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The Generation of Superoxide Anions in Glycation Reactions with Sugars, Osones, and 3-Deoxyosones[1]

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Glycoxidation is a process whereby glycated proteins chemically generate oxygen free radicals. Superoxide anion formation was measured by the superoxide dismutase-dependent reduction of ferricytochrome C in glycation reactions at pH 7.0 in the absence of transition metal ions. Assays were linear over 1 h, and most activity was seen after a 2 d incubation of 5 mM L-threose and 10 mM α-N-acetyl-lysine (N-Ac-Lys) or 10 mg/mL RNase A. Trioses, tetroses and their corresponding osones and 3-deoxyosones had the highest activity (12-16 nmoles $O_2^{\cdot-}$/hr/ml) with N-Ac-Lys. Osones and 3-deoxyosones alone generated considerable $O_2^{\cdot-}$, whereas aldose sugars largely did not. Xylosone and 3-deoxyxylosone produced 6 and 10 nmoles $O_2^{\cdot-}$/hr/ml respectively with N-Ac-Lys, however, xylose was inactive, as were glucose and fructose. Glycation assays with 3-deoxyglucosone and glyoxal showed no activity, however, methyl glyoxal generated 1.7 and 2.0 nmoles $O_2^{\cdot-}$/hr/ml with N-Ac-Lys and N-Ac-Arg, respectively. Therefore, Amadori compounds composed of lysine and short chain sugars can rapidly generate superoxide anion in the absence of metal ions.

Key Words: **superoxide anion formation; glycation; glycoxidation; osone; 3-deoxyosone.**

An accelerated formation of oxygen free radicals accompanies protein glycation in aging and diabetes (1-4), and has also been implicated in Alzheimer's disease (5), atherosclerosis (6), uremia (7) and cataract (8). The initial reaction of sugar carbonyl groups with protein amino groups results in the formation of rather benign Amadori adducts, however, these can give rise to superoxide anion ($O_2^{\cdot-}$) by glycoxidation in the presence of transition metal ions (9-14). $O_2^{\cdot-}$ can then dismutate to hydrogen peroxide which generates hydroxyl radicals by Fenton chemistry.

Superoxide anion formation by glycated proteins forms the basis for the fructosamine assay used for the detection of glycated proteins in serum (15). This assay, however, measures the rather non-specific reduction of nitroblue tetrazolium (NBT), and NBT reduction can also occur by the direct reaction with Amadori adducts at the pH of the assay (10.5) (16). Recently $O_2^{\cdot-}$ formation has been confirmed by EPR spectroscopy (10) and by the superoxide dismutase (SOD)-dependent reduction of cytochrome C, during glycation and by isolated glycated proteins.

The formation of advanced glycation endproducts (AGEs) has been shown to require oxygen free radicals (2) and consistent with this observation, AGE formation can be ameliorated by the administration of either SOD or catalase (17). The rate of superoxide formation by the glucose Amadori compound (fructosyl-lysine) is exceedingly slow, but can be accelerated by free Cu(II) in vitro (18). Since free transition metals do not likely exist in vivo, it is of interest to know the extent to which various glycated amino acids can produce superoxide anions in the presence of a transition metal chelator and at physiological pH.

The formation of most AGEs, are not likely synthesized directly from fructosyl-lysine, but rather from the glycoxidation of this Amadori compound to 3-deoxyglucosone; pentoses and tetroses (19). Autoxidation of glucose produces glyoxal and arabinose, which are also active in AGE formation (20). Similar AGE protein modifications can also be formed in vitro by ascorbic acid under oxidative conditions. The oxidation products of ascorbate include 3-deoxyxylosone (21) and a tetrose, possibly L-threose (22), and the latter can produce threosone and 3-deoxythreosone in the presence of lysine (23). Therefore, several short chain sugars and osone compounds may be major contributors to super-

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Abbreviations: AGE, advanced glycation endproduct; DTPA, diethylenetriaminepentaacetic acid; N-Ac-Lys, α-N-acetyl-lysine; NBT, nitroblue tetrazolium; $O_2^{\cdot-}$, superoxide anion; SOD, superoxide dismutase.

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oxide anion formation during glycation. Here we describe a system for measuring superoxide formation in vitro, and compare the reactivity of a variety of sugars, osones and 3-deoxyosones with N-Ac-Lys and N-Ac-Arg for this activity.

