Determination of pyrimidine 5'-nucleotidase (P5N) activity in whole blood as an index of lead exposure

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ABSTRACT A simple method for determining pyrimidine 5'-nucleotidase (P5N) activity in whole blood has been developed, inhibiting the plasma activity for UMP-hydrolysis by concanavalin (Con A). Con A specifically inhibits the activity of plasma 5'-nucleotidase (5N) but does not affect erythrocyte P5N activity. The anticoagulant EDTA partially inhibits 5N activity but slightly activates P5N. P5N activity determined by the present method with heparinised blood and Con A was comparable with that by the method reported previously and correlated well with blood lead concentrations. The mean value and SD for P5N activity in normal subjects ($n = 72$) not exposed to lead are 16.2 and 2.5 $\mu\text{mole/h/g Hb}$, respectively. The present method can eliminate not only the isolation step of RBC but also Hb determination, the activity being expressed as $\mu\text{mole/h/l blood or RBC}$. Thus the procedures are so simplified that the assay may be used as a routine test for mass screening of lead exposure.

Pyrimidine 5'-nucleotidase (P5N, EC 3.1.3.5) in the red cell cytosol catalyses the hydrolytic dephosphorylation of pyrimidine 5'-monophosphates but is ineffective on purine nucleotidase. The presence of P5N was first reported by Valentine *et al* in 1974 in their description of haemolytic anaemia.¹ Hereditary P5N deficiency results in a non-spherocytic haemolytic anaemia in which the erythrocytes contain extremely high concentrations of pyrimidine nucleotides and show pronounced basophilic stippling. Erythrocyte P5N activity is also known to decline in lead poisoning.²⁻⁴ The measurement of the enzyme activity has a diagnostic value in these disorders.

The conventional method for determining P5N activity is based on the liberation of inorganic phosphorus from pyrimidine 5'-nucleotides and requires time consuming and complicated procedures, limiting its application in the clinical laboratory. We have previously developed a rapid and sensitive method for P5N,⁵⁻⁶ in which the product (uridine) is separated from the substrate (UMP) and red cell constituents by means of high performance liquid chromatography (HPLC). Because the amount of endogenous uridine is non-detectable and the intensity of the absorption of uridine is high at 254 nm, the need to dialyse the sample is eliminated and the incubation time is

shortened. Nevertheless, the method still requires washed erythrocytes as the enzyme source, since serum 5'-nucleotidase (5N) non-specifically hydrolyses 5'-nucleotide to release inorganic phosphate at neutral condition. 5N activity is known to be increased in hepatobiliary disease and serves as a diagnostic tool for the disorder.⁷⁻⁸ In the present paper we describe P5N assay in whole blood, inhibiting 5N activity by a specific inhibitor, concanavalin A (Con A).^{9,10}

Materials and methods

Venous blood, treated with EDTA-2K or heparin as anticoagulants, was obtained from lead workers and from normal subjects with no history of exposure to lead. The lead workers were employed in secondary smelters, glass factories, and printing offices. Erythrocytes were separated from plasma, washed twice with a 10-fold volume of saline (0.9% NaCl), and then suspended in saline (about 50% suspension; RBC/saline). For the determination of P5N or 5N activity whole blood, or both, RBC/saline or plasma were used for the enzyme sources. P5N (and/or 5N) activity was determined by the method previously reported with some modifications^{5,6} as follows: the enzyme solution was prepared by diluting 50 μl of blood, RBC/saline, or plasma to 550 μl with distilled water. Commercially available UMP-Na (Sigma Chemical Co, Saint Louis, USA) was used as the substrate without purification

