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THE PLASMA-CELL PARTITION OF BLOOD LEAD, WITH A PHOTOMETRIC DITHIZONE METHOD FOR THE DETERMINATION OF LEAD IN BIOLOGICAL MATERIAL

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by

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THE PLASMA-CELL PARTITION OF BLOOD LEAD, WITH A PHOTOMETRIC DITHIZONE METHOD FOR THE DETERMINATION OF LEAD IN BIOLOGICAL MATERIAL

Lead has enjoyed wide use ever since ancient times, and certain toxic effects of the metal on those exposed to it have long been known. Hippocrates (370 B.C.) (1) described colic in a man who extracted metals, although he did not attribute the condition to lead specifically. Nicander (1), in the second century B.C., was perhaps the first to observe the relationship of constipation, abdominal pain, and palsy to the action of lead on the body. Pliny (2) mentioned white lead and described a colic caused by lead, and Celsus (3) knew the danger of its administration as a drug. Dioscordes (1), in the first century A.D., not only described the symptoms resulting from the introduction of lead into the stomach, but also stated that breathing lead emanations causes the same disorders. Galen (4), later on, described a colic identical with lead colic, but did not know its cause. Then, about 1000 A.D., Avicenna (1,5) gave a lengthy description of lead colic, defining it very clearly, and also indicated that lead fumes were dangerous.

During the middle ages no special tracts appeared on lead disorders until Citois(6), in 1616, described a colic prevalent in the vicinity of Poitou, later shown to be caused by the use of lead to decrease the acidity of wine. However, Citois was ignorant of the actual cause of the colic, although the symptoms were similar to those described in the articles mentioned above. The definite relation between lead and colic, and the danger associated with exposure to lead dust and fumes, were first clearly stated by Stockhausen (7) in 1656. He was physician at the lead mines of Goslar and is the father of the true history of plumbism. The recognition of lead disorders became much more common after the appearance of Stockhausen's work. The first instance in which lead in wine was pointed out as the cause of the colic previously considered epidemic in nature was in a letter by Wepfer (8) addressed to the government of Wurtemberg in 1671.

The eighteenth century produced many studies of plumbism, Henkel (9) proved by experiment that lead and its compounds are the only substances which produce lead colic, and in 1745 Dehaen (10), a Venetian physician, produced a full and exact description of the disorder. At

this same time Huxham (11), in England, described a disease prevalent in Devonshire and identical with lead colic, which was later traced by Sir George Baker and others to the contamination of cider with lead. A similar investigation in Spain was reported by Luzuriaga (12) in 1796, in which the lead-contributing substances were found to be adulterated wines and copper-tinned cooking vessels. Gardane (13), in 1768, published a collection of the works of the ancient writers on plumbism, in addition to his own contributions to the subject.

Shortly after 1800 Merat (14) discussed the clinical side of lead colic and Grisolle (15) described colic, the lead line, and encephalopathy very accurately. The definitive book of Tanquerel des Planches on lead diseases came out in 1838, in which the clinical picture of the disease was given in minute and precise detail. In fact, after this time little was added to our clinical knowledge of lead poisoning until the advent of sensitive and accurate analytical methods for lead determination.

The lead line, which had previously been described by Grisolle, Tanquerel, and others, has come to be known as the Burtonian line because of a description published by Burton in 1840. (16)

Actual experimental studies of the physiological

action of lead were first carried out by Orfila (17) in 1814, who administered lead intravenously and by mouth. Grisolle, Tanquerel, and others studied the toxicity, paths of entry, and distribution of lead in both human beings and experimental animals.

Lead in Tissues

Work on the quantity and distribution of lead in the body could not have much significance until sufficiently sensitive and accurate methods of lead determination were developed. However, Orfila (18) stated that lead is always found in human organs. He used the term "normal lead", which has undergone much criticism, since to some it conveys the idea that lead is indispensable to life. Before the method of Devergie was used in 1836, no lead had been found in the organs or excretions of human beings, even of those known to have been heavily exposed to lead compounds (19). At that time, lead was shown to be present in the organs of persons with no unusual exposure to lead. Devergie (20), in 1845, stated that all human organs contain traces of copper and lead, that the quantity present increases with the age of the subject, and that the amount of copper always exceeds that of the lead in normal cases while the reverse is true

in plumbism. Legrip (21) and Orfila (22), both in 1847, confirmed these findings; the former author also reported the presence of lead and copper in the organs of a cow, and the latter concluded that natural lead and copper constantly exist in the human body.

It is not surprising that lead was not detected in the blood until modern times. Millon (23), in 1848, was perhaps the first to report the presence of lead in normal blood. It is extremely interesting to note that he concluded that the lead was not distributed throughout the blood, but was held by the red cells. He also said that normal lead was combined with the globulin of the blood, and Cozzi (24), in 1844, stated that in plumbism the lead was combined with the albumin of the blood. Thus, almost one hundred years ago, workers thought that they had solved a problem which is still under discussion: namely, whether or not lead in plumbism is held in, or combined with, a different fraction of the blood than that involved in the case of normal lead.

At that time, and all through the years until quite recently, some investigators maintained that the blood and tissues of normal persons, that is individuals with no unusual lead exposure, contained no lead. Chevreul (25), and Danger and Flandin (26), contemporaries of

Devergie, Millon, Legrip, and Orfila, were the early exponents of this viewpoint. Meillere (27), in 1903, reported that he found traces of lead only occasionally in normal tissues. Later workers, especially the group associated with Aub, Fairhall, and Minot, usually found no lead at all in samples of human tissues and excreta. They expressed the definite conclusion that lead did not occur normally, and that the previous opinions to the contrary had resulted from the use of inadequate chemical methods. These workers reported such findings in 1926 (28), and as late as 1932 Fairhall (29) found no lead in the urine and feces of subjects wearing lead-weighted silk garments. In 1939 Fairhall (30) stated that at times there is no lead in the human urine, and in 1923 Minot (31) included as evidence against an electrolytic method of lead determination the fact that the method was sensitive enough to show the presence of lead in feces from normal subjects.

On the other hand, many workers have agreed with Devergie, Orfila, and Millon. Their argument, which in recent years has been proved to be true, was that lead is always present in biological material, and that analytical results which do not show its presence are due to the fact that the method used was not sensitive enough.

Kehoe, Thamann, and Cholak (32), in a series of papers in 1933, reported the results of an extensive study in which the lead content of foods, excreta, and blood of individuals living in primitive Mexican villages and also in modern circumstances were determined. They concluded that lead is always present and occurs naturally in biological material and does not necessarily result from the effect of modern civilization. A huge quantity of analytical data has accumulated concerning the lead content of normal and pathological tissues of human beings and lower animals, foods, and other substances related to physiology (32, 46, 97, 130, 131, 132).

LEAD METABOLISM

Any conclusions regarding the presence of lead in biological material have an important bearing on studies of the general metabolism of that metal. Lead can enter the body by the three main portals: the respiratory tract, the gastro-enteric tract, and the skin. Other paths, such as by absorption of lead injected subcutaneously or intraperitoneally, are mainly of experimental interest.

Absorption by Skin

It is well established that inorganic lead is

not absorbed in significant quantities by the intact skin. Many early writers, as Stockhausen (1649) (99), von Brambilla (1787) (100), and Canuet (1825) (101) felt that inorganic lead was absorbed in this way. They cited cases which would be considered poorly supported at the present time, such as the recollection of colic which had taken place ten years before, and poisonings attributed to the use of lead plasters or cosmetics containing litharge. With lead-bearing cosmetics, of course, one can never be certain that oral ingestion of lead has not taken place. Evidence of an indirect nature has been mentioned by Manrouvrier (1874) (102), Malherbe (1875) (103), and others, stating that the frequent appearance of paralysis in those parts most exposed to lead indicated cutaneous absorption of the metal. As recently as 1914 Oliver (104) suggested the possibility of the cutaneous absorption of lead from cosmetics.

Tanquerel (1838) cited both clinical and experimental evidence to show that lead was not absorbed by the intact skin. He observed that external applications of lead subacetate or diachylon plaster did not cause lead poisoning, and that the physicians of the Paris theaters asserted that actors who continually used lead-containing cosmetics suffered no ill effect from them. (105)

His experiments consisted of applying an ointment made of white lead (sometimes litharge) and animal fat to the intact shaved skin of two dogs and a rabbit. After repeated applications for as long as twelve days no signs of lead poisoning appeared. A test with potassium sulfide showed ample lead on the skin of the animals. He concluded that lead is not absorbed by the intact skin, at least not in quantities sufficient to cause clinical symptoms.

If the epidermis is abraded or removed, however, it is generally conceded that inorganic lead in considerable amounts can be absorbed there. All workers from Tanquerel's time on agree in this (106, 107, 108).

Certain organic lead compounds, especially fat-soluble substances such as tetraethyl lead, can be easily absorbed through the intact skin in dangerous quantities. Kehoe (109) reported that the lethal dose of tetraethyl lead, applied to the shaved skin of rabbits, is approximately 0.7 ml. of tetraethyl lead, equivalent to 0.7 g. of lead, per kilo. It is not unreasonable to believe that a lead compound which would not be absorbed by the skin under normal conditions might be rendered absorbable by the presence of another substance which penetrates the skin readily, such as methyl salicylate or nitrobenzene.

Alimentary Absorption

The path of entry of lead first considered by the early workers is the gastro-enteric tract. In Tanquerel's time many physicians regarded the absorption of lead by the digestive organs as the common and even the only way in which lead colic was caused (110). The stomach may absorb some lead; Meillere1(108), Brouardel (111), and Schmidt (112) believed that lead absorption occurs there, but only in small quantities. Legge and Goadby(113) and Shields, Mitchell, and Ruth (114) stated that almost no absorption takes place in the stomach. The upper portions of the small intestine are most important in lead absorption, according to Legge and Goadby(113), and Schmidt, Seiser, and Litzner (115).

The addition of hydrochloric acid to the diet increases the absorption of lead from the alimentary tract, so that gastric acidity is an important factor in the absorption of lead, according to Tompsett (116). Schmidt (112) described the probable steps in lead absorption, beginning with solution of at least part of the ingested lead by the hydrochloric acid of the stomach. In the presence of proteins a lead-protein compound is also probably formed. Such lead would be in a very finely divided or colloidal state, which may be absorbed by the

mucous membrane as easily as the lead ions themselves. The lead chloride and other lead compounds are changed upon entering the intestine by the alkaline intestinal juices, the bile, and the fats or fatty acids. Just as the absorption of fat is aided by the bile, so lead salts of fatty acids may be absorbed more readily in the presence of bile. According to Schmidt, the mucous membrane becomes impregnated with extremely fine lead particles, some of which are carried away at once through the intestinal capillaries of the portal system, and some through the lymph passages to the thoracic duct. In this way the lead can be carried through the body, with the liver becoming impregnated early in the process. Some workers believe that the reticulo-endothelial system plays a major part in collection of the hypothetical lead particles. It should be mentioned here that no definite evidence exists concerning the state of lead in the blood--whether it exists as collodial particles or as ions. Any theories of the actual mechanism of lead absorption, transport, and deposition must be only speculative in our present state of knowledge. However, it is important to recognize that the absorption of lead from the alimentary tract, even under the optimal conditions of

the ingestion of lead salts in solution, is incomplete and quite slow, most of the ingested lead passing through unabsorbed and appearing in the feces (144).

