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NORMAL VALUES OF LEAD CONCENTRATION IN HUMAN BLOOD

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In order to evaluate normal values of lead in blood for the local population of Zagreb and environs the concentrations of lead in blood have been determined in a random sample of 195 healthy subjects (100 men and 95 women) with no previous exposure to lead. The analyses were performed by a monocolour dithizone method. Two parallel samples of blood were analysed for each subject. It was found that the lead concentrations in blood of normal persons are log-normally distributed. 60 μg/100 ml is proposed as the upper normal limit of lead concentration in blood.

The interpretation of lead-in-blood values of subjects exposed to lead depends on the knowledge of the range of lead concentrations found in blood of normal, healthy individuals who have not been previously subjected to lead exposure other than the ordinary everyday contact (food, water etc.). There are numerous data in literature on the normal values of lead in blood which will be briefly reviewed in a later section of this paper. These data cover a very wide range. Although the main source of variation may undoubtedly be found in different analytical techniques used, it is certain that lead-in-blood values of unexposed individuals depend also on local conditions and habits, particularly with regard to food. Different statistical measures or lack of statistical treatment, and various definitions of the term »normal« may also be listed among factors which have influenced the reported values. It seemed, therefore, necessary to establish the range of normal values applicable to the local conditions of Zagreb and environs.

METHODS

Blood sampling

The blood specimens were obtained from 195 healthy individuals with no known previous exposure to lead. There was no system in the choice of the individuals and

for our purposes the group examined could be considered as a random sample of the population of Zagreb and environs. The age and sex distribution of the subjects is shown in Table 1, while Table 2 shows the occupations of the subjects.

Two 5 ml samples of venous blood were taken from each subject. All syringes and needles used for collecting blood samples were carefully examined for lead content; they were boiled in redistilled water for 30 min. and the water was analysed for lead by dithizone. The procedure was repeated several times. Before use all needles, syringes, and collection flasks were cleaned as described below. Following the introduction of the needle into the vein a small quantity of blood was sucked into the syringe in order to wash the needle, the syringe removed, and blood allowed to flow into a clean, dry test tube of Jena, Pyrex or Wirag glass. Therefrom a 5 ml sample of blood was immediately transferred by means of a pipette into a clean Kjeldahl flask. No anticoagulants were added.

Analytical method

The analytical method used was previously discussed (1). Therefore, only the procedure will be described in some detail.

Reagents and apparatus. The best quality reagents were used throughout. Double-distilled water was used in all aqueous solutions.

Preparation of reagents:

Dithizone stock solution: Dissolve 50 mg of dithizone in 50 ml of chloroform in a separating funnel; add 500 ml of aqueous ammonium hydroxide solution (1:4) and shake the mixture for 5 min. Separate the organic layer and acidify the aqueous phase with 10% aqueous hydrochloric acid until the colour changes to green. Extract dithizone with small portions of pure carbon tetrachloride (total quantity 250 ml), collect the extracts in another separating funnel, and wash several times with redistilled water. Store the stock solution in cold in a dark-glass bottle. *Dithizone reagent* is prepared by diluting 5 ml of the stock solution with 45 ml of pure carbon tetrachloride.

Carbon tetrachloride and chloroform: Add to the solvent (carbon tetrachloride or chloroform) some crystals of sodium thiosulphate and 20%-aqueous sodium hydroxide solution (about 50 ml per litre of solvent), and distill. Add some thiosulphate again and repeat the distillation. Dry the redistilled solvent with sodium sulphate and perform a vacuum distillation carefully avoiding exposure to sunlight. Vacuum distilled chloroform is mixed with 1 vol. % of absolute alcohol and stored in a cold and dark place. No addition of alcohol is necessary to carbon tetrachloride (20).

Acetate buffer solution (pH = 4.0–4.5): Place 100 ml of 1 N potassium hydroxide in a separating funnel, add some drops of thymolphthalein indicator, and neutralize the solution with 1 N acetic acid until the indicator just loses its colour. Extract this solution with small portions of dithizone reagent until two successive extracts show the unchanged green colour of the reagent. Add the rest of the acetic acid solution (total amount of acetic acid added is 200 ml of 1 N solution), and fill up to 1000 ml with redistilled water. Shake the buffer solution with small portions of pure carbon tetrachloride to extract finely dispersed dithizone precipitated during addition of acetic acid.

