Free. Rad. Res. Comms., Vol. 1, No. 3, pp. 189–199
8755-0199/86/0103-0189 $25.00/0

Printed in Great Britain

COMPARISON OF PHARMACOKINETIC AND CELL BINDING PROPERTIES OF TURKEY Cu-SOD AND E. COLI Mn-SOD

M. Spasic†, P. Durosay, K. Puget and A.M. Michelson††

Institut de Biologie Physico-Chimique
13, rue P. et M. Curie, 75005 Paris, France.

(Received May 24th 1985)

The pharmacokinetics in rats and cell fixation properties of *coli* Mn-SOD are compared with those of Cu-SODs extracted from turkey blood. Despite similarities in molecular weight and pl the different enzymes show different characteristics. The results are discussed with respect to the mechanism of anti-inflammatory activity of superoxide dismutase.

Key words: Turkey Cu-SOD, *coli* Mn-SOD, pharmacokinetics, cell fixation.

INTRODUCTION

We have previously reported the pharmacokinetic properties of various superoxide dismutases both free and in liposomal encapsulated forms[1,2]. Various approaches have been used to increase the anti-inflammatory activity of superoxide dismutase. Thus the enzyme has been coupled with macromolecules such as Ficoll, polyethylene glycol or albumin to increase the molecular mass and hence circulation lifetime[3]. Studies *in vitro* have shown that SOD protects the viability of phagocytosing neutrophills and very large differences were required in the concentrations of various SODs to provide equivalent protection. Pig Cu-SOD (basic pl of 7.6 by chromatofocalisation or 6.3 by isoelectric focalisation) was 100 times more effective than bovine Cu-SOD (pl 5.0). These differences were attributed to the net charges of the enzymes and indeed the protective capacity of bovine Cu-SOD was greatly enhanced by coupling with polylysine to give a net positive charge[4].

It may nevertheless be noted that pig Cu-SOD is *less* anti-inflammatory in the rat carageenin model than bovine Cu-SOD[5]. Since the true mechanism of anti-

† *Permanent address*: Institute for Biological Research, "S. Stanvic", Department of Endocrinology and Metabolism, 29 Novembra 142, 11000 Beograd, Yugoslavia.
†† *Senior Author for correspondence*: A.M. Michelson, I.B.P.C., 13, rue P. et M. Curie, 75005 Paris, France.

inflammatory activity of superoxide dismutase is not yet defined it was of interest to compare certain biological properties of two enzymes with similar molecular weights and pl. In this report we present some studies using *E. coli* Mn-SOD (pl 7.8 by chromatofocalisation or 6.4 by isoelectric focalisation, Mol. wt. 40 000) and two turkey Cu-SODs (pl 7.66 and 7.35 respectively by chromatofocalisation, Mol. wt. 33 000). These results are to be compared with the relative efficiency of SODs with different molecular weights (33 000–80 000), different metals as active centre (Cu, Mn, Fe) and different species origin. In view of the fact that homologous enzyme is *not* anti-inflammatory in the rat carageenin model we have already suggested that human SOD will prove disappointing when applied to human clinical disorders[6].

MATERIAL AND METHODS

Manganese Superoxide Dismutase (Mn-SOD) was purified from *E. coli* DF 1008[7] as described earlier[8] with slight modifications. The pl as determined by chromatofocalisation was 7.8. Isoelectric focalisation on gel gave a pl of 6.4.

