CL. THE DETERMINATION OF LEAD IN BIOLOGICAL MATERIALS

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(Received 22 June 1939)

FURTHER experience of the method of Tompsett & Anderson [1935] for the estimation of lead in human tissues and excreta has shown that in the analysis of different types of material modifications are an advantage in special cases. Also certain general improvements make it desirable to describe the method in more detail. A survey of the various methods for estimating minute amounts of lead in biological materials is not included as this has been done by us [1935] and more recently by Minot [1938].

The method consists essentially of three stages: (1) destruction of organic material, (2) extraction of Pb with ether as a complex with Na diethyldithiocarbamate, (3) colorimetric estimation of lead with dithizone.

Destruction of organic material

Two methods are available, viz. digestion with H_2SO_4 and some oxidant, e.g. HNO_3 or HClO_4 , and ignition.

The objection to the first method is that often large quantities of H_2SO_4 and oxidant are required and consequently large quantities of alkali are necessary for neutralization. This leads to a large blank even when the purest reagents are used.

Destruction by ignition, which avoids this high blank has been criticised on the grounds that Pb may be lost by volatilization. The writer has found that materials containing a large amount of ash, consisting in the main of phosphates, may be ignited in a silica dish over a bunsen burner without loss of Pb. When materials containing a low ash content are treated in this manner, a loss of Pb may occur. For this reason, the ash content of such materials is increased by the addition of Na phosphate. Biological materials are therefore divided into two classes:

(1) High mineral content—ignited without the addition of Na phosphate, e.g. urine, faeces, milk and bone.

(2) Low mineral content—Na phosphate added before ignition, e.g. blood, soft tissues.

The ignition method has been found to be quite satisfactory under these conditions and has been used throughout. Towards the end, ignition may be assisted by allowing the ash to cool, adding a little conc. HNO_3 and re-heating.

Separation of Pb with ether and Na diethyldithiocarbamate

Na diethyldithiocarbamate reacts with various metals to form complexes, many of which are soluble in organic solvents. Amongst the metals which occur in biological materials and react thus are Fe, Cu and Pb. Pb forms a complex which is soluble in ether. This complex is produced in acid and in alkaline solution and its formation is not inhibited by the presence of such substances as

citrate, pyrophosphate or cyanide. The formation of the Cu complex is inhibited if the solution is alkaline and contains cyanide in addition. The Fe complex is not formed if the solution is alkaline and contains in addition pyrophosphate, citrate or cyanide.

Before Pb may be estimated with the dithizone reagent it is essential to separate it from Fe and recent experience has shown that it is advantageous to separate it from Cu. Use of the above is made to achieve this. The separation is carried out by adding to a solution of the metals, Na citrate, NH_3 to make alkaline and then Na diethyldithiocarbamate. The Pb complex, which is the only one formed under these conditions, may then be extracted with ether. The presence of the Na citrate is essential so as to prevent the precipitation of hydroxides and phosphates in the alkaline medium.

This technique has been found to be quite satisfactory for urine, soft tissues and blood, but in the case of milk, bone and faeces, certain modifications have been found to be advantageous. The ash of the second class of material contains a considerable amount of Ca phosphate. As a result, sometimes in spite of the presence of citrate, troublesome precipitates of Ca phosphate appear when the mixture is made alkaline. Such precipitates may prevent the quantitative separation of Pb. For this reason, a twofold extraction is employed in the examination of these materials.

Na diethyldithiocarbamate is added to an acid solution of the ash. All the metallic complexes are formed and all are extracted with ether. The extracted metals are converted into the inorganic state and the process of extraction repeated but in an alkaline solution containing citrate and cyanide. Under these conditions, the Pb complex alone is formed, and this alone extracted by the ether.

The colorimetric estimation of Pb with dithizone

The extracted Pb is converted into the sulphate by digestion with H_2SO_4 . After digestion, water is added and then acetic acid. The mixture is then made alkaline by the addition of NH_3 . An alkaline reaction is essential for the occurrence of the reaction between Pb and dithizone and the presence of ammonium acetate ensures that the PbSO_4 is in solution.

