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TWO YEAR FEEDING STUDIES ON LEAD
Medical Research Project MR-787
HEMATOLOGIC AND BIOCHEMICAL RESPONSES OF RATS TO LEAD ACETATE IN THEIR DIETS

1. PROCEDURE:

Blood was taken from the tail vein of six male and six female rats selected at random from each group for an hematologic examination before lead acetate was added to the diets and again after approximately one, two and three months and at three-month intervals for the balance of the two year period. A 24-hour urine specimen was collected from each of these rats prior to the blood sampling for routine urinalysis and to measure the concentration of delta-aminolevulinic acid. A second 4-hour sample was collected to measure the urine glutamic-oxalacetic transaminase activity. Six other rats were sampled for biochemical measurements in the plasma at the same intervals. Four to six rats were sacrificed after 3, 6, 12 and 18 months and the tissues analyzed for lead. At the end of the study, tissues from all survivors were analyzed.

2. METHODS:

A) Blood and Urine:

Erythrocytes and leucocytes were counted electronically with a Coulter Counter; hemoglobin was measured by the cyanmethemoglobin method using certified reagent(1) and standard; hematocrits were measured in a capillary tube as microhematocrits. Blood films were prepared on cover slips and stained with Wright's stain for differential leucocyte counts (100 cells) and for counting the number of stippled cells (50 fields) and number of nucleated red blood cells (per 100 leucocytes).

(1) HYCEL, Inc., Houston, Texas.

The 24-hour urine volume was measured; the concentration in milliosmoles per liter was determined using a Fiske Osmometer; protein was measured quantitatively by the method of Karr et al.;[2] urine glutamic-oxalacetic transaminase activity by the method of Reitman and Frankel;[3] delta-aminolevulinic acid by the method of Mauzerall and Granick.[4] Sugar was measured semi-quantitatively with CLINITEST®[5] tablets. All specimens were tested for occult blood with OCCULTEST®[5] tablets; those giving a negative test were pooled and the sediment examined microscopically. The specimens giving a positive test for occult blood were examined separately.

Alkaline phosphatase activity was measured on blood taken from the tail using a modification of the method of Huggins and Marii.[6] Glutamic-pyruvic transaminase was measured spectrophotometrically using the method of Wroblewski and LaDue[7] adapted to a micro scale.

B) Lead:

The tissues were placed in individual plastic bags and frozen on solid carbon dioxide immediately following removal from the animal and then stored at -4° C. At the time of analysis, the samples were brought to room temperature, weighed into 35 milliliter Kjeldahl flasks and digested with 5 milliliters of a concentrated sulfuric-nitric acid solution (1:2) followed by 2 milliliters of a sulfuric-perchloric acid solution (1:1). After the digestion was completed, all nitrate and perchlorate were driven off by heating. The samples were diluted and an aliquot was taken for analysis by atomic absorption spectroscopy. The lead was extracted into an organic solvent with a chelating agent without pH adjustment and analyzed according

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(2) Karr, W.G., Reinhold, J.G. and Charnock, F. W. Manual of Clinical Chemistry, Stephenson Bros., Philadelphia,(1940).

(3) Reitman, S., and Frankel, S. Am. J. Clin. Path., 25:56 (1957).

(4) Mauzerall, D. and Granick, S., J. Biol. Chem. 219:435 (1956).

(5) ® Registered trademark Aines Co., Inc., Elkhard, Ind.

(6) Huggins, C. and Marii, S., J. Exptl. Med. 114:741 (1961).

(7) Wroblewski, F., and LaDue, S.J., Proc. Soc. Exptl. Biol. Med., 91:569 (1956).

to the methods recommended by the Perkin-Elmer Corporation.[8] Tissues taken at interim periods were pooled by groups; sample size and dilution were chosen to obtain one to ten micrograms of lead in the final aliquot. Livers from the animals remaining at the end of the experiment were analyzed individually and the kidneys were combined within groups in order to obtain an adequate sample.

Bones were stripped of soft tissue (cartilage caps remained), crushed, extracted in a Soxhlet with an ethyl alcohol-diethyl ether solution (1:1), dried and weighed into flasks. Digestion and analysis were similar to soft tissue. Blood and urine were analyzed by the method described by Berman.[9] Samples were pooled by groups at the interim sacrifices and analyzed individually at the end of the experiment.

