Calcium-ion-binding activity in human small-intestinal mucosal cytosol

Purification of two proteins and interrelationship of calcium-binding fractions

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Ca^{2+} -binding activity was investigated in human small-intestinal mucosal cytosol. Binding was detected in fractions with molecular weights of 28 000 and about 900 000, as determined by gel filtration. No binding was found at molecular weight 12 000–13 000 (the molecular weight of calcium-binding protein in lower mammalian species) until the cytosol had been subjected to a hollow-fibre-filtration step. The appearance of Ca^{2+} -binding at molecular weight 12 000–13 000 was associated with a decline in the 28 000-mol.wt. calcium-binding fraction. The 12 000–13 000-mol.wt. fraction contained two distinct calcium-binding proteins. One of these proteins had properties similar to those of pig calcium-binding protein. Antiserum to this protein reacted against the 28 000-mol.wt. calcium-binding fraction in cytosol from human small-intestinal mucosa and from human kidney. An immunoassay method for one of the calcium-binding proteins was established. In normal duodenal mucosa the concentration was $915 \mu\text{g/g}$ and in the ileum it was $443 \mu\text{g/g}$ of mucosa. A subject with hypercalcaemic sarcoidosis had $1200 \mu\text{g/g}$ of mucosa in the jejunum, and a subject with an undetectable concentration of plasma 25-hydroxycholecalciferol had concentrations of calcium-binding protein in the mucosa similar to those found in normal subjects.

Intestinal mucosal cytosol from many species possesses the ability to bind Ca^{2+} (Wasserman *et al.*, 1978). Although there are many proteins that could be responsible for this property, in many species, including the chick, in which the phenomenon was first described, the Ca^{2+} -binding appears largely to result from the presence of a vitamin D-dependent calcium-binding protein (Wasserman *et al.*, 1968, 1978). In many mammalian species the Ca^{2+} -binding in intestinal mucosal cytosol may be divided into a high-molecular-weight fraction and a low-molecular-weight fraction by gel chromatography (Hitchman & Harrison, 1972; Fuller & Wasserman, 1973), in contrast with a molecular weight of 27 000 in the chick (Wasserman *et al.*, 1968). The results in some animal species, including man, are, however, discordant. In the human, a low-molecular-weight Ca^{2+} -binding fraction has variously been detected at molecular weights of 13 000 (Hitchman & Harrison, 1972), of over 21 000 (Piazolo *et al.*, 1971) or of 20 000–25 000 (Alpers *et al.*, 1972). Further, this Ca^{2+} -binding fraction may also be related to a Ca^{2+} -binding

fraction (mol.wt. 27 000) found in the kidney. Antiserum to this protein fraction reacts with intestinal cytosol extracts (Morrissey & Rath, 1974), although the molecular weight of the Ca^{2+} -binding fraction in the gut was uncertain.

The vitamin D-dependence of the low-molecular-weight Ca^{2+} -binding fraction in mammals is controversial, although in the chick the 28 000-mol.wt. moiety appears to be dependent on vitamin D (Wasserman *et al.*, 1968), or more specifically on 1,25-dihydroxycholecalciferol (Spencer *et al.*, 1976). In the pig, however, calcium-binding protein was readily detectable in the intestine of animals in which the manifestations of vitamin D deficiency were apparent (Harrison *et al.*, 1975). Conversely, in the calf, a species in which most of the intestinal Ca^{2+} -binding is associated with the low-molecular-weight Ca^{2+} -binding fraction, rickets decreased this binding by about two-thirds (Wasserman *et al.*, 1978).

The present work describes the characterization of some of the constituents of Ca^{2+} -binding in human small-intestinal mucosal cytosol. An immunoassay method for one calcium-binding protein, which had properties in common with pig calcium-

Abbreviation used: SDS, sodium dodecyl sulphate.

binding protein, has been established to determine the relationship of this protein to other Ca^{2+} -binding fractions, and to determine the vitamin D-dependency of the protein, which, if confirmed, would provide a useful index of vitamin D status in man, as in the chick (Bar & Wasserman, 1974).

Materials and methods

Intestine

Whole small intestine was obtained within 5 h of death from cadavers of six humans aged 10–54 years whose kidneys had been used for transplantation. Intestine was also obtained from the body of a 16-month-old girl with idiopathic hypercalcaemia of childhood, who had received cortisone acetate (15 mg/day) before death. All intestine was processed at 4°C or below and stored at –20°C.

Jejunal biopsies were obtained by means of a Crosby capsule from a male aged 77 years with jejunal diverticulosis and an undetectable concentration of plasma 25-hydroxycholecalciferol, and from a male aged 34 years with sarcoidosis and hypercalcaemia (plasma 25-hydroxycholecalciferol concentration 62.5 nmol/l). In both instances the subjects were informed of the research nature of the investigation and their consent was obtained. Intestine was also obtained from a young baboon provided by Dr. W. A. Coward.

Preparation

Whole gut was opened up longitudinally and wiped free of luminal contents. The mucosa was scraped free with a blunt scalpel, weighed and then added to its own weight of 0.1 M-Tris/HCl buffer, pH 7.4. The mucosa was then homogenized in an MSE Ato-Mixer, with frequent intervals for cooling, and the resulting homogenate was centrifuged at 30 000 g for 30 min. All operations were performed at 0–4°C.

