LEAD STUDIES.

III. THE EFFECTS OF LEAD ON RED BLOOD CELLS.

PART 1. CHANGES IN HEMOLYSIS.

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Changes in the blood are often the first and most striking signs of lead poisoning in man. It is therefore probable that lead acts specifically upon the blood system. By studying the mechanism of this action two things might be learned: (1) the physiological, physical, and chemical reactions of lead on isolated cells, which would probably indicate its action on other body cells, and (2) the mechanism of the production of at least one type of secondary anemia. Studies of these problems are reported in this series of papers.

Much work on the subject of lead anemia has already been reported. This sign of the disease was probably first recognized by Laennec (1) who described its clinical aspects in 1831. Since then a vast amount of literature has dealt chiefly with the pathological picture, or the stippling of red blood corpuscles. Very few studies of the mechanism of the reaction have been published.

That lead causes marked anemia in man has been repeatedly demonstrated. Exposure of experimental animals is also followed by the same changes, as has been shown, in the case of rabbits, after intramuscular injection by Carcanaque and Maurel (2) and after oral administration by Key (3).

This anemia has been attributed either to marked destruction of blood or to a lesion in the bone marrow and a consequent deficient blood formation. Evidence for both processes appears in the literature. Stockman and Charteris, and Raimondi (4) observed in the bone marrow of rabbits a marked preliminary increase of leucoblasts with a disappearance of fat, which was later followed by definite gelatinous degeneration. Wolff (5) studied a case of severe lead poisoning in which the bone marrow indicated attempts to regenerate blood followed by deficient hematopoietic function. Meillère (6), Schnitter (7), and Sellers (8) thought lead definitely injured both blood corpuscles and bone marrow. But

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even though a late degeneration of the bone marrow may occur in lead poisoning, the primary appearance of anemia is probably due to abnormal destruction of the cells in the circulating blood—a fact first suggested by Bouchard (9), and later mentioned by various other writers including Rauch (10). The evidence for this is diverse. In early lead intoxication there is a hyperplasia of bone marrow (4); and many nucleated red cells in the circulation indicate regeneration. Key (3) has shown that within 24 hours after the ingestion of 1 gm. of lead the number of circulating red blood cells in rabbits decreased more than 20 per cent, and that many nucleated red cells appeared. During the onset of lead anemia Meillère (6) was able to show that the urinary excretion of iron, which is largely derived from red corpuscles, definitely increased. Working in this laboratory, Dr. J. Brady (11) has demonstrated that slow intravenous injection of lead acetate dissolved in saline solution causes marked hematuria and very marked increase in the excretion of bile pigments from the common duct. These unpublished experiments, which were performed on rabbits, indicate definitely and very clearly that small quantities of lead in the blood stream cause great destruction of blood, which could not be accounted for by any osmotic changes. In human cases Dr. Chester Jones (11) has obtained similar evidence. He found that the bile obtained by duodenal tube from several of our cases of plumbism, as well as the blood plasma, contained very high concentrations of bile pigments. All of these facts indicate the occurrence of marked peripheral destruction of blood caused by the ingestion of lead. But no good explanation for this increased destruction of blood has been previously suggested, because there has been very little thorough experimentation upon the subject.

Several phenomena associated with the blood in lead poisoning have, however, been noted. Since 1899, when Behrend (12) first observed the stippling associated with lead poisoning a great many reports have appeared in the literature. These have recently been summarized by Key (3). It seems probable that stippled cells are really young red corpuscles which are degenerating as a result of exposure to lead. But the mechanism of their development is not yet understood, and is particularly difficult to outline because stippling cannot be produced in vitro (3, 13).

Another change in red blood cells in lead poisoning is their increased resistance to hemolysis in dilute saline solution. As long ago as 1873 Malassez (14) reported not only that anemia was a symptom of lead poisoning, but that the blood cells were larger and more fixed than normal. Agasse-Lafont and Heim (15) are said to have found slightly increased globular resistance in cases of plumbism. von Liebermann (16) also demonstrated this well by a simple test which has been confirmed by Orbán (17) and Hayhurst (18). Fici (19) who has recently studied this reaction in vitro, found that lead acetate exerts a striking effect on the hemolysis of red cells in hypotonic salt solutions—which varies with concentration of lead and with temperature. His observations have been confirmed in some of the experiments reported here which were designed to investigate the action of lead on red blood cells in vitro. An explanation for this phenomenon has not, however, been advanced.
As the first step in a study of hemolysis by lead an investigation of the action of its salts on red blood cells in vitro was made.

