Histochemical Journal, 3 (1971), 463–469

Mercurous nitrate as a histochemical reagent for calcium phosphate in bone and pathological calcification and for calcium oxalate

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Received 21 July 1971

Synopsis. An aqueous solution of mercurous nitrate reacts with bone and tissue calcified sites with the formation of brown to black amorphous masses and feathery crystals, the last resembling the crystals formed from the action of an aqueous solution of mercurous nitrate on calcium orthophosphate. Calcium oxalate reacts with this mercurous nitrate solution to form brown to black deposits on the surface of the oxalate particles; this suggests an adsorption phenomenon. The brown deposits are blackened by ammonium hydroxide, gold chloride, and many sulphur-containing compounds.

Introduction

The histochemical use of the von Kossa (1901) silver nitrate technique for the recognition of calcium is well known. The procedure has been modified as to the concentration of silver nitrate, time exposure, presence and type of light (Pizzolato & McCrory, 1961) and the use of reducing agents. Hydrogen peroxide has been added for the detection of calcium oxalate (Pizzolato, 1964). Pizzolato & Lillie (1968) in a study on the uptake of metal salts by bone and calcified tissues found that the soluble mercurous salts produced black amorphous masses and feathery crystals at calcification sites. Since these reactions were not encountered previously, their characteristics were investigated further.

Material and methods

Normal adult human and mouse foetus bones as well as calcified sites such as human cardiac papillary muscle, pineal, choroid plexus, calcifying tumours, placenta, arteriosclerosis, gout, chronic pancreatitis, thyroid and renal oxalosis (human and experimentally produced in rats; Pizzolato & Pizzolato, 1966) were fixed in either unbuffered or acetate-buffered formalin and 80% alcohol. The tissues were processed through acetone, petroleum ether (b.p. range 60–

100°C) and paraffin (Lillie, 1965, p. 72) and attached to the slides with Mayer's albumen. An excess of albumen was avoided as there was a tendency for the formation of a finely granular black precipitate. Deparaffinized slides were washed three times, 5 min each in distilled water and placed in the mercurous nitrate solution. This solution was prepared 2 to 3 days in advance by adding approximately 0.5 g mercurous nitrate (reagent or purified grades were satisfactory) to 50 ml distilled water in a covered Coplin jar. It was shaken two or three times daily for 2 or 3 days, after which it was then ready for use without filtering. The colourless or pale yellow crystalline undissolved substance turned to a white amorphous mass and then to a yellow residue in a few days. The supernatant solution contained about 10 mg% solid and had a pH of 2.8. Deparaffinized sections were placed in this supernatant solution for 5 to 15 min, washed in distilled water for 10 min, counterstained with 1% w/v aqueous Alizarin Red S (C.I. 58005) for 1 min, washed in distilled water for 5 min, dehydrated, and mounted in cellulose caprate. The mercurous nitrate solutions can be used without filtering for six months and 100 slides can be processed through the same solution. Light and heat did not influence the reaction as staining occurred in total darkness and at 5°C. Some control sections were stained with 1% w/v aqueous Alizarin Red S or 1% w/v aqueous silver nitrate. Others were decalcified in 1% nitric acid to ensure that the reaction was due to the mineral salts and not proteins.

If calcium oxalate was suspected, 1% w/v mercurous nitrate in 1% v/v nitric acid was used. Deparaffinized sections were placed in this solution for 5 min, washed in distilled water and treated as above.

The darkness of the granules was enhanced by placing in 1% ammonium sulphide, 2% ammonium hydroxide or 5% sodium thiosulphate (all aqueous solutions w/v) for 5 min before counterstaining with Alizarin Red S. Gold chloride (0.1% w/v) for 5 min or diphenylcarbazone (1% w/v in 95% alcohol) for 5 min was also used.

Deparaffinized sections were also treated with a range of mercury salts and then extracted with various acids, alkalis, salts, etc. Test-tube and slide experiments were performed by adding the aqueous and acid solutions of mercurous nitrate to various metal carbonates, phosphates, oxalates and calcium salts.

