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EVIDENCE AS TO WHETHER DEPRESSION OF RED CELL δ-AMINOLEVULINIC ACID DEHYDRASE BY LEAD IN DOGS OCCURS "IN VIVO" OR IS AN "IN VITRO" ARTIFACT INHERENT IN METHODS USED TO MEASURE ITS ACTIVITY

Medical Research Project No. 10A11

HASKELL LABORATORY FOR TOXICOLOGY
AND INDUSTRIAL MEDICINE

Distribution List

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INTRODUCTION

The depression of red cell δ-aminolevulinic acid dehydrase (ALAD) activity, a phenomenon subsequent to absorption of lead, is assuming importance as a possible indicator of lead exposure (1-6). This indirect assay of lead absorption is based on the demonstration that the logarithm of ALAD activity has an inverse linear relation to blood lead concentration within the range of 5 μg-90 μg Pb/100 ml blood (3, 4, 7). Such a relationship predicts depression of red cell ALAD activity in association with very low concentrations of lead in blood, e.g. below 5 μg/100 ml (3, 7), and precludes a "no-effect" level for blood lead with respect to red cell ALAD.

For the individual exposed to lead, the significance of the depression of his red cell ALAD activity is dependent, in the final analysis, upon whether the depression occurred "in vivo", as is generally assumed (1-9), or whether it occurred <u>after</u> his blood was withdrawn for analysis, i.e. "in vitro". Since circulating lead is bound to the red blood cells, and since analyses of ALAD activity are performed on hemolysates of these cells (10), the possibility exists that the depression of ALAD activity as determined for such hemolysates may occur subsequent to the hemolysis.

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 activity effected by addition

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of inorganic lead to hemolysates of "normal" blood differs from the former in at least two respects (5):

(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 a lower pH, 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 addition of inorganic lead to hemolysates of "normal" blood 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 exposed individuals did occur "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 test whether the depression of the red cell ALAD activity of the blood from lead-exposed dogs, as measured by a commonly used method, is an "in vivo", i.e. occurred in the animal, or an "in vitro", i.e. occurred during the analysis, phenomenon.

In design, the experiments are simple. Whole blood from a lead-exposed dog, hence containing lead in whatever form it exists in

circulating blood, was added, in varying proportions, to whole blood from a "normal" (control) dog. The mixed samples were then analysed for resultant red cell ALAD activity and lead concentration. If the depression of ALAD activity of the blood of the lead-exposed dog occurred "in vivo", addition of this lead-containing blood should not affect the ALAD activity of "normal" blood other than by simple dilution. On the other hand, if the depression occurs during the analysis, i.e. "in vitro", the enzymes from both bloods should be affected, and the logarithm of the resultant ALAD activity should have an inverse linear relation to the resultant lead concentration.

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 ALAD activities ranging from 24 units to 39 units/ml RBC. 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:

*Three of these dogs were controls (no lead added to diet) in the previous study and the other two dogs (100 ppm lead) had been on lead-free diets for at least 62 weeks before starting the present experiments.

	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 microhematocrit method. Five such experiments were performed, each dog serving only once as a donor.

RESULTS

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*

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

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.

*Recommended by Dr. R. D. Snee, Engineering Department, E. I. Du Pont De Nemours and Company.

ALAD Activity of Samples of Normal Blood Diluted With Plasma

Preliminary to the main experiments, 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%, 60%, 50%, 40%, and 20% of normal blood (N). The effect of dilution of the blood on red cell ALAD activity is demonstrated by the results obtained for the first series of dilutions in Figures 1, A and B, in which the ordinate scale (Optical Density*) is linear (Figure 1A) or logarithmic (Figure 1B). The data show that when whole blood is diluted with plasma, the relationship between optical density (y) and the percentage of blood in the sample (x) conforms to the equation $y = a + bx$ (Figure 1A) rather than to the equation $\log y = a + bx$ (Figure 1B). The regression, calculated by the method of least squares, for the data in 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.