Turkey Copper Superoxide Dismutase (Cu-SOD) was prepared from turkey blood (2 l) collected on citrate. After lysis of the erythrocytes and precipitation of hemoglobin with chloroform-ethanol followed by addition of potassium phosphate and precipitation of protein with acetone[9] the crude enzyme was dialysed against 0.025 M ethanolamine-acetic acid pH 8.0. This solution (30 ml, total NBT/riboflavin units 72 330) was purified by chromatofocalisation on 50 ml PBE 94 eluting the column (in the same buffer as above at the beginning) with polybuffer 96 diluted 10 fold with water and adjusted to pH 6.0 with dilute acetic acid. An extremely complicated elution pattern of activities with six distinct peaks was observed in confirmation of the multibanded pattern on gel electrophoresis. Two major peaks of activity eluting at pH 7.67 and 7.35 were grouped separately. Minor peaks were observed at pH 7.2, 7.14, 7.07 and 7.02.

pl 7.67	45 ml	260 = 0.194 280 = 0.241	280/260 = 1.24	Total $\delta_{280} = 10.85$
	553 NBT units/ml		Total 24 885 units	2851 units/δ_{260} 2295 units/δ_{280}
pl 7.35	57.5 ml	260 = 0.137 280 = 0.102	280/260 = 0.74	Total $\delta_{280} = 5.86$
	811 NBT units/ml		Total 46 633 units	5920 units/δ_{260} 7951 units/δ_{280}

The protein in each fraction was precipitated by addition of $(NH_4)_2SO_4$ (700 g/l), collected by centrifugation and further purified by filtration on Sephadex 100 (50 ml) in PBS five fold diluted with water.

pl 7.67	11.3 ml	260 = 0.231 280 = 0.299	280/260 = 1.29	
	834 NBT units/ml		Total 9424 units	3610 NBT units/δ_{260} 2789 NBT units/δ_{280}
pl 7.35	11.6 ml	260 = 0.185 280 = 0.135	280/260 = 0.73	
	1120 NBT units/ml		Total 12 992 units	6054 NBT units/δ_{260} 8296 NBT units/δ_{280}

Gel electrophoresis showed that the fraction pl 7.35 was pure. Using a factor of 0.425 at 260 nm per mg the specific activity of fraction pl 7.67 was 1534 units/mg (i.e. about 60% pure) and of the fraction pl 7.35 was 2573 units/mg.

The UV spectrum of fraction pl 7.35 in PBS diluted five fold with water at 0.44 mg enzyme/ml is shown in Fig. 1.

Radioactive Labelling of Turkey Cu-SODS. A solution of the SOD (440 μg in 200 μl of 0.1 M borate pH 8.5) was added to 250 μC of ^{125}I Bolton and Hunter reagent (50 μl at 5 mc/ml previously evaporated to dryness) at room temperature with shaking. The mixture was then placed in ice for 1 hr with shaking every five min. Unlabelled SOD (1.7 mg) was added as carrier and the protein separated from reagents by filtration through a column of Sephadex 100 (30 × 1cm) in 5×10^{-3} M phosphate pH 7.2, eluting with this same buffer. Fractions containing the labelled protein were combined. The solution contained 0.425 mg protein/ml and after counting in a γ counter showed a specific radioactivity of 3224 cpm/μg for the Cu-SOD of pl 7.35 and 3163 cpm/μg for that of pl 7.67.

Labelling of E. coli Mn-SOD. The procedure was similar to that describd above. A solution of 230 μg pure Mn-SOD (30 μl of a solution 7.7 mg/ml, 1398 NBT units per mg) in 0.1 M borate pH 8.5 was added to 75 μC of ^{125}I Bolton and Hunter reagent. At the end of the reaction (60 min in ice) 1 ml of 0.1 M borate 0.2 M glycine pH 8.5 was added to destroy excess reagent and the labelled SOD separated by filtration on Sephadex 100 (1.4 × 25 cm) in 5×10^{-3} M phosphate pH 7.2. Active fractions were combined (3.0 ml) and 540 μg cold Mn-SOD added. The final solution had a specific radioactivity of 13211 cpm/μg.

