LEAD STUDIES.

III. The Effects of Lead on Red Blood Cells.

Part 3. A Chemical Explanation of the Reaction of Lead with Red Blood Cells.

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The first two papers of this series (1,2) have dealt with the effect of lead upon red blood cells and have suggested an interaction of the lead with the cell surface as the mechanism underlying the phenomena observed. All the changes probably have their origin in a chemical reaction and it is the purpose of this paper to report experiments designed to study this reaction.

An experiment described in Part 1 suggested a method of investigating this problem. It was found that lead, mixed with small amounts of blood serum, could no longer cause the usual change of resistance of red blood cells to hypotonic saline solution. In other words, some substance in the serum reacted with the lead to prevent further combination with red blood cells subsequently added. In order to ascertain which constituents were involved in this reaction, it was essential to determine the ability of each to combine with and to neutralize lead.

As far as is known at present, red blood cells are composed of inorganic salts, lecithin, cholesterol, albumin, euglobulin, pseudoglobulin, and hemoglobin (3). Each of these substances, in pure form and in amounts equal to its concentration in blood, was mixed with lead. After allowing time for a reaction to occur, red blood cells were added to this mixture and the usual hypotonic saline tests were carried out. If a normal curve was obtained, the sub-

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stance obviously had combined with the lead in the preliminary treatment, and thus prevented the latter from acting on the red cells. On the other hand, if the curve was that characteristic of "leaded" cells, the substance in question had had no effect on the lead. This test, therefore, provides a biological indicator of a chemical reaction involving amounts of lead as small as 0.01 mg. per cc. of red blood cells (1). In this work it was necessary, of course, to simulate as closely as experimental conditions permitted, conditions occurring normally in whole blood. The solutions of blood constituents were prepared with this in view, especially with respect to hydrogen ion concentration and osmotic pressure.

The Effect of the Inorganic Salts of Blood on Lead.

The first substances to be tested were the inorganic constituents of the red blood cells. Of these, the sulfates, carbonates, and phosphates are the most likely to unite with lead. Landolt, Börnstein and Roth (4) quote Böttger's solubility findings as follows:

Lead sulfate	42.5	mg. per liter.
Lead carbonate	1.43	" " "
Lead phosphate	0.13	" " "

From these figures it can be seen that the sulfate is relatively soluble and would not unite with lead in the presence of the more insoluble carbonate and phosphate. On the basis of solubility, moreover, small amounts of lead added to blood would be converted completely to phosphate, provided the reaction between lead and red blood cells were chemical in nature.

Carbonates.—In determining the effect of carbonates on lead, equal parts of double Ringer[1] and sodium carbonate solutions were mixed so that the final concentration was $\frac{M}{40}$ CO_2 which is approximately that of normal serum (5). The pH of this mixture was 6.9. Lead, exposed to this mixture for $\frac{1}{2}$ hour in amounts equivalent, respectively, to 0.02 and 0.2 cc. of serum, was added to washed red blood cells (0.01 mg. of lead per cc. of cells). After exposure for 1 hour, hypotonic saline tests were performed.

[1] By double Ringer solution is meant one with twice the concentration of salts, so that, when diluted with an equal volume of water, it is isotonic with blood.

In a previous paper (1) it was shown that 0.02 cc. of normal serum almost completely neutralizes the effect of 0.01 mg. of lead upon 1 cc. of corpuscles. In the present experiments an amount of carbonate, equivalent to that in 0.2 cc. of serum, only partially neutralized the lead, while that present in 0.02 cc. of serum had almost no effect (Table I). Therefore, union of lead with the carbonates of the corpuscles probably does not explain the "leading" of red blood cells.

Phosphates.—The presence of inorganic phosphates in red blood cells has been questioned recently (6), but it is generally accepted that inorganic phosphate is present in normal whole blood, in a

TABLE I.

Neutralization of the Effect of Lead on Red Blood Cells by Constituents of Blood.

Substance tested.	No. of cc. of solution, equivalent to blood in content of substance tested, necessary to show minimal neutralization of 0.01 mg. of Pb per cc. of red blood cells.	No. of cc. of solution, equivalent to blood in content of substance tested, necessary to show maximum neutralization of 0.01 mg. of Pb per cc. of red blood cells.
Carbonates.	0.02	0.2
Phosphates.	0.004	0.02
Sodium glycerophosphate.	No neutralization.	No neutralization.
Lecithin.	" "	" "
Cholesterol.	" "	" "
Albumin.	" "	" "
Euglobulin.	" "	" "
Pseudoglobulin.	" "	" "
Hemoglobin	" "	" "

concentration of approximately 3.0 mg. of phosphorus per 100 cc. of blood, and appears to be equally distributed between corpuscles and plasma (7).

