Journal of Chromatography, 166 (1978) 423–434

CHROM. 11,334

HYDROXYAPATITE FOR CHROMATOGRAPHY

I. PHYSICAL AND CHEMICAL PROPERTIES OF DIFFERENT PREPARATIONS

M. SPENCER
King's College Department of Biophysics, 26–29 Drury Lane, London WC2B 5RL (Great Britain)
and
M. GRYNPAS*
Department of Materials, Queen Mary College, Mile End Road, London E1 4NS (Great Britain)
(Received June 12th, 1978)

SUMMARY

With the aim of reducing the uncertainties experienced in preparing hydroxyapatite (HA) for column chromatography the properties of a number of preparations, made according to published procedures or purchased commercially, have been measured. Data are presented for BET surface area, crystallite dimensions, calcium to phosphorus ratio, binding capacity for bovine serum albumin (BSA) tRNA and two-stranded DNA, fractionation behaviour for a mixture of tRNAs, flow-rate, and appearance in the scanning electron microscope. Of commercial materials sold as hydroxyapatite, one proved to be octacalcium phosphate and another was whitlockite. The binding properties of HA appear unrelated to the flow-rate and Ca/P ratio. Binding capacities for protein and tRNA are closely correlated, but bear no relation to the capacity for DNA. The binding capacity for tRNA and the binding strength (as measured by elution with a salt gradient) are also largely unrelated; the binding capacity on its own is therefore of limited use in assessing material for chromatography. There is, however, some correlation between the binding strength and the crystallite size. Binding strength for tRNA levels off when crystallites reach about 60×20 nm; the faces of a hexagonal prism would then have widths comparable to the length of a molecule. For DNA the binding capacity is highest for smooth extended surfaces, and it is suggested that brushite, monetite and octacalcium phosphate merit further investigation as possible materials for separating long polynucleotides. For BSA and tRNA the higher surface area of HA gives it an advantage, though only up to 60% of the area measured by gas adsorption appears to be available.

* Present address: Children's Hospital Medical Center, 300 Longwood Avenue, Boston, Mass. 02115, U.S.A.

INTRODUCTION

Hydroxyapatite (HA) has been widely used for the fractionation of proteins and nucleic acids by column chromatography, and a number of different methods of preparing it have been published. However, there is no agreement on the best method of preparation, and commercial materials vary widely in their properties. Our early experience led us to concur with the verdict of Kothari and Shankar[1] that "hydroxyapatite is prepared under empirical conditions, which are often critical, and the whole procedure needs considerable familiarity. The preparation itself is laborious and not completely standardized and therefore it may not be possible to prepare the adsorbent repeatedly with comparable chromatographic characteristics."

With the aim of reducing these uncertainties we have measured the properties of a number of preparations, made according to published procedures or purchased commercially. In this paper we compare the properties listed in the Summary and examine what correlations exist between them. In Part II[2] the effects of varying certain parameters in a given preparation are discussed, and new preparations are described which we believe to be superior to others. In Part III[3] we describe their use in purifying single species of tRNA for crystallization.

MATERIALS AND METHODS

Chemicals

Reagent grade chemicals were mostly supplied by British Drug Houses (Poole, Great Britain). Hydroxyapatite was made according to published methods listed below, or purchased from Bio-Rad Labs. (Watford, Great Britain), British Drug Houses, Calbiochem (Bishops Stortford, Great Britain) and Clarkson (Williamsport, Pa., U.S.A.).

Surface areas

Surface areas were measured by nitrogen adsorption at 77 °K, using a Cahn microbalance. Samples were degassed either for 16 h at room temperature or 45 min at 200 °C. Data were interpreted by assuming the BET equation[4] with a nitrogen cross-section of 0.162 nm^2. Preliminary measurements showed that isotherms were of type I with a BET constant c of about 200. Subsequent areas were mostly calculated from single point measurements, assuming this value of c.

Crystallite sizes

Powder X-ray diffraction profiles of the 002 and 130 reflections were recorded, using either a Hilger diffractometer with copper radiation, or a Philips instrument with cobalt. Half-height widths were determined for 002 by computer smoothing, and for 130 by graphical subtraction of the overlapping 212 and 221 reflections. Instrumental broadening (about 0.10° for 002 with Cu K_a, and 0.21° for 130 with Co K_a) was measured with a powdered sample of mineral fluorapatite. Crystallite sizes were then calculated using the Scherrer equation with $K = 0.9$ and either Gaussian or Cauchy corrections for instrumental broadening[5]; since observed profiles were intermediate between these forms, the mean of the Gauss and Cauchy values was taken as a measure of the crystallite dimension L_{002} or L_{130}, ignoring heterogeneity and strain

effects. The 130 data did not justify use of more sophisticated Fourier techniques, and we preferred to use the same method of calculating both crystal dimensions. The uncertainty in the value obtained increased with the crystallite size; it varied from about 10% for size 10 nm to more than 40% above 100 nm.