Absorption Through Lungs

Recognition of the fact that lead may be absorbed from the respiratory tract dates from Stockhausen's time (7). Tanquerel (117) cited several cases of lead poisoning caused by the inhalation of lead dust, including that of a woman who lived over a shop in which white lead was ground and sifted; the floor of her room had holes and cracks in it, so that the dust from the shop entered her room. He also carried out an experiment in which lead oxide was administered to a dog through a tracheal cannula, causing fatal poisoning. In 1909, Goadby(118) showed that poisoning could be caused by one-tenth as much lead when inhaled as when taken by mouth.

The absorption of lead by the respiratory tract is influenced by the physical condition of the metal. Lead in the vapor state of an organic compound, as tetraethyl lead vapor, is readily absorbed. The fat-soluble molecules probably pass through the alveolar endothelium directly into the blood. The mechanism may well be somewhat

different in the case of particulate lead, either as the so-called "vapor" of the metal itself (partly the oxide) or as the fine dust of a lead compound. In entering the body, the greatest proportion of this particulate matter clings to the mucous membranes of the nose and naso-pharynx and in part is swallowed. A portion will penetrate to the pulmonary alveoli and be deposied there and a portion escapes in the exhaled air. According to Schmidt (112) this dust is taken up by the alveolar endothelium and is passed on to the capillaries and the blood. Part may be phagocytosed by the leukocytes, going either into the pulmonary alveoli or to the lymphatic system. It is worthy of mention that in the lungs no retarding filter organ such as the liver is present to delay the lead from being carried off to the greater circulation.

Lead entering the body through the respiratory tract is more dangerous than that entering through the gastro-intestinal tract (118, 119). In the latter case, most of the lead which is ingested is excreted without having been absorbed at all; even after absorption it may be caught by the liver and excreted in the bile without ever reaching the systemic circulation. Thus it is largely confined to a region where it causes relatively

little damage, while lead which enters by way of the lungs must involve transportation in the systemic blood in order to be absorbed, deposited, or excreted.

Lead Transport

Once in the body, the lead is ultimately transported by the blood. The answer to the way in which it is carried must, of course, wait until something is known of the state of the metal in the blood. However, in spite of the lack of primary facts with which to work, many theories of lead transport have been advanced. The earlier workers thought that lead was carried in the blood in combination with the proteins. It was mentioned in the first part of the paper that Millon believed the lead to be carried by the globulin and Cozzi by the albumin of the blood in plumbism. Views of this type prevailed until recent times, when Aub, Fairhall and their co-workers (36), confirmed by Brooks (120), stated that lead was probably carried in the blood as a colloidal phosphate. Their evidence related to the solubility behavior of the lead salts in the presence of excess phosphate and the peptizing effect of the serum on the lead phosphate. However, it must be remembered that these workers also felt that most of the lead in the blood was

held in the plasma instead of in the cells. Maxwell and Bischoff (121) believed that lead di phosphoglycerate or a closely related compound is formed when lead ions reach the red cells and that it is the compound concerned in lead poisoning. Their conclusions were based on studies of the effect of lead diphosphoglycerate upon hemolysis of the erythrocytes, the toxicity of the compound, and its solubility. Kehoe and Thamann(122) observed that colloidal lead phosphate is relatively inert in the body as compared to the compounds formed in the blood when soluble lead salts or colloidal lead are injected intravenously. This indicates that lead is not carried in the blood as a colloidal phosphate and so is contrary to Aub and Fairhall's and Brooks' views. Jowett(123)1 from in vitro studies, thought that lead was carried in the blood as a penta-lead-mono-chlor-triphosphate compound, possibly linked with a corresponding molecule containing calcium instead of lead.

The years 1911-1914 brought forth the "lead-stream" theory of Straub (124, 125) and Erlenmeyer (126), which enjoyed a vogue for a time. According to this theory, the lead is not bound to the cell substance but is in the fluid bathing them, and so forms a lead-albumin complex, enters the circulation, goes through the body and then the

lead is excreted in the urine and feces. The lead on its way through the body comes into contact with all the cells of the organism but is not retained by any portion of it in significant quantities. The quantitative factor concerning any organ lies in the density of the lead-stream passing through it, rather than in the retained lead or in the duration of the lead exposure. Erlenmeyer stated that lead is not stored in the specifically affected organs but that the final injury is in the result of repeated toxic effects of the lead stream circulating through the organs. The faulty conclusions in the lead-stream theory were probably due to inadequate analytical methods used in the experimental work, but even today certain features of the theory are still valid; for example, clinical symptoms of plumbism are usually associated with certain quantities of lead circulating in the blood.

Deposition of Lead

Consideration of the lead-stream theory brings one to the evidence regarding the deposition of lead in the organs and bones. As mentioned earlier, from the time the hydrogen sulfide test was developed, lead has been found in most body tissues by many workers. Once lead is absorbed it is distributed throughout the viscera,

but to the greatest extent in the liver, spleen, and kidneys in the period immediately following absorption (119). Promptly, however, after lead absorption much of the metal passes into the skeletal tissues and soon most of the lead in the body is found therein. Low concentrations remain in the soft tissues, especially the liver, kidneys, and blood, the pattern of the ditribution remaining substantially the same indefinitely except when a new source of rapid absorption develops. This characteristic distribution is approximately the same for inorganic (127,128), organic (122), or colloidal (122, 129) lead, regardless of the route of absorption. Even in the case of a fat-soluble compound like tetraethyl lead all of the lead in the tissues after ten days to two weeks is distributed in a manner characteristic of water-soluble lead compounds (109).

The total quantity of lead involved in the production of lead poisoning is small, for an adult with fatal lead poisoning need not have more than one gram of lead in the entire body, of which the greater portion is in the skeleton (119). Some of the lead which is taken from the circulation by the liver and spleen during rapid lead absorption is gradually given up and redeposited in the skeleton. This deposition is not a

permanent thing, for lead constantly enters and leaves the bones; under usual circumstances the loss is slow, but it appears to occur in accordance with a dynamic equilibrium. The flat bones usually show a higher lead content than the long bones (127, 134, 135); it is possible that lead is laid down more slowly in the long bones and if this is true it is to be expected that it would also be lost from them more slowly; conversely, lead should be deposited more readily in the more vascular bones, and lost more readily from them (136). According to Aub (119) and to Behrens and Baumann (128), the concentration of lead is greater in the bone trabeculae than in the hard bone cortex. In addition, the lead in the trabeculae is readily sent into the blood stream again.

Considerations of lead deposition in the bones have caused a search for a possible correlation between calcium, phosphorus, and vitamin D metabolism and lead metabolism. Aub (119) believes that the direction of the lead stream is similar to that of the calcium stream; when calcium is being deposited in the bones, lead is also being deposited there, and the same relation holds true in calcium and lead liberation. Acid-producing substances (137, 138), parathyroid extract(139), and

viosterol (140) have been reported to have increased the rate of excretion of lead. In dogs and rats, Grant and Calvery (131, 141) found that a low calcium diet favored lead storage in the bones. According to Tompsett and Chalmers (136) high calcium diets cause a decrease in blood lead, while low calcium diets, both with and without ammonium chloride, produce a rise in blood lead. Sobel et al (142, 143) introduced vitamin D into the calcium-phosphorus lead picture, and found that the administration of viosterol to rats on a low calcium lead-containing diet increased the blood lead concentration and also the lead deposition in the bones. With a high phosphorus, low calcium diet the concentration of blood lead was low, but the lead deposition in the bones was not significantly low. Kehoe and co-workers (144) found that the rate of accumulation of lead in the body of a subject kept on a daily dosage of 1 mg. of soluble lead in addition to dietary lead was not influenced by the ingestion of milk, apple powder (pectin) and Vitamin C, nor by gross variations in the calcium and phosphorus intake, in all the combinations that were employed to raise and lower the intake of those elements separately, in parallel, and in opposed relationship. These rather conflicting reports and conclusions indicate that more experimental work must be done before one can

feel sure whether or not there is any definite correlation between calcium metabolism and the factors influencing it and lead metabolism.

The presence of an abnormal quantity of lead in the the blood is usually accompanied by an increase in the basophilic granulation (stippling) in the erythrocytes of the peripheral circulation. Stippled erythrocytes occur in normal blood, and their numbers are increased in a variety of other physiological and pathological states. Due consideration must be given to the numbers present and to the other associated factors before stippling can be considered a sign of plumbism (145). The consistent presence of as few as one hundred stippled cells per million is held by some workers to indicate lead poisoning (146), while others have shown that even greater stippling often occurs in normal unexposed individuals, and that quantitative relationships are not specific except in the event of extreme findings (156). Key (147) has given evidence to show that stippled cells are young red corpuscles which have been exposed to lead. There are also indications that lead affects the blood-forming elements of the body; Schmidt believes (112) that it stimulates the blood-forming red bone marrow while a slow destructive effect is taking place

simultaneously in the circulation. In chronic plumbism a secondary anemia sets in later, as a result of this destructive effect coupled with the subsequent injury to the bone marrow.

An abrupt and maintained increase in lead absorption will result in the accumulation of a greater quantity of lead in the body (148). This accumulation of lead with consistent lead exposure within certain limits is apparently continuous, at least for long periods. However, it appears that when the lead intake is small - that is, within normal or little more than normal limits - the amount of lead excreted comes into balance with that ingested, so that the accumulated lead in the body will not increase progressively but will reach a stationary level. This proposition has been tested experimentally by Kehoe and his co-workers (148), who found that there was an approximate balance between the lead intake and output of normal subjects. Considering the small quantities of lead involved the balance was so nearly exact as to be quite convincing. When the normal lead intake was increased by 1.0 mg. and 2.0 mg of dissolved lead per day, respectively, for each of two subjects, it was found that some degree of lead retention (about 5% of the total lead ingested) occurred regularly

in both individuals. The retention in the subject taking 2.0 mg. of lead daily was greater for comparable periods of time than that in the other case. Certain factors (such as the excretion of lead by the perspiration) undoubtedly acted to decrease the actual magnitude of the apparent retention, and in any case one can conclude that only a very small proportion of the total lead ingested was retained by the subjects. At low levels of daily lead intake this quantity would seem to be insignificant.