The jejunal biopsies were weighed, mixed with their own weight of Tris/HCl buffer, pH 7.4, and homogenized in a 1.0 ml glass homogenizer. The homogenate was then centrifuged at 5000 g for 5 min, and the clear supernatant was used for further studies.

Kidney cytosol

Two human kidneys were obtained within 24 h of death and were homogenized with their own weight of 0.1 M-Tris/HCl buffer, pH 7.4, in the Ato-Mixer. The homogenate was heated at 60°C for 15 min and centrifuged at 30 000 g for 30 min. The clear supernatant was decanted for further study.

Buffers used in chromatography

The buffer used for gel chromatography was 0.1 M-Tris/HCl buffer, pH 7.4. Buffers for chromatography in the presence of 6 M-guanidinium

chloride were as described by Fish *et al.* (1969). Ion-exchange chromatography in the absence of Ca^{2+} was performed with the use of two buffers that were mixed to form a gradient of NaCl. The low-molarity buffer was 0.02 M-imidazole/HCl buffer, pH 7.0, containing 0.025 M-NaCl, and the high-molarity buffer contained 0.7 M-NaCl but was otherwise identical. Ion-exchange chromatography in the presence of Ca^{2+} was performed with 1 mM- CaCl_2 included in the low-molarity buffer. All buffers contained β -mercaptoethanol (1 mM).

Concentration and dialysis

To decrease the large volume of the whole-gut cytosol to manageable proportions, it was concentrated by an Amicon DC2 hollow-fibre concentrator (Amicon Corp., High Wycombe, Bucks., U.K.) fitted with fibre HDP10, with a stated 90% retention of proteins exceeding molecular weight 10 000. Concentration was otherwise performed in an Amicon stirred cell fitted with membrane UM2. Dialysis was performed with Visking dialysis tubing when the protein solution contained nothing with a molecular weight below 20 000. Otherwise buffers were changed in an Amicon stirred cell. All procedures were performed at 0–4°C.

Gel chromatography

This was performed with LKB Ultrogel Aca-22, -34 abd -54 gels (LKB Produkter, Bromma, Sweden) or with Sephadex G-50 (Pharmacia, Uppsala, Sweden). Gel filtration in non-denaturing conditions was performed at 0–4°C. Gel chromatography in the presence of guanidinium chloride was performed as described by Fish *et al.* (1969) at room temperature. Protein was applied by a flow adaptor on to Pharmacia columns, and elution rates were kept well below the maximum recommended by the manufacturer.

Ion-exchange chromatography

Whatman DE-52 pre-swollen ion-exchanger (W. and R. Balston, Maidstone, Kent, U.K.) was used. The protein was eluted with a gradient of low-molarity buffer and high-molarity buffer. Ion-exchange chromatography in the presence of CaCl_2 was performed by using the low-molarity buffer containing 1 mM- CaCl_2 until no further protein peaks were eluted. The column was then eluted with the high-molarity buffer. Chromatography was performed at 4°C.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Protein eluted from chromatography columns was monitored by means of transmittance at 280 nm (LKB Uvicord), except during chromatography in

the presence of 6 M-guanidinium chloride. In this case protein was identified by SDS/polyacrylamide-gel electrophoresis (see below) of the fractions.

Ca²⁺-binding

Ca²⁺-binding fractions eluted from the chromatography columns were detected either by adding ⁴⁵CaCl₂ (The Radiochemical Centre, Amersham, Bucks., U.K.) to the protein solution before chromatography and counting the radioactivity in the eluted fractions, or by the Chelex technique (Wasserman *et al.*, 1968).

Chelex (100–200 mesh) (Bio-Rad Laboratories, Bromley, Kent, U.K.) was extensively washed with 0.1 M-Tris/HCl buffer, pH 7.4, before use and was used in a 1:1 (w/v) proportion with this buffer. Activity in the Chelex technique was termed 'binding'. The test was performed with 0.1 ml of ⁴⁵CaCl₂ (300 000 c.p.m.; approx. 163 pmol), 0.1 ml of Chelex solution and 0.35 ml of the solution containing either protein in solution or buffer alone. The constituents were mixed and left for 15 min before centrifuging. The radioactivity of 0.1 ml of the supernatant was counted, and 'binding' was expressed as the percentage of ⁴⁵Ca in 0.1 ml of the supernatant from the protein-containing solution compared with the ⁴⁵Ca in the same volume but in the absence of Chelex. ⁴⁵Ca radioactivity was counted in a Packard 2425 liquid-scintillation counter. The scintillant used contained 0.75 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene, 12.5 g of 2,5-diphenyloxazole (Hopkin and Williams, Chadwell Heath, Essex, U.K.) and 1250 ml of Triton X-100 (Koch-Light Laboratories, Colnbrook, Bucks., U.K.) in 2½ litres of toluene. The solution containing ⁴⁵Ca was made up to 1.0 ml with water, and 10 ml of the scintillant was added.