The difference between the two equations is important for in human blood, the relation between red cell ALAD activity and the blood lead concentration appears to conform to the equation $\log y = a + bx$ (3,4,7). That this same relationship holds for dog blood is shown by the data in Figure 2, obtained on female dogs** in an earlier study (11).

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*Optical densities, rather than units of ALAD activity, are plotted against percentages of blood because ALAD activity, expressed as units/ml RBC, would be the same for all dilutions. In Figures 2-5, ALAD activity, as units/ml RBC, is used since the red cell volumes were essentially equal in all blood samples of a given series.

**For unknown reasons, the female dogs provided a more even distribution of blood lead concentrations than the males.

Effect of Lead in Blood on ALAD Activity of Normal Blood

The effect of adding lead-containing blood on the red cell ALAD activity of normal blood is shown by the data 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.

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 between predicted (x) and observed values (y) are shown by the regression, $y = a + bx$, with its $\pm$ 95% confidence limits, in Figure 4. The relatively close agreement between observed and predicted values strongly 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.

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).

DISCUSSION

If lead acts to depress red cell ALAD activity "in vitro" rather than "in vivo," all samples of blood as withdrawn (i.e. prior to hemolysis) would presumably have normal ALAD activities. The ALAD activity (y) of samples of mixed normal and lead-containing bloods would have the same relationship to resultant lead concentration (x) as has been shown to occur for unmixed samples obtained from lead-exposed individuals, i.e.

$$\log y = a + (-b)x.$$

The present data demonstrate, however, that the ALAD activities of the samples of mixed blood are not predictable by this relationship, but rather by

$$y = a + (-b)x$$

which indicates that a different mechanism is responsible for the observed reductions in ALAD activities.

The data presented in Figures 3, A and B, and in Figure 4 provide evidence that the reduction in ALAD activities of the samples of mixed normal and lead-containing bloods resulted from simple dilution of the ALAD activity contributed by the portion of normal blood in the mixture. Lead, presumably present in the form in which it exists in circulating blood, did not have a measurable depressant action on the activity of this "normal" ALAD.

Within the limits of the resultant lead concentrations of the mixtures, which ranged from 12 μg to 57 μg/100 ml, the present data support the generally - held concept that the red cell ALAD depression observed in lead-exposed individuals, as measured by a widely accepted method (12), occurred "in vivo," and is not an "in vitro" artifact inherent in the method.

SUMMARY

The reduction in red cell ALAD activity of normal blood, when mixed with lead-containing blood from a lead-exposed dog, may be attributed to simple dilution, rather than to a depressant action of the lead during or subsequent to the preparation of the hemolysates. This is interpreted as indicating that the depressant action of lead on the red cell ALAD of lead-exposed individuals occurs "in vivo," and is not an "in vitro" artifact inherent in the method of measurement of ALAD activity.