The reaction is as follows. KCN and CCl_4 are added to the alkaline acetate solution of Pb. Dithizone solution is then added and the whole well shaken. The Pb forms a complex which dissolves in the CCl_4 to produce a red solution. The solution of dithizone in ammoniacal solutions is brown and in CCl_4 green. After the shaking process, unchanged dithizone will be distributed between the aqueous and CCl_4 phases. The next stage is to separate the CCl_4 layer and to free it from unchanged dithizone by shaking it with KCN solution, when uncombined dithizone is removed and an extract containing the red coloured Pb complex alone is obtained. This reaction is quantitative and the Pb present may be estimated by colorimetric comparison with a standard.

Certain precautions are however necessary. Excess of dithizone must be used to ensure that all the Pb present has been converted into dithizone complex, but too great an excess must be avoided.

Dithizone is very susceptible to oxidation and a substance is produced which is soluble in CCl_4 to produce a yellow coloured solution. This substance cannot be removed from CCl_4 by cyanide solutions. It is produced when Fe or Cu salts are present and by bright sunlight. In the earlier work, the influence of Fe salts was realized and they were carefully eliminated. The effect of Cu was not then known but owing to its generally low concentration in biological materials, the

effect was almost negligible. Occasionally trouble was experienced and this was found to occur in materials having a high Cu content. Upon removal of the Cu, no trouble was experienced. It has been decided, therefore, to eliminate Cu as well as Fe.

Bright sunlight causes a rapid production of the yellow oxidation product. This does not take place in ordinary diffuse light. It is believed that it is the ultraviolet component of bright sunlight which is responsible for this reaction [Tompsett, 1936]. In ordinary diffuse light the CCl_4 solution of the red coloured Pb complex will remain stable for a very long period.

Apparatus and reagents

Pyrex glassware was used throughout. Silica dishes were washed with hot dilute HCl before use. Glass-distilled water was used and filter papers were washed with dilute HCl followed by water.

The reagents used were the same as those described by Tompsett & Anderson [1935] with the following exceptions:

Dithizone reagent. Commercial dithizone contains an oxidation product which must be removed. In earlier work a mass purification was done but this has now been abandoned. A 0.1% solution of 0.1% crude dithizone in CCl_4 is prepared. Just before use, a small volume of this is shaken with an equal volume of 0.5% NH_3 . Dithizone but not the oxidation product passes into the aqueous layer. After allowing the mixture to settle, the ammoniacal extract is separated and used directly.

Na_2HPO_4 —10% in water—lead-free. Before use the requisite volume of a stock solution is introduced into a separating funnel, sodium diethyldithiocarbamate added and the mixture shaken vigorously with ether. After settling, the lead-free aqueous layer is run off.

The method

In the following, details of the method as applied to various materials are described. The final colorimetric estimation is the same in each case.

A. Extraction of lead

Urine. 500 ml. of urine are evaporated to dryness in a silica dish in a hot air oven. The residue is then ashed by ignition over a bunsen burner in a fume cupboard.

The ash is dissolved in 100 ml. of water containing 5 ml. of conc. HCl. The solution is transferred to a separating funnel, 50 ml. of 20% Na citrate added and the mixture made alkaline by the addition of ammonia. 5 ml. of 10% KCN are added and the mixture cooled thoroughly. 5 ml. of 2% Na diethyldithiocarbamate are added and the mixture extracted 3 times with ether, 25 ml. on each occasion. The ether extracts which are separately washed with water are transferred to a hard glass round-bottomed flask.

The ether is evaporated off and the residue digested with 1 ml. conc. H_2SO_4 and 1 ml. HClO_4 to destroy organic matter.

The residue is diluted with water, 1 ml. glacial acetic acid added, followed by 5 ml. ammonia sp.gr. 0.88, and the mixture diluted to 25 ml. by the addition of water.

