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INCORPORATION OF D-GLUCOSAMINE-1-C-14 INTO SUBCELLULAR FRACTIONS OF LIVER AND WALKER 256 CARCINOSARCOMA CELLS OF THE RAT

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BY

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Oklahoma City, Oklahoma

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INCORPORATION OF D-GLUCOSAMINE-1-C-14 INTO SUBCELLULAR FRACTIONS OF LIVER AND WALKER 256 CARCINOSARCOMA CELLS OF THE RAT

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ACKNOWLEDGMENT

I wish to express my sincere appreciation and gratitude to Dr. Marvin R. Shetlar for his encouragement and guidance during the course of this work. I am also grateful to my husband, Dr. Yu-Teh Li, for his valuable discussion and suggestions.

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CHAPTER I

INTRODUCTION

The presence of protein-bound carbohydrate in the serum was recognized before the beginning of this century (1, 2). The major monosaccharide components of serum glycoproteins are galactose, mannose, glucosamine and sialic acid. Galactosamine and fucose are minor constituents of some serum glycoproteins. These carbohydrates are widely distributed in all major serum protein fractions with the possible exception of serum albumin, but are particularly prominent in the α-globulin fractions.

It is well known that the concentration of the glycoprotein in human serum is greatly increased in a number of physiological and pathological states (3). Very little is known at the present time about the metabolic alterations which lead to these changes. Our knowledge about the normal synthesis and destruction of glycoproteins in the animal body is also incomplete.

In the present investigation the mechanism of serum glycoprotein biosynthesis was studied in normal rats and in rats bearing Walker 256 carcinosarcoma. D-Glucosamine-1-C-14 was utilized as a means of following this biosynthetic process.

CHAPTER II

LITERATURE REVIEW

Since the work described in this dissertation includes studies on the biosynthesis of serum glycoprotein and the carbohydrate content of subcellular fractions in rat liver and Walker 256 carcinosarcoma, this section of the dissertation will review the studies concerned with the (1) biosynthesis of serum glycoprotein, and (2) cell fractionation and carbohydrate content of subcellular fractions in rat liver and tumor tissue.

Biosynthesis of Serum Glycoprotein

Although the presence of protein-bound carbohydrate in the serum was recognized before 1900 (1, 2), the progress in this field beyond this phase was rather slow. Renewed interest in the study of these protein-bound carbohydrates in the sera was initiated by the finding of a significant elevation of serum glycoproteins in various physiological and pathological states (3, 4, 5). Since the elevation of serum glycoproteins in several disease states is so prominent, efforts have been made by numerous investigators to explain the cause of elevated serum glycoproteins. A number of hypotheses concerning the physiological mechanism that produces the elevation of serum glycoproteins in disease states have been proposed.

Seibert, Seibert, Atno and Campbell (6) noted the prominent elevation of the α_2-globulin levels in pathological processes involving tissue destruction, and proposed that elevations of glycoprotein above the normal level reflect the processes of tissue destruction. In support of the view that tissue destruction contributes directly to the serum glycoprotein level, Lustig and Nassau (7) found that venous blood contained more glycoprotein than arterial blood. Catchpole (8) showed that connective tissue adjacent to transplanted tumors in mice contained more material with properties similar to seromucoid than did connective tissue remote from the tumor. He proposed that serum glycoproteins may arise as a result of the depolymerization of the ground substance of the connective tissue with the release of solubilized components into the circulation. In agreement with Catchpole's hypothesis, Engel (9) found that with the dissolution of bone following the administration of parathyroid hormone to rats, there was a pronounced increase in the seromucoid levels. It was presumed that the seromucoid was released as a result of the depolymerization of the ground substance of bone. Shetlar and his collaborators (10, 11), observed that elevations of serum glycoproteins are frequently associated with conditions in which tissue destruction is not pronounced and cell proliferations or protein synthesis is rapid. They suggested that an elevated serum glycoprotein level may reflect processes associated with tissue proliferation rather than with tissue destruction. Increased glycoprotein synthesis may be caused by either destruction or proliferation. The hypotheses mentioned above do not suggest where or how glycoproteins are synthesized in physiological and pathological states.