instead of buffered substrate as in the original method.⁵ The standard assay mixture contained 550 μ l of enzyme solution, 100 μ l of 0.2M Tris-HCl buffer (pH 7.7) containing 75 mM MgCl₂, 50 μ l of Con A (type IV, Sigma Chemical Co, Saint Louis, USA) solution (20 g/l), and 50 μ l of 50 mM UMP-Na. To determine the P5N activity without Con A, distilled water was added to the mixture instead of Con A solution. The reaction was started by adding substrate at 37°C. After 60 minutes the reaction was terminated by placing the tubes in boiling water for three minutes. Then 2.25 ml of distilled water were added to the mixture and agitated. After centrifugation, the resulting supernatant was used for the HPLC analysis. A Shimadzu liquid chromatograph (Shimadzu, Kyoto, Japan) consisting of a pump (LC-3A), an automatic sample injector (SIL-2A), a column oven (CTO-2A), a variable wavelength spectrophotometer (SPD-1), and an integrator (C-R1A) was used. Flow rate, oven temperature, and detector wavelength were set at 1.0 ml/min, 40°C, and 254 nm, respectively. The column (150 $\times$ 4 mm) used was packed with reversed phase (ODS) silica. The mobile phase was 5% methanol containing 5 mM KH₂PO₄ and 0.2 mM 1-decanesulphonic acid. Samples were cooled at 4°C during a series of analyses and 10 μ l were automatically injected at seven minute intervals. The unit (u) of P5N activity was expressed as μ mole/h/g Hb, μ mole/h/l RBC, or μ mole/h/l blood, and that of plasma 5N as μ mole/min/l. Serum 5N activity (μ mole/min/l) in some subjects was also determined by the method of Belfield *et al.*¹¹ Hb concentrations in RBC/saline or blood were determined using a haemoglobin counter (TOA Medical Electronic Co, Tokyo, Japan), which directly measured Hb concentrations spectrophotometrically as cyanmethaemoglobin. Blood lead concentrations (Pb-B) were determined by the method previously reported.¹²

Results

Figure 1 shows the effect of increasing amounts of inhibitor Con A on hydrolysis of UMP by blood from a normal subject whose 5N activity in serum was 21.3 μ mole/min/l. Zero to 5 mg of Con A are added to the reaction mixtures to produce final concentrations of 0 to 6.67 mg/l of Con A. The activity of heparinised blood declines in a linear fashion as the amount of inhibitor increases up to 0.5 mg but no further decline is observed when the amount of the inhibitor exceeds 0.5 mg. It appears that the difference between the activity in the presence and absence of Con A corresponds to the action of 5N in plasma, and that the residual activity with Con A exceeding 0.5 mg represents the hydrolysis of UMP by erythrocyte

Fig 1 Effect of increasing amounts of Con A on hydrolysis of UMP (μ mole/h/g Hb) by blood from a normal subject whose 5N activity in serum is 21.3 μ mole/min/l. Blood treated with EDTA ($\diamond$) or heparin ($\circ$) was used as enzyme sources. Zero to 5 mg of Con A was added to reaction mixture.

enzyme (P5N). The effect of Con A on UMP-hydrolysis of blood treated with EDTA differs considerably from that of heparinised blood. Even without addition of Con A, hydrolysis of UMP by blood treated with EDTA is weaker than that by heparinised blood. The activity of EDTA treated blood is further reduced with the addition of Con A. The residual activity, however, is slightly higher than that of heparinised blood. To discover the effects of EDTA or Con A, or both, on the enzymic hydrolysis of UMP by blood, plasma or erythrocytes from heparinised blood were examined separately. Table 1

Table 1 Effect of EDTA or Con A, or both, on the hydrolysis of UMP by plasma

	Activity (μ mole/min/l)
Control	10.88 $\pm$ 0.139
+ Con A	1.92 $\pm$ 0.113
+ EDTA	1.84 $\pm$ 0.106
+ EDTA + Con A	1.46 $\pm$ 0.073

Plasma pooled from heparinised blood was used as the enzyme source with or without treatment of EDTA, which was carried out by incubating plasma with EDTA-2K (8 mM) for 20 hours at 4°C. Con A (1 mg) was added to the reaction mixture. Results were the mean $\pm$ SD of triplicate determinations.

shows the action of EDTA or Con A, or both, on the activity of plasma (5N). The hydrolysis of UMP by plasma from a normal subject is depressed by both Con A and EDTA. The depressed activity is not restored by the addition of Mg ion to the reaction mixture at concentrations up to 70 mM (data not shown). In erythrocytes treated with EDTA the hydrolysis of UMP is slightly (about 10%) augmented, whereas Con A has no effect on the hydrolysis of UMP by erythrocytes (table 2). The augmentation of UMP hydrolysis is reversible, since the activity returns to the control level by rewashing the erythrocytes with saline (table 3).

