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

H. A. ROELS¹, J. P. BUCHET¹, R. R. LAUWERYS¹ and J. SONNET²

Industrial and Medical Toxicology Unit, Louvain University, Avenue Chapelle-aux-Champs 4, B-1200 Brussels, Belgium¹, and Unité de Biochimie Médicale, Louvain University, Cliniques Universitaires St-Pierre, Brusselsestraat 69, B-3000 Louvain, Belgium²

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 $\mu\text{mol/l}$ (15-40 $\mu\text{g}/100\text{ ml}$) (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 ($\text{Pb-B} < 5.8 \mu\text{mol/l}$ ($120 \mu\text{g}/100 \text{ ml}$)) 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 Cambiaghi, 1974). In the concentration range $1.2\text{--}4.8 \mu\text{mol/l}$ ($25\text{--}100 \mu\text{g Pb}/100 \text{ ml blood}$), lead primarily exerts a non-competitive inhibition of ALAD as shown for human (Granick *et al.*, 1973) and bovine (Hapke and Prigge, 1973) erythrocyte ALAD; similar inhibition properties of Pb^{2+} 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, 1967; 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 dithiotreitol (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 decreased 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; Vasiliu, 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 Sroczynski, 1963; Vergnano, Cartasegna, and Bonsignore, 1967). The biological significance of the

high intracellular GSH concentration ($2\text{--}2.5 \text{ mmoles/litre}$ packed erythrocytes = 0.4 to $0.5 \text{ mole/mole haemoglobin}$) 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 Bittenwieser, 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 Cd^{2+} 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 $1 \mu\text{mol Cd}^{2+}/\text{litre}$ (Icèn, 1967). A low concentration of Cd^{2+} (0.1 mmol/litre) also inhibits at low substrate concentrations ($<1 \text{ mmol/litre}$) purified beef-liver ALAD (Wilson *et al.*, 1972). Human erythrocyte ALAD in normal whole blood appears to be inhibited on *in vitro* addition of $0.5 \text{ mg Cd}^{2+}/\text{litre}$ (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 Pb^{2+} and/or Cd^{2+} 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 (Lauwerys *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 µ) 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 (Lauwerys *et al.*, 1973). The activity, corrected for the blood haematocrit, is expressed in units of absorbance for 2 cm light path:

$$(\text{A}_{\text{analysis}} - \text{A}_{\text{blank}}) \times 10/\text{hour/ml erythrocytes}.$$

Cd-B and Pb-B were determined by the ion exchange-atomic absorption spectrophotometric method of Vens and Lauwerys (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 phenyldiazene-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, Na_2HPO_4 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 (Cd^{2+} , Pb^{2+}) 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 to 200 µl) of aqueous solutions of PbCl_2 (106 µmol/l) and/or CdCl_2 (7.2 µmol/l) added beforehand. PbCl_2 and CdCl_2 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 Pb^{2+} (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 GLC-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 RC2-B 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,

TABLE 1
INTERRELATIONSHIPS BETWEEN DIFFERENT BIOLOGICAL PARAMETERS IN BLOOD OF
TOTAL STUDY POPULATION¹

Partial correlation coefficients			P	Regression equation
Factors correlated	Factor held constant	r		
log ALAD log Pb-B	log Cd-B	-0.760	<0.001	log ALAD = 2.640 - 0.982 (log Pb-B)
log ALAD log Cd-B	log Pb-B	-0.093	>0.05	
GSH . log Pb-B	log Cd-B	-0.423	<0.001	GSH = 85.74 - 13.87 (log Pb-B)
GSH . log Cd-B	log Pb-B	-0.050	>0.05	
log ALAD GSH	log Pb-B	+0.075	>0.05	
log ALAD log Pb-B	GSH	-0.748	<0.001	log ALAD = 2.639 - 0.983 (log Pb-B)

¹The total study population consists of 110 subjects, 26 controls and 84 workers.

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 azoester 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 ($\text{HPO}_3\text{--NaCl--EDTA}$). 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 Na_2HPO_4 were added to 1 ml of the supernatant. Fifteen minutes later 0.5 ml of a 1 mM 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 $\epsilon_M = 13\,600\text{ l mol}^{-1}\text{ cm}^{-1}$ 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

Total study population The mean value of lead and cadmium in whole blood of the total study population was 1.4 $\mu\text{mol/l}$ and 89 nmol/l (29.8 and 1.0 $\mu\text{g/100 ml}$) respectively; Pb-B ranged from 0.2 to 3.5

$\mu\text{mol/l}$ (4.5 to 72.7 $\mu\text{g/100 ml}$) and Cd-B from 4.45 to 457.5 nmol/l (0.05 to 5.14 $\mu\text{g/100 ml}$). Both parameters were not normally distributed (see below). The mean level $\pm$ 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 χ^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, $\log \text{Cd-B} = -0.2287$ (mean value of $\log \text{Cd-B}$ in the total study population); (○) 26 control subjects, (●) 84 workers from lead and cadmium producing plant. Conversion: Traditional to SI units—lead: 1 $\mu\text{g/100 ml} \approx 0.0483\text{ } \mu\text{mol/l}$.