Citrate solution: Dissolve 200 g of citric acid ($C_6H_8O_7 \cdot H_2O$) in 200 ml of tepid water containing some drops of thymolphthalein indicator, add concentrated ammonium hydroxide until blue colour appears, extract with small quantities of dithizone reagent and fill up to 1000 ml with redistilled water.

Potassium cyanide solution: Dissolve 50 g of potassium cyanide in water and extract with dithizone reagent until two successive extracts show the unchanged green colour. Dilute the aqueous phase with redistilled water to 500 ml.

Potassium cyanide-citrate-ammonium hydroxide buffer solution: Mix 100 ml of citrate solution, 200 ml of potassium cyanide solution and 200 ml of concentrated ammonium hydroxide, and fill up to 1000 ml. Prepare this solution fresh for each series of analyses.

Hydroxylamine-hydrochloride solution: Dissolve 200 g of hydroxylamine-hydrochloride in about 200 ml of water, add some drops of thymolphthalein indicator and sufficient amount of concentrated ammonium hydroxide to make the solution alkaline (blue colour). Extract with dithizone reagent. After extraction acidify the aqueous phase with concentrated hydrochloric acid and dilute to 1000 ml with redistilled water. Any dithizone precipitated during addition of the acid is removed by extracting the solution with carbon tetrachloride.

Cupferron solution: Dissolve 2 g of cupferron (Merck) in 100 ml of water, filter into a separating funnel, make alkaline with ammonium hydroxide and extract with dithizone reagent. Keep the solution in darkness and prepare it often fresh.

Ammonium hydroxide, concentrated, 24%, sp. gr. 0.91 of analytical purity, redistilled.

Hydrocloric acid, concentrated, sp. gr. 1.19, of analytical purity, redistilled.

Nitric acid, concentrated, sp. gr. 1.40, of analytical purity, redistilled.

Thymolphthalein indicator: Dissolve 0.1 g of thymolphthalein in 100 ml of 90% ethanol.

Hydrogen peroxide, of analytical purity.

All glassware used for the collection of specimens or in the course of analytical work was cleaned first in chromic sulphuric acid, rinsed with warm tap water and distilled water; then it was washed with hot 10% sodium hydroxide solution and rinsed with distilled water, and finally washed with hot nitric acid (1:2) and rinsed again with redistilled water.

A Beckman Model DU spectrophotometer, with 1 cm Corex cells, was used for extinction measurements.

PROCEDURE. To the Kjeldahl flask containing 5 ml of blood, 5 ml of concentrated nitric acid is poured, and the mixture is allowed to boil on electric heater or sand bath. At the beginning the reaction is rather vigorous and care must be taken until the reactions subsides. The boiling is continued until the content of the Kjeldahl flask evaporates to dryness. Another portion of 5 ml of nitric acid is added and the boiling continued till the appearance of nitrous fumes when a third portion of nitric acid (5 ml) is added and the volume of the mixture is reduced to 1 ml by boiling. The flask is then cooled and after addition of 2 ml of redistilled water the flask is heated until the dry residue on the walls dissolves. When the solution becomes completely clear and transparent, 10 ml of 30%-hydrogen peroxide is poured in, and the flask heated again to boiling and allowed to cool until the vigorous reaction subsides; the heating is then continued and the content of the flask allowed to evaporate almost to dryness. The dry residue on the walls is dissolved in some redistilled water (3–4 ml) and some nitric acid (2–3 drops), and the mixture is heated to obtain a clear solution.

The content of the Kjeldahl flask is quantitatively transferred into a separating funnel (125 ml, short stem), 2 ml of a 2% aqueous cupferron solution added and the mixture well shaken. The solution should be cold when adding cupferron and should not contain more than 1–2 ml of free nitric acid per 25 ml. The best results are obtained if the precipitation is carried out at pH=1. The precipitate of iron cupferrate is then extracted with carbon tetrachloride until two subsequent extracts remain colourless. The aqueous phase is then transferred into the same Kjeldahl flask which was used for digestion and evaporated to dryness; 2 ml of redistilled water and 1 ml of concentrated nitric acid are added and the evaporation repeated. This procedure is repeated several times until the excess of cupferron is destroyed. (The content of the flask remains colourless.)

