

CHROM. 11,335

HYDROXYAPATITE FOR CHROMATOGRAPHY

II. SOURCES OF VARIABILITY AND IMPROVED METHODS OF PREPARATION

M. SPENCER

King's College Department of Biophysics, 26-29 Drury Lane, London WC2B 5RL (Great Britain)

(Received June 12th, 1978)

SUMMARY

Hydroxyapatite (HA) made by published methods was analysed at intermediate stages in the preparations. In the standard Tiselius method conversion from brushite to HA was complete before the temperature reached 100°, but extensive boiling was necessary to ripen the crystallites to a size giving strong binding of tRNA. In a second method (due to Main, Wilkins and Cole) which involves conversion at pH 8, a short-lived intermediate compound, octacalcium phosphate (OCP), was detected. In both methods the extensive agitation involved was liable to give a low flow-rate. A new method of conversion at neutral pH was developed, in which a well-formed OCP intermediate was transformed into HA of large crystallite size without boiling. Such material gave flow-rates of 40–80 ml h⁻¹ in a 9 × 75 mm column under 250 mm head at room temperature, values well in excess of those given by commercial products of similar binding properties. Boiling was employed only when it was desired to reduce the particle size to obtain very high resolution on small columns; these preparations gave flow-rates of 10–15 ml h⁻¹ under the conditions described. The importance of brushite morphology was investigated, and two alternative methods of crystallization were developed for maximizing either the flow-rate or the resolution of the HA end product. For maximum flow rate the brushite preparation was seeded to produce stellate clusters of plates. HA made from this brushite was mechanically stable and could be freeze-dried and reconstituted without loss of flow-rate. Other topics investigated were the synthesis of OCP and monetite (dehydrated brushite), and the effect of carbonate incorporation on chromatographic behaviour. Up to 1% CO₃ had no effect.

INTRODUCTION

A troublesome feature of chromatographic hydroxyapatite (HA) is that both the flow-rate and the binding strength (as shown by the elution molarity of a macromolecule) can vary widely from one preparation to another¹; even when a satisfactory flow-rate is achieved by use of a standardised method, the elution molarity can be

unpredictable². The resolution between peaks can also vary considerably. In part I¹ of this series it was shown that the elution molarity for tRNA is correlated with the crystallite size of HA. In this paper I shall show that the crystallite size can change during a preparation, in a way that explains why empirically developed methods³⁻⁷ include periods of boiling.

This prolonged boiling, together with the extensive washing required to remove excess alkali, can often lead to an unfortunate loss of flow-rate. The great advance proposed by Tiselius *et al.*³ was the replacement of a calcium phosphate gel, containing submicroscopic microcrystals, by material in which compacted masses of crystals retained the gross morphology of the brushite from which they were derived. Much of this advantage can, however, be lost by excessive agitation in preparation. In the work described here, a new method of conversion was developed which considerably reduced the risk of mechanical breakdown, while also cutting down the time required. I shall also discuss the crucial importance of brushite morphology in connection with the flow-rate and the resolution between peaks in gradient elution. The possible effects of contamination by carbonate are also investigated.

MATERIALS AND METHODS

General

Reagent grade chemicals were supplied by British Drug Houses (Poole, Great Britain) and Fisons (Loughborough, Great Britain). Flow-rates were measured at room temperature with a 9×75 mm bed under a pressure head of 250 mm. Determination of tRNA elution profiles and analyses for calcium and phosphorus were as in Part I¹.

X-ray diffraction

Photographic records of powder diffraction were obtained with Cu K α radiation from a microfocus generator fitted with a graphite monochromator, feeding a cylindrical camera of diameter 58 mm with a 0.5 mm collimator. Powdered calcite was used for calibration. Samples taken from different stages of a preparation were neutralised and washed to arrest chemical changes, or simply pipetted directly onto a pad of filter paper. Samples were dried at a temperature not exceeding 85° (to avoid heat-induced changes) and were mounted in glass capillaries.

Carbonate contents

Measurements were made by microdiffusion using No. 1 Conway units⁸. The central chamber of a unit contained 1 ml 0.05 M Ba(OH)₂ and the outer chamber 0.1 g of sample, mixed with 2 ml 6 M H₂SO₄ immediately before closing the lid. After gentle agitation for 60 min the Ba(OH)₂ was titrated to neutrality with 0.25 N HCl under a stream of nitrogen, using BDH Universal Indicator. A water blank was also titrated. The limit of detection was about 0.05% CO₃.

