Comparison of *in vivo* effect of inorganic lead and cadmium on glutathione reductase system and δ-aminolevulinate dehydratase in human erythrocytes

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Roels, H. A., Buchet, J. P., Lauwerys, R. R., and Sonnet, J. (1975). *British Journal of Industrial Medicine*, 32, 181-192. Comparison of *in vivo* effect of inorganic lead and cadmium on glutathione reductase system and δ-aminolevulinate dehydratase in human erythrocytes. The activity of δ-aminolevulinate dehydratase (ALAD) of erythrocytes, the lead (Pb-B) and cadmium (Cd-B) concentration in whole blood, the content of reduced glutathione (GSH) in erythrocytes, and the regeneration rate of GSH by intact erythrocytes were measured during an epidemiological survey of 84 men employed in a Belgian cadmium and lead producing plant. A control group of 26 persons (students and laboratory staff) was also examined.

The logarithm of the ALAD activity is highly inversely correlated with log Pb-B (*r* = -0.760) but no correlation was found with log Cd-B. There exists a significant negative correlation between GSH and log Pb-B (*r* = -0.423) but not between GSH and log Cd-B. The apparently good relationship between log ALAD and GSH disappeared completely by holding log Pb-B constant, but log ALAD remained highly inversely correlated with log Pb-B when standardized for GSH concentration (*r* = -0.748).

*In vivo* investigation of the GSH regeneration rate of intact erythrocytes demonstrated clearly that the overall activity of the glutathione oxidation-reduction pathway is not impaired in Pb and Cd-exposed workers with significantly increased Pb-B and Cd-B, since their initial GSH regeneration rate (first 15 minutes) was identical with that of the control group. Results of similar *in vitro* experiments in which control whole blood was incubated beforehand with Pb²⁺ or Cd²⁺, or both, reinforce this conclusion.

Since increased Cd-B and Pb-B do not influence the glutathione reductase system of erythrocytes, and since endogenous erythrocyte GSH is not correlated with Cd-B, the moderate decrease in endogenous erythrocyte GSH found in Pb-exposed workers might result from a Pb-induced impairment of the erythrocyte mechanism for glutathione synthesis.

Of the heavy metals, cadmium, mercury, and lead, commonly recognized as toxic contaminants of our environment, only lead inhibits *in vivo* the human erythrocytic enzyme δ-aminolevulinate dehydratase (ALAD, 5-aminolevulinate hydro-lyase; EC 4.2.1.24) specifically (Lauwerys and Buchet, 1973; Lauwerys, Buchet, and Roels, 1973). This enzyme appears *in vivo* and *in vitro* to be extremely sensitive to the effect of inorganic lead (Pb), and its activity closely and inversely correlates with the concentration of lead in blood (Pb-B), even within the ‘normal Pb-B range’, that is 0.7-1.9 μmol/l (15-40 μg/100 ml) (Hernberg
and Nikkanen, 1972). A decreased activity of ALAD in mature erythrocytes of peripheral blood is considered to be a very early biological sign of exposure to lead (Hernberg et al., 1972) and under the usual environmental and occupational conditions (Pb-B < 5-8 μmol/l (120 μg/100 ml)) the activity as measured by test tube assay is a true reflection of the ALAD activity in vivo (Roels, Buchet, and Lauwerys, 1974). Although depressed ALAD activity in circulating erythrocytes can hardly have any significance for man's health, obviously it indicates indirectly a concomitant impairment of this enzyme by lead in haem-synthesizing tissues (Gibson and Goldberg, 1970; Millar et al., 1970; Kao and Forbes, 1973; Secchi, Erba, and Cambiaggi, 1974). In the concentration range 1-2-4-8 μmol/l (25-100 μg Pb/100 ml blood), lead primarily exerts a non-competitive inhibition of ALAD as shown for human (Granick et al., 1963) and bovine (Hapke and Prigge, 1973) erythrocyte ALAD; similar inhibition properties of Pb²⁺ were shown with purified beef-liver ALAD (Wilson, Burger, and Dowdle, 1972).

In addition, results of in vitro experiments with purified ALAD from different species strongly suggest that Pb-ions probably affect this enzyme by inactivating its essential sulphhydryl groups (Granick and Mauzerall, 1958; de Barreiro, 1969; Tomio, Tuzman, and Grinstein, 1968; Gibson and Goldberg, 1970; Granick et al., 1973).

The reversal of the ALAD deficit in Pb-poisoned erythrocytes of whole blood by preactivating the incubation mixture with thiol compounds has been demonstrated in vitro, for example, with reduced glutathione (GSH) (Lichtman and Feldman, 1963; Basecqz, Lauwerys, and Buchet, 1971; Hapke and Prigge, 1973), with cysteine (Heilmeyer, 1966), or with dithiothreitol (Granick et al., 1973). Similar observations were reported for rabbit liver ALAD after acute experimental lead-poisoning of the animals (de Barreiro, 1969). In vivo administration of GSH to workers with lead-intoxication improved the depressed erythrocyte ALAD activity to some extent (Nakao, Wada, and Yano, 1968). Several authors hypothesize that the endogenous concentration of GSH in erythrocytes or free -SH groups in liver cells play a regulating role in the activity of ALAD (de Barreiro, 1969; Moore et al., 1971; Granick et al., 1973).

The endogenous GSH concentration in erythrocytes is known to decrease moderately but significantly in workers intoxicated by lead (Shiraishi, 1952; Jonderko, 1961; Rubino et al., 1963; Bonsignore et al., 1967; Vasilii, Stavri, and Freund, 1969; Batolska and Marinova, 1970). Its reduction occurs also in rabbits experimentally poisoned with lead (Nagai, Huse, and Saikawa, 1956; Jonderko and Sroczyński, 1963; Vergnano, Cartasegna, and Bonsignore, 1967). The biological significance of the high intracellular GSH concentration (2-2-5 mmol/litre packed erythrocytes = 0-4 to 0-5 mole/mole haemoglobin) for the normal function and structure of mature erythrocytes has been greatly clarified by the discovery of persons with a hereditary defect in the enzyme systems (glucose-6-phosphate dehydrogenase (G-6-PD) and glutathione synthetase) involved in the maintenance of the original high GSH level (Beutler, Robson, and Buttenwieser, 1957; Prins and Loos, 1969). Deficiency in one enzyme system, for example G-6-PD, can cause hypersusceptibility to occupational (Granzoni and Rhomberg, 1965; Steiger, 1968; Saita and Lussana, 1971) or environmental (McIntire and Angle, 1972) lead-hazards.