After iron has been separated 10 ml of hydroxylamine hydrocloride solution are added and mixture boiled for 5–10 min. but not to dryness. To the hot solution 10 ml

of citrate solution are added. After cooling the solution is transferred to a separating funnel and pH adjusted to 10.5 (thymolphthalein indicator) by adding concentrated ammonium hydroxide and 20 ml of freshly prepared potassium-cyanide-citrate-ammonium hydroxide buffer. The extraction is performed with small portions of dithizone solution and continued until the last portion of dithizone reagent remains green. All extracts are collected in another separating funnel and well shaken with 40 ml of acetate buffer. By this operation lead is transferred to the aqueous phase. The foam appearing during this procedure may be removed by adding small amounts of carbon tetrachloride. Care should be taken, however, that subsequent separation of the organic phase is complete. This extraction procedure is repeated once more (starting with the adjustment of pH of the aqueous phase to 10.5 as described above), and the dithizone extracts collected into a centrifuge tube and centrifuged (3000 r. p.) to separate water from the organic phase. The clear organic phase is then transferred into a dry 25 ml volumetric flask and filled to the mark with pure carbon tetrachloride.

The extinction is measured at 520 mμ. The results are evaluated by means of a calibration curve prepared by adding known amounts of lead to the blood. Statistical analysis of several hundred analyses performed by this method showed that the standard error of the mean of duplicate analyses of the same sample did not exceed $\pm$ 5 μg for the concentration region from 25–500 μg/100 ml blood, as predicted from the regression analysis of the calibration line.

Statistical methods

Frequency distributions were investigated by means of arithmetic-probability and logarithmic-probability papers. As it was found that the concentration of lead in blood had a logarithmic normal distribution, all the calculations were performed using the logarithm of concentration as statistical metameter.

The mean and the standard deviation were estimated using the well known formulas:

$$\bar{x} = \frac{1}{N} \Sigma x_i \quad \text{and} \quad s^2 = \frac{1}{N-1} \left\{ \Sigma x_i^2 - \frac{1}{N} (\Sigma x_i)^2 \right\}$$

where $\bar{x}$ is the mean, s^2 the variance, N the number of observations, $x_i = \log c_i$ (c is the concentration of lead in blood in μg/100 ml). The coefficient of variation was computed by a formula derived by Sacher (2):

$$CV = (e^{a^2} - 1)^{1/2}$$

where CV is the coefficient of variation, e is the base of natural logarithms, and $a = 2.303\ s$. This formula gives an estimate of the coefficient of variation of the distribution of absolute concentrations of lead in blood.

The significance of differences of mean concentrations of lead in blood between different groups was investigated using t-test, the concentration of lead being treated as a logarithmic variable. For estimation of the upper normal limit of lead in blood one-sided tolerance limits were used defined as $U_\gamma = \bar{x} + K_\gamma s$; K_γ was chosen in such a way as to be 90% confident that under the conditions prevailing when the sample was collected at least 90% of lead-in-blood values in a population of unexposed individuals would lie below U_γ; K_γ coefficients were calculated as described by Wallis (3). The values of normal limits given in Table 5 were obtained by taking antilogarithms of U_γ.

RESULTS AND DISCUSSION

The age distribution of subjects is shown in Table 1. The majority of subjects were aged between 20 and 59 years. Since it was not likely that the age might influence the concentration of lead in blood of unexposed subjects (4), the association between these two variables was not further investigated. The distribution of subjects by occupation is given in Table 2.

Table 1

Age distribution of subjects

Age groups	Male	Female	Total
Under 19	2	0	2
20 to 29	54	22	76
30 to 39	22	36	58
40 to 49	18	34	52
50 to 59	4	2	6
60 years and over	0	1	1
Total	100	95	195

Table 2

Occupations of subjects

Occupational groups	Male	Female
Industrial workers	38	10
Agricultural workers	20	24
Housewives	—	33
Clerks	3	16
Recruits	21	—
Others	18	12
Total	100	95

The frequency distribution of concentrations of lead in blood of 195 individuals with no previous exposure to lead is shown in Figure 1.

Further analysis by means of probability paper showed that the distribution could be reasonably well represented by a normal frequency distribution curve if the concentration of lead was treated as a logarithmic variable.

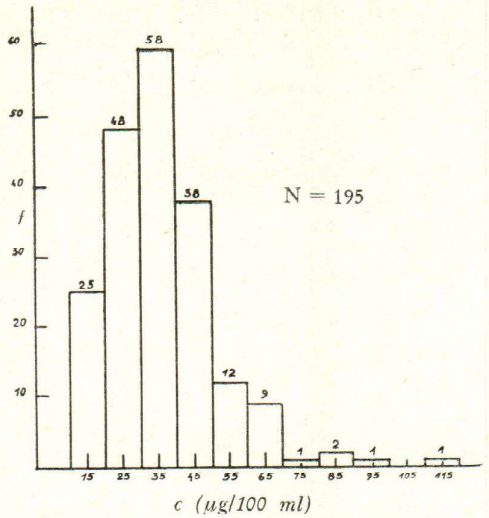

Fig. 1. Distribution of concentrations of lead in blood of 195 healthy subjects with no previous occupational exposure to lead. c = concentration of lead in blood; f = frequency

A skew distribution of lead in blood of normal subjects was noticed also by other authors (4), but they could not find a satisfactory frequency distribution curve to represent their results. Cumulative frequency distributions for male subjects, female subjects, and the total sample are plotted in Fig. 2.