Soft tissues. To 100 ml. of lead-free 10% Na_2HPO_4 in a silica basin are added 100 g. fresh tissue. After drying in a hot air oven, the material is ashed.

The ash is dissolved in 100 ml. water containing 10 ml. conc. HCl. The procedure is then as for urine.

Blood. To 100 ml. of lead-free 10 % Na_2HPO_4 in a silica dish are added 20 ml. blood. After drying in a hot air oven, the material is ashed.

The ash is dissolved in about 50 ml. water containing 5 ml. conc. HCl . After transference to a separating funnel, it is cooled, 5 ml. of 20 % Na citrate added and the mixture made alkaline to litmus by the addition of ammonia. 5 ml. of 10 % KCN are added, followed by 2 ml. of 2 % Na diethyldithiocarbamate. The mixture is extracted twice with ether, 20 ml. being used on each occasion. The ether extracts, which on each occasion are washed with water, are transferred to a 100 ml. hard glass round-bottomed flask.

The ether is evaporated off and the residue digested with 0.2 ml. conc. H_2SO_4 and 0.5 ml. HClO_4 . To the digest are added 3.5 ml. water, 0.2 ml. glacial acetic acid and 1.5 ml. ammonia, sp.gr. 0.88.

Faeces. 10 g. of dried faeces are ashed in a silica basin. The ash is dissolved in 100 ml. water containing 10 ml. conc. HCl . The solution is then made up to a volume of 200 ml. by the addition of water.

50 ml. of the ash solution are introduced into a separating funnel and 10 ml. of 2 % Na diethyldithiocarbamate added. The mixture is extracted 3 times with ether, 25 ml. on each occasion. The ether extracts are collected in a hard glass flask and the ether evaporated off. The residue is digested with 1 ml. conc. H_2SO_4 and 1 ml. HClO_4 .

The residue is diluted with water, 1 ml. conc. HCl added and the mixture heated to dissolve the digest. The solution is transferred to a separating funnel to make a volume of about 50 ml., 5 ml. of 20 % Na citrate are added and the mixture made alkaline to litmus by the addition of NH_3 . 5 ml. of 10 % KCN are added, followed by 5 ml. of 2 % Na diethyldithiocarbamate. The mixture is then extracted 3 times with ether, 25 ml. being used on each occasion. The combined ether extracts are transferred to a hard glass round-bottomed flask.

The ether is evaporated off and the residue digested with 1 ml. conc. sulphuric acid and 1 ml. perchloric acid. To the residue are added, water, 1 ml. glacial acetic acid and 5 ml. ammonia, sp.gr. 0.88, and the mixture is diluted to 25 ml. with water.

Bone. 20 g. of bone are ashed in a silica dish. The ash is dissolved in water containing HCl and the solution diluted to 200 ml., 50 ml. of this are taken and treated as in the case of faeces.

Milk. 500 ml. of milk are evaporated to dryness in a silica dish in a hot air oven and then ashed. The ash is dissolved in water containing HCl and treated as in the case of faeces.

B. The colorimetric estimation of lead

1. *Preparation of standard.* The following mixture is prepared. To 1 ml. conc. H_2SO_4 are added a little water, 1 ml. glacial acetic acid and 5 ml. ammonia, sp.gr. 0.88 and the whole diluted with water to a volume of 25 ml. giving ammonium acetate.

A known amount of Pb is added to 5 ml. of this mixture. To this are added 5 ml. of 1 % KCN and 10 ml. of CCl_4 . To this mixture is added drop by drop an ammoniacal solution of dithizone reagent, with constant shaking until excess has been added. Too great an excess must be avoided. Sufficient excess is indicated when the CCl_4 layer has reached its maximum intensity of redness and the aqueous layer is tinged brown. At this stage, the aqueous layer is removed and discarded. The CCl_4 layer, containing the red coloured lead complex, is shaken repeatedly with aliquots of 5 ml. of 1 % KCN until the aqueous layers

are no longer coloured. The CCl_4 layer may be passed through a filter to remove droplets of water and is then ready for comparison.