To confirm further the effects of the anticoagulant and of Con A on the hydrolysis of UMP by whole blood and erythrocytes, we determined the activity in

Table 2 *Effect of EDTA or Con A, or both, on the hydrolysis of UMP by RBC/saline*

	Activity ($\mu\text{mole/h/g Hb}$)
Control	13.7 $\pm$ 0.28
+ Con A	13.6 $\pm$ 0.26
+ EDTA	15.1 $\pm$ 0.21
+ EDTA + Con A	15.0 $\pm$ 0.25

RBC/saline from normal heparinised blood was used as the enzyme source with or without treatment of EDTA, which was carried out by incubating RBC/saline with EDTA-2K (8 mM) for 20 hours at 4°C. Con A (1 mg) was added to the reaction mixture. Results were the mean $\pm$ SD of triplicate determinations.

Table 3 *Reversible effect of EDTA treatment on the hydrolysis of UMP by RBC/saline from a normal subject and a lead worker*

	Activity ($\mu\text{mole/h/g Hb}$)	
	Normal subject	Lead worker
Control	15.6 $\pm$ 0.41	4.6 $\pm$ 0.09
EDTA treatment	16.1 $\pm$ 0.07	5.9 $\pm$ 0.11
Rewashing	15.2 $\pm$ 0.10	4.6 $\pm$ 0.09

RBC/saline was treated with EDTA as described in table 2 (EDTA treatment) and then rewashed with saline (rewashing). The results were the mean $\pm$ SD of triplicate determinations.

Table 4 *Various methods for P5N assay and comparison of data (y) with those (x) using the method (RH-) reported (5-6) in 16 lead workers*

Method	Enzyme source	Anti-coagulant	Con A	Activity ($\mu\text{mole/h/g Hb}$)		Level of significance	Linear regression analysis			Fig 2
				Mean	SD		Slope	y-intercept	r	
WH+	W	H	+	15.46	2.99	NS	1.009	0.0735	0.97	(e)
WH-	W	H	-	19.66	4.35	$p < 0.01$	0.923	5.38	0.63	(c)
WE+	W	E	+	16.39	3.07	NS	1.011	0.752	0.98	(f)
WE-	W	E	-	18.33	3.81	$p < 0.05$	1.003	2.439	0.81	(d)
RH+	R	H	+	15.23	3.01	NS	1.002	-0.259	0.99	(b)
RH-	R	H	-	15.46	2.99					
RE+	R	E	+	15.52	2.95	NS	0.961	0.666	0.97	
RE-	R	E	-	15.37	2.95	NS	0.975	0.295	0.99	(a)

W = Whole blood, R = RBC/saline, H = heparin, E = EDTA, NS: Not significant.

samples from 16 lead workers (mean Pb-B $\pm$ SD, 12.4 $\pm$ 3.7 $\mu\text{g}/100\text{ g}$) under various conditions as shown in table 4. With the exception of activities in whole blood without Con A (WH-, WE-), the data obtained are comparable with those of the method reported earlier⁶ in which erythrocytes from heparinised blood (RH-) are used as enzyme source. Data

Fig 2 *Relations between P5N activity (y) by various methods as in table 4 and that with heparinised RBC without inhibitor (x, RH-). Correlation coefficients and regression equations are also shown in table 4. Both regression (—) and identical (---) lines are shown.*

obtained by the various methods in table 4 correlate well with those of the method reported previously (fig 2). Figure 2a shows that the data with RBC from EDTA treated blood (RE-) are similar to those of the method (RH-) reported previously, indicating that the activating effect of EDTA on the erythrocyte enzyme is eliminated by washing erythrocytes with saline. It is also confirmed that Con A has no effect on the erythrocyte enzyme (fig 2b). The activities obtained with blood treated with EDTA (WE-) or heparin (WH-) are higher (27.2% or 21.8%, respectively) than those using the previous method (RH-, figs 2c and d). It appears that the differences between activity with whole blood and with erythrocytes are due mainly to the plasma action (5N) of hydrolysis. The amount of EDTA used as anticoagulant seems to be not sufficient totally to inhibit the activity of plasma (fig 2d). The concentration should not be so high as in the experiments reported in table 1. Activities determined with heparinised blood and Con A (WH+) agree well with those of the earlier method (RH-, fig 2e), whereas data from EDTA treated blood and Con A (WE+) are slightly higher (RH-, fig 2f). The slightly raised activity in EDTA treated blood (WE+) is due to the weak activation of erythrocyte enzyme by EDTA. Based on these findings, it appears that Con A is useful as a differential inhibitor of enzymic hydrolysis of UMP by plasma 5N and erythrocyte P5N.