Elimination of Lead

Lead is excreted in the urine, perspiration, and feces. Absorbed lead only is excreted by the first two routes; the feces contain unabsorbed lead which was present in the food and water, some portion of the lead which was excreted in the bile, and also, probably, some quantity of lead actually excreted into the alimentary tract. The history of the dispute regarding the presence of lead in the urine and feces of normal subjects has been covered in the early part of this paper. In recent years it has been clearly shown that, given an analytical method of sufficient sensitivity, lead can always be found in any biological material, regardless

of whether the organism had suffered an undue exposure to lead or not.

It has been found (148) that in the case of lead taken by mouth, about 94% of the lead eliminated in the feces and urine is in the feces. When lead is administered orally, the quantity in the feces increases on the first day after the beginning of the administration and reaches its maximal level within only a few days. After discontinuance of administration, the fecal lead falls in a few days to the normal level. These facts indicate that most of the ingested lead passes directly through the alimentary tract without absorption. On the other hand, the urinary lead output increases slowly and gradually during the period of administration, the rate of increase depending upon the quantity of lead administered; afterward, it falls, first rapidly, then gradually. For instance, in the experiment cited in the preceding paragraph, the urinary lead of the subject receiving 1 mg. of lead daily increased progressively for a period of almost four years, while that of the subject receiving twice as much lead increased more rapidly and continued to increase for about two years, after which time the administration was discontinued.

From these and other facts developed in the

foregoing observation it appears that the excretion of lead from the tissues during and following periods of abnormal lead intake is handled to a large extent by the kidneys, rather than by the liver and the alimentary tract (144).

Lead absorbed by the respiratory tract, especially when the lead is in the form of a fat-soluble organic compound, causes a urinary lead excretion which is disproportionately high in relation to the fecal excretion (149). In the case of exposure to lead dust some absorption takes place in the lungs and some lead is always swallowed; the ratio of the lead found in the urine to that in the feces will therefore be higher than it will be where the total lead exposure is due to ingested lead alone.

Some quantitative figures regarding the occurrence of lead may be mentioned here. The normal lead content of the present diet in the United States is about 0.32 mg. of lead per day (148), including both food and drink. The daily fecal lead output averages about the same as the quantity of lead in the food and drink. The lead concentration in normal urine averages about 0.030 mg. per liter (46) and that in normal blood is about 0.025 mg. per 100 g. (46). The quantity of lead in tissues varies

greatly; some workers believe that this quantity increases with age even in normal individuals (67), while others have not found this to be the case (46, 132). In the experiment mentioned previously, Kehoe et al (148) found that in the case of the subject taking 1 mg. of lead daily in addition to his normal diet the urinary lead concentration reached a value of 0.068 mg. per liter, with a blood level of 0.059 mg. per 100 g.; with the subject taking 2 mg. of lead the urinary value was 0.087 mg. per liter and the blood lead was 0.063 mg. per 100 g. Lead analyses of urine, blood, and feces are of great diagnostic value in cases of suspected plumbism.

Clinical Deleading

Some clinicians resort to a deleading procedure to hasten the elimination of lead from the body after exposure to the metal has ceased. Tanquerel used croton oil to hasten this lead excretion; many workers have used magnesium sulfate. However, it is probable that the administration of cathartics does not greatly influence the lead excretion (151); it probably merely empties the alimentary tract more rapidly and thereby prevents reabsorption of lead. Potassium iodide, which has been used as a deleading agent since 1840 (151) seems to

increase lead excretion. According to Aub et al, it is the iodide ion which has this effect, since potassium chloride does not show it, but ammonium iodide does (151). Except for a few recent observations under carefully controlled conditions, the experimental data of the literature is of little value in relation to this question. The work of Tompsett seems to demonstrate that ammonium chloride, potassium iodide, or sodium bicarbonate causes mobilization of lead on both high and low calcium diets (116). The actual changes in the lead concentrations in the blood and urine were quite small, however, and are not wholly convincing. A common treatment for the purpose of deleading consists of a low calcium diet with the addition of phosphoric acid, vitamin D, and magnesium sulfate (152). Regardless of whether this deleading procedure actually is effective or not, one should bear in mind that actual deleading is a slow process, so that any treatment lasting for only a few weeks can dispose of only a few milligrams of lead more than the body would excrete without special treatment, and the net effect of the deleading may not be important (150).

The effect of lead on certain physiological units may aid in the formulation of an adequate theory of the mechanism of lead poisoning. For instance, lead has a

definite action on enzyme systems, and this may be considered as a way in which the metal interferes with normal processes in living tissues (153). Small amounts of lead have been found to hasten the rate of cleavage in autolyzing liver, while higher concentrations decrease the rate (154). Dolowitz, Fazekas, and Himwich (155) studied the effect of lead on tissue oxidation, dehydrogenation, and glycolysis, using the Warburg apparatus and Thunberg tubes. Lead caused a decrease in oxygen consumption and changes in respiratory quotients in brain, kidney, liver, and testis. In brain tissue lead inhibited hydrogen transfer and glycolysis.

Plasma-Cell Partition of Blood Lead

Opposing views exist in the question of the plasma-cell partition of blood lead. The earliest reference to the distribution of lead in blood is the one given above by Millon. He found the lead in the cells, and it seems that he was probably correct in this conclusion. Riva (33), in 1912, and Erlenmeyer (34), in 1913, reported that in the blood of experimentally poisoned animals the lead could be found only in the globulin fraction of the proteins, while all other fractions, as the nucleo-protein fraction, remained lead-free. Schmidt (35), in 1909, found

lead in the clot of a blood sample taken from a poisoned rabbit. Aub, Fairhall, Minot, and Reznikoff (36), in their book (1926) on lead poisoning, stated that the greater part of the lead in the blood is held by the plasma and but little is carried by the cells; one experiment indicated that 80% of the total blood lead was carried by the plasma. Behrens and Pachur (37) (1927) reported that the lead is present chiefly in the serum, although when a lead salt is added to the whole blood the greater part of the lead is taken up by the red cells. They postulated that this is an adsorptive process, involving the surface of the erythrocyte. A different view was held by Schmidt, Seiser, and Litzner (38), in their book on lead poisoning (1930). They cited the conclusion of Aub et al given above, but added that they could not check the American workers, since they always found more lead in the blood clot than in the serum. Schmidt and Barth (38), in experiments on birds, found that the lead was largely fixed in the cell nuclei, and so was concentrated in the erythrocytes.

Bischoff et al (39), using lead compounds administered by injection, reported that when colloidal lead compounds were used, ten times as much lead was carried by the cells as by the serum, while the reverse

was true for highly ionized lead salts.

Behrens (40) modified his earlier conclusion regarding the plasma-cell partition of blood lead, for in 1934 he said that the largest portion of the lead is bound to the erythrocytes and the plasma contains a comparatively small quantity. Weyrauch (41) reported that the serum from the blood of a lead-poisoned goat contained less than 10% of the blood lead. Teisinger (42), in 1935-36, found lead almost equally divided between the plasma and cells of patients. Blumberg and Scott (43) stated that the greater part of the lead, usually about 90%, appeared in the clot, and that if an anticoagulant (heparin) were used the plasma showed only about 10% of the total lead. Schmitt and Taeger (44) (1937) cited numerous analyses of the blood of patients undergoing various types of treatments and found that the plasma (using "Novirudin" for an anticoagulant) contained about one-third of the total blood lead. Willoughby and Wilkins (45),in 1938, reported that in 90% of the samples of blood from normal subjects no detectable amount of lead was found in the serum, and only insignificant quantities appeared in the serum of the remaining samples.

Kehoe, Cholak, and Story (46) stated in 1940 that the normal plasma is almost free from lead, containing

less than 10% of the total blood lead in almost all instances. They used sodium citrate and heparin as anticoagulants. In 1941 Tompsett and Anderson (47), using potassium oxalate, found that the plasma of normal subjects and of patients suffering from plumbism contained about 40% of the total blood lead. Also, using normal and lead poisoned rabbits, about half of the total blood lead was found in the plasma.

A somewhat different viewpoint has been presented by Smith, Rathmell, and their co-workers (48,49,50,51). They contend that lead is not present in the serum of normal individuals but is present in the serum only in plumbism, so that the essential way in which the blood of healthy persons differs from that of clinical cases of lead poisoning lies in the appearance of lead in the serum. Differential diagnoses of lead poisoning, with regard to latent and acute plumbism, can be carried out with those factors in mind.

Thus the literature contains widely divergent views regarding the plasma-cell, or serum-clot, partition of blood lead. Conclusions of workers from the earliest to the most recent seem to fall into three classes; those who believe that the plasma contains most of the

blood lead, those who think that the lead is about equally distributed between the cells and the plasma, and those who hold to the other alternative, saying that the lead is associated almost entirely with the cells.

The validity of any conclusions concerning this problem depends upon the significance of the analytical figures obtained when lead is determined in the blood fractions. For that reason it seems advisable at this point to take up a detailed study of methods for determining lead in biological material.

METHODS FOR LEAD DETERMINATION

The precipitation of lead as the sulfate was one of the first analytical methods (52). However, the relatively high solubility of this salt (53,54) even in the presence of alcohol (55) limited the usefulness of the method. Lead sulfide, which is one of the most insoluble lead compounds, formed the basis for most of the analytical procedures until the last few years. Zoller (56), in 1707, used an aqueous extract of orpiment (arsenous sulfide) and lime water to precipitate lead sulfide. Fourcroy and Hahnemann (57),

at about the close of the eighteenth century, were probably the first to use hydrogen sulfide with hydrochloric acid as the precipitant. Devergie (19), who introduced his method in 1836, prepared the dried sample by ashing, dissolving the ash in acid, and precipitating the lead with hydrogen sulfide. When only very small quantities of lead are present the sulfide will often not precipitate, but will remain in a colloidal state and impart a brownish color to the solution. This property has been used by many workers, beginning with Pelouze (58), in 1842. The method of Millon, whose results have been mentioned previously, was particularly well adapted to the analysis of blood (59). The blood was mixed with chlorine water, which precipitated the organic matter (especially the proteins) and left the salts, including the lead and other metals, in solution. This solution could then be concentrated and treated with hydrogen sulfide in the usual way. One of the earliest workers to use cyanide in order to prevent the interference of iron, copper, and other metals in the sulfide test was Warington, in 1893 (60).

Other insoluble lead salts, as the molybdate (61,62, 63, 64) have been used for lead determination, but most of these methods are not applicable to the

analysis of biological material because of interfering ions. Modern methods, depending upon the precipitation of lead as the chromate after an initial sulfide separation, will be described later.