We are indebted to Whipple and his co-workers for their studies

of the formation of plasma proteins. Their work indicates that most of the plasma proteins with the possible exception of γ-globulin were synthesized by the liver (12, 13, 14). Miller and Bale (15) presented direct evidence for the dominant role of the liver in the biosynthesis of plasma proteins in a study of the isolated perfused rat liver.

Since the liver plays a dominant role in the plasma protein biosynthesis, it is natural for investigators to suggest that the liver is the organ responsible for glycoprotein synthesis. In 1949, Werner (16) concluded that the liver was the site of serum glycoprotein formation on the basis of experiments which showed that the concentration of serum protein-bound glucosamine increased in rabbits after bleeding. This phenomenon did not occur if the liver had been damaged with phosphorus or benzene. Greenspan et al. (17, 18, 19, 20, 21) working on this problem observed that the serum glycoprotein level was lowered in patients with infectious hepatitis, homologous serum hepatitis or portal cirrhosis. On the bases of these observations, they suggested that the liver is involved in the synthesis of serum glycoprotein.

Studies of serum glycoprotein biosynthesis utilizing radioactive glucose as a tracer were made by Becker and Day (22) and Spiro (23) in rats and by Boström, Rodén and Yamashina (24) in guinea pigs. In 1953, Becker and Day (22) first found that C-14 was present in the glucosamine isolated from the rats fed glucose-1-C-14. In 1959, Boström et al. (24) isolated radioactive α_1-acid-glycoprotein from the plasma of guinea pigs at different times after intraperitoneal injection of glucose-U-C-14. They found that the incorporation of radioactivity from glucose-U-C-14 into α_1-acid-glycoprotein reached a maximum approximately 24 hours after

injection. The greatest incorporation of radioactivity was observed in the neutral sugar component of the carbohydrate moiety. They also noted that sialic acid and glucosamine were labelled. The results of Boström et al. (24) only indicated that carbohydrates in plasma glycoproteins were synthesized from glucose. Their experimental results did not indicate how and where the plasma glycoproteins were synthesized. In the study of the incorporation of radioactivity from glucose-U-C-14 into the protein-bound glucosamine of various tissues of the intact rat, Spiro (23) noted that liver and serum showed the most rapid synthesis of glucosamine from glucose. By measuring the specific radioactivity and total radioactivity of liver and serum protein-bound glucosamine in rats at varying time intervals after the injection of a tracer dose of glucose-U-C-14, Spiro was able to obtain evidence that serum glycoproteins are synthesized in the liver.

In 1961 Shetlar and his co-workers (25, 26) first utilized glucosamine-1-C-14 as a tracer to demonstrate that the radioactivity was rapidly taken up by rat liver and released to the serum as a protein-bound glucosamine without appreciable destruction. By measuring the changes of radioactivity in serum proteins and in the liver protein after intraperitoneal injection of glucosamine-1-C-14 into rats, Shetlar (25) found that the activity curve of bound glucosamine reached a peak more quickly in the liver than it did in the serum and suggested that a sizable fraction of the serum glycoproteins are synthesized by the liver. The result of this work was compatible with the work of Spiro (23) who used glucose-U-C-14 as a tracer. The work of Shetlar's group was confirmed in 1962 by Kohn, Winzler and Hoffmann (27) who reported the incorporation of D-glucosamine-1-C-14 into labeled trichloroacetic acid-

precipitable glucosamine and sialic acid of the rat liver. In the same year, Shetlar and his co-workers also reported the incorporation of radioactivity into various tissues after parenteral administration of glucosamine-1-C-14 to the rats (28). Also in the same year, Sarcione (29) used a perfusion technique to demonstrate that the isolated rat liver incorporated glucose-U-C-14 into plasma glycoprotein. It was at this stage (January, 1963), the author joined Dr. Shetlar's group to undertake the studies on the mechanism of the biosynthesis of serum glycoprotein. Since then, the following studies related to the glycoprotein biosynthesis have appeared in the literature.

