# Analysis of Nucleic Acid Derivatives at the Subnanomolar Level. II. Quantitative Analysis of Ribose Derivatives by Tritium Labeling[1,2]

Quantitative and sequential analysis of unlabeled ribonucleic acids and their derivatives at the subnanomolar level has not been possible so far due to the lack of sufficiently sensitive procedures. This communication reports on a new procedure for ultramicroanalysis of ribose derivatives, which is considerably more sensitive than conventional methods. The procedure involves oxidation of the ribose moiety of nucleosides, followed by reduction of the resulting nucleoside dialdehydes with tritiated sodium borohydride to nucleoside trialcohols. These are separated chromatographically and evaluated by liquid scintillation counting.

*Experimental.* $NaBH_4$-($^3H$) (sp. act. 200 mC/mmole) was a product of New England Nuclear Corp. All operations involving this compound were carried out under a hood. 100 mC were dissolved in 1 ml 0.1 *N* NaOH and stored at −90 °C. Carrier nucleoside trialcohols were prepared according to a modification of the procedure of KHYM and COHN[4].

Periodate oxidations and borohydride reductions were carried out in the dark. Nucleosides were treated with a 2-fold molar excess of $NaIO_4$, the resulting dialdehydes were reduced with a 20- to 25-fold molar excess of $NaBH_4$-($^3H$), and the samples were prepared for analysis as described in footnote [a] of the Table. 1-µl aliquots were applied to Eastman No. 6064 cellulose sheets at 2.5 cm from the lower edge. After the starting areas had been dried in a stream of hot air, the chromatograms were developed by ascending irrigation with ethyl acetate–*n*-butanol–isopropanol–7.5 *N* aqueous ammonium hydroxide (3:1:2:2, by vol.) until the solvent front reached 12–14 cm from the origin. The solvent was prepared fresh for each run (no tank saturation). After the chromatograms had been dried thoroughly in a stream of hot air, the separated compounds (Rf values: 0.56 for AR'[5], 0.45 for CR', 0.34 for UR' and 0.23 for GR') were located under a short-wave UV-light. Rectangles of equal size containing the compounds were cut from the sheets and transferred, layer side down, to counting vials. Elution was carried out in the vial with 500 µl water for 60 min at room temperature. 100 µl 4 *N* ammonium hydroxide and 11 ml scintillation fluid[6] were added, the vials were shaken, and the radioactivity was measured by liquid scintillation counting. For calculating the composition of the mixture, it was assumed that the resulting radioactivity of each derivative was directly proportional to the concentration of the corresponding nucleoside in the original solution. Preliminary experiments had shown this to be the case.

*Results.* The Table summarizes data from 5 typical experiments, in which a ribonucleoside mixture of the same composition was subjected to the labeling procedure at 5 different concentrations ranging from 0.00002 to 0.005 *M*. The composition of the original nucleoside mixture (expressed as nmole of individual nucleoside/nmole

[1] This work was supported by grants-in-aid from the U.S. Atomic Energy Commission (No. AT(30-1)2643), the U.S. Public Health Service (No. CA 05018), and the American Cancer Society (No. E-51-J). This is publication No. 1325 of the Cancer Commission of Harvard University.

[2] The first paper of this series[3] described a procedure for detecting subnanomolar quantities of mono- and oligonucleotides on PEI-cellulose thin layers.

[3] K. RANDERATH, Analyt. Biochem. *21*, 480 (1967).

[4] J. X. KHYM and W. E. COHN, J. Am. chem. Soc. *82*, 6380 (1960).

[5] Abbreviations: AR, adenosine; CR, cytidine; UR, uridine; GR, guanosine; AR', CR', UR', GR', the corresponding nucleoside trialcohol derivatives obtained by periodate oxidation and borohydride reduction.

[6] C. F. GORDON and A. L. WOLFE, Analyt. Chem. *32*, 574 (1960).