Fixation of Enzymes on Erythrocytes. Blood was removed on citrate from MS. Countage showed 4.98×10^9 erythrocytes per ml and estimation of SOD gave 84.5 μg per ml. Two ml of blood were centrifuged at 3000 g for 10 min and the plasma separated from the erythrocytes. These were washed with 4 ml PBS and after centrifugation the erythrocytes (1 ml) were resuspended in 3 ml of PBS. To 400 μl of the suspension was added ~500 μl of the ^{125}I labelled SOD (~200 μg for the Cu-SOD and 100 μg or 200 μg for the Mn-SOD) in a centrifuge tube (volume 3.5 ml). The mixture was gently shaken at room temperature. After 5 min or 1 hour PBS (1 ml) was added and the suspension centrifuged for 6 min at 3000 g. The erythrocytes were washed a further three times with 1 ml PBS (supernatants were counted in a γ counter to follow the washing) and the radioactivity in the total erythrocytes (corresponding to 200 μl blood i.e. 16.9 μg Cu-SOD in 0.996×10^9 erythrocytes) were determined.

Two preparations of ^{125}I Bolton and Hunter labelled protein were used for each enzyme with the characteristics shown in Table I. For each preparation two studies of fixation were performed. The results shown in Table II are thus the average of four experiments for each enzyme.

RESULTS AND DISCUSSION

Comparison of Fixation of coli Mn-SOD and Turkey Cu-SOD on Human Erythrocytes

Turkey Cu-SOD with a pl of 7.35 was used whereas coli Mn-SOD is slightly more basic (pl 7.8). Both have a similar molecular weight (33 000 for the Cu-SOD and 40 000 for

