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Studies on the Site of Biosynthesis of Acidic Glycoproteins of Guinea-Pig Serum

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1. Studies were carried out to determine the cellular and subcellular site of biosynthesis of components of fraction I, an α-globulin fraction containing acidic glycoproteins isolated from guinea-pig serum. L-[U-^{14}C]Leucine or -valine and D-[1-^{14}C]glucosamine were used as precursors. 2. A lag of about 10min. occurred before appreciable label appeared in fraction I of serum after injection of leucine or glucosamine. Label in fraction I after 60min. labelling with glucosamine was present almost entirely in hexosamine and sialic acid. 3. Site of synthesis was investigated by studies *in vivo* up to 17min. after injection of precursor. Particulate subcellular fractions isolated from liver, spleen and kidney or homogenates of the latter two tissues were extracted with Lubrol. Extracts were allowed to react by double diffusion with antisera to fraction I or to subfractions isolated from it, and gels were subsequently subjected to radioautography. With either amino acid or glucosamine as precursor, only extracts of the microsome fraction of liver formed precipitin lines that were appreciably radioactive. 4. The role of the microsome fraction of liver in the synthesis of these glycoproteins was confirmed by immunological studies after incubation of liver slices with leucine or glucosamine. Incorporation of leucine was also investigated in a cell-free microsome system. 5. Material was also precipitated from certain Lubrol extracts of liver microsomes by direct addition of antiserum and its radioactivity measured. Degradation of material thus precipitated and use of heterologous immune systems showed that labelling of precipitin lines represented biosynthesis. 6. A study of extraction procedures suggested that the substances present in the microsome fraction of liver that react with specific antisera are associated with membranous structures. 7. Most or all precipitin lines formed by Lubrol extracts of liver microsomes interacted with precipitin lines given by guinea-pig serum or fraction I, immunological identity being apparent with some lines. The microsome-bound substances thus represent serum glycoproteins or precursors of them. 8. The distribution of label in various tissues and in the protein of subcellular fractions of liver after administration of [^{14}C]glucosamine to the guinea pig was also studied. Some variation in results obtained with liver was found depending on the fractionation medium used.

Previous papers have described work on the characteristics and structure of components of an α-globulin fraction containing acidic glycoproteins, fraction I, isolated from guinea-pig serum (Simkin, Seshadri & Skinner, 1964*a*; Simkin, Skinner & Seshadri, 1964*b*; Cunningham & Simkin, 1966). Certain resemblances in properties between components of the fraction were found in these investigations. The present paper describes studies on the site of biosynthesis of components of this fraction. Though considerable evidence has accumulated that comparable serum α-globulins are produced in other species by the liver (e.g. Hochwald, Thorbecke & Asofsky, 1961; Asofsky & Thorbecke 1961; Richmond, 1963; Sarcione, 1963; Miller, Hanavan, Titthasiri & Chowdhury, 1964), work on the biosynthesis of the carbohydrate prosthetic groups of these and other glycoproteins, in particular with respect to the subcellular site of formation, has been restricted largely to the study of crude mixtures of proteins. We have investigated the cellular and subcellular site of synthesis of components of fraction I by studies *in vivo* and *in vitro* with labelled leucine or valine and glucosamine. Immunological procedures involving the use of antisera to fraction I or to subfractions of it were used to characterize products of synthesis. In most experiments, labelled material was allowed to

react with antiserum by double diffusion in agar, followed by radioautography (cf. Morgan, Perlmann & Hultin, 1961). In other work, material precipitated from solution by antiserum was taken for measurement of radioactivity and was also subjected to degradative procedures. By use of such immunological techniques, it is possible to study, individually or collectively, the synthesis of a small number of definite glycoproteins about which there is some knowledge of characteristics and structure. The immunological approach also has the advantage that it might detect possible precursors of glycoproteins containing incomplete carbohydrate groups, since several serum glycoproteins bearing partially degraded prosthetic groups can still react with antibody (e.g. Athineos, Thornton & Winzler, 1962; Bergmann, Levine & Spiro, 1962; Barker & Whitehead, 1963). Glucosamine was used to study formation of the carbohydrate groups because *N*-acetylglucosamine residues participate in linkage with polypeptide in components of fraction I (Cunningham & Simkin, 1966) and also occur in more peripheral positions in the groups (Simkin *et al.* 1964*a*). In addition, studies in the rat had shown that glucosamine is used efficiently for synthesis of serum glycoproteins, with label found almost exclusively in hexosamine and sialic acid (e.g. Robinson, Molnar & Winzler, 1964; Macbeth, Bekesi, Sugden & Bice, 1965). Opportunity was taken to study some general aspects of glucosamine metabolism in the guinea pig as information on this species was not available.

A preliminary account of some of this work has been published (Simkin & Jamieson, 1964).

EXPERIMENTAL

Materials

These were obtained as follows: L-[U-^{14}C]leucine, L-[U-^{14}C]valine, *n*-[1-^{14}C]hexadecane and L-[4,5-$^{3}H_2$]-leucine, The Radiochemical Centre, Amersham, Bucks.; D-[1-^{14}C]glucosamine, New England Nuclear Corp., Boston, Mass., U.S.A.; crystamycin (a mixture of sodium penicillin G and streptomycin sulphate), Glaxo Laboratories Ltd., Greenford, Middlesex; phosphoenolpyruvate (silver barium salt) and pyruvate kinase, C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; puromycin and puromycin aminonucleoside, Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; iso-octane (spectroscopic grade) and 1-fluoro-2,4-dinitrobenzene, British Drug Houses Ltd., Poole, Dorset; Lubrol W, a gift kindly provided by Imperial Chemical Industries Ltd., Dyestuffs Division, Manchester; sodium deoxycholate (special enzyme grade), Mann Research Laboratories Inc., New York, N.Y., U.S.A.; pancreatic ribonuclease, either crystalline grade, C. F. Boehringer, or chromatographically purified, Seravac Laboratories Ltd., Maidenhead, Berks.; Ionagar no. 2, Oxo Ltd., London; Dextran 80, Pharmacia, Uppsala, Sweden; trypsin (twice-crystallized, salt-free, freeze-dried), Worthington Biochemical Corp., Freehold, N.J., U.S.A.; Hyamine hydroxide, 2,5-diphenyloxazole, 1,4-bis-(5-phenyloxazol-2-yl)benzene and naphthalene, Nuclear Enterprises (G.B.) Ltd., Edinburgh; bovine γ-globulin, Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex. Other materials were as used in previous papers (Simkin *et al.* 1964*b*; Cunningham & Simkin, 1966).

Animals

Guinea pigs, 450–625g. body wt., of either sex, were purchased from A. J. Tuck and Son Ltd., Rayleigh, Essex, and were starved overnight before use.

Preparation of serum fractions

Fractionations were carried out at 2°.

Guinea-pig fraction I and subfractions. Unless otherwise stated, fraction I was prepared as described by Simkin *et al.* (1964*b*). For preparation of fraction I_b, DEAE-cellulose was first washed with 0·5N-NaOH–0·5M-NaCl and then water. After adjustment of pH to 8·4 with 5M-NaH_2PO_4, the exchanger was washed with 41mM-NaH_2PO_4–31mM-$Na_2B_4O_7$, pH8·4. After application of 90mg. of fraction I to a 40cm.×2cm. column of exchanger, the column was eluted stepwise with 250ml. of the same buffer, 530ml. of 60mM-NaH_2PO_4–54mM-$Na_2B_4O_7$, pH8·4, and 290ml. of 102mM-NaH_2PO_4–94mM-$Na_2B_4O_7$, pH8·4. The protein content of fractions was determined by measurement of extinction at 280mμ. Appropriate fractions eluted by 114mM-buffer were pooled, dialysed against water and freeze-dried. To prepare fraction I_{b2}, 20mg. of fraction I_b was applied to an 87cm.×1·4cm. column of Sephadex G-100 (bead form). The column was equilibrated and eluted with 80mM-NaH_2PO_4–21mM-Na_2HPO_4, pH6·2. Appropriate fractions (see the Results section) were pooled, dialysed against water and freeze-dried.

