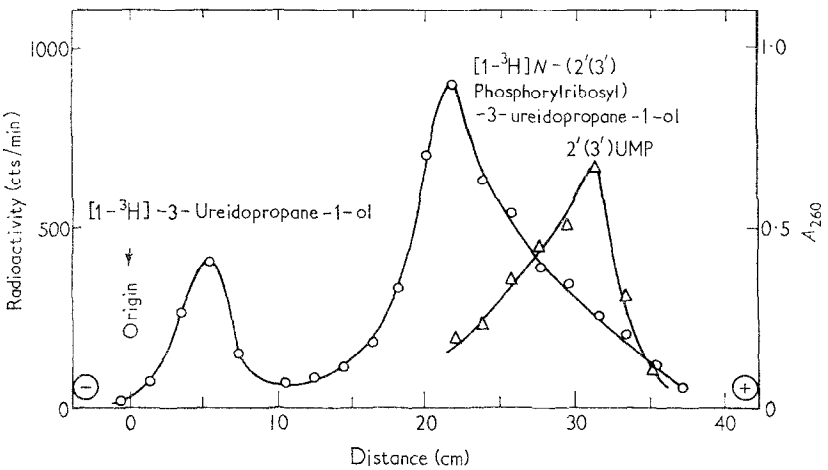

FIG. 4. Electrophoretic separation of the components in the 0·05 N-HCl eluates from Dowex 50W-X4(H^+) of the alkaline digests of yeast tRNA, exposed to [^{3}H]$NaBH_4$. (For experimental details see Materials and Methods, section (c) (ii)).
—○—○—, Radioactivity; —△—△—, absorbance, A_{260}.

The time-course of the incorporation of tritium into the fraction containing 2′(3′)-UMP and [^{3}H]phosphoribosylureidopropanol is presented in Fig. 5 (for experiments at pH 9·8 to 10·1, 0·14 M-Na^+). After a fast uptake of tritium during the first hour of exposure to [^{3}H]$NaBH_4$, the reaction slowed down but did not come to a complete stop even after prolonged treatment with the reducing agent (24 hours). No significant effect of 0·02 M-Mg^{2+} was observed in these experiments. The rate for the initial uptake of tritium by the polymer and the rate of the disappearance of dihydrouridine-2′(3′)-phosphate (A_{233}, 0·1 N-NaOH) upon treatment with the reducing agent, were found to be of equal magnitude.

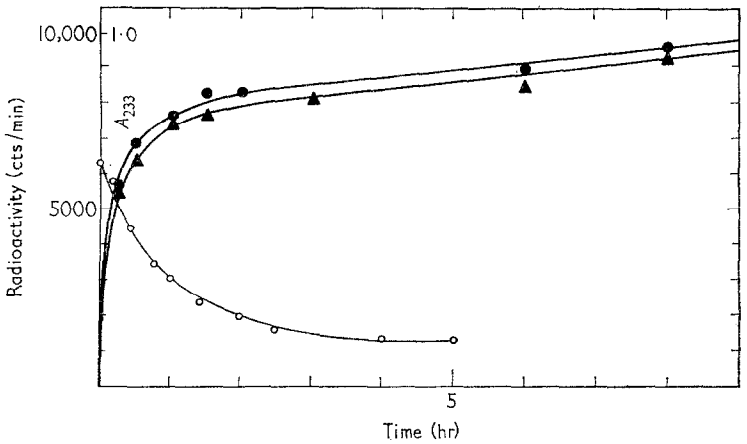

FIG. 5. Time-course of incorporation of tritium into the first fraction (containing 2′(3′)-UMP and [1-^{3}H]*N*-(2′(3′)-phosphorylribosyl)-3-ureidopropane-1-ol) of the Dowex 50W-X4(H^+) column used in the separation of the alkaline digests of yeast tRNA exposed to [^{3}H]$NaBH_4$ at pH 10. (For experimental details see Materials and Methods, section (c) (ii)).
—●—●—, Yeast tRNA in 0·14 M-Na^+; —▲—▲—, yeast tRNA in 0·14 M-Na^+–0·02 M-Mg^{2+}; —○—○—, dihydrouridine-2′(3′)-phosphate–$NaBH_4$, A_{233} (0·1 N-NaOH).