**Methods.**

Unless otherwise stated, the blood was gently defibrinated and the red blood cells were freed from plasma by washing three times in carbonate- and phosphate-free Ringer solution. Enough of this Ringer solution was then added to the cells to make the volume that of the original quantity of whole blood. This suspension was divided into fractions, one of which served as control. Lead or other metallic salts in Ringer solution were added to the other fractions, allowed to stand for 1 hour, and then washed off with an excess of Ringer solution. The control was treated similarly but with Ringer solution alone. Comparison of this control with the other fraction showed the results of the experiment. In every case the pH of all solutions was carefully kept between 6.4 and 6.8, and nearly always at 6.5. This range of variation produced no demonstrable changes in our results.

All manipulations were so standardized that they did not vary from day to day. In every experiment the control tests allowed direct comparisons—a method which gives more accurate results than would comparisons with an average normal.

The so called “fragility,” or hypotonic, tests of hemolysis were made in the usual manner. 0.1 cc. of washed blood cells was added to 1 cc. of hypotonic sodium chloride solution. The difference in the concentration of salt in adjacent tubes was 0.025 per cent. The results proved to be similar whether NaCl, Ringer, or blood serum dilutions were used. Therefore, simple sodium chloride solutions were employed because of ease in preparation; but it is certain that the absence of calcium or other ions plays no significant rôle in the results which were obtained after the tubes had stood for 15 hours in the cold room. The degree of hemolysis was determined from a set of standard tubes which contained known dilutions of the test blood and distilled water. The amount of hemolysis in a given saline concentration could thus be recorded as a percentage of the total. To check the readings in these tubes the amount of unhemolyzed cells was carefully observed. 100 per cent hemolysis is indicated by a clear solution without a sediment of intact red cells. This method is quite accurate in tubes where hemolysis is less than 60 per cent, but where it is greater than that an error of 10 per cent must be allowed.

The Ringer solution was made up as follows: NaCl, 0.9 per cent, KCl, 0.042 per cent, CaCl₂, 0.024 per cent. No carbonate or phosphate was added, to avoid the formation of their relatively insoluble lead salts. The pH was kept at 6.5 throughout in order to avoid the formation of lead hydroxide.

The concentration of these salts is expressed as the weight of the metallic constituents and is so very low—approximately 1 part per 100,000—that no demonstrable changes in osmotic tension can be ascribed to these variations. Where higher concentrations are used, the concentration of NaCl is proportionally reduced.
LEAD STUDIES. III

OBSERVATIONS.

When normal red blood cells were exposed to varying concentrations of lead as lead chloride for 1 hour, the variations in their resistance to these hypotonic saline solutions were very marked (Text-fig. 1). Cells exposed to a concentration of only 2 parts of lead by weight per million showed a definite increase in resistance, while those in a solution of 1 part per 100,000 showed so marked an

Text-fig. 1. Sodium chloride hemolysis. The effect of varying the quantity of lead. The curves show the effect on hemolysis of previous exposure for 1 hour of washed red blood cells to:

B, 1 cc. of red blood cells exposed to 0.002 mg. of Pb as chloride.
C, 1 cc. of red blood cells exposed to 0.004 mg. of Pb as chloride.
D, 1 cc. of red blood cells exposed to 0.01 mg. of Pb as chloride.
E, 1 cc. of red blood cells exposed to 0.03 mg. of Pb as chloride.
F, 1 cc. of red blood cells exposed to 0.08 mg. of Pb as chloride.

A shows the action of the control normal red blood cells in Ringer solution. This chart indicates the per cent of hemolysis in each concentration of salt solution.

effect that many of the cells were not even completely hemolyzed in 0.1 per cent saline solution, and some remained unhemolyzed in 0.05 per cent saline. Normal cells, on the other hand, averaged complete hemolysis in 0.25 per cent salt solution. It is striking that in three tubes, corresponding to those in which marked destruction of normal cells first appeared, there was no hemolysis of the least resistant
"leaded" corpuscles. When greater concentrations of lead are used (Text-fig. 1) two phenomena become evident. The resistance to hemolysis increases; but some cells are so injured that they hemolyze upon standing even in normal Ringer solution, although the controls do not. These facts are still more sharply demonstrated in tubes of hypotonic saline where a dark ring of hemolysis lies directly over the cellular sediment, and the supernatant fluid is relatively clear. This indicates that there is no prompt hemolysis on exposure to diminished osmotic tension, but that after they have settled these cells break up more quickly than normal cells. The action of lead is therefore double: (1) it increases cellular resistance to reduced osmotic tension, and (2) increases the rate of hemolysis of the cells. These effects have now been observed as constant findings in the blood of thirty-five different individuals, nearly all normal, whose blood belongs in the various isoagglutinating groups. In eight experiments, in which many different concentrations of lead were tried, all results were similar, and there was a definite quantitative relationship between the amount of lead used and the extent of its effect. Three of these eight bloods were obtained from patients suffering from plumbism. The maintenance of a similar quantitative relationship in all cases indicates that the chemical reaction is uninfluenced by immunity or susceptibility. Since the reaction is, therefore, quantitative, a fairly accurate prediction may be made as to the degree a given quantity of lead will influence like numbers of red cells. This is also true of cases where the reticulated count was high—as in a case of lead poisoning with 16 per cent reticulated cells. In the blood of two cases of hemolytic jaundice in which the reticulated cell count was as high as 26 per cent and 16 per cent, and in which hemolysis took place with abnormal ease, we observed that the difference between the curves for control and "leaded" cells was approximately that found in experiments with normal blood. It is therefore probable that this phenomenon appears constantly in human red cells washed free from plasma, and gives no indication of susceptibility to lead intoxication or lead anemia.