Gel electrophoresis

The 7%-polyacrylamide-gel electrophoresis was performed with a solution containing 0.98 M-acrylamide, 0.023 M-bisacrylamide (Bio-Rad Laboratories), 0.35 M-Tris/HCl buffer, pH 8.8, 1 mM-β-mercaptoethanol, 4 mM-ammonium persulphate and 2 mM-NNN'-tetramethylethylenediamine, the last two substances being added to initiate polymerization. The solution was also made 1 mM with respect to EDTA or to CaCl₂, according to the conditions under which the gel was being run. A stacking gel was used composed of 0.72 M-acrylamide, 0.02 M-bisacrylamide, 0.065 M-Tris/HCl buffer, pH 6.5, 1 mM-β-mercaptoethanol, 4 mM-ammonium persulphate and 2 mM-NNN'-tetramethylethylenediamine with 1 mM-EDTA or CaCl₂ as appropriate. Reservoir buffer contained 0.05 M-Tris, 0.38 M-glycine and 1 mM-CaCl₂ or EDTA as indicated by the gel. Bromophenol Blue was used as the tracker dye. The sample was applied to the top of

the stacking gel and mixed with Bromophenol Blue and 3 drops of 2 M-sucrose. Electrophoresis was performed at 4°C with a current of 0.14 mA/cm². Gels were stained in a mixture containing Coomassie Blue (0.25%), acetic acid (9.2%), methanol (45.2%) and water (45.2%). SDS/polyacrylamide-gel electrophoresis was performed as described by Swank & Munkres (1971) with 12.5%-acrylamide gels.

Molecular-weight determinations

Proteins of known molecular weight used for gel chromatography were (molecular weights in parentheses): ferritin (540 000), catalase (240 000), fructose biphosphate aldolase (158 000), ovalbumin (45 000), chymotrypsinogen (25 000) cytochrome *c* (12 500) (all from Boehringer, Mannheim, Germany) and myoglobin (17 500) (from Sigma Chemical Co., Poole, Dorset, U.K.). Also used were soya-bean trypsin inhibitor (21 500) and lima-bean trypsin inhibitor (9500) (from Sigma Chemical Co.), which gave reasonable peaks on gel chromatography although they were not pure on gel electrophoresis. The elution volume of each standard protein (*V_e*) was measured, and the void volume of the column (*V₀*) was measured by Blue Dextran. *V_e*/*V₀* was plotted against log (molecular weight) of the protein and yielded a straight line.

For chromatography in the presence of guanidinium chloride the molecular-weight-marker proteins were myoglobin (17 500), ribonuclease (13 700), glucagon (3600) and insulin (all from Sigma Chemical Co.). Insulin was found to be eluted in two peaks, which were taken to be the constituent chains (3400 and 2300). Over the molecular-weight range under consideration, *V_e*/*V₀* correlated well with log (molecular weight).

For SDS/polyacrylamide-gel electrophoresis the molecular-weight markers were cytochrome *c* (12 500), aprotinin (6500) and the insulin B-chain (3400) (all from Boehringer). The movement of the protein relative to that of Bromophenol Blue was plotted against log (molecular weight) of the protein and yielded a straight line.

Isoelectric focusing

This was performed on LKB PAG plates pH 3.5–9.5, and the pH gradient was measured across the plate at the end of focusing.

Antisera

Antiserum was prepared by injecting New Zealand White rabbits with material obtained to a single band of purity on SDS/polyacrylamide-gel electrophoresis from CaDE52 peak 1 (see the Results section). A 1.5 ml volume of solution containing 1.4 mg of protein was emulsified in complete Freund's adjuvant (1:1, v/v) in an MSE ultrasonic

vibrator. The emulsion was injected into the buttock muscles, and a repeat injection of 0.7 mg of protein in complete Freund's adjuvant (1:1, v/v) was administered 21 days later. Antibodies were detected after a further week, and this antiserum was designated An1. Antiserum was raised in a similar fashion against the protein found in CaDE52 peak 2 (see the Results section) and was designated An2.

Antigen was detected by the 'rocket' method of Laurell (1966). A 1% solution of agarose (Bio-Rad laboratories) was made in a buffer containing 0.07 M-Tris, 0.024 M-barbitone and 3.0 mM- NaN_3 (pH 8.45). After solubilization by boiling, the agarose was cooled to 60°C and the antiserum was added. The combined antibody/agarose mixture was poured on to glass plates (20 cm $\times$ 10 cm) to a depth of 0.15 cm. Holes were punched at 1 cm centres and allowed 5 μl of sample to be introduced. A potential difference of 4 V/cm was applied across the plate for 16 h at room temperature, after which time the non-precipitated protein was removed by pressing wet filter paper on to the agarose. The gel was dried, stained with Coomassie Blue stain for 2 min and destained. The height of the 'rocket' was measured from the top of the punched hole to the top of the 'rocket'.

U.v. spectrophotometry was performed on a Pye Unicam SP. 1800 spectrophotometer.

Chemicals were normally of AnalaR grade (BDH Chemicals, Poole, Dorset, U.K.), except for the urea used in SDS/polyacrylamide-gel electrophoresis, which was of Aristar grade.