Feed and feces were dried, ground through a 20 mesh screen and analyzed by the method described for soft tissue. Hair was washed with acetone, water, then acetone again, dried, ground through a 20 mesh screen and treated the same as soft tissue.

Standards containing 1 to 10 micrograms of lead per milliliter were prepared from lead nitrate in 1 normal sulfuric acid. One milliliter of each was chelated and extracted into methyl-isobutyl ketone without pH adjustment and aspirated into the flame. These standards were used for all digested tissue as there was no appreciable difference between standards prepared in 1 normal sulfuric acid and those prepared in 0.1 normal sulfuric acid. Standards for blood and urine samples were prepared according to Berman.

The limit of measurability for digested samples was approximately 0.1 microgram per gram while that of blood and urine was 0.1 microgram per 10 milliliters. Only a single analysis of each sample was performed, but samples containing added lead were included with each batch of tissues. When recovery of added lead was less than 85%, the entire batch was re-analyzed.

The lead content of the fat, muscle, spleen and testis was low in comparison to the minimum measurable levels and had no consistant relationships to dietary lead. For this reason, fat was not analyzed after six months; muscle, spleen and testis were not analyzed after eighteen months.

8. Analytical Methods for Atomic Absorption Spectroscopy, The Perkin-Elmer Corporation, Norwalk, Conn. 1964

9. Berman, E., Atomic Absorption Newsletter, 3 (9): 111 (1964)

3. STATISTICAL ANALYSIS:

All of the quantitative and semi-quantitative measurements made on the rats were analyzed statistically by D. W. Marquoit of the Applied Statistics Group, Engineering Department. A description of the procedure and a tabulation of the results are included in the appendix.

Two matrices of the simple correlation coefficients were computed for all pairs of variables; the four fixed (sex, group, time and level of lead in the feed) and the 26 observed responses. One matrix included the level of lead in the diet expressed as micrograms of lead per gram of feed (ppm) as determined by chemical analysis; the second included the calculated level of lead in the diet expressed as a dose in mg/kg of body weight. The portion of either matrix of interest is the correlation of observed response with the level of lead. As might be expected, various responses were strongly correlated with other responses (i.e. hemoglobin with erythrocyte count) but are useful in this study only in that they serve to reflect the consistancy of the data. Although it is also possible that these normal interrelationships might be interfered with by added lead in the diet, there was no indication of such an effect by the analysis.

The matrices were supplimented by a graphical analysis of a number of responses whose correlation coefficient indicated some relationship with the level of lead in the feed. Finally, to separate the effect of sex and age from the effect of the lead in the diet on the measured response, multiple regression coefficients were computed for those responses that were likely to have been affected as suggested by the scatter plots and magnitude of the regression coefficient.

The differences between groups for those responses that were significantly affected by the level of dietary lead were tested by the "t" test to determine the effect level.

Regression coefficients were calculated for those measurements that changed as the animals aged using all of the data when there was no difference between males and females. Separate regression coefficients were calculated when the value for one sex was higher or lower than that of the other.

An "F" test was used to determine whether these coefficients differed significantly.

A result was considered significant when the probability was less than 5% that such an effect would be found in random data. When this probability was less than 1% the effect was considered highly significant. It must be remembered that the conclusions drawn are based upon the differences between averages in a relatively large sample population. Considerable individual variation was observed in almost all measurements made, and for most of the responses the effect of lead reported was not detected in all animals in the group at every examination.

4. RESULTS:

The average value and its standard deviation for each of the measurements made on the blood and urine during the two-year period are listed in Table I. The average value observed at each sampling interval for these measurements and for the tissue analyses are found in the appendix.

The measured responses that were significantly affected by lead in the diet as indicated by the computed correlation coefficient matrices are listed in Table II. These responses and those for which there was a probable effect of lead as indicated by the multiple regression analysis and scatter plots are discussed below:

A) Stippled Cells:

Basophilic stippling of erythrocytes, or punctate basophilia, is the term given to the appearance of bluish or bluish-black granules in red corpuscles stained with Wright's stain. These granules have been found to contain ribonucleic acid and their formation is attributed to the clumping of RNA around the mitochondria in the cytoplasm of cells. They are considered to be evidence of defective erythrocytes produced in the bone marrow and released into the circulating blood.