The crystallite dimensions for each sample were also used to calculate a surface area per unit mass for comparison with the BET value; it was assumed that each crystallite was a cylinder of height L_{002}, diameter L_{130} and specific gravity 3.2.

Ca/P ratios

Calcium was determined by dissolving material in dilute HCl, adding excess EDTA, adding ammonia to pH 10.5 and back-titrating with magnesium acetate solution in the presence of Eriochrome Black T[6]. Calibration with standard solutions showed slight interference from phosphorus, which was allowed for. Phosphorus was determined by the molybdenum blue reaction[7] using differential spectrophotometry against a standard phosphate solution. Sample and reference solutions were incubated together for 30 min and measured at 600 nm. Using about 0.1 g of sample for each determination, values of Ca/P agreed with those obtained gravimetrically within $\pm$ 0.03.

Binding capacities

Binding capacities were measured for bovine serum albumin fraction V (BSA) (Sigma, Kingston upon Thames, Great Britain), tRNA from *E. coli* K12 CA265 (Microbiological Research Establishment, Porton, Great Britain), and high molecular weight double-stranded DNA (gift of Dr. L. D. Hamilton). HA samples of 25 mg or more were dispersed in 5 ml 0.05 *M* sodium cacodylate buffer adjusted to pH 6.8 $\pm$ 0.1. Concentrated stock solutions were then added to give values of 0.4%, 0.2% and 0.01% for BSA, tRNA and DNA, respectively. The mixtures were gently agitated for 30 min at room temperature and then pelleted. Solute bound was estimated from the optical density of the supernatant, subtracting any absorbance measured in a blank containing no solute. Extinction coefficients ($E^{1\%}_{1\,cm}$) were assumed to be 6.6 for BSA 280 nm and 200 for tRNA and DNA at 260 nm. The amount of HA added was adjusted in successive experiments until the ratio of free to bound solute lay between 1.5 and 5.0; below 1.5 the binding decreased, while above 5.0 the accuracy deteriorated. With suitable precautions against volumetric errors (particularly necessary with cold, viscous stock solutions) a reproducibility of $\pm$ 10% was obtained.

Fractionation of tRNA

A column of bore 9 mm and length 150 mm was packed with HA and tested for uniformity with a methyl orange marker. A total of 2 mg tRNA was then applied and eluted at 5 °C with a linear gradient of 0–0.2 *M* sodium phosphate buffer (pH 7.0), total volume 120 ml. The hydrostatic head was about 1 m, reduced in some cases of high flow-rate. Ultraviolet absorbance and conductivity were recorded continuously, and the molarity of phosphate corresponding to the principal peak in absorbance[2] was noted. The value appeared to be independent of flow-rate over the range used, which was up to 50 ml h^{-1}.

