Studies on Bone Matrix Glycoproteins

INCORPORATION OF [1-¹⁴C]GLUCOSAMINE AND PLASMA [¹⁴C]GLYCOPROTEIN INTO RABBIT CORTICAL BONE

By JAMES T. TRIFFITT and MAUREEN OWEN

Bone Research Laboratory, The Churchill Hospital, Headington, Oxford OX3 7LJ, U.K.

(Received 26 March 1973)

The radioactively labelled constituents present in bone matrix were compared 12 days after injection of either [¹⁴C]glucosamine or plasma [¹⁴C]glycoprotein. Both precursors are utilized in the synthesis of organic matrix by bone tissue. Cortical bone from animals injected with [¹⁴C]glucosamine contains radioactivity derived from glucosamine and plasma glycoproteins and all glycoprotein fractions are labelled. Plasma [¹⁴C]glycoprotein labels the less acidic glycoproteins to a greater extent than the more acidic components. An antibody has been raised against the less-acidic-glycoprotein fraction of bone. The latter contains a glycoprotein of α -mobility that appears to be concentrated specifically in bone tissue and which is present also in plasma. This α -glycoprotein accounts for a large proportion of the components labelled and retained in bone matrix after [¹⁴C]glucosamine injection.

The organic matrix of bone contains collagen as the major constituent and smaller quantities of glycoproteins, proteoglycans, glycosaminoglycans, lipids and peptides. The glycoproteins (Gottschalk, 1966) make up the largest proportion of the non-collagenous proteins of bone (Herring, 1972). The acidic fractions of bovine and rabbit bone have been isolated and characterized (Herring, 1964; Burckard *et al.*, 1966) and recently studies of the less-acidic-glycoprotein fractions have been made (Ashton *et al.*, 1971). Glycoproteins are present in high concentration in osteoid at sites where mineralization is occurring (Lapierre & Nusgens, 1970; Pugliarello *et al.*, 1970) and are thought to play a role in biological calcification (Nusgens *et al.*, 1972; Weinstock *et al.*, 1972).

Radioautographic studies (Owen & Shetlar, 1968; Owen & Triffitt, 1972) have shown that, 12 days after an injection of either [¹⁴C]glucosamine or plasma [¹⁴C]glycoprotein, radioactive material is in calcified bone in a position corresponding to the matrix synthesized immediately after injection. The present work is an extension of these studies and was designed to investigate the nature of the components of bone matrix derived from these two precursors during bone formation.

Experimental

Materials

D-[1-¹⁴C]Glucosamine hydrochloride (specific radioactivity 3.1 mCi/mol) was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.).

Plasma [¹⁴C]glycoprotein was obtained from Dutch rabbits (400 g) 5–6 h after an intraperitoneal injection of 250 μ Ci of [1-¹⁴C]glucosamine. Previous work has shown that this label is incorporated mainly into glucosamine residues (at least 70% of the total label) with the remainder in sialic acid moieties (Shetlar *et al.*, 1961, 1964; Winzler, 1965; Capps *et al.*, 1966; Robinson, 1968). Blood was obtained by cardiac puncture and plasma separated by centrifugation. Plasma was dialysed against 0.16 M-NaCl overnight, stored at –20°C and centrifuged before use. This solution contained approximately 4 μ Ci of ¹⁴C/ml.

Methods

Injection schedules. Dutch rabbits (600–700 g) were used and food and water were given *ad libitum* during the experiments. The animals were progeny of stock obtained from Goodchild Bros., Crawley, Sussex, U.K.

[¹⁴C]Glucosamine (100 μ Ci) was injected intraperitoneally into each of three animals followed by a similar injection 6 h later. Three other rabbits each received four intravenous injections of 4 ml of plasma [¹⁴C]glycoprotein at 24 h intervals via the ear vein. In each case, 12 days after the last injection, the animal was anaesthetized with ether, and blood was obtained by cardiac puncture. Blood plasma was prepared by centrifugation, the femora, tibiae and humeri shafts were dissected and the compact bone was cleaned thoroughly of all contaminating soft tissue and marrow.

Tissue extraction. Two types of extraction procedure were used. (1) Cortical bone was powdered

at liquid N₂ temperature (Spex Freezer Mill; Spex Industries Inc., New Jersey, U.S.A.). Portions of this material (2g) and plasma [¹⁴C]glycoprotein (2ml) were subjected to a procedure similar to that developed for the study of bovine bone glycoproteins (Oldroyd & Herring, 1967). The samples were placed in dialysis tubing (The Scientific Instrument Centre Ltd., London WC1X 9HZ, U.K.) together with 5ml of 2.5M-NaCl and dialysed for 1 week against 200ml portions of EDTA (10%, w/v) solution titrated to pH7.2 with NaOH. This EDTA solution was changed daily. Dialysis was continued against distilled water and the volume decreased with Carbowax 20M (Searle Scientific Services, High Wycombe, Bucks., U.K.). After dialysis against 0.05M-Tris-HCl buffer, pH7.2, (containing 0.3M-calcium acetate and 0.001% phenylmercuric acetate) for 24h with two changes, the non-diffusible sac contents were digested with 0.5mg of collagenase (Sigma type III; Sigma Chemical Co., London SW6, U.K.) at 37°C for 24h. The digest was dialysed exhaustively against 0.05M-Tris-HCl buffer, pH7.2, and stored at -20°C until required. These collagenase digests of total bone powder contained the non-collagenous proteins and were used for DEAE-cellulose and dextran-gel chromatography.