MATERIALS AND METHODS

Materials. Osones were prepared from their corresponding aldoses and ketoses by oxidation with cupric acetate according to described methods (24,25). Where needed, further purification of the osones from unreacted sugars was accomplished by the method of Vuorinen and Serianni (25). 3-Deoxyosones were synthesized according to the method of Madson and Feather (26). The purity of all preparations was confirmed to be at least 95% by thin layer chromatography and NMR spectroscopy. All preparations were treated with chelex resin to remove any metal impurities. Sugars and all other reagents were purchased from Sigma Chemical Co., St. Louis, MO.

Glycation incubations. Mixtures containing 5.0 mM sugar, osone or 3-deoxyosone, 10 mM N-Ac-Lys or N-Ac-Arg and 50 μM diethylenetriaminepentaacetic acid (DTPA) in 50 mM chelex-treated phosphate buffer, pH 7.0 were incubated for 2 d at 37°C. Control incubations were also carried out with sugar alone or with other acetylated amino acids. Incubations were also made with increasing sugar, increasing N-Ac-Lys, or with 10 mg/ml RNase A.

Superoxide assays. Superoxide assays (1.5 ml) contained 0.4 ml of the incubation mixture, 1.5 mg ferricytochrome c, and 50 μM DTPA in 50 mM phosphate buffer, pH 7.0. The superoxide formed was calculated as the increase in absorbance at 550 nm ($E_m = 21 \times 10^3$) less the absorbance increase seen in the presence of 250 units of SOD. All assays were conducted in triplicate, and repeated in triplicate with fresh reagents.

Other assays. Hydrogen peroxide formation was determined by the method of Jiang, Woollard and Wolff (27). Incubations of 5.0 mM [1-^{14}C]threose and 10 mM N-Ac-Lys were analyzed by thin layer chromatography with isopropanol:ethyl acetate:water (83:11:6) as irrigant.

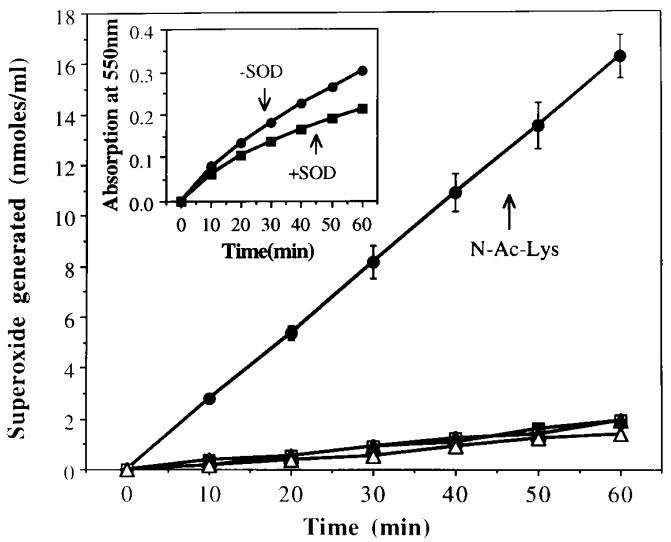

FIG. 1. The generation of superoxide anion by 5 mM L-threose alone (open triangles) or in the presence of 10 mM N-Ac-Lys (filled circles), N-Ac-Arg (filled triangles), N-Ac-His (open boxes) or N-Ac-Lys (filled boxes). Inset shows a 1 h assay with L-threose and N-Ac-Lys with and without SOD. All reactions were carried out in 50 mM Chelex-treated phosphate buffer containing 50 μM DTPA.

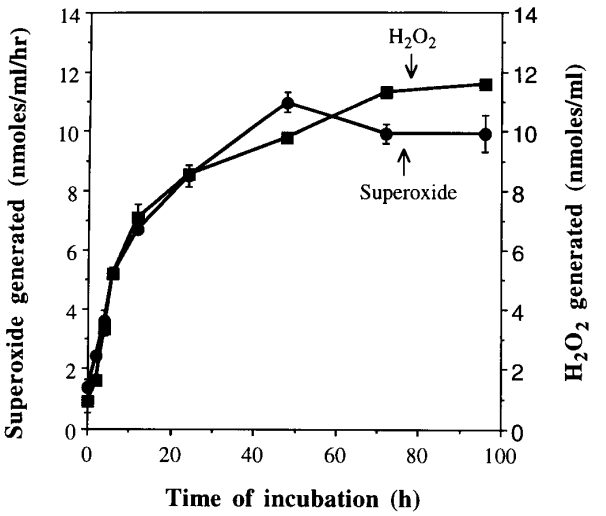

FIG. 2. Superoxide anion and H_2O_2 formed by aliquots from an incubation containing 5.0 mM L-threose and 10 mM N-Ac-Lys over 4 d (96 h) of incubation.