Brushite preparations

2.0 l 0.5 M CaCl₂ were added at 50 ml min⁻¹ to 2.4 l 0.5 M sodium phosphate buffer (pH 6.7). Mixing was in a 9-l bucket with a bath recirculator (B. Braun, Mel-

sungen, G.F.R.) having an impeller pump; the CaCl_2 was fed through a tube ending above the impeller orifice. For high flow-rate (type HF) preparations, the supply voltage was reduced to 130 V to give a circulation rate of about 6 l min^{-1} , and 1.0 g brushite seed powder was added to the phosphate solution before starting. Seed material was made by grinding a wet paste of brushite in a mechanical pestle and mortar until the particle size was about $3 \mu\text{m}$; it was either stored at 5° as a wet suspension, or dried at 85° and kept in a desiccator. For general-purpose preparations (type GP) and those for high resolution (type HR) no seed was added, and before starting the apparatus was cleaned by washing briefly in 0.1 N HCl . The mixer was in this case run at full voltage but the impeller orifice was reduced to half its full horizontal extent, giving a circulation rate of about 4 l min^{-1} ; this helped to break up crystal clusters.

After mixing the crystals were allowed to settle and washed in 5 l distilled water to remove soluble salts. Settled volumes were about 500 ml for type HF and 700 ml for GP and HR.

Conversion to HA

The brushite suspension described above was transferred (after removing the supernatant) to a well-lagged 5-l beaker fitted with a $50 \times 100 \text{ mm}$ paddle stirrer rotatable at 200 rpm. For type HF material, 5 ml 0.5 N HCl were added to inhibit the formation of OCP in the next stage, which (for all types of preparation) was the rapid addition with stirring of 2.5 l boiling distilled water. The temperature was now $70\text{--}75^\circ$ and the pH $5.0\text{--}5.5$. The stirrer was stopped and the sediment allowed to settle. The supernatant was removed by suction without delay and a further 5 ml 0.5 N HCl added to all preparations.

A further 2.5 l boiling water were then added, followed by the addition of about 72 ml 20% NaOH through a delivery tube reaching to the base of the beaker. The pH was monitored continuously using high-temperature electrodes with a platinum resistance thermometer, and the delivery rate of alkali was regulated (using a variable-speed peristaltic pump) so that the pH stayed at 7.0 ± 0.2 after the first 0.5 min, during which there was an initial drop to $5.5\text{--}6.0$ due to the rapid formation of OCP intermediate. Typical values of delivery rate were as follows: 0–0.5 min, 35 ml min^{-1} ; 0.5–1 min, 50 ml min^{-1} ; 1–2 min, 30 ml min^{-1} . During this time the temperature fell by about 6° from an initial value of $85\text{--}90^\circ$. Delivery was stopped when the pH stayed steady at 7.2.

After the sediment was allowed to settle and the supernatant removed the material was washed twice in 2.5-l aliquots of boiling water. After the final re-suspension the beaker was covered and transferred without removal of supernatant to an insulated cabinet, where it was allowed to cool slowly overnight; the initial rate was $0.3^\circ \text{ min}^{-1}$. The settled volume was about 550 ml for type HF and 750 ml for types GP and HR. HR material was further treated by diluting a portion of sediment with an equal volume of water and boiling vigorously for 30 min on a hotplate equipped with a magnetic stirrer. Stirring was continued during cooling after removal from the hotplate.

RESULTS AND DISCUSSION

Changes in properties during standard preparations

Small-scale preparations were made by two published methods^{3,6} and samples taken at different stages. The starting material was brushite, $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, made as described under Materials and methods. It gave the diffraction pattern shown in Fig. 1a; the photographic record shows rings that are "spotty" because of the large sizes (up to $50 \mu\text{m}$) of the crystallites.