To obtain a solution with an inorganic phosphorus content equal to that of blood, 0.0232 gm. of NaH_2PO_4 was dissolved in 100 cc. of water and diluted with an equal volume of double Ringer solution. The pH of this solution was then brought to 6.5 with $\frac{N}{10}$ sodium hydroxide. Table I shows that after 0.01 mg. of lead is treated with a quantity of this mixture equivalent to 0.02 cc. of serum, it has no effect on red blood cells added subsequently (1 cc. of cells per 0.01 mg. of Pb). This indicates that the lead was rendered

completely inactive by the preliminary treatment with inorganic phosphate. In these concentrations, therefore, the effect of lead on red blood cells can be entirely explained by the lead-phosphate reaction.

In vivo a preliminary reaction between lead and inorganic phosphate in the absence of red blood cells does not occur (1). Therefore the next point to be considered was the neutralizing effect of phosphate when added to corpuscles or whole blood before exposure to lead. Preliminary tests showed that much more phosphate is required in these cases to affect the action of lead. This, of course, is to be expected because the lead reacts simultaneoulsy with red blood cells and inorganic phosphate—conditions similar to those *in vivo* (see Part 1).

In one series of experiments varying amounts of phosphate were added to washed red blood cells, and after standing for ½ hour the mixtures were treated with 0.01 mg. of lead per cc. of corpuscles. Neutralization of the lead was first observed with an amount of phosphate equal to that in 0.02 cc. of serum, and was found to be almost complete with a quantity of phosphate equal to 0.12 cc. of serum (Table II). It is very significant that concentrations of phosphate greater than that contained in 0.12 cc. serum have no additional neutralizing effect. This shows that some red blood cells are affected by lead no matter what the neutralizing value of the serum may be, provided corpuscles, lead, and serum are all present at the same time. Such a condition, of course, occurs *in vivo* and accounts for the inability of serum to overcome completely the action of lead on the cells.

A similar experiment was carried out with defibrinated whole blood. Varying quantities of phosphate were added to whole blood and after ½ hour the mixture was treated with 0.04 mg. of lead per cc. of blood. It has been shown (1) that 0.04 mg. of lead has nearly the same effect on 1 cc. of whole blood as 0.01 mg. of lead on 1 cc. of washed corpuscles. Phosphate, when added to whole blood in a quantity equivalent to that found in 0.48 cc. of serum, failed to neutralize the lead completely (Table II). Therefore, a mixture of phosphate and whole blood can neutralize less lead than a mixture of the same amount of phosphate and washed

cells from an equal quantity of blood. This fact suggests the possibility that phosphate in Ringer solution is more available to react with lead than is phosphate added to serum. Here again, the failure of excess phosphate to neutralize completely the lead, which acts simultaneously on the corpuscles, illustrates the condition *in vivo*.

There is further evidence that lead reacts with inorganic phosphate. About 0.02 cc. of normal serum can unite with 0.01 mg. of lead to neutralize completely the effect of the lead on 1 cc. of red blood

TABLE II.

Relation of Inorganic Phosphate in Blood to Neutralization of the Effect of Lead on Washed Red Blood Cells and on Whole Blood.

Experiment.	No. of cc. of phosphate solution, equivalent to serum in inorganic phosphorus content, necessary to show minimal neutralization of effect of 0.01 mg. of Pb per cc. of red blood cells.	No. of cc. of phosphate solution, equivalent to serum in inorganic phosphorus content, necessary to show maximum neutralization of effect of 0.01 mg. of Pb per cc. of red blood cells.
1. Phosphate added to lead, before adding red blood cells.	0.002	0.02 (complete neutralization).
2. Phosphate added to red blood cells, before adding lead.	0.02	0.12 (neutralization incomplete).
3. Phosphate added to whole blood before adding lead.	0.04	0.15–0.48 (neutralization less complete than in (2)).

0.01 mg. of lead per cc. of red blood cells and 0.04 mg. of lead per cc. of whole blood are equivalent in effect.

cells (1). Serum with an abnormally high inorganic phosphate content should have a greater neutralizing value. Among several specimens tested, a few were obtained which illustrate this point. In these experiments the serum was diluted with Ringer solution so that small amounts could be added conveniently. The diluted serum was mixed with lead and, after $\frac{1}{2}$ hour, normal red blood cells were added to determine the degree to which the serum neutralized the lead. Three cases are reported here.