Human fraction I. This was prepared in a similar way to guinea-pig fraction I; after dialysis of serum against acetate buffer, 8ml. samples were applied to 8cm.×3cm. columns of CM-cellulose.

Incorporation studies in vivo

Labelled compounds dissolved in saline were administered except where noted by injection into an ear vein.

Incorporation of precursors into serum fraction I. After administration of 20μc of [^{14}C]leucine (10·7mc/m-mole) to a guinea pig, samples (about 0·8–1·0ml.) of blood were collected from an ear vein at appropriate times. After 60min., the animal was killed by a blow on the head and blood collected after severing the jugular veins. The sera were dialysed for 6hr. against 0·15M-NaCl–10mM-DL-leucine, for 19hr. against 50mM-acetate–10mM-leucine, pH5·0, and for 2hr. against acetate buffer without leucine. Fraction I was prepared as usual, either 10cm.×1cm. or 10cm.×2cm. columns of CM-cellulose being used as appropriate. Suitable fractions were pooled, dialysed against 10mM-DL-leucine for 24hr. and against water for 48hr., and then freeze-dried. After administration of 10μc of [^{14}C]glucosamine (12·1mc/m-mole), guinea pigs were killed by a blow at appropriate times and blood was collected. Fraction I was isolated from samples (5ml.) of serum essentially as described above; 10cm.×2cm.

columns were used, and the unlabelled leucine present in the dialysis medium before and after isolation was replaced by D-glucosamine.

For measurement of specific radioactivity, samples were removed from aqueous solutions of fraction I for scintillation counting (see below) and determination of protein (Lowry, Rosebrough, Farr & Randall, 1951); bovine serum albumin was used as standard in protein assays.

Incorporation into tissue proteins. In most experiments with labelled amino acids, 75–100μc of [^{14}C]leucine (8–25 mc/m-mole) or [^{14}C]valine (26mc/m-mole) was administered. After 15–17min., animals were killed by a blow, samples of blood collected and appropriate tissues removed. In two experiments, animals were anaesthetized as described by Simkin & Sutton (1960) and 100 or 75μc of [^{14}C]leucine (42 or 155mc/m-mole respectively) was injected into the portal vein. After 75 or 95sec. respectively, the portal vein was clamped and the liver rapidly excised. In experiments with [^{14}C]glucosamine, usually 12–20μc (2·4–12·1mc/m-mole) was administered. When required, urine passed during an experiment was collected and that remaining in the bladder at death was removed with a syringe.

Incorporation studies with liver slices. Slices were cut by a method similar to that of Hultin, Arrhenius, Löw & Magee (1960); the template used had a groove 7mm. wide and 0·36mm. deep. Iced 0·15M-NaCl containing 100mg. of crystamycin/l. was used as medium during preparation of slices. Slices were transferred to the medium of Marsh & Drabkin (1958) containing 100mg. of crystamycin/l.; the medium was changed several times by aspiration before the slices were blotted and weighed. Samples (400mg. wet wt.) of slices were incubated under O_2+CO_2 (95:5) for 20–100 min. with shaking at 37° with 2·5ml. of medium–crystamycin containing 25mM-D-glucose and 6–7·5μc of [^{14}C]leucine (10·7–30mc/m-mole) or 2·5–3μc of [^{14}C]glucosamine (2·4–12·1mc/m-mole); other additions were as indicated in the Results section. After incubation, samples were chilled and 2·5ml. of iced 0·15M-NaCl–crystamycin containing 20mM-DL-leucine or D-glucosamine was added. The slices were removed and washed further at 2° with this solution and with 0·25M-sucrose. The slices were then homogenized with 2ml. of 0·25M-sucrose (see below) and the microsome fraction was isolated as described below (procedure 1) after initial centrifuging for 10min. at 11500$g_{av.}$. To provide a large-granule fraction, the 11500g sediment was washed twice with 0·25M-sucrose.

Incorporation studies with a cell-free system

Samples (5g.) of liver mince were homogenized with 12·5ml. of medium A (see below). Homogenates were centrifuged for 10min. at 10000g (see procedure 1 below). The top 9ml. of supernatant was removed as the microsome–cell-sap fraction. Incubation mixtures (1·8ml. total volume) contained 1·25ml. of microsome–cell-sap fraction, 0·18M-sucrose, 50mM-tris–32mN-HCl buffer, pH7·8, 25mM-KCl, 4mM-$MgCl_2$, 10mM-potassium phosphoenolpyruvate, 50μg. of pyruvate kinase/ml. and 2μc of [^{14}C]leucine (10·6–30 mc/m-mole). Other additions are given in the Results section. After incubation for 20min. at 37° in air, samples were chilled and 4ml. of iced medium A containing 20mM-DL-leucine was added. Microsome and cell-sap fractions were separated as in procedure (1) below.

Subcellular fractionation

All procedures were carried out at 0–4°. The g values given refer to $g_{av.}$.

Liver. Liver was minced and, unless otherwise stated, was homogenized with 5vol. of 0·25M-sucrose or medium A (0·25M-sucrose, 50mM-tris–32mN-HCl buffer, pH7·8 at 25°, 25mM-KCl, 4mM-$MgCl_2$) for 30sec. with a Potter–Elvehjem homogenizer (about 17 passes of the pestle; for other details see Simkin & Work, 1957). One of three fractionation procedures was then applied. Centrifuges supplied by Measuring and Scientific Equipment Ltd. (Crawley, Sussex) were used as follows: Magnum centrifuge, preparation of nuclear fractions; 13 centrifuge, preparation of mitochondrial fractions in (3); 40 centrifuge, all other instances, a 25ml. angle head being used for centrifuging at 12200rev./min. and a 10ml. or 25ml. angle head for centrifuging at 40000rev./min. (1) Homogenate was centrifuged for 10min. at 10000g. Most of the supernatant was removed and centrifuged for 90min. at 106000g to give microsome and cell-sap fractions. (2) This procedure was normally applied only to homogenates prepared with 0·25M-sucrose. Nuclear and mitochondrial fractions were prepared by centrifuging for 10min. at 750g and 10500g respectively. The sediments were washed twice with 0·25M-sucrose and the washings discarded. Microsome and cell-sap fractions were prepared from the 10500g supernatant as in (1). (3) This was applied only to homogenates prepared with medium A. Nuclear, heavy-mitochondrial and light-mitochondrial fractions were prepared by centrifuging for 10min. at 750g, 8min. at 4000g and 10min. at 10800g respectively. Each sediment was washed twice with medium A, the first wash of a sediment being pooled with the initial supernatant separated from that sediment, and the second wash was discarded. Microsome and cell-sap fractions were prepared from the 10800g supernatant as in (1).

Microsome subfractions were prepared by two methods. A Spinco model L ultracentrifuge with a no. 40 rotor was used, and the values of $g_{av.}$-min. employed followed those of the authors cited. (*a*) Liver mince was homogenized with 5vol. of 0·88M-sucrose and fractionated by the procedure involving iso-octane described by Hallinan & Munro (1965). (*b*) Liver mince was homogenized with 4vol. of 0·25M-sucrose and fractionated by a method involving CsCl and $MgCl_2$ similar to that of Dallner (1963).

Spleen and kidney. Spleen and kidney were fractionated by a method based on (1) above. The 10000g sediments were washed twice to provide large-granule fractions.