The Tiselius method³⁻⁵ involves boiling brushite in an excess of NaOH. The

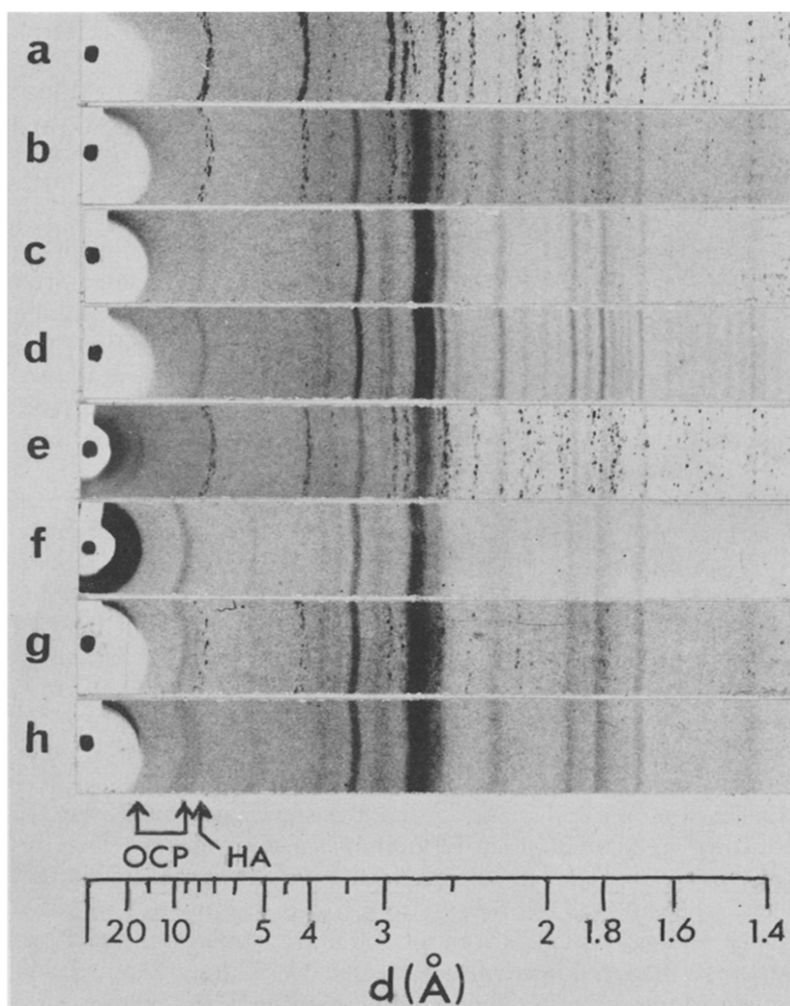


Fig. 1. Powder X-ray diffraction patterns. (a) Brushite, $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$; (b) partial conversion to HA by heating to 67° in an excess of alkali; (c) complete conversion to HA; (d) after 60 min boiling and 5 stages of resuspension, boiling, and decantation of supernatant³; (e) brushite heated to 50° at pH 8 (ref. 6); (f) reference sample of OCP; (g) and (h) stages in the conversion of brushite to HA at neutral pH, at temperatures of $70\text{--}80^\circ$. JCPDS file no.s: brushite 9-77, HA 24-33, OCP 26-1056.

present work confirmed and explained the observation of Jenkins⁵ that this must be added before heating is started; X-ray diffraction showed that boiling without alkali leads to a rapid partial conversion of brushite to other forms of calcium phosphate⁹, with disruption of the brushite morphology that is needed for adequate flow-rate. While heating in the presence of alkali the material showed a mixture of brushite and HA as in Fig. 1b; the HA rings are distinguished by their uniform density, due to the much smaller crystallite size of the HA phase. By the time the temperature reached 70–80° (10–15 min from the start of these experiments) the conversion to HA appeared complete, but the diffuseness of the rings indicated that the HA crystallites were still very small (Fig. 1c). Beyond this stage titration of samples showed that there was little further incorporation of alkali, but after the repeated boiling in dilute phosphate buffer that is a feature of this method the HA rings were considerably sharper (Fig. 1d).