RESULTS

The formation of superoxide anion by aliquots from a 2 d incubation of L-threose and N-Ac-Lys was linear over 60 min in the cytochrome c assay as shown in Fig. 1. The incubation with 10 mM N-Ac-Lys produced 16.2 nmoles $O_2^{\cdot-}$/ml/hr compared to 1.8 nmoles $O_2^{\cdot-}$ with threose alone or with threose and 10 mM N-Ac-Arg, N-Ac-His or N-Ac-Leu. The inset shows the absorbance increase measured with and without SOD. The reduction of cytochrome c in the presence of SOD may represent direct reduction by the Amadori compound. Similar assays conducted at pH 6.0, 7.0 and 8.5 produced 5.1 ± 0.2, 10.3 ± 1.8 and 26.1 ± 0.7 nmoles $O_2^{\cdot-}$/ml/h respectively, consistent with the effect of pH on the dismutation rate of $O_2^{\cdot-}$ to H_2O_2.

Aliquots from an incubation containing 5 mM threose and 10 mM N-Ac-Lys were removed over time and assayed for both $O_2^{\cdot-}$ and H_2O_2 formation. Fig. 2 shows that superoxide formation increased rapidly with time of incubation of L-threose with N-Ac-Lys, reaching a maximum of 11 nmoles $O_2^{\cdot-}$/ml/h at 48 h. Hydrogen peroxide values, increased in a similar manner, reaching a value more than twice the predicted amount (half of the superoxide at pH 7.0). This is likely due to the accumulation of the more stable hydrogen peroxide in the incubation mixture prior to the assay. Aliquots reduced with $NaBH_4$ showed the accumulation of threitol-lysine proportional to the $O_2^{\cdot-}$ formation by thin layer chromatography. Unreduced aliquots showed only L-threose (data not shown). Increasing N-Ac-Lys (0-5 mM) with 10 mM L-threose resulted in a linear increase in superoxide formed after 2 d of incubation (Fig. 3A). Likewise, increasing L-threose (0-5 mM) incubated with either 10 mM N-Ac-Lys or 10 mg/ml