Basophilic stippling of erythrocytes has been reported in lead intoxications. Their appearance, however, is not restricted to poisoning with lead but also occurs in certain anemias, leukemia, after exposure to analine, carbon monoxide, benzene and to other heavy metals. Occasional stippled cells are found in the blood of normal, healthy subjects.

The correlation coefficient (stippled cells vs. lead) and the multiple regression analysis indicated a significant ($p > 0.01$) effect on the erythrocytes from the lead added to the diet. Usually more stippled cells were found in the females than the males and there was a tendency for the number observed to decrease with time. The scatter plot of the data, however, suggested that the numerical value of the correlation coefficient was grossly inflated because of the heavy concentration of data in the lower left corner (0 cells, low lead intake) of the diagram. When the data was transformed and the log of lead intake is plotted against the log of the number of stippled cells a very clear dose relationship was evident (Figure 1). Although there was a significant effect on erythrocytes as indicated by the analysis of the data, there was considerable variation from time to time in the number of stippled cells seen in the control animals and the animals receiving the lower lead supplements. For this reason, a definite punctate basophilia is considered to have occurred only in the rats receiving the highest lead supplement in their diet.

B) delta-Aminolevulinic Acid:

The biosynthesis of heme occurs principally in the erythroblasts in the bone marrow. Glycine is condensed with succinyl-coenzyme A to form aminolevulinic acid in the initial step of the synthesis. Lead has been found to interfere with the normal synthesis of heme and the consequence of this interference results in an increased excretion of delta-aminolevulinic acid (δ-ALA) in the urine.

In this study the coefficient of correlation (urine δ-ALA vs. lead in the diet) and the multiple regression analysis indicated that lead added to the diet of rats had a highly significant ($p > 0.01$) effect on delta-ALA excretion. The output of delta-ALA was higher in the males than in the females and there was a tendency for the output to decrease as the rats aged. There was only a slight increment in the urinary ALA with increasing amounts of lead in the diet through the 100 ppm level of lead supplement, but at the highest lead level the output of delta-ALA increased markedly.

This tended to distant the scatter plots because of the heavy cluster of data points in the lower left corner (low delta-ALA, low lead) and suggested that the numerical value of the correlation coefficient might be exagerated. A "t" test showed the mean for the control groups and the groups fed the highest lead level (sexes tested separately) were significantly different ($p > 0.01$). Since the amount of delta-ALA eliminated will change as the animals age, the slopes of the regression, delta-ALA vs. time, were compared and found to be affected by the level of lead in the diet only at the highest level of added lead.

These results indicate that the addition of lead to the diet of rats at levels up to 100 ppm produces no clearly discernable effect on the amount of delta-ALA excreted. When 500 ppm is added to the diet a significant increase in delta-ALA excretion occurs, presumably as a result of impaired heme synthesis.

C) Other Changes in the Blood:

The multiple regression analyses that were computed for some of the responses on the basis of the correlation coefficient and scatter plots indicated that there was a very small, but nevertheless significant, effect due to lead in the diet on the segmented neutrophils and on alkaline phosphatase activity. Both of these have their origins in the bone, the former in the marrow in common with heme synthesis and erythropoiesis, and the latter in the osteoblasts. Because of the demonstrated effect on marrow activity and the concentration of lead in bone, more importance can be attributed to these very small differences than their numerical significance would indicate.

Neutrophilia, an increase in the neutrophilic granulocytes, has been found to occur following intoxication by a number of chemical agents and drugs, including lead. Many of these compounds will also cause a neutropenia. The effect appears to be dependent on the degree of the intoxication; a neutrophilia occuring when the effect is temporary or less severe. The results reported here are probably only indicative of the beginning of a trend toward the abnormal for a definite neutrophilia did not occur at any level of lead added to the diet. The increase in neutrophilic granulocytes that

normally occurs with time was affected only in the males at the highest level of lead intake.

The decrease in alkaline phosphatase activity of the plasma is less easily explained. It is noteworthy only because of its association with the bone and may be another indication of an effect of lead in this tissue. A decrease in alkaline phosphatase activity normally occurs with age in rats. The more than usual decrease that appeared to occur in the lead-treated rats was statistically significant only in the females fed the highest lead supplement.

