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.

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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

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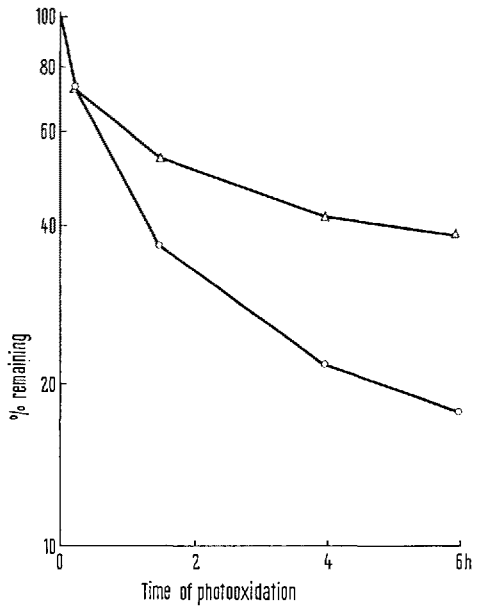
than at 10 °C. Other proteins behave similarly during photooxidation[9,10] indicating that the reaction is not purely photochemical. Most attention should be given to experiments performed at low temperature, since it is reported that the lower the temperature the more specific is the reaction for destroying histidyl residues[10].

The effect of increasing the pH was to increase the rate of loss of PPD oxidase activity (Table). This also has been found with other enzymes[11,12], and has been attributed to the fact that only the free imidazole base is photooxidized. Histidine is the only photosensitive amino acid whose destruction exhibits pH dependence in the pH 4–8 region[12].

Two explanations are possible for the observed loss of PPD oxidase activity by ceruloplasmin when it is subjected to photooxidation. The first is that photooxidation causes specific destruction of histidyl residues including those in the active site. The second is that destruction of histidyl and other residues leads to changes in the conformation of the whole protein molecule which cause distortion and therefore inactivation of the active site[13]. First order kinetics for loss of activity during photooxidation have been observed for a number of enzymes[11,12], and in some cases it has been possible to show that the rate constants for loss of activity and for destruction of certain histidyl residues are identical[11]. In the case of ceruloplasmin first order kinetics for the loss of PPD oxidase activity were not observed, except possibly for the first part of the reaction curve. Nevertheless, the relationship between the destruction of histidyl residues and loss of PPD oxidase activity (Figure) strongly suggests that histidyl residues are essential for activity. That extensive changes in the tertiary structure of the protein did not take place may be inferred from the fact ceruloplasmin which had been photooxidized for 2 h still reacted with a specific antiserum in immunoelectrophoresis, though the mobility of the protein was increased.



Time course of loss of PPD oxidase activity (O) and destruction of histidine (Δ) during photooxidation of human ceruloplasmin at 11 °C. The Warburg flasks contained 10 mg ceruloplasmin and 0.1 mg methylene blue in 2 ml 0.2 *M* phosphate buffer, pH 8.0.

Photooxidation of human ceruloplasmin in the presence of methylene blue under different conditions of temperature and pH

Conditions during photooxidation	Amount of PPD oxidase remaining after 30 min (%)	90 min (%)	Amount of copper remaining after 90 min (%)
Phosphate, pH 8.0, 10 °C	57.8	31.6	29.1
Phosphate, pH 8.0, 25 °C	26.0	5.1	11.8
Phosphate, pH 8.0, 37 °C	9.0	2.7	2.6
Acetate, pH 6.0, 10 °C	80.3	66.8	52.6
Phosphate, pH 7.0, 10 °C	64.3	44.1	41.8
Phosphate, pH 8.0, 10 °C	51.1	25.7	21.2
Borate, pH 9.2, 10 °C	10.0	0.0	8.8

The reaction mixture consisted of 4 mg ceruloplasmin in 2 ml buffer in the temperature-dependence experiments, and 6 mg ceruloplasmin in 2.5 ml buffer in the pH-dependence experiments. Methylene blue, 0.1 mg/flask, was present in all cases, and all buffers were 0.2 *M*.

Reaction of histidine residues with the diazonium reagent diazo-1-H-tetrazole[14] (DHT) also resulted in loss of PPD oxidase activity. DHT was added to $2.65 \times 10^{-6} M$ ceruloplasmin in 0.67 *M* bicarbonate buffer, pH 8.8, up to a final concentration of $3 \times 10^{-2} M$. After 20 min at room temperature the absorbance at 480 nm, which is due to *bis*-azotized histidine, was recorded. PPD oxidase activity was measured after dialysis of the reaction mixture against distilled water.

DHT caused 50% inhibition of PPD oxidase activity at a concentration of $7.5 \times 10^{-4} M$, and 100% inhibition at a concentration of $3 \times 10^{-3} M$. At the point where 50% inhibition occurred, it was calculated that about 1.5 histidyl residues/molecule of protein had been *bis*-azotized. Complete loss of activity occurred well before *bis*-azotization of tyrosyl residues.

These findings are difficult to interpret by themselves because DHT forms colourless *mono*-azo-derivatives before *bis*-azotization of histidyl residues commences[14]. However, it does indicate that histidyl, rather than tyrosyl residues are important for the PPD oxidase activity of ceruloplasmin, as may be deduced from photooxidation experiments[15].

Zusammenfassung. Mit Photooxydation und Diazotierung wird gezeigt, dass Histidinreste an der Bindung von Kupfer an Caeruloplasmin beteiligt sind.

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