Extraction of subcellular fractions

Except where noted, about 1·0–1·4ml. of extractant was used/10mg. of protein present. Extraction with 1% Lubrol W was at 2°. The volume used to extract microsome material from liver slices was about 1·5–2·0ml./10mg. of protein. For extraction of spleen and kidney *in toto*, 0·7g. of minced tissue was homogenized with 6ml. of 1% Lubrol. In some experiments (see the Results section), Lubrol extracts were concentrated by ultrafiltration with concurrent dialysis against 1% Lubrol; the appropriate unlabelled precursor (10mM) was added to the Lubrol used for the first 6hr. of dialysis. With sodium deoxycholate, about 0·5–0·7ml. of a 2·5% solution in medium A was used/10mg. of

protein at 2°. With the following, extraction was for 60min. at 37°: 50mM-tris–40mN-HCl, pH7·5, 20μg. of ribonuclease/ml. of the same buffer, and 25mM-EDTA, pH7·5. When material insoluble in Lubrol or deoxycholate was extracted with these reagents, the volume used was similar to that of the original extractant. For extraction with ultrasonic vibrations, microsome material (about 10mg. of protein) was suspended in 0·5ml. of 0·15M-NaCl and kept in ice. A type 1130A Soniprobe (Dawe Instruments Ltd., London) fitted with a 3mm. tip was used for 2min. at 30–40w. An equal volume of NaCl was added before centrifuging. Insoluble material was removed from all extracts by centrifuging for 120min. at 40000rev./min. (MSE 40 centrifuge).

Immunological methods

Antisera. These were prepared as described by Simkin *et al.* (1964*b*). All were pooled samples of serum from bleedings made at different times on an individual rabbit, except with anti-(fraction I_b), when an antiserum from a second rabbit was used for some later experiments. Anti-(hen's-egg white) was kindly supplied by Dr J. E. Fothergill.

Double-diffusion analysis. This was carried out at room temperature. In some experiments, the procedure used was that of Simkin *et al.* (1964*b*). In most work with tissue extracts, however, antigen and antibody solutions diffused into the gel from reservoirs. These were lengths of glass tubing 20mm. high and 5mm. internal diam. from the lower end of which a segment of glass 1·5mm. high and about 5mm. wide had been removed. The reservoirs were placed in a 2mm.-deep layer of molten agar gel, and after setting most of the agar within the reservoirs was removed with a cork-borer.

Immunoelectrophoresis. This was carried out on 3in. × 1in. glass slides with equipment supplied by Shandon Scientific Co. Ltd., London. The buffer contained 55mM-sodium acetate–48·5mM-sodium diethylbarbiturate–65mN-HCl, pH8·6; the gel was prepared from Ionagar no. 2 and contained buffer at one-third concentration.

Radioautography of gels. Gels were washed at room temperature for 2 days with 0·15M-NaCl, 3 days with tap water and 2 days with distilled water; the wash liquids were changed continuously. The gels were dried at 25°, placed on Kodirex X-ray film (Kodak Ltd., London), usually for 3–12 weeks, and then stained for protein with Nigrosine (Bodman, 1960).

Precipitin technique. For isolation of material for measurement of radioactivity, Lubrol extracts of liver microsomes were concentrated sixfold by ultrafiltration with concurrent dialysis against 0·15M-NaCl–1% Lubrol containing appropriate unlabelled precursor (10mM). Mixtures (total volume 0·45ml.) were prepared containing 0·2–0·3ml. of Lubrol extract, 0·15M-NaCl, 1mM-sodium azide, 1% Lubrol and 4·7% Dextran 80. After addition of 15μg. of human fraction I and 0·03ml. of anti-(human fraction I), mixtures were stood at 37° for 45min. and 2° for 24hr. After centrifuging at 2°, a similar amount of human fraction I and anti-(human fraction I) was added to the supernatants and the mixtures were stood as before. After centrifuging, 0·05ml. of anti-(fraction I_b) was added to the supernatants, the mixtures were stood at 37° for 45min. and 2° for 48hr. and then centrifuged. All precipitates were washed at 2° with 0·3ml. of 0·15M-NaCl–1% Lubrol–4% Dextran 80 and 3 × 0·3ml. of 0·15M-NaCl, and were then dissolved in 0·1N-NaOH and samples removed for determination of protein (Lowry *et al.* 1951) and radioactivity by scintillation counting (see below). For isolation of material to be subjected to degradation (see below), the above procedure was scaled up 15-fold, and precipitation with the heterologous system carried out only once.

Isolation of protein for measurement of radioactivity

After labelling with amino acids, protein was isolated by the method of Simkin & Work (1957). The appropriate unlabelled amino acid (20mM) was added before application of the procedure, and was also added to the N-NaOH used to dissolve protein before reprecipitation. After use of [^{14}C]glucosamine, an equal volume of 10% (w/v) trichloroacetic acid was added to soluble samples or suspensions of particulate preparations containing 5mg. or more of protein/ml. and unlabelled D-glucosamine (20mM). Precipitates were collected by centrifuging and washed as follows, the volume of each wash solution being equal to that of the material taken for precipitation and washing carried out at room temperature except where noted: once with 5% trichloroacetic acid–10mM-glucosamine; once with 5% trichloroacetic acid; once with 0·1M-NaCl–80% (v/v) acetone (cf. Weinfeld & Tunis, 1960); twice with ethanol–ether–chloroform (2:2:1, by vol.) for 15min., the first wash being at 50°; and twice with ether. After drying in air, the material was suspended at about 10mg./ml. in a solution of 100μg. of ribonuclease/ml. in 100mM-tris–80mN-HCl, pH7·5, and shaken for 90min. at 37°. Samples were then dialysed for 24hr. against water at 2° and freeze-dried. Phosphotungstic acid-insoluble material was isolated from trichloroacetic acid-soluble fractions by addition of 0·2vol. of 5% (w/v) phosphotungstic acid in 2N-HCl, and was washed as for glucosamine-labelled material, except that the trichloroacetic acid used contained 0·2vol. of phosphotungstic acid reagent. For preparation of trichloroacetic acid-soluble fractions in glucosamine distribution studies, tissue homogenate or serum was diluted with 0·5vol. of water and 10% trichloroacetic acid equal in volume to the mixture added.

Degradation of labelled proteins

Fraction I from serum. Two experiments were carried out: 5 and 15μc of [^{14}C]glucosamine respectively were injected intraperitoneally and the animals killed after 60min. Fraction I was isolated as in incorporation studies described above. (1) Fraction I (2·5mg.) was hydrolysed with 50mN-H_2SO_4 and sialic acid isolated by chromatography with Dowex 2 (formate form) (Simkin *et al.* 1964*b*). A sample of the sialic acid fraction was taken for determination of radioactivity (see below) and the remainder chromatographed on paper with butan-1-ol–acetic acid–water (4:1:5, by vol.) as solvent. Appropriate areas of the chromatogram as determined by guide strips were removed for counting. Two other samples of fraction I (2mg.) were hydrolysed with Dowex 50–40mN-HCl and fractionated to give neutral sugar and hexosamine fractions as described by Simkin *et al.* (1964*b*). (2) Fraction I (4·5mg.) was heated with 25mN-H_2SO_4 for 60min. at 90° and then dialysed against water and freeze-dried. Small samples of treated

and untreated material were taken for determination of protein and radioactivity. The remainder of the treated material was heated with 4N-HCl for 4hr. at 100°. HCl was removed *in vacuo*. The residue was dissolved in 0·3N-HCl and chromatographed on a column of Dowex 50 (H^+ form) (Gardell, 1953). Fractions were analysed for hexosamine (Rondle & Morgan, 1955), and samples of hexosamine-containing fractions taken for counting after removal of HCl.

Liver proteins. Liver proteins isolated from subcellular fractions after administration of [^{14}C]glucosamine were hydrolysed with 50mN-H_2SO_4 (see above). One or both of two procedures were then applied: (1) The soluble material was fractionated with Dowex 2 as above and the radioactivity of the sialic acid compared by scintillation counting with that of the corresponding untreated protein dissolved in NaOH. (2) The hydrolysate was dialysed against water. The non-diffusible material was freeze-dried and its radioactivity compared with that of a suitable control by counting at infinite thickness.