However, the relationship between ALAD activity of lead-poisoned erythrocytes and their level of endogenous GSH has been interpreted by several authors in different ways: (a) if a control mechanism for ALAD involving GSH exists, it might have been affected in intoxication with lead (de Barreiro, 1969); (b) the lead-induced loss of ALAD activity should scarcely be influenced by the endogenous GSH concentration (Granick et al., 1973); or (c) lead should not act directly on GSH, and the inhibition of ALAD would not be due to reduced levels of GSH (Cartasegna, Vergnano, and Ardoino, 1967; Vergnano et al., 1967). Concerning the effects of lead on the activity of erythrocyte G-6-PD (measured in haemolysates) conflicting results have been reported on occupationally lead-intoxicated workers (Rubino et al., 1963; Molé et al., 1965) and on experimentally lead-poisoned animals (Molé et al., 1965; Erich and Waller, 1967; Rogers et al., 1971).

Little is known about the interference of cadmium (Cd) with the in vivo activity of erythrocyte enzymes, but it is interesting to notice that the activities of both glutathione reductase and ALAD are affected in vitro by low Cd²⁺ concentrations. Indeed, purified human erythrocyte glutathione reductase (active form) which possesses sulphhydryl group(s) in its active centre is inhibited by 50% in the presence of 1 μmol Cd²⁺/litre (Icén, 1967). A low concentration of Cd²⁺ (0-1 mmol/litre) also inhibits at low substrate concentrations (<1 mmol/litre) purified beef-liver ALAD (Wilson et al., 1972). Human erythrocyte ALAD in normal whole blood appears to be inhibited in in vitro addition of 0-5 mg Cd²⁺/litre (Abdulla and Haeger-Aronsen, 1973).

Challenged by some of these conflicting data in the literature and the opposite hypothesis derived from them, we investigated during an epidemiological survey among workers exposed to lead and cadmium dust the interrelationships between three biological parameters, the concentration of Pb and Cd in blood, the activity of erythrocyte ALAD and the endogenous GSH level of erythrocytes. Furthermore, in vivo and in vitro experiments were carried out with
intact washed erythrocytes to evaluate the influence of increased blood lead and/or cadmium concentrations on the multi-enzyme system involved in the regeneration of endogenous GSH after its intracellular oxidation with methyl phenylazoformate (Kosower, Song, and Kosower, 1969a). Indeed, if cadmium or lead or both should significantly influence one or more enzymes involved in GSH regeneration, it would be reflected by the overall GSH regeneration rate of intact erythrocytes. In connexion with these in vivo and in vitro experiments we studied the distribution of Pb and Cd in whole blood of workers exposed to both metals and in control whole blood to which Pb²⁺ and/or Cd²⁺ were added in vitro. Finally, two methods, a chemical isolation (Beutler, Duron, and Kelly, 1963) and an enzymatic assay (Tietze, 1969), for determining glutathione in erythrocytes were compared.

**Materials and methods**

The ALAD activity of erythrocytes, the cadmium (Cd-B) and lead (Pb-B) concentration in whole blood, the content of reduced glutathione (GSH) in erythrocytes, and the regeneration rate of GSH by intact erythrocytes were measured during an epidemiological survey of 84 men employed in a Belgian cadmium and lead producing plant that differs from that mentioned in our previous paper (Lauwers et al., 1973). A control group of 26 students and laboratory staff (including five women) was also examined. About 20 ml of blood was obtained by venepuncture, immediately heparinized in capped polyethylene tubes, and kept at 4°C in a portable refrigerator.

Respirable dusts (aerodynamic diameter < 5 μm) were collected at different work places with the use of a gravimetric dust sampler (Casella, London, type 113A). The respirable Cd and Pb concentrations were measured by atomic absorption spectrophotometry; they ranged from 7.5 to 65 and from 3 to 26 μg/m³ respectively.

ALAD activity in whole blood was always measured 24 hours after blood withdrawal according to our method described previously (Lauwers et al., 1973). The activity, corrected for the blood haematocrit, is expressed in units of absorbance for 2 cm light path:

\[
\text{Activity} = \left( \frac{A_{\text{analyzed}} - A_{\text{blank}}}{10} \right) \times \text{hour/ml erythrocytes}. 
\]

Cd-B and Pb-B were determined by the ion exchange-atomic absorption spectrophotometric method of Vens and Lauwers (1972) and are expressed in nanomoles per litre (micrograms per 100 ml) and micromoles per litre (micrograms per 100 ml) of whole blood respectively. In some cases where the metal concentrations were very low, that is, in Pb and Cd uptake and distribution experiments, the Pb and Cd containing fractions of the eluate were extracted with ammonium pyrrolidine dithiocarbamate and 4-methyl-2-pentanone to concentrate the metals before the spectrophotometric reading.

Regeneration of GSH by intact erythrocytes was studied using a slight modification of the method of Kosower et al. (1969a). After centrifugation and removal of plasma and buffy coat, the erythrocytes from at least 6 ml whole blood were washed twice with ice-cold 0.15 M NaCl and their packed volume was adjusted with ice-cold 0-15 M NaCl in order to obtain an 80 to 90% erythrocyte suspension. Portions of 0.5 μl of methyl phenylazene-carboxylate (azoester, methyl phenylazoformate) (Calbiochem, Los Angeles) were delivered into small test tubes (5 ml) with a Hamilton syringe (Hamilton Co., Whittier, Calif., USA). A few seconds before the addition of the erythrocytes, the azoester was suspended in 0.3 ml of ice-cold isotonic buffer (NaCl 0.133 M, Na₂HPO₄ 0.01 M, glucose 0.01 M) used as the incubation medium, then 0.3 ml erythrocyte suspension was added with a constriction pipette and mixed gently. This mixture was kept in an ice-bath for 10 minutes to allow the intracellular oxidation of GSH by azoester, and then incubated at 37°C. Aliquots of 0.1 ml were removed from this mixture to determine the level of GSH at zero time and after 15, 30, and 60 minutes of incubation. Preliminary experiments have indicated that an almost complete oxidation, and similar and satisfactory (more than 70%) yields of regenerated GSH were obtained with oxidation periods (in an ice-bath) of 3, 6, 10, 20, 30, and 60 minutes. For practical reasons an oxidation period of 10 minutes was chosen.