TABLE I

PROPERTIES OF CALCIUM PHOSPHATE PREPARATIONS

For details of measurements see Materials and Methods

Prep. No.	*Description*	*Ref.*	*BET surface area* ($m^2 g^{-1}$)	*Powder X-ray diffraction*			*Molar ratio Ca/P*	*Weight-specific binding capacity* ($mg g^{-1}$)			*Area-specific capacity** ($mg m^{-2}$)			*tRNA profile peak* (*M*)	*Flow-rate* ($ml h^{-1}$)
				L_{002} (*nm*)	L_{130} (*nm*)	*Surface area* ($m^2 g^{-1}$)		*BSA*	*tRNA*	*DNA*	*BSA*	*tRNA*	*DNA*		
1	Brushite ($CaHPO_4 \cdot 2H_2O$)	2	<2**	–	–	–	1.00	<1	<1	0.4	<1	<1	≥0.4	<0.01	40
2	Monetite ($CaHPO_4$)	2	≥3	–	–	–	1.04	5	3	0.9	≥2	≥1	≥0.3	0.050	12
3	Tiselius HA	9	68	53	23	66	1.56	49	30	2.0	0.72	0.44	0.029	0.090	12
4	Bio-Rad HTP (control 13879)	9	62	74	25	58	1.52	55	32	1.6	0.89	0.52	0.026	0.096	17
5	DNA-HTP (control 13621)	9	67	72	26	56	1.51	53	33	1.7	0.79	0.49	0.025	0.109	2
6	Calbiochem HA (lot 610012)	9	29	96	39	39	1.47	60	26	2.0	2.07	0.90	0.069	0.110	<1
7	Main *et al.* HA (method CPA)	8, 13	68	62	19	78	–	78	46	2.7	1.15	0.68	0.040	0.097	6
8	Main *et al.* HA (method CPA)	8, 13	–	–	–	–	1.55	–	–	–	–	–	–	0.085	28
9	BDH HA (lot 1751110)	8, 14	92	61	18	79	1.53	50	34	1.2	0.54	0.37	0.013	0.105	6
10	Cold KOH HA	15	56	30	10	147	1.47	53	33	2.7	0.95	0.59	0.048	0.042	14
11	Cold NaOH HA	15	67	40	16	96	1.49	31	20	3.3	0.46	0.29	0.049	0.050	15
12	New process HA (NH_3 conversion)	2	80	52	16	92	1.56	55	36	2.4	0.69	0.45	0.030	0.098	34
13	New process HA (method "HF")	2	–	57	28	56	1.52	46	26	2.4	0.82	0.46	0.043	0.102	52
14	New process HA (method "HR")	2	–	77	34	46	1.54	90	51	4.2	1.96	1.10	0.091	0.108	11
15	Directly precipitated HA	16	45	101	46	33	1.59	–	44	–	–	0.97	–	–	1
16	Directly precipitated HA	16	–	105	33	44	1.62	57	28	3.6	1.30	0.64	0.082	0.085	<1
17	Hypatite C (lot 6513)	–	64	–	–	–	1.35	68	42	0.5	1.06	0.66	0.008	0.048	100
18	Octacalcium phosphate	2	11	–	–	–	1.37	22	12	3.9	2.0	1.1	0.36	0.056	49
19	Spheroidal "HA" (lot 2399870)	–	<2	–	–	–	–	2	1	0.1	–	–	–	<0.01	49

* Obtained by dividing weight-specific binding capacity either by BET surface area, or by X-ray surface area where no BET value is listed.

** 1 $m^2 g^{-1}$ calculated from microscopic measurements (see text).

Flow-rate

A method similar to that of Main, Wilkins and Cole[8] was used: material was packed in a column of bore 9 mm to a height of 75 mm, and the flow-rate measured at room temperature with a pressure head of 250 mm.

Scanning electron microscopy

Gold-coated specimens were examined in a JEOL model JXA-50A at a magnification factor of 10^4.

RESULTS AND DISCUSSION

Table I summarizes the numerical data. Brushite (preparation No. 1) was studied because it is the type of starting material used in the well-known Tiselius preparation of HA[9–11]. Our brushite[2] was prepared by adding $CaCl_2$ slowly to a phosphate solution adjusted to pH 6.7[8], and monetite (No. 2) was prepared by boiling a sample of brushite[2]. Both these preparations differ from the HAs listed below them in having much smaller surface areas and binding capacities. This is to be expected because the conversion involves a transition from relatively large brushite plates of approximately 30 μm length to compacted masses of submicroscopic HA needles. What is striking, however, is that the area-specific binding capacities are quite comparable; indeed, for DNA both brushite and monetite give exceptionally high values (we shall return to this point later). Furthermore for our monetite, which is more finely divided than the brushite, the elution molarity for tRNA is as high as for the poorer preparations of HA. Our first conclusion is, therefore, that it might be useful to explore further the chromatographic properties of these materials, particularly for DNA and other large polynucleotides. For proteins and tRNA the higher capacity of HA probably ensures that it will remain preferable.

Comparison of chromatographic properties for HA

Preparation No. 3 was made on a small scale according to Tiselius *et al.*[9] except that the brushite crystals were prepared as above. Nos. 4–6 are commercial products made by the Tiselius method. The binding properties are all very similar, but there are wide variations in the flow-rate. For Bio-Rad materials the properties are within the manufacturers' specification for these batches; the "DNA grade" has a much lower flow-rate but the resolution between peaks in the tRNA profile is greater. (This point is not illustrated here but will be discussed in Part II[2]).