Electrolytic Methods

Lead, like other metals, can be separated by electrolysis. The pioneer in this field was probably Gusserow (65) who in 1861 plated the lead on the cathode from a hydrochloric acid solution of the sample. The lead was then brought into solution and precipitated as the sulfide. He found lead in the liver, kidney, and other organs, and even regarded the uniform presence of lead in the bones as a kind of depot, which was a significant conclusion for those days. Lehmann (66) reported in 1882 that lead in urine could be determined by electrolyzing the sample directly. However, other workers could not confirm this, and so destruction of the organic matter and preliminary separation of the lead by hydrogen sulfide or as the oxalate was resorted to (67, 68, 69, 70, 71). Cooksey (72) was one of the later workers who used electrolysis to separate the lead from the unashed sample but his work also lacks confirmation. When the organic matter was destroyed,

either by ashing or by wet digestion with sulfuric acid, it was found to be necessary to separate the lead from phosphates and other salts before beginning the electrolysis (73, 77). For that reason electrolytic methods have never enjoyed much popularity in this field.

Bambach and Cholak were the first to show that lead could be separated quantitatively from a solution containing the ash of biological material by the use of citrate (74). If deleaded ammonium citrate is added to the prepared sample and the solution is made neutral or slightly alkaline with ammonium hydroxide, the interference of phosphate and other salts is prevented and the lead will be deposited on the cathode when the solution is electrolyzed. In other more lengthy electrolytic methods, employing a preliminary chemical lead separation, the lead is deposited on the anode as lead dioxide. In any case, the lead can be dissolved and determined by dithizone, by titration, or in other ways.

Chromate Methods

The chemical method for lead determination which probably was the most widely used, at least in this country, before the advent of dithizone is that developed by Fairhall (56). Biological samples, except

in the case of urines, are prepared by driving off the water by heat in the presence of nitric acid and ashing the residue in a muffle furnace at a carefully controlled temperature. Lead can be lost by volatilization if the temperature is allowed to go above 500 C. The ash is dissolved in hydrochloric acid or a mixture of hydrochloric and tartaric acids, neutralized with sodium hydroxide, and then made just acid to methyl orange with hydrochloric acid. The solution is saturated with hydrogen sulfide, allowed to stand over night, filtered, and the precipitate is washed with water. It is dissolved in nitric acid, hydrogen sulfide is expelled by boiling, andthe solution is made just alkaline to phenolphthalein with sodium hydroxide. Acetic acid is then added until the solution is acid and the lead is precipitated with potassium chromate. After standing over night the solution is filtered, the precipitate is washed with warm water and dissolved in dilute hydrochloric acid.

Determination of the lead in the hydrochloric acid solution is done indirectly by estimation of the chromate ion, either by titration with sodium thiosulfate or colorimetrically. In the first procedure, an excess of potassium iodide is added and the liberated iodine

is titrated with 0.005 N sodium thiosulfate solution. The colorimetric method consists of adding s-diphenylcarbazide in glacial acetic acid and comparing the color produced with that made by known amounts of chromate ion.

The method used in the determination of lead in urine is somewhat shorter (75), because in this case the sample need not be dried and ashed. Instead, ammonium hydroxide is added to the urine, precipitating the calcium and magnesium phosphate, and the lead phosphate is carried down quantitatively by entrainment. The phosphate precipitate is then added, the ash is dissolved in acid, and the lead is precipitated by hydrogen sulfide and determined as the chromate in the usual way. It is possible, of course, that this phosphate precipitation method may give slightly low results, since it would not determine the lead present in urine as a soluble unionized organic compound.

This hydrogen sulfide-chromate-diphenylcarbazide method was used fairly successfully with some modifications by many investigators (29, 32, 76, 78). One modification worthy of mention is that introduced by Letonoff and Reinhold (79). The ashed sample is dissolved in hydrochloric acid, sodium citrate and acetic acid

added, and lead chromate precipitated directly without previous separation of the lead as the sulfide. According to these workers, a double chromate of lead and potassium, $PbCrO_4 . K_2CrO_4$, is precipitated within a pH range of 7.4 to 6.6. This precipitate can be washed with ammonium acetate solution and the lead determined indirectly by the color developed with s-diphenylcarbazide, as described previously. In this case, however, a factor of two is used because of the presence of the double chromate.

Dithizone Methods

The greatest advance in chemical methods for lead determination began when Hellmut Fischer announced the use of dithizone (diphenylthiocarbazone) as a reagent for lead (80). Dithizone forms colored complexes with many metals; these complexes are soluble in organic solvents, and so this reagent provides a way of separating and concentrating the metal, in addition to determining it colorimetrically. Specificity is attained by the use of other compounds which form stronger complexes with some of the metals which would otherwise interfere, and by proper regulation of the pH of the extraction. Thus, in alkaline solution and in the presence of cyanide,

dithizone will extract only lead, divalent tin, monovalent thallium, and bismuth (81). Zinc, mercury, cadmium, gold, silver, and copper are prevented from reacting with the dithizone by the complex ions they form with cyanide. In the sample preparation generally used, tin and thallium (if any is present) are oxidized to their higher valence states, so that the only metal which will interfere is bismuth. This metal can be separated from lead by extracting the aqueous solution at pH 2-3 with dithizone; at this pH bismuth forms a stable complex and is extracted, while the lead stays behind (82).

The various dithizone methods all employ similar procedures for the lead isolation, but the determination of the lead is done in three ways: titrimetrically, colorimetrically, and photometrically. Wilkins, Willoughby, and Kraemer (83) have devised an excellent titrimetric-extraction procedure. The sample is prepared by wet oxidation with sulfuric, nitric, and perchloric acids, evaporated to a small volume, taken up in sodium chloride-hydrochloric acid solution, and made alkaline to litmus with ammonium hydroxide. Citric acid and potassium cyanide are added, and the solution is again made alkaline. The lead is extracted by dithizone in chloroform and then transferred to the aqueous phase

by shaking the chloroform extract with dilute nitric acid. Potassium cyanide is added to the lead nitrate solution, the pH is set at 7.5-8.3, and the titration is begun. This titration is carried out by adding from 15 to 25 per cent of the volume of dithizone solution estimated to be required (from the initial extraction), the lead is extracted, and the chloroform solution is drained off. Then smaller portions of the dithizone solution are added until the last portion retains its unchanged green color.

In single-color colorimetric methods the excess dithizone must be removed from the solution containing the lead complex before the colorimetric reading is made, for otherwise the solution has a mixed color due to the presence of both the red complex and the green dithizone. Fischer and Leopoldi (84) extracted the lead with dithizone in the usual way, washed out the excess dithizone with ammonium hydroxide and potassium cyanide, and converted the resulting pure lead complex into green dithizone by shaking it with dilute acid. The intensity of the green color was then measured in a colorimeter and found to bear a linear relationship to the quantity of lead. Winter and co-workers (85) based their method on this procedure, but they did not decompose the lead-dithizone

complex. Instead, they took readings of the red complex, even though the colorimeter readings-quantity of lead relationship was not linear, and compared these readings to a calibration curve. Methods of this type have the disadvantages that they are not very accurate, and also that fading of the color frequently occurs after the excess dithizone has been removed from the solution containing the lead complex.

Photometric methods are probably the most rapid and practical for routine work in lead determination by dithizone. The so-called "mixed-color" procedures are well adapted for use with photometers and with them it is not necessary to remove the excess dithizone from the standard solution containing the lead-dithizone compound. Clifford and Wichmann (86) developed a procedure using a neutral wedge photometer, which formed the basis for the method employed in this research. In Clifford and Wichmann's procedure the sample is prepared by ashing at 500 C, the ash is dissolved in hydrochloric acid, and citric acid and potassium cyanide are added with sufficient ammonium hydroxide to bring the pH to about 8.5. The lead is extracted by shaking with successive portions of dithizone in chloroform, then stripped from the dithizone solution by shaking it with dilute nitric

acid. The nitric acid solution is then filtered into another funnel, an ammonia-cyanide buffer solution is added to bring the pH to 9.5, and the proper dithizone standard solution is introduced. After shaking the funnel for one minute the dithizone solution is filtered into an absorption cell and the reading is taken on a suitable photometer. The authors of the procedure designed a wedge photometer for this purpose, but various commercial photometers, both visual and photoelectric, are now available which do the work very well. Various improvements and modifications of Clifford and Wichmann's method have appeared (87, 88, 89), showing that the procedure is widely used at present.

Physical Methods

Spectrographic and polarographic methods for the determination of lead in biological material are based upon physical rather than chemical phenomena. The spectrographic method of Cholak (90, 91) was one of the first procedures sensitive and accurate enough for this type of work. A solution of the ashed sample containing a known quantity of bismuth as an internal standard is placed in the crater of a purified graphite electrode and the arc spectrum photographed while a five-step

logarithmic sector is being rotated before the slit of the spectrograph. The lead line (2833.07 Å.) and the bismuth line (2898.1 Å.) on the plate are then compared by means of a densitometer. In the evaluation of very weak lines the determination of opacity instead of the usual density measurement is used. The lead in the prepared sample need not be isolated or concentrated in this method.

With the polarograph, on the other hand, both lead isolation and concentration must be employed, since the method is not sensitive enough to detect normal quantities of lead in prepared samples of urine or blood. For that reason the polarographic methods so far worked out differ mainly in their preliminary treatment of the prepared sample. Two ways of concentrating and isolating the lead have been employed in the polarographic method developed by this laboratory (92). One method involves a preliminary dithizone extraction, similar to the extraction used in ordinary dithizone procedures, and the other was of concentrating the lead by electrolysis. This is carried out in the presence of citrate to prevent the interference of phosphate, as mentioned previously in the discussion of electrolytic methods for lead. In either case a known quantity of cadmium is added to the

solution to be polarized in order to serve as an internal standard. The height of the cadmium step compared to the height of the lead step indicates the quantity of lead present. Changes in temperature, supporting electrolytes, or size of the mercury drops affect the height of the lead and cadmium steps similarly, so that the resulting lead determination is independent of these variable factors.

Experimental

A modification of the dithizone-photometric method proposed by Clifford and Wichmann was used in this work. Hubbard (87) had changed the original method, making it more suitable for the analysis of biological samples by introducing a test for bismuth and by using a double extraction so that mixed foods and feces, which contain large quantities of various salts, could also be analyzed. This last method was quite satisfactory, but it was a rather lengthy procedure and occasional difficulty was experienced with blood and other samples containing iron, due to oxidation of the dithizone extraction solution. In addition, the bismuth test was not very sensitive, so that there was danger that small quantities of bismuth would not be detected and would be estimated as lead.

Accordingly, the procedure as outlined in the description of Clifford and Wichmann's method was shortened by omitting the two filtrations, where the nitric acid solution containing the lead is filtered into another funnel, and where the standard dithizone solution is filtered into an absorption cell before taking the photometer reading. Each step was carefully tested and it was found that the omission of these two filtrations made no appreciable difference in the analytical results; in fact, chances for lead contamination from filter papers and from the extra manipulations were decreased. The second filtration through paper into the cell was especially dangerous, since not only lead but many other metals, as zinc and copper, can cause a color change in the standard dithizone solution at this point. It was found that even acid-washed filter papers frequently contained traces of these metals.

