**Table 1** Activities of phosphofructokinase and three marker enzymes in a chloroplast extract and cytoplasmic fraction from spinach leaves

| | Chloropiast extract | | Cytoplasmic fraction | | Ratio: |
|---|---|---|---|---|---|
| | Total activity* | Specific activity† | Total activity* | Specific activity† | s.a. chloroplast / s.a. cytoplasm |
| Phosphofructokinase | 1.9 | 0.25 | 31 | 1.0 | 0.25 |
| Non-reversible GAPDH | 0.40 | 0.05 | 72 | 2.4 | 0.02 |
| Chloroplast GAPDH | 320 | 45 | 1200 | 39 | 1.1 |
| FDP-aldolase | 110 | 14 | 310 | 10 | 1.4 |

* Measured as μmol per h per 10 g fresh weight of leaves.
† Expressed as μmol per mg protein per h.
Enzyme activities were determined at 22 °C. The reaction mixtures for phosphofructokinase contained, in a final volume of 1 ml, 50 μmol imidazole-HCl buffer (*p*H 7.7), 2.5 μmol $MgCl_2$, 2.5 μmol dithioerythritol, 1.5 μmol D-fructose-6-phosphate, 0.5 μmol ATP, 0.08 μmol NADH, 1 unit aldolase, 12 units triosephosphate isomerase, 1 unit α-glycerophosphate dehydrogenase and 50–100 μl extract. The composition of reaction mixtures for the determination of FDP-aldolase and chloroplast GAPDH activities were based on those described earlier[2]; the buffers used in the present experiment were imidazole-HCl (*p*H 7.7) and triethanolamine-NaOH (*p*H 8.0) respectively. Non-reversible GAPDH was assayed as described previously for cell-free plant extracts[9]. All assay enzymes were dialysed before use to remove $(NH_4)_2SO_4$. Activities were calculated from changes in extinction of reaction mixtures at 340 nm measured spectrophotometrically. Protein was determined by the Biuret procedure as described previously[9].

relatively high concentrations of ATP inhibited the chloroplast phosphofructokinase activity: phosphoenolpyruvate at 0.1 mM produced more than 70% inhibition, while the activity with 4.8 mM ATP was less than half that with the optimum ATP concentration of 0.1 mM. Inhibition with increased levels of ATP is a common property of phosphofructokinase[6] and sensitivity to inhibition by phosphoenolpyruvate is a notable feature of plant phosphofructokinase[7]. Some of these regulatory phenomena may explain why phosphofructokinase activity was not found in chloroplasts in earlier studies[1-4] particularly if ATP levels used in those assays were too high.

Preliminary experiments in which intact chloroplasts were added to a solution containing the substrates for phosphofructokinase showed that the product fructose-1,6-diphosphate was not formed when the solution was isotonic (containing 0.33 M sorbitol) and the chloroplasts remained intact, but product was formed at a rate of approximately 2 μmol per mg chlorophyll per h when sorbitol was omitted and the chloroplasts were broken. These results not only confirmed that the phosphofructokinase was contained within the chloroplast, but also demonstrated an activity compatible with the amount of starch expected to be metabolised in chloroplasts. It is not inconceivable that chloroplast phosphofructokinase catalyses a regulatory step in the degradation of starch in the chloroplast. Efforts are now being made to separate and partially purify the chloroplast and cytoplasmic phosphofructokinases from leaves with a view to relating their regulatory properties to the carbon metabolism of photosynthesis. Initial results are encouraging.

This work was assisted by the Deutsche Forschungsgemeinshaft.

G. J. KELLY
E. LATZKO

*Abteilung Chemische Pflanzenphysiologie,*
*Technische Universität München,*
*8050 Weihenstephan, Germany*

Received May 28; accepted June 15, 1975.

