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Relationship between the pentose phosphate shunt and methemoglobin reductase activity in human erythrocytes: Effect of aging on methemoglobin reductase activity

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Summary. The increase in methemoglobin reductase activity in human erythrocytes upon incubation with inosine, phosphate, pyruvate occurs only in the presence of methylene blue. No difference in activity of the methemoglobin reductases was observed between enzyme extracts of fresh cells and aged cells.

It was reported earlier[2] that human erythrocytes incubated with inosine (10 mM), pyruvate (10 mM) and phosphate (5 mM) (IPP) showed an increase in methylene blue catalyzed methemoglobin reductase activity which was somewhat more prominent in fresh cells than in those stored for 16-30 days in ACD. Inosine is believed to influence red cell metabolism by providing substrate for the pentose phosphate shunt which in turn increases the flow of substrate into the glycolytic pathway[3].

The method used to estimate methemoglobin reductase activity involved following the rate of reduction of methemoglobin in the presence of methylene blue and either lactate or glucose as substrate after oxidation of the red cells with nitrite[4]. There is considerable evidence that the catalytic effect of methylene blue involves an NADPH-dependent methemoglobin reducing system[5,6]. Since methylene blue is known to increase pentose phosphate shunt activity which in turn supplies NADPH the work reported here was undertaken to explore further the relationships between inosine and methylene blue stimulation of methemoglobin reductase activity.

The lack of effect of IPP incubation on methemoglobin reductase activity in the absence of methylene blue shown in figure 1A and the large increase in activity in the presence of methylene blue (figure 1B) is consistent with the idea that the effects of both inosine and methylene blue are operative through the pentose phosphate shunt. The reduction of methylene blue by the NADPH produced by IPP incubation is known to be more rapid than the reduction of methemoglobin directly[5]. The reduced methylene blue can also reduce the methemoglobin nonenzymatically as has been shown by Beutler and Baluda[5] and Sass et al.[6].

Earlier work[7] with rabbit erythrocytes indicated that the loss of methemoglobin reductase activity through hemolysis was much less when the hemolysis was carried out in the presence of nicotinamide and NADPH. The results in figures 2A and 2B show that the low methemoglobin reductase activity in 26-day-old erythrocytes and their hemolysates is increased markedly for cells incubated with IPP and their hemolysates. Thus if the increased reductase activity upon incubation is due to production of NADPH

Comparison of methemoglobin reductase activity of 'crude enzyme extract' in fresh and aged erythrocytes

Extract	Pyridine nucleotide	$\frac{\mu\text{moles DCIP min}^{-1}}{\text{mg protein}} \times 10^3$
Red cells 30 days old	NADH	376
	NADPH	156
Fresh red cells	NADH	333
	NADPH	143

Assay performed by method of Sugita et al.[12]; NADPH dependent activity was determined in the absence of KCl.

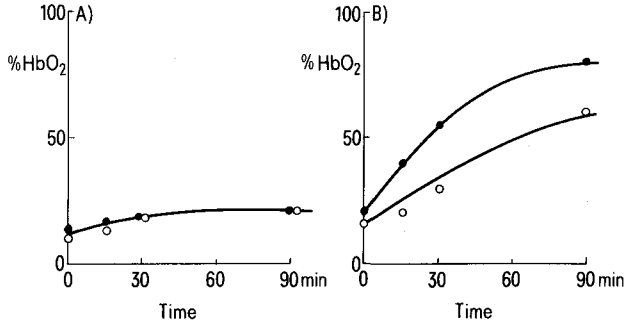

Fig. 1. Effect of incubation of human erythrocytes (stored 6 days in ACD buffer) with inosine (10 mM), pyruvate (10 mM), and phosphate (5 mM) on the rate of reduction of methemoglobin in 0.05 Tris HCl buffer, pH 7.4, 0.1 M NaCl, T = 37 °C: *A* in presence of glucose 20 mM; *B* glucose 20 mM, methylene blue, 5×10^{-5} M; ●, red cells incubated with IPP; ○, red cells without incubation.