It was dissatisfaction with the unpredictable flow-rate of the Tiselius product[12] that led some workers to use the later method of Main *et al.*[8,13] and this has been developed for use on a large scale[14]. Nos. 7 and 8 of Table I are small-scale preparations made from brushite crystallized as above[2], while No. 9 is a commercial sample. Again the flow-rates cover a wide range, although the best value is greater than for any of the Tiselius preparations. Atkinson *et al.*[14] obtained a flow-rate of 25 ml h^{-1} in a test similar to ours, while Muench[13] obtained 21 ml h^{-1} in a 10 $\times$ 40 mm column under 300 mm head (equivalent to about 8 ml h^{-1} in our test).

Nos. 10 and 11 of Table I were made by alkaline conversion of brushite at room temperature; this method[15] is claimed to avoid the problems inherent in boiling during conversion, which is a feature of both methods discussed above. We again

used pH 6.7 brushite[2]. The binding capacities are comparable to those found in samples discussed above, being relatively high for DNA, but the elution molarities for tRNA are low and the resolution of peaks poor. These differences, and the reasons for them, are discussed more fully in Part II[2]; it is sufficient for now to point out that both Nos. 10 and 11 have unusually small crystallite dimensions, and the surface areas computed from them are appreciably greater than those measured by nitrogen adsorption. This implies that in material of this type the crystallites are closely compacted together so that not all their surfaces are exposed, whereas in other preparations there is reasonable agreement between the two values for surface area.

Preparations 12–14 are typical of the new methods described in Part II[2], which are designed to combine the best features of earlier methods; they include the heat treatment which seems to be essential, but avoid repeated washing or boiling and are correspondingly quicker to carry out.

Nos. 15 and 16 are samples of HA prepared by direct precipitation; the materials were not intended for chromatography, being of the kind used in the study of tooth and bone growth[16], but we include them for comparative purposes (supplied by Mr. D. J. Langdon, London Hospital Medical College). As expected they have low flow-rates and the Ca/P ratios are higher. The other data, apart from a somewhat high capacity for DNA in No. 16 and a large crystallite size for both, lie within the range covered by other samples.

Other calcium phosphates

The last group of Table I, Nos. 17–19, contains samples which powder X-ray diffraction showed not to be hydroxyapatite, but some related calcium phosphate. No. 17 (Hypatite C; Clarkson) showed a remarkably high flow-rate. However, it gave the diffraction pattern of octacalcium phosphate (OCP), $Ca_8H_2(PO_4)_5 \cdot 5H_2O$ (JCPDS file No. 26-1056), a result confirmed by its Ca/P ratio, and the resolution of a mixture of tRNAs was rather poor. The manufacturers do not describe how this material is made, but we obtained OCP ourselves when conversion was carried out slowly with insufficient alkali[2]; No. 18 is a sample of such material. Both Nos. 17 and 18 show binding capacities comparable to those of HA, and No. 18 has a DNA capacity higher than for any other sample, despite having a very small surface area. This point is discussed in connection with the electron microscopy. Although the peak elution molarity for tRNA is lower than with HA, this might be useful for those proteins that bind very strongly. As with brushite and monetite, the chromatographic properties of OCP appear to merit more investigation.

Finally, No. 19 proved to be whitlockite, β-$Ca_3(PO_4)_2$ (JCPDS file No. 9-169). This material, which was purchased from British Drug Houses as "spheroidal hydroxylapatite", is made by high-temperature fusion into larger particles from HA similar to preparation No. 9 of Table I. The process seems (at least for the batch tested by us) to have greatly reduced the surface area and hence the binding capacities, although the flow-rate has improved. The binding strength for tRNA appears negligible.

Scanning electron microscopy of brushite and HA

Figs. 1a and 1b show the appearance of brushite before conversion to HA. With the crystallization method used[2] it forms stellate clusters of plates up to 40 μm