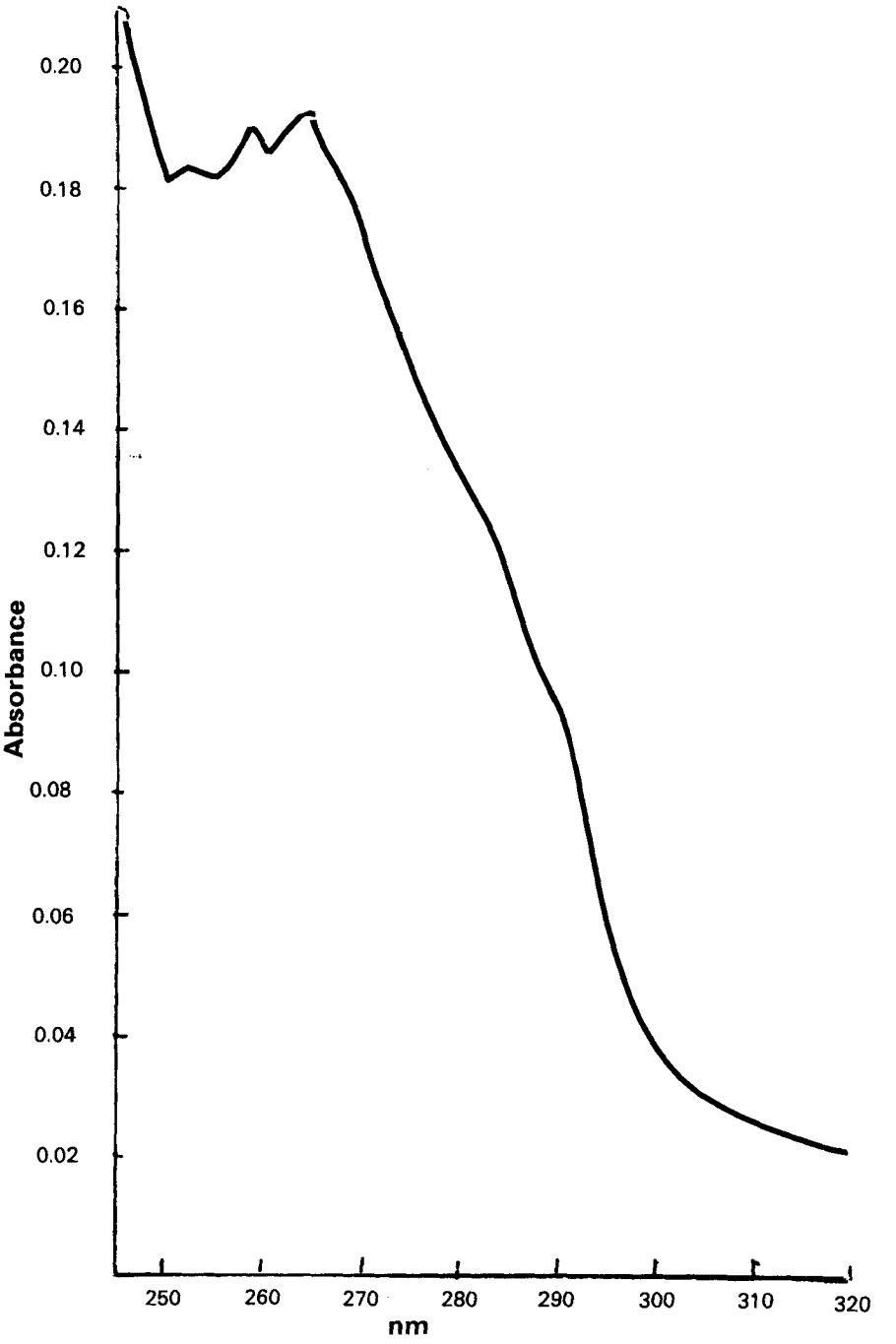

FIGURE 1 Ultraviolet absorption spectrum of turkey.Cu-SOD pl 7.35. The solution contains 0.44 mg enzyme per ml in PBS diluted five fold with water.

TABLE I

	E. coli Mn-SOD		Turkey Cu-SOD pl 7.35	
	1	2	1	2
δ_{260}	0.184	0.173	0.186	0.165
δ_{280}	0.331	0.324	0.126	0.113
280/260	1.80	1.87	0.68	0.68
Protein (μg/ml)	186	182	437	388
cpm/μg	6604	7302	1904	2434
Input	90 μg	91.1 μg	211 μg	200 μg

TABLE II

	E. coli Mn-SOD		Turkey Cu-SOD pl 7.35
Ratio input/endogenous SOD	5.4	12.3	12.1
μg exogenous fixed (0.996×10^9 erythrocytes)	0.1413	0.3472	3.82
μg fixed per 10^9 erythrocytes	0.1417	0.3486	3.84
% Total exogenous fixed	0.157	0.174	1.91
% Exogenous/endogenous SOD	0.836	2.14	22.60

the Mn-SOD). It was therefore of interest to compare the fixation properties of the two enzymes on human erythrocytes.

Fixation as a percentage of endogenous SOD is 10.6 times more efficient for turkey Cu-SOD (pl 7.35) than *coli* Mn-SOD (pl 7.8). Under similar conditions fixation of bovine Cu-SOD (pl 5.0) is about 3% of the endogenous enzyme, i.e. slightly more than *coli* Mn-SOD but eight times less than turkey Cu-SOD. When a lower ratio of input to endogenous SOD is used fixation is reduced as may be expected as shown in the first column of Table II.

Pharmacokinetics in Rats

The various SODs were injected in the vein of the penis of Wistar rats ($\sim$ 300 g) at time zero and the animals sacrificed at different intervals. Organs were removed and the general procedure previous described was followed. Quantities injected per rat were as follows: turkey Cu-SOD pl 7.35, 247 μg at 3224 cpm/μg; turkey Cu-SOD pl 7.67, 206 μg at 3163 cpm/μg; *coli* Mn-SOD, 82.8 μg at 13211 cpm/μg or 200 μg at 5276 cpm/μg.

Exponential decay times in different organs and in the plasma and the erythrocytes are given in Table III. In general two exponentials are observed — a rapid phase followed by a much slower rate of decay.

It can be seen that with respect to the rapid phase both Cu-SODs are eliminated in general (except for the erythrocytes) some three to four times faster than the Mn-SOD whereas with the second slow phase differences are not particularly significant except that clearance from the spleen is again more rapid for the Cu-SODs but slower with respect to the heart.