Results

After treatment with an aqueous solution of mercurous nitrate a dark brown to black deposit with a peripheral zone of black feathery crystals was formed in areas of normal and pathological calcification (Figs. 1, 2 & 3). Calcium oxalate yielded only dark brown amorphous or cuboidal masses with weak birefringency. If the mercurous nitrate was dissolved in 1% nitric acid, oxalate crystals appeared as brownish black and they lost their birefringency (Fig. 4). The other calcium deposits dissolved. The background was colourless or exhibited fine black granules. No colour changes were encountered in decalcified sections. When mercurous nitrate-treated calcified deposits or oxalate-containing tissues were treated with ammonium sulphide, ammonium hydroxide, sodium thiosulphate, sodium sulphide (2%), thiourea (5%), thioacetic acid (5%), thiolactic acid (5%), thioacetamide (5%), gold chloride (0.1%), sodium hydroxide (1% in 70% alcohol), or 2-mercaptoethanol (8% v/v in propyl alcohol), the brown masses turned black. Prolonged exposures (beyond 1 hr) to these agents results in some loss of the crystals and amorphous material. Similar blackening was noted with 2% aniline, morpholine, ethylcyclohexylamine or isopropylhexylamine in 95% alcohol. The masses and crystals were soluble in bromine water, 5% potassium cyanide and Gram's iodine solution, but were

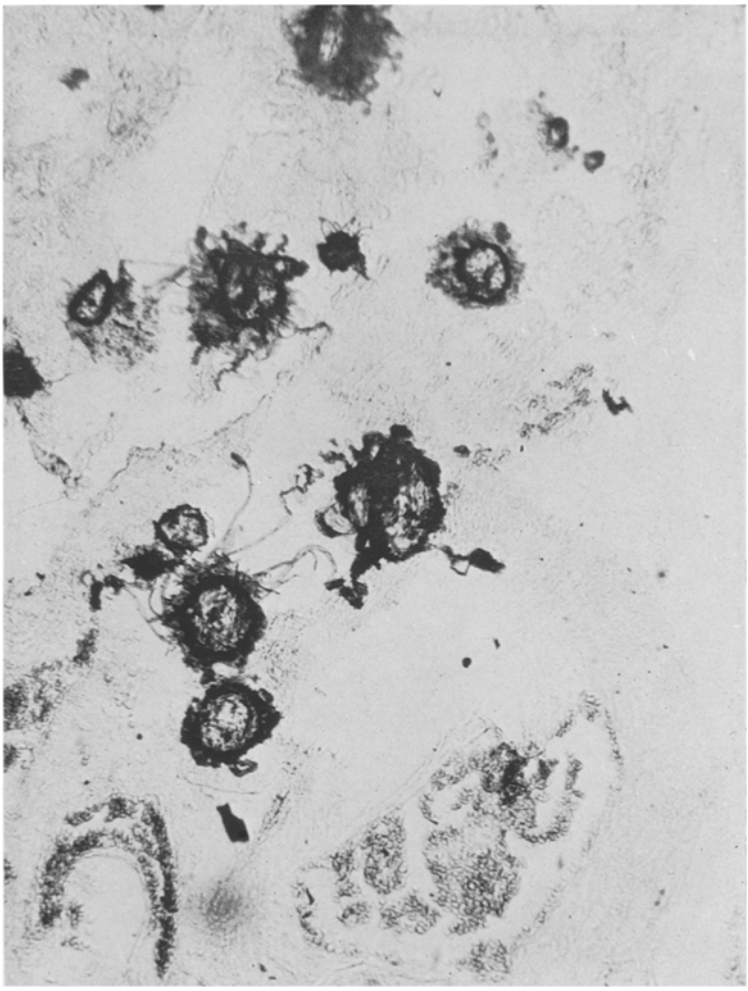

Figure 1. Choroid plexus treated with saturated mercurous nitrate solution for 5 min and washed in water. Corpora amylacea are stained as dark brown amorphous masses. No counterstain. × 160

insoluble in 1% v/v aqueous nitric, hydrochloric, sulphuric, phosphoric or picric acids, 5% acetic acid, 5% potassium iodide, 2% sulphosalicylic acid and 1% mercuric nitrate (all within 15 min).