Material precipitated by anti-(fraction I_b). After application of the procedure described above to a Lubrol extract of a liver microsome fraction labelled with [^{14}C]leucine in a cell-free system, one-third of the precipitate obtained was suspended in 1·5ml. of 0·1M-KCl containing 2·5mg. of fraction I. The pH was adjusted to 8·5 with KOH, 0·07ml. of 1-fluoro-2,4-dinitrobenzene added, and the pH maintained at 8·5 by addition of KOH (Biserte, Holleman, Holleman-Dehave & Sautière, 1959). When addition of alkali was complete, the mixture was extracted with ether before and after acidification. The aqueous material was dialysed against water. The non-diffusible material was evaporated to dryness *in vacuo* and then heated with 0·3ml. of 6N-HCl for 15hr. at 105°. The hydrolysate was diluted to 1·7ml. with water and extracted with 3 × 1ml. of ether. The pooled ether extracts were evaporated to dryness and dissolved in 5N-NH_3. Samples of ether-soluble and ether-insoluble material were counted at infinite thinness (see below). The remaining precipitate obtained from the Lubrol extract was mixed with 2·5mg. of fraction I_b isolated as described above from an animal killed 90min. after intraperitoneal injection of 400μc of [^{3}H]leucine. Sialic acid was removed by heating with 1·4ml. of 25mN-H_2SO_4 for 60min. at 85°. The hydrolysate was dialysed against water. Then $(NH_4)_2CO_3$ was added to the non-diffusible material to 1% followed by trypsin equal to about 1% of the weight of protein. After incubation for 5hr. at 37°, trypsin equal to one-half that originally used was added and incubation continued for 17hr. The digest was freeze-dried. The residue was dissolved in water and centrifuged. The soluble material was streaked on Whatman no. 3MM paper and electrophoresis carried out with 240mM-pyridine–17mN-acetic acid, pH6·4, for 2·2hr. at about 20v/cm. Peptides were located by staining the edges of the separated material with ninhydrin, eluted (Ingram, 1963) with 100mN-acetic acid, and their radioactivity was measured. A mixture of a precipitate obtained from a Lubrol extract prepared after labelling with [^{14}C]glucosamine *in vivo* and 5mg. of fraction I was heated with 2·5ml. of 25mN-H_2SO_4 for 60min. at 85°. After dialysis against water, the non-diffusible material was freeze-dried and digested with Pronase as described by Cunningham & Simkin (1966), except that the amount of Pronase added was increased by one-third. The digest was fractionated on a column (81cm. × 1·5cm.) of Sephadex G-50 (medium grade) with 100mN-acetic acid as eluent. Samples of fractions were analysed by the orcinol and ninhydrin methods (Cunningham & Simkin, 1966) and for radioactivity.

Determination of radioactivity

Except where noted, protein samples isolated from tissues were plated on 0·3cm.^{2} or 1cm.^{2} planchets. When only small quantities were available, proteins were dissolved in 5N-NH_3; one sample (100–600μg. of protein) was transferred to a 2cm.^{2} planchet containing 75μg. of Brij 35 and evaporated to dryness *in vacuo*, and another taken for estimation of protein (Lowry *et al.* 1951). When necessary, correction to infinite thickness was made by use of an empirically determined curve. Planchets were counted with a thin end-window Geiger–Müller counter. Under the conditions used, 1cm.^{2} of [^{14}C]methacrylate resin of 1mμc/mg. gives 1000counts/min. at infinite thickness. Samples of trichloroacetic acid-soluble fractions and urine obtained in glucosamine distribution studies and fractions resulting from the degradation of serum fraction I were assayed by scintillation counting adsorbed on 2cm. × 2·5cm. pieces of Whatman no. 3MM filter paper. After evaporation of solvent, papers were suspended in 20ml. silica vials containing 10ml. of NE213 scintillator and counted with an NE8301 scintillation spectrometer [Nuclear Enterprises (G.B.) Ltd.]. Efficiency, as determined with [^{14}C]glycine of known activity applied to papers in the presence and absence of samples, was about 50%. In all other instances, samples (up to 0·5ml.) were added to a mixture of 1ml. of M-Hyamine hydroxide in methanol and 10ml. of the scintillation mixture of Schall & Turba (1963); with samples from the fractionation of trypsin and Pronase digests of material isolated by the precipitin technique, Hyamine was omitted. Radioactivity was measured with a model 3003 Tri-Carb scintillation spectrometer (Packard Instrument Co., La Grange, Ill., U.S.A.). Efficiency, determined with [^{14}C]hexadecane in the presence and absence of samples, was about 78%; values in the Results section have been corrected to 100% efficiency.

With few exceptions, the standard deviation of the net count rate was not greater than ±5%.

RESULTS

Preparation of subfractions of fraction I. Antiserum to fraction I contains an appreciable number of antibody components, about ten precipitin lines being formed on double-diffusion analysis with undiluted guinea-pig serum (Simkin *et al.* 1964*b*). Moreover, as previously reported, anti-(fraction I) contains antibody to guinea-pig serum albumin, from which carbohydrate is absent. To simplify interpretation of the results of double-diffusion analysis connected with work on the biosynthesis of glycoprotein components of fraction I, subfractions of fraction I were prepared and used for production of antisera. The first fractionation involved chromatography on DEAE-cellulose with stepwise elution with three borate–phosphate buffers of pH8·4; the concentrations used were based on

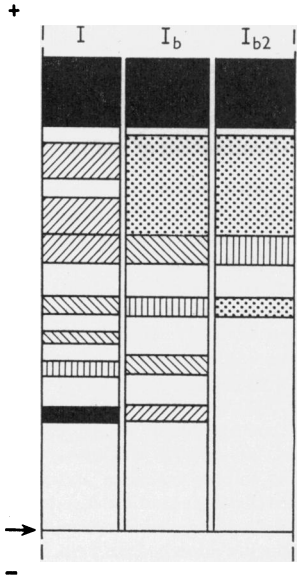


Fig. 1. Starch-gel electrophoresis with the pH9 buffer system of fractions I, I_b and I_{b2}. Intensity of staining (in decreasing order) is indicated by: ■, ■, ■, ▥, ▩.

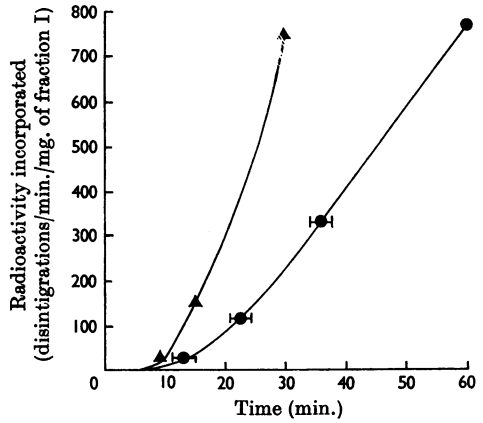


Fig. 2. Incorporation of radioactivity into fraction I isolated from serum after intravenous injection of [^{14}C]-glucosamine (▲) and [^{14}C]leucine (●). Results for glucosamine were obtained with separate guinea pigs, whereas those for leucine were obtained with a single animal, the width of bars indicating the period over which the blood sample was obtained.

results obtained with gradient elution (J. L. Simkin & E. R. Skinner, unpublished work). Fig. 1 shows that, with fraction I_b, the major fraction obtained, eluted by 114mM-buffer, there is a reduction in the minor bands relative to the main band seen on starch-gel electrophoresis of fraction I at pH9 (cf. Simkin *et al.* 1964*b*). Fraction I_b was fractionated by chromatography on Sephadex G-100. Two peaks were obtained: the smaller and larger were of V_e/V_0 1·01 and 1·31 respectively (V_e, elution volume; V_0, void volume, taken as the elution volume of the faster peak obtained with bovine γ-globulin). Sephadex G-100 separated components of faster and slower mobility on starch-gel electrophoresis, the major fraction, fraction I_{b2}, containing the faster components (Fig. 1).