A range of standards may be prepared but the writer prefers to use a standard containing 0.02 mg. Pb, and to take the requisite amount of the unknown to conform to this. This standard may be obtained by using 2 ml. of a standard solution of lead acetate, containing 0.01 mg. Pb per ml.

2. *Preparation of unknown urine, faeces, soft tissues, bone and milk.* The extracted Pb is contained as PbSO_4 in a solution of ammoniacal ammonium acetate having a volume of 25 ml. For the estimation, 5 ml. of the solution are taken and to this are added 5 ml. of 1% KCN and 10 ml. of CCl_4 . The colour is developed as described above.

In the event of the Pb content of the unknown being low, 10 ml. of the solution are used, the amounts of the other reagents being the same. If the Pb content of the unknown is high, a smaller volume than 5 ml. is used. In this case the solution is diluted to 5 ml. by the addition of an ammoniacal ammonium

Table I. *The recovery of added Pb*

	Initial Pb content mg.	Pb added mg.	Total Pb found mg.	Pb recovered mg.
Urine (500 ml.)	0.120	0.050	0.163	0.043
	0.120	0.100	0.215	0.095
	0.120	0.200	0.308	0.188
	0.120	0.250	0.362	0.242
	0.120	0.500	0.625	0.505
	0.042	0.020	0.079	0.037
	0.042	0.050	0.096	0.054
	0.042	0.100	0.140	0.098
	0.155	0.200	0.360	0.205
	0.155	0.400	0.565	0.410
Faeces (10 g.)	0.030	0.200	0.235	0.205
	0.030	0.500	0.549	0.519
	0.030	1.000	1.036	1.006
	0.042	0.030	0.079	0.037
	0.042	0.050	0.096	0.054
	0.042	0.100	0.140	0.098
	0.031	0.040	0.072	0.041
	0.031	0.080	0.115	0.084
Milk (500 ml.)	0.031	0.120	0.154	0.123
	0.050	0.050	0.105	0.055
	0.050	0.100	0.145	0.095
	0.050	0.200	0.248	0.198
Liver (100 g. fresh)	0.050	0.500	0.560	0.510
	0.243	0.050	0.299	0.056
	0.243	0.100	0.350	0.107
	0.243	0.200	0.440	0.197
Blood (20 ml.)	0.243	0.400	0.640	0.397
	$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$
	16	10	27	11
	16	20	37	21
	16	40	58	42
	19	10	28	9
	19	20	39	20
	19	40	58	39
	12	10	22	10
	12	20	34	22
	12	40	53	41

acetate solution, having the same composition as that used to prepare the standard. The amounts of the other reagents are the same. The development of the colour is best carried out in glass-stoppered tubes.

Blood. In the case of blood, the whole of the Pb-containing solution is used. To the mixture containing the Pb in the flask used for the digestion, add 5 ml. 1% KCN and 10 ml. CCl_4 . The colour is developed as above.

C. Blank

A blank should always be done on a new set of reagents. In estimating the blank, the complete process is carried out. The blank is small and is thus difficult to estimate accurately so that the following method has been adopted. Before colorimetric estimation, 0.02 mg. Pb is added to the blank. This, after the development of the colour with dithizone, is compared with a standard containing 0.02 mg. Pb. The blank is then calculated from the difference.

A series of recovery experiments is shown in Table I.

DISCUSSION

It will be seen from the Table that added Pb could be estimated accurately. The method is fairly quickly performed. It is designed so that little apparatus is required and so that large scale purification of reagents is avoided.

The accurate estimation of Pb is of some importance as Pb is a "normal" hazard which at times may become unduly large, and it is also an important industrial hazard. In a recent publication Tompsett & Anderson [1939] have shown the value of Pb estimations, especially in the blood, in the diagnosis of plumbism.

SUMMARY

The estimation of lead in biological materials is described. The method comprises an earlier one in which are incorporated certain modifications.

In conclusion I wish to thank Dr A. B. Anderson for his helpful criticism and advice.

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