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Reaction of Uric Acid and 3-*N*-Ribosyluric Acid with 1,1-Diphenyl-2-picrylhydrazyl[1]

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Antioxidants can be assayed by their reaction with 1,1-diphenyl-2-picrylhydrazyl (DPPH), which results in a decrease in absorbance at 517 nm of the DPPH. Both uric acid and 3-ribosyluric acid reacted with DPPH to produce about the same change in absorbance at 517 nm as an equal concentration of ascorbic acid. Fourteen related purines, pyrimidines, and their nucleosides, including xanthine and xanthosine, failed to give a reaction with DPPH at the same concentration as the urates or at 10 times this concentration. When DPPH interacted with [2-^{14}C]uric acid, it was converted to allantoin. Cold trichloroacetic acid extracts of bovine blood contained two major compounds that reacted with DPPH, ribosyluric acid and glutathione. These compounds were found only in the red cells and not in the plasma.

INTRODUCTION

3-*N*-Ribosyluric acid is the predominant nucleoside in the erythrocytes of cattle (*1*). Although the metabolic role of ribosyluric acid is unknown, it was suggested that it might function as a free radical scavenger in the bovine red cell (*2*). Uric acid was reported to protect linoleate from oxidation at a much lower concentration than that of other purines, pyrimidines, or α-tocopherol (*3*). Proctor (*4*) suggested that uric acid may have taken over some of the functions of ascorbate in primates. Ames *et al.* (*5*) recently reported that uric acid protected human red cell membranes against oxidation by t-butylhydroperoxide. These workers suggested that uric acid may act as a free radical scavenger *in vivo* and be important in reducing cancer and affecting aging.

Blois (*6*) has shown that antioxidants such as cysteine, ascorbate, and α-tocopherol react with the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), convert it to 1,1-diphenyl-2-picrylhydrazine, and change the color of the DPPH from violet to yellow. Glavind (*7*) used this reaction to assay for antioxidants in animal tissues, and Smith *et al.* (*8*) used it to test the ability of barbiturates to act as free radical scavengers. The present study was initiated to determine whether DPPH would react with uric acid, ribosyluric acid, and related purines and pyrimidines.

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MATERIALS AND METHODS

Chemicals

Tri-n-octylamine, DPPH, uric acid, and other bases and nucleosides were purchased from Sigma Chemical Company. The trichlorotrifluoroethane was purchased from Fisher Scientific Company. Ribosyluric acid was prepared from washed bovine red cells by the method of Forrest *et al.* (*9*). The [2-^{14}C]uric acid (specific activity, 57 mCi/mmol) was purchased from Amersham.

Antioxidant Assay

The DPPH was dissolved in 95% ethanol to give a concentration of 35 μM. The compound to be assayed was dissolved in water and 0.4 ml of this solution or of water was added to 5 ml of DPPH. The absorbance of the DPPH was read at 517 nm with a Beckman Model 25 spectrophotometer at 5 min after the addition of the compound to be assayed. The spectra of DPPH and the product formed after the DPPH reacted with the antioxidant were determined from 300 to 700 nm with 95% ethanol in the reference cuvette.

Product of the Reaction between [2-^{14}C]Uric Acid and DPPH

The degradation of [2-^{14}C]uric acid by DPPH was carried out by adding 0.2 ml of [2-^{14}C]uric acid (2 μCi, 36 nmol) in water to 1.0 ml of 95% ethanol containing either 20 or 40 nmol of DPPH and incubating at 25°C for 20 min. Duplicate 40-μl samples of both incubation mixtures plus allantoin, allantoic acid, unlabeled uric acid, and unreacted [2-^{14}C]uric acid were spotted on Whatman 3 MM filter paper and chromatographed ascending in nine solvents. After chromatography, the paper was dried and the uric acid located with a mineralight uv lamp. The lanes containing allantoin and allantoic acid were cut out and these compounds were detected by dipping the sections in a solution of 95% ethanol : concentrated HCl (100 : 10) containing 1% dimethylaminobenzaldehyde. The radioactivity was detected by exposing Kodak no-screen X-ray film to the chromatograms for 1 week and then developing the film. The radioactive areas of the chromatogram were cut out and eluted in 5 ml of water for 2 hr. The extracts were filtered and 0.2 ml of the filtrate was mixed with 10 ml of a toluene : Triton X-100 (2 : 1) mixture with 6 g/liter of a mixture of 98% 2,5-diphenyloxazole and 2% *p*-bis-(*O*-methylstyryl) benzene and counted for 10 min in an Isocap/300, 6868 liquid scintillation system from Searle Analytic.