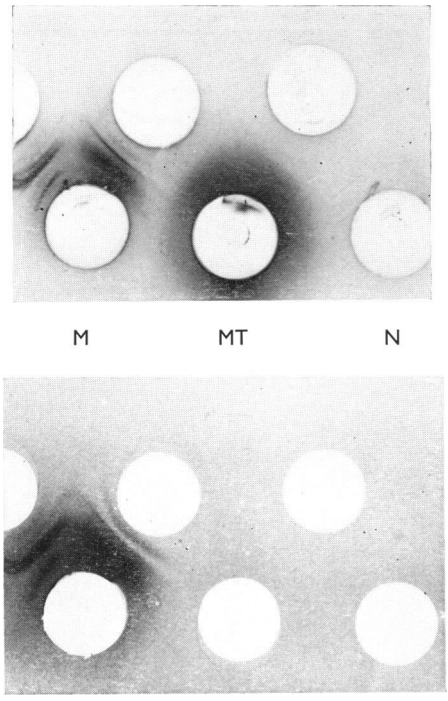
Incorporation of precursors into serum fraction I. After injection of [^{14}C]leucine or [^{14}C]glucosamine into guinea pigs, little label appeared in fraction I present in the serum for about 10min. (Fig. 2); thereafter, there was a rapid rise in specific activity. Because results with labelled amino acid at short times after injection varied somewhat from animal to animal, the values shown in Fig. 2 were obtained with a single animal; some error may, however, arise with this technique from the use of small samples of serum.

Immunodiffusion studies on biosynthesis of fraction I: incorporation of ^{14}C-labelled amino acids. In initial studies to determine the cellular and subcellular site of synthesis of the polypeptide moieties of components of fraction I, tissues were removed from animals killed 15–17min. after intravenous injection of [^{14}C]leucine. Since there is negligible label in serum fraction I at this time-interval after injection (see above), the presence in intracellular structures of labelled material capable of reaction with anti-(fraction I) should provide information on the site of synthesis of these serum proteins. The tissues investigated on the basis of previous work (e.g. Miller & Bale, 1954; Hochwald *et al.* 1961; Asofsky & Thorbecke, 1961; Espinosa, 1961) were: liver, the site of synthesis of many serum proteins;

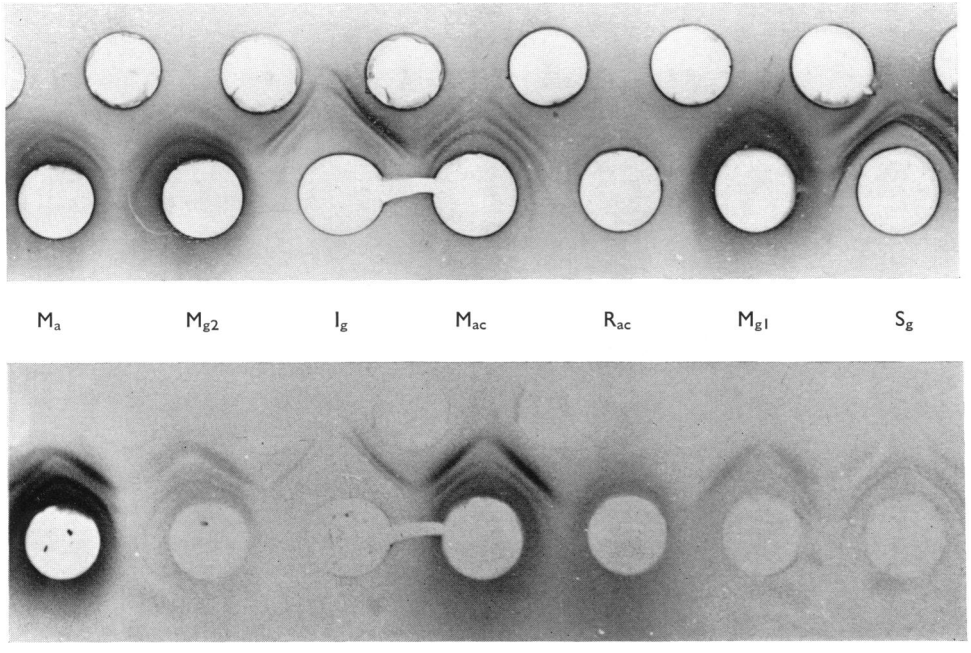
EXPLANATION OF PLATE I

(*a*) Double-diffusion analysis with anti-(fraction I_b) of Lubrol extracts of microsomal (M), mitochondrial (MT) and nuclear (N) fractions from liver of guinea pig killed 17min. after intravenous injection of [^{14}C]valine. The homogenization medium was 0·25M-sucrose. Bottom, radioautograph of gel; top, gel stained for protein. (*b*) Results of double-diffusion analysis with anti-(fraction I_b). Except where noted, labelling was *in vivo*. M_a, Lubrol extract of liver microsome fraction, 15min. labelling with [^{14}C]leucine; M_{g1} and M_{g2}, Lubrol extract of liver microsome fraction, 10 and 14min. labelling with [^{14}C]glucosamine respectively; M_{ac}, Lubrol extract of liver microsome fraction labelled in cell-free system with [^{14}C]leucine; R_{ac}, ribonuclease extract of Lubrol-insoluble material corresponding to M_{ac}; I_g and S_g, fraction I (0·3mg./ml.) and serum (1:40 dilution) respectively, 60min. labelling with [^{14}C]glucosamine. The homogenization medium was 0·25M-sucrose, except for M_{ac} and R_{ac}. Bottom, radioautograph of gel; top, gel stained for protein.

(*a*)



(*b*)



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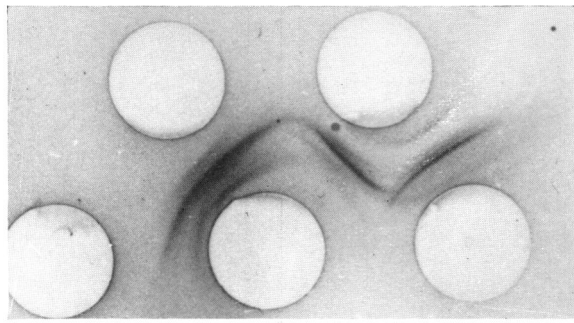
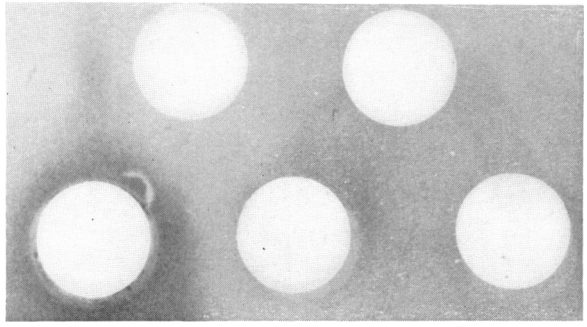
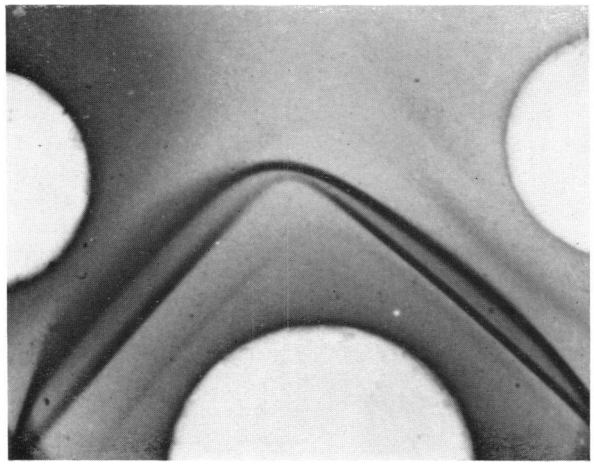


M MH H



(*b*)

M



S

spleen, which can synthesize some serum proteins; and, as a control, kidney, which has little capacity for synthesis of serum proteins. Livers were fractionated into three or four particulate fractions with use of 0·25 M-sucrose or medium A respectively, and the fractions extracted with the non-ionic detergent Lubrol W. Extracts and cell sap were allowed to react with anti-(fraction I) by double diffusion in agar and the gels were subsequently radioautographed. Only extracts of microsome fractions formed an appreciable number of precipitin lines that were well labelled. Precipitin lines were formed by cell sap and to a smaller extent by extracts of nuclear and mitochondrial fractions (cf. Plate 1*a*), but all showed little or no radioactivity. Examination with anti-(fraction I_b) gave essentially similar results (Plate 1*a*; in the Plates, faint precipitin lines may not be revealed by the photographs presented) (see also below). Precipitin lines formed by Lubrol extracts of spleen or kidney homogenates or of large-granule and microsome fractions from these tissues also contained little or no radioactivity. Results with [^{14}C]valine (Plate 1*a*) were similar to those with [^{14}C]leucine. Precipitin lines formed by sera from the animals used were not radioactive, confirming the delay in labelling of serum fraction I found after isolation with CM-cellulose.