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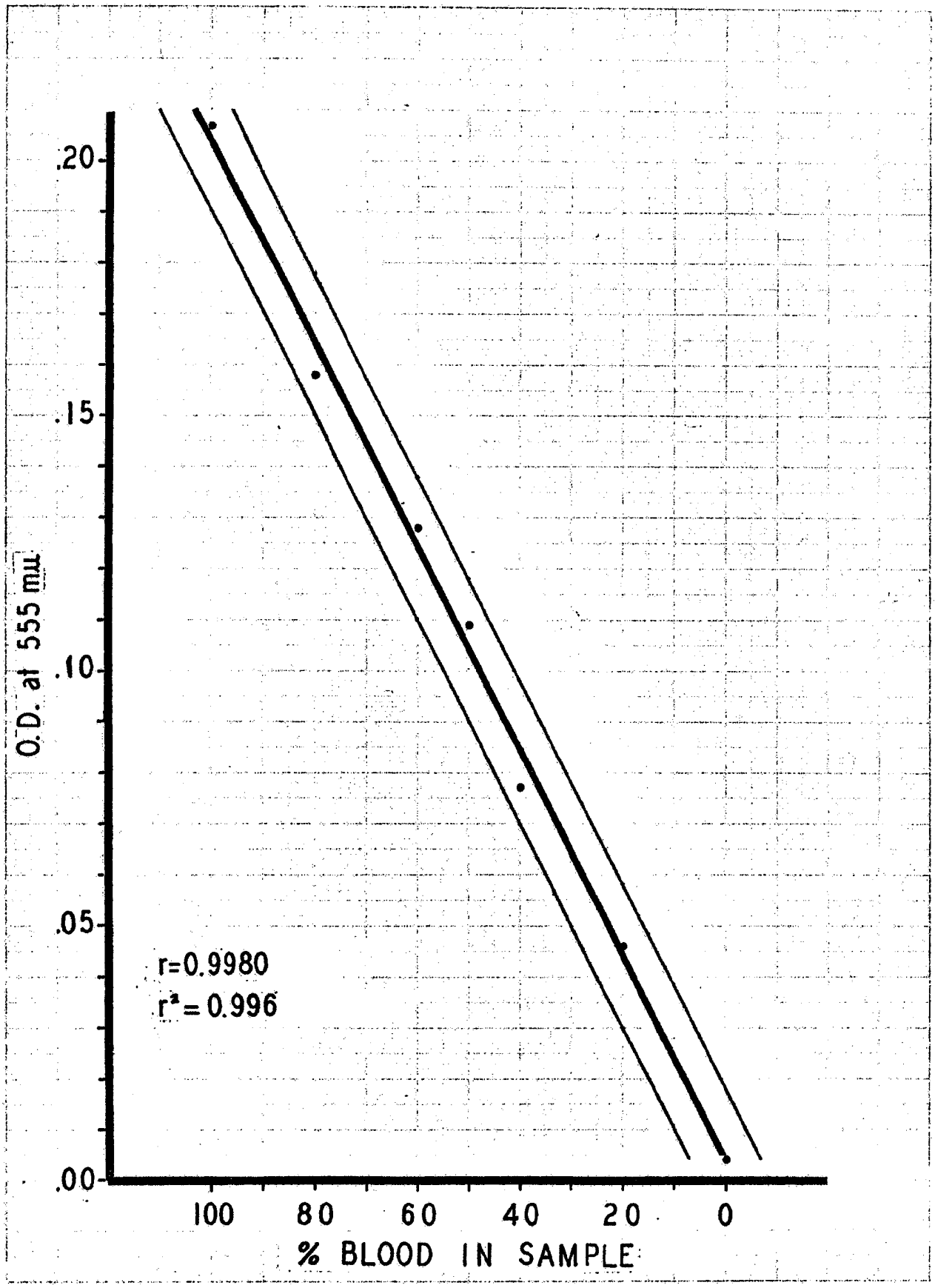
Figures 1, A and B

The effect of whole blood dilution with plasma upon the normal red cell ALAD activity.

A: Optical density (O.D.), on linear scale, vs. percent of blood contained in sample.
Calculated Regression: $y = a + bx$ ━━━
$\pm$ 95% confidence limits: ———

B: Log optical density vs. percent of blood contained in sample.
Curve fitted to data "by eye."