In view of the significantly greater number of defective erythrocytes and the evidence of impaired heme synthesis in the group fed the highest level of lead, an effect on the hemoglobin concentration might be expected. Although the multiple regression analyses indicated that there was no effect on hemoglobin, the "F" test of the regression coefficients (hemoglobin vs. time) showed that the highest lead supplement did cause a more rapid than usual decrease in hemoglobin concentration as the animals aged.

D) Lead Excretion:

The lead excreted in the urine had the highest correlation, of all the responses measured, with the amount of lead added to the diet. There was also a very high correlation between the lead in the diet and the lead eliminated in the feces, but this is of less biological significance since most of the fecal lead represents the unabsorbed lead passing through the gastrointestinal tract.

Figure 2 shows the correlation of lead in the urine with lead added to the diet. Neither sex excreted more lead than the other, nor was there any apparent change in the amount of lead excreted as the animals aged. The coefficient of correlation, the scatter plots and the multiple regression analysis all indicated a very significant effect on the level of urinary lead by the addition of lead to the diet. A "t" test of the difference between the mean of the control groups (0.2 ± 0.14 µg Pb/10 ml) and the group fed a diet containing 10 ppm of added lead (0.4 ± 0.25 µg Pb/10 ml) showed that the rats fed even the lowest lead supplement excreted significantly more lead.

Although a statistically significant difference in lead excretion was found when only 10 ppm of lead was added to a control diet, an increase in lead excretion is not likely to be detected in a single specimen, particularly a casual one, from an individual receiving such a small supplement of lead. The difference reported represents an average difference in an aliquot of a 24-hour specimen collected from a relatively large number of rats. Of all the measurements made, nevertheless, the amount of lead in the urine was the most sensitive response to added dietary lead.

E) Lead in Tissues:

The tissues that showed a significant increase in lead concentration are listed in Table 3 . Because of the need to pool many of the tissues in order to measure the lead content, the resulting small number of samples did not provide sufficient data to be included in the statistical analyses described. Correlation coefficients were calculated, however, for tissue lead vs. dietary lead using group averages.

There was a high correlation between the lead in the tissue and the lead in the diet for bone kidney, blood, liver and brain. The highest concentrations were found in the bone and the least in the brain. For all but the kidney, there was a similar relative increase in tissue lead with increasing amounts of lead added to the diet. This is shown graphically in Figure 3 using transformed data (log tissue lead vs. log lead in diet).

If the tissues from the control rats (those receiving no added lead) are used as a base line and a level of more than twice this is considered a significant increase, then 50 ppm of added lead resulted in an increase in the kidney and brain, and 100 ppm resulted in an increase in the bone, blood and liver.

The lead content of the hair has been suggested as an industrial hygiene procedure for detecting an exposure to lead. In this experiment, the variation within groups for the lead content of the hair was greater than for any other tissue analyzed. For this reason, the average lead content

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Y. Suzuki, K. Nishiyama and Y. Matsuka, Tokushima J. Exptl. Med. 5:111 (1958).

of the hair of the rats receiving even the highest added lead was less than twice that of the controls. The cause of this wider variation was not investigated. Contamination of the samples during the collection and processing for analysis may have contributed. Analysis of the hair to detect an exposure to lead seems to be a less reliable or less sensitive method than the more usual analysis of blood or urine.