To evaluate the method for determining P5N activity with whole blood and Con A, we adopted it to the larger group of lead workers ($n = 37$). Figure 3 shows the relations between Pb-B and P5N activity determined with whole blood in the presence or absence of Con A. When P5N activity is determined with whole blood in the presence of Con A (figs 3a, c), correlation coefficients of Pb-B ν log P5N are improved by comparison with those in the absence of inhibitor (figs 3b, d). In the presence of Con A the activities with EDTA treated blood (fig 3c) are slightly higher (about 10%) than those with heparinised blood (fig 3a). Thus in the present study the method with heparinised blood and Con A (WH+) was selected for determining P5N activity in lead workers. The activity may be expressed in $\mu\text{mole/h/l}$ blood or RBC, omitting the measurement of Hb. In the expression of $\mu\text{mole/h/l}$ RBC, however, Hct determination is necessary. In 83 lead workers we compared the expression of the three types of unit with regard to the relation of Pb-B ν P5N activity (fig 4). A strong negative correlation was observed between Pb-B and log P5N activity expressed in $\mu\text{mole/h/l}$ RBC ($r = -0.91$) and in $\mu\text{mole/h/l}$ blood ($r = -0.88$) and when expressed in conventional units ($r = -0.91$). The three units may be converted using the equations given in table 5.

In the present method with heparinised blood and

Fig 3 Relations between Pb-B ($\mu\text{g}/100\text{ g}$) and P5N activity ($\mu\text{mole/h/g Hb}$) in blood from 37 lead workers. P5N activity was determined by four types of method in table 4. Regression lines (—) and 95% predictive interval of individual values (---) are shown in figures. (a) WH+, $y = -0.212 \times + 18.22$ ($r = -0.85$) or $\log y = -0.00887 \times + 1.303$ ($r = -0.89$), (b) WH-, $y = -0.236 \times + 22.37$ ($r = -0.80$) or $\log y = -0.00721 \times + 1.373$ ($r = -0.85$), (c) WE+, $y = -0.227 \times + 18.97$ ($r = 0.87$) or $\log y = -0.00927 \times + 1.33$ ($r = -0.92$), (d) WE-, $y = -0.235 \times + 21.15$ ($r = 0.83$) or $\log y = 0.00801 \times + 1.36$ ($r = -0.86$).

Con A the reference value was determined in a control group of non-exposed men ($n = 72$). The arithmetic mean and SD of P5N activity were 16.2 and 2.5 $\mu\text{mole/h/g Hb}$, respectively. The age of the subjects ranged between 19 and 55 (mean $\pm$ SD, 34.4 ± 6.0). No significant relation was found between age (x) and P5N activity (y) ($y = 0.0192x + 15.5$, $r = 0.09$). Within assay and between assay CVs are shown in table 6, indicating that the precision of the present method is sufficiently accurate to detect decreased activity in subjects exposed to lead.

Fig 4 Relations between Pb-B ($\mu\text{g}/100\text{ g}$) and P5N activity expressed in three types of unit by method "WH+" in 83 lead workers. P5N activity was corrected with Hb (a, $\mu\text{mole/h/g Hb}$) or with Hct (b, $\mu\text{mole/h/l RBC}$), or not corrected (c, $\mu\text{mole/h/l blood}$). Regression lines (—) and 95% predictive interval of individual values (---) are shown in figures. (a) $y = -0.209 \times + 18.4$ ($r = -0.88$) or $\log y = -0.00811 \times + 1.29$ ($r = -0.91$), (b) $y = -71.09 \times + 6147$ ($r = -0.88$) or $\log y = -0.0084 \times + 3.82$ ($r = -0.91$), (c) $y = -34.788 \times + 2898$ ($r = -0.85$) or $\log y = -0.0091 \times + 3.49$ ($r = -0.88$).

Table 5 Relations among P5N activities expressed in three types of unit

x	y	Regression equation	r
$\mu\text{mole/h/l RBC}$	$\mu\text{mole/h/g Hb}$	$y = 0.00295 \times + 0.339$	0.99
$\mu\text{mole/h/l blood}$	$\mu\text{mole/h/g Hb}$	$y = 0.00569 \times + 1.708$	0.98
$\mu\text{mole/h/l blood}$	$\mu\text{mole/h/l RBC}$	$y = 1.929 \times + 468$	0.98

Samples used were the same in fig 4 ($n = 83$).