The relative quantities in different organs as a function of time for the different

TABLE III
Exponential decay times to 50% of maximal

	E. coli Mn-SOD		Turkey Cu-SOD pl 7.35		Turkey Cu-SOD pl 7.67	
	1st exponential	2nd exponential	1st exponential	2nd exponential	1st exponential	2nd exponential
Plasma	0h44′	2h43′	0h09′	2h44′	0h14′	3h25′
Erythrocytes	0h55′	4h55′	0h18′	5h30′	0h19′	4h05′
Liver	2h15′	5h35′	0h43′	3h42′	0h39′	5h13′
Heart	1h40′	3h55′	0h26′	5h58′	0h37′	7h00′
Spleen	1h58′	>10h	0h54′	4h06′	0h31′	1h51′
Lungs	2h06′	—	0h34′	3h28′	0h49′	4h52′

SODs expressed as a percentage of total input per g of tissue are given in Table IV and per total organ in Table V. It can be seen that Mn-SOD is concentrated significantly in the lungs in contrast with the Cu-SODs, and judging by the pharmacokinetics in the kidneys is cleared from the animal much less rapidly (4 hr to reach maximum concentration compared with $\leqslant 30$ min for the Cu-SODs. A much larger fraction of Mn-SOD is also retained in the heart. With respect to circulation the Mn-SOD is again retained in the plasma (and the erythrocytes) to a much larger extent than the Cu-SODs (and also has a longer first exponential life time). Mn-SOD is also present in the liver and the spleen to a greater degree than turkey Cu-SOD pl 7.35. However this situation is completely inversed with the Cu-SOD pl 7.67 which shows very high accumulation in both organs.

It may be concluded that despite the similarities of the three SODs the pharmacokinetics and organ localisations are very different. To be noted also is that *in vitro* the Cu-SOD binds to erythrocytes (human) much more efficiently than Mn-SOD whereas *in vivo*, possibly the result of various plasmatic factors, at least in the rat, the inverse is true.

A comparative analysis of erythrocyte SOD and glutathione peroxidase and serum glutathione transferase in turkey and other species is shown in Table VI.

CONCLUSIONS

It is clear that circulating life times are not correlated with the pl of the SOD considered, nor are the pharmacokinetic properties in general. Human Mn-SOD has a much longer half time (~ 7 h) in the plasma than *coli* Mn-SOD despite the fact that both have practically the same pl. This may in part be attributed to the larger molecular weight (80 000 compared with 40 000). A major role of pl is also excluded by the fact that bacteriocupreine (pl 8.7) has a very short circulating lifetime[1] despite the higher pl and in fact shows pharmacokinetic characteristics very similar to bovine Cu-SOD (pl 5.0).

These studies were initiated to understand the role of various parameters and the mechanisms of anti-inflammatory action of SOD. At least in inflammation models in the rat, homologous rat Cu-SOD is *not* anti-inflammatory and perhaps a certain distance between endogenous and exogenous enzyme, in terms of sequence homology, is necessary[10]. Fixation on cell membranes is perhaps an important step in