In vitro experiments were carried out with intact erythrocytes in order to evaluate the effect of added heavy metal ions (Cd²⁺, Pb²⁺) on the GSH regeneration rate after intracellular oxidation of GSH with azoester. Portions of 7 ml heparinized normal whole blood (Pb-B 0.94 μmol/l (19.4 μg/100 ml) and Cd-B 22.3 nmol/l (0.25 μg/100 ml)) were transferred in polyethylene tubes and mixed gently with small volumes (50-200 μl) of aqueous solutions of PbCl₂ (106 μmol/l) and/or CdCl₂ (7.2 μmol/l) added beforehand. PbCl₂ and CdCl₂ were dissolved in 0.15 M NaCl. Pb-B levels up to 6.8 μmol/l (140-7 μg/100 ml) (with a constant Cd-B level of 22.3 nmol/l (0.25 μg/100 ml)) and Cd-B levels up to 371 nmol/l (4.17 μg/100 ml) (with a constant Pb-B level of 0.94 μmol/l (19.4 μg/100 ml)) were prepared. In two other samples the concentration of both metals was increased. The tubes were kept at 37°C for one hour and at 4°C overnight. The GSH level of the washed erythrocytes and their GSH regeneration rate were then assayed.

Similar blood samples were prepared to estimate the uptake and distribution of lead and cadmium added in vitro. The distribution of lead in normal whole blood charged in vitro with Pb²⁺ (Pb-B increased from 0.94 to 2.95 μmol/l (19.4 to 61 μg/100 ml)) was also compared with that in whole blood from a worker occupationally exposed to Pb (Pb-B of 3.3 μmol/l (68 μg/100 ml)). Plasma was separated from the erythrocytes by centrifugation of the blood samples (about 10 ml) at room temperature in a Sorvall GR-1 (10 min, 2600 rev/min) and the erythrocytes were washed twice with ice-cold 0.15 M NaCl. The erythrocytes were haemolysed by adding 100 ml ice-cold deionized water, and the mixture was kept for 30 minutes at −20°C. The haemolysate was subsequently centrifuged in a Sorvall RC-2B at 0°C (20 min, 20,000 rev/min, rotor SS-34); the supernatant cytosol was decanted and the pellet (erythrocyte ghosts) washed with portions of 30 ml ice-cold deionized water until the supernatant became nearly colourless (four washings). The cytosol and the first wash were combined, while the three other subsequent washes were kept apart. Plasma,
combined saline washes, erythrocyte ghosts, cytosol, and the washes of the erythrocyte ghosts were analysed for lead and cadmium.

GSH was determined according to the metaphosphoric acid method of Beutler et al. (1963) using ice-cold 2-7 mM ethylenediaminetetraacetic acid, disodium salt (EDTA), for haemolysis of the erythrocytes. The GSH content of washed erythrocytes not treated with azeoster was determined with 0·3 ml erythrocyte suspension added to 5·7 ml EDTA solution, and that of treated erythrocytes with 0·1 ml added to 0·9 ml EDTA solution. The haemolysates were kept in an ice-bath until addition of the protein-precipitating reagent (HPO3-NaCl-EDTA). The coagulated proteins were removed by centrifugation at room temperature (Sorvall GLC-1, 10 min, 2600 rev/min) and then 4 ml of 0·3 M Na2HPO4 were added to 1 ml of the supernatant. Fifteen minutes later 0·5 ml of a 1 M, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (UCB, Brussels, Belgium) solution in 1% sodium citrate was added; 2 minutes later the absorbance was measured with a Zeiss PMQ2 spectrophotometer at 412 nm in a 2 cm cuvette. The amount of GSH was calculated using $\varepsilon_{412} = 13,600$ l mol$^{-1}$ cm$^{-1}$ and is expressed in milligrams per 100 ml of packed erythrocytes.

The total glutathione content (reduced + oxidized glutathione) in washed erythrocytes and whole blood was also measured according to the glutathione reductase method of Tietze (1969) by means of a single beam spectrophotometer equipped with a thermostated cuvette compartment. Reduced glutathione (Sigma Chemical Company, St-Louis, Mo., USA) was used for the standard calibration line (50 to 200 ng GSH) and a blank was run several times to check the reagents (NADPH and yeast glutathione reductase; Boehringer, Mannheim, West Germany). The reactions were performed at 37°C using potassium phosphate as buffer salts and 1 mM NADPH as the final concentration.

**Results**

**In vivo experiments**

<table>
<thead>
<tr>
<th>Partial correlation coefficients</th>
<th>P</th>
<th>Regression equation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Factors correlated</strong></td>
<td><strong>Factor held constant</strong></td>
<td><strong>r</strong></td>
</tr>
<tr>
<td>log ALAD log Pb-B</td>
<td>log Cd-B</td>
<td>-0.760</td>
</tr>
<tr>
<td>log ALAD log Cd-B</td>
<td>log Pb-B</td>
<td>-0.093</td>
</tr>
<tr>
<td>GSH log Cd-B</td>
<td>log Pb-B</td>
<td>-0.423</td>
</tr>
<tr>
<td>log ALAD GSH</td>
<td>log Pb-B</td>
<td>-0.050</td>
</tr>
<tr>
<td>log ALAD log Pb-B</td>
<td>GSH</td>
<td>-0.748</td>
</tr>
</tbody>
</table>

The total study population consists of 110 subjects, 26 controls and 84 workers.

The mean level ± standard deviation (SD) of reduced glutathione in washed erythrocytes was 66·2 ± 7·43 per 100 ml erythrocytes.