(2) The shafts of the long bones were cut into annular pieces (0.1–0.5g) and extracted at 4°C with successive portions (2ml) of NaCl (0.16M), EDTA (10%, w/v) and collagenase solutions as described previously (Owen *et al.*, 1973). Samples of skin (0.5g), muscle (1.5g), kidney (2g), intestine (0.7g) and aorta (0.1g) were treated by the same procedure. The separate extraction solutions were dialysed exhaustively against distilled water and freeze-dried. These materials will be referred to as NaCl extract, EDTA extract and collagenase extract and were used for polyacrylamide-gel electrophoresis and immunochemical experiments. Losses of material during the extraction and freeze-drying procedures were estimated by adding trace amounts of ¹²⁵I-labelled albumin prepared as described by Owen *et al.* (1973).

DEAE-cellulose chromatography. Bone and plasma collagenase digests and untreated plasma were dialysed against 0.05M-Tris-HCl buffer, pH7.2. Portions of these equivalent to 0.2–0.3g of original bone powder and 0.25ml of plasma were chromatographed on separate 2ml columns (0.5cm diam.) of DEAE-cellulose (DE-32; W. and R. Balston Ltd., Maidstone, Kent, U.K.) in 0.05M-Tris-HCl buffer, pH7.2. The columns were washed with this buffer until the *E*₂₈₀ reached low values. Elution was continued by using a total volume of 50ml of a linear gradient from 0–0.6M-NaCl. The elution rate was controlled at 8ml/h with a peristaltic pump and 1ml fractions were collected by using a fraction collector. Samples of each fraction were examined

for protein (*E*₂₈₀), sialic acid (Aminoff, 1961) and uronic acid (Bitter & Muir, 1962). Radioactivity was measured by dissolving samples in dioxan-based scintillant (Bray, 1960).

Gel column chromatography. Columns (68cm × 1.5cm) of Sephadex G-100 (Pharmacia G.B. Ltd., London W5 5SS, U.K.) were equilibrated with 0.05M-Tris-HCl buffer, pH7.2, and samples of bone and plasma collagenase digests (equivalent to 0.1–0.4g of original bone or 0.1ml of plasma) and plasma (0.1ml) applied. Fractions (2ml) were collected and examined for protein (*E*₂₈₀) and radioactivity after addition of scintillant (Bray, 1960).

Specificity of collagenase. The collagenase used to prepare the non-collagenous fraction of powdered bone was checked for non-specific protease activity. Plasma proteins labelled with ¹⁴C were prepared by injection into rabbits of 200μCi of [¹⁴C]glucosamine or [U-¹⁴C]leucine (The Radiochemical Centre) and 3h later the plasma was prepared and dialysed exhaustively against 0.16M-NaCl. Samples (0.5–1.0ml) of the dialysis residue were incubated with 1ml of 0.025M-Tris-HCl buffer, pH7.2, containing 0.3M-calcium acetate with or without collagenase (1.5mg) at 37°C and without enzyme at 4°C. Phenyl mercuric acetate (0.001%) was used as bacteriostatic agent. This was followed by ultrafiltration through dialysis tubing by centrifugation for 30min at 2000g at 4°C. Portions of ultrafiltrate and retained material were prepared for radioactivity measurement by dissolution in 0.5ml of Soluene (Packard Instrument Ltd., Wembley, Middx., U.K.) at 50°C before adding 10ml of scintillant (Bray, 1960). The results were obtained as the proportion of radioactivity in the incubated solutions associated with low-molecular-weight components appearing in the ultrafiltrate. Collagenase released 1.8% of the total radioactivity from plasma protein labelled after [¹⁴C]glucosamine injection and 4% of that from plasma proteins labelled by [¹⁴C]leucine. After collagenase digestion of powdered bone from animals labelled with [¹⁴C]glucosamine or plasma [¹⁴C]-glycoprotein 91% or 95% respectively of radioactive label subjected to the procedure was recovered. These results indicate that only a small amount of non-specific protease activity was associated with the collagenase.

Antisera. Antisera to rabbit serum proteins prepared in goat (Wellcome Reagents Ltd., Beckenham, Kent, U.K.) and sheep (Behringwerke-AG, Frankfurt, Germany) were obtained. Antisera to bone fractions and rabbit serum albumin (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) were prepared in guinea-pigs by injection of 0.2–2mg of protein homogenized in Freund's complete adjuvant in one subcutaneous injection. Then 60 days later 0.2–2mg of protein dissolved in 1ml of 0.16M-NaCl was injected subcutaneously and 14 days later blood

was collected by exsanguination and sera were prepared.

