IN VITRO EFFECT OF LEAD IN BLOOD ON THE DETERMINATION OF DELTA-AMINOLEVULINIC ACID DEHYDRASE

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Abstract

Whole blood, as withdrawn, from lead-exposed dogs was added in varying proportions, to whole blood, as withdrawn, from normal (non-exposed) dogs. The resultant red cell ALAD activities and lead concentrations of the mixtures were then determined. It was found that the resultant ALAD activities could be predicted on the basis of simple dilution of the blood with normal ALAD activity by the blood with severely reduced ALAD activity. The observed values for ALAD of the mixtures related to resultant lead concentration according to the equation: $y = a + bx$, rather than to the equation $\log y = a + bx$ as reportedly observed for samples of blood from lead-exposed humans, and from lead-fed dogs. There was no evidence that the ALAD activity contributed by the portion of normal blood in the mixture was affected by the lead contributed by the blood from the lead exposed dog. This is considered as evidence that the reduction in red cell ALAD activity observed in lead-exposed dogs is not an "in vitro" artifact inherent in the method used to determine ALAD activity.

MEM/jtd
10/23/72

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Within the last few years the depression of red cell δ-aminolevulinic acid dehydrase (ALAD), associated with elevated lead concentrations in the blood from lead-exposed individuals, has been considered as an effect of lead on this enzyme system in the body (1-9). Recently, however, caution in the interpretation of this depressant action as representative of an "in vivo" effect of lead, has been expressed (10). Since the determination of ALAD activity is an "in vitro" assay performed on hemolysates of the red blood cells, one question that arises is whether this depression actually occurs "in vivo," or whether it occurs during the assay itself, e.g. during or following the hemolysis. If the latter should be demonstrated to occur, the depression of red cell ALAD activity, as commonly measured, would be an "in vitro" artifact inherent in the method used for its determination.

In support of the contention that the depression of red cell ALAD activity in individuals exposed to lead occurs "in vivo," is the demonstration that the depression of ALAD effected by addition of inorganic lead to hemolysates of normal blood differs from the former in at least two respects (5):

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1. The optimum pH, 6.4, for red cell ALAD activity following addition of inorganic lead to hemolysate is the same as for normal blood, whereas the optimum pH for ALAD activity of blood from lead-exposed individuals has shifted to 5.8.
2. The depression of red cell ALAD activity of the blood from lead-exposed individuals is reversed by heat-denaturation, whereas the depression due to the addition of inorganic lead to hemolysate is not so reversed.

However convincing as these differences may be, they do not unequivocally prove that the depression of red cell ALAD activity observed for blood from lead-exposed individuals occurred "in vivo." Rather, these differences may reflect differences between an "in vitro" depressant action due to lead ions and that due to a lead complex in which form the lead may exist in its attachment to circulating red blood cells. The experiments herein reported were designed to obtain data which might provide evidence as to whether the depressant action of lead on red cell ALAD activity occurs "in vivo," or may be attributed to an "in vitro" artifact inherent in the method used to measure ALAD activity.

In design, the experiments are simple. Blood (N), as withdrawn (i.e. not a hemolysate) from a normal (non-exposed) dog was mixed in varying proportions with lead-containing blood (L), as withdrawn, from a lead-exposed dog. The samples of mixed bloods were then analysed for resultant red cell ALAD activity and lead concentration.

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1. If the depressant action of lead on red cell ALAD, as usually determined, occurs "in vivo," the lead present in a sample of mixed bloods should not affect the ALAD activity of the red cells contributed by the portion of normal blood in the mixture. The resultant ALAD activity should be predictable on the basis of dilution of red cells with normal ALAD activity by red cells with reduced ALAD activity.
2. In contrast, if the depression of ALAD occurs subsequently to some procedure in the analysis of ALAD activity, i.e. is an "in vitro" artifact, the lead-containing blood, as well as the normal, presumably would provide red cells with normal ALAD activity. At some stage in the analysis, the lead would exert its depressant action on the ALAD of the red cells, regardless of whether these cells were from normal or lead-exposed dogs. The resultant ALAD activity would then relate to the resultant lead concentration as observed for unmixed samples obtained from lead-exposed individuals, i.e. in accordance with the equation (3,4,7)

$$\log y = a + bx$$

where y is ALAD activity and x is the lead concentration of the sample. The slope, b, would be negative. Inherent in this argument is the assumption that, if the depression of ALAD does occur during the analysis, the ALAD in both mixed and unmixed (i.e. from a single individual) blood samples will be equally depressed by a given concentration of lead. Although this assumption is logical, it may be difficult to verify.