This observation suggests that the main effect of the boiling stages—recommended by Tiselius *et al.*³ without explanation and followed by all authors since—is to ripen the HA crystallites to a size giving optimum binding strength¹. It was stated by Levin⁴ that even the final preparation contains incompletely converted brushite crystals, but the present X-ray results do not support this; the low Ca/P ratio of Tiselius material^{1,3} compared with the stoichiometric value of 1.67 is now believed to be due to deficiencies in the lattice¹⁰. The data in Table I give quantitative support for the ripening hypothesis: there is a parallel increase in both crystallite size and elution molarity as the preparation proceeds. The Ca/P ratio also changes during the preparation, but we have seen in Part I¹ that this does not appear to be directly correlated with the binding properties; also a change of the order shown would not affect very much the surface density of calcium ions. The binding capacities for protein, tRNA and DNA¹ were also measured, but did not change greatly during the preparation.

It is now possible to explain why the cold alkali method of making HA¹¹ gives smaller crystallites and low binding strength¹; the absence of heat treatment means that the initially formed crystallites do not grow larger. A very long incubation at room temperature might be equivalent to heating, but this has not been tested. In the cold alkali method, as in that of Tiselius, X-ray diffraction shows a direct conversion from brushite to HA.

Similar experiments were carried out with the CPA method of Main *et al.*^{6,7}. This is a shorter procedure than that of Tiselius, but it still involves a period of boiling and there are numerous washing stages. Again there is an increase in elution molarity and crystallite size on boiling (Table I), but in this case Ca/P changes very little. It is noteworthy that at the start of the boiling period this material gave stronger binding than Tiselius HA at a similar stage. Furthermore, X-ray diffraction showed that the conversion to HA proceeded differently. The method uses ammonia which is added continuously so as to maintain a pH of about 8. At a very early stage of the conversion, when the temperature had risen only to 50° (6 min from the start of the author's experiment), X-ray diffraction (Fig. 1e) showed brushite mixed not with HA but with octacalcium phosphate (OCP), $\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$. There was also amorphous material, giving a high background. Fig. 1f shows the diffraction of pure OCP for comparison; it is mainly distinguished from that of HA by a strong reflection at 18.7 Å, and a weaker pair of rings at 9.36 and 9.05 Å in

TABLE I
CHANGES IN HYDROXYAPATITE DURING PREPARATIONS
Measurements made by methods of Spencer and Grynepas¹

<i>Method</i>	<i>Stage in preparation</i>	<i>rRNA elution peak (M)</i>	<i>Crystallite size L₀₀₂ (nm)</i>	<i>Molar ratio Ca/P</i>
Tiselius <i>et al.</i> ³	Initial heating, when temp. reached 100° (45 min from start)	0.05	45	1.47
	After 60 min boiling	0.06	—	—
Main <i>et al.</i> ⁶	After 5 stages of boiling in phosphate buffer	0.09	53	1.56
	When temp. reached 100° (11 min from start)	0.07	49	1.54
	After 30 min boiling and buffer washes	0.10	62	1.55
pH 7 conversion (see text)	NaOH added at 80°	0.10	63	1.52
	After boiling for 30 min	0.11	77	1.54

place of the 8.17 Å of HA. At later stages (not shown) the patterns showed HA mixed with a trace of OCP which finally disappeared.

This sequence of transitions has been reported before, but only in the absence of added alkali⁹. The present results show that it can occur under mildly alkaline conditions as well; it may occur in the Tiselius procedure too, but the higher pH could have made the OCP stage too transient for it to be recorded. It seems clear that a more rapid production of suitably sized HA crystallites is associated with a detectable OCP intermediate. However, the procedure shares the disadvantage of the Tiselius method that prolonged boiling and washing can lead to a breaking up of the massed crystallites with consequent reduction in flow-rate. The example in Table I actually increased in flow-rate upon boiling, but this was not always so; there is always a risk of "bumping" which varies in extent with the apparatus employed, and the process is not easy to control. Furthermore, increases in flow-rate due to aggregation are liable to be only temporary, as the aggregates can break up when the material is packed in a column.

Conversion to HA at neutral pH

In an effort to improve on the flow-rate and reproducibility of standard methods a new procedure was developed empirically that retained heat treatment but avoided boiling, and the studies reported in Part I¹ gave insights which enabled the effects of the different variables to be clearly identified. The essential difference from other methods is that conversion from brushite to HA takes place at neutral pH, even lower than the pH 8 of Main *et al.*⁶. This has two advantages: firstly it is unnecessary to wash out excess alkali at the end of the preparation, and (even more importantly) there is a rapid production of large HA crystallites without extensive boiling and changes of buffer. It appears to arise because the production of an OCP intermediate is more pronounced than at higher pH values.