The first (J. B. C.) was one of lead poisoning complicated by rather severe nephritis. The inorganic phosphate content was increased to 6.7 mg. of phos-

phorus per 100 cc. of whole blood. His serum neutralized lead between two and three times as effectively as normal serum (Table III).

The second specimen was taken from a patient (O. M. B.) suffering from uremia. The whole blood contained 15 mg. of inorganic phosphorus, 165 mg. of non-protein nitrogen, and 8.01 mg. of creatinine per 100 cc. This serum was four times as effective as normal in its neutralization of lead (Table III).

A third case (S.S.) was one of lead poisoning uncomplicated by nephritis. The blood of this patient contained 4.4 mg. of inorganic phosphorus and 35.7 mg. of non-protein nitrogen per 100 cc. The result shows that 0.012 cc. of this plasma was almost as effective as 0.02 cc. of normal serum. Although in this case the phosphate content was only slightly higher than normal, the neutralizing effect is increased (Table III).

TABLE III.

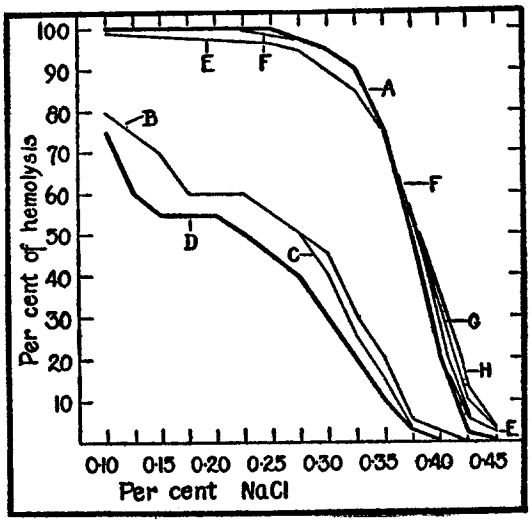
Relation of Inorganic Phosphorus Content of Blood to Amount of Serum Necessary to Neutralize the Effect of 0.01 Mg. of Lead on 1 Cc. of Red Blood Cells.

Serum.	Mg. of inorganic phosphorus per 100 cc. of whole blood.	No. of cc. of serum necessary to obtain minimal neutralization of 0.01 mg. of lead per cc. of red blood cells.	No. of cc. of serum necessary to obtain practically complete neutralization of 0.01 mg. of lead per cc. of red blood cells.
1. L. F. (control).	3.0	0.002	0.02
2. J. B. C.	6.7	0.0006	0.012
3. O. M. B.	15.0	0.0004	0.005
4. S. S.	4.4	0.001	0.012

Other observations point to the importance of phosphate in the union of lead and corpuscles. Experiments were performed in which washed red blood cells were permitted to stand at 30° C. for varying intervals. Without being washed free from the Ringer solution in which they stood, the corpuscles were then treated with lead (0.01 mg. per cc. of cells). Text-fig. 1 illustrates one of seven such experiments and shows that, while the action of lead is at first increased, after 2 hours, small concentrations of lead are no longer effective. The same result was obtained in three experiments performed at room temperature, but it was necessary to allow the cells to stand for longer periods before adding lead in order to prevent the usual effect of the latter.

This change might be due to diffusion of substances from the red blood cells into the Ringer solution. To determine this, washed corpuscles were centrifuged after standing for various lengths of

time and the supernatant fluid was tested for phosphate, carbonate, and protein. The Ringer solution used for washing contained only sodium, potassium, and calcium chlorides; therefore, any other



TEXT-FIG. 1. The effect of lead on red blood cells which have stood at 30°C. for varying intervals before the addition of lead.

A, red blood cells.

B, red blood cells at 30°C. in Ringer solution for 15 min.; then, 0.01 mg. of Pb per cc.

C, red blood cells at 30°C. in Ringer solution for 30 min.; then, 0.01 mg. of Pb per cc.

D, red blood cells at 30°C. in Ringer solution for 1 hr.; then, 0.01 mg. of Pb per cc.

E, red blood cells at 30°C. in Ringer solution for 2 hrs.; then, 0.01 mg. of Pb per cc.

F, red blood cells at 30°C. in Ringer solution for 3 hrs.; then, 0.01 mg. of Pb per cc.

G, red blood cells at 30°C. in Ringer solution for 4 hrs.; then, 0.01 mg. of Pb per cc.