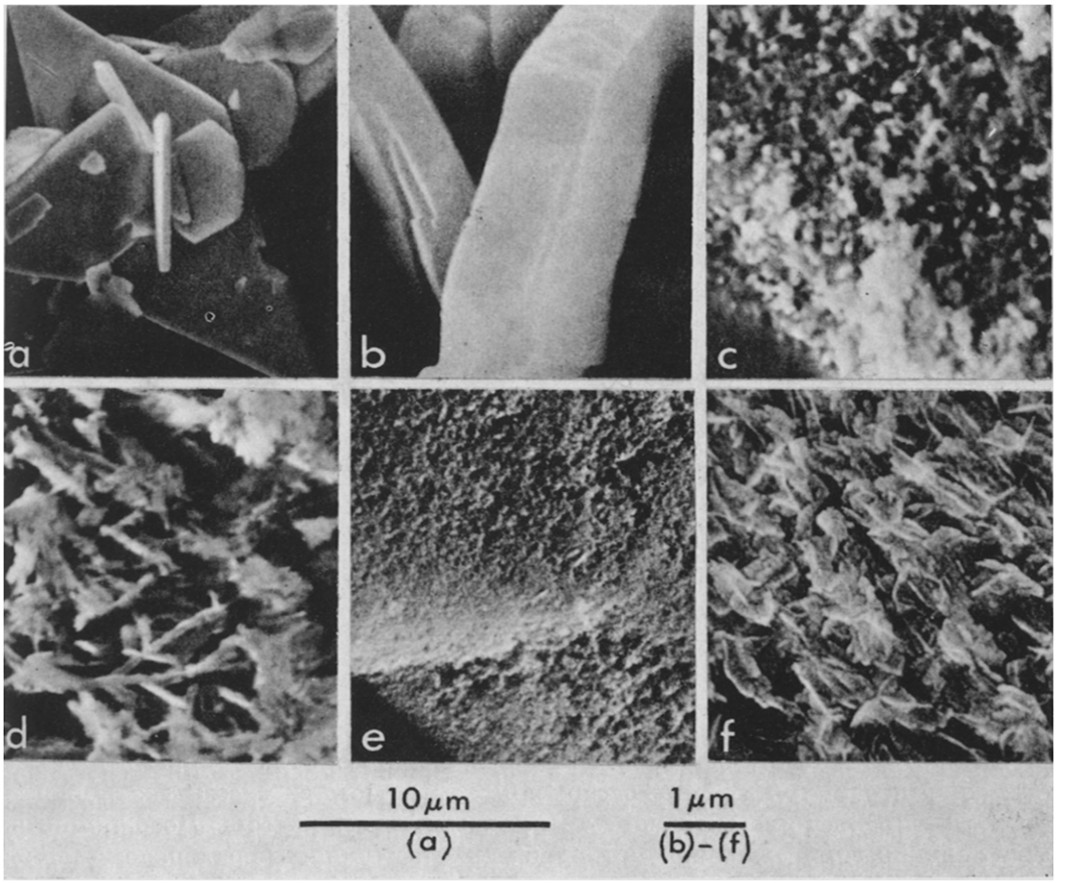

Fig. 1. Scanning electron micrographs. (a) and (b) brushite (similar to No. 1 of Table I); (c) Tiselius HA[9] (No. 3 of Table I); (d) Main *et al.* HA[8] (No. 7 of Table I); (e) cold KOH HA[15] (No. 10 of Table I); (f) new method HA[2] (similar to No. 12 of Table I).

in length and of thickness 1 μm, giving a calculated surface area of about 1 $m^2 g^{-1}$. On conversion the surface is changed into a finely divided structure which varies considerably with the method used (Figs. 1c–1f). In no case is it possible to resolve individual crystallites of the sizes deduced from diffraction; the fine structure seen must represent aggregates of crystals, which are sintered together so as to retain the gross outline of the brushite plates. This suggests that a sizeable fraction of the surface area of the HA is likely to be unavailable for interaction with macromolecules, a conclusion supported by the binding data of Table I: one can estimate that a monolayer of tRNA[17] would give a theoretical binding capacity of about 1.6 mg m^{-2}, whereas the HA samples of Table I show values not exceeding 60% of this. The directly precipitated HA samples are no different in this respect, and an earlier study[18] of the binding of amino acids to directly precipitated HA gave 30% of theoretical coverage. The higher capacity of HA for BSA compared with tRNA (Table I) can be explained by the greater mass per unit area in a monolayer of BSA.