1. The Quantitative and Specific Resistance to Reduced Osmotic Tension.—The most striking results which were found in the first series of experiments showed that after exposure to 0.01 mg. of Pb per
cc. (1 part per 100,000) the power of red blood cells to resist lowered osmotic tension was definitely increased, and that they did not hemolyze within 18 hours. This concentration was therefore employed in all further experiments, even though it did not exercise a maximal effect on the resistance.

If the results of a series of ten such experiments are plotted in a distribution chart (Text-fig. 2) in which each point represents the increase in per cent of cells hemolyzed, it appears clearly that the cells are unevenly "leaded," or that the phenomenon involves two reactions, one of which permits hemolysis in concentrations above 0.225 per cent salt solution, while the other prevents hemolysis until the concentration of salt falls to 0.100 per cent.

**Text-Fig. 2.** The distribution of hemolysis. The average of ten experiments with cells exposed to 0.01 mg. of lead as lead chloride per cc. of washed red blood cells for 1 hour. Each point represents the per cent increase of hemolysis over that in the next tube of greater saline concentration.

- - - Normal cells. x---x "Leaded" cells.
In another series of twenty consecutive experiments an average of only 69 per cent of all the "leaded" cells hemolyzed at this saline dilution. The invariable movement toward the left of the curve representing hemolysis of "leaded" cells suggests that the normal cells which hemolyze most easily are those which are most susceptible to lead and are made more resistant by its action. Just which cells these may be—reticulated or mature, young or old—has not yet been definitely demonstrated (20); but the chemical reaction which probably causes the change in hemolysis will be discussed in the third paper of this series.

Attempts to reverse this reaction, and to "de-lead" the red cells proved unsuccessful. They consisted in soaking "leaded" cells in isotonic solutions of sodium acetate, sodium glycerophosphate, and sodium tartrate, and then determining their resistance to hypotonic saline solution. The results of this procedure, as well as those obtained from repeated washing of cells in Ringer solution, indicate that the lead reaction is irreversible with such substances as might dissolve a lead precipitate without destroying the blood cells.