1 Smillie, R. M., *Can. J. Bot.*, **41**, 123–154 (1963).
2 Latzko, E., and Gibbs, M., *Z. PflPhysiol*, **59**, 184–194 (1968).
3 Schnarrenberger, C., and Oeser, A., *Eur. J. Biochem.*, **45**, 77–82 (1974).
4 Kachru, R. B., and Anderson, L. E., *Pl. Physiol., Lancaster*, **55**, 199–202 (1975).
5 Heldt, H. W., and Rapley, L., *FEBS Lett.*, **10**, 143–148 (1970).
6 Lowry, O. H., and Passonneau, J. V., *Archs exp. Path. Pharmak.*, **248**, 185–194 (1964).
7 Kelly, G. J., and Turner, J. F., *Biochim. biophys. Acta*, **208**, 360–367 (1970).
8 Lilley, R. McC., Walker, D. A., and Holborow K., *Biochim. biophys. Acta*, **368**, 269–278 (1974).
9 Kelly, G. J., and Gibbs, M., *Pl. Physiol., Lancaster*, **52**, 111–118 (1973).

# Localisation of plasma $\alpha_2$HS glycoprotein in mineralising human bone

THE protein of the organic matrix of bone consists mainly of collagen in association with a small proportion of non-collagenous proteins. When bone is decalcified, much of the non-collagenous protein can be solubilised in conditions of neutral *p*H but the collagen remains insoluble. Extracts of decalcified bone prepared in this manner contain small amounts of plasma proteins which derive in part from the presence of blood vessels within the tissue[1,2]. There is evidence, however, that some plasma proteins, including albumin, form an integral part of the calcified matrix[3]. When $^{14}$C-glucosamine or $^{14}$C-glucosamine-labelled rabbit total plasma protein is injected into a rabbit, some activity is incorporated into the bone[4]. Using the procedure of Herring[5] to separate the EDTA-soluble proteins of the bone matrix, most of the activity was shown to be present in the less acidic glycoprotein fraction, much of this in one glycoprotein which was also present as a minor component of blood[4]. This G2B glycoprotein had the electrophoretic mobility of an α globulin and a molecular weight by SDS gel electrophoresis[6] of about 50,000. Studies by Ashton *et al.* (private communication) support the view that the G2B glycoprotein they have investigated in rabbit[4] and bovine bone[6] is the analogue of human $\alpha_2$HS glycoprotein.

In this report we provide evidence by immunoprecipitation and passive haemagglutination techniques that $\alpha_2$HS glycoprotein (an α globulin of estimated molecular weight 49,000 by ultracentrifugal analysis[10] present in human plasma at a concentration of about 60 mg $dl^{-1}$) is concentrated extravascularly in the matrix of normal human bone and can be located in areas of mineralisation in bone using an immunofluorescent antibody-staining technique.

The non-collagenous proteins of normal adult human cortical bone were isolated by sequential extraction of the powdered bone with 0.5 M EDTA at *p*H 7.6. The concentrated extract, dialysed free of mineral salts, and human plasma each gave a single precipitin line of complete identity when they were diffused against the antiserum to plasma $\alpha_2$HS glycoprotein. The antiserum used in these studies was raised in rabbits against human plasma $\alpha_2$HS glycoprotein and was specific for the latter, as judged by immunoelectrophoresis and double diffusion against plasma proteins. Tanned sheep erythrocytes, coated with the non-collagenous bone protein as described previously[1], agglutinated in the presence of antiserum to $\alpha_2$HS glycoprotein but not with antisera to two other human plasma α globulins, $\alpha_1$ antitrypsin and $\alpha_2$ macroglobulin (Table 1). The latter two proteins are present in plasma at concentrations approximately three times higher than $\alpha_2$HS glycoprotein in normal individuals. This suggested that the identification of only the latter protein in bone in these conditions did not derive from the presence of blood within the tissue. The antisera to the three α globulins were each adsorbed with lyophilised bone protein and then incubated with human plasma protein-coated tanned sheep erythrocytes. These preparations had negligible effect (Table 1) on the haemagglutination titres of two of the antisera but the activity of that against $\alpha_2$HS glycoprotein was destroyed. By radial immunodiffusion in an agarose gel containing antibody, the concentration of $\alpha_2$HS