Quantitative analysis of ribonucleoside mixtures by tritium labeling[a]

| | | Experiment 1 | | Experiment 2 | | Experiment 3 | | Experiment 4 | | Experiment 5 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.00002 *M*[c] | | 0.0001 *M*[c] | | 0.0005 *M*[c] | | 0.001 *M*[c] | | 0.005 *M*[c] | |
| Nucleoside | Composition by spectrophotometry[b] | Composition by labeling (± s[d]) | $s_{rel}$[e] (%) | Composition by labeling (± s[d]) | $s_{rel}$[e] (%) | Composition by labeling (± s[d]) | $s_{rel}$[e] (%) | Composition by labeling (± s[d]) | $s_{rel}$[e] (%) | Composition by labeling (± s[d]) | $s_{rel}$[e] (%) |
| AR | 0.246 | 0.252 ± 0.0049 | 1.9 | 0.247 ± 0.0026 | 1.0 | 0.244 ± 0.0028 | 1.1 | 0.241 ± 0.0025 | 1.0 | 0.243 ± 0.0033 | 1.4 |
| CR | 0.283 | 0.286 ± 0.0042 | 1.5 | 0.282 ± 0.0018 | 0.6 | 0.281 ± 0.0023 | 0.8 | 0.283 ± 0.0023 | 0.8 | 0.283 ± 0.0027 | 1.0 |
| UR | 0.284 | 0.277 ± 0.0069 | 2.5 | 0.284 ± 0.0037 | 1.3 | 0.288 ± 0.0028 | 1.0 | 0.288 ± 0.0024 | 0.8 | 0.286 ± 0.0028 | 1.0 |
| GR | 0.187 | 0.185 ± 0.0030 | 1.6 | 0.186 ± 0.0021 | 1.1 | 0.187 ± 0.0022 | 1.2 | 0.187 ± 0.0017 | 0.9 | 0.187 ± 0.0011 | 0.6 |

[a] The mixture contained per nmole ribose moiety 0.246 nmole adenosine, 0.283 nmole cytidine, 0.284 nmole uridine and 0.187 nmole guanosine. The total nucleoside concentration, prior to the addition of $NaIO_4$, ranged from 0.00002 *M* (experiment 1) to 0.005 *M* (experiment 5). Periodate and borohydride reactions were carried out for 30 min each in experiments 2, 3, 4 and 5 and for 120 min each in experiment 1. A 2-fold molar excess of $NaIO_4$ in 5 µl water was added to 50 µl aqueous nucleoside solution. After the specified time 1 µl of a solution of $NaBH_4$-($^3H$) in 0.1 *N* NaOH containing a 20-fold molar excess of borotritiide (200 mC/mmole) was added. After the specified time 50 µl 1 *N* acetic acid was added to destroy the residual borotritiide. When the liberation of tritium gas had ceased (10–15 min) the solution was evaporated to dryness in a stream of filtered air. The residue was taken up in 300 µl water and again dried. The residue was finally taken up in 100 µl of an aqueous solution containing 1–1.5 nmoles/µl each of carrier AR', CR', UR' and GR'. 1-µl aliquots of this solution were used for chromatography and subsequent analysis by liquid scintillation counting (see text). Each value is the mean of 11 determinations. The composition by labeling is expressed as count rate of each nucleoside derivative/sum of count rates of all derivatives. [b] Expressed as nmole of individual nucleoside/nmole of total nucleoside. [c] Total nucleoside concentration (prior to addition of $NaIO_4$). [d] Standard deviation. [e] Relative standard deviation, $s_{rel} = (s \cdot 100/\text{mean})\%$.

of total nucleoside) is accurately reflected by the relative radioactivities of the corresponding derivatives (expressed as count rate of each nucleoside derivative/total count rate of all derivatives). At a total nucleoside concentration below 0.0001 $M$, oxidation and reduction were incomplete in 30 min; reaction times were extended therefore to 120 min in experiment 1. The quantities determined ranged from about 1.8 picomoles (guanosine in experiment 1) to about 700 picomoles (uridine and cytidine in experiment 5).

*Discussion.* The data presented in the Table demonstrate that the labeling technique makes possible a quantitative analysis of ribose derivatives at the subnanomolar level. The main characteristics of the novel method are (1) its extreme sensitivity and (2) its accuracy and precision:

(1) The quantities determined in experiment 1 were 1.8–2.8 picomoles/compound. The lower limit for the labeling technique is 0.7–1.0 picomole/compound. Conventional optical analysis of nucleic acid derivatives on thin layers requires approximately 5000 times more material.

(2) The greatest deviation from the expected values was found to be about 2.5% (adenosine and uridine in experiment 1). Most values were accurate within ± 1%. Relative standard deviations were ± 0.6–1.4% in experiments 2–5 and ± 1.6–2.5% in experiment 1. In our experience, the labeling method is more precise at the picomolar level than optical methods at the nanomolar level.

*Zusammenfassung.* Es wird eine Methode zur quantitativen Analyse von Ribosederivaten beschrieben, welche etwa fünftausendmal empfindlicher ist als die spektrophotometrischen Standardverfahren.