The next problem investigated was the effect of various lead salts and other metals. From Table I it is clear that all the soluble lead salts studied affected red blood cells similarly, while the salts of other metals showed no comparable effect after exposures of 1 hour. As already demonstrated by Gunn (21), Hill (22), and Greenthal and O'Donnell (23), arsenic shows some effect, but even with such a relatively high concentration as 1.5 mg. per cc. this is very slight in comparison with that of lead. Mercury, likewise, affects the cells as Detre and Sellei (24) first demonstrated, but only at the high concentration of 1 per cent, when it "fixes" the cells by precipitation of their proteins. A different mechanism apparently causes hemolysis after exposure to mercuric chloride. It is therefore probable that the action of lead on the resistance of red blood cells to hypotonic salt solution is an isolated phenomenon which appears only with the various lead salts which were tested, at least under the conditions and in the concentrations of these experiments. It is not evidence of a
<table>
<thead>
<tr>
<th>Substance used</th>
<th>No. of experiments</th>
<th>Concentration of substance per cc. of red blood cells (mg.)</th>
<th>Start of hemolysis</th>
<th>Completion of hemolysis</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pb as acetate.</td>
<td>16</td>
<td>0.025</td>
<td>0.550</td>
<td>0.300</td>
<td>Slight effect between 0.300 and 0.550.</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>0.15</td>
<td>0.550</td>
<td>0.400</td>
<td>Marked effect.</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>0.25</td>
<td>0.550</td>
<td>0.300</td>
<td>“ “</td>
</tr>
<tr>
<td>Pb as nitrate.</td>
<td>1</td>
<td>0.08</td>
<td>0.500</td>
<td>0.200</td>
<td>pH 6.5. No effect except agglutination. With</td>
</tr>
<tr>
<td>Cerium as chloride.</td>
<td>1</td>
<td>0.2</td>
<td>0.400</td>
<td>0.250</td>
<td>stronger solutions only effect is increased</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.1</td>
<td>0.400</td>
<td>0.250</td>
<td>hemolysis of test cells, and agglutination.</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.0</td>
<td>0.250</td>
<td>0.250</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>As as chloride.</td>
<td>1</td>
<td>0.004</td>
<td>0.450</td>
<td>0.275</td>
<td>pH 3.9. Stronger concentrations caused agglu-</td>
</tr>
<tr>
<td>Ca &quot; &quot;</td>
<td>1</td>
<td>0.36</td>
<td>0.475</td>
<td>0.225</td>
<td>tination and hemolysis.</td>
</tr>
<tr>
<td>As as arsenious acid.</td>
<td>1</td>
<td>1.5</td>
<td>0.425</td>
<td>0.250</td>
<td>pH 6.6. Very slight protection down to 0.025.</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.15</td>
<td>0.450</td>
<td>0.250</td>
<td>Hemolysis in increased concentrations.</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3.00</td>
<td></td>
<td></td>
<td>No effect.</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.60</td>
<td>0.500</td>
<td>0.200</td>
<td>Very slight effect.</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.75</td>
<td>0.525</td>
<td>0.200</td>
<td>Moderate effect between 0.200 and 0.500.</td>
</tr>
<tr>
<td>As as sodium arsenite.</td>
<td>1</td>
<td>0.095</td>
<td>0.450</td>
<td>0.200</td>
<td>No effect.</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.08</td>
<td>0.450</td>
<td>0.200</td>
<td>Slight effect between 0.200 and 0.450.</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.09</td>
<td>0.20</td>
<td>0.450</td>
<td>0.450</td>
</tr>
<tr>
<td>------------------</td>
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</tr>
<tr>
<td>As sodium arsenate.</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.07</td>
<td>0.25</td>
<td>0.525</td>
<td>0.200</td>
</tr>
<tr>
<td>As Fowler's solution.</td>
<td>2</td>
<td>0.07</td>
<td>0.25</td>
<td>0.525</td>
<td>0.200</td>
</tr>
<tr>
<td>Cd as chloride.</td>
<td>2</td>
<td>0.4</td>
<td>0.25</td>
<td>0.525</td>
<td>0.200</td>
</tr>
<tr>
<td>Cu “ “</td>
<td>1</td>
<td>3.0</td>
<td>0.50</td>
<td>0.500</td>
<td>0.225</td>
</tr>
<tr>
<td>Hg as mercuric chloride.</td>
<td>1</td>
<td>0.02</td>
<td>0.45</td>
<td>0.450</td>
<td>0.200</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.001</td>
<td>0.45</td>
<td>0.450</td>
<td>0.200</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>14.8</td>
<td>0.45</td>
<td>0.450</td>
<td>0.900</td>
</tr>
<tr>
<td>Serum.</td>
<td>1</td>
<td>0.04 cc.</td>
<td>0.45</td>
<td>0.450</td>
<td>0.250</td>
</tr>
<tr>
<td>Formalin.</td>
<td>1</td>
<td>0.4%</td>
<td>0.45</td>
<td>0.450</td>
<td>0.200</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.04%</td>
<td>0.45</td>
<td>0.450</td>
<td>0.200</td>
</tr>
<tr>
<td>Tannic acid.</td>
<td>1</td>
<td>0.02%</td>
<td>0.50</td>
<td>0.500</td>
<td>0.225</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.002%</td>
<td>0.50</td>
<td>0.500</td>
<td>0.225</td>
</tr>
<tr>
<td>Sodium tannate.</td>
<td>1</td>
<td>0.008%</td>
<td>0.40</td>
<td>0.400</td>
<td>0.200</td>
</tr>
</tbody>
</table>

* Below 0.300.
† “ 0.100.
‡ Hemolysis in 0.900.
§ 20 per cent hemolysis in 0.900
|| 20 “ “ “ 0.100
general reaction with metals, or merely of a given valency or ionization, for if these were the governing influences, then other substances—particularly trivalent metals like aluminum—would cause a similar reaction.

2. Effect of Time, Temperature, and Diffusion.—If this reaction is chemical it is to be expected that the establishment of equilibrium would take time, and that a high temperature would increase its speed. This proved to be true. Text-fig. 3, which represents one of three similar experiments, shows that the reaction is very incomplete after 30 minutes but nearly complete after 1 hour exposure to lead at room temperature. Observations after exposures lasting 5 and 7 hours, which are not shown in the chart, demonstrate the tendency to rapid destruction and hemolysis even in high concentrations of salt. In the observations made after 9 hours exposure this reaction was slightly increased. This experiment is important, for it is added

Text-FIG. 3. Sodium chloride hemolysis. Effect of varying the time of exposure to lead. The curves show the effect on the hemolysis of washed red blood cells of previous exposure to:

- B, 1 cc. of red blood cells exposed for 15 min. to 0.01 mg. of Pb as chloride.
- C, 1 cc. of red blood cells exposed for 30 min. to 0.01 mg. of Pb as chloride.
- D, 1 cc. of red blood cells exposed for 1 hr. to 0.01 mg. of Pb as chloride.
- E, 1 cc. of red blood cells exposed for 3 hrs. to 0.01 mg. of Pb as chloride.
- F, 1 cc. of red blood cells exposed for 9 hrs. to 0.01 mg. of Pb as chloride.