H, red blood cells at 30°C. in Ringer solution for 5 hrs.; then, 0.01 mg. of Pb per cc.

substances present after washing must have diffused from the corpuscles, as there was no evidence of hemolysis. To test for phosphate, molybdic acid was used; for carbonates, 1:1 hydrochloric acid;

and for proteins, both the heat and acetic acid test and Millon's reagent. Table IV shows the results. The failure of lead to act on red blood cells which have stood in Ringer solution for several hours is probably connected with the diffusion of some substance from the cell into the surrounding medium. It is especially to be noted that the phosphate is one of these diffusing substances.

The question arises, as to whether greater concentrations of lead can affect cells from which diffusion has taken place. To test this, washed corpuscles were permitted to stand in Ringer solution at 30°C. for 6 hours. After centrifuging, the supernatant fluid was discarded and the suspension of cells was brought back to its original volume with fresh Ringer solution. Several samples of these erythrocytes were treated with 0.01, 0.02, 0.04, and 0.2

TABLE IV.

Diffusion from Red Blood Cells Standing in Ringer Solution.

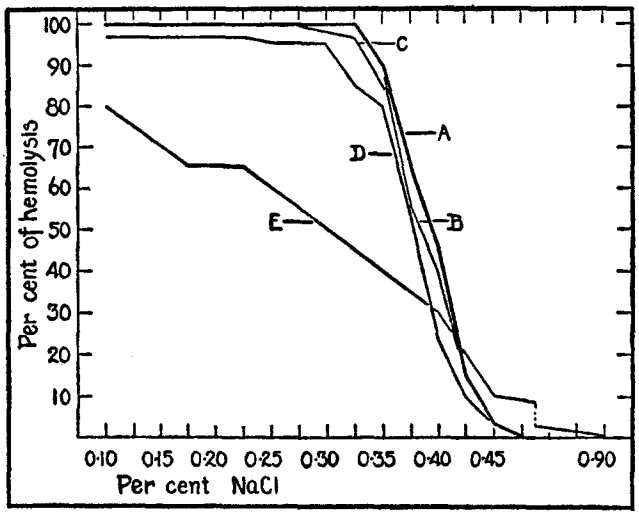
Solution tested.	Phosphates.	Carbonates.	Proteins.	
			Heat and acetic acid.	Millon's reagent.
Ringer solution.	Negative.	Negative.	Negative.	Negative.
Supernatant fluid after 3 hrs.	++	+	"	+
" " " 6 "	+++	++	Very slight trace.	++

mg. of lead per cc. of red blood cells, respectively. The results obtained are shown graphically in Text-fig. 2 and show that (*a*) 0.01 mg. of lead has practically no effect, (*b*) 0.02 mg. begins to "lead" the cells very slightly, (*c*) while 0.04 mg. has a distinct, and (*d*) 0.2 mg., a very marked action.

This failure of small quantities of lead to affect corpuscles which have stood in Ringer solution might be due to neutralization of lead by phosphate which has diffused from the cells. To investigate this point experiments were performed in which red blood cells stood in Ringer solution for varying lengths of time. Part of these were then "leaded" in the original Ringer and part were washed twice with fresh Ringer. In Text-fig. 3 are shown the results of one of three experiments, which illustrate quite conclusively that

when the cells are washed free from the original Ringer they can be affected again by small quantities of lead.

All these experiments point to the importance of inorganic phosphate in the interaction of lead and red blood cells. They show that inorganic phosphate, in the same concentration as normally occurs in serum, combines with as much lead as the whole serum. This



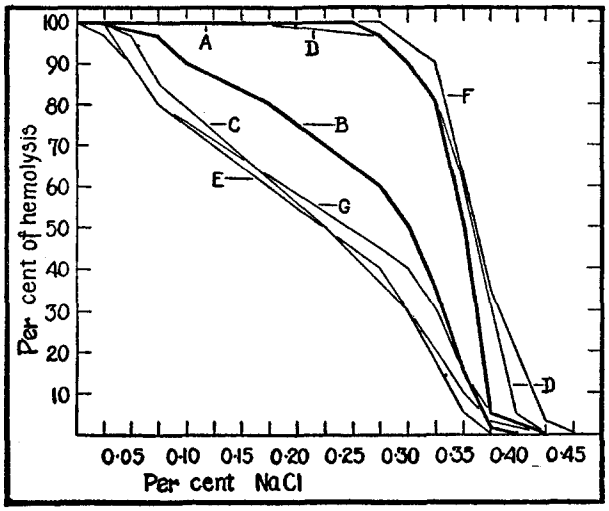
TEXT-FIG. 2. The effect of increasing concentrations of lead on red blood cells which have stood in Ringer solution for 6 hours.