Polyacrylamide-gel disc electrophoresis. Gels (7%, w/v, 6.5 cm × 0.5 cm) were prepared with Cyanogum 41 (BDH Chemicals Ltd., Poole, Dorset, U.K.) and the simple discontinuous gel system of Hjerten *et al.* (1965) was employed with 38 mM-glycine adjusted to pH 8.2 with Tris solution as the tank buffer, and 377 mM-Tris titrated to pH 8.9 with HCl as the gel buffer. The various bone extracts were dissolved in tank buffer (16–20 mg/ml) and centrifuged to remove small amounts of insoluble material. Samples of plasma (5–10 μ l) or bone protein solutions (10–50 μ l) were applied to the gels. After electrophoresis at 3 mA/gel for 1½ h the gels were stained for protein with Amido Black (Gordon, 1969) and for carbohydrates by the periodic acid–Schiff reagent (Zacharias *et al.*, 1969).

Preparation of a less-acidic-glycoprotein fraction from rabbit bone. Pooled rabbit cortical bone (80 g) was decalcified by prolonged dialysis against EDTA (10%, w/v) adjusted to pH 7.2 with NaOH by the method of Herring (1964). The non-diffusible material was dialysed against a buffer of 0.05 M-Tris adjusted to pH 7.2 with HCl which contained also 0.4 M-NaCl. A column of DEAE-cellulose (7 cm × 2 cm) was equilibrated with this buffer and the bone extract (50 ml) was applied. The column was washed with 200 ml of equilibration buffer and the eluate collected, leaving the acidic constituents adsorbed to the cellulose column. The eluate was dialysed against polyethylene glycol (Carbowax 20 M) to decrease the volume to approximately 20 ml, followed by exhaustive dialysis against 0.05 M-Tris-HCl buffer, pH 7.2, before application to a second column (7 cm × 2 cm) of DEAE-cellulose equilibrated with this buffer. The column was washed thoroughly and the effluent discarded. Adsorbed proteins were eluted with 1000 ml of a gradient from 0–0.4 M-NaCl and the fraction eluted from 0–0.15 M-NaCl was concentrated and freeze-dried after diafiltration with an XM50 ultrafiltration membrane (Amicon Ltd., Lexington, Mass. 02173, U.S.A.). A sample (20 mg) of this less-acidic-glycoprotein fraction from rabbit bone was passed through a concanavalin A-agarose column (6 ml) (Miles-Yedah, Yehoveth, Israel); non-reacting material was eluted with 0.01 M-sodium phosphate buffer, pH 7.3, containing 0.16 M-NaCl. The adsorbed glycoprotein was eluted with 5% (w/v) methyl- α -D-mannopyranoside (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) in the column buffer, diafiltered against 25 vol. of distilled water with a UM2 membrane (Amicon Ltd., High Wycombe, Bucks., U.K.) and freeze-dried. This isolated fraction was the less-acidic-glycoprotein fraction of rabbit bone and will be referred to as the less-acidic-glycoprotein fraction.

Immunochemical methods. Immunoprecipitation

studies were performed on plasma [14 C]glycoprotein and EDTA extracts of 14 C-labelled bone. Commercial antisera and the antisera prepared against the less-acidic-glycoprotein fraction of bone were used. In the latter studies EDTA extract and plasma [14 C]-protein were centrifuged at 3000g for 30 min. Portions of the clear supernatants from EDTA extracts (1 mg in 0.5 ml of 0.05 M-Tris buffer containing 0.16 M-NaCl and adjusted to pH 7.2 with HCl) and from plasma [14 C]glycoprotein (10 μ l) were incubated with an excess amount of antisera estimated from radial immunodiffusion studies (Mancini *et al.*, 1965). Incubation at 37°C for 2 h was followed by incubation at 4°C overnight before centrifugation at 2000g for 30 min and collection of the precipitate and supernatant. The supernatant was subjected to precipitation with antisera as before and this was repeated. The precipitates were washed four times with 0.05 M-Tris buffer containing 0.16 M-NaCl and adjusted to pH 7.2 with HCl. The supernatants and precipitates were dissolved separately in 1 ml of Soluene (Packard Instrument Ltd., Wembley, Middx., U.K.) and 10 ml of scintillant (Bray, 1960) was added before measurement of radioactivity. A similar procedure was employed with commercial antisera to rabbit plasma proteins but 1 ml of antiserum was used and immunoprecipitation was repeated once with this volume of antisera.

Double immunodiffusion (Ouchterlony, 1958) or immunoelectrophoresis (Scheidegger, 1955) were performed in 1 or 2% (w/v) agar gels (Ionagar no. 2; Oxoid Ltd., London SE1 9HF, U.K.) in sodium barbitone buffer, pH 8.6. Radioautography after immunoelectrophoresis was performed by a 3–12 week exposure of the plate to X-ray film (Kodirex; Kodak Ltd., Hemel Hempstead, Herts., U.K.) at room temperature. Single radial immunodiffusion (Mancini *et al.*, 1965) was used to estimate the amount of antigen in bone fractions and plasma.