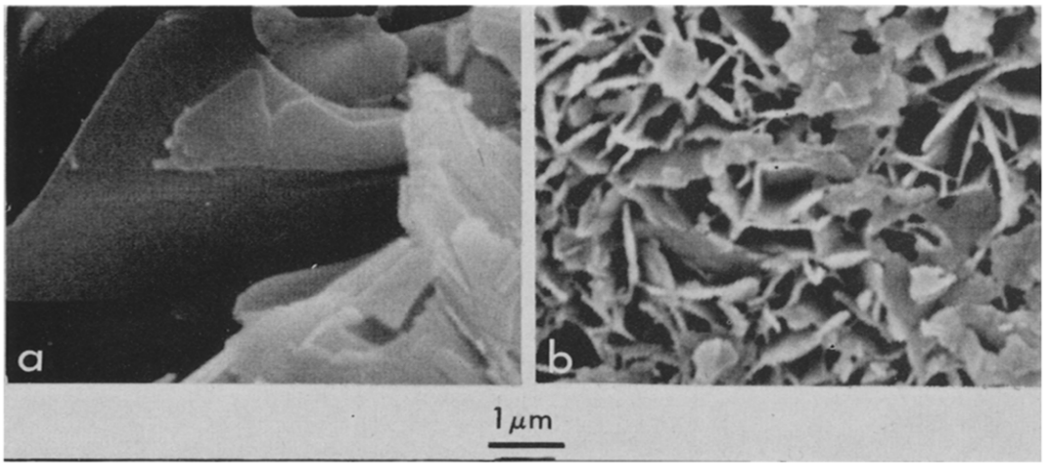

Fig. 2. Scanning electron micrographs of octacalcium phosphate. (a) Preparation No. 18 of Table I, DNA capacity 3.9 mg g^{-1}, 0.36 mg m^{-2}. (b) Hypatite-C (No. 17 of Table I), DNA capacity 0.5 mg g^{-1}, 0.008 mg m^{-2}.

Surface topography and the DNA binding process

Fig. 2 sheds some light on the question of why certain non-HA samples show high binding capacities, particularly for DNA, and why the DNA capacity of all samples is so much smaller than for BSA or tRNA, when the theoretical values are of the same order. Fig. 2a (OCP) shows extended thin plates, in contrast with Fig. 2b and the examples in Fig. 1c–1f. This OCP has an exceptional capacity for DNA, and the area-specific capacity is an order of magnitude greater than that of HA. The same is true of brushite and monetite (Table I). The high capacity of the Fig. 2a material is not due to its being OCP; the sample shown in Fig. 2b is also OCP but it is much more finely divided, and its DNA capacity is exceptionally low. Now the length of an extended double-stranded DNA molecule of molecular weight 10^7 is about 5 μm. Such molecules could adsorb without distortion onto surfaces such as that of Fig. 2a, but it may well be imagined that they could fail to penetrate the interstices of more irregular structures. This raises the interesting question of whether the discrimination between ordered and disordered polynucleotides, which is such a useful property of HA[11], is also to be expected with the other calcium phosphates discussed here. If Martinson[19] is correct in concluding that variations in configurational entropy loss on adsorption are responsible, then brushite and some forms of OCP might even be superior to HA.

Correlations between properties

As discussed above, there is evidence that only a certain fraction of crystallite surface area is available for adsorption of BSA and tRNA, and the fraction is much smaller for DNA. It is not therefore surprising that binding properties show only a rough correlation with surface area, since the fraction available may vary with the method of preparation. The high area-specific capacity for all solutes of No. 18 of Table I could arise from a free availability of more of the surface (Fig. 2a).

The Ca/P ratio (Table I) varies from 1.47 to 1.62 for HA; as found by others, all preparations are calcium-deficient. There is no obvious correlation with any of the

other properties: it seems, therefore, that the binding process is not related to that of catalytic activity, which depends strongly on Ca/P[20].

Binding capacities for different solutes are plotted against each other in Fig. 3. For BSA and tRNA, which have molecular weights of the same order of magnitude, there is close correlation between the different binding capacities of a given sample of HA (Fig. 3a). The capacity for DNA is, however, quite unrelated to that for tRNA (Fig. 3b). This is consistent with the conclusion drawn from microscopy, that DNA adsorption is critically dependent on the larger-scale topography of the surface.

The binding capacity for tRNA is not closely correlated with the binding strength, as measured by the elution molarity (Fig. 4a). The same is true when area. specific capacity is plotted (not shown). This is not too surprising when we recall tha- in the classical Langmuir theory of adsorption[18] the two parameters are independentt The results show that binding capacity, which is all that some manufacturers quote in the specification, is not on its own sufficient as a guide to chromatographic behaviour.