Results

Molecular weights of the calcium-binding fractions in mucosal cytosol

The molecular weights of the calcium-binding fractions in the cytosol from human intestinal mucosa were determined by chromatography on Ultrogel Aca-54. Two peaks of activity were eluted (Fig. 1a): one appeared at the void volume, and the second was eluted at a position corresponding to a molecular weight of 28 000. No calcium-binding fraction was eluted at a molecular weight below 28 000 in any of the six intestines investigated in this manner. Prior removal of endogenous Ca^{2+} from the cytosol by means of Chelex did not affect the distribution of the Ca^{2+} -binding activity. Ca^{2+} -binding activity was also followed in the eluate from gel-filtration columns after the addition of $^{45}\text{CaCl}_2$ to the cytosol before gel filtration. A similar pattern of Ca^{2+} -binding activity emerged (Fig. 1b).

Concentration of cytosol

The considerable volume of cytosol had to be concentrated to manageable quantities before further investigation of the Ca^{2+} -binding. This was achieved

Fig. 1. Ca^{2+} -binding activity (a, Chelex technique; b, $^{45}\text{CaCl}_2$ method) eluted from gel filtration of intestinal mucosal cytosol

In (a) 46 mg of protein was applied to a 1.6 cm $\times$ 75 cm column of Ultrogel Aca-54, and 2.0 ml fractions were collected. In (b) 84 mg of protein was applied to a 1.6 cm $\times$ 69 cm column of Ultrogel Aca-54, and 1.3 ml fractions were collected. For full experimental details see the text., Ca^{2+} -binding activity (a) by the Chelex technique, (b) by the $^{45}\text{CaCl}_2$ method; —, transmittance at 280 nm (arbitrary scale); elution positions of marker proteins (indicated by molecular weight) are shown at the top in each case.

by using an Amicon DC2 hollow-fibre concentrator, which split the cytosol into two fractions: that retained by the filter, which was termed the DC2 retained fraction, and that passing the filter, which was termed the DC2 filtrate. Ca^{2+} -binding activity was detected in both the DC2 retained fraction and the DC2 filtrate, the proportions being $79.3 \pm 54.6\%$ and $20.7 \pm 26.3\%$ (means $\pm$ s.d.) respectively. The Ca^{2+} -binding activity was further investigated by means of the procedures outlined in Table 1.

Properties of the DC2 retained fraction

The DC2 retained fraction was chromatographed on Ultrogel Aca-54. In comparison with whole-gut mucosal cytosol there was a marked decline in the Ca^{2+} -binding activity occurring at molecular weight 28 000 (see Fig. 2 for typical profile). Considerable Ca^{2+} -binding activity was still eluted at the void volume. This Ca^{2+} -binding activity was eluted in a single peak at 0.1–0.15 M-NaCl on ion-exchange chromatography. There was no further elution of

Table 1. Preparation of proteins from intestinal mucosa

For experimental details see the text. Ca²⁺-binding refers to Ca²⁺-binding in the Chelex supernatant/mg of protein in the supernatant, expressed as a ratio of the binding in the cytosol. The ratios are means for the numbers of observations shown in parentheses.

Preparation	Ca ²⁺ -binding
Intestinal mucosa	
↓ Ato-Mix homogenization	
Homogenate	
↓ Centrifugation	
Cytosol	1.0 (6)
↓ DC2 hollow-fibre concentration	
DC2 retained fraction	
↓ DE-52 DEAE cellulose chromatography	
Ca ²⁺ -binding peak	
↓ Ultrogel Aca-34 ↓ Ultrogel Aca-22	
Ca ²⁺ -binding peak of mol.wt. 900 000	
DC2 filtrate	0.9 (6)
↓ DE-52 DEAE-cellulose chromatography	
Ca ²⁺ -binding peak	2.1 (6)
↓ DE-52 DEAE-cellulose chromatography with Ca ²⁺	
CaDE52 peak 1	49.2 (2)
CaDE52 peak 2	15.1 (2)
CaDE52 peak 3

Fig. 2. Ca²⁺-binding activity (⁴⁵CaCl₂ method) eluted from gel filtration of the DC2 retained fraction
A 50 mg portion of protein was applied to a 1.6 cm × 70 cm column of Ultrogel Aca-54, and 3.6 ml fractions were collected. For full experimental details see the text., Ca²⁺-binding activity; —, transmittance at 280nm (arbitrary scale). The position of 28 000-mol.wt. calcium-binding protein is indicated at the top. Use of the Chelex technique yielded a similar distribution of Ca²⁺-binding activity.

Ca²⁺-binding activity up to 0.5 M-NaCl in the two preparations examined in this way. However, the yield of Ca²⁺-binding activity in this peak was low, being only 12% and 25% of the binding activity in the DC2 retained fraction.

Gel filtration of the preparation on Ultrogel Aca-34 still showed that Ca²⁺-binding activity was eluted at the void volume, but retention was achieved by using Ultrogel Aca-22. The molecular weight of the Ca²⁺-binding fraction exceeded that of the highest-molecular-weight-marker protein. However, if the curve connecting the standard proteins were extrapolated, this fraction was eluted at a molecular weight corresponding to 900 000 (Figs. 3a and 3b).

The material associated with the Ca²⁺-binding activity eluted from the Ultrogel Aca-22 had an A₂₈₀/A₂₆₀ ratio of 1.5.