Measurement of bone blood volume with 51 Cr-labelled erythrocytes. Sodium chromate (51 Cr) solution (100–300 μ Ci/mg of Cr) was obtained from The Radiochemical Centre, and erythrocytes from donor rabbits were labelled by the procedure of Gray & Sterling (1950) as modified by Huggins *et al.* (1966). Weighed portions were injected intravenously by ear vein into four young rabbits (600–700 g, aged 5 weeks) and into two older animals (2 kg, aged 14 months) and 30 min later the animals were killed. The haematocrit of the blood was determined in microhaematocrit tubes (Sherwood Medical Industries Inc., St Louis, Mo., U.S.A.) and blood and bone samples were assayed for 51 Cr γ -radioactivity for calculation of the blood volume of the bone tissue.

Quantitation of albumin and less-acidic-glycoprotein fraction in tissues. The animals which had been used for bone blood volume measurement were used in these studies. Samples of freeze-dried NaCl extracts,

EDTA extracts and collagenase extracts of bone pieces and soft tissues were dissolved in sodium barbitone buffer, 10.075, pH 8.6, to give solutions of 15–40 mg/ml. Portions (1 μ l) were assayed for content of albumin and the less-acidic-glycoprotein fraction by the radial immunodiffusion method of Mancini *et al.* (1965), with antisera concentrations of 1–3% in 1% agar.

Radioactivity determinations. All β -ray radioactivity assays were performed on a Packard Tri-Carb liquid-scintillation spectrometer (Model 3320, Packard Instrument Ltd., Wembley, Middx., U.K.) and samples corrected for quenching by internal standardization. A well-type sodium iodide crystal scintillation system was used for γ -ray radioactivity measurement of ^{125}I and ^{51}Cr .

Results

DEAE-cellulose column chromatography

Fig. 1(a) shows the elution pattern from DEAE-cellulose of plasma [^{14}C]glycoproteins subjected to EDTA and collagenase treatment. A similar elution pattern was obtained for plasma protein without pretreatment (results not shown). The radioactivity was present in two peaks emerging with the buffer and in the early part of the gradient. Fig. 1(b) shows the elution profile obtained by DEAE-cellulose chromatography of the collagenase digest of bone from animals injected 12 days earlier with plasma [^{14}C]glycoprotein. The radioactivity appeared in positions in the gradient which corresponded closely to those of the main radioactivity peaks of the plasma [^{14}C]glycoproteins (Fig. 1a). The peaks that were more acidic than any of the plasma constituents (fractions 30–59, Fig. 1b) contained a smaller proportion of the radioactivity. A DEAE-cellulose chromatograph of the collagenase digest of rabbit bone 12 days after injection of [^{1-14}C]glucosamine is presented in Fig. 1(c). Comparison with Fig. 1(b) shows that more radioactivity was associated with the more acidic constituents. The latter contained the majority of the sialic acid and uronic acid (Fig. 1c) and are analogous to the sialoprotein and chondroitin sulphate fractions of bovine bone (Herring, 1964). However, the final peak also contains material with a maximum u.v. absorption at 260 nm and minimum absorption at 230 nm, presumably either nucleic acids or purine and pyrimidine bases bound in some other macromolecular form.

Dextran-gel column chromatography

Typical Sephadex (G-100) gel chromatograms of plasma [^{14}C]glycoprotein and collagenase digests of labelled bone are illustrated in Fig. 2. The labelled

plasma protein was eluted in two major peaks (Fig. 2a). The elution patterns of bone digests labelled with plasma [^{14}C]glycoprotein and [^{14}C]glucosamine are shown in Figs. 2(b) and (c). In both cases a large part of the radioactivity was eluted with the higher-molecular-weight components in a similar position to the plasma proteins. The lower-molecular-weight components, fractions 35–65, were also labelled (Figs. 2b and c). In bone labelled after [^{14}C]glucosamine injection (Fig. 2c) these low-molecular-weight components were more highly labelled than in bone labelled by plasma [^{14}C]glycoprotein.

Preparation of less-acidic-glycoprotein fraction from bone

The plasma glycoproteins in bone were present in a less-acidic-glycoprotein fraction (Figs. 1a and b) and therefore the separation of this fraction was undertaken. The 7% (w/v) polyacrylamide-gel disc patterns of the fractions obtained during the preparation of this fraction are shown in Fig. 3 stained by Amido Black. The total EDTA extract contained albumin and several other components with lower mobility (Fig. 3a). The 0–0.15 M-NaCl eluate from DEAE-cellulose showed the presence of two major bands that stained diffusely for protein (Amido Black, Fig. 3b) and also for carbohydrate (periodic acid–Schiff reagent). The band of highest mobility coincided with that of albumin but contained a component that stained for carbohydrate. Purified albumin was stained negligibly by periodic acid–Schiff reagent. Albumin was detected in the fraction by immunodiffusion and immunoelectrophoresis. The other band stained intensely with periodic acid–Schiff reagent and ran with α -globulin mobility. Affinity chromatography on concanavalin A-agarose removed the albumin (Aspberg & Porath, 1970) and left the major α -glycoprotein contaminated by a minor glycoprotein component in the albumin region (Fig. 3c). This purified less-acidic-glycoprotein fraction (Fig. 3c) was used for the preparation of antisera in guinea-pigs.