The bismuth test was made more sensitive by the introduction of a step in which the initial dithizone extraction containing the lead is washed with water. Apparently, various ions (particularly cyanide, citrate, and chloride) entrained by the dithizone extraction solution and transferred to the dilute nitric acid solution used for stripping the lead from the dithizone,

inhibit the extraction of bismuth by pH 2-3 (93). Since the bismuth test depends upon this extraction, the sensitivity of the test is decreased greatly by the presence of these entrained ions. Therefore the washing step, which removes the undesirable ions, increased the sensitivity of the bismuth test so that three micrograms can easily be detected.

Samples containing iron, as blood, mixed food, and fecal samples, frequently were difficult to analyze by the Clifford-Wichmann-Hubbard methods because oxidation of the dithizone occurred during the initial lead extraction. This oxidation was probably caused by the ferricyanide in the sample solution. Fischer and Leopoldi (84) and Behrens and Taeger (94) prevented this oxidation by using hydroxylamine hydrochloride in the sample solution. However, it was felt that hydroxylamine might reduce at least a small part of the large quantity of tin which is usually present in fecal and mixed foods samples, so that interference due to stannous tin could result. Normally, of course, tin is completely oxidized to the stannic form during the sample preparation, so that it does not react with dithizone. In order to determine whether hydroxylamine would reduce tin and cause interference in the lead determination, prepared

samples of feces and mixed foods were analyzed by Hubbard's method with and without the addition of hydroxylamine, and were also analyzed for tin and lead by a spectrographic method (95). Table I gives the results, showing that hydroxylamine causes no interference, even though relatively large quantities of tin may be present.

Table I

Effect of Hydroxylamine

Sample	Lead (Hubbard's Method)*		Spectrographic Method	
	Without Hydroxyl-amine mg.	With Hydroxyl-amine mg.	Lead mg.	Tin mg.
Food, 1886	0.16	0.16	0.16	3.75
Food, 2198	0.34	0.34	0.35	7.8
Feces, 2243	0.56	0.55	0.56	3.0

*Aliquots of one tenth of the sample were used.

This work showed that hydroxylamine hydrochloride could be added routinely to prepared samples of biological material to prevent oxidation of dithizone during the initial extraction, without fear of causing interference. Thousands of samples have since been run without any indication of interference due to hydroxylamine.

The significance of the results given by microanalytical methods depends largely upon the magnitude of the reagent blank, especially where very small quantities are to be determined. If adventitious contamination is practically eliminated, a low blank can be attained by careful purification of all reagents. In the case of lead, contamination is kept to a minimum by providing a relatively dust-free atmosphere in the laboratory, by using Pyrex glassware throughout and by scrupulous attention to cleaning all glassware with nitric acid and double-distilled water. In the course of this investigation it was found possible to delead the reagents so that the reagent blank remained between nil and 0.1 microgram, which is probably the lowest consistent blank for lead analyses yet reported. Reagent blanks of 2 to 5 micrograms are most frequently encountered (45, 50, 85, 86, 96, 97). In this work, where a difference of less than 1 microgram of lead in the analytical results on the plasma may mean that 30% instead of 10% of the total blood lead is found in the plasma, it is apparent that a reagent blank of several micrograms would decrease the significance of the final results and conclusions.

PROCEDURE

Preparation of Sample

The blood sample is put into a small silica dish, about 3ml. of distilled nitric acid are added, and the sample is dried on a hotplate. It is then ignited in a muffle furnace at 500 C., the ash moistened with nitric acid, dried, and ignited again, and finally dissolved in a few ml. of a mixture of distilled hydrochloric and nitric acids.

Extraction of Lead

Ammonium hydroxide: Reagent ammonium hydroxide is distilled into double-distilled water which is chilled in an ice bath.

Ammonium citrate solution: Four hundred grams of citric acid are dissolved in water and sufficient reagent ammonium hydroxide to make the solution alkaline to phenol red and water added to 1 liter. The solution is purified by shaking it with repeated portions of a solution of dithizone in chloroform until the dithizone does not change in color.

Hydroxylamine hydrochloride solution: Twenty grams of hydroxylamine hydrochloride are dissolved in

sufficient water to make about 65 ml. and a few drops of m-cresol purple indicator solution are added. Concentrated ammonium hydroxide is next added until a yellow color results. Sodium diethyldithiocarbamate in water (an approximately 4 per cent solution) is added in sufficient quantity to combine with all the lead (and most other metals) present and to leave a considerable excess. After a few minutes the organo-metallic complexes and the excess reagent are completely extracted with chloroform. The absence of a yellow color in the chloroform when a portion of the chloroform extract is shaken with a dilute solution of copper salt indicates when this point is reached. Redistilled hydrochloric acid is then added to the hydroxylamine hydrochloride solution until the indicator turns pink, and redistilled water is added to make the final volume 100 ml. It is not necessary to filter the solution.

Potassium cyanide solution : A substantially saturated solution containing 50 grams of potassium cyanide in sufficient water to make 100 ml. is repeatedly shaken with portions of dithizone in chloroform (30 mg. per liter) until the lead is removed. Part of the dithizone dissolves in the aqueous phase but sufficient remains in the chloroform to color it and to

indicate when the lead has been completely extracted. Most of the dithizone in the aqueous phase can be removed, if desired, by repeated extractions with pure chloroform. The strong potassium cyanide solution is then diluted with redistilled water to the proper strength (10 grams per 100 ml.). It is not necessary to filter the solution. (If instead of the concentrated solution, the final one is shaken with dithizone in chloroform in an attempt to delead it, the increased alkalinity of this dilute solution causes the removal of all the excess dithizone from the chloroform and renders the complete extraction of the lead more difficult.)

Dilute nitric acid: Ten milliliters of nitric acid, sp.gr. 1.40, are diluted to 1 liter with redistilled water; or approximately 9.1 ml. of reagent nitric acid may be used instead of the other acid.

Dithizone extraction solution: One liter of chloroform is shaken with 100 ml. of water containing about 0.5 g. of hydroxylamine hydrochloride and which has been made alkaline to phenol red with ammonium hydroxide. The chloroform is drained off, and 30 mg. of dithizone are dissolved in the chloroform. Approximately 5 ml. of absolute alcohol are added to the solution if

part of it is to be kept for several days. The quantity of dithizone solution to be used for one day is shaken with about 100 ml. of dilute nitric acid just before use.

The solution of the prepared sample is poured directly into a clean separatory funnel, 15 ml. of ammonium citrate solution , 5 ml. of potassium cyanide solution, and 1 ml. of hydroxylamine hydrochloride solution are added, and the mixture is made alkaline to phenol red with ammonium hydroxide. Five ml. of dithizone extraction solution are added and the funnel is shaken vigorously. At this point it is necessary to estimate by the color of the solution whether or not the quantity of lead present is over 10 micrograms. Then another 5-ml. portion of dithizone solution is added, the funnel is shaken again, and the 10 ml. of chloroform are drained into another clean separatory funnel. The lead is extracted completely with repeated 5-ml. portions of dithizone solution, draining each portion into the second funnel, until two successive portions of dithizone remain an unchanged color after shaking. The sample solution is then discarded and the funnel is washed.

The collected dithizone extract is shaken

with 50 ml. of redistilled water, drained into the first funnel, and the water is washed with 5 ml. of pure chloroform. This chloroform washing is added to the first dithizone extract and the water is discarded. Then the dithizone solution, which contains the lead, is shaken with 50 ml. of dilute nitric acid. The lead is immediately stripped from the dithizone by this operation and the chloroform is drained off and discarded.

The dilute nitric acid solution containing the lead is brought to pH 2-3 (orange color of m-cresol purple) with distilled ammonium hydroxide and shaken with 0.5 ml. of dithizone extraction solution. If no bismuth is present the dithizone remains unchanged in color; if bismuth is present it is extracted by the dithizone. Repeated extractions are then necessary at this point to remove the bismuth completely. All dithizone is drained off, 5 ml. of pure chloroform are added, and the funnel is shaken. The aqueous solution is then ready for the final lead estimation.

Final Estimation of Lead

The chloroform added in the last step is drained out completely, and the proper standard dithizone solution is added to the separatory funnel. Two standard

dithizone solutions are used: a solution for the range 0-10 micrograms of lead (it contains 5 mg. dithizone per liter), and one for the range 10-50 micrograms (containing 10 mg. dithizone per liter). Ten ml. of the first solution are used for each sample, in conjunction with a 2-inch photometer cell. A third standard dithizone solution which can combine with 100 micrograms of lead is used with some samples, but it was not needed in this research.

Seven ml. of ammonia-cyanide solution (20 g. potassium cyanide and 150 ml. of distilled ammonium hydroxide, sp. gr. 0.90, per liter) are added and the funnel is shaken for one minute. The dithizone solution is then drained into the proper photometer cell and the reading is taken. This reading is referred to a standard curve which has been made by taking known quantities of lead contained in 50 ml. of dilute nitric acid and carrying them through the procedure beginning with the words " The dilute nitric acid solution containing the lead....."

Discussion of Method

The primary lead standard used in this work was a solution of recrystallized lead nitrate in the dilute nitric acid specified in the list of reagents,

containing 1 mg. of lead per ml. The standard solution was checked against standard sodium oxalate obtained from the National Bureau of Standards, using the method given in the United States Pharmacopoeia XI for the assay of lead acetate. More dilute standard lead solutions were made from this primary standard by ordinary volumetric dilution with dilute nitric acid. Bureau of Standards calibrated glassware was used throughout.

Two photometers were used; the first was a neutral wedge photometer constructed according to the description given by Clifford and Wichmann (86), with a glass filter giving maximum transmission at 510 millimicrons. The second photometer was similar to the Pulrich step photometer in principle; it consisted of the light source, photometer, and eyepiece of a Bausch and Lomb spectrophotometer, with a glass filter in the eyepiece. Both instruments were very satisfactory, but the second one was somewhat superior optically.

This procedure is quite accurate for a micromethod; errors can be kept below 3% with quantities of lead over 5 micrograms. With less than 5 micrograms of lead the error is about 0.2 microgram. The figures in Table II indicate the accuracy of the method. They

represent analyses made on samples of deleaded salts simulating the salts of normal urine (each sample is equivalent to 100 ml. of ashed urine) (91), to which had been added quantities of lead unknown to the analyst.