Low-molecular-weight Ca²⁺-binding fraction

Ca²⁺-binding activity was also found in the DC2 filtrate. The activity was eluted on gel-filtration chromatography at a molecular weight of 13 000, and was associated with the main protein peak (Fig. 4). Neither protein nor Ca²⁺-binding activity was present at a higher molecular weight, indicating that the filter was performing close to its speci-

Fig. 3. Ca^{2+} -binding activity (Chelex technique) eluted from gel filtration of partially purified Ca^{2+} -binding component of the DC2 retained fraction

In (a) 42.4 mg of protein from the Ca^{2+} -binding fraction obtained after ion-exchange chromatography of the DC2 retained fraction was applied to a $2.6\text{ cm} \times 92\text{ cm}$ column of Ultrogel Aca-34, and 4.7 ml fractions were collected. In (b) 49 mg of protein from the Ca^{2+} -binding fractions obtained from the gel chromatography shown in (a) was applied to a $2.6\text{ cm} \times 73\text{ cm}$ Ultrogel Aca-22 column, and 3.0 ml fractions were collected. At the top of (b) are shown the elution volumes of (1) ferritin, (2) catalase and (3) fructose biphosphate aldolase plotted according to their molecular weights (top right-hand scale). Extrapolation of the line connecting the molecular weights of these marker proteins indicates that the molecular weight of the Ca^{2+} -binding activity eluted is about 900 000., Ca^{2+} -binding activity; —, transmittance at 280 nm (arbitrary scale) in (a) and absorbance at 280 nm in (b).

cation. The molecular-weight distribution of the protein was also examined by SDS/polyacrylamide-gel electrophoresis of the whole-gut mucosal cytosol and of the DC2 filtrate (Fig. 5). These gels (taken from a series underloaded or overloaded with protein) show that the whole-gut mucosal cytosol has little protein with molecular weight below 12 000, whereas most of the protein in the DC2 filtrate has a molecular weight below this value. The distribution of the protein bands in the gels also suggests that the filter (but not SDS) is responsible for an alteration in the protein pattern of the cytosol.

Fig. 4. Ca^{2+} -binding activity eluted from the DC2 filtrate

A 40 mg portion of protein was applied to a $1.6\text{ cm} \times 66\text{ cm}$ column of Ultrogel Aca-54, and 2.2 ml fractions were collected. The positions of the elution of standard marker proteins (indicated by their molecular weights) are shown at the top., Ca^{2+} -binding activity ($^{45}\text{CaCl}_2$ method); —, transmittance at 280 nm (arbitrary scale).

The Ca^{2+} -binding activity in the DC2 filtrate was also investigated by ion-exchange chromatography (Fig. 6). The main peak of Ca^{2+} -binding activity (arrowed) was eluted at between 0.09 M- and 0.18 M- NaCl , and, although the activity was partially separated into two peaks, no useful separation was achieved by using this system. The fractions in the two peaks were therefore combined and the buffer was changed to a low-molarity ion-exchange buffer containing 1 mM- CaCl_2 in an Amicon stirred cell. This fraction was applied to an ion-exchange column (Whatman DE-52) and eluted with low-molarity buffer containing 1 mM- CaCl_2 . Under these conditions two peaks of protein were eluted (Fig. 7), the first peak being designated CaDE52 peak 1 and the second CaDE52 peak 2. When no further protein was eluted with this buffer, the column was stripped with high-molarity ion-exchange buffer, and a further peak of protein was eluted and denoted CaDE52 peak 3. The fractions constituting each peak were collected, and the Ca^{2+} was removed by EDTA. Both CaDE52 peaks 1 and 2 were shown to have Ca^{2+} -binding activity.

Gel-electrophoresis studies of CaDE52 peaks 1 and 2 (Fig. 8)

Both CaDE52 peaks 1 and 2 were investigated by polyacrylamide-gel electrophoresis in the presence either of EDTA or of CaCl_2 (see the Materials

Fig. 5. SDS/polyacrylamide-gel electrophoresis of whole-gut mucosal cytosol and of the DC2 filtrate

In (a) is shown the distribution of bands after application of whole-gut mucosal cytosol; in (b) is shown the distribution of bands from a sample of the DC2 filtrate. Alongside (b) is shown the mobility expected of cytochrome *c* (1), aprotinin (2) and insulin B-chain (3) when run in this system. Migration is from top to bottom. For full experimental details see the text.

and methods section). Protein from CaDE52 peak 1 migrated as three bands, two of which contained most of the protein. In the presence of EDTA migration was rapid, whereas the presence of CaCl₂ retarded motility to a marked degree. The protein in CaDE52 peak 2 moved at an intermediate rate, which was not influenced by the presence or absence of Ca²⁺. CaDE52 peak 3 contained several bands, one of which corresponded to the CaDE52 peak 2 protein, but there was no band that corresponded to the bands of CaDE52 peak 1 protein.

On SDS/polyacrylamide-gel electrophoresis protein from CaDE52 peak 1 migrated as a single band with a molecular weight equivalent to 7200. Protein from CaDE52 peak 2 migrated as a separate distinct band with a molecular weight of 11 500. The

Fig. 6. Ca²⁺-binding activity eluted from ion-exchange chromatography of the DC2 filtrate

A 1.33 g portion of protein was applied to a 2.6 cm × 64 cm column of Whatman DE-52 DEAE-cellulose and 3.6 ml fractions were collected. For full experimental details see the text. The main Ca²⁺-binding region referred to in the text is arrowed. ---, Ca²⁺-binding activity (Chelex technique); —, transmittance at 280 nm (arbitrary scale) ○, NaCl molarity gradient (right-hand scale).