Polyacrylamide-gel electrophoresis

Total collagenase digests of powdered bone prepared by the method of Oldroyd & Herring (1967) were poorly resolved by polyacrylamide-gel disc electrophoresis. The freeze-dried materials from NaCl, EDTA and collagenase extracts of intact bone pieces were well separated on the gels. Examples of these gell patterns are shown in Plate 1. To some extent the extracts represent selective extraction from different compartments of bone tissue. NaCl solutions remove mainly the contents of the blood vessels and soluble components from the tissue

EXPLANATION OF PLATE I

Polyacrylamide-gel disc electrophoretograms of plasma and bone extracts

(a) Plasma, arrow indicates albumin, (b) NaCl extract, (c) EDTA extract, arrow indicates α -glycoprotein, (d) collagenase extract. The bone extracts were obtained by the method of Owen *et al.* (1973). Further details are given in the Experimental section, and the direction of migration is indicated by the arrow.

EXPLANATION OF PLATE 2

Immunoelectrophoretograms of rabbit plasma and bone extracts prepared by the method of Owen et al. (1973), allowed to react with various antisera

(a) Top and bottom wells, bone less-acidic-glycoprotein fraction; centre trough, anti-(less-acidic-glycoprotein fraction; antiserum) (b) centre well, rabbit plasma; top trough, anti-(less-acidic-glycoprotein fraction) antiserum; bottom trough, antiserum to rabbit plasma; (c) centre well, NaCl extract of bone; top trough, antiserum to rabbit plasma; bottom trough, anti-(less-acidic-glycoprotein fraction) antiserum; (d) centre well, collagenase extract of bone; top trough, anti-(less-acidic-glycoprotein fraction) antiserum; bottom trough, antiserum to rabbit plasma; (e) centre well, EDTA extract of bone; top trough, anti-(less-acidic-glycoprotein fraction) antiserum; bottom trough, antiserum to rabbit plasma; (f) radioautograph of (e).

Fig. 1. DEAE-cellulose column chromatography of the non-collagenous fractions of bone and plasma

The non-collagenous fractions were prepared by collagenase digestion of EDTA extracts of bone powder and plasma samples by the method of Oldroyd & Herring (1967). Elution patterns of these materials from 2 ml columns of DEAE-cellulose are given for (a) plasma proteins labelled from [^{14}C]glucosamine, (b) bone labelled after injection of plasma [^{14}C]glycoprotein 12 days earlier, (c) bone labelled after injection of [^{14}C]glucosamine 12 days earlier. ●, Radioactivity (c.p.m.); ○, protein (E_{280}); △, sialic acid, ▲, uronic acid contents are of the total fractions. Other details are given in the Experimental section.

fluid of bone, EDTA solutions extract the bulk of the glycoproteins from the calcified phase and collagenase digestion releases the components closely associated with collagen (Owen *et al.*, 1973). Many components were removed from cortical bone

by the first extraction with NaCl (0.16M) solution (Plate 1b) and the electrophoretic pattern showed some similarities with that from plasma, the main component being albumin (Plate 1a). The EDTA extract (Plate 1c) also contained albumin but the component present in greatest concentration stained strongly with periodic acid-Schiff reagent and ran in the position of the α -component of the less-acidic-glycoprotein fraction (Fig. 3c). Immunodiffusion after polyacrylamide-gel electrophoresis showed that this major band, with α -mobility, present in the EDTA extract (Plate 1c) contained the immunoreactive component of the less-acidic-glycoprotein fraction (J. T. Triffitt, unpublished work). Final treatment of the extracted bone fragments with collagenase released other protein components of high mobility (Plate 1d) but the α -component seen in the EDTA extract (Plate 1c) was absent.

Immunoelectrophoresis

The commercial anti-(rabbit plasma) antisera used failed to give any detectable precipitin reaction with the less-acidic-glycoprotein fraction prepared from bone. Therefore this latter fraction and EDTA extract of bone were used to prepare antisera in guinea-pigs. Both antigens elicited immune responses and the precipitating antibodies produced were specific to a component with α -mobility present in the less-acidic-glycoprotein fraction (Fig. 3c and Plate 2a). The antisera also reacted strongly with rabbit plasma (Plate 2b). The EDTA extract of bone showed a major component reacting with anti-(less-acidic-glycoprotein fraction) antiserum (Plate 2e), but this was absent in the bone collagenase extract (Plate 2d). It was detectable in immunoelectrophoretograms of the NaCl extract of bone (Plate 2c). Albumin was present in both collagenase extracts and EDTA

Fig. 2. Dextran-gel chromatography of the non-collagenous fractions of bone and plasma

The non-collagenous fractions were prepared by collagenase digestion of EDTA extracts of bone powder and plasma samples according to the method of Oldroyd & Herring (1967). Samples were applied to a column (68cm $\times$ 1.5cm) of Sephadex G-100 and eluted with 0.05M-Tris-HCl buffer, pH 7.2. The total fractions (2ml) were analysed for protein (E_{280} , $\circ$) and radioactivity ($\bullet$). Other details are given in the Experimental section. (a) Plasma proteins labelled from [^{14}C]glucosamine, (b) bone labelled 12 days earlier with plasma [^{14}C]glycoprotein, (c) bone labelled 12 days earlier with [^{14}C]glucosamine.