A, control of red blood cells in Ringer solution for 6 hrs.
B, 2.5 cc. of red blood cells and 0.01 mg. of Pb per cc. of red blood cells.
C, 2.5 " " " " " " 0.02 " " " " " " " " " "
D, 2.5 " " " " " " 0.04 " " " " " " " " " "
E, 2.5 " " " " " " 0.2 " " " " " " " " " "

reaction between lead and phosphate is also suggested by the fact that Ringer solution, into which phosphate has diffused from red blood cells, can neutralize lead. Furthermore, serum with a high inorganic phosphate content, and either red blood cells or whole blood to which inorganic phosphate has been added, can react with much more lead than does normal serum or blood.

The Effect of the Blood Lipoids on Lead.

Lecithin.—The organically bound phosphorus compounds of red blood cells were next investigated. Lecithin is the principal of these. Preliminary tests were performed with sodium glycerophosphate. Accepting the formula for lecithin suggested by Levene



TEXT-FIG. 3. The effect of washing red blood cells which have stood in Ringer solution for varying intervals before the addition of lead.

A, red blood cells.

B, red blood cells and lead (0.01 mg. of Pb per cc.). Red blood cells at 31.5°C. for 1 hr. before addition of lead.

C, red blood cells at 31.5°C. for 1 hr.; twice washed before addition of lead.

D, red blood cells at 31.5°C. for 2 hrs. before addition of lead.

E, red blood cells at 31.5°C. for 2 hrs.; twice washed before addition of lead.

F, red blood cells at 31.5°C. for 4 hrs. before addition of lead.

G, red blood cells at 31.5°C. for 4 hrs.; twice washed before addition of lead.

(8), and Abderhalden's figure (9) for the lecithin content of serum—1.675 gm. per liter—a solution was prepared equivalent in phosphorus content to that of the blood by dissolving 0.1048 gm. of sodium glycerophosphate in 100 cc. of Ringer solution. The pH

was brought to 6.7. After treating 0.01 and 0.12 mg. of lead with 0.4 and 2 cc. of the glycerophosphate solution, respectively, for ½ hour, 1 cc. of washed red blood cells per 0.01 and 0.12 mg. of lead was added. In the concentrations used, sodium glycerophosphate had no effect on the subsequent action of lead on the red blood cells (Table I).

Blum (10) has suggested the possibility that a combination of lead with lecithin may be responsible for the symptoms of plumbism. Bloor (11) gives as the concentration of lecithin and cholesterol in human blood 0.30 and 0.25 gm. per 100 cc., respectively. In these experiments an emulsion of lecithin,[2] equivalent to 0.25 gm. per 100 cc., was made in Ringer solution and gave no positive test for inorganic phosphate. 0.4 and 2 cc. of this lecithin preparation (equivalent to 0.03 and 0.17 cc. of blood) were treated with 0.01 and 0.04 mg. of lead, respectively, for ½ hour. Then 1 cc. of red blood cells per 0.01 and 0.04 mg. of lead was added and the results showed that lecithin alone modified the permeability of the cells, but did not alter the usual effect of lead (Table I). It is interesting to note that Thudichum (12) could precipitate all the lipoids of the brain except lecithin with basic lead acetate.

Cholesterol.—Although cholesterol is insoluble in Ringer solution, it can be dissolved in an emulsion of lecithin (3). An excess of cholesterol (Eimer and Amend's C.P.) was added to the lecithin emulsion described above. After filtration, 1 cc. of the lecithin-cholesterol emulsion was found by Bloor's method (11) to contain 0.5 mg. of cholesterol—about the amount present in 0.25 cc. of blood. In the experiment 0.2 and 0.04 cc. of the emulsion were used. These quantities were equivalent in cholesterol content to 0.05 and 0.01 cc. of blood, respectively. These mixtures stood with 0.01 mg. of lead for ½ hour and then 1 cc. of corpuscles per 0.01 mg. of lead were added. The result showed that lead retained its original effect (Table I). Knowing the action of lecithin alone, it seems reasonable to conclude from these observations that cholesterol cannot combine with lead.

[2] Obtained from the Abbott Laboratories, Chicago, and assayed 93 per cent pure.

As a check, lecithin was dissolved in alcohol and an aqueous solution of lead chloride (0.81 mg. of lead per cc.) was added until a precipitate formed. Filtration removed the great bulk of the precipitate, but the filtrate was somewhat turbid. The precipitate was washed repeatedly with acidulated water until the washings were free from lead when tested with hydrogen sulfide. The residue and filter paper were ashed, dissolved in hydrochloric acid and tested for lead. The same procedure was carried out with cholesterol, using acetone as a solvent. In this case the filtrate was clear. With neither lecithin nor cholesterol was lead found in the residue. This indicates that the lipoids of blood did not form an insoluble compound with lead.