Fig. 7. Elution of partially purified DC2 filtrate Ca²⁺-binding fraction from ion-exchanger in the presence of Ca²⁺

A 13.5 mg portion of protein from the Ca²⁺-binding fractions depicted in Fig. 6 was applied to a 1.6 cm × 26 cm column of Whatman DE-52 DEAE-cellulose equilibrated with 1 mM-CaCl₂. For experimental details see the text. At (A) elution with low-molarity ion-exchange buffer containing 1 mM-CaCl₂ was started; at (B) elution with high-molarity ion-exchange buffer was started. (1) refers to the peak denoted CaDE52 peak 1 in the text, (2) to the peak denoted CaDE52 peak 2 and (3) to the peak denoted CaDE52 peak 3.

Fig. 8. Polyacrylamide-gel electrophoresis of CaDE52 peak 1 and CaDE52 peak 2 proteins

In (a) is shown protein from CaDE52 peak 1 separated by polyacrylamide-gel electrophoresis in the presence of 1 mM-EDTA; in (b) is shown protein from CaDE52 peak 1 separated by polyacrylamide-gel electrophoresis in the presence of 1 mM- CaCl_2 ; in (c) is shown protein from CaDE52 peak 1 appearing as a single band on SDS/polyacrylamide-gel electrophoresis; in (d) is shown protein from CaDE52 peak 1 and CaDE52 peak 2 separated by SDS/polyacrylamide-gel electrophoresis. The band labelled (1) refers to CaDE52 peak 1 protein and the band labelled (2) to protein from CaDE52 peak 2. Migration is from top to bottom. The band labelled D indicates the position of the Bromophenol Blue tracker dye. For experimental details see the text.

properties of the calcium-binding fractions are summarized in Table 2.

Studies with antiserum

Antiserum was raised against protein from CaDE52 peak 1 (termed An1), and a separate antiserum was obtained that reacted against CaDE52

Fig. 9. Reaction of antiserum An1 with fractions eluted from kidney cytosol or intestinal mucosal cytosol by gel filtration

In (a) 54.6 mg of protein from a preparation of kidney cytosol was applied to an Ultrogel Aca-54 gel-filtration column (1.6 cm $\times$ 69 cm). In (b) 40 mg of intestinal mucosal cytosol protein was applied to the same column. $\blacksquare$ — $\blacksquare$, Ca^{2+} -binding ($^{45}\text{CaCl}_2$ technique); —, transmittance at 280 nm (arbitrary scale). The hatched areas correspond to fractions reacting with antiserum An1 measured by the 'rocket' technique (see the text). The height of the vertical dotted lines indicates the height of the 'rocket' (right-hand scale).

peak 2 protein (termed An2). Cross-reactions of the antisera are shown in Table 3.

To investigate the molecular-weight distribution of antigen in whole-gut mucosal cytosol and in kidney cytosol, antiserum An1 was used to detect antigen in the eluate from Ultrogel-Aca-54-gel chromatography of these cytosols (Figs. 9a and 9b). By use of the Laurell (1966) 'rocket' procedure, antigen was found only in the region coincident with the 28000-mol.wt. Ca^{2+} -binding fraction. A further intestinal cytosol was chromatographed on Ultrogel Aca-54 in the presence of $2\text{ }\mu\text{M}$ - CaCl_2 and $^{45}\text{CaCl}_2$, and the eluate was investigated with both antisera An1 and An2. There was a clear distinction between the molecular weights of the fractions against which each antiserum was reacting (Fig. 10).

Quantitative studies with antiserum An1

By using the Laurell (1966) 'rocket' procedure an assay method was established that used antiserum An1. In view of the findings described in the preceding section, this assay method was taken to measure calcium-binding protein associated with the 28000-mol.wt. Ca^{2+} -binding fraction. A linear relationship existed between 'rocket' height and the weight of the calcium-binding protein. The minimum quantity of calcium-binding protein that could be assayed was 100 ng, and at 500 ng of calcium-

binding protein the standard deviation/mean ratio was 0.108 (six observations). This assay method was used to investigate the concentrations of

calcium-binding protein in intestine and in kidney (Table 4). The highest value obtained was found in the jejunal mucosal cytosol from the subject with hypercalcaemic sarcoidosis. The concentration in the jejunal mucosa from the subject with jejunal diverticulosis was, however, not low.

Protein band pattern of jejunal mucosal cytosol on polyacrylamide-gel electrophoresis

Since the subject with hypercalcaemic sarcoidosis had the highest concentration of calcium-binding protein and would be expected to have high concentrations of 1,25-dihydroxycholecalciferol (see the Discussion section), this cytosol might be expected to show CaDE52 peak 1 protein. The presence of this protein was investigated by gel electrophoresis in this subject, and it was compared with protein from the jejunal mucosa of the subject with jejunal diverticulosis and the mucosa from a normal subject (Fig. 11). The only cytosol to show a band equivalent to that of CaDE52 peak 1 protein was that from the subject with jejunal diverticulosis.