In order to check whether the measured biological parameters were normally distributed in the total study population, we applied the curve fitting $x^2$ method to the obtained values. Only those of GSH showed a normal distribution, but the values of Pb-B, Cd-B, and ALAD needed a logarithmic transformation to make their distribution normal. Table 1 shows the interrelationships between the biological parameters measured in the total population.

In view of the significant correlation coefficient between log Pb-B and log Cd-B ($r = 0.404$) we

![FIG. 1. Relationship in total study population (n = 110) between the level of reduced glutathione (GSH) in erythrocytes and lead in blood (log scale) standardized for a given level of cadmium in blood, log Cd-B = -0.2287 (mean value of log Cd-B in the total study population); (O) 26 control subjects, (●) 84 workers from lead and cadmium producing plant. Conversion: Traditional to SI units—lead: 1 µg/100 ml = 0.0483 µmol/l.](http://oem.bmj.com/content/81/2/184)
had to compute partial correlation coefficients between these two parameters and GSH or log ALAD in order to distinguish unambiguously the effect of both heavy metals. Log ALAD is highly inversely correlated with log Pb-B (r = -0.76; p < 0.001) but not with log Cd-B, confirming our previous results (Lauwerys et al., 1973). The partial correlations between GSH and log Pb-B (standardized for log Cd-B) and between GSH and log Cd-B (standardized for log Pb-B) demonstrated a significant negative correlation between GSH and log Pb-B (r = -0.423; p < 0.001) (Fig. 1) but no relationship between GSH and log Cd-B (Fig. 2).

The significant correlation coefficient found between log ALAD and GSH (r = 0.414; p < 0.001) could be fortuitous, since both parameters are significantly correlated with log Pb-B. Therefore the partial correlation coefficients between log ALAD and GSH (standardized for log Pb-B) and between log ALAD and log Pb-B (standardized for GSH) were calculated (Table 1). The apparently good relationship between log ALAD and GSH disappeared completely by holding log Pb-B constant (r = 0.075; p > 0.05) but log ALAD remained highly inversely correlated with log Pb-B when standardized for GSH concentration (r = -0.748; p < 0.001).

Subgroups Besides the control group (n = 26) the workers of the cadmium and lead producing plant can be classified into two subgroups according to their Pb-B; that is, subgroup I (n = 58; upper individual limit of Pb-B ≤ 2.1 μmol/l (42.5 μg/100 ml); group average Pb-B = 1.3 μmol/l (27.1 μg/100 ml)) and subgroup II (n = 26; 2.1 < Pb-B ≤ 3.5 μmol/l (42.5 < Pb-B ≤ 72.7 μg/100 ml); group average Pb-B = 2.5 μmol/l (52.2 μg/100 ml)). Both subgroups are age-matched. A Pb-B of 2.1 μmol/l (42.5 μg/100 ml) as criterion of classification looks reasonable, since a Pb-B of 1.9-2.2 μmol/l (40-45 μg/100 ml) can be considered a critical threshold value (Zielhuis, 1973; Lauwerys et al., 1974). According to Zielhuis’ (1973) proposal, subgroup I can be considered a population representative of exposure to environmental conditions (public health) whereas subgroup II represents occupational exposure (occupational health).

Table 2 gives the mean values of the biological parameters measured in the control group and the two subgroups of workers. By comparison with the control group (Pb-B = 0.6 μmol/l (13.3 μg/100 ml)) subgroups I and II show a statistically significant decrease of haematocrit, of ALAD activity, and of

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**TABLE 2**

**BIological Parameters of Different Subgroups in Study Population**

(mean ± SEM)

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>No. of subjects</th>
<th>Age (years)</th>
<th>Haematocrit</th>
<th>ALAD*</th>
<th>Pb-B (μmol/l)</th>
<th>Cd-B (nmol/l)</th>
<th>GSH*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>26</td>
<td>25.9 ± 1.0</td>
<td>45.3 ± 0.4</td>
<td>43.8 ± 2.1</td>
<td>0.64 ± 0.03</td>
<td>40.9 ± 4.5</td>
<td>71.8 ± 1.2</td>
</tr>
<tr>
<td>I</td>
<td>58</td>
<td>40.7 ± 2.3*</td>
<td>43.9 ± 0.3*</td>
<td>19.5 ± 1.2*</td>
<td>1.31 ± 0.05*</td>
<td>75.7 ± 11.6</td>
<td>66.0 ± 0.9*</td>
</tr>
<tr>
<td>II</td>
<td>26</td>
<td>43.2 ± 2.0*</td>
<td>43.6 ± 0.5*</td>
<td>9.0 ± 0.8†</td>
<td>2.52 ± 0.08†</td>
<td>165.5 ± 24.0†</td>
<td>60.9 ± 1.0†</td>
</tr>
</tbody>
</table>

SEM = standard error of the mean

*ALAD activity expressed in absorbance units × 10/hour/ml erythrocytes for 2 cm light path

*GSH: glutathione content of erythrocytes expressed in mg per 100 ml erythrocytes

*Means significantly different from the control value (P < 0.05)
†Means significantly different from subgroup I and the control value (P < 0.05)

Conversion: SI to Traditional units—lead 1 μmol/l ≈ 20.7 μg/100 ml

            —cadmium 1 nmol/l ≈ 0.0112 μg/100 ml
GSH content of the erythrocytes, and the average Pb-B level is raised to 1.3 and 2.5 μmol/l (27.1 and 52.2 μg/100 ml) respectively. Moreover, the mean values of ALAD activity and GSH content of subgroup II are statistically significantly lower than those of subgroup I. The haematocrit in both subgroups of workers is the same and only the Cd-B mean value of subgroup II differs significantly from that of the two other groups.