Fig. 3. Diagrammatic representation of polyacrylamide-gel electrophoretograms obtained at stages in the preparation of less-acidic-glycoprotein fraction from rabbit cortical bone

The direction of migration is indicated by the arrow. Protein was stained by Amido Black (Gordon, 1969). (a) Total EDTA extract of powdered compact bone, arrow indicates albumin, (b) fraction eluted from DEAE-cellulose by a linear gradient of 0–0.15M-NaCl, (c) less-acidic-glycoprotein fraction eluted from concanavalin A-agarose column, arrow indicates glycoprotein with α -mobility. The different stippling, ■, ▨, ▩ and ▪ indicates decreasing density of the stain.

extracts of bone (Plates 2d and e) in agreement with previous findings (Owen *et al.*, 1973).

The bone EDTA extract in Plate 2(e) was from animals injected with [^{14}C]glucosamine 12 days previously. A radioautograph of this plate shows the presence of radioactivity in the component reacting

with anti-(less-acidic-glycoprotein fraction) antiserum but none in the albumin arc from (anti-rabbit plasma) antiserum (Plate 2f).

Immunoprecipitation

The anti-(less-acidic-glycoprotein fraction) antisera precipitated 6% of the total radioactivity present in portions of plasma from rabbits injected 5h earlier with [^{14}C]glucosamine. EDTA solutions extracted the majority (71%) of the radioactivity from the bone of animals labelled with [^{1-14}C]glucosamine or plasma [^{14}C]glycoprotein, with smaller amounts extracted by 0.16M-NaCl (6%) and the remainder was present in the collagenase digest. Immunoprecipitation of the EDTA extracts of bone, labelled 12 days earlier with [^{14}C]glucosamine or plasma [^{14}C]glycoprotein, with anti-(less-acidic-glycoprotein fraction) antisera showed that of the total radioactivity present 57% of the former and 42% of the latter was precipitable. Therefore these values represent 40% and 30% respectively of the total radioactivity in bone. Negligible radioactivity was precipitated from the EDTA extracts by any commercial anti-(rabbit plasma) antisera used, and these antisera precipitated at a maximum only 55% of the radioactivity from plasma [^{14}C]glycoprotein samples.

Quantitation of less-acidic-glycoprotein fraction and albumin in bone and other tissues

The amounts of less-acidic-glycoprotein fraction in tissue extracts and plasma were determined by radial immunodiffusion with freeze-dried less-acidic-glycoprotein fraction as standard. The less-acidic-glycoprotein fraction was measurable only in the EDTA extract of bone and was not detectable by this method in the NaCl extract or the collagenase extract. None of the soft tissue extracts analysed contained detectable amounts of this fraction. The results obtained for the amounts of less-acidic-glycoprotein fraction and albumin in the bone and plasma of four young rabbits and two older animals are shown in Table 1. The plasma volume of the bone pieces, determined by using ^{51}Cr -labelled erythrocytes, is given also for these animals. The vascularity of young bone was greater than that in older animals, as found in the rat (Brady & Cutright, 1971). Relative to albumin the less-acidic-glycoprotein fraction is more highly concentrated in bone tissue than in plasma by a factor of approximately 30 times in young animals and 60 times in old animals. From the measured blood volumes of the bone tissue, it was calculated that the amount of less-acidic-glycoprotein fraction in blood plasma contributes approximately 0.5% of the total less-acidic-glycoprotein fraction in young rabbits and 0.2% in older animals. In the case

Table 1. *Plasma volumes and concentrations of less-acidic-glycoprotein fraction and albumin in blood and bone tissues of old and young rabbits*

Bone blood volumes were determined by using ^{51}Cr -labelled erythrocytes and the plasma volume obtained from the haematocrit. Concentrations of less-acidic-glycoprotein fraction and albumin were measured by single radial immunodiffusion. The ratio, G/A, is calculated from the expression

$$\frac{\text{less-acidic-glycoprotein fraction in bone}}{\text{albumin in bone}} \bigg/ \frac{\text{less-acidic-glycoprotein fraction in plasma}}{\text{albumin in plasma}}$$

Other details are given in the Experimental section.

Age (weeks)	Body wt. (kg)	Plasma vol. of bone ($\mu\text{l/g}$)	Less-acidic- glycoprotein fraction in plasma (mg/ml)	Less-acidic- glycoprotein fraction in bone (mg/g)	Albumin in plasma (mg/ml)	Albumin in bone (mg/g)	G/A
5	0.69	10.04	2.40	4.8	48.9	2.85	34.3
5	0.68	10.34	2.90	4.5	51.5	3.08	25.9
5	0.68	7.84	2.82	6.4	51.8	3.06	38.4
5	0.69	9.86	2.91	5.7	51.5	2.55	39.5
60	2.6	2.05	1.49	2.3	42.2	1.09	59.9
60	2.4	3.55	1.46	2.3	39.7	1.03	60.9

of albumin, plasma contributes 10–20% of the total albumin present in bone tissue. The concentration of less-acidic-glycoprotein fraction in plasma of young rabbits was about twice that in the plasma of older animals, and its concentration in bone also decreased with age.