Estimates of some statistical parameters of distribution of lead in blood values for normal subjects are presented in Table 3, for the total sample and for different groups of population. The attention is drawn to the coefficient of variation which is essentially the same for all groups except for the group of 21 recruits. This fact indicates the preponderant influence of living habits on the level and variability of lead in blood values. In order to test the hypothesis that different living habits, particularly with regard to food, had influence on the concentration of lead in blood, the means of different groups were compared using t-test. The results are shown in Table 4. The only statistically significant difference

was found between the samples of urban and rural population, both male and female, and between the recruits and the rest of the male population. We have not investigated the reason for these differences, but it is very likely that they could be explained by different amounts of lead taken in food.

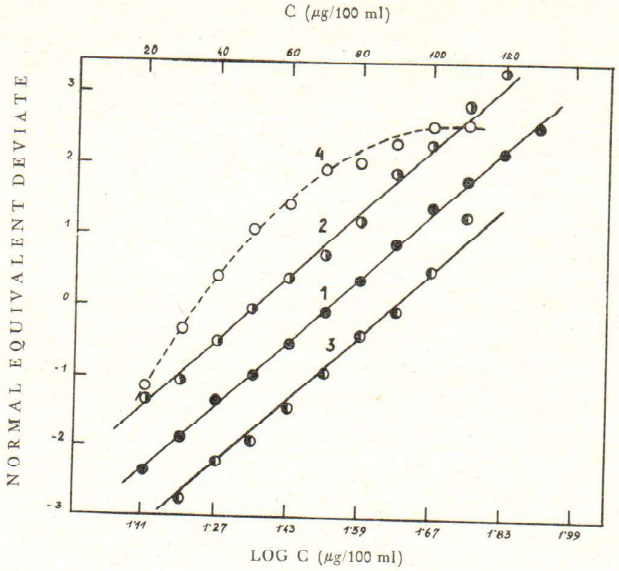

Fig. 2 Cumulative frequency distribution of concentrations of lead in blood of »normal« subjects. Abscissa. logarithm of concentration (for 1, 2, 3), concentration (for. 4); ordinate: normal equivalent deviate (N. E. D.) calculated from the percentages of the cumulative frequency. 1. total sample; 2. female subjects only; 3. male subjects only; 4. total sample (N. E. D. v. concentration) showing that the frequency distribution is not normal.

The estimates of upper limits for normal values, computed by the method described previously (see section on statistical methods) are shown in Table 5. It is thus reasonable to suppose that for the population of Zagreb and environs a value of lead in blood exceeding 60 μg/100 ml would indicate an abnormal exposure to lead of occupational or other origin. The differences in the upper normal limit between

the rural and the urban population have no practical significance. It should be pointed out that these normal values are strictly applicable only if the blood sampling and analysis are carried out using methods described in this paper.

For the sake of comparison we have listed in Table 6 some data on normal values in blood as recorded by other authors. This table has only illustrative purpose and no claim is being made for completeness. No detailed analysis of these results was carried out to make them directly comparable with our results. It is doubtful whether such an analysis may be performed as there are great differences in analytical methods used by different authors and in most cases reliable data on the sensitivity and precision of the analytical method are not available.

Table 3

Estimates of some statistical parameters for the distribution of concentrations of lead in blood (in μg/100 ml) (c = concentration of lead in blood)

1	2	3	4	5	6	7	8
Group	Number of subjects	Mean (log c)	Standard deviation (log c)	Mean (c)	Median (c)	Mode (c)	Coefficient of variation
Total sample	195	1.518	0.176	35.8	33.0	28.0	0.421
Male	100	1.543	0.180	38.0	34.9	29.4	0.434
Female	95	1.493	0.172	33.7	31.1	26.6	0.411
Urban population	100	1.550	0.183	38.7	35.5	29.7	0.440
Male	52	1.569	0.187	40.6	37.1	30.8	0.449
Female	48	1.529	0.178	36.8	33.8	28.6	0.428
Rural population	74	1.456	0.173	31.0	28.6	24.4	0.415
Male	27	1.457	0.199	31.8	28.6	23.2	0.483
Female	47	1.455	0.168	30.5	28.5	25.0	0.401
Recruits	21	1.587	0.0775	39.3	38.6	37.4	0.181