Extraction of Bovine Blood and Detection of Its Antioxidants

Blood was collected from three adult cattle immediately after slaughter when the jugular vein was cut. The blood was either added directly to an equal volume of ice-cold 10% trichloroacetic acid or to the anticoagulant acid–citrate–dextrose (ACD). The ACD-treated blood was cooled in an ice bath and centrifuged at 3200*g* for 20 min. The plasma and buffy coat were removed by aspiration; the erythrocytes were resuspended in 4 vol of 0.9% sodium chloride and the suspension was

centrifuged at 4000*g* for 20 min. The saline wash was removed and 1 vol of the washed erythrocytes was added to 3 vol of ice-cold trichloroacetic acid.

The trichloroacetic acid extracts were centrifuged at 1500*g* for 5 min and the supernatant solution was decanted and was mixed repeatedly with an equal volume of 0.5 *M* tri-n-octylamine in trichlorotrifluoroethane to remove the trichloroacetic acid from the extract (*10*). The extract was centrifuged at 1500*g* for 5 min and 400 μl of the supernatant solution plus uric acid, ribosyluric acid, glutathione, and ascorbic acid were spotted on Whatman 3 MM filter paper and chromatographed ascending in four solvents. The antioxidants in the extracts were detected by dipping the dried chromatograms in a solution of 0.1 mg/ml of DPPH in methanol and allowing the chromatograms to air dry (*11*). The antioxidants appeared as yellow spots on a violet background.

RESULTS

The addition of either uric acid or 3-ribosyluric acid to DPPH resulted in rapid oxidation of these compounds with a concomitant decrease in the absorbance at

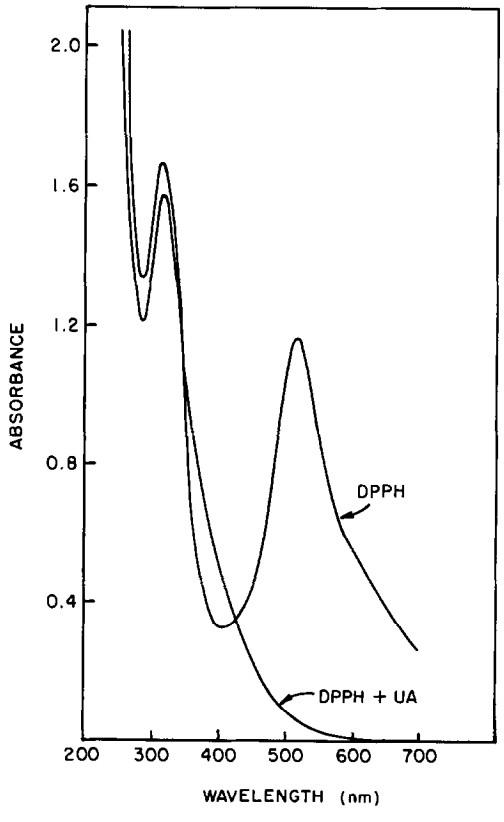

FIG. 1. The absorption spectra of 1,1-diphenyl-2-picrylhydrazyl (DPPH) before and after the addition of uric acid (UA).

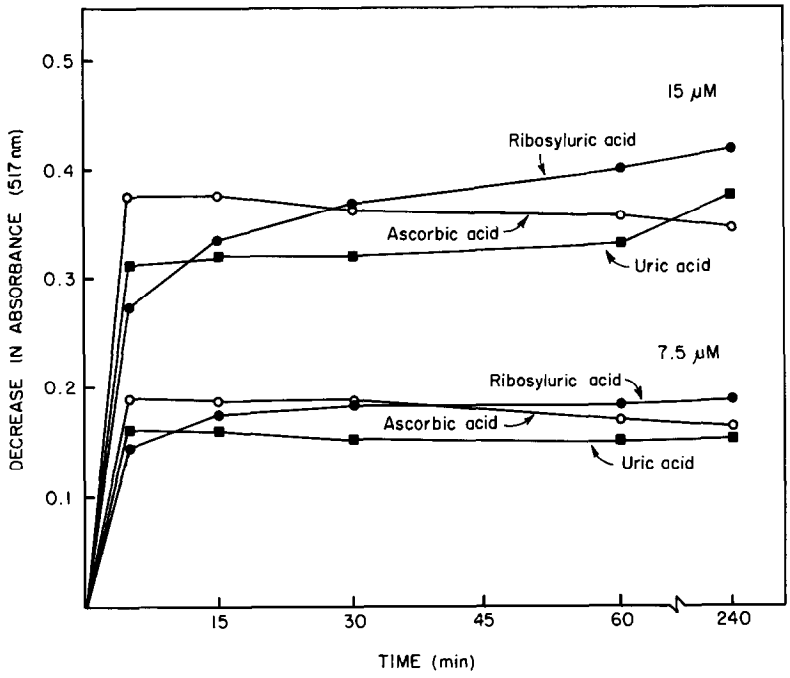

FIG. 2. Rate of decrease in absorbance at 517 nm of 1,1-diphenyl-2-picrylhydrazyl treated with either 7.5 or 15.0 μ*M* ribosyluric acid (●), ascorbic acid (○), or uric acid (■).