To investigate whether a different pattern of labelling existed at a much shorter exposure to amino acid, extracts were prepared from fractions isolated from livers removed 75 and 95 sec. after intraportal injection of [^{14}C]leucine. Essentially similar results were obtained as with labelling for 15 min.

These results suggest that the microsome material of liver contains the site of synthesis of the polypeptide moieties of components of fraction I. Confirmatory evidence was sought in experiments with liver slices and a cell-free microsome system. Examination was made with anti-(fraction I) and anti-(fraction I_b). The latter gives essentially similar results to the former, except that two or three minor components are missing, including anti-(serum albumin). After reaction of liver microsome extracts with anti-(fraction I), the line resulting from precipitation of albumin is readily identifiable and shows negligible labelling with [^{14}C]glucosamine; a faint line close to that formed by albumin also showed a low labelling with glucosamine. Both lines have therefore been excluded from consideration in the results discussed below.

Lubrol extracts of microsome fractions isolated after incubation of liver slices or a microsome–cell-sap system with [^{14}C]leucine gave precipitin lines that were radioactive (e.g. Plate 1*b*). Labelling was markedly inhibited by addition of 50–75 μM-puromycin, but puromycin aminonucleoside produced little inhibition at a similar concentration. Labelling in the cell-free system was almost completely abolished by omission of phosphoenolpyruvate. In one experiment, cell sap and a Lubrol extract of the large-granule fraction isolated from liver slices were examined and, as *in vivo*, the precipitin lines contained little radioactivity.

All precipitin lines showed radioactivity in experiments both *in vivo* and *in vitro* (e.g. Plate 1*b*); occasionally, however, the labelling of a very faint line could not be established unequivocally. The labelling of lines does not appear to be due to non-specific adsorption or precipitation of labelled material since, as shown in Plate 2(*a*), significant precipitin lines were not formed on reaction of Lubrol extracts of liver microsome material with anti-(human fraction I), and lines formed on reaction of a mixture of Lubrol extract and human fraction I with this antiserum contained little or no radioactivity (human fraction I consists of a group of α-globulins and has a total hexose/protein ratio slightly higher than guinea-pig fraction I; J. L. Simkin & E. R. Skinner, unpublished work). Similar negative results were obtained with hen's-egg-white anti-(egg white) as a heterologous immune system.

Study of immunological correspondence between precipitin lines formed by Lubrol extracts of liver microsome fractions and lines formed by guinea-pig serum or fraction I was made difficult by the complex patterns of lines found, by differences in relative concentration between tissue and serum constituents, and by the use of reservoirs with exits covering an arc of about 120° set in a triangular pattern (e.g. Plates 1*a* and 1*b*). Some improvement in results was obtained by concentrating Lubrol extracts about twofold before reaction with anti-(fraction I_b). Extracts concentrated tenfold were also examined with this antiserum without the use of reservoirs, and, though all minor components were not apparent, immunological correspondence was easier to establish. These methods indicated that the two principal and several of the minor precipitin lines formed by the Lubrol extracts gave reactions of identity with lines formed by serum (Plate 2*b*). In other instances, a clear result on immunological

EXPLANATION OF PLATE 2

(*a*) Double-diffusion analysis with anti-(human fraction I) of: M, Lubrol extract of liver microsome fraction from guinea pig killed 15 min. after injection of [^{14}C]leucine; MH, mixture of M and human fraction I; H, human fraction I. Bottom, radioautograph of gel; top, gel stained for protein. (*b*) Double-diffusion analysis with anti-(fraction I_b) of: M, Lubrol extract of liver microsome fraction isolated from cell-free system; S, guinea-pig serum (1:15 dilution). The gel was stained for protein.

correspondence could not be obtained. Comparison with fraction I, although less extensive, and examination of mixtures of Lubrol extracts with serum or fraction I supported these findings, as did use of anti-(fraction I_{b2}). This antiserum gives fewer well-defined precipitin lines with guinea-pig serum than anti-(fraction I_b) and several constituents of the latter are missing; the pattern of lines formed with Lubrol extracts was rather similar to that with anti-(fraction I_b), except for a smaller number of minor lines.

The microsome fraction of liver is heterogeneous with respect to morphological constituents (e.g. Siekevitz, 1963), and, in an attempt to establish the origin of the labelled substances present that react with anti-(fraction I), extracts obtained by application of various procedures to microsome material labelled with [^{14}C]leucine either for 1·25–17min. *in vivo* or in a cell-free system were examined with this antiserum or anti-(fraction I_b). Such experiments might also reveal the presence of precursors of the glycoproteins investigated. Material extracted by deoxycholate formed several precipitin lines, but it was difficult to establish the number of lines present and their immunological identity because of considerable non-specific precipitation. The substances released by ultrasonic vibrations were qualitatively similar to those extracted with Lubrol, and sometimes were obtained in a yield similar to that found with the latter. Incubation with 150mM-sodium chloride at 0° or 50mM-sodium chloride–25mM-EDTA–50mM-tris buffer, pH7·5, or ribonuclease at 37° extracted appreciably less material than Lubrol. Incubation of Lubrol- or deoxycholate-insoluble material with EDTA–tris buffer or ribonuclease at 37° released little material that reacted with anti-(fraction I_b) (Plate 1*b*), but material labelled for 1·6min. or less *in vivo* or in the cell-free system gave two highly labelled lines when allowed to react with anti-(fraction I), one of which showed immunological identity to a line produced by guinea-pig serum albumin.

Metabolism of [^{14}C]*glucosamine in the guinea pig.* Preliminary to immunological studies on incorporation of glucosamine into tissue constituents, the distribution of label in guinea-pig tissues and the relative labelling of the trichloroacetic acid-insoluble material of subcellular fractions of liver after administration of [^{14}C]glucosamine were investigated. At about 15min. after intravenous injection of glucosamine, about one-sixth and one-third of the label administered were present in the trichloroacetic acid-soluble fractions of serum and liver respectively, and one-third had been excreted in the urine (mean of three experiments). Of other tissues examined, only kidney contained appreciable label in acid-soluble form, about 5% of the dose being present. About 1·5% of the dose was present in the trichloroacetic acid-insoluble fraction of liver and a negligible quantity in the corresponding fraction of serum. At 60min. after intraperitoneal injection of 5μc of [^{14}C]glucosamine (one experiment), the main differences from the results just described were that there was very little radioactivity in the trichloroacetic acid-soluble fraction of serum and about 4·5 and 6% of the dose were present in the trichloroacetic acid-insoluble fractions of serum and liver respectively.

With guinea-pig liver subfractions after labelling with [^{14}C]glucosamine for 10min. *in vivo*, protein soluble in trichloroacetic acid but insoluble in phosphotungstic acid had a higher specific activity (four times or less) than protein insoluble in trichloroacetic acid (cf. Helgeland, 1965; Sinohara & Sky-Peck, 1965). Provided, however, that protein was present during precipitation and washing with trichloroacetic acid at more than about 2·5mg./ml., only 4% or less of the total protein was soluble in trichloroacetic acid but insoluble in phosphotungstic acid, and little error with regard to incorporation into total glycoprotein should thus result from omission of the use of phosphotungstic acid in subsequent work.

When 0·25M-sucrose was used for subcellular fractionation of liver, a large part of the radioactivity incorporated into the trichloroacetic acid-insoluble material was present in the microsome

Table 1. *Radioactivity incorporated into trichloroacetic acid-insoluble material of subcellular fractions of guinea-pig liver after intravenous injection of* [^{14}C]*glucosamine*

Except where noted, the values given are the means of experiments involving 8–15min. labelling and are expressed in terms of the ratio of the specific activity of a given subfraction to the specific activity of the microsome fraction isolated with 0·25M-sucrose from the same liver. The numbers of values are given in parentheses.