This is illustrated by Figs. 1g and 1h, in which ammonia was added to a hot suspension of brushite. The first change is from brushite to OCP, and the intensities of the two innermost sets of OCP rings (at 9.2 and 18.7 Å) are high com-

pared with the background, showing that the OCP intermediate is better crystallized than in Fig. 1e. In Fig. 1h there is still a strong OCP component, though all brushite spots have disappeared and the 8.2-Å spacing of HA is prominent. The final preparation showed only HA as in Fig. 1d.

In agreement with the conclusion reached above it was found that the binding strength for tRNA could further be increased by incubation at high temperature, without resorting to boiling. The new standard preparations described under Materials and methods incorporate such treatment, and their properties are described later in this paper.

The difference between these and other preparations is illustrated quantitatively by the last set of data in Table I. The preparation chosen was intended for high resolution between peaks (see later) and was boiled to improve this property. However, even before boiling the crystallite size and binding strength for tRNA were comparable to those of other preparations after prolonged boiling and washing.

Variables in brushite production

The method used to make brushite influenced the HA end product in several ways; for instance the flow-rate after conversion depended critically on the morphology of the brushite. In the Tiselius method³ the brushite is made by mixing equal streams of 0.5 M solutions of CaCl₂ and Na₂HPO₄. This was found to give clusters of brushite plates whose longest dimension was about 100 μm, with a high flow-rate of about 100 ml h⁻¹ (see Materials and methods). However, this material was found by X-ray diffraction to contain a small proportion of OCP which rendered it unsuitable for conversion to HA at neutral pH: it gave an excessive early production of OCP, nucleated by the pre-existing OCP, and conversion to HA was inhibited. When converted to HA by the usual Tiselius method, this brushite also tended to break up and give an unacceptably low flow-rate. Most brushite preparations were therefore made by the method of Main *et al.*^{6,7} in which CaCl₂ solution is added to an excess of sodium phosphate.

In this method the pH of the phosphate solution was found to determine the chemical composition of the end product: at pH values above neutrality the first precipitate was OCP, with increased production of brushite as the pH fell during mixing. With an initial pH of 9.5 (that of Na₂HPO₄) the end product contained an appreciable proportion of OCP and its flow-rate was very low. At initial pH values below neutrality the material was entirely brushite and the flow-rate much higher, but the yield was lower because of the cessation of crystallization at low pH. In the present work most preparations were made with an initial phosphate pH of 6.7, which yielded approximately 50% of the available calcium in the form of brushite. Contrary to a statement made by Main *et al.*⁶, this material invariably gave the diffraction pattern of CaHPO₄·2H₂O and not that of the anhydrous form.

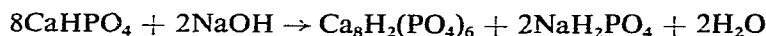
Attempts to increase the yield by simultaneous addition of alkali to maintain constant pH were not promising; the crystal clusters were very dense, and they tended to break up on conversion to HA. The "unregulated" pH 6.7 preparation gave a final pH of about 3.2, and this seemed to favour the production of suitably thin plates of brushite.

A most important variable in this method was the degree of seeding. Apparatus used repeatedly without cleaning acquired a deposit of brushite which gave seeding

that increased with time, while rigorous cleaning gave a delayed start to crystallization. For reproducible production of uniform crystal clusters it was found best to add to the phosphate solution a portion of brushite previously ground in a mortar; this gave a reproducibly high flow-rate suitable for large columns. Over-seeding gave excessively large clusters with a lower elution molarity after conversion. HA made from seeded brushite gave a poor resolution between peaks when used with gradient elution in smaller columns (e.g., 10 × 150 mm); for such applications it was preferable to make brushite without seeding to obtain larger, less clustered crystals. The converted HA could also be boiled for a limited period to further reduce the effective particle size. The HA preparations derived from these alternative brushites are discussed later.