A shows the action of the control normal cells in Ringer solution.
evidence for the short life of "leaded" cells. It demonstrates that
t heir resistance to decreased osmotic tension gradually increases, and
that later there is increased hemolysis of some cells even in higher
concentrations of salt. This rapid change in reaction does not occur
with the normal cells of the control.

Another experiment shows the relationship between concentration
of lead and time. Three concentrations were used (Table II) and
the rate of reaction was noted at intervals. Those cells exposed to
the highest concentration of lead (0.08 mg. of Pb per cc.) reached their
maximum "fixation" or greatest resistance in 25 minutes. All tubes
when tested at the end of 2 hours showed a definite increase of hemo-
lysis. Cells exposed to 0.04 mg. of lead reached maximum "fixation"
after 1 and 2 hours and at that time showed signs of hemolysis. Those

<table>
<thead>
<tr>
<th>Concentration of lead chloride per cc.</th>
<th>Per cent hemolysis after exposure for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>½ hr.</td>
</tr>
<tr>
<td>mg.</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>0.08</td>
<td>20</td>
</tr>
<tr>
<td>0.04</td>
<td>35</td>
</tr>
<tr>
<td>0.008</td>
<td>100</td>
</tr>
</tbody>
</table>

exposed to 0.008 mg. of Pb per cc. manifested progressively increasing
degrees of "fixation" up to 2 hours. When examined after 6 hours
exposure to lead they still showed maximal "fixation" without evi-
dence of general hemolysis. One experiment demonstrated the
occurrence of a slight acceleration of these phenomena when the cells
were kept at 30°C instead of at room temperature.

3. Evidences of this Reaction in Vivo.—The questions which at once
arise are whether this phenomenon can occur in the presence of blood
plasma, and whether it occurs in life. Two types of experiments
were performed to determine whether normal plasma neutralizes the
lead and prevents its action on red blood cells. In the first of these,
the solution of lead is mixed with serum before being added to the red
corpuscles; in the second, a solution of lead is mixed directly with
whole blood. When lead and serum are first united and allowed to stand at room temperature for 30 minutes, the serum neutralizes the lead completely. In a series of twenty-two experiments—in six of which the subjects had lead poisoning—the combining power of serum and lead was found to be surprisingly constant. Generally 0.022 to 0.035 cc. of blood serum counteracted the effect of 0.01 mg. of lead upon 1 cc. of red blood corpuscles; smaller quantities of serum eliminated only part of the lead. Text-fig. 4 illustrates a typical experiment and demonstrates that some constituent in the serum may so react with the lead salt that it no longer affects the red corpuscles.¹

Similar evidence for the chemical binding of lead is obtained by mixing red blood cells with the lead solution. After standing for 30 minutes this mixture is centrifuged and the supernatant fluid pipetted

¹ The mechanism of this reaction is discussed in Part 3 of this series.
off. The relative volumes are so arranged that the supernatant fluid should then contain 0.01 mg. of lead per cc. When other red cells are exposed to this, however, no evidence of the effect of lead can be found. This again shows that the lead has been bound by some constituent of the red cells. Hemolyzed red cells have a similar action. Two experiments showed that if a given volume of cells is laked in distilled water, to which double strength Ringer solution is later added to bring the final concentration of salt to 0.9 per cent, their power to neutralize lead is the same as that of an equal volume of intact cells. It is interesting to note that the red blood cells from a unit volume of blood have approximately the same power to combine with lead as has the serum from the same unit quantity.

When lead is added to defibrinated whole blood, however, the serum does not completely inhibit its reaction with the blood cells. In four such experiments it was clear that part of the lead united with the red blood cells, although it was necessary to add more than four times the usual amount of lead salt to cause an effect comparable to that in washed cells. Text-fig. 5 shows figures from a typical experiment of this series. The apparent interpretation depends upon the fact that the reaction with lead may occur simultaneously with serum and cells, and that both are therefore involved.