The investigation of the GSH regeneration rate in intact erythrocytes after intracellular oxidation of the endogenous GSH by azoester is summarized in Figure 3. The mean level of endogenous GSH amounted to 71.8, 66.0, and 60.9 mg/100 ml erythrocytes (see Table 2) respectively for the control group, subgroup I, and subgroup II of the workers. The azoester oxidized GSH efficiently, since the amount of GSH at zero time never exceeded 5% of the endogenous amount found before azoester treatment. After 15 minutes of incubation at 37°C the GSH levels of the three groups rise to almost the same values (mean ± SD), that is, 27.3 ± 3.5 mg for the control group, and 26.4 ± 4.1, and 26.6 ± 3.1 mg for subgroups I and II respectively. This indicates that the initial rate of GSH regeneration of the glutathione reductase system is similar for the three groups, whatever their content of Pb and Cd in blood. The GSH levels increased further in the next 15 minutes and then reached almost a plateau during the next 30 minutes. The statistically significant differences between the GSH levels after 60 minutes of regeneration reflect those observed before azoester treatment. Approximately 70% of the original GSH level was regenerated, which corresponds to (mean ± SD) 51.3 ± 5.7 mg for the control group, and to 45.1 ± 6.2 and 42.2 ± 4.9 mg for subgroups I and II respectively. In view of the molar ratio azoester:GSH = 5, a 70% yield of regenerated GSH could be expected (see also Kosower et al. (1969b) and Yawata and Tanaka (1973)).

### In vitro experiments

**Effect of Pb⁺⁺ and Cd⁺⁺ added in vitro on GSH regeneration** Table 3 shows the results obtained for GSH regeneration by intact erythrocytes after treatment with azoester. The erythrocytes were isolated from control whole blood of which the Pb-B and/or Cd-B was increased by direct addition of small volumes of a PbCl₂ and/or CdCl₂ solution. The added amounts of Pb⁺⁺ and/or Cd⁺⁺ were such that the new Pb-B and Cd-B levels remained comparable with levels currently observed in the general population and in workers occupationally exposed to these heavy metals. The GSH levels of the treated samples before azoester addition and those after various regeneration periods following azoester addition did not differ significantly from the corresponding GSH levels of the control sample (no heavy metal added). It can therefore be concluded that this concentration range of *in vitro* added Pb⁺⁺ and Cd⁺⁺ has no effect on the glutathione reductase system of intact erythrocytes, and this result reinforces that obtained *in vivo*.

**Uptake and distribution of Pb⁺⁺ and Cd⁺⁺ added in vitro** The conclusion drawn from the preceding results can be valid only if under the actual experimental conditions the erythrocytes took up *in vitro* substantial amounts of Pb and Cd. When increasing amounts of Pb⁺⁺ and Cd⁺⁺ were added *in vitro* to control whole blood (Pb-B of 0.9 μmol/l (19.4 μg/100 ml) and Cd-B of 22.3 nmol/l (0.25 μg/100 ml)) to increase Pb-B and Cd-B up to a level of 3.5 μmol/l and 194 nmol/l (71.8 and 2.18 μg/100 ml) respectively, we observed that uptake and distribution of added Pb differed remarkably from that of added Cd. At least 80% of the added amount of Pb...
### TABLE 3

**Regeneration of GSH by Erythrocytes after Addition of Pb\(^{2+}\) and/or Cd\(^{2+}\) to Control Whole Blood in vitro**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pb-B ((\mu)mol/l)</th>
<th>Cd-B ((\mu)mol/l)</th>
<th>GSH (mg/100 ml erythrocytes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before addition of</td>
<td></td>
<td>Regeneration period (min)</td>
</tr>
<tr>
<td></td>
<td>azoester</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control(^1)</td>
<td>0.94</td>
<td>22.3</td>
<td>66.3 ± 1.4</td>
</tr>
<tr>
<td>Pb(^{2+}) added</td>
<td>1.66</td>
<td>22.3</td>
<td>67.8</td>
</tr>
<tr>
<td></td>
<td>2.38</td>
<td>22.3</td>
<td>66.8</td>
</tr>
<tr>
<td></td>
<td>3.84</td>
<td>22.3</td>
<td>68.1</td>
</tr>
<tr>
<td></td>
<td>6.80</td>
<td>22.3</td>
<td>66.8</td>
</tr>
<tr>
<td>Cd(^{2+}) added</td>
<td>0.94</td>
<td>71.2</td>
<td>64.9</td>
</tr>
<tr>
<td></td>
<td>0.94</td>
<td>170.0</td>
<td>68.4</td>
</tr>
<tr>
<td></td>
<td>0.94</td>
<td>371.1</td>
<td>67.5</td>
</tr>
<tr>
<td>Pb(^{2+}) and Cd(^{2+}) added</td>
<td>1.67</td>
<td>72.1</td>
<td>66.8</td>
</tr>
<tr>
<td></td>
<td>3.13</td>
<td>170.9</td>
<td>65.6</td>
</tr>
</tbody>
</table>

\(^1\)The GSH values for the control sample are mean ± SD values from four determinations; all the others are single values

**Conversion:** SI to Traditional units—lead 1 \(\mu\)mol/l ≈ 20-7 \(\mu\)g/100 ml
cadmium 1 \(\mu\)mol/l ≈ 0.0112 \(\mu\)g/100 ml

was taken up by the erythrocytes, whereas about 75% of the added amount of Cd was found in the plasma and only 10 to 20% was taken up by the erythrocytes in vitro. Although the Cd level of the erythrocytes increased by three to four times, it never reached levels as high as those found in erythrocytes of persons occupationally exposed to this metal (for example, workers of subgroup II). On the contrary, approximately 90% of the total amount of lead was found to be associated with the erythrocytes—on average, 5% with the erythrocyte ghosts and 85% with the soluble erythrocyte constituents; the remainder was found partially in the plasma and partially in the combined saline washes. Similar results are obtained for in vivo distribution of lead in blood (Table 4).