Production of OCP and monetite

It became apparent during development of the neutral-pH conversion process that if the pH fell below neutrality the favoured reaction (at 70–80°) was conversion to OCP rather than HA. By adding alkali at a reduced rate so as to keep the pH at about 6, OCP alone could be produced. The reaction can be written as



and uses 0.25 moles of alkali per mole of calcium. Addition of a further 0.35 moles of alkali to a neutral endpoint can give conversion to HA, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, with release of more phosphate. However, once OCP has formed large crystallites this reaction appears to be slow.

Nucleation of OCP was inhibited below pH 6, but once crystallization began it would proceed rapidly and, in the absence of added alkali, the pH would fall to about 4 before the reaction ceased. This reaction also explained the observation referred to earlier, of a drastic physical change when brushite was added to water at 80–100° (0.05 moles Ca^{2+} in 500 ml); although the proportion of OCP produced in the absence of added alkali was very small, the very thin crystals produced gave a fivefold increase in settled volume and a large reduction in flow-rate. Boiling for 30 min, however, led to a progressive recrystallization as anhydrous secondary calcium orthophosphate (monetite, CaHPO_4). On cooling, this material (like brushite) was stable over a long period when kept as a wet suspension. X-ray diffraction and light microscopy showed this monetite to have crystals of size about 10 μm ; material of this kind was analyzed in Part I (No. 2 of Table I¹), and found to have appreciable binding capacity for DNA. A microcrystalline monetite could also be produced by heating dried brushite to 200° but in this case the DNA binding was negligible, supporting the hypothesis¹ that DNA binding demands extended crystalline surfaces.

These experiments, together with those on brushite and HA production, illustrate the remarkable complexity of the equilibria between various forms of calcium phosphate. Similar problems arise in applications to bone growth^{10,12–15} and soil science^{16,17}.

The effect of carbonate incorporation

It is well known that carbonate ions can be incorporated into HA in significant

proportions¹⁰, so it was decided to investigate what effect, if any, a carbonate impurity had on the chromatographic properties of HA. Preparations made during development of the new methods described here, without special precautions to exclude CO₂, contained less than 0.05% CO₃ (the detection limit of the analyses). The use of CO₂-free solutions and the addition of nitrogen bubblers to the conversion vessel made no difference to the binding capacities¹ or to the chromatographic behaviour with tRNA. When CO₂ was bubbled through the suspension during conversion up to 1% CO₃ was incorporated into the HA, but again no significant change in properties was detected. It may therefore be concluded that CO₃ contents of up to 1% have no effect on the chromatographic properties of HA.

New standard methods of making HA

In early trials the conversion was carried out with ammonia, as in the CPA method of Main *et al.*⁶. These authors reported unsatisfactory results when ammonia was replaced by NaOH, and the same seemed initially true in the present work. However, it became apparent with increased understanding of the variables involved that this difference was fortuitous, and in the final forms of the preparation NaOH