The Effect of the Blood Proteins on Lead.

Euglobulin.—The first protein to be tested was serum euglobulin.[3] Normal whole blood contains 50 gm. of globulin per liter, of which 41 per cent or 20.5 gm. is euglobulin (13). According to Hardy (14) this protein contains 0.07 to 0.08 per cent phosphorus.

In these experiments the preparation used was made by Dr. E. J. Cohn (15) from horse serum, contained no inorganic phosphates, a very faint trace of chlorides, and had a pH of 6.49. The nitrogen content was 0.26 gm., equivalent to 1.6 gm. of protein, per liter. When diluted with an equal volume of double Ringer, 1 cc. of the solution contained the same amount of euglobulin as occurs in 0.04 cc. of blood.

Varying quantities of this protein were treated with 0.01 and 0.12 mg. of lead, and the mixtures tested against 1 cc. of red blood cells. Table I shows that the protein has a slight modifying influence on the behavior of red blood cells in hypotonic saline but as much euglobulin as is present normally in 0.16 cc. of blood has no neutralizing action on lead.

Pseudoglobulin.—The pseudoglobulin used was prepared from horse serum (15). It was salt-free and had a nitrogen content

[3] The serum euglobulin, pseudoglobulin, and hemoglobin were obtained from the Laboratory of Physical Chemistry, Harvard Medical School. We take this opportunity to express our sincere thanks to Dr. E. J. Cohn for these materials and for his many helpful suggestions.

of 1.115 mg. per cc., or 7 gm. per liter. The pH was raised to 6.2 and an equal volume of double Ringer solution added. Since normal blood contains 29.5 gm. of pseudoglobulin per liter (13), this procedure gave us a solution 9.4 cc. of which contained an amount of pseudoglobulin equivalent to that normally found in 1 cc. of blood. Treating 0.01 and 0.04 mg. of lead with the amount of pseudoglobulin present in 1 cc. of blood rendered it no less effective than untreated lead in its action on red blood cells (Table I). Pseudoglobulin has a marked water-binding power and therefore modifies the shape of the hypotonic saline curve. With lead the effect is additive.

Hemoglobin.—The hemoglobin employed was prepared from horse blood according to the method of Ferry (16). It was in aqueous solution (0.126 gm. of hemoglobin per cc.). The pH was 7. This was diluted with an equal volume of double Ringer. Since normal blood contains 16.7 per cent of hemoglobin by weight (9), 2.6 cc. of our hemoglobin-Ringer mixture was equivalent to 1 cc. of blood. Experiments showed that hemoglobin in solutions equivalent to 0.2 and 0.75 cc. of blood fails to neutralize the effect of 0.01 and 0.04 mg. of lead, respectively, per cc. of red blood cells (Table I). Hemoglobin alone, like the other proteins, somewhat alters the hypotonic saline curve, but does not affect the action of lead on red blood cells.

Albumin.—Albumin was prepared from horse serum as follows: The serum was half saturated with $(NH_4)_2SO_4$ to precipitate the globulins, and the mixture filtered twice. To the chilled filtrate $\frac{N}{10}$ H_2SO_4, half saturated with $(NH_4)_2SO_4$, was added until the pH of the solution was 5.2. When this solution was allowed to stand overnight crystalline albumin separated out. The crystals were dissolved in water and were twice reprecipitated with saturated $(NH_4)_2SO_4$ solution, the pH being kept constant to allow complete separation of the albumin. Between precipitations the albumin was dissolved in a small volume of water and filtered. The aqueous solution was dialyzed in Dr. Cohn's laboratory until free from all sulfate. It contained 6.22 mg. of nitrogen or 38.87 mg. of albumin per cc.

Since in blood there are approximately 29.4 gm. of albumin per liter (13), 1 cc. of blood contains the same quantity of albumin as 0.76 cc. of our solution. In these experiments isotonic mixtures were made with Ringer solution so that 1 cc. of the mixture was equivalent to 0.1 and 0.5 cc. of blood. The pH of these two mixtures was 6.6. It was found that albumin, even in quantities as great as occur in 1.5 cc. of serum, had no effect on the action of 0.01 mg. of lead upon 1 cc. of red blood cells (Table I).