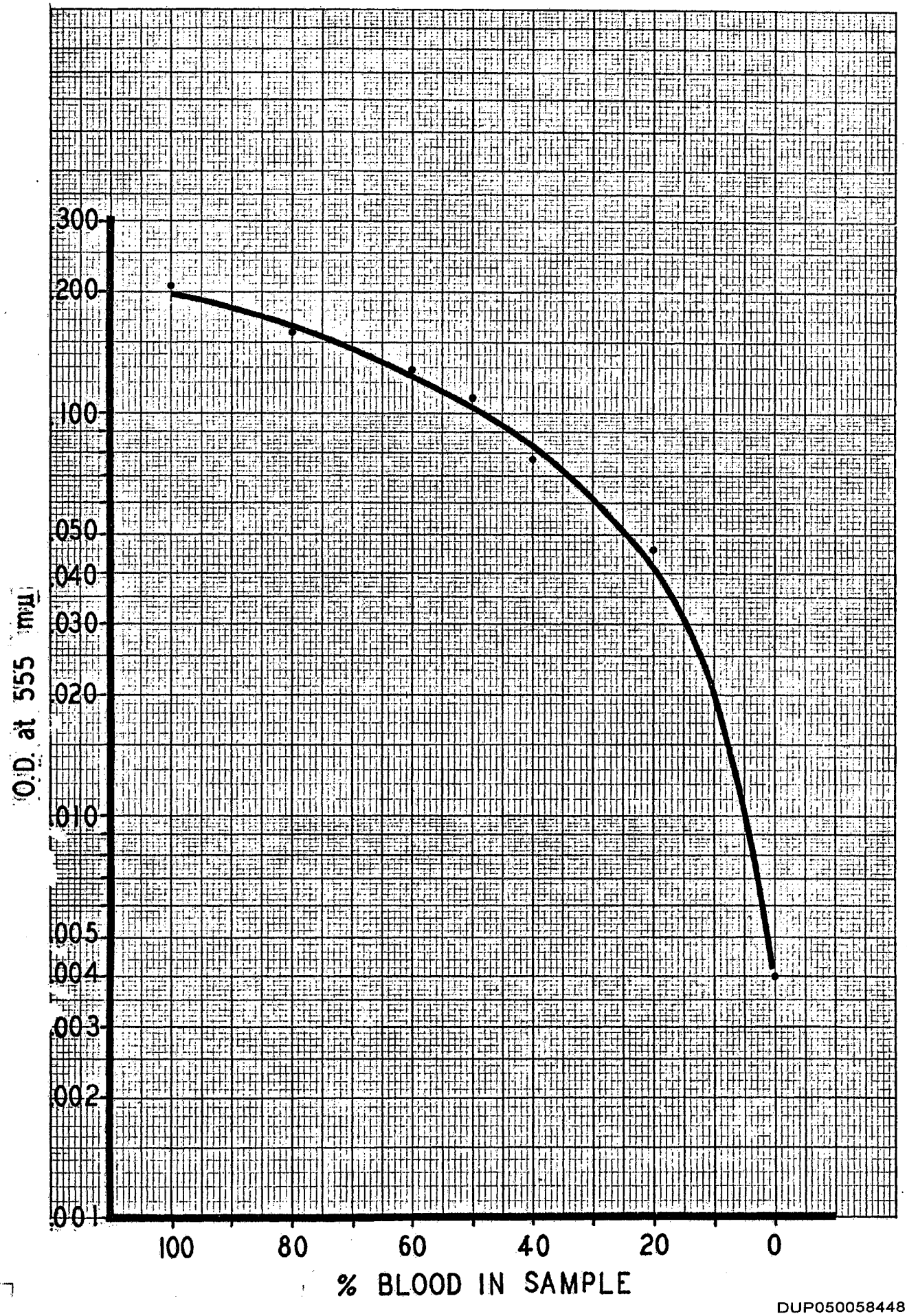


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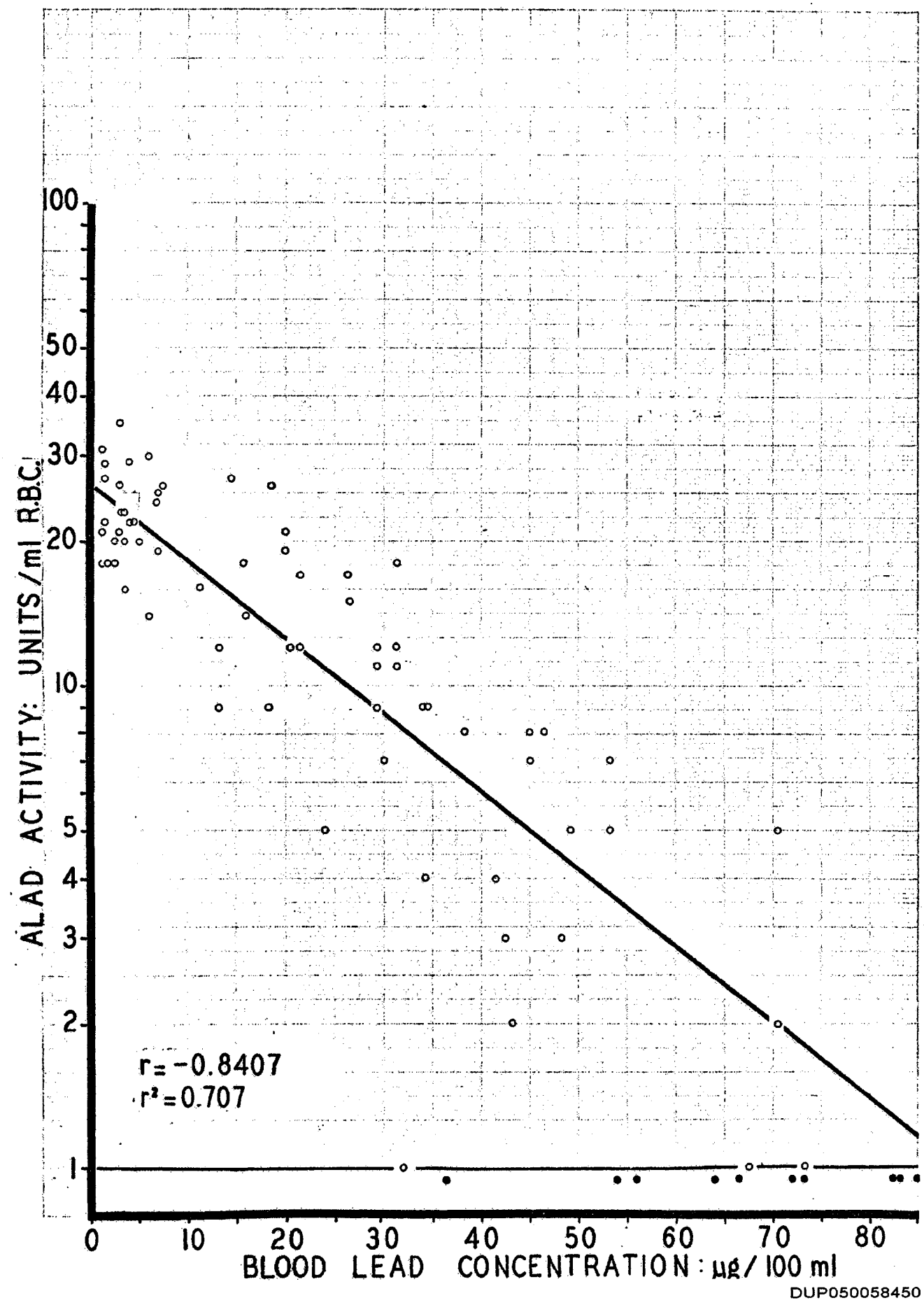
Figure 2

Relationship between log red cell ALAD activity and blood lead concentration, female dogs. Data obtained in previous study (11).

Calculated regression: $\log y = a - bx$ ▬▬▬

●: ALAD activity not measurable. These points, arbitrarily placed below the limit of sensitivity ————, were omitted in the calculation of the regression.