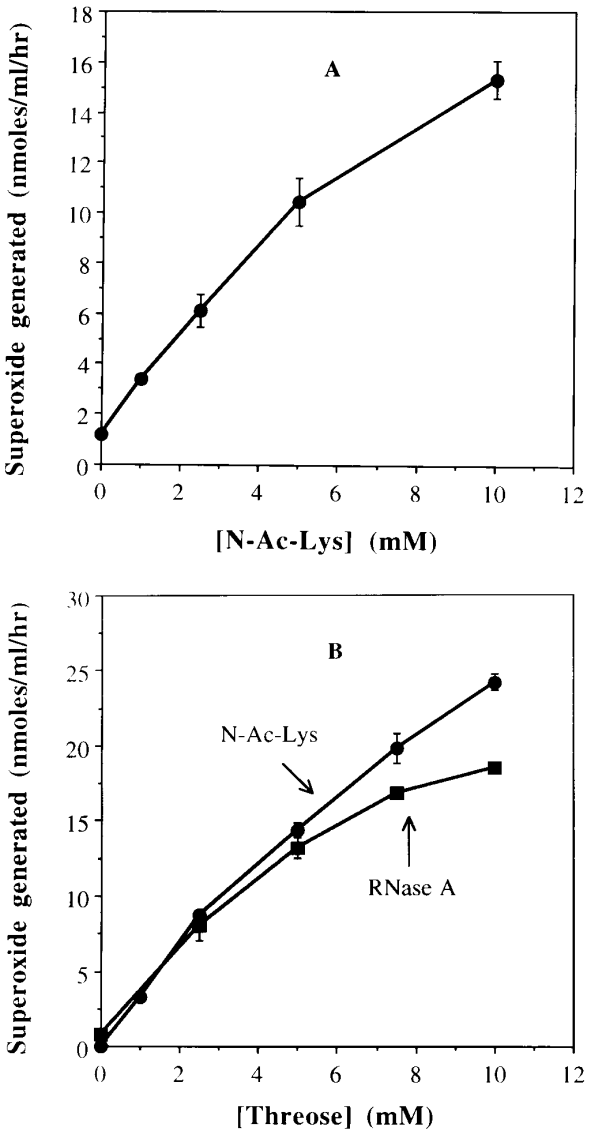

FIG. 3. The effect of increasing N-Ac-Lys with 5.0 mM L-threose (panel A), and increasing L-threose with 10 mM N-Ac-Lys or 10 mg/ml RNase A (Panel B) on the formation of superoxide anion during a 1 h assay.

RNase A resulted in a linear increase in superoxide formation (Fig. 3B) in the 1 h assay. A similar incubation of 5.0 mM L-threose with 10 mg/ml RNase A generated 15 nmoles $O_2^{\cdot -}$/ml/hr. After dialysis to remove free sugar and dissociate any Schiff base structures, the glycated RNase A produced 10 nmoles $O_2^{\cdot -}$/ml/hr. These assays were carried out in the absence of transition metal ions, and in fact, the addition of either Fe(III) or Cu(II) up to 10 mM had no stimulatory effect on superoxide production in the L-threose and N-Ac-Lys system (data not shown).

Table I shows the ability of several sugars, osones and deoxyosones (5.0 mM) to generate superoxide anion following a 2 d incubation with 10 mM N-Ac-Lys, 10 mM N-Ac-Arg or with no added amino acid. Among the aldoses, glyceraldehyde and threose were most active with N-Ac-Lys, whereas xylose and glucose were inactive. The ketose, erythrulose, was more active than fructose. Considerable $O_2^{\cdot -}$ was formed in reactions of osones and deoxyosones with amino acids, but most of this activity was also seen in the absence of added amino acid. Significant increases in $O_2^{\cdot -}$ were seen with hydroxypyruvaldehyde, threosone and xylosone with N-Ac-Lys, whereas only threosone showed significant activity with N-Ac-Arg. Similar results were obtained with the 3-deoxyosones tested. In only a few cases did osones and 3-deoxyosones demonstrate increased activity with N-Ac-Arg, even though they are known to form imidazolones and lysine crosslinks (28,29). The compounds thought to be of primary importance in AGE formation in vivo, 3-deoxyglucosone, glyoxal and methylglyoxal, were considerably less active than short chain aldose sugars, but their activity was higher than glucose or fructose, which had no activity in this assay. Controls with N-Ac-Lys and buffer, glucose and buffer, threitol and buffer or buffer alone were no different than glucose and N-Ac-Lys. The activity shown (0.1 nmoles $O_2^{\cdot -}$/h/ml) represents an average SOD dependent increase in absorbance at 550 nm of 0.001/h or less. In fact no reduction of cytochrome c was seen in these assays. Incubations extended to 8 d showed no increase in the activity for the glucose and N-Ac-Lys

TABLE I

Superoxide Generated by Various Carbohydrates with and without Either 10 mM *α-N*-Ac-Lys or *α-N*-Ac-Arg