Subfraction	Radioactivity incorporated	
Fractionation medium	Sucrose	Medium A
Nuclear	0·08 (5)	0·16 (1)*
Mitochondrial	0·04 (5)	0·32 (1)*
Microsomal	1·00 ‡	0·68 (5)
Lubrol-soluble	1·34 (5)	1·34 (1)*†
Lubrol-insoluble	0·52 (5)	0·50 (1)*†
Deoxycholate-soluble		1·22 (5)†
Deoxycholate-insoluble		0·29 (5)†
Cell sap	0·25 (5)	

* 12min. labelling; values obtained for nuclear and mitochondrial fractions isolated with 0·25M-sucrose from the same liver were 0·13 and 0·04 respectively.

† Ratio in relation to microsome fraction isolated from same liver with medium A.

‡ Specific activity 0·052–0·240mμc/mg.

fraction after injection of [^{14}C]glucosamine (Table 1). There was no obvious change in subcellular distribution within the 8–15min. time-period studied. When, however, medium A was used with either fractionation procedure (2) or (3), both the total and specific radioactivity of the microsome fraction decreased as compared with the corresponding values obtained with the same liver when 0·25M-sucrose was used for fractionation. The radioactivity of the more rapidly sedimenting fractions increased correspondingly (Table 1); the increase was most evident with the light-mitochondrial fraction. Lubrol-soluble fractions of microsome material had a higher specific activity than Lubrol-insoluble fractions, and this difference was more marked with deoxycholate-soluble and -insoluble fractions (Table 1). Incorporation into smooth- and rough-membrane microsome subfractions was also studied. After use of the method of Hallinan & Munro (1965), the smooth subfractions had a specific activity 1·9 and 1·7 times as great as those of the rough subfractions after 8 and 15min. incorporation respectively. When the method of Dallner (1963) was used after 15min. labelling, the Mg^{2+}-free smooth subfraction had a specific activity 2·7 times that of the rough subfraction.

Distribution of label in serum fraction I after administration of [^{14}C]*glucosamine.* For immunological investigations involving incorporation of glucosamine to be meaningful for study of the biosynthesis of carbohydrate prosthetic groups, label must be present largely or entirely in sugar residues. Fraction I isolated from serum 60min. after administration of [^{14}C]glucosamine was therefore degraded and the distribution of label determined. Sialic acid and neutral hexose fractions contained about 25–30 and 2% respectively of the total radioactivity, and hexosamine accounted for most of the remaining radioactivity. The nature of the radioactive material in the sialic acid and hexosamine fractions was checked by paper and ion-exchange chromatography respectively.

Immunodiffusion studies on biosynthesis of fraction I: incorporation of [^{14}C]*glucosamine.* The results just described show that the incorporation of glucosamine indicates the occurrence of biosynthesis of carbohydrate prosthetic groups, and the presence of label in a precipitin line demonstrates that the substance precipitated is a glycoprotein. When serum or fraction I obtained after 60min. labelling with [^{14}C]glucosamine *in vivo* was allowed to react with anti-(fraction I_b), all of the precipitin lines were labelled (Plate 1*b*). Examination with anti-(fraction I_{b2}) and application of immunoelectrophoresis supported these findings; with anti-(fraction I), certain minor components contained little or no label (see above). Thus all of the principal components of fraction I are glycoproteins, confirming suggestions made in earlier studies (Simkin *et al.* 1964*b*).

After labelling *in vivo* for 8–15min. with [^{14}C]-glucosamine, Lubrol extracts of liver subfractions or spleen and kidney homogenates and sera were allowed to react with anti-(fraction I) or anti-(fraction I_b). As with labelled amino acids, only Lubrol extracts of the microsome fraction of liver gave precipitin lines that contained appreciable radioactivity. All of the precipitin lines formed by Lubrol extracts of liver microsomes were labelled (Plate 1*b*), including those given with anti-(fraction I_{b2}), with exceptions noted above with anti-(fraction I); control experiments with a heterologous immune system were negative. Precipitin lines formed by Lubrol extracts of the more rapidly sedimenting liver subfractions isolated with medium A showed slightly more label than those given by the corresponding extracts obtained after use of 0·25M-sucrose; some effect of this kind was also found after labelling with leucine. Though only a very approximate estimate of specific radioactivity can be made with the technique employed, results were obtained that suggested that precipitin lines formed by Lubrol extracts of smooth subfractions of microsome material had a higher specific activity than those given by extracts of rough subfractions.

Precipitin lines formed by Lubrol extracts of microsome fractions isolated from liver slices after incubation with [^{14}C]glucosamine were radioactive. In contrast with results with leucine, puromycin did not cause marked inhibition of incorporation, but as estimates of specific activity are approximate the occurrence of a small effect cannot be excluded.

Investigation of material precipitated from liver extracts by antiserum. To obtain an approximate estimate of the specific radioactivity of the material precipitated from Lubrol extracts of liver microsome fractions on addition of anti-(fraction I_b) and to investigate whether label is incorporated into such material in a manner consistent with the occurrence of biosynthesis, precipitates were collected after direct addition of antiserum to extracts. Human fraction I and anti-(human fraction I) were added before anti-(fraction I_b) to reduce non-specific precipitation with the latter; Table 2 shows that a second addition of this heterologous system removed little further labelled material. As expected from the heterogeneity of the system, when extracts were titrated with antiserum, plots of amount of precipitate formed versus volume of extract added did not give smooth curves. Dextran was therefore added in an attempt to minimize resolution of any components present in a state of antigen excess (cf. Hellsing & Laurent, 1964). Despite this, it is unlikely that there was complete precipitation of reactive material. Volumes of extracts corresponding to a region of the

precipitin curve in which the amount of material precipitated and its specific radioactivity approximated to a plateau were used for the results shown in Table 2. Results obtained with fluorescent-labelled fraction I suggest that the precipitates contain about 12–15% of radioactive material. The results obtained (Table 2) thus showed that the material precipitated has a higher specific radioactivity than the total protein of the corresponding Lubrol extract. When the precipitin procedure was applied to samples labelled with [^{14}C]leucine in a cell-free system, precipitates isolated from incubation mixtures from which phosphoenolpyruvate had been omitted or puromycin or puromycin aminonucleoside added had specific activities 20, 20 and 70% respectively of the control system, thus confirming the results of immunodiffusion studies described above.

A precipitate isolated from a Lubrol extract after labelling with [^{14}C]leucine in a cell-free system was degraded in two ways. A sample was treated with 1-fluoro-2,4-dinitrobenzene and hydrolysed with acid. Less than 5% of the total radioactivity of the hydrolysate was soluble in ether. A second sample was mixed with fraction I_b labelled with [^{3}H]leucine *in vivo*, sialic acid removed (cf. Cunningham & Simkin, 1966), and the material was incubated with trypsin. The digest was fractionated by paper electrophoresis at pH 6·4, peptides were eluted and their contents of ^{14}C and ^{3}H measured. [^{3}H]-labelled Fraction I_b was added to locate peptides originating from fraction I_b in the presence of non-radioactive material deriving from rabbit γ-globulin. Of 16 ninhydrin-positive bands detected, 13 contained significant amounts of both isotopes. A precipitate isolated from a Lubrol extract after labelling with [^{14}C]glucosamine *in vivo* was mixed with unlabelled fraction I and sialic acid removed. A digest of the treated material with Pronase was fractionated with Sephadex G-50 (cf. Cunningham & Simkin, 1966). Significant radioactivity was present only in fractions that contained glycopeptides derived from fraction I.

DISCUSSION

The preparation of fractions I_b and I_{b2} involved chromatography on DEAE-cellulose and Sephadex G-100 respectively. The elution volume of fraction I_{b2} on the latter was smaller than expected from the value of approx. 50000 previously reported for the molecular weight of the major constituents of fraction I (Simkin *et al.* 1964*b*), the value of V_e/V_0 obtained being close to that of bovine serum albumin (1·34). This finding may result from the high content of carbohydrate of the glycoproteins investigated (cf. Andrews, 1965). Detailed studies on the carbohydrate composition of fractions I_b and I_{b2} have not been carried out, but fraction I_b shows a similar and fraction I_{b2} a slightly higher content of total hexose and sialic acid in relation to polypeptide as compared with fraction I.