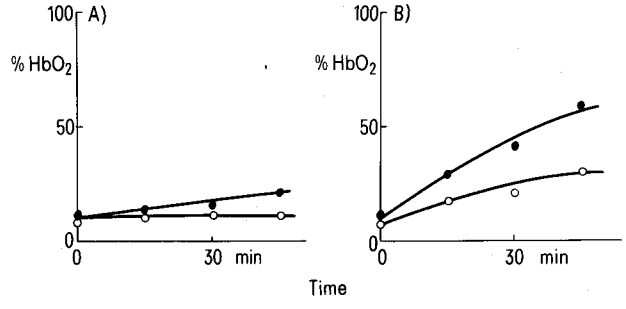

Fig. 2. Rate of reduction of methemoglobin in red cells from blood stored 26 days in ACD at 4 °C and their hemolysates: *A* before incubation of the cells with IPP; *B* after incubation with IPP. Conditions: glucose 20 mM, methylene blue 5×10^{-5} M, 0.05 Tris HCl buffer, pH 7.4, NaCl 0.1 M, T = 37 °C. ●, intact erythrocytes; ○, after hemolysis in 10 vol distilled water, centrifugation and resuspension in Tris buffer.

then it is reasonable that the effect survives the hemolysis process to a large extent.

To investigate the reason for the previously observed[2] decrease in the effect of IPP incubation on reductase efficiency for aged cells, extracts were made of fresh and 30-day cells, according to the method of Hegesh et al.[8]. A comparison of the methemoglobin reductase activity of the 'crude enzyme extract' for fresh and aged erythrocytes is shown in the table. The similarity in reductase activity of the 2 extracts indicate that the activity of the reductases does not decrease during 30 days storage. Control experiments in which the reductase activity of 26-day-old red cells before and after incubation with IPP were compared and indicated no change in activity caused by the incubation. Further, disc gel electrophoresis of the 'crude enzyme extracts' were carried out according to the method of Kaplan[9] and showed no significant differences between the reductases from fresh and 26-day-old cells or between IPP incubated and nonincubated 26-day-old cells. Since the difference in the effect of IPP incubation between fresh and aged cells does not lie with the methemoglobin reductase then perhaps one of the enzymes in the pentose phosphate pathway is responsible.

The activity of glucose-6-phosphate dehydrogenase has been reported to decline upon aging of the red cell[10]. Further, methylene blue is known to have less effect on reductase activity in cells deficient in glucose-6-phosphate dehydrogenase activity[11].

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Production of antibodies against bradykinin

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Summary. High-titer antibodies against bradykinin were raised in rabbits. 2 different conjugates of bradykinin were used for immunization: bradykinin coupled to human serum albumin via 1,5-difluoro-2,4-dinitrobenzene and bradykinin coupled to edestin via 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide. The sensitivity of the radioimmunoassay method is in the range of 1–50 pg of bradykinin. Cross-reaction of anti-bradykinin antisera occurred with kallidin and met-lys-bradykinin.

The sensitivity and specificity of a radioimmunoassay depends mainly on the sensitivity and the specificity of the reaction between the antigenic determinant(s) of the antigen, and the specific binding sites of the antibody population in the antiserum. Consequently, for establishing a sensitive radioimmunoassay, the production of an antiserum with a high specific antibody titer is of primary importance. Various factors, including the immunogenicity of the antigen used[1], are of significance for the specificity of the antibodies. Methods of producing antibodies against bradykinin have been described by various investigators[2–9], but in most cases, very low concentrations of antibodies were obtained (titers up to 1:1000).

The purpose of the present study was to reinvestigate the coupling methods used so far, in order to obtain an antigen of high immunogenicity.

Conjugation methods for the preparation of bradykinin immunogens

Method	Tager[10]	Goodfriend and Ball[2]
Bradykinin triacetate (BK)	10 mg	35 mg
Solvent for BK	1.0 ml 0.1 M potassiumphosphate + 7 M guanidine HCl pH 7.0	1.5 ml water
Coupling reagent	150 mg 1,5-difluoro-2,4-dinitrobenzene (DFDNB)	50 mg 1-ethyl-3(3-dimethyl-amino-propyl)carbodiimide HCl (EDC)
Solvent for coupling reagent	5.0 ml methanol	1.0 ml water
Protein carrier	20 mg human serum albumin	70 mg edestin
Solvent for protein carrier	1.0 ml 0.4 M sodiumboratebuffer pH 10.0	3.0 ml 0.1 M Tris-HCl pH 5.34
pH of reaction mixture	9.6	4.75
Reaction time	24 h at R.T.	1 h at 18 °C
Coupling rate	70–80%	10–25%
Number of BK-molecules/ molecule of protein carrier	10–11	6–15