Discussion

Our results show that both [^{14}C]glucosamine and plasma [^{14}C]glycoprotein are utilized in the synthesis of the glycoproteins of bone matrix. DEAE-cellulose and dextran-gel chromatography demonstrate that the various non-collagenous constituents of bone tissue are labelled to different extents by the two precursors and suggest that some of the glycoprotein components are similar to serum glycoproteins whereas others are synthesized by the bone cells. Radioactivity from plasma [^{14}C]glycoprotein is retained mainly in the least-acidic and highest-molecular-weight fractions, which behave similarly to the injected plasma protein. The most-acidic and also the lowest-molecular-weight fractions are labelled to a greater extent by [^{14}C]glucosamine than by plasma [^{14}C]glycoprotein. The acidic fractions are analogous to the sialoprotein and chondroitin sulphate fractions isolated from bovine bone (Herring, 1964). In the rat these components are rapidly labelled with [^{14}C]glucosamine (Shetlar *et al.*, 1972).

A glycoprotein which is immunologically identical with a plasma glycoprotein is present in the less-acidic-glycoprotein fraction isolated from bone

matrix. This fraction has been resolved into two glycoprotein components by polyacrylamide-gel electrophoresis. The major constituent migrates with α -mobility and the minor one migrates in the albumin region. An antiserum prepared against the less-acidic-glycoprotein fraction showed a precipitin arc that corresponded to an α -component when extracts of rabbit bone and rabbit plasma were subjected to immunoelectrophoresis. By using the freeze-dried weight of less-acidic-glycoprotein fraction as standard the amount of α -glycoprotein in bone was estimated to be about 0.5% of fresh bone weight and the concentration in plasma to be from 1–3 mg/ml. These values are likely to be high because of the unknown water content of the less-acidic-glycoprotein fraction and the presence of a contaminant. However, the immunoreactive α -glycoprotein in the less-acidic-glycoprotein fraction must account for a large proportion of the glycoproteins of bone on the basis of the immunoprecipitation results. In the EDTA extract of bone labelled by [^{14}C]glucosamine, about 57% of the radioactivity is precipitated by the antiserum to the less-acidic-glycoprotein fraction and this is equivalent to about 40% of the total radioactivity in bone. In the case of plasma [^{14}C]glycoprotein, about 6% of the radioactivity is precipitated by the antiserum to the less-acidic-glycoprotein fraction.

In bone labelled after injection of plasma [^{14}C]glycoprotein there is some radioactivity in components which are not of plasma glycoprotein nature by the criteria of ion-exchange and dextran-gel chromatography. This suggests that there is some breakdown and reutilization by bone tissue of the

^{14}C label from the injected plasma [^{14}C]glycoprotein. In the present experiments there has been little conversion of radioactivity into amino acids for collagen synthesis since over 90% of the ^{14}C label in the bone from both injected groups was recovered in the dialysis residue after collagenase digestion. In the rat amino sugars and glycopeptides do not appear in the plasma as a result of catabolism of plasma glycoproteins and there is no evidence for their reutilization in biosynthesis by the liver (Robinson, 1969). The local reutilization of products of plasma glycoprotein catabolism at sites in bone tissue, however, may occur and it is possible that in addition to the accumulation of certain plasma glycoproteins in bone matrix some are used in the synthesis of other matrix components.

Other authors have detected plasma proteins in EDTA extracts of rabbit cortical bone, but have attributed their presence to the plasma contents of the blood vessels (Burckard *et al.*, 1966). This is not an explanation of our findings since the volume of plasma in the bone was measured and it can be calculated that the contents of the blood vessels account for less than 0.5% of the α -glycoprotein in bone tissue. In other studies in our laboratory a similar α -glycoprotein has been isolated from bovine bone matrix and found to occur in plasma (B. A. Ashton, J. T. Triffitt & G. M. Herring, unpublished work). The α -glycoprotein is present in both bovine and rabbit bone in a much higher proportion relative to albumin than in the plasma. In the present work the α -glycoprotein was not detectable in skin, kidney, intestine, muscle and aorta and it may be uniquely concentrated in calcified tissue. The circumstantial evidence for the involvement of glycoproteins in biological calcification is therefore of interest (Nusgens *et al.*, 1972; Weinstein *et al.*, 1972). The amount of osteoid in bone diminishes with age and calcification (Lapiere & Nusgens, 1970; Pugliarello *et al.*, 1970). The observed decrease with age of the less-acidic-glycoprotein fraction in cortical bone may reflect this lower osteoid content.