This negative result is interesting because a lead albuminate is known to exist. If lead is added to serum (0.5 mg. of lead per cc. of serum) a precipitate of lead albuminate is formed if the solution is between pH 5.8 and 6.0 (17); but if the hydrogen ion concentration varies beyond these limits in either direction the precipitate dissolves. Furthermore in the presence of inorganic phosphates, small amounts of lead would combine not with the albumin, but with the phosphate.

The Explanation of the Effect of Lead.

How can the changes in red blood cells caused by lead be best explained? Apparently they depend upon the union of lead with inorganic phosphate, and are largely surface phenomena (2). But is the mere removal of ionized phosphate by lead sufficient to cause these effects? The diffusion experiments prove this improbable. When red blood cells are permitted to stand in Ringer solution for several hours, considerable phosphate diffuses out; nevertheless, despite this loss in phosphate, there is no variation of the hypotonic saline curve from normal.

The possibility that lead phosphate is precipitated on the surface of the cell and has a modifying influence must be considered. It is very probable that phosphate unites with lead as $Pb_3(PO_4)_2$. This may be precipitated in the membrane, where its very presence may change the colloidal properties. That it forms a complete coating on the outside of the cell, however, is hardly probable, because of the very small amount of lead used. Determination of the possible magnitude of this reaction cannot be made, for all the cells are not equally affected by lead and therefore calculations of the amount of lead available for each cell or of the amount of phosphate involved

per unit of cell surface are not justified. A rough approximation made by Dr. Fairhall should, however, be mentioned. It shows that the area of a red cell is at least of the order of magnitude of 10^{-7}, while that of the lead molecules available for that cell is at most of the order of 10^{-12}. Such a great difference makes the possibility of a superficial coating of the cells even by a single molecular layer highly improbable.

TABLE V.

Agglutination of Normal and "Leaded" Red Blood Cells by Colloidal Iron and Arsenic Solutions.

0.1 cc. of corpuscles per cc. of colloid.

Strength of colloid (diluent, isotonic saccharose).	Colloidal iron.*		Colloidal arsenic.*	
	Normal red blood cells.	"Leaded" red blood cells.	Normal red blood cells.	"Leaded" red blood cells.
Full strength.	+	++	++++	++++
1:2	++	++	++++	+++
1:4	++	++++	++++	++
1:8	+++	++++	++++	++
1:16	++++	+++	++++	+
1:32	++++	+++	++++	+

++++ Very marked agglutination.
+++ Marked agglutination.
++ Moderate "
+ Slight "
0 No "

* The colloids were prepared according to directions from Holmes, H. N., Laboratory manual of colloidal chemistry, New York and London, 1922.

Such phenomena as the disappearance of agglutination and the loss of stickiness of the cells (2) suggest a change in the charge of the cell surface as a possible explanation of the action of lead. Rather marked differences in the agglutination of normal and "leaded" blood cells may be observed when tested with colloidal iron or colloidal arsenic. Table V gives the results of one of five experiments.

In view of these variations with colloidal solutions it seemed desirable to determine directly the charge of normal and "leaded" cells by cataphoresis measurements. This was done with an apparatus described by Dr. E. J. Cohn and his coworkers (18). In

the experiments red blood cells were washed free from plasma, part then being "leaded" and part being used as controls. Both control and "leaded" corpuscles were washed three times with isotonic saccharose solution. Distinct differences in direction or rate of migration could not be detected between normal and "leaded" cells, at pH 6.5 or 5. There certainly was no reversal of charge. It must be remembered that the methods of determination are relatively crude and that there are many unavoidable sources of error inherent in cataphoresis measurements of red blood cells. The microscopic method described by Northrop (19) might show differences between normal and "leaded" cells but no attempt has been made to use the method.

Some work by Fairhall (17) introduced another factor in the probable explanation of the action of lead. He found that on mixing lead chlorides and disodium phosphate, a precipitate of lead phosphate is formed and that there is a marked rise of the hydrogen ion concentration to at least pH 2.5. This increase in acidity is due to the liberation of free HCl in the reaction

$$2\ Na_2HPO_4 + 3\ PbCl_2 \rightarrow Pb_3(PO_4)_2 + 4\ NaCl + 2\ HCl$$

Could this acid be of importance in the effect of lead on red blood cells? Although buffer action would prevent any appreciable change in the pH of the blood, could there be a marked local change at the cell surface? If this were the case, there would be a disturbance of colloidal equilibrium with precipitation of the proteins at their isoelectric points, and even coagulation.