4. Discussion

Little is known about the biological role of the minor nucleotides and their participation in the different active sites of tRNA. The selective chemical modification of minor nucleotides in tRNA offers an approach for the study of this problem.

Carbon *et al.* (1965) have demonstrated that oxidation of the thiopyrimidine nucleotides in *E. coli* tRNA with iodine leads to a partial loss of the amino acid acceptor function. A change in codon recognition in the Leder & Nirenberg binding assay was observed for lysine tRNA from liver upon oxidation with iodine (Carbon & Hung, 1966). Treatment of *E. coli* tRNA with acrylonitrile, which reacts preferentially with pseudouridine and 4-thiouridine and less readily with uridine and inosine (Chambers, 1965; Ofengand, 1965; Yoshida & Ukita, 1965), resulted in a rapid first-order loss of the acceptor activity for all the amino acids tested (Ofengand, Chu & Schaefer, 1966; Rake & Tener, 1966).

The reduction of dihydrouridine with sodium borohydride described in this paper offers a method for the modification of these residues in RNA with high selectivity. N^4-Acetyl-cytidine and possibly the N^6-(*N*-formylaminoacyl)-adenosines are so far the only additional components of yeast tRNA shown to be susceptible to sodium borohydride under the conditions used for the modification of dihydrouridine.

N^4-Acetyl-cytidine is also readily deacetylated at pH 9 or 10 in the absence of sodium borohydride. A similar behavior towards mild alkali was observed for the N^6-(*N*-formylaminoacyl)-adenosines (Hall & Chheda, 1965). Discharging of tRNA by incubation at pH 10·3 (e.g. for three hours at 36°C), digestion with venom phosphodiesterase at pH 9 (e.g. for 24 hours at 37°C) and alkaline digestion of RNA (e.g. 0·3 N-KOH for 18 hours at 37°C) are therefore questionable procedures if these residues are to be preserved.

In experiments with nucleotide mixtures containing dihydrouridine-2′(3′)-phosphate and with yeast tRNA, a slow uptake of tritium continues after the initial rapid reaction, even after prolonged exposure to sodium borotritiide. It is presently not understood if this reaction represents a slow reductive degradation of phosphoribosylureidopropanol or of an unknown side-product of the dihydrouridine-2′(3′)-phosphate reduction. The structure elucidation of a second, minor component detected in the hydrolysis mixture of the reduction products of dihydrouridine-2′(3′)-phosphate could be of importance in this connection. For the case of tRNA the slow reaction of an unknown minor component with sodium borohydride has also to be considered. The reduction of N^4-acetyl-cytidine proceeds at a higher rate than the reduction of dihydrouridine and can hardly be the basis for the continuing tritium incorporation.

Dihydrouridine in polymers is not able to form a stable base-pair with a matching counterpart, probably largely due to its non-planar structure (Cerutti *et al.*, 1966). In models of the secondary structure of the fully or partially elucidated tRNA's from yeast, it is generally assumed that the dihydrouridine region forms a loop of ten to twelve nucleotides ($tRNA_{ala}$, Holley *et al.*, 1965; $tRNA_{ser\ I\ \&\ II}$ and $tRNA_{tyr}$, Zachau *et al.*, 1966). This hypothesis obtains support from our experiments. No significant difference in the rates of the tritium uptake by tRNA was found in the presence or absence of 0·02 M-Mg^{2+} upon treatment with sodium borotritiide.

The selective modification of dihydrouridine provides a method for the detection and possible quantitation of these residues in different RNA species and oligonucleotides. This analytical aspect is being investigated in this laboratory using highly purified yeast alanine tRNA as a standard.

Our studies on the effects of $NaBH_4$ reduction on the biological activity of purified $tRNA_{ser}$ and $tRNA_{ala}$ will be published elsewhere.

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