In addition to these in vitro experiments the distribution study of Pb and Cd in blood from workers of subgroup II confirmed data in the literature, that in vivo both Cd (Carlson and Friberg, 1957; Nordberg, Piscator, and Nordberg, 1971) and Pb (Bruenger et al., 1973) are hardly detectable in plasma. Haemoglobin appeared to serve as binding protein for Pb (Barltrop and Smith, 1971; Bruenger

### TABLE 4

**Distribution of Pb in Whole Blood of a Pb-intoxicated Worker and in Normal Whole Blood with Pb\(^{2+}\) added**

<table>
<thead>
<tr>
<th>Amount of Pb</th>
<th>Pb-intoxicated worker (Pb-B 3.28 (\mu)mol/l)</th>
<th>Normal with Pb(^{2+}) added (Pb-B 2.95 (\mu)mol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>In original sample(^1) ((\mu)mol)</td>
<td>0.0347 (\mu)mol</td>
<td>0.0311</td>
</tr>
<tr>
<td>In the different fractions ((\mu)mol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>0.0008 (2.4%)(^3)</td>
<td>0.0017 (5.1%)(^3)</td>
</tr>
<tr>
<td>Saline washes</td>
<td>0.0006 (1.8%)</td>
<td>0.0011 (3.3%)</td>
</tr>
<tr>
<td>Erythrocyte ghosts</td>
<td>0.0078 (23.6%)</td>
<td>0.0019 (5.7%)</td>
</tr>
<tr>
<td>Erythrocyte constituents</td>
<td>0.0237 (72.2%)</td>
<td>0.0292 (85.9%)</td>
</tr>
<tr>
<td>Total recovered ((\mu)mol)</td>
<td>0.0329</td>
<td>0.0339</td>
</tr>
<tr>
<td>Recovery</td>
<td>95%</td>
<td>109%</td>
</tr>
</tbody>
</table>

\(^1\)About 10 ml blood was used for the fractionation

\(^3\)Within parentheses are the percentages for the Pb-amounts relative to the total amount of Pb recovered

**Conversion:** SI to Traditional units—lead 1 \(\mu\)mol/l ≈ 20-7 \(\mu\)g/100 ml
cadmium 1 \(\mu\)mol/l ≈ 0.0112 \(\mu\)g/100 ml
et al., 1973) and Cd (Carlson and Friberg, 1957; Nordberg et al., 1971), but more than 60% of Cd seemed to be bound to erythrocyte metallothionein (Nordberg et al., 1971). It is therefore quite astonishing still to find in a recent publication that the utility of measuring Cd concentration in plasma as an index of exposure is stressed (Colucci et al., 1973).

Comparison of assays for the determination of erythrocyte glutathione

Two methods were compared, Beutler’s and Tietze’s. With the former, only chemically isolated GSH is measured spectrophotometrically; with the latter, the total content of glutathione (GSH + GSSG) is measured enzymatically with yeast glutathione reductase. Preliminary results showed that ergothionein, of which an appreciable amount is present in the erythrocytes, did not react with DTNB in the conditions in which erythrocyte GSH is determined by Beutler’s method. Table 5 compares the glutathione determinations carried out on washed erythrocytes and on whole blood from three different control subjects. Glutathione levels in washed erythrocytes given by Beutler’s method are lower than those found with Tietze’s method; the values for whole human blood correspond with those reported by Tietze (1969). Although oxidized glutathione (GSSG) is included in the erythrocytic glutathione levels obtained with Tietze’s method, it cannot explain the striking difference between the two methods, since GSSG accounts for less than 0.5% of the total erythrocyte glutathione content (Srivastava and Beutler, 1967; Tietze, 1969). Although Pihl, Eldjarn, and Bremer (1957) ruled out the possibility that mixed disulphides containing glutathione can serve as direct substrate for glutathione reductase, nevertheless a number of investigators have questioned the specificity of this enzyme (Icén, 1967; Tietze, 1969). Furthermore, it has been shown that human erythrocyte glutathione reductase can catalyse the reduction of dihydrolipoic acid (Scott, Duncan, and Ekstrand, 1963) and the release of GSH from the haemoglobin-GSH mixed disulphide (Srivastava and Beutler, 1970).

Discussion

The moderate decrease of GSH concentration in erythrocytes correlates significantly with increasing Pb-B (r = -0.423) but not with increasing Cd-B (Table 1; Figs 1 and 2). Despite the rather low correlation coefficient, the internal dose (Pb-B) response (% of individuals with GSH < X - 2 SD of the controls) relationship shows that erythrocyte GSH concentration can be decreased already in the ‘normal Pb-B range’ but only in a limited number of subjects. With increasing Pb-B the relative number of affected subjects only slightly increases up to about 40 to 50% in the high Pb-B range. In regard to erythrocytic GSH level the non-response internal dose of Pb in blood appeared to be 0.9-1.2 μmol/l (20-25 μg/100 ml). Hence it appears that the reliability of a threshold GSH value of 54 mg/100 ml erythrocytes (Bonsignore et al., 1967) or 32.9 mg/100 ml whole blood (Vasiliu et al., 1969) to differentiate between Pb-intoxicated and normal individuals can be questioned. We conclude that the erythrocyte GSH concentration can hardly be used as a diagnostic or discriminating criterion for estimation of the degree of exposure to lead.

Pb-ions are known to have affinity for -SH groups, less strongly however than do Hg-ions (Passow, 1970), and to form stable chelate complexes with sulphhydryl containing compounds (Lenz and Martell, 1964; Doornbos and Faber, 1964). One of the adverse effects of lead on cellular metabolism arises from the interaction of Pb with enzyme -SH groups in such a way that they are not available to certain enzymes that require free -SH group(s) for their activity, for example, enzymes involved in the biosynthesis of haem. In the living organism under most conditions this inhibition is apparently partial (Chisolm, 1971). The strong inactivating action of Pb on ALAD in

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**TABLE 5**

**COMPARISON OF BEUTLER’S AND TIEZTE’S METHOD FOR THE DETERMINATION OF GLUTATHIONE IN HUMAN ERYTHROCYTES**

(mean ± SD)¹

<table>
<thead>
<tr>
<th>Subject</th>
<th>Washed erythrocytes (mg GSH/100 ml erythrocytes)</th>
<th>Whole blood (μg GSH/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beutler’s method</td>
<td>Tietze’s method</td>
</tr>
<tr>
<td>TS</td>
<td>89.5 ± 0.8</td>
<td>99.2 ± 7.7</td>
</tr>
<tr>
<td>HR</td>
<td>72.8 ± 1.2</td>
<td>87.7 ± 9.9</td>
</tr>
<tr>
<td>JPB</td>
<td>71.5 ± 1.0</td>
<td>85.4 ± 5.7</td>
</tr>
</tbody>
</table>

¹Mean ± SD for three determinations
erythrocytes, presumably through chelation of essential sulphhydryl groups, is however as demonstrated by the present investigation not countered by the endogenous erythrocytic GSH concentration which at least with respect to Pb probably does not play a regulating role in the ALAD activity of erythrocytes. Even if Pb should act directly on endogenous GSH, the loss would represent no more than 0.5% for extreme cases of Pb-exposed workers of our study population. Our results therefore support the hypothesis of Cartasegna et al. (1967), Vergnano et al. (1967), and Granick et al. (1973) that inhibition of ALAD in vivo by Pb is not due to decreased levels of endogenous erythrocytic GSH. Indeed, the ALAD activity in our study population remained highly inversely correlated with increasing Pb-B \( r = -0.748 \) when the erythrocyte GSH concentration was held constant, but on the contrary, the apparently significant correlation between ALAD activity and GSH level completely disappeared by keeping Pb-B constant (Table 1).