If Alizarin Red S was used as a counterstain after the aqueous solution of mercurous nitrate, the centre of some large calcified sites stained red whereas the 'background' was coloured pale orange-red to yellow-red. No red particles were encountered when the nitric acid solution was used. Gold chloride and diphenylcarbazone acted as counterstains giving a pink to pale red 'background'.

Other mercurous salts as the acetate, sulphate and perchlorate gave similar but lighter brown amorphous and feathery crystalline deposits at calcified areas; mercurous chloride and mercuric salts did not show these characteristics.

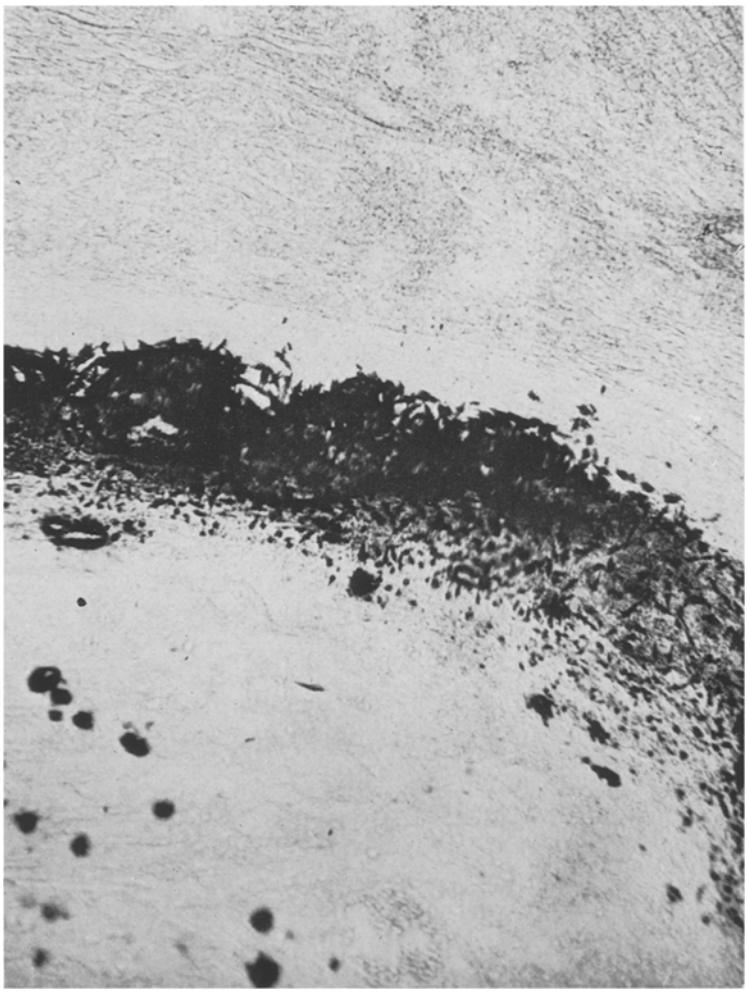

Figure 2. Calcified artery treated with saturated mercurous nitrate solution for 5 min, washed in water and in 2% ammonium hydroxide. No counterstain. The calcified sites show black amorphous material and small black feathery crystals. × 160

The phosphomolybdic acid and the ferric ferrocyanide tests of Feigl (1958) for metals were negative for the amorphous and crystalline material.