Fig. 10. Reaction of antiserum An1 and antiserum An2 with fractions of intestinal mucosal cytosol eluted by gel filtration

A 71mg portion of protein from an intestinal mucosal preparation was applied to a 1.6 cm x 66 cm column of Ultrogel Aca-54. Before chromatography 10 μ Ci of ⁴⁵CaCl₂ was added to the cytosol, which was also made 2 μ M with respect to CaCl₂., Ca²⁺-binding (⁴⁵CaCl₂ method); —, transmittance at 280nm (arbitrary scale). The open rectangles indicate the fractions reacting with antiserum An1, and the shaded rectangles those fractions reacting with antiserum An2; the height of the rectangles is equivalent to the height of the 'rocket' (see the text) obtained (right-hand scale).

Table 3. Reactions of antisera An1 and An2

Preparation	Antiserum An1	Antiserum An2
Human whole-gut mucosal cytosol	+	+
DC2 retained fraction	+	+
DC2 filtrate	+	+
Human kidney cytosol	+	Weak +
Human plasma	—	+
Intestinal mucosal cytosol from baboon (<i>Papio</i> sp.)	+	Not tested
Chick intestinal calcium-binding protein	—	—

Table 2. Properties of CaDE52 peak 1, peak 2 and peak 3 proteins
For experimental details see the text. Numbers of observations are shown in parentheses.

Property	CaDE52 peak 1 protein	CaDE52 peak 2 protein	CaDE52 peak 3 protein
Mol.wt. (gel filtration)	9500, 10 500 (2)	11 000–12 000 (4)	12 000 (2)
(gel filtration in 6 M-guanidinium chloride)		9400 (1)	
(SDS/polyacrylamide-gel electrophoresis)	7200 (4)	11 500 (2)	
Isoelectric point	5.0 (2)	5.4 (2)	
Mobility on non-SDS polyacrylamide-gel electrophoresis			
(a) In EDTA	0.75* (4)	0.54 (4)	
(b) In CaCl ₂	0.04* (4)	0.54 (4)	
A ₂₈₀ /A ₂₆₀ ratio	1.1–1.3 (3)	1.3, 1.4 (2)	
Tryptophan absorption	Absent	Present	
Wt. recovered from intestine (mg)	2.8–5.8 (4)	5.0–13.0 (4)	

* Refers to the major band.

Table 4. Concentrations of calcium-binding protein (investigated by antiserum An1) in intestine of four subjects. For experimental details see the text. Subjects: 1, 34-year-old male, normal; 2, 7-year-old male, jejunal diverticulosis; 3, 44-year-old male, hypercalcaemic sarcoidosis; 4, 16-month-old female, idiopathic hypercalcaemia of childhood (on cortisone).

Subject	Concentration of calcium-binding protein							
	Duodenum		Proximal jejunum		Distal jejunum		Ileum	
	($\mu\text{g/g}$ of mucosa)	($\mu\text{g/mg}$ of cytosol protein)	($\mu\text{g/g}$ of mucosa)	($\mu\text{g/mg}$ of cytosol protein)	($\mu\text{g/g}$ of mucosa)	($\mu\text{g/mg}$ of cytosol protein)	($\mu\text{g/g}$ of mucosa)	($\mu\text{g/mg}$ of cytosol protein)
1	915	16.9	834	17.0	1141	27.1	443	24.6
2		33.1						
3	1200	60.0						
4		33.2		27.8		20.5		20.0

Fig. 11. Polyacrylamide-gel electrophoresis of intestinal mucosal cytosol preparations obtained from normal subjects and from subjects with disturbances of vitamin D metabolism

Cytosol protein preparations were applied to polyacrylamide gels, and electrophoresis was performed in the presence of 1 mM-EDTA (see the text). (I) CaDE52 peak 2 protein (labelled 2); (II) normal intestinal mucosal cytosol; (III) jejunal mucosal cytosol from subject with jejunal diverticulosis; (IV) jejunal mucosal cytosol from subject with hypercalcaemic sarcoidosis; (V) normal jejunal

Discussion

The large quantity of high-molecular-weight Ca^{2+} -binding fraction, the absence of Ca^{2+} -binding activity at molecular weight 12000 and the presence of such a peak at molecular weight 28000 distinguish human small-intestinal mucosal cytosol from that of other species (Hitchman & Harrison, 1972; Fullmer & Wasserman, 1973) apart from the rat, in which Ca^{2+} -binding activity at molecular weight 28000 is found (Hosoya *et al.*, 1977). Many purification procedures include a heating step, which diminishes high-molecular-weight Ca^{2+} -binding activity in human preparations (M. Davie, unpublished work). Part of the high-molecular-weight Ca^{2+} -binding fraction may be composed of Ca^{2+} -binding goblet-cell mucin, which would not be eluted from ion-exchange resin with ionic strength of 0.7 M (Forstner & Forstner, 1975).