Lead-induced shortening of the life-span of mature erythrocytes has been studied in vivo and in vitro by investigating alterations in membrane properties, for example, changes in permeability to K\(^+\) (Hernberg, 1967; Passow, 1970; Secchi, Alessio, and Cambiaghi, 1973) and in mechanical and osmotic fragility (Waldron, 1966). The presence of free membrane sulphhydryl groups (Jacob and Jandl, 1962a, b) and a relatively high endogenous GSH level (Prins and Loos, 1969) have been clearly demonstrated to be indispensable for the structural and functional integrity of erythrocytes. Much less attention however has been paid to possible interferences of Pb and Cd with erythrocytic enzyme systems known to be involved in the maintenance of a high endogenous GSH level, that is, the system of GSH regeneration and that of the continuous GSH resynthesis. The most frequently studied enzyme in the former system is G-6-PD, but its activity is measured in haemolyzates. Our present in vivo investigation of GSH regeneration rate was performed however with intact erythrocytes that provide more or less normal physiological working conditions for the enzymes. The observed overall activity of the glutathione oxidation-reduction pathway showed clearly no impairment of the enzymatic reduction of GSSG \( \rightarrow 2 \) GSH in exposed workers with significantly increased Pb-B and Cd-B (subgroup II), since their initial GSH regeneration rate (first 15 min) was identical with that of the control group (Fig. 3). Results of similar in vitro experiments in which control whole blood was incubated beforehand with Pb\(^{2+}\) or Cd\(^{2+}\), or both, reinforce this finding (Table 3). Although Rubino et al. (1963) observed in 30 lead-poisoned patients significant decreases in erythrocyte G-6-PD activity, and in the level and stability of GSH, their hypothesis that these changes were caused by the action of Pb on the oxidation-reduction system of erythrocytes lacks confirmation from the literature. On the contrary, our conclusion that within the Pb-B (and Cd-B) range reported in the present paper no impairment of the glutathione redox system occurs is supported by Saita and Lussana's (1971) clinical follow-up of an occupationally Pb-intoxicated woman with erythrocytic G-6-PD deficiency, and by other studies on erythrocytes from Pb-intoxicated workers showing no significant changes in G-6-PD (Molé et al., 1965; Shafer and Tague, 1970) or in glutathione reductase activity (Shafer and Tague, 1970). Similar results were obtained with experimentally lead-poisoned rabbits (Molé et al., 1965; Rogers et al., 1971). In spite of a significant decrease of erythrocytic G-6-PD activity in acute lead-poisoned rats, Erich and Waller (1967) concluded from their own investigations and those of others that the premature splenic sequestration of circulating erythrocytes in lead-intoxication is a consequence of disturbances in the erythrocyte membrane (formation of Pb-complexes with membrane proteins) caused by lead rather than of changes in metabolism. Studies on enzymes and metabolism of erythrocytes from lead-intoxicated persons (Hernberg, Nurminen, and Hasen, 1967; Shafer and Tague, 1970) and experimentally lead-poisoned rabbits (Rogers et al., 1971) revealed that, apart from ALAD, the activities of many enzymes (among them methaemoglobin reductase, G-6-PD, 6-phosphogluconic dehydrogenase, pyruvate kinase, triose phosphate isomerase, glutathione reductase, and diaphorase) were not affected, nor the overall activities of the Embden-Meyerhof and hexose monophosphosphate shunt pathways.

An alternative explanation for the normally functioning GSH regeneration system in erythrocytes of workers with significantly increased Pb-B and Cd-B, for example, those of subgroup II, might be that in spite of a 15% decrease, the endogenous erythrocytic GSH level still remains sufficiently high to regulate the normal physiological activity of the hexose monophosphosphate shunt pathway (Jacob and Jandl, 1966).

Unlike the inhibition in vitro by low Cd\(^{2+}\) concentrations, neither ALAD nor glutathione reductase seemed to be affected by a substantial increase of Cd-B in vivo (Table 1, Fig. 3). In vivo metallothionein (Nordberg et al., 1971) presumably acts within the erythrocytes as a Cd-chelating agent protecting both enzymes, whereas purified enzymes lack this protection. Haemoglobin, which also binds Cd (Carlson and Friberg, 1957; Nordberg et al. 1971), could at least partially be responsible also for such a protective effect (İcên, 1967).

Since our results demonstrate that increased Pb-B does not impair the glutathione reductase system of erythrocytes, the moderate decrease in endogenous
erythrocyte GSH found in Pb-exposed workers could result from an inhibition by lead of the erythrocyte mechanism for glutathione synthesis. Recent experiments showed that human erythrocytes in whole blood may renew their whole GSH content in about six days (Bondi et al., 1973) by de novo glutathione synthesis in which two enzymes, γ-glutamylcysteine synthetase and glutathione synthetase, are involved (Prins and Loos, 1969). The activity of the former enzyme depends most probably on a sulphhydril group that is essential for the enzymatic activity, whereas the latter does not (Majerus et al., 1971).

Although little is known about the regulation of GSH synthesis in erythrocytes, it might be that small lasting disturbances of the in vivo activity of these enzymes induced by lead may have moderate drawbacks on GSH synthesis and consequently to some extent on the GSH depending defence mechanism of erythrocytes and on their life-span in cases of lead intoxication.