The addition of an aqueous solution of mercurous nitrate to a solution of sodium dihydrogen orthophosphate or *di*-sodium hydrogen orthophosphate resulted in a white precipitate of fine acicular crystals. *Tri*-sodium orthophosphate gave an amorphous dark grey precipitate. In the test-tube a suspension of the three varieties of calcium phosphate exhibited no reaction to the aqueous mercurous nitrate solution. However, when the mercurous nitrate solution was added directly to the dry crystals of the three calcium phosphates on a slide, calcium orthophosphate [$Ca_3(PO_4)_2$] yielded small black feathery crystals similar to those encountered in calcified tissues. The 'mono' and 'di' calcium phosphates (calcium tetrahydrogen di-ortho-

Figure 3. Heart muscle treated with saturated mercurous nitrate solution for 5 min, washed in water and toned with 0.1% gold chloride. The calcified muscles exhibit black amorphous material. × 160

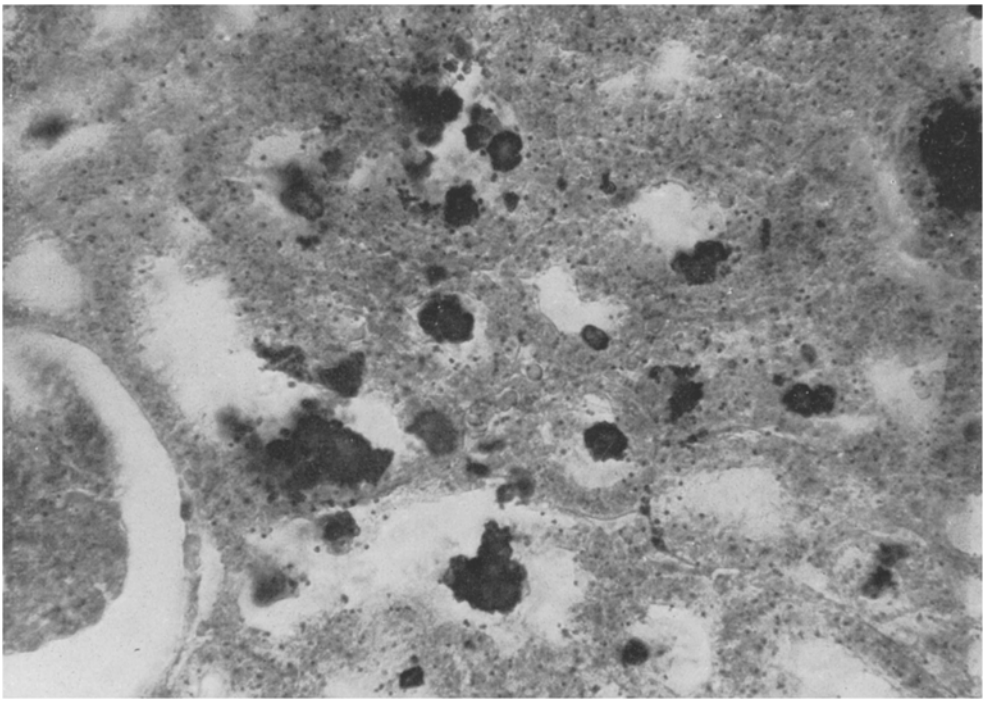

Figure 4. Kidney from patient with oxalosis treated with 1% mercurous nitrate for 5 min, rinsed in 2% ammonium hydroxide and counterstained with Alizarin Red S. Calcium oxalate is represented as black amorphous masses. × 200

phosphate and calcium dihydrogen orthophosphate respectively) produced needle-like crystals which gradually developed into black, small, irregular feather-like crystals. Aqueous solutions of mercurous nitrate and ammonium oxalate yielded a white precipitate composed of pale brown amorphous material.

Most insoluble salts showed no gross or microscopic reaction towards mercurous nitrate. If to a suspensions of various salts, an aqueous solution of mercurous nitrate was added, the addition of ammonium hydroxide to the thoroughly washed residue resulted in blackening in the cases of calcium carbonate and sulphate, and magnesium, calcium, strontium and barium oxalates (Table 1).