Table 4

*Differences and significance of differences between means of lead-in-blood concentrations for various groups of population**

1	2	3	4	5
Groups compared	Diff.	t	ν	P
Total sample[1]: Male and female	0.038	1.30	172	> 0.10
Rural population: Male and female	0.0020	0.047	72	> 0.80
Urban population: Male and female	0.040	1.12	98	> 0.20
Total sample[1]: Rural and urban	0.094	3.43	172	< 0.001
Male population: Rural and urban	0.112	2.47	77	< 0.02
Female population: Rural and urban	0.074	2.08	93	< 0.05
Recruits and total male[1]	0.056	2.01	88	< 0.05

* The concentration of lead in blood is treated as logarithmic variable.

[1] The homogenous group of rectruits excluded.

Notation: $t = \frac{\text{observed difference between means}}{\text{estimate of the S. E. of difference}}$

ν = degrees of freedom

P = probability that (t) given in column 3 is exceeded

Table 5

Estimates of normal values of lead in blood

Group	Number of subjects	Upper 90% »normal« limit (in μg/100 ml)
Total sample	195	59.3
Rural population	74	53.3
Urban population	100	67.5

Table 6

Normal content of lead in blood

Authors	Number of subjects	Mean µg/100 ml	S. D. µg/100 ml	Range µg/100 ml	Analytical method	Upper limit recommended
Bass (5)	24	20	—	10—50	Electrolytic-Colorimetric	—
Kehoe, Thamann, Cholak (6)	—	58	—	100	Spectrographic	—
Kehoe, Thamann Cholak (7)	77	38	20	—	Spectrographic	—
Willoughby, Wilkins (4)	—	25	—	90	Dithizone	—
Tompsett, Anderson (8)	29	—	—	—	—	100 µg/100 ml
Tompsett, Anderson (9)	—	—	—	0—200	—	—
Straube, Beck (10)	22	—	—	5—20	Electrolytic-Colorimetric	—
Chalmers (11)	70	57	—	30—90	Dithizone	—
Kehoe, and others (12)	—	—	—	60—70	—	—
Kaplan, McDonald (13)	126	31	12	50—60	Dithizone	—
Cholak, Bambach (14)	188	30 µg/100 g	9 µg/100g	—	—	—
Kehoe (15)	—	—	—	10—55	—	—
Stalker (16)	—	—	—	—	—	70 µg/100 g
Castrop (17)	—	—	—	10—60	—	60 µg/100 g
Schrader (18)	—	—	—	—	Spectrographic	100 µg/100 ml
Sitgreaves, May (19)	226	24.7	3.8	15.1—39.7	Dithizone	—

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Sadržaj

NORMALNE VRIJEDNOSTI OLOVA U KRVI

Radi određivanja normalnih vrijednosti olova u krvi izvršili smo analizu krvi kod 195 zdravih osoba (100 muškaraca i 95 žena) iz Zagreba i okolice, koji u svom zvanju nisu dosad bili eksponirani olovu.

Analize smo izvršili jednobojnom ditizonskom metodom (Weber, Voloder, Vouk, Arh. hig. rada, *3* (1952) 296). U području od 10–500 μg Pb/100 ml krvi preciznost te metode iznosi od $\pm$ 4,6 μg do $\pm$ 5,6 μg (preračunano po 100 ml krvi). Taj se podatak odnosi na aritmetičku sredinu rezultata dviju paralelnih analiza istog uzorka. Za svakog ispitanika izvršili smo po dvije paralelne analize. Za pojedinu analizu potrebno je 5 ml krvi.

Našli smo, da su koncentracije olova u krvi kod neeksponiranih osoba raspodijeljene logaritamski normalno. Kao gornju granicu normalnih vrijednosti uzeli smo gornju 90%-nu granicu tolerancije (koeficijent pouzdanosti 0.95). Tako definirane normalne vrijednosti iznose za ukupni uzorak 60 μg/100 ml.

Našli smo također, da stanovnici sela imaju statistički značajno niže vrijednosti olova u krvi nego stanovnici grada, ali te razlike nemaju praktične vrijednosti za dijagnozu profesionalne ekspozicije olovu.

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