Amicon DC2 hollow-fibre concentration led to loss of the 28000-mol.wt. Ca^{2+} -binding fraction and to the appearance of a lower-molecular-weight Ca^{2+} -binding fraction. One of the proteins associated with this derived fraction had antigen in common with the 28000-mol.wt. fraction. The derived protein (CaDE52 peak 1 protein) had properties in common with pig calcium-binding protein [lack of tryptophan absorption, molecular weight on SDS/polyacrylamide-gel electrophoresis and differential mobility on polyacrylamide-gel electrophoresis in the presence and in the absence of Ca^{2+} (Hitchman *et al.*, 1973, 1974)]. The lack of CaDE52 peak 1 protein in unprocessed cytosol contrasts with animal studies. The older age and prolonged adequate vitamin D

mucosal cytosol. Migration is from top to bottom. The numbers alongside the gels indicate the positions of the following proteins: 1, band with mobility equivalent to CaDE52 peak 1 protein; 2, band with mobility equivalent to CaDE52 peak 2 protein; 3, band equivalent to human albumin. The band labelled D in gel (V) indicates the position of the Bromophenol Blue tracker dye.

status of the human subjects may be important factors.

Use of antiserum An1 showed that CaDE52 peak 1 protein was distinct from CaDE52 peak 2 protein; antiserum An1 reacted with renal 28000-mol.wt. Ca²⁺-binding fraction but not with chick calcium-binding protein. Anti-(chick calcium-binding protein) antibody reacts with human renal Ca²⁺-binding fraction but not with the intestinal calcium-binding protein (Wasserman *et al.*, 1978). It is thus possible that antiserum An1 was reacting with a different part of the 28000-mol.wt. calcium-binding protein than was anti-(chick calcium-binding protein) antibody.

Concentrations of calcium-binding protein in intestinal segments were more akin to the pig (Arnold *et al.*, 1975) than to the cow (Wasserman *et al.*, 1978). The highest concentration occurred in sarcoidosis, a condition associated with elevated 1,25-dihydroxycholecalciferol concentrations (Papapoulos *et al.*, 1979). Since concentrations of this hormone may be maintained even when plasma 25-hydroxycholecalciferol concentrations are low (Eastwood *et al.*, 1979), the presence of calcium-binding protein in the intestine of the subject with jejunal diverticulosis is not unexpected, although the relationship of calcium-binding protein to osteomalacia is controversial (Harrison *et al.*, 1975). The presence of a band on polyacrylamide-gel electrophoresis coincident with CaDE52 peak 1 in this mucosa (Fig. 11) may, however, reflect a change in the form of calcium-binding protein in response to vitamin D deficiency.

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References

- Alpers, D. A., Lee, S. W. & Avioli, L. V. (1972) *Gastroenterology* **62**, 559–564
- Arnold, B. M., Kuttner, M., Willis, D. M., Hitchman, A. J. W., Harrison, J. E. & Murray, T. M. (1975) *Can. J. Physiol. Pharmacol.* **53**, 1135–1140
- Bar, A. & Wasserman, R. H. (1974) *J. Nutr.* **104**, 1202–1207
- Eastwood, J. B., de Wardener, H. F., Gray, R. W. & Lemann, J. L. (1979) *Lancet* **i**, 1377–1378
- Fish, W. W., Mann, K. G. & Tanford, C. (1969) *J. Biol. Chem.* **244**, 4989–4994
- Forstner, J. F. & Forstner, G. G. (1975) *Biochim. Biophys. Acta* **386**, 283–292
- Fullmer, C. S. & Wasserman, R. H. (1973) *Biochim. Biophys. Acta* **317**, 172–186
- Harrison, J. E., Hitchman, A. J. W. & Brown, R. G. (1975) *Can. J. Physiol. Pharmacol.* **53**, 144–149
- Hitchman, A. J. W. & Harrison, J. E. (1972) *Can. J. Biochem.* **50**, 758–765
- Hitchman, A. J. W., Kerr, M. K. & Harrison, J. E. (1973) *Arch. Biochem. Biophys.* **155**, 221–222
- Hitchman, A. J. W., Hui, A., Hofmann, T., Murray, T. M., Arnold, B., Dorrington, K. J., Kerr, M. K. & Harrison, J. E. (1974) *Calcium-Binding Proteins* (Drabikowski, W., Strzelecka-Golaszewska, H. & Carafoli, E., eds.), pp. 771–790, Elsevier, Amsterdam
- Hosoya, N., Yamanouchi, T. & Moriuchi, S. (1977) *Calcium-Binding Proteins and Calcium Function* (Wasserman, R. H., Corradino, R. A., Carafoli, E., Kretsinger, R. H., MacLennan, D. H. & Siegel, F. L., eds.), pp. 376–378, North-Holland, New York
- Laurell, C. B. (1966) *Anal. Biochem.* **45**, 45–52
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Morrissey, R. L. & Rath, D. F. (1974) *Proc. Soc. Exp. Biol. Med.* **145**, 699–703
- Papapoulos, S. E., Clemens, T. L., Fraher, L. J., Lewin, I. G., Sandler, L. M. & O'Riordan, J. L. H. (1979) *Lancet* **i**, 627–630
- Piazolo, P., Schleyer, M. & Franz, H. E. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 1480–1486
- Spencer, R., Charman, M., Wilson, P. W. & Lawson, D. E. M. (1976) *Nature (London)* **263**, 161–163
- Swank, R. T. & Munkres, K. D. (1971) *Anal. Biochem.* **39**, 462–477
- Wasserman, R. H., Corradino, R. A. & Taylor, A. N. (1968) *J. Biol. Chem.* **243**, 3978–3986
- Wasserman, R. H., Fullmer, C. S. & Taylor, A. N. (1978) *Vitamin D* (Lawson, D. E. M., ed.), chapter 4, Academic Press, London