There has been a recent statement that dithizone methods for lead are not specific (98), so that quantities of lead in urine as indicated by dithizone measurements may be greater than the true lead content. While thousands of workers all over the world are using dithizone for such

Table II

Lead Added Micrograms	Lead Found Micrograms
0	0.2
3	2.9, 3.0
5	5.0, 5.1
6	6.0, 6.2
9	9.0, 9.2
17	17.0,17.2
25	24.5,25
50	49.5,50
75	74 ,75
100	100 ,100

determinations with success and with no indication that the methods, correctly applied, are not specific for lead, a direct proof that at least the method used in this work is specific is afforded by comparative analytical results obtained by this method and two other independent procedures. Table III shows the results on samples of biological material given by the dithizone method described above and the polarographic (92) and spectrographic (91) methods previously mentioned. When three methods, based on entirely different and independent principles, yield closely concordant results, one can only conclude that the methods are specific as applied and that figures obtained by them are significant.

BLOOD ANALYSES

Studies on the partition of lead in blood have all been made on serum and clot, or on plasma and cells with the use of anticoagulants, except in one instance where Blumberg and Scott (43) used a sample of hemophilic blood. No record could be found of a series of lead analyses on plasma and cells where no anticoagulant was used. In addition, no data was found concerning the partition in arterial blood compared with that in venous blood. For that reason a technique was worked out by means

Table III

Comparative Results on Common Biological Material

Material	Lead by Polarographic Method: Electrolytic isolation and concentration mg.	Lead by Polarographic Method: Combined extraction and electrolysis mg.	Lead by Dithizone Method mg.	Lead by Spectrographic Method mg.
Feces[a]	0.55	0.56	0.58	0.58
	0.29	0.29	0.30	0.32
Food[b]	0.21	0.20	0.22	0.22
	0.44	0.39	0.43	0.41
	mg./l.	mg./l.	mg./l.	mg./l.
Urine	0.035	0.036	0.038	0.043
	0.170	0.160	0.170	0.170
	0.086	0.082	0.087	0.087
	mg./100g.	mg./100g.	mg./100g.	mg./100g.
Blood	0.065	.....	0.070	0.065
	0.080	.....	0.095	0.085
	.....	0.071	0.071	0.075
	.....	0.060	0.064	0.070

[a]Twenty-four hour samples
[b]Mixed food samples duplicating that eaten by experimental subject in 24 hours.

of which arterial and venous blood samples could be obtained and separated into cells and plasma with and without the use of anticoagulants.

Adult female rabbits were given lead acetate by spraying a solution of the salt on the pellets of a prepared rabbit food. Sufficient lead acetate was used so that each rabbit was receiving about 150 mg. of lead daily. After eleven days, a blood sample showed 0.04 mg. of lead per 100 g. of blood; this level increased to 0.06-0.08 mg. per 100 g. after another week, and did not rise significantly as long as the same amount of lead acetate was used (three months).

Procedure

The technique of obtaining blood samples from the rabbits is as follows:

A staimless steel needle with a stainless steel hub (not the usual brass hub) is used, with a clean Pyrex hypodermic syringe from which the metal spring clip or plunger guide has been removed. About 1 ml. of heavy mineral oil is put in the dry syringe and some of the oil is expelled through the needle. The blood sample is taken from the left or right heart, depending upon whether arterial or venous blood is desired. If an

anticoagulant is used the blood is expelled into a centrifuge tube containing the dry anticoagulant, mixed by agitation, and centrifuged for five to ten minutes. If no anticoagulant is used the blood is poured (with as little disturbance as possible) into a chilled paraffined centrifuge tube and held in an ice bath for one minute. It is then centrifuged for five minutes.

The plasma is removed as completely as possible by a pipette, expelled into a dry silica dish, and the cells (with the small quantity of plasma remaining) are poured into another tared dish. Both dishes are weighed and are then ready for the usual sample preparation and lead determination. All tubes, pipettes, dishes, etc, are cleaned with dilute nitric acid (1 + 1) and double distilled water and every possible precaution is taken to avoid lead contamination from dust or other sources.

Analytical Results

Assuming that the blood is half plasma and half cells by weight, the quantity of lead in the total plasma is calculated and subtracted from the lead in the total sample in order to obtain the quantity in the cells.

An example will illustrate this calculation:

Weight of plasma .....3.7 g.

Weight of cells (+ some plasma)...6.3 g.

Total weight of sample .....10.0 g.

Weight of total plasma .....5.0 g.

Weight of total cells .....5.0 g.

Lead analyses:

3.7 g. plasma contained 0.3 microgram of lead

6.3 g. cells (+ some plasma) contained 8.1 micrograms

Total lead in sample = 8.4 micrograms, or 0.085 mg. lead per 100 grams

5.0 g. plasma would contain 0.4 micrograms of lead

8.4 minus 0.4 = 8.0 micrograms of lead in the cells

Proportion of total lead in the plasma = $\frac{0.4}{8.4}$ = 5%

Table IV gives the results on the lead partition between plasma and cells in arterial and venous blood.

The plasma-cell partition of lead in rabbit blood in the presence of anticoagulants is given in Tables V, VI, and VII. In the first table about one milligram of a very powerful preparation of heparin was used for each 10 gram sample of blood. Sodium citrate

Table IV

Lead Partition in Arterial and Venous Blood

Rabbit Number	Sample	Wt. of Sample grams	Total Lead micrograms	Total Lead mg./100 g.	Lead in Plasma micrograms	Lead in Cell Fraction micrograms	% of Total Lead in Plasma
1	Arterial	10.0	4.8	0.05-	0.6	4.2	12.5
1	Venous	10.3	4.0	0.04-	0.3	3.7	7.5
2	Arterial	10.9	7.7	0.07+	0.4	7.3	5
2	Venous	5.0	4.1	0.08	0.4	3.7	10
2	Arterial	9.8	7.3	0.075	0.2	7.1	3
2	Venous	10.0	8.5	0.085	<0.1	8.5	<1
4	Arterial	9.3	7.0	0.075	0.1	6.9	1.5
4	Venous	9.3	6.0	0.065	0.4	5.6	7
5	Arterial	10.4	11.1	0.11-	0.8	10.3	7
5	Venous	9.7	11.2	0.11+	1.7	9.5	15
6	Arterial	11	8.5	0.08-	0.7	7.8	8
6	Venous	9.7	8.1	0.085-	0.5	7.6	6

Table V

Effect of Anticoagulants upon Plasma-cell Pb Partition

Heparin

Rabbit Number	Sample	Wt. of Sample Grams	Total Pb micro-grams	Total Pb mg./100 g.	Pb in Plasma micro-grams	Pb in Cell Fraction micro-grams	% of Total Lead in Plasma
3	No anticoagulant	10.6	6.6	0.06+	0.3	6.3	5
3	With heparin	8.7	5.7	0.065	<0.1	5.7	<2
4	No anticoagulant	9.8	8.6	0.09-	0.9	7.7	10
4	With heparin	9.1	7.6	0.085	0.6	7.0	8
5	No anticoagulant	10.3	7.5	0.07+	0.35	7.15	5
5	With heparin	9.0	6.6	0.075-	0.3	6.3	5
6	No anticoagulant	10.3	8.5	0.08+	0.6	7.9	7
6	Withheparin	9.0	7.2	0.08	0.8	6.4	11
7	No anticoagulant	8.5	4.7	0.055	<0.1	4.7	<2
7	With heparin	9.0	5.1	0.055	<0.1	5.1	<2

Table VI

Effect of Anticoagulants upon Plasma-Cell Pb Partition

Sodium Citrate

Rabbit Number	Sample	Wt. of Sample grams	Total Pb micrograms	Total Pb mg./100 g.	Pb in Plasma micrograms	Pb in Cell Fraction	% of Total Lead in Plasma
2	No anticoagulant	9.2	5.7	0.06+	0.3	5.4	5
2	With citrate	8.9	7.5	0.085	0.7	6.8	9
2	No anticoagulant	10.8	6.2	0.06-	0.5	5.7	8
2	With citrate	8.6	6.1	0.07	0.7	5.4	11
3	No anticoagulant	9.9	7.0	0.07+	0.3	6.7	4
3	With citrate	7.7	4.9	0.065	0.2	4.7	4
4	No anticoagulant	10.3	9.0	0.085	0.3	8.7	3.5
4	With citrate	8.2	6.9	0.085	0.5	6.4	7
4	No anticoagulant	10.0	8.4	0.085	0.4	8.0	5
4	With citrate	8.8	7.2	0.08+	0.4	6.8	6
7	No anticoagulant	10.0	6.7	0.065	0.1	6.6	1.5
7	With citrate	9.1	5.7	0.065	<0.1	5.7	<2

Table VII

Effect of Anticoagulants upon Plasma-Cell Pb Partition

Potassium Oxalate

Rabbit Number	Sample	Wt. of Sample grams	Total Pb micro-grams	Total Pb mg./100 g.	Pb in Plasma micro-grams	Pb in Cell Fraction micro-grams	% of Total Lead in Plasma
3	No anticoagulant	10.1	6,2	0.06+	0.3	5.9	5
3	With oxalate	9.4	5.9	0.065	0.1	5.8	2
4	No anticoagulant	10.4	8.4	0.08+	0.9	7.5	11
4	With oxalate	11.0	8.6	0.08-	0.1	8.5	1
7	No anticoagulant	10.0	6.7	0.065	<0.1	6.7	<2
7	With oxalate	8.7	5.5	0.065	<0.1	5.5	<2
8	No anticoagulant	9.9	4.6	0.045	<0.1	4.5	<2
8	With oxalate	9.1	3.8	0.04+	<0.1	3.8	<2
8	No anticoagulant	8.5	3.5	0.04	<0.1	3.5	<3
8	With oxalate	6.6	2.9	0.045	0.1	2.8	3

Table VIII

Plasma-Cell Partition of Lead in Human Blood

Sample	Wt of Sample grams	Total Pb micro-grams	Total Pb mg./100 g.	Pb in Plasma micro-grams	Pb in Cell Fraction micro-grams	% of Total Lead in Plasma
No anticoagulant	9.5	3.5	0.035+	0.2	3.3	6
With heparin	10.0	3.5	0.035	0.1	3.4	3
No anticoagulant	10.6	3.4	0.03+	<0.1	3.4	<3
With heparin	10.3	3.5	0.035	0.1	3.4	3
No anticoagulant	11.6	3.6	0.03+	<0.1	3.6	<3
With heparin	10.9	3.6	0.035	<0.1	3.6	<3
No anticoagulant	10.5	15.2	0.14+	0.8	14.4	5
With heparin	9.8	13.8	0.14	0.9	12.9	7

was used in the analyses given in Table VI. A solution of reagent quality sodium citrate (30 g. per 100 ml.) was deleaded by treatment with dithizone, the chloroform driven off by heat and 0.3 ml. of the solution were put into a centrifuge tube and dried. In the analyses given in Table VII approximately 10 mg. of reagent quality potassium oxalate were used with each 10 grams of blood. The heparin and the potassium oxalate were not deleaded because the quantities used were so small that the lead contained in them was insignificant.