Table 6 Precision of the simplified method

Sample	Within assay		Between assay	
	Mean $\pm$ SD ($\mu\text{mole/h/g Hb}$)	CV (%)	Mean $\pm$ SD ($\mu\text{mole/h/g Hb}$)	CV (%)
Normal subject	16.0 ± 0.35	2.1	15.2 ± 0.83	5.5
Lead worker	6.2 ± 0.29	4.7	6.4 ± 0.50	7.8

$n = 8$.

Discussion

Con A is a jack bean lectin that binds to α -D-mannose and related saccharide residues. Reversible inhibition of 5N by Con A is thought to result from a direct interaction of Con A to saccharide residues on the glycoprotein enzyme molecules.⁹⁻¹⁰ Zygowicz *et al* reported that Con A was a useful reagent in clinical chemistry for differentiating enzymic hydrolysis of 5'-AMP by 5-N from that by alkaline phosphatase in serum, since Con A does not inhibit non-specific hydrolysis of 5'-AMP (adenosine 5'-monophosphate) by alkaline phosphatase.¹⁰ Although hydrolysis of AMP by erythrocyte 5N was reported to be partially inhibited by Con A,¹⁰ we found that erythrocyte P5N was not affected by the reagent (table 2). When whole blood was used as the enzyme source in P5N assay, substrate UMP was hydrolysed not only by erythrocyte P5N but also by plasma 5N under neutral conditions. 5N activity was almost inhibited by Con A in amounts exceeding 0.5 mg (fig 1). The sample used in fig 1 showed the highest 5N activity among those we experienced in the present study. Therefore 1 mg of Con A was selected to be sufficient to inhibit 5N in the assay of P5N activity using whole blood. Using the present method with whole blood and Con A we obtained data comparable with those obtained by the method reported previously in which isolated RBC were used as the enzyme source.⁶

Serum 5N was known to have greater resistance to inactivation by EDTA compared with non-specific alkaline phosphatase.¹³ We found, however, that UMP-hydrolysis by plasma from heparinised blood was suppressed by EDTA treatment in neutral conditions (table 1).

All of the methods reported so far require washed

RBC as the enzyme source. Washing erythrocytes is one of the most time consuming steps for assaying P5N activity so the main purpose of the present study was to eliminate the step of RBC isolation. We successfully determined P5N activity in whole blood without washing erythrocyte, inhibiting the plasma activity for UMP hydrolysis by Con A. P5N activity with the present method with whole blood was comparable with that with washed RBC and correlated well with blood lead concentrations. In the method with whole blood Hb determination may also be omitted and the activity expressed as $\mu\text{mole/h/l}$ blood or $\mu\text{mole/h/l}$ RBC rather than $\mu\text{mole/h/g}$ Hb. The correlation between Pb-B and log P5N in $\mu\text{mole/h/l}$ blood is slightly lower than that between Pb-B and log P5N in other units because the activity in $\mu\text{mole/h/l}$ blood is not corrected to the red cell volume. The activity may be determined even when the blood is partly haemolysed during storage at 4°C , this has little effect on the activity,^{6,14} and is expressed as $\mu\text{mole/h/l}$ blood. Thus the present method eliminates not only isolation of RBC but also Hb determination, and is so simple that it may be used for the mass screening of lead exposure.

In a previous paper we showed that the P5N activity in Japanese subjects⁶ was almost twice that in American and European subjects.^{1,2,4,15-17} Sato *et al*¹⁸ explained the higher activity in Japanese subjects as due to the lower Pb-B concentrations.¹⁸ In the present study the reference value for P5N activity determined in the control groups was 16.2 ± 2.5 (arithmetic mean $\pm$ SD) but the Pb-B values were not determined. These data are similar to those reported by Sato *et al*,¹⁸ taking into account that the substrate used in our method is UMP. By contrast, Cook *et al*¹⁹ recently reported normal values $\pm$ SD for P5N activity as 12.0 ± 0.71 ($n = 14$), using an HPLC method with UMP as the substrate.¹⁹ The mean Pb-B $\pm$ SD in their control group was $0.37 \pm 0.02 \mu\text{M}$ ($7.6 \pm 4.0 \mu\text{g}/100 \text{ g}$) which was not so high and similar to that of Japanese subjects.¹⁸ The normal value for P5N activity by Cook *et al*¹⁹ is similar to those reported by American and European investigators^{1,2,4,15-17} but somewhat lower than those reported here or by Sato *et al*.¹⁸ It seems that the difference in P5N activity is not only due to the different concentrations of Pb-B but also to other factors, including genetic differences.

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