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Figures 3, A and B

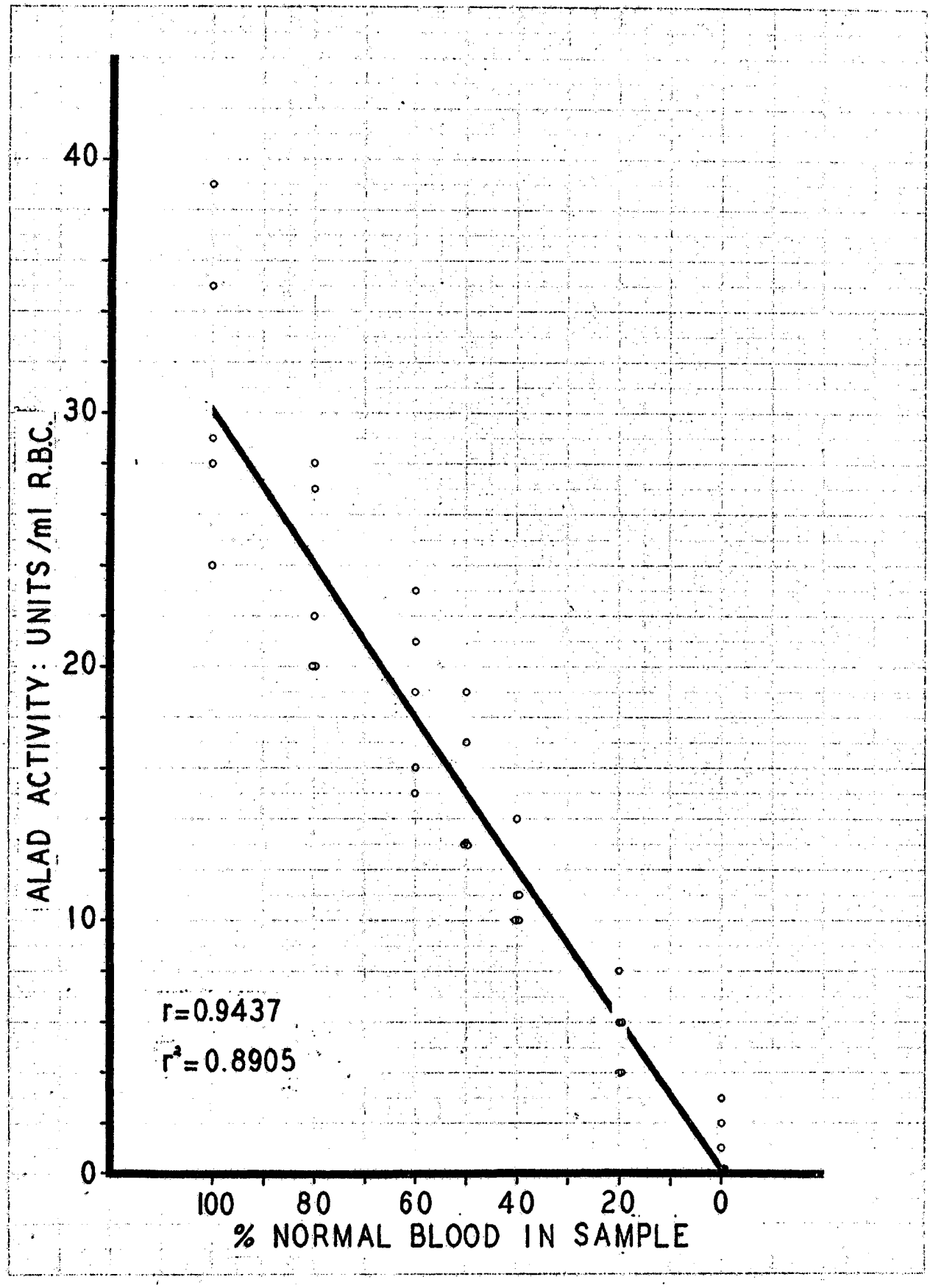
The effect of lead-containing blood on the red cell ALAD activity of normal blood.

A: Units of ALAD activity, or linear scale, versus percent of normal blood contained in sample. Calculated regression, $y = a + bx$ ——

●: ALAD activity not measurable. Not included in calculation of regression.

B. Log units ALAD activity versus percent of normal blood contained in samples. Curve fitted to data "by eye."

●: ALAD activity not measurable. Arbitrarily placed below limit of sensitivity ——