TABLE IVa
% cpm injected per gram of tissue

Organ		30′	1h30′	4h	5h	7h
Liver	Coli Mn-SOD	0.47	0.34	0.30	—	0.21
	Turkey Cu-SOD pl 7.35	0.18	0.07	—	0.04	—
	Turkey Cu-SOD pl 7.66	1.12	0.41	0.12	—	0.08
Kidney	Coli Mn-SOD	2.50	4.97	7.03	—	3.28
	Turkey Cu-SOD pl 7.35	5.19	2.38	—	1.24	—
	Turkey Cu-SOD pl 7.66	7.21	4.21	2.13	—	1.26
Spleen	Coli Mn-SOD	0.50	0.35	0.37	—	0.28
	Turkey Cu-SOD pl 7.35	0.17	0.09	—	0.05	—
	Turkey Cu-SOD pl 7.66	2.67	0.76	0.27	—	0.09
Heart	Coli Mn-SOD	0.70	0.49	0.19	—	0.09
	Turkey Cu-SOD pl 7.35	0.15	0.03	—	0.02	—
	Turkey Cu-SOD pl 7.66	0.27	0.1	0.04	—	0.03
Intestines	Coli Mn-SOD	0.50	0.40	0.15	—	0.09
	Turkey Cu-SOD pl 7.35	0.46	0.11	—	0.02	—
	Turkey Cu-SOD pl 7.66	0.34	1.26	0.11	—	0.11
Muscle	Coli Mn-SOD	0.10	0.10	0.08	—	0.05
	Turkey Cu-SOD pl 7.35	0.04	0.01	—	0.01	—
	Turkey Cu-SOD pl 7.66	0.04	0.02	0.01	—	0.01
Lungs	Coli Mn-SOD	1.14	0.93	0.56	—	0.13
	Turkey Cu-SOD pl 7.35	0.20	0.06	—	0.03	—
	Turkey Cu-SOD pl 7.66	0.55	0.24	0.17	—	0.10

TABLE IVb
% cpm injected per ml

		5′	30′	1h30′	4h	5h	7h
Coli Mn-SOD	Plasma	8.19	5.21	1.83	0.79	—	0.35
	Erythrocytes	0.91	0.41	0.15	0.08	—	0.10
Turkey Cu-SOD pl 7.35	Plasma	3.69	0.59	0.17	—	0.07	—
	Erythrocytes	0.25	0.09	0.02	—	0.02	—
Turkey Cu-SOD pl 7.66	Plasma	4.27	1.22	0.49	0.22	—	0.12
	Erythrocytes	0.31	0.13	0.11	0.05	—	0.04

TABLE Va
% cpm per organ

Organ		30′	1h30′	4h	5h	7h
Liver	Coli Mn-SOD	4.86	3.45	2.45	—	1.57
	Turkey Cu-SOD pl 7.35	2.20	0.80	—	0.36	—
	Turkey Cu-SOD pl 7.66	12.71	4.34	1.17	—	0.80
Kidney	Coli Mn-SOD	3.87	7.60	10.13	—	5.28
	Turkey Cu-SOD pl 7.35	9.39	3.85	—	2.29	—
	Turkey Cu-SOD pl 7.66	12.83	7.75	3.59	—	2.01
Spleen	Coli Mn-SOD	0.29	0.21	0.24	—	0.18
	Turkey Cu-SOD pl 7.35	0.12	0.05	—	0.02	—
	Turkey Cu-SOD pl 7.66	1.63	0.43	0.17	—	0.06
Heart	Coli Mn-SOD	0.45	0.33	0.11	—	0.06
	Turkey Cu-SOD pl 7.35	0.10	0.02	—	0.01	—
	Turkey Cu-SOD pl 7.66	0.20	0.06	0.02	—	0.02
Lungs	Coli Mn-SOD	1.22	0.92	0.61	—	0.14
	Turkey Cu-SOD pl 7.35	0.23	0.06	—	0.03	—
	Turkey Cu-SOD pl 7.66	0.63	0.26	0.19	—	0.12

TABLE Va *cont'd*

Organ		30′	1h30′	4h	5h	7h
Thyroid	Coli Mn-SOD	0.05	0.05	0.09	—	0.37
	Turkey Cu-SOD pl 7.35	0.07	0.08	—	0.52	—
	Turkey Cu-SOD pl 7.66	0.12	0.25	0.82	—	1.21

TABLE Vb
% cpm injected, total blood content per animal.