Carbohydrate (5.0 mM)	Superoxide anion formation (nmoles/ml/hr)[a]		
	+ N-Ac-Lys	+ N-Ac-Arg	CHO Only
Sugars			
Glycoaldehyde	4.1 ± 0.3	3.4 ± 0.1	2.7 ± 0.2
Glyceraldehyde	13.6 ± 1.5	6.0 ± 0.3	6.7 ± 0.3
Threose	14.6 ± 0.5	2.0 ± 0.2	2.0 ± 0.5
Xylose	0.5 ± 0.1	1.0 ± 0.1	0.7 ± 0.1
Arabinose	1.0 ± 0.3	0.9 ± 0.1	0.6 ± 0.2
Glucose	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.1
Erythrulose	4.8 ± 0.6	0.2 ± 0.3	3.3 ± 0.1
Fructose	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
Controls	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
Osones			
OH-Pyruvaldehyde	18.0 ± 0.0	11.5 ± 0.4	12.3 ± 0.1
Threosone	12.1 ± 0.5	15.4 ± 0.1	9.9 ± 0.6
Xylosone	12.4 ± 0.0	7.2 ± 0.4	5.7 ± 5.7
Glucosone	12.7 ± 0.9	10.6 ± 0.5	11.0 ± 0.4
Glyoxal	1.4 ± 0.2	0.4 ± 0.1	1.4 ± 0.0
3-Deoxyosones			
Methylglyoxal	3.7 ± 0.2	4.0 ± 0.2	2.0 ± 0.2
3-D-Threosone	16.2 ± 0.5	nd[b]	nd
3-D-Xylosone	19.5 ± 0.6	9.3 ± 0.4	9.6 ± 0.7
3-D-Glucosone	1.3 ± 0.4	1.8 ± 0.1	1.4 ± 0.1

[a] 1.0 nmole/h superoxide anion is equal to a ΔA_{550} of 0.006/h.
[b] nd, not determined.

mixture. Xylosone and 3-deoxyxylosone, putative ascorbate oxidation products, exhibited high activity for superoxide formation. Xylose, however, was almost completely inactive.

DISCUSSION

Gillery et al. (9) were able to show that serum from diabetic patients generated increasing levels of superoxide anion in vitro using both the NBT and cytochrome C assays. This activity was SOD-dependent, and was also demonstrated with glucose-modified proteins, however, assays of several days in length were employed in the presence of phosphate buffers, which are known to contain Fe and Cu impurities. Mullarkey et al. (10) demonstrated cytochrome c reduction by glycated RNase in the presence of chelators after several weeks of incubation. After 2 days and 8 days of incubation, however, our assays detected no significant superoxide formation with glucose either with or without N-Ac-Lys or N-Ac-Arg.

Osones and deoxyosones are known to react with Arg to form imidazolones, however, these compounds generated little or no additional superoxide anion in the presence of N-Ac-Arg. Osones and deoxyosones produced considerable superoxide anion without added amino acids. Even cyclic structures of osones retain a carbonyl group and can form the requisite enediols for $O_2^{\cdot -}$ formation. Because of this, it is possible that the sugar Amadori compounds generated additional $O_2^{\cdot -}$ oxidation by the released osones. Mossine et al. (18) have shown that 1.0 mM fructosyl-lysine was able to generate only 57 nM $O_2^{\cdot -}$/hr under these conditions, but this activity was increased more than 100-fold in the presence of Cu(II). Similarly, Cheng and Kawakishi (30) observed the formation of glucosone and arabinose from glucose Amadori compounds, but only in the presence of Cu(II). Recent evidence, however, shows that various proteins are employed by the cell to bind and transport Cu(II) through the cytoplasm (31), and other workers argue for the absence of free metals in cells (32). Our data show that transition metals are not required to generate superoxide anion with the shorter chain sugars and N-Ac-Lys or with osones and deoxyosones, arguing that glycoxidation could procede in the absence of free Cu (II) or Fe (III) in vivo.

Superoxide formation requires enolization of the Amadori compound, which occurs readily with short chains sugars. Consistent with this was the observation that the 2 carbon sugars, glycolaldehyde and glyoxal, as well as methylglyoxal were less active than the 3 and 4 carbon sugars. Simple Amadori compounds with xylose, glucose and fructose form pyranose and furanose ring structures, which appear to require Cu(II) for superoxide formation. In this regard it is important that the addition of Cu(II) up to 10 μM did not increase superoxide formation with L-threose and N-Ac-Lys, possibly due to the inherent instability of the threose Amadori compound. Amadori compounds with short chain sugars release superoxide anion, and likely result in the release of osones from the protein, which can produce another superoxide anion spontaneously. Since it has been shown that metal-catalyzed oxidation is essential for the formation of carboxymethyllysine, pentosidine and protein crosslinks (2), this may reflect a role for metals in hydroxyl radical formation from H_2O_2 more than in the original superoxide production. Since the oxidation of glucose and/or ascorbate is required for AGE formation, and since both oxidations give rise to shorter chain sugars, which can function efficiently in superoxide formation even in the absence of transition metal ions, this mechanism may play a vital role in the oxidative protein damage seen in diabetes.

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