In 1963, Sarcione (30) reported the synthesis of α_1-acid glycoprotein by the isolated rat liver perfused with D-glucose-U-C-14 and L-leucine-U-C-14. Both glucose and leucine were incorporated into plasma α_1-acid glycoprotein by perfused liver in vitro.

In 1964, Shetlar, Capps and Hern (31) reported the incorporation of radioactive glucosamine into the serum proteins of intact rats and rabbits. They reported that when D-glucosamine-1-C-14 was administered to rats and rabbits by intraperitoneal injection, maximum radioactivity of the trichloroacetic acid soluble fraction was found in rat liver within 30 minutes and in rabbit liver within 1 hour after the injection. Maximum protein-bound radioactivity in the serum was found at 3 hours for the rat and at 7 hours for the rabbit. The efficiency of the incorporation into serum proteins at the maximum level of radioactivity was 24% and 28% of the administered dose for rats and rabbits, respectively. About 22% of the bound radioactivity in serum was associated with sialic acid in the rat and most of the remaining 78% activity was associated with glucosamine. Negligible amounts of radioactivity were found in the hexoses.

Macbeth, Bekesi, Sugden and Bice (32) also published a similar study in 1965. They reported that the administered glucosamine-1-C-14 was present as bound hexosamine and N-acetylneuraminic acid in the serum glycoprotein and in various tissues of the intact rat. Glucosamine-1-C-14 was shown to be a precursor in the biosynthetic pathway of both protein-bound hexosamine and N-acetylneuraminic acid. From the experiment using hepatectomized rats, the conclusion was made that the liver was the major, if not the sole, site of biosynthesis of serum glycoproteins in the normal rat.

In 1964, Robinson, Molnar and Winzler (33) reported that glucosamine-1-C-14 administered intravenously to rats was rapidly converted to protein-bound components of liver microsomes and mitochondria and then released to the plasma without appreciable accumulation in the cytoplasmic protein of the liver. Molnar, Robinson and Winzler (34) showed that the incorporation of glucosamine-1-C-14 into rat liver and plasma protein was markedly inhibited by puromycin. By adding glucosamine-1-C-14 and galactose-1-C-14 into the perfusing blood to isolated rat liver in vitro, Sarcione (35, 36) found that the deoxycholate-soluble (membranous) protein fraction of microsomes was a major site of sugar incorporation. He reported virtually no incorporation of hexose into ribosomal proteins. In contrast to Sarcione's results Molnar, Robinson and Winzler (37) found a definite labeling of ribosomes, which reaches a maximum in specific radioactivity somewhat earlier than the microsomal membrane. Athineos, Kukral and Winzler (38) administered glucosamine-1-C-14 into normal and hepatectomized dogs, and found that α_1-acid glycoprotein became highly labeled in normal dogs with almost all of the radioactivity residing in the glucosamine component, while the glyco-

protein isolated from hepatectomized dogs was not radioactive and concluded that the liver is the site of biosynthesis of α_1-acid glycoprotein.