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METHODS

Ten male beagle dogs, available from a previous study (11), supplied the blood used in the present experiments. Five of these dogs, fed the basic diet of ground Purina® dog meal, provided normal (N) blood, with lead concentrations ranging from "0" (not-detectable) to 7 μg/100 ml and red cell activities ranging from 24 units to 39 units/ml RBC. (Three of these dogs were controls -- no lead added to diet -- in the previous study, and the other two dogs -- which had received 100 ppm Pb, had been on lead-free diets for at least 62 weeks before starting the present experiments). After approximately one month on the basic diet to which 1000 ppm lead, as the acetate, was added, the other five dogs provided blood (L) with lead concentrations ranging from 52 μg to 70 μg/100 ml and red cell ALAD activities ranging from "0" to 3 units/ml RBC.

For each experiment, duplicate series of mixtures of whole blood (as withdrawn, i.e. not hemolysates) were prepared by mixing blood, N, from one of the normal dogs with blood, L, from one of the lead-fed dogs, to provide mixtures containing the following proportions of N and L bloods:

	Percentage						
N Blood:	100	80	60	50	40	20	0
L Blood:	0	20	40	50	60	80	100

The mixtures were analysed for resultant blood lead concentration, using an atomic absorption spectrophotometer (Model 303, Perkin-Elmer Corp., Norwalk, Conn.), and for red cell ALAD activity, using the method of Bonsignore et al (12). Hematocrit ratios were determined by the micro-hematocrit method. Five such experiments were performed, each dog serving only once as a donor.

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RESULTS

Accuracy of Measurement.

The determination of the accuracy of the measurements, including mixing of blood samples, was based on the differences between the two values obtained for duplicate mixtures, and was calculated according to the equation (recommended by Dr. R. D. Snee, Engineering Department)

$$\text{Standard Deviation} = \pm \sqrt{\frac{\Sigma(m_1 - m_2)^2}{2xN}}$$

where m_1 and m_2 are the values for the duplicate mixtures, and N is the number of pairs of duplicates. The standard deviations so calculated are: Blood lead concentration: $\pm$ 2.8 μg, ALAD activity: $\pm$ 2.2 units. The data presented in the figures are the averages of the values obtained for the duplicate determinations.

Relation of Red Cell ALAD Activity to Lead Concentration in Blood From Lead-Exposed Dogs.

As observed for blood samples obtained for humans exposed to lead (3,4,7), the red cell ALAD activity (y) in samples of blood obtained from lead-fed dogs during a previous study (11) was related to blood lead concentration (x) in accordance with the equation

$$\log y = a + bx$$

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for which the slope, b, is negative. This relationship is demonstrated by the data obtained on the female dogs in Figure 1 (for unknown reasons, the female dogs provided a more even distribution of lead concentrations than the males). The regression applied only to data which were with the measurable ranges for ALAD activity and blood lead concentration, for datum points dependent upon "0" (not measurable) values for ALAD or blood concentration were excluded from the calculation.

ALAD Activity of Samples of Normal Blood Diluted With Plasma.

Preliminary to the main experiments in the present study, the effect of simple dilution of whole blood on red cell ALAD activity was determined for two series of dilutions. In each series, whole blood (not hemolysates) withdrawn from a normal dog was diluted with the dog's own plasma to provide samples consisting of 80%, 50%, 40%, and 20% of normal blood (N). The effect of dilution of the blood on red cell ALAD activity, expressed in this series as optical density (ALAD activity, as units/ml red blood cells, would be the same for all dilutions of a given blood sample), is demonstrated by the results obtained for the first series of dilutions in Figures 2, A and B, in which the ordinate scale (Optical Density) is linear (Figure 2A) or logarithmic (Figure 2B). The data demonstrate that when whole blood is diluted with plasma, the relationship between optical density (y) and the percentage of blood (x) in the sample conforms to the equation $y = a + bx$ (Figure 2A) rather than to the equation $\log y = a + bx$ (Figure 2B). The regression, calculated by the method of least squares, for the data in

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Figure 1A has a correlation coefficient (r) of 0.9980, and accounts for 99.6% of the variance in optical densities. The regression calculated for the data of the second dilution series, has a correlation coefficient of 0.9986, and accounts for 99.7% of the variance.