TABLE 1

AVERAGE VALUE FOUND FOR THE MEASUREMENTS MADE ON BLOOD AND URINE OF RATS FED LEAD ACETATE FOR TWO YEARS

				MALE					FEMALE				
				I	II	III	IV	V	I	II	III	IV	V
	N	$\bar{X}$	± S.D.	milligrams LEAD/gram FEED					milligrams LEAD/gram FEED				
				5	18	62	141	548	5	18	62	141	548
Erythrocytes $X10^6/mm^3$	714	6.518	1.047	6.66	6.64	6.81	6.60	6.87	6.35	6.13	6.41	6.23	6.37
Hemoglobin gm/100 ml	713	15.32	1.63	15.5	15.3	15.5	15.5	14.9	15.2	15.2	15.5	15.2	15.2
Hematocrit %	716	42.4	4.7	44	43	43	43	42	42	42	42	41	42
Leucocytes $X10^3/mm^3$	690	13.43	5.37	14.8	14.3	15.9	1.57	14.9	10.6	11.5	12.3	12.0	11.5
Neutrophils, Segmented %	716	30.3	13.9	29.4	29.8	32.1	32.3	34.6	28.5	28.3	30.7	30.2	30.8
Neutrophils, Juvinile %	716	1.38	1.62	1.0	1.6	1.4	1.5	1.5	1.3	1.2	1.6	1.6	1.6
Myelocytes %	716	0.13	0.40	0.1	0.2	0.2	0.1	0.2	0.1	0.1	0.1	0.1	0.1
Lymphocytes %	716	62.0	15.3	64.4	61.6	59.0	59.5	57.2	64.8	64.1	61.4	61.9	61.4
Eosinophils %	716	3.0	2.1	2.6	3.5	3.2	3.6	3.3	3.1	2.6	2.9	2.5	2.7
Monocytes %	716	2.3	1.8	1.8	2.5	2.9	2.3	2.3	2.2	2.3	2.7	2.5	2.6
Basophils %	716	0.2	0.5	0.1	0.2	0.3	0.2	0.4	0.2	0.1	0.2	0.2	0.2
Atypical Cells %	716	0.6	1.0	0.6	0.7	0.9	0.7	0.6	0.5	0.6	0.7	0.5	0.9
Nucleated RBC'S/100 WBC'S	716	0.1	0.5	0.1	0.1	0.2	0.1	0.3	0.1	0.2	0.1	0.3	0.3
Stippled Cells/50 mf	716	0.54	1.17	0.06	0.17	0.23	0.57	1.63	0.06	0.20	0.42	0.42	2.60
Urine Volume ml/24 hr.	716	20.1	10.7	22.5	21.9	20.4	21.9	22.3	18.2	16.3	18.7	20.2	18.1
Osmolality mOs/L	571	1430	586	1612	1601	1611	1504	1517	1397	1433	1340	1335	1468
Protein mg/24 hrs.	716	49	109	81	58	67	54	77	21	25	25	48	16
Urine GOT Units/ml	549	24.3	11.6	27	26	30	24	25	21	21	21	19	22
delta-Aminole-vulinic Acid mg/24 hrs.	665	0.088	0.059	0.082	0.087	0.089	0.093	0.188	0.056	0.059	0.056	0.064	0.134

TABLE 1 (Continued)

MALE

	N	$\bar{X}$	± S.D.	I	II	III	IV	V	I	II
				milligrams LEAD/gram FEED						millig
				5	18	62	141	548	5	18
Alkaline Phosphatase /m pNP/ml/hr.	717	4.43	2.23	5.1	4.9	4.9	4.5	4.6	4.3	4.5
GPT units/ml.	364	25.8	13.0	29.4	-	-	-	23.4	23.3	-
Lead in Urine /g/10 ml.	197	1.23	1.72	0.23	0.40	0.85	1.51	4.71	0.18	0.4
Lead in Feces /g/gm.	120	381	601	59	80	199	361	1338	58	81

TABLE 2
SIGNIFICANT SIMPLE CORRELATION COEFFIENTS OF RESPONSE VS. LEAD IN FEE

		N	STIPPLED CELLS	δ-ALA	LEAD IN URINE	
Lead in Feed μg/gm	All data	≤720	0.571	0.542	0.880	
	Group data	≤120	0.775	0.682	0.888	
	Males	≤360	0.525	0.569	0.840	
	Females	≤360	0.585	0.542	0.874	
Lead in Feed $\frac{mg}{kg\ body\ wt.}$	All data	≤720	0.624	0.529	0.812	
	Group data	≤120	0.852	0.683	0.829	
	Males	≤360	0.572	0.575	0.805	
	Females	≤360	0.611	0.641	0.840	

TABLE 3
AVERAGE VALUE FOUND FOR THE LEAD CONTENT OF TISSUES FROM RATS FED LEAD ACETATE FOR TWO YEARS

	I	II	III	IV	V	r	$\bar{y}$	Slope X on Y	Intercept	Slope logx on logy
	milligrams LEAD/gram FEED									
	5	18	62	137	548					
Bone μg/gm	6.4	6.4	11.2	16.2	45.1	0.918	16.70	0.067	6.26	0.422
Blood μg/10 ml	0.28	0.27	0.43	0.79	1.86	0.969	0.75	0.003	0.30	0.419
Kidney μg/gm	0.32	0.54	1.32	3.19	17.90	0.937	4.64	0.033	0.42	0.855
Liver μg/gm	0.24	0.21	0.37	0.66	1.56	0.919	0.63	0.002	0.28	0.422
Brain μg/gm	0.08	0.13	0.21	0.24	0.66	0.954	0.26	0.001	0.11	0.426
Muscle (a) μg/gm	0.2	0.1	0.1	0.2	0.4					
Spleen (a) μg/gm	>0.1	>0.1	>0.1	0.1	0.1					
Fat (b) μg/gm	0.2	0.2	0.2	0.3	0.4					
Hair μg/gm	10.9	6.0	5.3	7.2	16.3					