Table 1. Effect of an aqueous solution of mercurous nitrate on solutions or suspension of calcium salts, phosphates and oxalates.

Salts	*Gross changes*	*Microscopic changes*	*Action of 2% ammonium hydroxide on washed mercurous nitrate-treated sediment*
NaH_2PO_4	W ppt	G acicular	Bk acicular
Na_2HPO_4	W ppt	G acicular	Bk acicular
Na_3PO_4	G ppt	Bk amorphous	Bk amorphous
$Na_4P_2O_7$	W ppt	Bk amorphous	Bk amorphous
$Ca(H_2PO_4)_2$	W ppt	Bk needles and small feathery crystals	
$Ca(HPO_4)$	—	—	Bk amorphous
$Ca_3(PO_4)_2$	—	—	Bk & G amorphous
$Ca(CO_3)$	Y ppt	Y amorphous	Bk & G tiny cubes
$CaSO_4$	—	G acicular	Bk & G acicular
CaF_2	—	—	—
CaC_2O_4	—	—	Bk amorphous
MgC_2O_4	—	—	Bk amorphous
BaC_2O_4	—	—	Bk amorphous
SrC_2O_4	—	—	Bk amorphous
$(COONH_4)_2$	W ppt	Br amorphous	Bk amorphous
$BaSO_4$	—	—	—
$PbSO_4$	—	—	—

Abbreviations: — = No change; W = white; G = grey; Bk = black; Y = yellow; Br = brown; ppt = precipitate.

Discussion

When mercurous nitrate is added to water, hydrolysis occurs and produces a variety of slightly soluble hydroxynitrates of varying composition (Cox, 1929; Denham & Fife, 1933). However, sufficient salts of some kind are formed that react with bone and calcified sites to yield dark brown to black amorphous masses and feathery crystals. The features of these crystals are suggestive of those obtained by Chamot & Mason (1931) with mercurous chromate. The crystals resemble those produced when an aqueous solution of mercurous nitrate is added to calcium orthophosphate on a slide but not with a suspension of the phosphate in a test-tube, probably as a result of dilution.

The action of mercurous nitrate on calcium oxalate appears to be an adsorption phenomenon. No characteristic reaction was observed by mixing mercurous nitrate with calcium or magnesium oxalate and only a brown amorphous material was obtained with ammonium oxalate. The addition of ammonium hydroxide to thoroughly washed mercurous nitrate that had been treated previously with calcium or magnesium oxalate results in a blackening of the suspended particles.

The nature of the brown amorphous material produced by the action of mercurous nitrate on calcified tissues is still under investigation but appears to be reaction products such as mercurous phosphates, sulphides, oxides or free finely divided mercury resulting from the action of protein or some other tissue-reducing agents. Some of the black material is soluble in 2-mercaptoethanol and in sodium sulphide, the last being in agreement with the findings of Barber & Taylor (1933) with mercurous sulphide.

From the nature of the crystalline patterns, the reaction appears specific for tissue calcium orthophosphate but the desired localization cannot be ascertained precisely from the resulting growth of crystals. Eisenstein *et al.* (1961) had difficulties in localizing calcium deposits with chloranilic acid. Similar difficulties with diffusion of crystals were encountered with the technique of Lillie (1965) using sulphuric and oxalic acids. In the case of calcium oxalate, localization is precise and the black mercury deposits assumed the configuration and birefringency of the original oxalate crystal.

Acknowledgements

The aid of Mrs Carolyn Berger in the preparation of material and conduct of histochemical experiments, Mrs Frances McCabe in the preparation of this manuscript, and Mr Saul Weiss for the photographs is gratefully acknowledged. I am also especially indebted to Dr R. D. Lillie for his reading and criticism of this report. I also wish to thank Evans Chemetics, Inc., New York, for the thioacetic and thiolactic acids and Abbott Laboratories, North Chicago, Ill., for the ethylcyclohexylamine and isopropylcyclohexylamine.

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