K. Randerath and E. Randerath

*The John Collins Warren Laboratories of the Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, Boston (Massachusetts 02114, USA), 8 July 1968.*

## The Binding of Copper in Human Ceruloplasmin

About 90% of the serum copper is bound to the protein ceruloplasmin. It is generally acknowledged that ceruloplasmin plays an important role in the maintenance of normal copper balance. The chronic copper toxicity of Wilson's disease is almost invariably associated with a deficiency of ceruloplasmin[1]. The mode of binding of copper to ceruloplasmin is clearly an important problem in experimental medicine which needs investigating.

Ceruloplasmin contains 8 atoms of copper/molecule and shows oxidase activity towards a number of aromatic polyamines and polyphenols, as well as to ascorbic acid and a number of organic and inorganic reducing agents[1]. Removal of copper from the protein causes loss of enzyme activity[2,3]. Kasper and Deutsch[4] found that the acid-base titration curves of ceruloplasmin and apoceruloplasmin suggested that histidyl, and either tyrosyl or lysyl residues could be involved in copper binding. Furthermore their results from spectrophotometric titrations did not appear to implicate tyrosine.

If histidyl residues are involved in copper binding, photooxidation of the protein may be expected to lead to loss of copper and of enzyme activity, since these residues are readily destroyed in the process[5]. The results obtained in the present work indicate that photooxidation does cause a rapid loss of enzyme activity concomitantly with loss of copper.

Ceruloplasmin (copper content 0.32–0.34%) was photooxidized in the presence of methylene blue and oxygen in Warburg flasks essentially as described by Weil and Buchert[5]. The flasks contained 4–10 mg ceruloplasmin and 0.1 mg methylene blue dissolved in 2.0–2.5 ml buffer. The gas phase was air. The flasks were irradiated from below by means of 150 W lamps placed 12 cm from the flasks. The procedure was carried out in a darkened room. At the end of a period of photooxidation the solutions were removed from the flasks and 0.1 ml portions were used for the estimation of *p*-phenylenediamine (PPD) oxidase activity[6]. The remainder of the sample was treated with a small amount of charcoal to remove the dye, and then dialysed against several changes of deionized water at 4 °C. Portions were then used for the determination of copper[7], tryptophan[8], and of histidine and tyrosine by means of a Technicon amino acid analyser after hydrolysis in 6 $N$ HCl for 22 h at 106 °C.

The Figure shows the rate of loss of PPD oxidase activity and histidine residues with time of photooxidation. This experiment was performed with 10 mg ceruloplasmin in 0.2 $M$ phosphate buffer, pH 8.0, at 11 °C. When the residual PPD oxidase activity was less than 20%, about 60% of the histidine had been destroyed. At this point (6 h photooxidation; oxygen consumption 6.33 µmoles) 7% of the tyrosine and 60% of the tryptophan had been destroyed. After dialysis against deionized water and passage through a column of chelating resin (Chelex 100, Sigma Chemical Co., St. Louis, Mo., USA) the amount of copper remaining was found to be 15.9% of that in the non-irradiated control sample.

The photooxidation reaction was found to be strongly dependent on both temperature and pH (see Table). The rate of oxygen uptake was 2–3 times more rapid at 37 °C

[1] I. H. Scheinberg, in *The Biochemistry of Copper* (Ed. J. Peisach, P. Aisen and W. E. Blumberg; Academic Press, New York and London 1966), p. 513. – S. Osaki, J. A. McDermott, D. A. Johnson and E. Frieden, in *The Biochemistry of Copper* (Academic Press, New York and London 1966), p. 559.
[2] F. L. Humoller, M. P. Mockler, J. M. Holthaus and D. J. Mahler, J. Lab. clin. Med. *56*, 222 (1960).
[3] J. Marriott and D. J. Perkins, Biochim. biophys. Acta *117*, 395 (1966).
[4] C. B. Kasper and H. F. Deutsch, J. biol. Chem. *238*, 2325 (1963).
[5] L. Weil and A. R. Buchert, Archs Biochem. Biophys. *34*, 1 (1951).
[6] H. A. Ravin, J. Lab. clin. Med. *58*, 161 (1961).
[7] G. Curzon and L. Vallet, Biochem. J. *74*, 279 (1960).
[8] J. R. Spies and D. C. Chambers, Analyt. Chem. *21*, 1249 (1949).