Another explanation for the observed GSH decrease might be that, in particular, lead catalyses the formation of Heinz-bodies. An increased prevalence of erythrocytes with Heinz-bodies has been observed in children living in the neighbourhood of lead processing plants (Ghelberg et al., 1966). The apparent participation of disulphides in Heinz-body formation has focused attention on the role of haemoglobin-GSH complexes (mixed disulphide with the readily accessible thiol group of cysteine-93 of the haemoglobin β-chain) in haemolytic disease (Jacob, Brain, and Dacie, 1968). Further experimental work is necessary to distinguish between these two hypotheses.

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References


krise bei Mangels an Glukose-6-phosphatdehydrogenase und 

and glutathione with delta-aminolevulinic acid 


Hernberg, S. (1967). Life span, potassium fluxes and 
membrane ATPases of erythrocytes from subjects 
exposed to inorganic lead. *Dissertation, Work-
Environment Health, 3*, Suppl. 1, p. 74.

——, and Nikkanen, J. (1972). Effect of lead on 
δ-aminolevulinic acid dehydratase. A selective review. 
*Pracovni lekařství, 24*, 77-83.

——, Nurminen, M., and Hasan, J. (1967). Oxygen and 
glucose consumption and lactate production of 
erthrocytes of workers exposed to inorganic lead. 
*Internationales Arch für Gewerbepathologie und 
Gewerbehygiene, 23*, 117-126.

Erythrocyte δ-aminolevulinic acid dehydratase in new 
lead exposure. A longitudinal study. *Archives of 

Icén, A. (1967). Glutathione reductase of human ery-
throcytes. Purification and properties. *Scandinavian 
Journal of Clinical and Laboratory Investigation, 
Suppl. 96*, 1-57.

Jacob, H. S., Brain, M. C., and Dacie, J. V. (1968). 
Altered sulfhydryl reactivity of hemoglobin and red 
blood cell membranes in congenital Heinz body 
hemolytic anemia. *Journal of Clinical Investigation, 
47*, 2664-2677.

——, and Jandl, J. H. (1962a). Effects of sulfhydryl 
inhibition on red blood cells. I. Mechanism of 
hemolysis.*Journal of Clinical Investigation, 41*, 779-792.

——, ——— (1962b). Effects of sulfhydryl inhibition on red 
blood cells. II. Studies in vivo. *Journal of Clinical 
Investigation, 41*, 1514-1523.

——, ——— (1966). Effects of sulfhydryl inhibition on red 
blood cells. III. Glutathione in the regulation of the 
hexose monophosphate pathway. *Journal of Biological 
Chemistry, 241*, 4243-4250.

Jonderko, G. (1961). Diagnostic value of the determine-
ation of the blood glutathione level in chronic poison-
ing in human subjects. *Polskie Archiwum Medycyny 
Wewntrznej, 31*, 647-655.

——, and Sroczynski, J. (1963). Behaviour of reduced 
glutathione concentration in blood in experimental 
lead poisoning in rabbits. *Postepy Higieny i Medycyny 
Dowsiadzialnej, 17*, 615-618.

on heme-synthesizing enzymes and urinary δ-aminole-
levulinic acid in the rat. *Proceedings of the Society for 
Experimental Biology and Medicine, 143*, 234-237.

Kosower, N. S., Song, K.-R., and Kosower, E. M. (1969a) 
Glutathione. I. The methyl phenylglycinecarboxylate 
(azoester) procedure for intracellular oxidation. 
*Biochimica et Biophysica Acta, 192*, 1-7.

——, ——— (1969b). Glutathione. III. Biological 
aspects of the azoester procedure for oxidation within 
the normal human erythrocyte. *Biochimica et Bio-

exposure to mercury vapors and biological action. 
*Archives of Environmental Health, 27*, 65-68.

of effect of inorganic lead and cadmium on blood 
δ-aminolevulinic dehydratase in man. *British 
Journal of Industrial Medicine, 30*, 359-364.

between urinary δ-aminolevulinic acid excretion 
and the inhibition of red cell δ-aminolevulinic 
dehydratase by lead. *Clinical Toxicology, 7*, 383-388.

some sulfur-containing amino acids. *Biochemistry, 3*, 
745-750.

and porphyrin synthesis in lead poisoning and iron 

Majerus, P. W., Brauner, M. J., Smith, M. B., 
erthrocytes. II. Purification and properties of the 
enzymes of glutathione biosynthesis. *Journal of Clinical 
Investigation, 50*, 1637-1643.

McIntire, M. S. and Angle, C. R. (1972). Air lead: 
Relation to lead in blood of black school children 
deficient in glucose-6-phosphate dehydrogenase. 
*Science, 177*, 520-522.

Millar, J. A., Cumming, R. L. C., Battistini, V., Carswell, 
F., and Goldberg, A. (1970). Lead and δ-aminole-
levulinic acid dehydratase levels in mentally retarded 
children and in lead-poisoned suckling rats. *Lancer, 
2*, 695-698.

Gli enzimi glicolitici del globulo rosso nel saturnismo 
uman e experimentale. *Folia Medica (Napoli), 48*, 
44-48.

Moore, M. R., Beattie, A. D., Thompson, G. G., 
and Goldberg, A. (1971). Depression of δ-aminoleva-
linic acid dehydratase activity by ethanol in man and rat. 
*Clinical Science, 40*, 81-88.

of blood glutathione level in experimentally lead-
poisoned rabbits. *Journal of Science of Labour 
(Japan), 32*, 390-403.

Nakao, K., Wada, O., and Yano, Y. (1968). δ-Amino-
levulinic acid dehydratase activity in erythrocytes 
for the evaluation of lead poisoning. *Clinica Chimica 
Acta, 19*, 319-325.

On the distribution of cadmium in blood. *Acta 
Pharmacologica et Toxicologica, 30*, 389-395.

Passow, H. (1970). In *Effects of Metals on Cells, 
Subcellular Elements, and Macromolecules*, edited by 
J. Maniloff, J. R. Coleman, and M. W. Miller, p. 291. 
Thomas, Springfield, Illinois.

of action of X-ray protective agents. III. The enzy-
matic reduction of disulfides. *Journal of Biological 
Chemistry, 227*, 339-345.

*Biochemical Methods in Red Cell Genetics*, edited by 
and London.


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