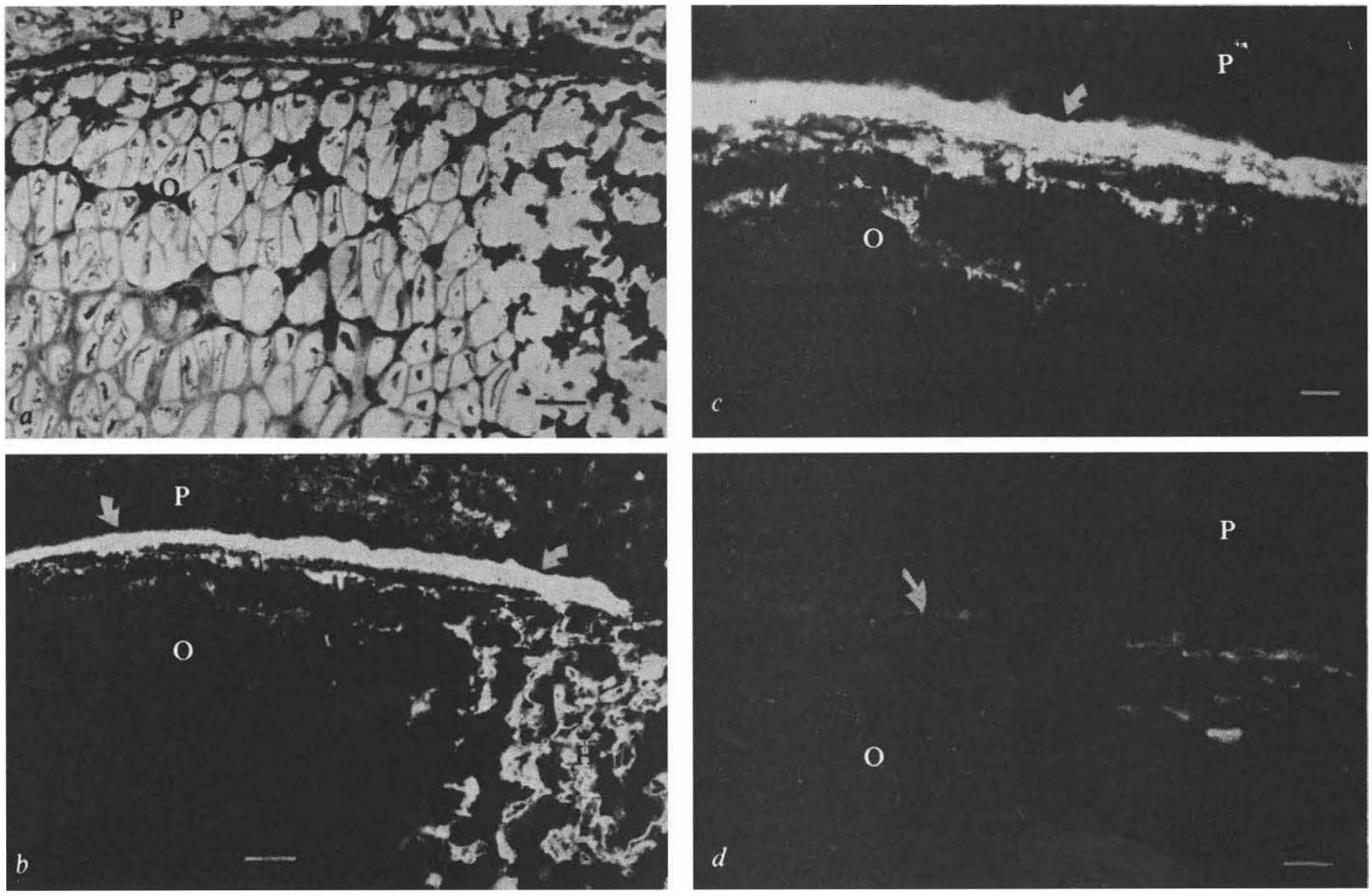


**Fig. 1** Longitudinal 6 μm thick sections of femur. Frozen sections were fixed for 5 min in formaldehyde as described previously[8]. Sections were then washed in phosphate-buffered saline (PBS) for 30 min. *a*, Stained with von Kossa's reagent and counterstained with haematoxylin and chromotrope. A band of brown/black staining (arrows) in the periosteum (P) overlays staining for calcium in the hypertrophic cartilage which is either heavily ossified (H) or partially ossified (O). *b–d*, Immunohistochemical localisation of $\alpha_2$HS glycoprotein. Sites of binding of rabbit antibody Fab′ to $\alpha_2$HS glycoprotein in tissue sections were detected by subsequently using fluorescein isothiocyanate-labelled pig antibody Fab′ directed against rabbit Fab′. Thus sections were initially treated with pepsin digests (at 1.6 mg ml$^{-1}$) of 33% $(NH_4)_2SO_4$ immunoglobulin G-rich fractions isolated from the antiserum to $\alpha_2$HS glycoprotein (*b* and *c*) or non-immune rabbit serum (control); *d*, as described previously[9]. Sections were washed in PBS and finally stained with Fab′ isolated from a pig antiserum to rabbit Fab′, and labelled with fluorescein isothiocyanate at a concentration of 2 mg ml$^{-1}$. Sections were finally washed, mounted and examined (for details see ref. 9). *b* and *c*, Intense green extracellular fluorescence can be seen in the calcified band in the periosteum (arrows) and in the heavily ossified sites in the shaft (H) is magnified region of *b*. Less intense staining is present in sites of partial ossification (O). *d*, No intense green fluorescence was detected and only weak non-specific cellular staining was seen. Comparable sites are indicated as in *b* and *c*. Scales: *a* and *b*, 50 μm; *c* and *d*, 20 μm.

glycoprotein was estimated as 0.1% of the organic matrix of bone.

To establish whether the protein was incorporated into bone during the process of mineralisation, attempts were made to localise the protein by immunohistochemical techniques. The tissue used was the femur of a human foetus aged approximately 12 weeks (obtained after a therapeutic abortion). The femur was placed in a solution of 7% gelatin and 0.09% NaCl at 30°C and then rapidly frozen in liquid nitrogen before final storage at −20°C. Frozen sections were fixed and then