(a) 3, 6, 12, 18 month samples, averaged
(b) 3 and 6 month samples, averaged

FIGURE 1

Increase in the number of stippled cells with increasing levels of lead in the diet

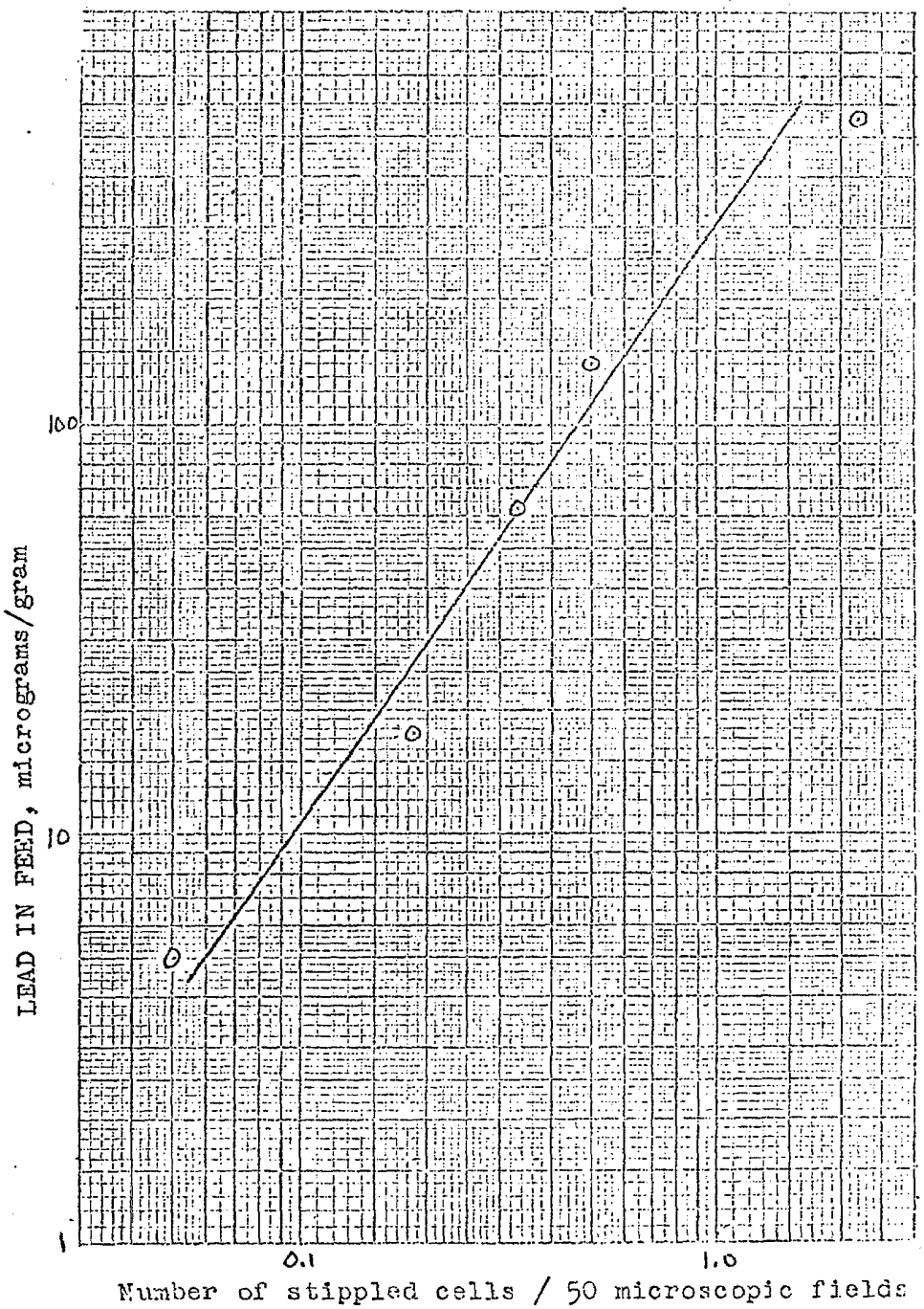

FIGURE 2

Increase in the concentration of lead in the urine with increasing levels of lead in the diet