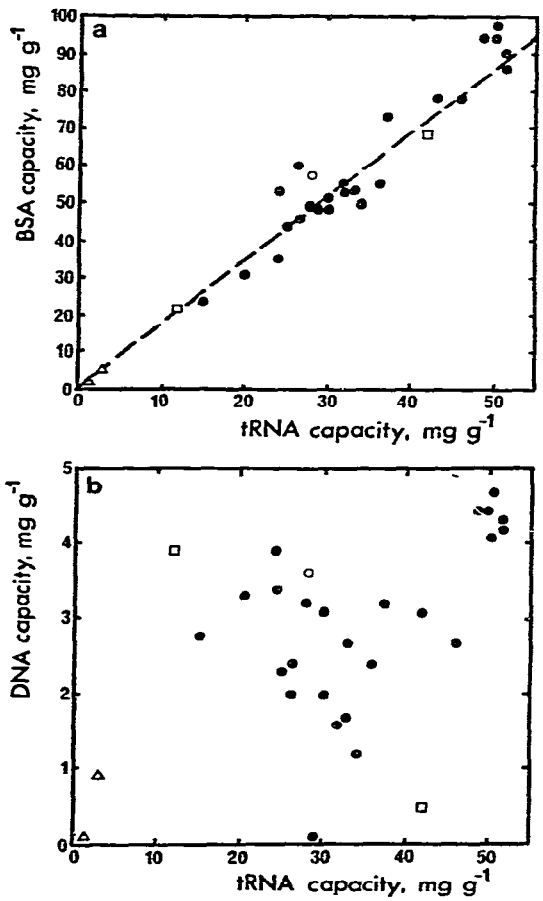

Fig. 3. tRNA binding capacity plotted against capacity for (a) BSA, (b) DNA. The data plotted cover all samples in Table I, together with some from intermediate stages in the preparations[2]. ●, Chromatographic HA; ○, directly precipitated HA; □, OCP; △, other calcium phosphates. For details of measurements see Materials and Methods.

There is, however, some degree of correlation between the tRNA elution molarity and the crystallite size (Fig. 4b). We have furthermore found[2] that crystallite size and elution molarity increase together during the formation of HA by standard methods. There seems to be an upper limit to the binding strength, reached when L_{002} exceeds about 60 nm, beyond which a further increase in crystallite size has no effect. The data of Table I show that for most samples the smaller dimension L_{130} is about one-third of L_{002}, so the plateau region corresponds to an L_{130} of about 20 nm. Now L_{130} is an average thickness measured at right angles to the long axis of the crystals, and the width of crystal faces could be smaller than this; if, for instance, the crystals

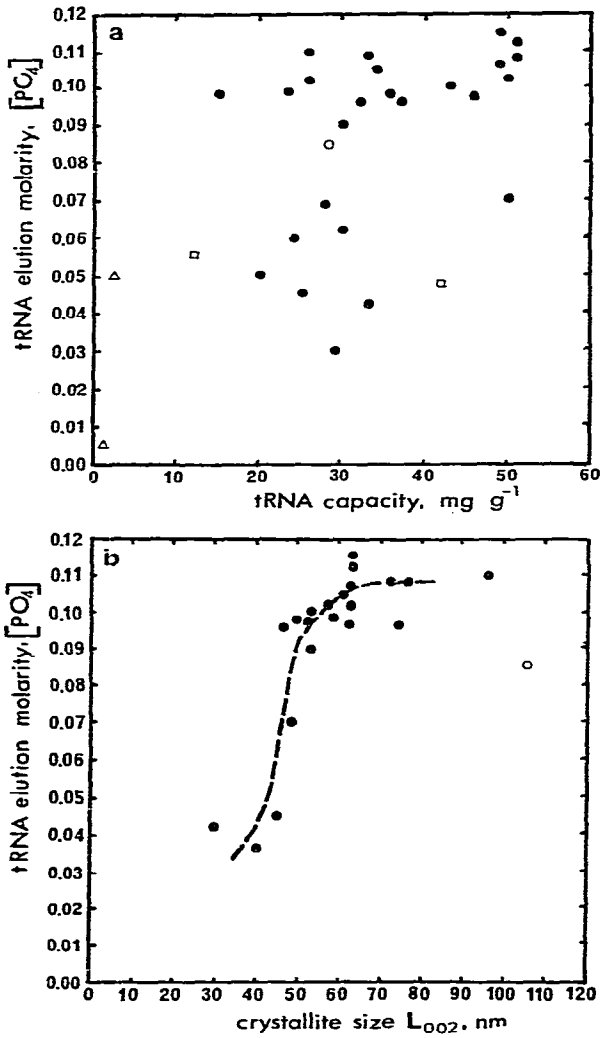

Fig. 4. tRNA elution molarity plotted against (a) tRNA binding capacity, (b) crystallite size (HA only). Same samples as in Fig. 3; ●, chromatographic HA; ○, directly precipitated HA; □, OCP; △, other calcium phosphates. Samples containing a proportion of brushite or OCP have been omitted from (b).

were needles of hexagonal cross-section[21], each face would be of width 11 nm. Since the longest dimension of $tRNA^{phe}_{E.\ coli}$ (one of the smaller species) is about 9 nm[17], this suggests that for optimum binding the smallest dimension of the crystallite faces needs to exceed the size of the molecule. This is entirely consistent with the usual concept of binding to HA by maximised apposition of charged groups.