In 1965, Helgeland (39) re-examined the incorporation of glucosamine-1-C-14 into subcellular fractions of rat liver and reported that 69% of the total radioactivity was associated with the ultrasonic extract of the microsomal pellet, 28% in the subsequent deoxycholate extract and 3% associated with the ribosomes. Sinohara and Sky-Peck (40) failed to demonstrate *in vitro* incorporation of glucosamine-1-C-14 into the soluble ribonucleic acid of mouse liver. They found that during the course of glucosamine-1-C-14 incorporation into the microsomal fraction *in vivo* by way of uridine diphosphate-N-acetylglucosamine, the soluble ribonucleic acids isolated from the high-speed supernatant fraction of liver were not labeled. By using Ehrlich ascites carcinoma cells, Cook, Laico and Eylar (41) examined the incorporation of labeled glucosamine and amino acid into the smooth and rough endoplasmic reticulum. They found that the labeled hexosamine of microsomes was only associated with the membrane, and not with the ribosomes. Molnar, Lutes and Winzler (42) and Molnar, Teegarden and Winzler (43) reported a rapid incorporation of glucosamine-1-C-14 into the trichloroacetic acid-insoluble fraction of Ehrlich ascites carcinoma cells. Among the subcellular fractions, the microsome was the one most highly labeled.

Fractionation of Subcellular Particles and Their Carbohydrate Content

Biochemical studies of the subcellular components have been achieved by isolating the particulate fractions from disrupted cells by differential centrifugation. This technique is mainly based on the assumption that the particulate components of tissue homogenates have

different sedimentation coefficients and densities. Obviously, the sedimentation coefficients and densities are greatly affected by the size and shape of the particle as well as the viscosity of the suspension. Much work has been done on establishing the scheme of the cell fractionation. The pioneer works were the study of Bensley and Hoerr (44) in 1934. They disrupted the cell membrane mechanically in a suitable medium and used the resulting homogenate for the isolation of mitochondria by means of differential centrifugation. A few years later, Claude (45, 46, 47) subjected normal and tumor tissue homogenates in dilute electrolyte solution to differential centrifugation and obtained a fraction, after sedimentation of the ordinary mitochondrial fraction, which was rich in ribonucleic acid and phospholipids. He introduced a descriptive name, microsome, i.e. "small bodies" to the newly found particle. The use of salt media did not yield morphologically intact mitochondria and often led to agglutination of the particles. An important step forward was made when Hogeboom, Schneider and Palade (48) introduced sucrose as a medium in the fractionation procedure. Hypertonic sucrose (0.88 M) gave the morphologically most satisfactory results. However, it was soon found that this high concentration of sucrose inhibits certain metabolic processes, and isotonic sucrose (0.25 M) was therefore substituted by Schneider (49). Beginning with Hogeboom's group (50, 51), the sucrose medium became the one most often used in tissue fractionation.

The identification of microsomes as fragments of the endoplasmic reticulum was achieved by Palade and Siekevitz (52, 53). They performed systematic electron-microscope study of intact rat liver, liver homogenates, and isolated microsomes, as well as submicrosomal fractions prepared with deoxycholate. The morphological observations were correlated with

chemical analysis of the various fractions and it was found that the rat liver microsomes consist of "rough-surfaced" vesicles with small, dense particles attached to their outer surface and "smooth-surfaced" vesicles, devoid of such particles. Deoxycholate in a final concentration of 0.5% solubilized the membrane and the contents of the vesicles, but not the particles. This experiment also provided clear evidence that the microsomal vesicles are derived from the endoplasmic reticulum. The majority of components, the rough vesicles or rough tubules, are the result of fragmentation of the rough-surfaced endoplasmic reticulum, and the smooth vesicles originate, at least partly, from the region of the endoplasmic reticulum which lacks attached ribosomes.

By using the sucrose medium and the differential centrifugation described by Hogeboom, Schneider and Palade (48), De Duve found that acid phosphatase was detected in the microsomal fraction, but by using the modified technique of Novikoff, Podber, Ryan and Noe (54) or Tsuboi (55), the same enzyme was identified in the mitochondrial fraction. This inconsistent localization of an enzyme led De Duve, Pressman, Gianetto, Wattiaux and Appelmans (56) to establish a modified scheme by which they were able to fractionate, besides mitochondria and microsomes, one group of new particles having the density between mitochondria and microsomes. These newly separated particles were named "lysosomes" by De Duve's group. Today the scheme described by De Duve et al. (56) is the one most widely used.