Effect of Lead in Blood on ALAD Activity of Normal Blood.

In the five experiments in which blood from a normal dog was mixed, in varying proportions, with blood from a lead-exposed dog, the resultant ALAD activities are expressed as units/ml red blood cells, since the percent red cell volumes were essentially the same for all samples (including 100 percent normal blood and 100 percent lead-containing blood) in a given series of mixtures.

The effect of adding lead-containing blood on the red cell ALAD activity of normal blood is shown by the data obtained for the five series of mixtures in Figures 3, A and B, in which units (Figure 3A) or log units (Figure 3B) of ALAD activity are plotted against the percentage of normal blood in each mixture. As when normal blood was diluted with plasma, the relationship between ALAD activity (y) and the percent of normal blood in the mixture (x) is expressed by the equation $y = a + bx$, rather than by $\log y = a + bx$. The effect of adding the lead-containing blood appears to be that of simple dilution of the blood with normal ALAD activity.

On the assumption that the reduction in ALAD activity of the normal blood was due to simple dilution by blood with low ALAD activity, the resultant activity for each blood mixture was predicted. The agreement

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between predicted (x) and observed values (y) are shown by the regression, $y = a + bx$, with its $\pm$ 95% confidence limits, in Figure 4A. The relatively close agreement between observed and predicted values supports the contention that the only significant effect of mixing lead-containing blood with normal blood was to dilute the ALAD activity of the latter.

Additional support for simple dilution is provided by the data in Figure 4B in which observed values (y) for both blood lead concentration and ALAD activity are plotted against corresponding values (x) predicted on the basis of dilution. The datum points for ALAD show considerable overlap with those for lead concentration, indicating that the resultant ALAD activity, as well as the lead concentration, of a sample of mixed bloods may be predicted on the basis of simple dilution. The calculated regression, $y = 0.145 + 0.996\ x$, for lead concentration almost coincides with the "ideal" regression, i.e. $y = x$ at all points on the line, or $y = 0 + 1.000\ x$. The regression calculated for ALAD activity, $y = -1.147 + 0.990\ x$, closely parallels those for lead concentration and the "ideal," but has a lower elevation, indicating observed values tend to be lower than predicted values. In comparison with the "ideal" line for ALAD, the lower elevation of the regression for ALAD is significant at the five percent level, but the error which this represents, and which appears to be systematic, approximates only 1-1.5 units of ALAD activity. For individual comparisons, the error exceeded the accuracy of measurement in only 7 of the 25 pairs. The magnitude of the error showed no correlation with, or any pattern relating to, percentage of normal blood in the mixture, the level of activity of ALAD, hematocrit ratios, lead concentration, etc.

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ALAD Versus Blood Lead Concentration in Samples of Mixed Bloods.

The relationship between the red cell ALAD activities of the mixtures and the corresponding lead concentrations is shown in Figures 5, A and B. It is obvious from these graphs that the inverse linear relationship between the logarithm of the ALAD activity (y) and blood lead concentration (x) as usually described, does not apply to these mixtures of normal and lead-containing bloods (Figure 5B). Rather, the relationship conforms to the equation: $y = a + bx$, for which in this case, the slope b has a negative value (Figure 5A).

The nonconformity of the present data to the equation $\log y = a + bx$ was confirmed by using this equation to predict the ALAD activities (y) of the samples from their lead concentrations (x). Unpublished data previously obtained on dogs in this laboratory provided values for the constants a and b, but observed values were considerably higher than predicted values except at very low levels (less than 5-6 units) of ALAD activity. The predictions were less discrepant when values for a and b, calculated for the data obtained on the limited number of samples of unmixed blood (i.e. 100% normal and 100% lead-containing) available in this experiment, were substituted into the equation so that

$$\log y = 1.55 - 0.019\ x$$

In Figure 6, observed values (y) are plotted against the values predicted (x) by this equation, and demonstrate that the observed values in ALAD activity of samples of mixed bloods are not predictable in accordance with the equation $\log y = a + bx$.