Diffusion of substances out of the red cells is also important in the reaction with lead. This was tested by allowing washed red cells to lie in Ringer solution for varying lengths of time, and then centrifuging them once before exposure to lead. In five out of six experiments the usual solution of lead (0.01 mg. per cc.) produced no alteration in the normal resistance to hypotonic solutions of cells which had been in Ringer solution for 6 hours. More concentrated solutions (0.1 mg. of Pb per cc.) however, still increased the cellular resistance. After the saline solution surrounding these cells was thoroughly removed by repeated washing and centrifuging, the red cells reacted as before with the usual low concentrations of lead. It is, therefore, evident that something which usually reacts with the lead has diffused from the cells into the surrounding media, and has there reacted with the lead. These experiments are more fully outlined in the third paper of this series (page 194). They are reported here merely to indicate further evidence of a chemical explanation of the action of lead on the blood.
If this reaction between lead and red corpuscles has any great significance its occurrence in life must, of course, be demonstrable. That it is, has already been shown in man by von Liebermann (16), by Orbán (17), and by Hayhurst (18), who found that corpuscular resistance increased in acute lead poisoning. However, the resistance of corpuscles was further tested in this laboratory in six rabbits before and after the ingestion of 1 gm. of lead acetate. Five of these animals showed similar reactions to the lead, the sixth showed no change either in resistance of the red cells, in anemia, or in stippling. Text-

![Text-Fig. 5. Sodium chloride hemolysis. Effect of lead added to whole blood. A, control of red blood cells washed in Ringer solution. B, 1 cc. of washed red blood cells exposed to 0.01 mg. of Pb as chloride. C, 1 " " whole blood exposed to 0.04 mg. of Pb as chloride. D, 1 " " " " " " 0.01 " " " " " " ]

fig. 6 shows the characteristic, though most marked effect obtained. This rabbit had suffered from a secondary anemia due to repeated bleeding prior to the ingestion of lead (see Key (3)), and it died with a marked anemia 2 days after lead was given. The chart demonstrates that the corpuscles are affected just as in vitro, that many resist the strain of hypotonic saline better than the control cells, but that many hemolyze quickly even in normal saline solution. These experiments justify the assumption that the phenomena studied here in vitro depict reactions similar to those which may occur in life. They
are most probably related to development of the anemia of lead poisoning. This is further indicated by the fact that the effect of lead on hemolysis in hypotonic solutions seems to run parallel to the appearance of stippling and anemia which occurs in different species of animals during life.

These phenomena are not found in all species, as can be seen in Table III, but in the species studied all or none have been observed. The results of the in vitro experiment, therefore, run parallel to those found after lead has been allowed to act in vivo. Thus, stippling and anemia are easily produced in man, rabbits, guinea pigs, and rats; and diminished hemolysis in hypotonic salt solution is also marked.

![Text-Fig. 6. Sodium chloride hemolysis. The effect of acute lead poisoning on red blood cells in vivo. The curves represent the action of red blood cells washed in Ringer solution. A, before lead was given. B, 19 hours after 1 gm. of lead acetate was given by mouth. C, 43 " " 1 " " " " " " "

The red cells of horses, dogs, and cats do not hemolyze after exposure to these dilute solutions of lead; and, although for some species the data here presented are incomplete, it is clear that the cat shows no symptoms of anemia until the end of the disease, and that no definite stippling has been observed in its blood. The causes for this variation among different species are not yet clear.

Occasional granulation of the red cells was seen, but this was indefinite in outline, did not stain darkly, and was rare. This is similar to the experience of Nehring (25).
LEAD STUDIES. III

In two experiments tests were made to determine whether lead reacts with cat blood even though there is no change in resistance to hypotonic salt solutions. These tests were made by adding 0.1 mg. of lead in 1 cc. of Ringer solution to 5 cc. of thoroughly washed red cells from cat blood. After standing for an hour this mixture was centrifuged and the supernatant fluid pipetted off. If no reaction had taken place the supernatant fluid should react with washed red cells from man, evidence for the reaction being found by testing with hypotonic salt solutions. 1.5 cc. of the supernatant fluid (i.e. 0.025 mg. of lead), were, therefore, added to 2.5 cc. of washed human red corpuscles. No change in hemolysis in hypotonic salt solution resulted. Chemical reaction had, therefore, taken place with the cat corpuscles, but without affecting their resistance to hypotonic

### TABLE III.
The Parallelism between Blood Manifestations in Various Animal Species.
The Effect of Lead in Vivo on the Appearance of Anemia and Stippling in Blood, and in Vitro, on the Hemolysis in Hypotonic Salt Solution.

<table>
<thead>
<tr>
<th>Species</th>
<th>Distortion of hypotonic saline curve to left following exposure to lead.*</th>
<th>Appearance of anemia in &quot;leaded&quot; animals,†</th>
<th>Appearance of stippling in &quot;leaded&quot; animals,†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man</td>
<td>+ +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rabbit</td>
<td>+ +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rat</td>
<td>+ + +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>+</td>
<td>+‡</td>
<td>+‡</td>
</tr>
<tr>
<td>Dog</td>
<td>Negative.</td>
<td>Negative until just before death.</td>
<td>No clear cut stippling. Rare mottling of cells.</td>
</tr>
<tr>
<td>Cat</td>
<td>Negative.</td>
<td>Negative until just before death.</td>
<td>No clear cut stippling. Rare mottling of cells.</td>
</tr>
<tr>
<td>Horse</td>
<td>“</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Chicken</td>
<td>“</td>
<td>?</td>
<td>Negative.†</td>
</tr>
</tbody>
</table>