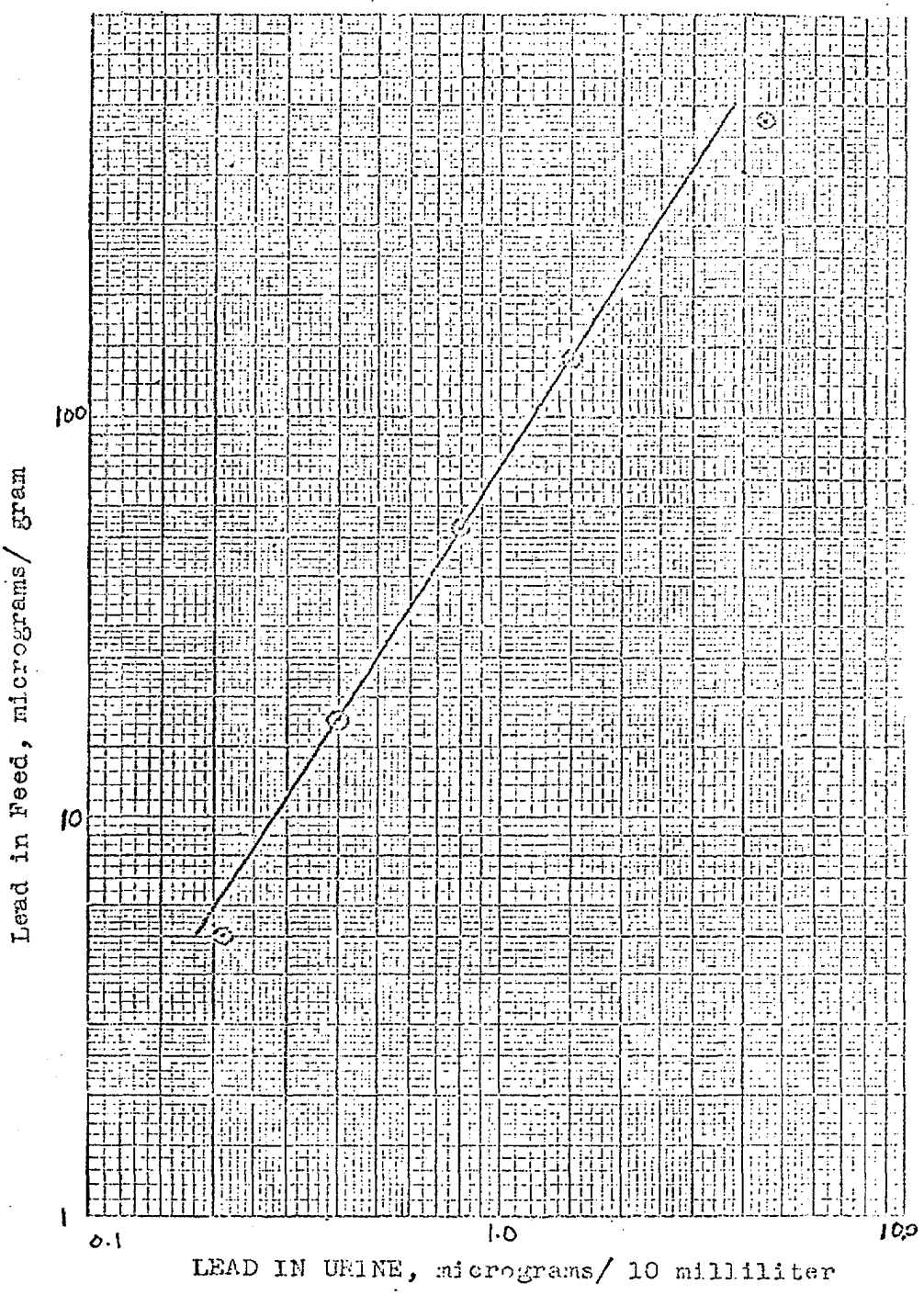

FIGURE 3

Increase in the level of lead in rat tissue
with increasing levels of lead in the diet