The objection may be raised that normally the buffers of the blood are quite adequate to take care of any acid produced. It must be remembered, however, that we are here dealing with a cell surface capable of local reactions and variations in diffusion. Furthermore, the principal buffer, the phosphate, interacts with lead to form insoluble phosphate and free acid, thus diminishing its buffer action. A series of experiments with collodion sacs illustrates this. Mixing equal volumes of Ringer solution containing $PbCl_2$ (0.1 mg. of Pb per cc.) and serum diluted three times with Ringer solution, produces no macroscopic precipitate. If, however, the diluted serum alone is placed in a collodion sac which is suspended in the lead solution,

a heavy precipitate forms at the membrane. Moreover, if methyl red is added to each solution, both remain alkaline to the indicator but the membrane itself turns red. This demonstrates a local formation of acid. The same result is obtained if phosphate solution is substituted for serum.

These facts would imply that anything which changes the colloidal state of the cell surface affects its properties. The two reactions due to lead, are the precipitation of insoluble lead phosphate and the production of acid. These local reactions may not be the only causes of colloidal changes, but they are very important factors in producing the phenomena described in the two preceding papers.

CONCLUSIONS.

The possible reaction of lead with each of the pure chemical constituents of the red blood cell has been studied. As the indicator of chemical combination the effect of lead on the behavior of red blood cells in hypotonic saline was used.

1. By means of such tests it was found that lecithin, cholesterol, euglobulin, pseudoglobulin, hemoglobin, and albumin do not interfere with the reaction.

2. Sodium bicarbonate can neutralize lead; but ten times as much is required as occurs in an equivalent neutralizing quantity of serum. This carbonate can therefore play only a small rôle in the reaction.

3. Inorganic phosphate in the same concentration as is present normally in serum, neutralizes the same quantity of lead as does the whole serum itself. This can, therefore, completely account for the action of lead on red blood cells.

4. Adding inorganic phosphate to red blood cells or to whole blood greatly decreases the action of lead on the cells but cannot eliminate it completely. This indicates that there is a simultaneous reaction between lead and serum and lead and corpuscles when all are present, and explains the action of lead *in vivo*.

5. Serum with increased inorganic phosphate content, obtained from two patients, neutralized the effect of lead to a high degree. In one case serum with a high normal phosphorus content had a similar though less marked action.

6. Diffusion of inorganic phosphate from red blood cells into the surrounding Ringer solution prevents the action of lead upon the corpuscles. This is due to neutralization of the lead by the diffusate. Washing these same cells free from diffusate again allows lead to react with them.

7. All the findings indicate that the action of lead upon red blood cells can be entirely explained by the union of lead with the inorganic phosphate of the cell.

8. Chemical tests are cited which show that interaction of lead salts and phosphate results in the formation of a very insoluble lead phosphate ($Pb_3(PO_4)_2$) and free acid (Fairhall).

9. When a collodion membrane separates solutions of lead chloride and phosphate or serum, lead phosphate is precipitated in the membrane with a local formation of acid.

10. It is suggested that this acid, which is formed as the result of the union of lead and phosphate, may change the physicochemical state of the colloids of the cell surface.

11. Macroscopic cataphoresis determinations demonstrated no difference between the migration of "leaded" and normal cells.

12. In agglutination experiments with colloidal iron and arsenic a marked difference was observed between "leaded" and normal cells. Thus, further evidence of changes in cell surface is added to that reported in former papers.

GENERAL SUMMARY.

These three papers have developed the following facts, which suffice to explain completely the anemia of lead poisoning.

In vitro, the exposure to a very small amount of lead greatly changes the surface of the red blood cells. Their permeability to water is so altered that they shrink and are incapable of swelling as much as normal cells. With this is associated a marked increase in the resistance to different osmotic surroundings—demonstrated by far less hemolysis than normal in salt solution of very low concentrations. The "leaded" cells, however, are relatively short lived, and hemolyze readily as the result of slight trauma. These observations can also be demonstrated *in vivo* as experiments with rabbits with acute lead poisoning show. In addition to these effects on permeability, lead

alters the physical properties of red blood cells so that they lose their normal stickiness and are no longer agglutinated by the sera of different isoagglutinating groups. All these changes are evidence of an effect on the surface of the cell; the interior of the cell does not undergo disturbances; at least, the physiological properties of the hemoglobin remain normal. The chemical reaction which causes these physical changes in the cell is a precipitation of insoluble lead phosphate and a formation of acid. This causes the "leaded" red blood cell to change from an elastic distensible sac, to one which is contracted, relatively inelastic and brittle. In such condition the cell can poorly withstand the trauma involved in circulation of the blood, and this lack of resistance probably explains the marked destruction of peripheral blood in lead poisoning.

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