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 ($r = -0.760$; $P < 0.001$) 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).

FIG. 2. Relationship in total study population ($n = 110$) between the level of reduced glutathione (GSH) in erythrocytes and blood cadmium (log scale) standardized for a given level of lead in blood, log Pb-B = 1.4104 (mean value of log Pb-B in the total study population); (○) 26 control subjects, (●) 84 workers from lead and cadmium producing plant. Conversion: Traditional to SI units—cadmium: $1 \mu\text{g}/100 \text{ ml} \approx 89.0 \text{ nmol/l}$.

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 $\leq 2.1 \mu\text{mol/l}$ ($42.5 \mu\text{g}/100 \text{ ml}$); group average Pb-B = $1.3 \mu\text{mol/l}$ ($27.1 \mu\text{g}/100 \text{ ml}$)) and subgroup II ($n = 26$; $2.1 < \text{Pb-B} \leq 3.5 \mu\text{mol/l}$ ($42.5 < \text{Pb-B} \leq 72.7 \mu\text{g}/100 \text{ ml}$); group average Pb-B = $2.5 \mu\text{mol/l}$ ($52.2 \mu\text{g}/100 \text{ ml}$)). Both subgroups are age-matched. A Pb-B of $2.1 \mu\text{mol/l}$ ($42.5 \mu\text{g}/100 \text{ ml}$) as criterion of classification looks reasonable, since a Pb-B of $1.9\text{--}2.2 \mu\text{mol/l}$ ($40\text{--}45 \mu\text{g}/100 \text{ ml}$) 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 = $0.6 \mu\text{mol/l}$ ($13.3 \mu\text{g}/100 \text{ ml}$)) subgroups I and II show a statistically significant decrease of haematocrit, of ALAD activity, and of

TABLE 2
BIOLOGICAL PARAMETERS OF DIFFERENT SUBGROUPS IN STUDY POPULATION
(mean $\pm$ SEM)

Subgroup	No. of subjects	Age (years)	Haematocrit ¹ (%)	ALAD ²	Pb-B ($\mu\text{mol/l}$)	Cd-B (nmol/l)	GSH ³
Controls	26	25.9 ± 1.0	45.3 ± 0.4	43.8 ± 2.1	0.64 ± 0.03	40.9 ± 4.5	71.8 ± 1.2
I	58	$40.7 \pm 1.3^*$	$43.6 \pm 0.3^*$	$19.5 \pm 1.2^*$	$1.31 \pm 0.05^*$	75.7 ± 11.6	$66.0 \pm 0.9^*$
II	26	$43.2 \pm 2.0^*$	$43.6 \pm 0.5^*$	$9.0 \pm 0.8^\dagger$	$2.52 \pm 0.08^\dagger$	$165.5 \pm 24.0^\dagger$	$60.9 \pm 1.0^\dagger$

SEM = standard error of the mean

¹The controls include five women who were not taken into account for the haematocrit

²ALAD activity expressed in absorbance units $\times 10/\text{hour}/\text{ml}$ erythrocytes for 2 cm light path

³GSH: 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 $1 \mu\text{mol/l} \approx 20.7 \mu\text{g}/100 \text{ ml}$

—cadmium $1 \text{ nmol/l} \approx 0.0112 \mu\text{g}/100 \text{ ml}$

GSH content of the erythrocytes, and the average Pb-B level is raised to 1.3 and 2.5 $\mu\text{mol/l}$ (27.1 and 52.2 $\mu\text{g}/100\text{ ml}$) 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

FIG. 3. Regeneration rate of GSH by intact erythrocytes from two groups of workers employed in a lead and cadmium producing plant; (○) subgroup I (58 subjects) and (□) subgroup II (26 subjects), and from a control group of 26 subjects (△) without occupational exposure. Intact erythrocytes were preincubated (10 min at 0°C) with the incubation medium containing methyl phenylazoformate, and then incubated at 37°C for 60 min. Aliquots were removed after 0, 15, 30, and 60 minutes of incubation to determine the amount of regenerated GSH. The symbols (▲, ■, ●) represent the endogenous erythrocyte GSH amount of the respective groups before the addition of methyl phenylazoformate; the concentrations of lead and cadmium in blood (Pb-B and Cd-B in $\mu\text{g}\%$) are also given for each group. Mean values $\pm$ standard error of the mean are shown. Conversion: Traditional to SI units—lead 1 $\mu\text{g}/100\text{ ml} \approx 0.0483\text{ }\mu\text{mol/l}$; cadmium 1 $\mu\text{g}/100\text{ ml} \approx 89.0\text{ nmol/l}$.