The delay in appearance of label in fraction I present in serum after administration of leucine or glucosamine is similar to that found in other studies on serum proteins (e.g. Peters, 1962; Richmond, 1963; Robinson *et al.* 1964; Macbeth *et al.* 1965). Fraction I shows a greater incorporation of [^{3}H]-leucine than serum albumin (J. L. Simkin & J. C. Jamieson, unpublished work), as with comparable α-globulins of other species (e.g. Richmond, 1963; Sarcione, 1963; Miller *et al.* 1964).

The distribution of label in tissues of the guinea pig after administration of [^{14}C]glucosamine is roughly comparable with that found in the rat (e.g. Robinson *et al.* 1964; Macbeth *et al.* 1965), except that the percentage of label present in the trichloroacetic acid-insoluble fraction of liver was somewhat smaller than in the rat. An experiment

Table 2. *Incorporation of precursors into material precipitated from Lubrol extracts of guinea-pig liver microsome fractions*

Labelling was for 15 min. *in vivo* (IV) with either [^{14}C]leucine or [^{14}C]glucosamine or for 20 min. in a microsome–cell-sap system (CF) with [^{14}C]leucine. Precipitates were collected after two additions of human fraction I and anti-(human fraction I) and after addition of anti-(guinea-pig fraction I_b). Trichloroacetic acid-insoluble material was isolated from separate samples of the Lubrol extracts. Results are expressed as: (*a*) total radioactivity of precipitate in disintegrations/min.; (*b*) specific radioactivity of precipitate in disintegrations/min./mg. of protein.

Precursor	Radioactivity incorporated						
Precipitant.....	Anti-(human fraction I)				Anti-(guinea-pig fraction I_b)		Trichloroacetic acid
	1		2				
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*b*)
Leucine (IV)	101	1090	29	284	468	2560	5050
Leucine (CF)	52	494	9	74	250	1310	2280
Glucosamine (IV)	6	58	2	15	98	377	454

with rats suggested that this difference was not due merely to altered experimental conditions. An appreciable proportion of the dose was excreted in the urine, as found in the rat by Kohn, Winzler & Hoffmann (1962). These workers did not, however, study rate of excretion; the present investigation indicates that excretion is rapid.

The pattern of incorporation of [^{14}C]glucosamine into the trichloroacetic acid-insoluble material of subcellular fractions of liver shows similarities to the results of other workers (e.g. Robinson *et al.* 1964; Molnar, Robinson & Winzler, 1965; Sinohara & Sky-Peck, 1965). As in previous studies (e.g. Sarcione, Bohne & Leahy, 1964; Molnar *et al.* 1965; Sinohara & Sky-Peck, 1965), most of the label present in microsomes is solubilized by detergents. The proportion of label present as sialic acid in protein from liver subfractions was lower than found in the rat (Helgeland, 1965; Macbeth *et al.* 1965; Molnar *et al.* 1965). This may reflect a species difference or might result at least in part from the greater manipulation of samples that occurred in the present work. It is not clear why the addition of salts to 0·25M-sucrose used for subcellular fractionation causes more labelled material to sediment with the nuclear and mitochondrial fractions. The finding that both the total and specific radioactivity of the microsome fraction decreases suggests that the loss may reflect a change in the sedimentation properties of a component that does not form a large part of the microsome fraction but that has a relatively high specific radioactivity. The fact that the protein of smooth-microsome subfractions is more highly labelled than total microsome protein after administration of [^{14}C]-glucosamine in the guinea pig (present work) and in the rat (Hallinan, 1965; Molnar *et al.* 1965) shows that morphological components of the microsome fraction are labelled to different extents.

Patterns of precipitin lines given by Lubrol extracts of liver microsomes isolated after experiments *in vivo* or with cell-free extracts were similar. Not only were the lines labelled with both amino acid and glucosamine in intact cell systems and with amino acid in a cell-free system, but identity was directly shown in several instances by joins between amino acid- and glucosamine-labelled material. Moreover, several amino acid-labelled lines joined with lines formed by glucosamine-labelled components of serum or fraction I (Plate 1*b*). Coupled with the results of degradation of fraction I isolated after administration of [^{14}C]-glucosamine, these findings indicate that the substances precipitated by antibody represent serum glycoproteins synthesized by the liver or are precursors of them. Perfusion of a liver with saline before fractionation did not significantly reduce the intensity of precipitin lines produced by a Lubrol extract of the microsome fraction, suggesting that the material associated with the latter is not derived largely from residual serum present in the liver. Although differences occur in relative concentration between substances associated with liver microsomes and the substances present in serum with which they show immunological correspondence, the principal precipitin lines given by the tissue extracts join to well-defined lines formed by serum (Plate 2*b*). Both experiments with heterologous immune systems and degradation studies on material precipitated from Lubrol extracts by anti-(fraction I_b) indicate that the labelling of precipitin lines represents biosynthesis. Further, incorporation of [^{14}C]leucine into immunologically characterized glycoprotein in the systems used *in vitro* was strongly inhibited by puromycin. There was no marked inhibition of incorporation of glucosamine in liver slices, but the effect of puromycin on the synthesis of other glycoproteins has been variable (e.g. Richmond, 1963; Molnar, Robinson & Winzler, 1964; Spiro & Spiro, 1966).

As would be predicted from more general studies on the biosynthesis of serum glycoproteins (e.g. Molnar *et al.* 1965), both the polypeptide and carbohydrate moieties of individual glycoproteins present in fraction I are produced by components of the microsome fraction of guinea-pig liver. The nature of the procedures that release from microsome material substances that react with specific antisera suggest that these substances are associated with membranous structures or are present within vesicles enclosed by membranes (cf. e.g. Campbell, 1961). This may be explained by the occurrence of one or both of two processes: (1) transport via membrane systems to the exterior of the cell (e.g. Bruni & Porter, 1965); (2) synthesis of prosthetic groups associated with membranous structures (cf. e.g. Sarcione, 1964; Sarcione *et al.* 1964; Molnar *et al.* 1965; Droz, 1966; O'Brien, Canady, Hall & Neufeld, 1966).

The usefulness of the immunological procedures employed for study of the mechanism of biosynthesis of the glycoproteins investigated depends on whether the molecules can react with antibody if part or all of their carbohydrate is absent. As with other serum glycoproteins (see above), components of fraction I still react with antibody after removal of sialic acid with acid or neuraminidase, and at least some appear to retain immunological activity after removal of a greater proportion of their carbohydrate by use of periodate (J. L. Simkin & J. C. Jamieson, unpublished work). Thus, although microsome-bound substances can show immunological identity with components of fraction I present in serum, it is possible that these substances represent precursors of serum glycoproteins rather than complete molecules (cf. O'Brien *et al.* 1966).

It is very likely that the polypeptide moieties of serum glycoproteins are synthesized by ribosomes. Attempts to show precursors of components of fraction I associated with ribosomes by extraction of Lubrol- or deoxycholate-insoluble material of liver microsomes were inconclusive, although a highly labelled substance that reacts with anti-albumin is present in such extracts. This substance is comparable with one isolated from rat liver ribosomes (e.g. Hirokawa & Ogata, 1962). The nature of the material in these extracts giving the second highly labelled but faint line on reaction with anti-(fraction I) is uncertain; it shows little labelling with glucosamine and attempts to demonstrate a relationship between the material and a serum glycoprotein have so far been unsuccessful. It is, however, not yet known whether immunological procedures would in fact detect the polypeptide chain of a glycoprotein with little or no attached carbohydrate. In agreement with some (e.g. Helgeland, 1965; Molnar *et al.* 1965) but not all (e.g. Sarcione *et al.* 1964) workers, we have found that the deoxycholate-insoluble material of liver microsome fractions contains significant label after administration of [^{14}C]glucosamine. These studies, however, have not been carried out on ribosome preparations that have been purified, e.g. by density-gradient centrifuging, nor has the presence of traces of residual membrane been excluded (cf. Tsukada & Lieberman, 1965).

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