incubated with a pepsin digest of either rabbit anti-(human $\alpha_2$HS glycoprotein) immunoglobulin G or a rabbit non-immune globulin as control, and then with the fluorescein-labelled Fab′ fragment of a pig antibody to rabbit immunoglobulin G Fab′. Some sections were fixed and stained by von Kossa's method for calcium, being counterstained with haematoxylin and chromotrope. Staining for calcium revealed a broad band of calcification in the periosteum adjacent to the hypertrophic cartilage of the shaft (Fig. 1*a*). There was also extensive calcification within the shaft and early signs of calcification in the hypertrophic cartilage. Wherever extracellular calcium was detected we observed very intense green fluorescent staining in sections which had been initially treated with the digest of the immune globulin (Fig. 1*b* and *c*). The intensity of this immunohistochemical staining was greatest where staining with von Kossa's reagent was most concentrated. Sections which had been treated with the control non-immune immunoglobulin digest showed almost no staining associated with extracellular

**Table 1** Haemagglutination titres of various antisera against three plasma α globulins

| Protein | Plasma concentration | Reciprocal heamagglutination titre | | |
|---|---|---|---|---|
| | | Bone protein-coated cells | Serum protein-coated cells | |
| | | | Control | Adsorbed |
| $\alpha_2$ macroglobulin | 180 mg dl$^{-1}$ | ⩽4 | 2048 | 1024 |
| $\alpha_1$ antitrypsin | 150 mg dl$^{-1}$ | ⩽4 | 512 | 512 |
| $\alpha_2$ HS glycoprotein | 60 mg dl$^{-1}$ | 512 | 128 | ⩽4 |

*Average concentrations in adult human plasma of three α globulins. Antisera against those proteins were incubated with tanned sheep erythrocytes coated with human bone proteins; only antiserum against $\alpha_2$HS glycoprotein caused haemagglutination. If these antisera were first absorbed with protein this inhibited the capacity of the antiserum against $\alpha_2$HS glycoprotein to haemagglutinate tanned sheep erythocytes coated with either bone protein (not shown) or serum protein compared with the non-adsorbed antiserum control.

sites, including those where calcification had taken or was taking place (Fig. 1*d*). These control specimens did exhibit a very low level of non-specific green fluorescent cellular staining of the kind also seen in the test sections.