* All exposed to same concentration, i.e. 0.01 mg. of Pb per cc. of washed red blood cells. Plus means that the curve of "leaded" cells indicated distinctly increased resistance to hemolysis in dilute saline solutions.
† Concentrations of Pb per kg. of animal not constant.
‡ Data from Sabrazés, Bourret, and Léger (26), and from Beresina and Engling (26).
§ Stated by White and Pepper (28) to be positive, though not typical. This observation has not been repeated.
∥ Data from Key (3) and also from Meyer and Speroni (27).
salt solutions. The results of in vitro experiments, therefore, are parallel to those obtained with lead in vivo.

4. The Effect of Lead on the Length of Life of the Red Cells.—In Text-figs. 1 and 4 two phenomena of red cells can be seen (1) the increase in their resistance to lowered osmotic tension, and (2) the increased rate of destruction of blood even in normal salt solution. This latter phenomenon is striking when red corpuscles are exposed to higher concentrations of lead. But in the following experiments such a concentration was used (0.01 mg. of Pb per cc.) that there was no more hemolysis after standing for several hours in normal saline, than was found in control cells. After being treated with lead the corpuscles were put in one tonometer, and normal washed red blood cells in another. The controls were treated in every way exactly like “leaded” cells, except for exposure to normal salt solution instead of to normal salt solution plus lead. All manipulations were carried out simultaneously in the same apparatus. Neither control nor “leaded” cells hemolyzed markedly before they were put in the tonometers. Both groups of cells should, theoretically, resist rotation equally well, but when the suspensions were centrifuged, after being slowly rotated for 5 minutes, hemolysis, due to the rolling of the corpuscles on glass, was markedly increased in the lead suspensions and absent in the controls (nine experiments). Shaking the tonometers in a shaking machine gave similar results (one experiment).

In another group of experiments we exposed cells intermittently to atmospheres rich in CO₂, or to alveolar air, as well as to room air, because it was thought that perhaps the gaseous exchange, by changing the volume of the corpuscles (29), would cause greater destruction of “leaded” cells. The technique used was as follows:

Washed red blood cells were put into four tonometers, two containing “leaded” cells, and two, normal washed cells. The cell counts of the blood in all tubes in each experiment were similar. Air was slowly blown through the control tubes while an equal amount of gas rich in CO₂ was passed through the others. Then, at a constant temperature of 37° or 25° all four were slowly rotated in the same machine for a short while. After blowing air through all four tonometers for a given period, rotation was again begun. This whole procedure was repeated three times. Since the cell counts in all tubes agreed closely, any differences in degree of final hemolysis must have been due to the action of lead on the cells or to the gas mixtures, which were the only variables in the experiments. All manip-
ulations during centrifugalization and rotation were performed very carefully to prevent all possible trauma.

After the rotation necessary to establish equilibrium, all the "leaded" solutions showed greater hemolysis than the controls. In seven of the nine experiments the exposure to high concentrations of CO₂ did not cause a greater percentage increase of hemolysis in the tubes containing lead than in the saline controls. Perhaps more frequent exposure to CO₂ might cause greater differentiation, but no experiments to test this were performed because of the marked hemolysis in "leaded" cells following slight rotation alone. Hemolysis of both normal and "leaded" cells was always more complete after alternate exposure to CO₂ and room air, than after exposure to room air only.

In the last three experiments the per cent of hemolysis of the different samples of blood was:

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Type of cells</th>
<th>Amount of lead per cc. of defibrinated blood</th>
<th>Analysis of CO₂-rich gas mixture</th>
<th>Duration of total rotation</th>
<th>Before rotation or exposure to CO₂</th>
<th>After rotation and exposure to CO₂ and room air</th>
<th>After rotation in atmospheres of CO₂ and room air</th>
</tr>
</thead>
<tbody>
<tr>
<td>162</td>
<td>Normal</td>
<td>0.2 mg.</td>
<td>2% O₂</td>
<td>15 min.</td>
<td>0.5</td>
<td>2.0</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>&quot;Leaded.&quot;</td>
<td></td>
<td>8.5% CO₂</td>
<td>15</td>
<td>5.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>190</td>
<td>Normal</td>
<td>0.2 mg.</td>
<td>Pure CO₂</td>
<td>9</td>
<td>0.25</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>&quot;Leaded.&quot;</td>
<td></td>
<td></td>
<td>9</td>
<td>10.00</td>
<td>12.0</td>
<td>16.0</td>
</tr>
<tr>
<td>191</td>
<td>Normal</td>
<td>0.05 mg.</td>
<td>Pure CO₂</td>
<td>6</td>
<td>0.0</td>
<td>0.0</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>&quot;Leaded.&quot;</td>
<td></td>
<td></td>
<td>6</td>
<td>0.5</td>
<td>1.7</td>
<td>2.5</td>
</tr>
</tbody>
</table>

These figures were checked in Experiment 162 by determination of the oxygen-combining power of the supernatant fluid. This method gave approximately the same results as did the colorimetric test.