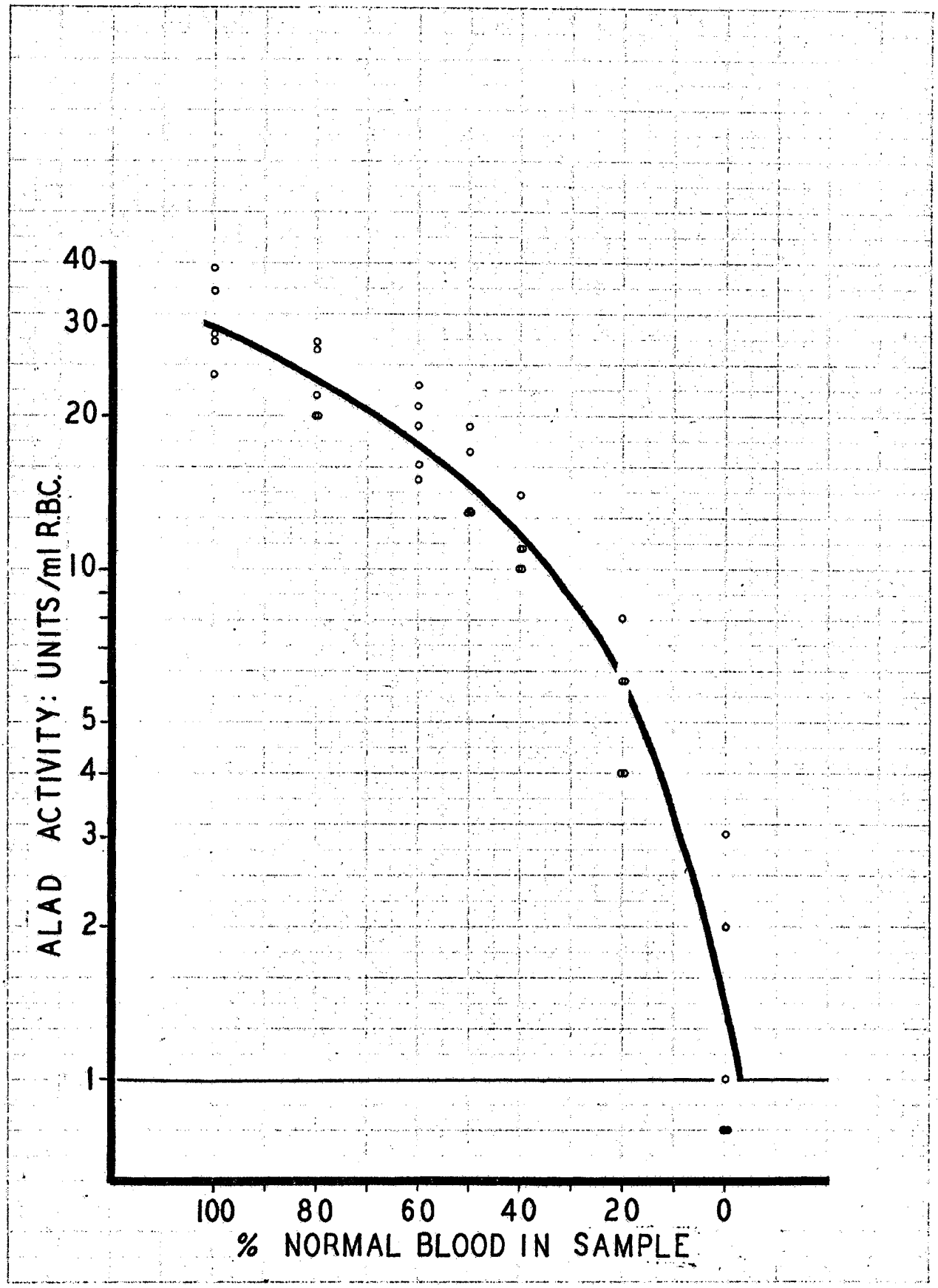


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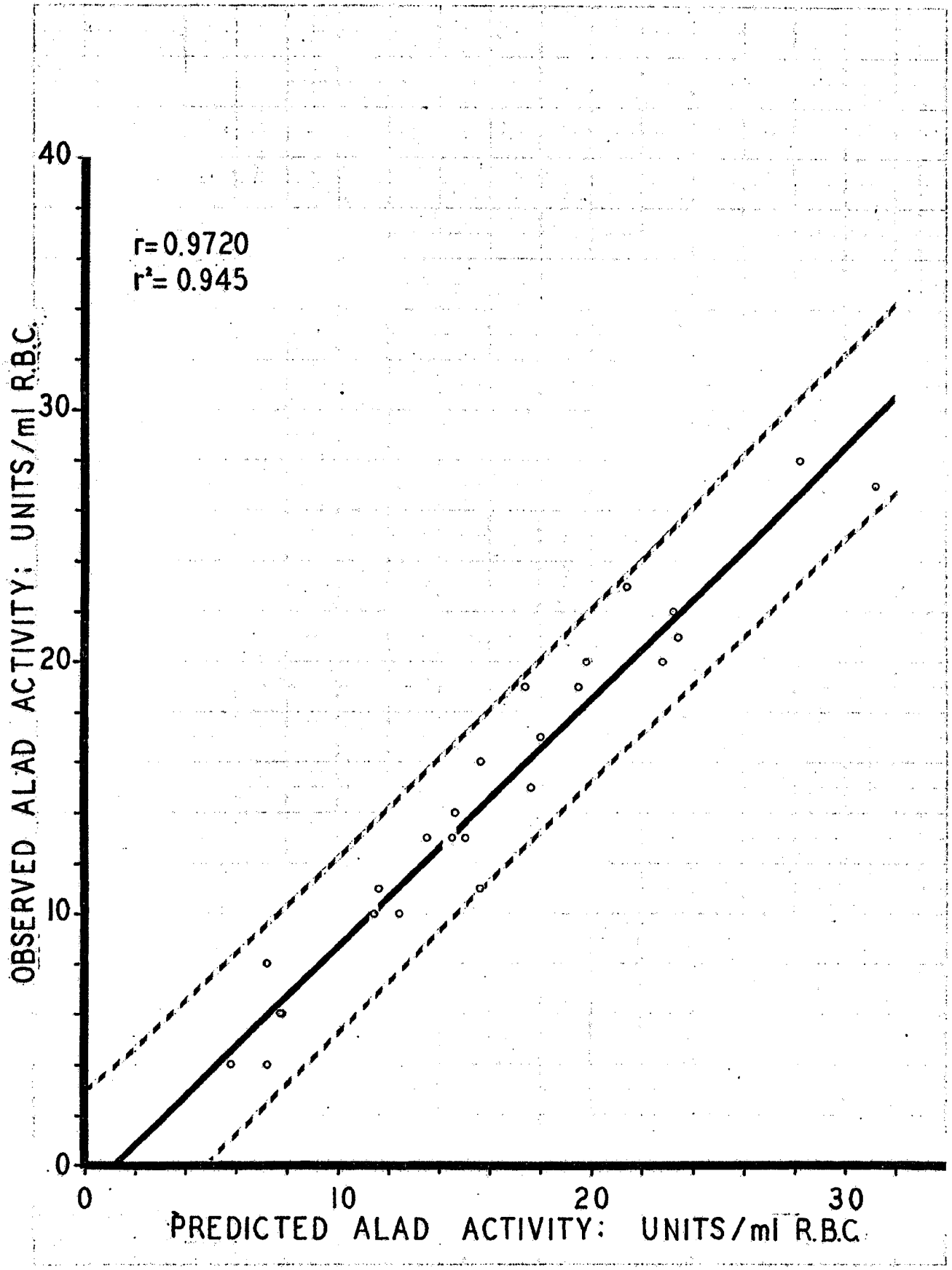
Figure 4

Observed ALAD activity of mixtures of normal and lead-containing bloods versus predicted values.

Calculated regression $y = a + bx$ ——————

$\pm$ 95% confidence limits - - - - - -



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Figures 5, A and B

Red cell ALAD activity of mixtures of normal and lead-containing bloods versus resultant lead concentration of the mixtures.

A. Units of ALAD activity, a linear scale, versus blood lead concentration.
Calculation regression ▬▬▬▬▬▬

●: ALAD activity not measurable. Arbitrarily positioned below sensitivity limit ————. Data not included in calculation of regression.

B. Log units of ALAD activity versus blood lead concentration.

●: ALAD activity not measurable. Arbitrarily positioned below sensitivity limit ————.
Curve fitted to data "by eye."