These results show therefore that a plasma protein, $\alpha_2$HS glycoprotein, is a constituent of both foetal and adult human bone matrix and is incorporated and concentrated into bone presumably through interactions with one of the other components of the mineralising matrix. The function of this protein in bone tissue has not yet been established but it has been observed[7] that human plasma $\alpha_2$HS glycoprotein possesses opsonic properties, as demonstrated by its capacity to promote increased phagocytosis of *Staphylococcus aureus* and *Escherichia coli* by human neutrophils *in vitro*. This would account for our own observations that plasma levels of $\alpha_2$HS glycoprotein, measured by radial immunodiffusion, fall markedly after various surgical procedures (I. R. D., unpublished); in the case of surgical damage to bone, plasma levels of this glycoprotein, corrected for plasma volume changes, fall to as little as half their pre-operative values. There is no evidence that this protein is present in areas where pre-osseous cartilage is degraded so that, if the opsonic property is utilised, it will most likely be in mediating the eventual resorption of the bone.

We thank Dr E. Kodicek for advice and support, and Dr C. Alper and Mr D. Evans for antisera. This work was supported in part by the NIH.

I. R. DICKSON

*Strangeways Research Laboratory, Wort's Causeway, Cambridge, UK*

A. R. POOLE

*Division of Immunology, Department of Pathology, University of Cambridge, UK*

A. VEIS

*Northwestern University Medical School, 303 East Chicago Avenue, Chicago, Illinois 60611*

Received April 15; accepted June 17, 1975.

1 Dickson, I. R., *Calcified Tiss. Res.*, **16**, 321–333 (1974).
2 Burchard, J., Havez, R., and Dautrevaux, M., *Bull. Soc. chim. Biol.*, **48**, 851–861 (1966).
3 Ashton, B. A., Herring, G. M., Owen, M. E., and Triffitt, J. T., *Israel J. med. Sci.*, 7, 409–411 (1971).
4 Triffitt, J. T., and Owen, M., *Biochem. J.*, **136**, 125–134 (1973).
5 Herring, G. M., Ashton, B. A., and Chipperfield, A. R., *Prepar. Biochem.*, 4, 179–200 (1974).
6 Ashton, B. A., Triffitt, J. T., and Herring, G. M., *Eur. J. Biochem.*, **45**, 525–533 (1974).
7 van Oss, C. J., Gillman, C. F., Bronson, P. M., and Border, J. R., *Immun. Commun.* 3, 329–355 (1974).
8 Poole, A. R., Dingle, J. T., and Barrett, A. J., *J. Histochem. Cytochem.*, **20**, 261–265 (1972).
9 Poole, A. R., in *Methological Developments in Biochemistry*, 4 (edit. by Reid, E.), 1 (Longmans, London, 1974).
10 Schmid, K., and Bürgi, W., *Biochim. biophys. Acta*, **47**, 440–453 (1961).