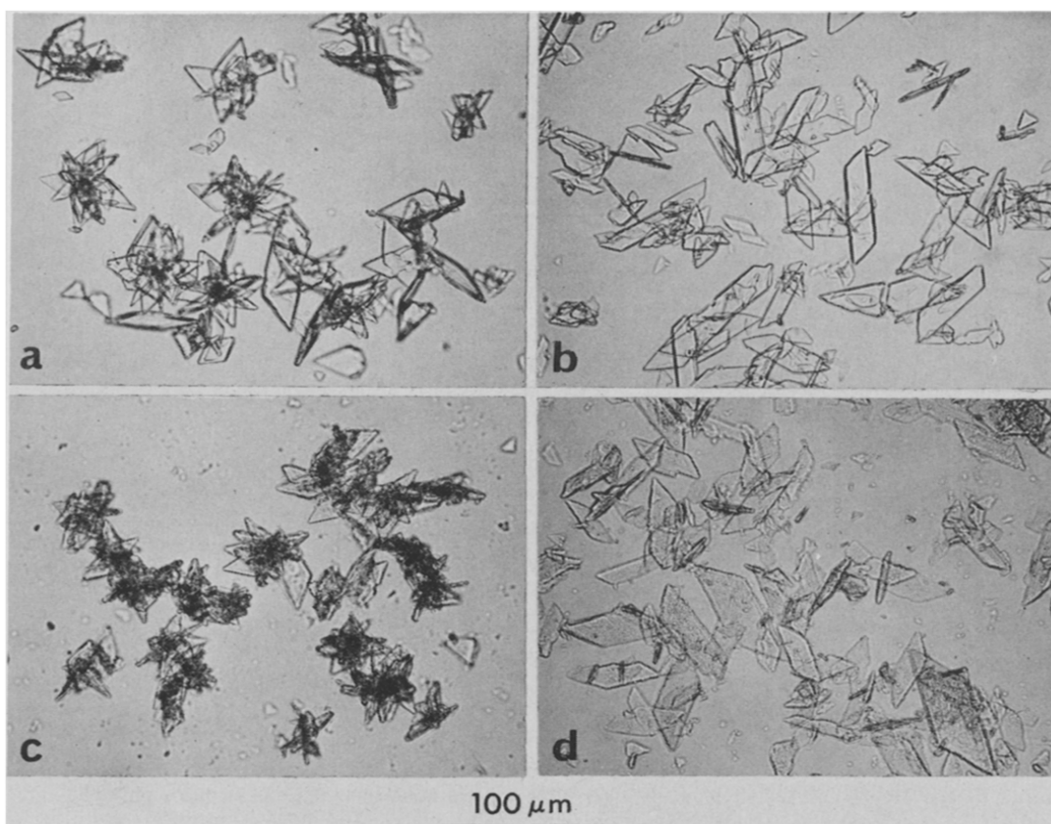


Fig. 2. Light microscopy of brushite and HA. (a) Brushite of type HF; (b) types GP and HR; (c) HA of type HF; (d) type HR. Recorded with $\times 16$ objective using bright-field illumination and reduced aperture stop.

was used exclusively. This avoided the uncertainties and hazards of working with a highly volatile alkali.

It also became clear that flow-rate and resolution could not be maximized in the same preparation. Homogeneity of packing, as tested with a methyl orange marker, was no guarantee of resolution between peaks, and although this could be slightly improved by restricting the flow-rate and increasing gradient volumes, there was no substitute for reducing the effective particle size.

Three variants were therefore developed: a very high flow-rate preparation (type HF) suitable for batch fractionations and gradient elution from columns of diameter exceeding 15 mm, a general-purpose preparation (type GP) of intermediate properties, and a high-resolution preparation (type HR) having a lower flow-rate.