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DISCUSSION

It should be emphasized that all determinations of ALAD activity were carried out under controlled conditions. Temperature, pH, substrate concentration, and incubation time were the same for all samples. The proportion of red cells (hematocrit ratios) in the samples of any one series of mixtures, including those of the 100 percent normal and 100 percent lead-containing bloods, were essentially constant. For four of the five series, the standard deviation from the mean of the series ranged from $\pm$ 0.3 to $\pm$ 0.9 volumes percent. For the fifth series, the standard deviation was $\pm$ 2 volumes percent, attributable to a high reading for one sample.

The data presented in Figures 5 and 6 indicate that the ALAD activity (y) of a sample of mixed bloods may be predicted for a given lead concentration (x) in accordance with the equation

$$y = a + bx$$

rather than with the equation

$$\log y = a + bx$$

which applies to the data obtained on blood from lead-exposed humans (3,4,7) and dogs, Figure 1. This difference in the relationship between ALAD activity and lead concentration indicates that the mechanism underlying the reduction in ALAD activities of the samples of mixed bloods differs from that underlying the depression of the enzyme in blood from lead-exposed individuals.

The data in Figures 3 and 4 provide evidence that the reductions in the ALAD activity of the samples of mixed bloods may have been due to simple dilution of the blood with normal ALAD activity by blood with severely

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depressed ALAD activity. This explanation implies that, within the concentration range, 12 μg to 57 μg/100 ml, of the samples of mixed bloods, lead, as it exists in circulating blood, does not have a measurable "in vitro" depressant action on the ALAD activity of normal blood. The depression of ALAD observed in blood from lead-exposed individuals is, then, unlikely to be an "in vitro" artifact inherent in the method used for determination of ALAD activity.

The present data do not exclude alternative explanations favoring an "in vitro" depressant action of lead on ALAD. For example, the resultant ALAD in a sample obtained by mixing normal blood with blood from a lead-exposed individual may differ from the ALAD (with normal activity) in the blood from a lead-exposed individual, hence the mechanisms underlying an "in vitro" depressant action of lead may differ in the two cases. However, until it is demonstrated that mixing bloods from normal and lead-exposed dogs interferes with the mechanism of an "in vitro" depressant action of lead on the resultant ALAD, the data obtained in the present experiments are considered as evidence that the depression of ALAD observed in blood from lead-exposed dogs is an "in vivo" phenomenon.

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SUMMARY

When blood from a normal (non-exposed) dog was mixed, in varying proportions, with blood from a lead-exposed dog, the resultant red cell ALAD activity (y) of the mixture was found to relate to its lead concentration (x) according to the equation: $y = a + bx$. Since the red cell ALAD activity of blood (unmixed) samples from lead-exposed dogs relates to blood lead concentration according to the equation $\log y = a + bx$, it is concluded that the mechanism of the reduction in ALAD activity in the samples of mixed bloods differs from the mechanism responsible for the depression of ALAD observed in lead-exposed individuals. Data, which indicate that the reduction in ALAD activity observed in the samples of mixed bloods may be explained on the basis of simple dilution of blood with normal ALAD activity by blood with reduced ALAD activity, are presented. The data in this report may be considered to support the contention that, within the limits of lead concentrations between 12 μg and 57 μg/100 ml, the depressant action of lead on the red cell ALAD activity of lead-exposed dogs, occurs "in vivo," rather than as an "in vitro" artifact inherent in the method used for measuring the ALAD activity.

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ACKNOWLEDGMENTS

We wish to take this opportunity to thank Dr. John A. Zapp, Jr., Director of Haskell Laboratory, for his interest in this project, and Dr. John R. Barnes, Chief of Biochemistry and Dr. Alex Azar, Research Manager of Environmental Sciences, for their advice and critism during the preparation of this text.

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DUP040002078