Human blood from a subject who had been exposed to lead but whose level had dropped to a normal value, and from another subject with severe lead exposure, was analyzed in the same way as the rabbit blood in Table V. In this case the blood was drawn from an arm vein with a stainless steel needle and oiled Pyrex syringe and treated exactly as the rabbit blood samples.

In order to determine the plasma-cell partition of lead added to normal blood in vitro, a heparinized sample of rabbit blood was used. Lead chloride solution was added to this blood sample, which was stirred gently and kept warm in the hand for 15 minutes. It was then centrifuged and the plasma and cell fractions analyzed for lead in the usual way (Table IX).

The volume of lead chloride solution added to the blood was purposely kept low in order to prevent significant alteration of the water content of the blood and to prevent hemolysis. The chloride was used instead of the nitrate or acetate to avoid addition of a foreign ion to the blood.

All workers who have studied the plasma-cell partition of blood lead have separated the two fractions obtained by centrifugation and have assumed that the lead found in the cell fraction was lead which had been connected with the cells in some way, either by being in the erythrocytes or by being adsorbed on their surface. Instead of being actually connected with the cells it is possible, but not probable, that the lead could be present in the blood in the form of a dispersed compound, either organic or inorganic, and that when the blood is centrifuged this compound, being heavier than the plasma, settles to the bottom part of the centrifuge just as the cells do. Washing the cells, as Blumberg and Scott (43) did , does not rule out this possibility, for the cells are separated from their suspension in the washing medium by centrifuging the mixture, and this hypothetical compound could follow them to the bottom again. It was felt that other manipulations, such as hemolysis, would

not settle the point because any treatment which would hemolyze the cells could also change this possible compound in some way.

Evidence to prove or to disprove the connection of lead found in the cell fraction with the actual cells was obtained by centrifuging duplicate 10 ml. samples of heparinized normal rabbit blood. The plasma was drawn off the two samples and combined, and lead chloride solution was added to it. The same quantity of the same solution was used that was added to one of the whole blood samples in Table IX.

The plasma was stirred and kept warm for 15 minutes, then centrifuged at the same speed and for the same length of time as the samples in Table IX. The top half of the plasma was drawn off, just as in the case of a whole blood sample, and analyzed for lead. The bottom half was mixed with the erythrocytes (from which the white cells had been removed) from one of the two 10-ml. whole blood samples mentioned above, kept warm for 15 minutes, and centrifuged as before. The plasma and cell fractions of this sample were then analyzed as usual, together with the cell fraction left from the other duplicate sample. The results are given in Table X.

Table IX

Lead Added to Blood in Vitro

Sample	Wt. of Sample grams	Total Pb micro-grams	Total Pb mg./100 g.	Pb in Plasma micro-grams	Pb in Cell Fraction micro-grams	% of Total Pb in Plasma
Normal Blood - no Pb added	10.4	1.3	0.01+	0.1	1.2	8
Pb Added	10.7	9.4	0.09	0.9	8.5	10.5
Pb Added	10.0	20.0	0.20	2.5	17.5	12.5
Pb Added	11.5	40.4	0.35	3	37.4	7.5
Pb Added	9.9	54	0.55	11	43	20

Table X

Sample	Wt. of Sample grams	Total Pb micro-grams	Pb micro-grams per g.
I			
Cell Fraction - no Pb added	6.7	1.5	0.2
Top half of centrifuged plasma, Pb added	5.3	17.5	3.3
Lower half of plasma, mixed with cells, then centrifuged - plasma portion	3.7	6.9	1.9
Cells from above mixture	5.5	11.5	2.1
II			
Cell Fraction - no Pb added	4.8	1.0	0.2
Top half of centrifuged plasma, Pb added	5.3	18.5	3.5
Lower half of plasma, mixed with cells, then centrifuged - plasma portion	5.0	13.0	2.6
Cells from above mixture	3.2	8.3	2.6

Discussion

The application of modern analytical methods to problems of lead metabolism has made experimental results much more significant than they were in the past. The dithizone method described in this paper, with its accuracy, specificity, and extremely low blank, is a useful tool for the study of such problems. The conflicting opinions in the literature on the occurence of normal lead, the plasma-cell partition of blood lead, and other phases of lead physiology are no doubt due to conclusions drawn from misleading experimental results which have come from the use of inadequate analytical methods.

The results in Table IV indicate that there is no significant difference between the plasma-cell lead partition in arterial blood and that in venous blood. At least 90% of the total blood lead is found in the cell fraction; it is apparent that the quantities of lead in the plasma are so small that variations of a fraction of a microgram cause relatively large variations in the absolute percentage of the total lead contained in the plasma. It is possible that even the small quantity of lead found in the plasma, especially at lower lead levels, is the result of a contamination which is

unavoidable in the present state of analytical technique. Constant use of the dithizone method described has indicated that the lead determination itself, exclusive of the sample preparation procedure, is precise to 0.2 microgram. However, lead contamination may occur while the sample is being dried and ignited, so that with quantities of lead below 10 micrograms in the original sample the total error may be as large as 0.5 microgram.

Anticoagulants may be used in the plasma-cell separation of blood, at least within the lead levels investigated, without causing significant change in the lead partition, as shown by Tables V, VI, and VII. Heparin, sodium citrate, and potassium oxalate were used in these experiments. The most satisfactory anticoagulant for this purpose seems to be heparin; potassium oxalate suffers from the disadvantage that lead oxalate is a very insoluble salt and could conceivably cause precipitation of lead in the plasma. However, it is probably not insoluble enough to account for the very small quantity of lead found in the plasma; four ml. of water will dissolve six micrograms of lead as the oxalate (157), while in 4-5 g. samples of oxalated plasma not over 0.1 microgram of lead was found.

The investigation of the plasma-cell lead

partition was extended to human blood and the results in Table VIII confirm the findings of Blumberg and Scott (43), Willoughby and Wilkins (45), and Kehoe, Cholak, and Story (46). Here again practically all of the lead was found in the cell fraction, and no significant difference was seen in the partition when no anticoagulant and when heparin was used. Unfortunately, only one human blood sample at higher lead levels was available, but in the future when such samples are received the plasma-cell lead partition will be determined on them.

Other workers have added lead salts to blood *in vitro* (37, 42), but have not given any actual data regarding this work. They have merely stated that the added lead was found in the cell fraction without mentioning the quantities employed. Table IX shows that when lead was added to normal rabbit blood (heparinized), most of the lead was found in the cell fraction, and that the ratio was approximately the same as that found in the blood of rabbits which were ingesting lead. When sufficient lead was added to the blood to be equivalent to a lead level of 0.35 mg. per 100 g., which was higher than that reached in any feeding experiment, at least 90% of the total lead was still found with the cells.

However, when this added lead was increased so that the lead level reached 0.55 mg. per 100 g., the fraction of the total lead associated with the cells decreased to 80 %, which seems to indicate that the lead concentration of the cells reached a saturation value near that point. Some hemolysis occurred in all the samples reported in Table IX (especially in the last one) so that the conditions were not exactly comparable to cases in which the lead had reached the blood through physiological absorption, which may explain why the lead extraction by the cells was not as complete as it is in the living animal.

As mentioned in the previous section, all of this work on the plasma-cell partition of blood lead has been based upon separation of the blood fractions by the centrifuge, with the assumption that the lead found in the cell fraction was actually associated with the cells and had not settled to the bottom of the centrifuge tube merely because it was a heavy lead compound. The results in Table X indicate that this assumption is probably correct, since if a heavy compound containing lead had been formed in the plasma and had settled out during centrifugation, the top half of the plasma would have contained very little lead (quantities of the order of the plasma analyses in Table IX). Instead,

the top half of the plasma contained about as much lead as the sum of the other two analyses (especially after allowance was made for the normal lead already in the cells), showing that the lead was homogeneously distributed throughout the plasma after centrifugation. This indicates rather strongly that in plasma-cell analyses the lead found in the cell fraction is actually associated with the cells in some way, perhaps by being in the cells themselves or by being adsorbed on their surface.

Unexpectedly, it was found at this point that when the cells were returned to the plasma to which lead had been added they did not remove the lead from the plasma, as they did when the same lead solution was added to whole blood. This is shown by the last two analyses in each experiment in Table X, in which the amount of lead per gram in the lower half of the plasma after being mixed with the cells was about the same as that in the cell fraction. This indicates that the lead was equally distributed throughout the mixture and was not taken out of the plasma by the cells.

The inability of the cells to remove the lead may be due to two factors: the cells were injured in some way when they were centrifuged so they could no

longer adsorb lead; or the lead, when added to the plasma alone, was rendered inactive by the plasma so that the cells could have no effect upon it. The second possibility at first glance seems rather unlikely, since lead, when added to whole blood, must surely pass through a thin layer of plasma to reach (and become associated with) each cell. However, if the inactivating reaction of the lead with the plasma were slower than that which takes place with the cells the second possibility would be explained. In any case, the fact that lead, when added to whole blood, is removed by the cells indicates that the lead-cell association or compound is more insoluble than the possible compound formed when lead is added to plasma alone.

That the ability of the cells to adsorb lead was not injured when they were separated by centrifugation was shown in two ways:

(1) Normal rabbit blood (heparinized) was separated into plasma and cells, then the two fractions were mixed together thoroughly and after a few minutes the lead chloride solution was added to the recombined whole blood. After mixing as usual for 15 minutes and centrifugation the plasma and cell fractions were analyzed and the lead partition compared with that

obtained when the same amount of lead solution was added to a duplicate sample of normal blood. It was found that an equal proportion of the total added lead was removed by the cells in both instances, indicating that cells which had undergone centrifugation could still adsorb lead in some way.

(2) Normal rabbit blood (heparinized) was separated into plasma and cells; the plasma was removed and discarded, and the cells were washed with an equal volume of an isotonic saline solution containing a known quantity of lead chloride solution. The cells and the saline were separated by centrifugation and analyzed, and it was found that most of the lead in the saline had been removed by the cells, giving results similar to these obtained in the previous experiment. All analytical figures are given in Table XI.