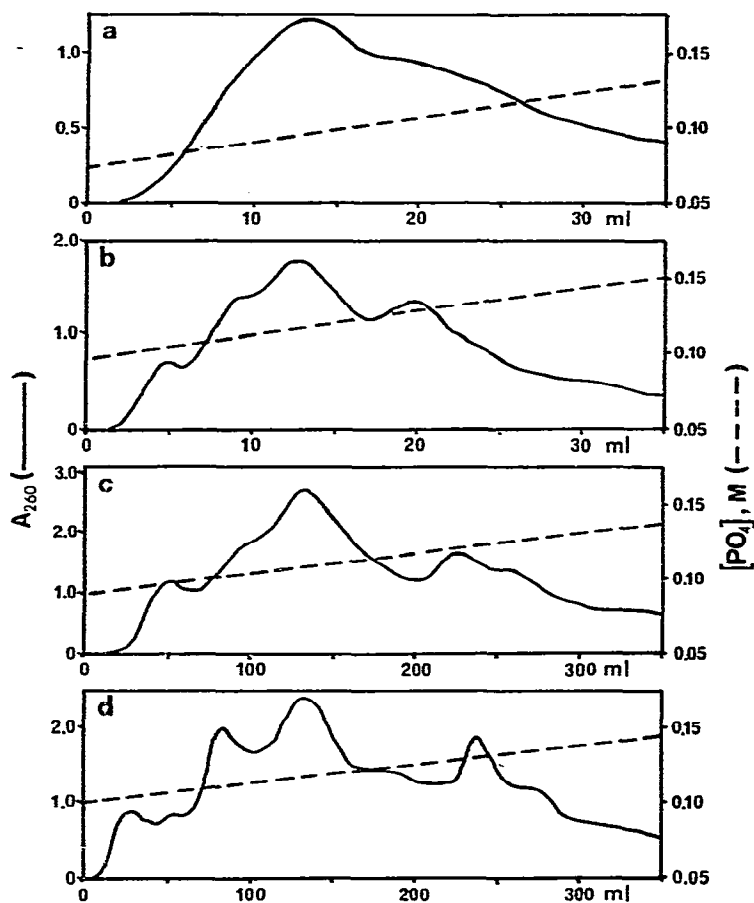


Fig. 3. tRNA elution profiles of HA. (a) Type HF in a 9×150 mm column loaded with 29 A_{260} units *E. coli* tRNA, eluted at 5° with a $60 + 60$ ml gradient of 0–0.20 M sodium phosphate (pH 7.0), flow restricted to 21 ml h^{-1} ; (b) type HR run as in (a) with 35 A_{260} units at 5 ml h^{-1} ; (c) type HF in a 16×900 mm column loaded with 770 A_{260} units, eluted with $600 + 600$ ml of 0.05–0.20 M phosphate at 37 ml h^{-1} under a hydrostatic head of 1.8 m; (d) type HR run as in (c) with 700 A_{260} units at 12 ml h^{-1} .

Even type HR material, however, gave a flow-rate well in excess of that given by some of the commercial products analysed in Part I¹. Full details of the preparations are given under Materials and methods.

Preparation HF. The brushite was in the form of stellate clusters of plates of length up to 40 μm and thickness 1 μm (Fig. 2a and ref. 1); its flow-rate was 60–150 ml h^{-1} . After conversion to HA (Fig. 2c) the flow rate was 40–80 ml h^{-1} , which is much higher than that of any commercial HA having comparable binding properties¹. The tRNA elution profile at 5° gave a peak at $0.100 \pm 0.005 M$ sodium phosphate (pH 7.0); the resolution between peaks was poor with a 9×150 mm column (Fig. 3a) but greatly improved with a 16×900 mm column (Fig. 3c). For maximum resolution a larger column still would be required. The material could be stored at 5° as a wet suspension without change in properties. Samples placed for 24 h at room temperature in an end-over-end shaker operating at 30 rpm showed no drop in flow-rate. Material could be freeze-dried and reconstituted with water without loss of flow-rate, but air-drying led to a large reduction if the resulting lumps were ground up. Binding capacity data are given in Part I¹.

Preparation GP. The brushite (Fig. 2b) contained more isolated plates than type HF and their length was up to 70 μm . The flow-rate was 20–40 ml h^{-1} both before and after conversion. The resolution (not illustrated) was somewhat better than with type HF.

Preparation HR. HA flow rates were 10–15 ml h^{-1} (Fig. 2d). The tRNA elution profile peaked at a slightly higher phosphate molarity, due to the boiling stage, and gave much better resolution than types GP and HF (Figs. 3b and 3d). Stability was similar to that of type HF. The binding capacities were about twice those of type HF¹.

CONCLUSION

Most of the problems commonly encountered in the making of HA may be attributed to inadequate control of certain stages in the procedure, such as the formation of the brushite starting material. If unsuitable brushite is used, or fragmentation occurs during conversion, this can lead to a very low flow-rate. Mixing with inert material to increase the flow-rate¹⁸ is hardly a solution because of the consequent increase in column size and fraction volume. Other problems (discussed earlier) are inherent in the preparations, particularly where these include stages of boiling.

The new methods described here offer better scope for control of the end product, and can be used to vary the properties according to need; they are also less time-consuming¹⁹. Part III²⁰ will describe the application of one of these types of HA to the fractionation of tRNA, at two different pH values and with two phosphate salts for elution.

The occurrence of an OCP intermediate at neutral pH and elevated temperature shows interesting parallels with the biosynthesis of HA in calcified tissues^{10,13,14}, where OCP is thought to be a natural precursor. The results reported here also confirm the conclusion of others¹⁵ that the formation of a given type of calcium phosphate depends not only on the chemical environment but on both the nature and the crystal size of pre-existing seeds.

ACKNOWLEDGEMENTS

At different stages in this work a number of skilled assistants have greatly contributed: they include Jan Rickard, Robert Ireland, Rajinder Johal, Elizabeth Neave, Matthew Binns and Assanah Mydin.

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