Recently we have demonstrated that albumin is a component of bone matrix and that it escapes from the vessels in bone into the perivascular connective tissue space and passes across the cellular membrane which covers bone surfaces into the bone interstitial fluid (Owen *et al.*, 1973). Some of the albumin is incorporated into the calcified matrix at sites of bone formation and the remainder drains away from the tissue fluid. This is substantiated by previous studies with fluorescent labels (Lipp, 1967). It is possible that the α -glycoprotein present in the less-acidic-glycoprotein fraction follows a similar route in the passage from blood to bone tissue.

It is not known whether the α -glycoprotein which is present in both bone tissue and plasma is produced

in bone and released into the plasma or whether it enters the bone from the plasma. The relatively large proportion of the radioactivity in the α -glycoprotein in bone after plasma [^{14}C]glycoprotein injection suggests the possibility that the glycoprotein is accumulated from the plasma.

The authors are Members of the Medical Research Council External Scientific Staff and thank Miss F. Schofield, Mrs. S. Fearn and Mrs. F. Bates for skilful assistance.

References

- Aminoff, D. (1961) *Biochem. J.* **81**, 384–392
- Ashton, B. A., Herring, G. M., Owen, M. E. & Triffitt, J. T. (1971) *Isr. J. Med. Sci.* **7**, 409–411
- Aspberg, K. & Porath, J. (1970) *Acta Chem. Scand.* **24**, 1839–1841
- Bitter, T. & Muir, H. M. (1962) *Anal. Biochem.* **4**, 330–334
- Brady, J. M. & Cutright, D. E. (1971) *Anat. Rec.* **170**, 143–146
- Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285
- Burckard, J., Havez, R. & Dautrevaux, M. (1966) *Bull. Soc. Chim. Biol.* **48**, 851–861
- Capps, J. C., Shetlar, M. R. & Bradford, R. H. (1966) *Biochim. Biophys. Acta* **127**, 194–204
- Gordon, A. H. (1969) in *Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T. S. & Work, E., eds.), vol. 1, pp. 1–149, North-Holland Publishing Co., Amsterdam and London
- Gottschalk, A. (1966) *Glycoproteins*, pp. 20–28, Elsevier, Amsterdam
- Gray, J. S. & Sterling, K. (1950) *Science* **112**, 179–180
- Herring, G. M. (1964) *Clin. Orthop.* **36**, 169–183
- Herring, G. M. (1972) in *The Biochemistry and Physiology of Bone* (Bourne, G. H., ed.), 2nd edn., pp. 127–189, Academic Press, London and New York
- Hjerten, S., Jerstedt, S. & Tiselius, A. (1965) *Anal. Biochem.* **11**, 211–218
- Huggins, R. A., Smith, E. L. & Deavers, S. (1966) *Amer. J. Physiol.* **211**, 283–287
- Lapiere, Ch. M. & Nusgens, B. V. (1970) in *Chemistry and Molecular Biology of the Intercellular Matrix* (Balazs, E. A., ed.), vol. 1, pp. 55–79, Academic Press, London and New York
- Lipp, W. (1967) *Histochemie* **9**, 339–353
- Mancini, G., Carbonara, A. O. & Heremans, J. F. (1965) *Immunochemistry* **2**, 235–254
- Nusgens, B., Chantraine, A. & Lapiere, Ch. M. (1972) *Clin. Orthop.* **88**, 252–274
- Oldroyd, D. & Herring, G. M. (1967) *Biochem. J.* **104**, 20p
- Ouchterlony, O. (1958) *Progr. Allergy* **5**, 1–78
- Owen, M. & Shetlar, M. R. (1968) *Nature (London)* **220**, 1335–1336
- Owen, M. & Triffitt, J. T. (1972) in *Calcium, Parathyroid Hormone and the Calcitonins* (Talmage, R. V. & Munson, P. L., eds.), pp. 316–326, Excerpta Medica, Amsterdam

- Owen, M., Triffitt, J. T. & Melick, R. A. (1973) *Hard Tissue, Growth, Repair and Remineralization, Ciba Found. Symp.*, pp. 263–285.
- Pugliarello, M. C., Vittur, F. & de Bernard, B. (1970) *Calcif. Tissue Res.* **5**, 108–114
- Robinson, G. B. (1968) *Biochem. J.* **108**, 275–280
- Robinson, G. B. (1969) *Biochem. J.* **114**, 635–640
- Scheidegger, J. J. (1955) *Int. Arch. Allergy Appl. Immunol.* **7**, 103–110
- Shetlar, M. R., Hern, D. L., Bradford, R. H. & Endecott, B. (1961) *Biochim. Biophys. Acta* **53**, 615–616
- Shetlar, M. R., Capps, J. C. & Hern, D. L. (1964) *Biochim. Biophys. Acta* **83**, 93–101
- Shetlar, M. R., Shurley, H. & Hern, D. (1972) *Proc. Soc. Exp. Biol. Med.* **139**, 340–344
- Weinstock, A., Weinstock, M. & Leblond, C. P. (1972) *Calcif. Tissue Res.* **8**, 181–189
- Winzler, R. J. (1965) *Clin. Chem.* **11**, 339–347
- Zacharias, R. M., Zell, T. E., Morrison, J. H. & Woodlock, J. J. (1969) *Anal. Biochem.* **30**, 148–152