Other factors concerning the unexpected inability of the cells to remove lead which had been added to the plasma were investigated. It was felt that if the lead-plasma compound were a loose combination with a protein the addition of another metallic ion, such as sodium, might cause a replacement of the lead by sodium and thus allow the cells to extract the lead. Accordingly, lead chloride solution was mixed with the

Table XI

Sample	Wt. of Sample g.	Total Pb micro-grams	Total Pb mg./100 g.	Pb in Plasma micro-grams	Pb in Cell Fraction micro-grams	% of Total Pb in Plasma
Pb added directly to whole blood	9.9	54	0.55	11	43	20
Pb added to centrifuged remixed blood	9.7	55.5	0.57	10.5	45	19
Cells washed with Pb-containing saline		20.9		(saline) 3.9	17	(in saline) 19

Table XII

Sample	Wt. of Sample g.	Total Pb micro-grams	Total Pb mg./100g.	Pb in Plasma micro-grams	Pb in Cell Fraction micro-grams	% of Total Pb in Plasma
Cells added to contaminated plasma immediately, then separated after 15 minutes	9.1	22	0.24	10	12	45
Cells added to contaminated plasma after 10 minutes, then separated after 15 minutes	8.2	21.3	0.26	10.5	10.8	49
Cells added to contaminated plasma after 10 minutes, then separated after 2 hours	9.0	22	0.24	10	12	45
Cells added to contaminated plasma immediately, then separated after 24 hours	9.2	14.6	0.16	3.3	11.3	23
Cells added to contaminated plasma after 10 minutes, then separated after 24 hours	9.8	13.9	0.14	3.7	10.2	27

plasma obtained from 9 g. of heparinized blood, then after 10 minutes 50 mg. of sodium chloride were added. After another short wait the cells were returned to the plasma, mixed, and after 15 minutes were separated again. It was found that the lead remained almost equally distributed between the cells and the plasma, and that the sodium chloride had had no effect in allowing the cells to remove the added lead.

The time factor was investigated next. When the lead was added to the plasma, stirred, and the cells poured in as soon as possible, there was no significant difference between this lead partition and that found in those experiments where about ten minutes had been allowed to elapse before the cells were added to the contaminated plasma. This indicates that the reaction which inactivates the lead when it is added to the plasma is rather rapid.

It was felt that the length of time the cells were allowed to remain in the contaminated plasma might have some effect upon the lead partition. Experiments were carried out in which the lead was added to the plasma, the cells stirred in, and then the mixture was allowed to stand with agitation for two hours and for twenty-four hours. In the two-hour sample the

concentration of lead in the cells was not significantly greater than that in the plasma, but in the twenty-four hour sample about three-fourths of the total lead was found in the cell fraction, indicating that if sufficient time is allowed to elapse the cells will gradually remove lead from the plasma, even though that lead has previously formed a relatively inactive complex with the constituents of the plasma. Longer periods of equilibration were not tried because of the degenerative changes that appear in blood after it is shed. The results of these experiments are given in Table XII.

In order to detect any possible localization of lead in leucocytes, a sample of heparinized blood from one of the lead-exposed rabbits was separated into plasma, buffy layer, and red cell fractions. No significant difference was found between the lead concentration in the buffy layer and that in the red cell fraction, while the plasma, as usual, contained only a slight amount of lead.

During the course of the analyses on plasma to which lead had been added it was found that a small quantity of lead sometimes went into the pores of the silica dish used for igniting the sample at 500 C. This lead could not be removed by washing or even by

soaking the dish for days in nitric acid, but portions of it would go into the ash of subsequent analyses, causing abnormally high results. The dishes were finally cleaned by repeated treatment with deleaded salts, simulating the ash from a normal urine, igniting the salts at 500 C., at which point fusion had occurred. This difficulty has never been encountered in the analysis of urine, whole blood, or of the cell fractions, probably due to the greater quantity of ash in those cases. With blood plasma taken from normal or lead-poisoned animals the quantity of lead in the plasma is so small that no absorption by the dish takes place, but where an unnatural amount of lead is added to the plasma a small quantity is sometimes lost in the dish, resulting in a possible source of contamination not often recognized.

Increased quantities of lead were added to the food of the experimental rabbits in order to raise their blood lead above the levels reported in Tables IV, V, VI, and VII. They had ingested approximately 150 mg. of lead per animal per day for three months; this quantity was increased to about 250 mg. for one month, and since the blood level showed no marked rise during that time, a second increase to 500 mg. of lead per animal per day was tried. However, the rabbits did not eat this highly

contaminated food well and began to lose weight; therefore after two weeks the lead content was decreased so that each animal then ingested about 250 mg. of lead daily. The blood level showed a significant rise in each rabbit during this time, yet the percentage of lead in the plasma still remained below 10% of the total lead. A few determinations of the serum-clot lead partition were also made, which showed no significant departure from the plasma-cell partition, so that all of these results can probably be compared to those obtained by workers using the serum-clot separation. Analytical figures are given in Tables XIII and XIV.

The results reported in these various tables do not agree with those given by Tompsett and Anderson (47), which were mentioned previously. They found about one-third of the total human blood lead in the plasma, using potassium oxalate as an anticoagulant. This was true in both normal and lead poisoning cases. With normal rabbits and those receiving lead orally, from one-third to over one-half of the total blood lead was found in the serum. It is probable that this disagreement in the results is due to differences in the analytical methods used. However, in justice to the figures given in this paper, it should be mentioned that they are in substantial

Table XIII

Plasma-Cell Partition of Blood Pb - Higher Pb Levels

Rabbit Number	Sample	Wt. of Sample g.	Total Pb	Total Pb mg./100 g.	Pb in Plasma	Pb in Cell Fraction	% of Total Pb in Plasma
	Pb in food - approx. 250 mg./rabbit/day						
2	No anticoagulant	13.0	12.8	0.10	0.9	11.9	7
4	No anticoagulant	12.0	10.3	0.085	0.3	10	3
	Pb in food increased to approx. 500 mg./day						
2	Heparin	12.2	14.7	0.12	2.2	12.5	15
2	No anticoagulant	9.5	13.5	0.14	1.0	12.5	7.5
3	No anticoagulant	11.1	11.1	0.10	1.2	9.9	11
4	No anticoagulant	11.9	16.6	0.14	1.6	15	10
7	No anticoagulant	11.0	32.3	0.29	1.4	30.9	4
	Pb in food decreased to approx. 250 mg./day						
2	No anticoagulant	10.5	15	0.14	<0.1	15	<1
2	No anticoagulant	10.5	14.7	0.14	0.7	14	5
3	No anticoagulant	10.2	7.4	0.07+	0.4	7	5
7	No anticoagulant	10.9	31.3	0.29	1.3	30	4
8	No anticoagulant	11.1	10.5	0.095	0.5	10.	5
8	No anticoagulant	10.7	8.1	0.075	0.6	7.5	8

Table XIV
Comparison of Plasma-Cell and Serum-Clot Lead Partitions

Rabbit Number	Sample (No Anti-Coagulants Used)	Wt. of Sample g.	Total Pb micro-grams	Total Pb mg./100 g.	Pb in Plasma or Serum micro-grams	Pb in Cell Fraction or Clot micro-grams	% of Total Pb in Plasma or Serum
2	Plasma-Cell	10.5	15	0.14	<0.1	15	<1
	Serum-Clot	10.1	14.5	0.14	0.5	14	3
2	Plasma-Cell	10,5	14.7	0.14	0.7	14	5
	Serum-Clot	11.3	14.2	0.13	0.7	13.5	5
3	Plasma-Cell	10.2	7.4	0.07+	0.4	7.0	5
	Serum-Clot	10.7	7.3	0.07-	0.4	6.9	6
7	Plasma-Cell	11.0	32.3	0.29	1.4	30.9	4
	Serum-Clot	10.6	31.4	0.30	1.9	29.5	6
7	Plasma-Cell	10.9	31.3	0.29	1.3	30	4
	Serum-Clot	10.4	30.0	0.29	1.0	29	3
8	Plasma-Cell	11.1	10.5	0.095	0.5	10	5
	Serum-Clot	10.8	10.3	0.095	0.3	10	3
8	Plasma-Cell	10.7	8.1	0.075	0.6	7.5	8
	Serum-Clot	11.7	8.9	0.075	0.9	8.0	10

agreement with those obtained by dithizone by Willoughby and Wilkins (45) and spectrographically by Blumberg and Scott (43) and by Kehoe, Cholak, and Story (46). In addition, the conclusions stated here have been based upon a very large number of closely agreeing analyses, apparently much greater in number than those cited by workers who have reported opposing figures.

The reaction of lead with blood plasma which seems to inactivate the lead has been noticed by others. For instance, when red cells are exposed to lead which has been previously mixed with serum the characteristic effects on fragility and stickiness of the cells are no longer produced (158). Aub and Reznikoff (159) and Orskov (160) believe that the inactivating substance in serum is inorganic phosphate. It is possible that this so-called inactivation is similar to that noticed in this work when the lead which had been added to blood plasma was not readily removed from the plasma by the red cells mixed in later.

Summary

This research has included the development of a photometric dithizone method for the determination of lead in biological material, with a comparison of the

results obtained by this method with those given by two other independent procedures. The dithizone method was shown to be accurate and to have satisfactory specificity.

The plasma-cell partition of blood lead was investigated and the following points were brought out:

(1) It is probably correct to assume that the lead found in the cell fraction when rabbit blood is centrifuged is there by virtue of an actual association with the cells, and not simply because some lead compound had been formed which had settled out with the cells during the centrifugation. If some lead compound of this type is present in blood it is formed only in the presence of the cells, not with plasma alone.

(2) There is no significant difference between the plasma-cell lead partition in arterial blood and that in venous blood.

(3) The use of heparin, potassium oxalate, or sodium citrate does not cause a significant change in the plasma-cell lead partition.

(4) The plasma-cell and the serum-clot partitions of blood lead in rabbits do not differ significantly.

(5) At least 90% of the total blood lead of rabbits is associated with the cells; this applies both to normal animals and to those exposed to large quantities of lead.

At least 90% of the total blood lead of human subjects is found in the cell fraction.

(6) When lead chloride solution is added to the whole blood (heparinized) of normal rabbits most of the lead is taken up by the cells; over 90% of the total lead is found in the cell fraction with lead levels as high as 0.35 mg. of lead per 100 g. of blood, while at 0.55 mg. per 100 g. at least 80% of the lead is associated with the cells.

(7) When lead chloride solution is added to the heparinized plasma of the blood of normal rabbits the lead is inactivated by the plasma in some way, so that when the cells are returned to the plasma they will not remove the lead in a short period of time, but require 24 hours to remove as much as 75% of the total lead.

(8) This inability of the cells to remove lead which has been added to blood plasma is not due to some injury suffered by the cells during centrifugation, but is caused by some inactivating reaction (as yet unknown) of the lead with the plasma.

(9) These facts give rise to a possible explanation of the above observations: when lead is added to whole blood a rapid reaction with the cells takes place before a somewhat slower reaction with the plasma has time to

occur. This reaction with the cells results in the formation of a more insoluble or more non-reactive compound than that which takes place with the plasma. Therefore, when lead is added to plasma alone and the cells are introduced later, the inactivating lead-plasma reaction (which has already taken place) gradually reaches a more stable equilibrium in the lead-cell reaction, so that after sufficient time has elapsed most of the added lead will be found with the cells.

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