517 mm as 1,1-diphenyl-2-picrylhydrazine was formed (Fig. 1). The reaction was very rapid and essentially complete in 5 min with uric acid and ascorbic acid (Fig. 2). With ribosyluric acid, however, the change in absorbance continued to in-

TABLE 1

PAPER CHROMATOGRAPHY OF THE MAJOR PRODUCT FORMED AFTER REACTION OF [2-^{14}C]URIC ACID WITH 1,1-DIPHENYL-2-PICRYLHYDRAZYL

Solvent	R_f			
	Uric acid	Allantoic acid	Allantoin	Major ^{14}C compound
1. t-Butanol:methylethylketone:formic acid:water (8:6:3:3)	0.17	0.62	0.32	0.32
2. 95% Ethanol:1 *M* ammonium acetate, pH 3.8 (7:3)	0.39	0.67	0.57	0.57
3. 95% Ethanol:1 *M* ammonium acetate, pH 7.5 (7:3)	0.24	0.66	0.54	0.54
4. Isopropanol:HCl:water (170:41:39)	0.22	0.67	0.49	0.49
5. Butanol:pyridine:water (1:1:1)	0.24	0.46	0.46	0.46
6. 5% Na_2HPO_4, $7H_2O$ saturated with isoamyl alcohol	0.44	0.86	0.86	0.86
7. Butanol:formic acid:water (77:10:13)	0.09	0.28	0.11	0.10
8. 4% Trisodium citrate	0.28	0.87	0.88	0.88
9. Isobutyric acid:NH_4OH:water (66:1:33)	0.40	0.44	0.47	0.47

TABLE 2

PAPER CHROMATOGRAPHY OF COMPOUNDS IN BOVINE ERYTHROCYTES WHICH GAVE A POSITIVE REACTION WITH 1,1-DIPHENYL-2-PICRYLHYDRAZYL

Solvent	R_f				
	Compounds which reacted with DPPH	Ribosyluric acid	Glutathione	Uric acid	Ascorbic acid
1. 5% KH_2PO_4 saturated with isoamyl alcohol	0.61, 0.90	0.61	0.90	0.49	0.87
2. 95% Ethanol:1 *M* ammonium acetate, pH 3.8 (7:3)	0.36, 0.45	0.46	0.36	0.31	0.61
3. Butanol:formic acid:water (77:10:13)	0.15	0.12	0.16	0.15	0.34
4. 95% Ethanol:acetic acid:water (65:1:34)	0.56	0.55	0.58	0.44	

crease slowly for up to 4 hr. The effect of the concentration of uric acid, ribosyluric acid, and ascorbic acid on the decrease in absorbance of DPPH at 517 mm is given in Fig. 3. These data are for readings taken at 5 min. The decrease in absorbance was very similar for these three compounds. In addition, adenine,