		5′	30′	1h30′	4h	5h	7h
Coli Mn-SOD	Plasma	70.19	42.80	14.95	6.34	—	2.83
	Erythrocytes	7.78	3.35	1.26	0.67	—	0.82
Turkey Cu-SOD pl 7.35	Plasma	36.51	5.68	1.57	—	0.64	—
	Erythrocytes	2.47	0.85	0.23	—	0.18	—
Turkey Cu-SOD pl 7.66	Plasma	41.73	11.55	4.38	2.00	—	1.12
	Erythrocytes	3.06	1.20	1.00	0.45	—	0.38

the biological activity shown by certain SODs. However some caution must be exercised in that *in vivo* studies are much more relevant than use of cell cultures or suspensions of erythrocytes or neutrophils. Thus considerably more Mn-SOD rests in circulation in the plasma (and attached to the erythrocytes) than the Cu-SODs after injection into rats, despite the similarity of pl and the *in vitro* results which would suggest less fixation to erythrocytes. Since fixation is a function of the concentration of exogenous SOD increased fixation to erythrocytes *in vivo* of Mn-SOD could be explained at least partially by the increased plasmatic enzyme compared with Cu-SOD. However other factors undoubtedly are involved.

In addition, there is no correlation between anti-inflammatory activity and the level of circulating exogenous SOD. Both *coli* Mn-SOD and human Mn-SOD are basic proteins but, in the rat caraageenin model, the human enzyme is inefficient whereas the bacterial enzyme (with half the molecular weight) shows anti-inflammatory activity[5]. It may thus be concluded that no correlation can be made between anti-inflammatory activity and pl or circulation life time or indeed pharmacokinetic characteristics in general and that the mechanism of this activity *in vivo* is not yet elucidated. It has been demonstrated that for fibroblasts, protection remains even when the exogenous SOD is removed from the cell culture medium[11]. Removal of plasmatic superoxide is perhaps much less important than fixation to cell membranes. With respect to human use *in vivo*, increased intracellular protection (due to penetration) at the dose rates used (about 1/1000 of the total endogenous SOD), whether free SOD or liposomal SOD, is most unlikely.

The very wide range of anti-inflammatory activity shown by divers SODs from different sources in the rat suggests that the major role is *not* to remove excessive superoxide from the circulation[5]. Certainly, in human pathologies in which large

TABLE VI

	Hb g %	SOD μg/ml blood	SOD μg/g Hb	NBT units per ml blood	NBT units/ g Hb	Cyt units/ ml blood	Cyt units/ g Hb	GPX u/ml blood	GPX u/mg Hb	GST u/ml serum
MICHELSON	15.74	88.30	561	235	1496	596	3747	1580	10.04	2.50
DUROSAY	17.83	81.25	456	217	1216	540	3313	1850	10.62	2.08
SPASIC	17.28	84.50	489	225	1304	558	3406	1702	9.84	2.29
PIG	13.94	62.50	449	167	1197	400	2614	3767	27.02	4.80
TURKEY	18.55	45.40	245	—	—	—	—	573	3.09	14.4

Analysis of erythrocyte SOD and glutathione peroxidase and serum glutathione transferase in a British, French and Yugoslav human, pig and turkey. The first four subjects show a close similarity in SOD whereas in the pig much higher glutathione peroxidase and transferase levels are observed. In contrast the turkey has much lower SOD and glutathione peroxidase but very high glutathione transferase. The results indicate that pigs should have large amounts of fatty tissue with a high lipid metabolism and turkeys should have low fat content with reduced fatty acid metabolism.

amounts of *homologous* SOD are found in the plasma (10–20 fold increase in normal levels) no increased protection is observed, and indeed high plasmatic SOD levels are an excellent indication of various hepatic disorders. A large increase of plasmatic Cu-SOD is found in trisomy 21 subjects[12] and very high levels are also found in certain cases of hemolytic anemia (for whom injections of bovine Cu-SOD are beneficient[13]) again suggesting that in humans homologous human Cu-SOD whether prepared from erythrocytes or produced by genetic engineering techniques will not necessarily be very efficient. However, it will certainly be a useful enzyme for the treatment of rats, rabbits and ruminants since in these cases the protein will be heterologous.

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Accepted by Dr. J.V. Bannister