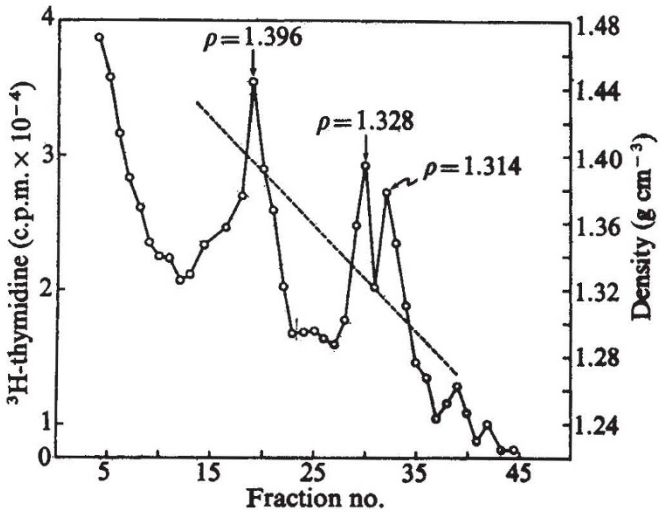


**Fig. 1** Isopycnic centrifugation of HWV grown in tissue culture. BE cells were infected with HWV that has been passaged in tissue culture 11 times. $^3$H-thymidine (2 μCi ml$^{-1}$) was added to the culture fluid 2 h after infection. Rounded, detached cells along with culture medium were collected 5 d later; cells were disrupted by repeated freeze–thawing, clarified at 800 r.p.m., and virus was concentrated by centrifugation (SW27; 2 h, 25,000 r.p.m.). Resuspended viral pellet was centrifuged in CsCl (ρ 1.32 g cm$^{-3}$) in an SW50 rotor for 72 h at 40,000 r.p.m.; fractions were collected from the bottom of the tube. Gradient density was determined by measuring refractive index of various fractions.

# Propagation of human wart virus in tissue culture

HUMAN wart (papilloma) virus (HWV) is an authentic human tumour virus. Based on morphology, it has been classified as a member of the papova group[1] of animal tumour viruses. Using electron microscopy, HWV has been found to be associated with human skin warts[2], laryngeal and venereal papillomas[3,4] and epidermodysplasia verruciformis[5]. Moreover, experiments done with human volunteers at the beginning of this century[6–8], showed that filtered extracts of warts, laryngeal and venereal papillomas can cause skin warts in man. Since these early experiments, there has been little increase in our knowledge of HWV as a causative agent of either benign tumours or those which can progress to malignancy[5,9], mainly because of the lack of a system in which to propagate HWV *in vitro*[10]. This report describes the propagation of HWV in a tissue culture system.

HWV is epitheliotropic. It infects epidermal cells, a wart probably being a clone of transformed descendants[11] of a single infected cell. Virus progeny are produced in epithelial cells *in vivo*[2]. Our first choice was, therefore, to select a human epithelioid cell strain in which to attempt virus propagation *in vitro*. We have used a cell strain established by Dr M. Bean (Bean epithelial, BE) which, to our knowledge, is unique, as no other epithelioid cell strains have been established from human skin.

Virus was obtained from surgically removed samples of human skin warts, collected in tissue culture medium, incubated at 37 °C for 27 h, and either stored at −80 °C or used immediately. Pooled samples of wart tissue were minced and homogenised with carborundum with a mortar and pestle. Suspensions (10% w/v) were prepared according to a procedure described by Pass and Marcus[12].

To infect BE cells successfully *in vitro*, it was necessary to use cells in the logarithmic phase of growth and to allow the virus to absorb to the cells at low *p*H (6.5), and then to stress the cells by raising the *p*H rapidly to an alkaline level (8.0), after which the *p*H was allowed to fall slowly in a $CO_2$ incubator to *p*H 7.0–7.2. Alkaline *p*H stress was repeated 24 h after infection, and the HWV was thus successfully passaged 14 times in series. Unless cells were treated each time with these three successive *p*H stresses, serial passage of HWV with cytopathic effect (CPE) failed. The following changes were considered to constitute CPE. At 4–5 d after infection many rounded cells appeared which, in stained preparations, were seen to have intranuclear inclusions accompanied by nuclear vacuolisation. Infected cultures at this time also contained many clear areas surrounded by rounded cells that could be shown by immunofluorescence to contain viral antigens. Rounded cells eventually became detached and died; however, even with continued incubation for 3–4 weeks, only about 50% of the adjacent flattened cells showed similar changes. When the rounded cells were shaken off and examined by indirect immunofluorescence, using hyperimmune rabbit antisera prepared against virus purified from human warts, 90% showed positive nuclear fluorescence. When the remaining attached cells were removed with trypsin and similarly examined, 2–4% were positive. Mock-infected controls treated