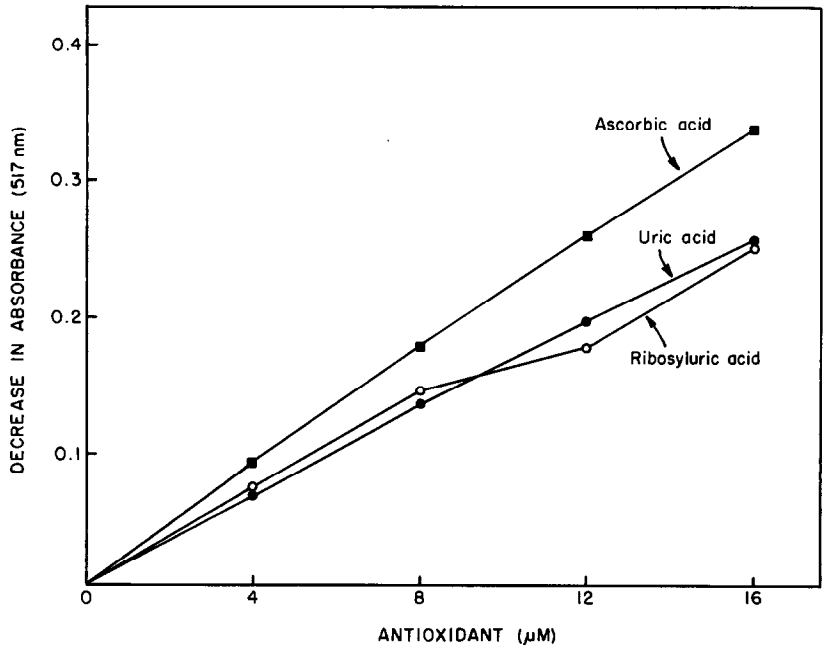

FIG. 3. Decrease in absorbance at 517 nm of 1,1-diphenyl-2-picrylhydrazyl treated with varying concentration of ascorbic acid (■), uric acid (●), or ribosyluric acid (○).

adenosine, allantoic acid, allopurinol, alloxan, guanine, guanosine, hypoxanthine, inosine, orotic acid, uracil, uridine, xanthine, and xanthosine were also tested at the same concentration as uric acid and ribosyluric acid and at 10 times this concentration. None of these compounds brought about a reduction in color of the DPPH.

When [2-^{14}C]uric acid (18 nmol) was mixed with DPPH (20 nmol) for 20 min, the products chromatographed on paper, and radioautograms prepared, all of the original [2-^{14}C]uric acid had disappeared. One major and several minor radioactive compounds were eluted from the chromatograms and counted. Over 70% of the radioactivity recovered had the same R_f value as allantoin (Table 1); 10–15% of the radioactivity recovered was in allantoic acid. Most of the solvents used separated uric acid from allantoin and allantoic acid, and several solvents separated allantoin and allantoic acid. When the amount of DPPH used was reduced to 10 nmol, about 35% of the uric acid was recovered unchanged, and the major product was still allantoin.

Glavind and Holmer (*11*) used DPPH to detect antioxidants such as α-tocopherol on thin-layer chromatograms. When cold trichloroacetic acid extracts of either whole bovine blood or washed bovine erythrocytes were chromatographed, the chromatograms dried, and dipped into a DPPH solution, two yellow spots were detected that corresponded to ribosyluric acid and reduced glutathione (Table 2). Neither uric acid nor ascorbic acid was detected by this method. No spots were detected in trichloroacetic acid extracts of plasma.

DISCUSSION

Matsushita *et al.* (*3*) and Ames *et al.* (*5*) have shown that uric acid can protect unsaturated fatty acids and red cell membranes from oxidation, and it was postulated (*5*) that uric acid may be important in protecting humans from oxygen radicals. Matsushita *et al.* (*3*) reported that uric acid would decolorize DPPH, but no quantitative data were given. In the present work it was shown that both uric acid and ribosyluric acid react with DPPH, a reagent used to assay for antioxidants. The reactivity of uric acid and ribosyluric acid with DPPH was very similar to that of ascorbic acid. Since ribosyluric acid is a major low molecular weight component of the bovine erythrocyte, it may be of importance as an antioxidant in the bovine erythrocyte. One advantage of ribosyluric acid over uric acid as a cellular antioxidant is that the ribosyluric acid is not permeable to the red cell membrane; and thus, once it is synthesized, it will remain in the erythrocyte. Ribosyluric acid is also more water soluble than uric acid (*12*).

Since the radioactive product formed when [2-^{14}C]uric acid interacted with DPPH was primarily allantoin, it is probable that the product of the oxidation of ribosyluric acid is the riboside of allantoin. This reaction appears to be similar to that observed when uric acid or ribosyluric acid are adsorbed to charcoal and then eluted (*13*). In both cases, the uric acid and ribosyluric acid are degraded; the uric acid primarily generates allantoin. Allantoin was also formed when uric acid was treated with hydrogen peroxide in the presence of hemoglobin or hematin (*14*).

The data obtained with the cold trichloroacetic acid extracts of bovine red cells showed that both glutathione and ribosyluric acid are major antioxidants in the bovine red cell. If the primary function of ribosyluric acid in the bovine red cell is that of an antioxidant, the question still remains: Why is it present only in the red cells of cattle and bison (*15*)? The level of orotate phosphoribosyltransferase, the enzyme which synthesizes ribosyluric acid 5′-monophosphate (the precursor of ribosyluric acid), is much richer in bovine red cells than it is in other species that have been assayed (*16*). Bovine red cells have about 50 times as much of the enzyme as human erythrocytes. The enzyme has an altered specificity which allows it to use uric acid as a substrate to synthesize 3-*N*-ribosyluric acid 5′-monophosphate, the immediate precursor of ribosyluric acid (*17*).

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