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 $\pm$ SD), that is, $27.3 \pm 3.5\text{ mg}$ for the control group, and 26.4 ± 4.1 , and $26.6 \pm 3.1\text{ mg}$ 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 $\pm$ SD) $51.3 \pm 5.7\text{ mg}$ for the control group, and to 45.1 ± 6.2 and $42.2 \pm 4.9\text{ mg}$ 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^{2+} and Cd^{2+} 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 PbCl_2 and/or CdCl_2 solution. The added amounts of Pb^{2+} and/or Cd^{2+} 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^{2+} and Cd^{2+} has no effect on the glutathione reductase system of intact erythrocytes, and this result reinforces that obtained *in vivo*.

Uptake and distribution of Pb^{2+} and Cd^{2+} 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^{2+} and Cd^{2+} were added *in vitro* to control whole blood (Pb-B of 0.9 $\mu\text{mol/l}$ (19.4 $\mu\text{g}/100\text{ ml}$) and Cd-B of 22.3 nmol/l (0.25 $\mu\text{g}/100\text{ ml}$)) to increase Pb-B and Cd-B up to a level of 3.5 $\mu\text{mol/l}$ and 194 nmol/l (71.8 and 2.18 $\mu\text{g}/100\text{ ml}$) 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 Pb²⁺ AND/OR Cd²⁺ TO
CONTROL WHOLE BLOOD *in vitro*

Sample	Pb-B ($\mu\text{mol/l}$)	Cd-B (nmol/l)	GSH (mg/100 ml erythrocytes)				
			Before addition of azoester	Regeneration period (min)			
				0	15	30	60
Control ¹	0.94	22.3	66.3 $\pm$ 1.4	2.7 $\pm$ 0.6	36 $\pm$ 2.1	51.1 $\pm$ 2.4	49.5 $\pm$ 2.0
Pb ²⁺ added	1.66	22.3	67.8	0.7	36.6	50.5	49.5
	2.38	22.3	66.8	2.1	37.0	50.7	50.7
	3.84	22.3	68.1	3.0	36.2	53.4	51.7
	6.80	22.3	66.8	3.1	33.1	51.7	49.0
Cd ²⁺ added	0.94	71.2	64.9	2.8	39.0	53.5	48.3
	0.94	170.0	68.4	2.7	34.0	51.7	48.0
	0.94	371.1	67.5	2.7	35.4	49.8	49.0
Pb ²⁺ and Cd ²⁺ added	1.67	72.1	66.8	3.4	36.8	46.9	47.2
	3.13	170.9	65.6	2.5	34.9	49.0	47.2

¹The GSH values for the control sample are mean $\pm$ SD values from four determinations; all the others are single values
Conversion: SI to Traditional units—lead 1 $\mu\text{mol/l} \approx 20.7 \mu\text{g}/100 \text{ ml}$
—cadmium 1 $\text{nmol/l} \approx 0.0112 \mu\text{g}/100 \text{ ml}$

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 Pb²⁺ ADDED

Amount of Pb	Pb-intoxicated worker (Pb-B 3.28 $\mu\text{mol/l}$)	Normal with Pb ²⁺ added (Pb-B 2.95 $\mu\text{mol/l}$)
In original sample ¹ (μmol)	0.0347 μmol	0.0311
In the different fractions (μmol)		
Plasma	0.0008 (2.4%) [*]	0.0017 (5.1%) [*]
Saline washes	0.0006 (1.8%)	0.0011 (3.3%)
Erythrocyte ghosts	0.0078 (23.6%)	0.0019 (5.7%)
Erythrocyte constituents	0.0237 (72.2%)	0.0292 (85.9%)
Total recovered (μmol)	0.0329	0.0339
Recovery	95%	109%

¹About 10 ml blood was used for the fractionation

^{*}Within parentheses are the percentages for the Pb-amounts relative to the total amount of Pb recovered

Conversion: SI to Traditional units—lead 1 $\mu\text{mol/l} \approx 20.7 \mu\text{g}/100 \text{ ml}$
—cadmium 1 $\text{nmol/l} \approx 0.0112 \mu\text{g}/100 \text{ ml}$

TABLE 5
COMPARISON OF BEUTLER'S AND TIETZE'S METHOD FOR THE DETERMINATION OF
GLUTATHIONE IN HUMAN ERYTHROCYTES
(mean $\pm$ SD)¹

Subject	Washed erythrocytes (mg GSH/100 ml erythrocytes)		Whole blood (μ g GSH/ml)
	Beutler's method	Tietze's method	Tietze's method
TS	89.5 $\pm$ 0.8	99.2 $\pm$ 7.7	549 $\pm$ 37
HR	72.8 $\pm$ 1.2	87.7 $\pm$ 9.9	485 $\pm$ 24
JPB	71.5 $\pm$ 1.0	85.1 $\pm$ 5.7	470 $\pm$ 51

¹Mean $\pm$ SD for three determinations

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 (Icn, 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 $< \bar{X} - 2$ 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 μ mol/l (20-25 μ g/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

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 haemolysates. 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 $GSSG \rightarrow 2\text{ GSH}$ 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 Hasan, 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 monophosphate 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 erythrocyte GSH level still remains sufficiently high to regulate the normal physiological activity of the hexose monophosphate 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 (Icn, 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 sulphhydryl 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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