We suggest, therefore, that a generally useful test of HA for chromatography of proteins and small nucleic acids would be to measure L_{002}; for maximum affinity this should then be about one order of magnitude greater than the expected size of the molecules to be fractionated. It is possible, of course, that where there are size differences between molecules it might be best to choose HA of an intermediate crystallite size, so as to weaken the binding of the larger species; this argument may also apply in the case of large polynucleotides, but in that case the structure of crystal aggregates is more relevant. It is clear that there is unlikely to be a "best" preparation of HA for all purposes.

CONCLUSION

Above a certain minimum standard, the binding capacity of HA is of less interest than its binding strength, because columns are usually operated well below their maximum capacity. Our experiments with tRNA[2,3] confirm that for optimum separation of a mixture of similar molecules it is best to use material having a high affinity for all of them. We have suggested above that crystallite size is a useful criterion in such applications. In Part II[2] we shall go on to describe how this and other parameters change in the course of a preparation, and how variability in the end-product can greatly be reduced through an understanding of the processes involved.

ACKNOWLEDGEMENTS

We thank Dr. N. D. Parkyns, Miss A. de Koranyi ,Miss S. Bruce, Dr. D. Puxley and Mr. J. Laker of the British Gas London Research Station for facilities and advice in the measurement of surface area and crystallite size; Dr. P. Barnes of Birkbeck College, London for additional X-ray facilities; and Mr. R. L. Whitenstall for taking electron micrographs. We are indebted to Mr. D. J. Langdon of the London Hospital Medical College for supplying HA samples. We also thank Drs. W. B. Gratzer, E. G. Richards and J. C. Elliott for helpful discussions. tRNA fractionation was carried out with the skilled assistance of Mrs. G. Neave.

REFERENCES

1 R. M. Kothari and V. Shankar, *J. Chromatogr.*, 98 (1974) 449.
2 M. Spencer, *J. Chromatogr.*, 166 (1978) 435.
3 M. Spencer, E. J. Neave and N. L. Webb, *J. Chromatogr.*, 166 (1978) 447.
4 K. S. W. Sing, in G. D. Parfitt and K. S. W. Sing (Editors), *Characterization of Powder Surfaces*, Academic Press, London, 1976, p. 1.
5 H. P. Klug and L. E. Alexander, *X-ray Diffraction Procedures*, Wiley, New York, 1974, p. 618.
6 S. S. Barton and B. H. Harrison, *J. Colloid Interface Sci.*, 55 (1976) 409.
7 L. Jaenicke. *Anal. Biochem.*, 61 (1974) 623.

8 R. K. Main, M. J. Wilkins and L. J. Cole, *J. Amer. Chem. Soc.*, 81 (1959) 6490.
9 A. Tiselius, S. Hjertén and Ö. Levin, *Arch. Biochem. Biophys.*, 65 (1956) 132.
10 Ö. Levin, *Methods Enzymol.*, 5 (1962) 27.
11 G. Bernardi, *Methods Enzymol.*, 21 (1971) 95.
12 K. H. Muench and P. Berg, *Biochemistry*, 5 (1966) 982.
13 K. H. Muench, *Procedures Nucleic Acid Res.*, 2 (1971) 515.
14 A. Atkinson, P. A. Bradford and I. P. Selmes, *J. Appl. Chem. Biotechnol.*, 23 (1973) 517.
15 H. W. Siegelman, G. A. Wieczorek and B. C. Turner, *Anal. Biochem.*, 13 (1965) 402.
16 N. C. Blumenthal and A. S. Posner, *Calcif. Tiss. Res.*, 13 (1973) 235.
17 J. L. Sussman and S. H. Kim, *Science*, 192 (1976) 853.
18 M. Kresak, E. C. Moreno, R. T. Zahradnik and D. I. Hay, *J. Colloid Interface Sci.*, 59 (1977) 283.
19 H. G. Martinson, *Biochemistry*, 12 (1973) 2731.
20 S. J. Joris and C. H. Amberg, *J. Phys. Chem.*, 75 (1971) 3167.
21 J. A. S. Bett, L. G. Christner and W. K. Hall, *J. Amer. Chem. Soc.*, 89 (1967) 5535.