Such experiments indicate that slight trauma destroys "leaded" cells far more readily than normal cells, but give no good evidence that the change in volume which occurs in each cycle of the circulation, causes a greater destruction of "leaded" red corpuscles than of normal
J. C. AUB, P. REZNIKOFF, AND D. E. SMITH

cells. Rous and Robertson (30) and recently Broun (31) have attributed destruction of the blood to mechanical trauma; and these experiments provide an illustration of a state in which cells injured by lead would be very much more fragile than normal and, therefore, far more susceptible to the traumata involved in circulation. This is probably a very important factor in the increased destruction of blood in plumbism.

The Effect of Lead on Other Forms of Hemolysis.

The effect of lead upon other types of hemolysis was also tried. Five experiments were carried out with saponin hemolysis. Three of these showed a similar though far less marked effect than that seen in the hypotonic salt test. The “leaded” cells hemolyzed less readily than did the control cells. The other two experiments were essentially negative. These results are not striking enough to be much emphasized, and give but little new indication of the reactions involved in the phenomenon. Even though saponin probably acts by dissolving the lipoids of the cells, these experiments do not necessarily indicate that the action of lead is upon these lipoids, as any other effect upon the cell membrane might well distort the effect of saponin.

The effect of anti-human serum was also tried on “leaded” cells. Three experiments were done with the serum of a rabbit which had been immunized against human blood. The amboceptor was diluted 1 to 15, 25, and 40 in the different experiments. In the three experiments the “leaded” cells showed more rapid and complete hemolysis than did the control cells. The significance of this is not yet clear, but the data may be important in the explanation of mechanisms of various reactions which occur in hemolysis.

CONCLUSIONS.

It appears, from the investigations in other laboratories, that the anemia observed in cases of lead poisoning is due to destruction of blood rather than to diminished production of blood. The method of poisoning cells in vitro with lead was adopted in order to study this

7 These experiments were made possible by the cooperation of Dr. William A. Hinton. We take this opportunity to thank him for furnishing the immune serum and for his help in the experiments.
phenomenon, and distinct effects were thereby obtained, even when only 0.001 mg. of lead is added to approximately 5 billion washed red corpuscles. In order to obtain optimum results the usual dosage employed was ten times this or 0.01 mg. per 5 billion cells. The following changes were observed in cells so treated.

1. Such a marked increase in the resistance to hypotonic salt solution develops that complete hemolysis does not occur until the cells are exposed to a saline solution of 0.05 per cent. Untreated cells are completely hemolyzed in 0.25 or 0.225 per cent saline.

2. This reaction is quantitative and varies with the concentration of lead used. Under the conditions of our experiments this phenomenon seems to be unique. The effects of arsenic are very slight in comparison.

3. While from this reaction it may be concluded that lead increases cellular resistance, it also appears that it shortens the life of blood cells. This may be demonstrated by the much more rapid appearance of hemolysis than normal when the cells are merely allowed to stand in Ringer solution of any dilution.

4. In rabbits with acute lead poisoning these same phenomena may be noted in vivo.

5. Both phenomena may be changed in vitro by varying the time and temperature of the reaction and the concentration of lead, as Fici has already pointed out.

6. If normal cells stand in Ringer solution for 6 hours something diffuses into the solution which largely reduces the action of lead. After repeated washing these cells react with lead in the usual manner.

7. Small amounts of serum react with lead and eliminate its effects. Red blood cells, treated with a mixture of lead and blood serum, show normal hemolysis in hypotonic salt solution.

8. When lead is added to whole blood the only evidence of this neutralization is a marked decrease in the intensity of the reaction. This is probably explained by the fact that since both serum and cells are present together, the lead reacts with both simultaneously. This in turn explains how the reaction may occur in vivo.

9. The change in hemolysis does not appear in the blood of all species of animals. In the species in which it does occur, anemia and stippling of the red cells also tend to develop readily during lead intoxication.
10. **In vitro**, cells which have been exposed to lead are far more fragile than normal blood cells. Slight trauma causes them to hemolyze. **In vivo** this is probably an important causal factor in the increased destruction of blood and in the anemia of acute lead poisoning.

11. The serum of a rabbit which has been immunized to human blood, causes more rapid and complete hemolysis in cells treated with lead than in the control cells. No satisfactory explanation for this has been found.

**BIBLIOGRAPHY.**