Although many papers have been published on the distribution of enzymic activities in various subcellular particles, very little work has been reported on the distribution of carbohydrate in these particles. In 1961, Wallach and Eyler (57) determined the distribution of sialic

acid in subcellular fractions of Ehrlich ascites carcinoma cells and reported the following distribution: 69.5% in microsomes, 17.3% in mitochondria, 13.6% in supernatant and 3.2% in the nuclear fraction. In 1962, Patterson and Touster (58) determined the distribution of sialic acid in subcellular fractions of rat liver and found that sialic acid was distributed throughout the cell, with the exception of the nuclear fraction. The highest concentration was in the microsomal and supernatant fraction. They also found that the sialic acid in the supernatant fraction is in bound form as indicated by a negative thiobarbituric acid test prior to hydrolysis. In 1964, Yamashina, Isumi and Naka (59) studied the intracellular distribution of hexosamine and sialic acid in rabbit liver. They found that hexosamine and sialic acid in rabbit liver were localized mainly in the microsomal and the mitochondrial fractions. No work can be found in the literature related to the analysis of neutral sugars in subcellular fractions.

CHAPTER III

MATERIALS AND METHODS

Preparation of Subcellular Fractions

Male albino rats of the Holtzman strain (200-300 gm) were used. The animals were fed with Rockland laboratory diet ad libitum. Walker 256 carcinosarcoma was carried in the subcutaneous tissue of the rats 7 to 8 days. All animals were fasted for 24 hrs before sacrifice. The animals were exsanguinated from the abdominal aorta, and the livers were perfused in situ with physiologic saline via the portal vein. Both liver and tumor tissues were fractionated by the procedures of De Duve et al. (56) with slight modifications. The tissues were homogenized in a Potter-Elvehjem type of homogenizer in 5 volumes of 0.25 M sucrose-0.001 M EDTA (ethylenediaminetetraacetate). The homogenate was centrifuged at 1000 X g for 10 minutes in a Servall refrigerated centrifuge. The sedimented fraction was saved for the preparation of nuclei, and the supernatant was centrifuged at 3300 X g for 10 minutes to obtain mitochondria. The resulting sediment was washed with 3 volumes of the sucrose-EDTA solution to give the final mitochondrial fraction. A small lysosomal-rich fraction was sedimented at 12,000 X g for 10 minutes and washed once with 3 volumes of sucrose-EDTA solution. The microsomal fraction was sedimented at 105,000 X g for 60 minutes in a Spinco Model L ultracentrifuge. The nuclei were prepared from 1000 X g sediment by

the method of Hogeboom, Schneider and Striebich (60). Sediment obtained from 1000 X g centrifugation was homogenized with 5 volumes of 2.2 M sucrose and centrifuged at 40,000 X g for 30 minutes to obtain nuclei. The cell fragments accompanying the unbroken cells floated on top of the fluid. These floating materials were defined as debris. The purity of mitochondrial and microsomal fractions was checked by determination of cytochrome oxidase and glucose-6-phosphatase activities, as described by De Duve et al. (56). α-Mannosidase was used as an enzyme marker for the lysosomal fraction (61). The purity of the nuclear fraction was checked by microscopic examination. Those fractions with minimum cross contamination of subcellular fractions were used for the subsequent studies.

For the determination of protein-bound carbohydrates in subcellular fractions, the protein from all the fractions was precipitated with 10% trichloroacetic acid; lipids were removed from all of the fractions by washing successively with 95% ethanol, 100% ethanol, chloroform-methanol (2:1), benzene, and finally with ethyl ether, as described by Busch et al. (62). The lipid-free protein powders were used for subsequent chemical analysis.

Microsomal membrane was prepared from the microsomal fraction by extracting with sodium deoxycholate as described by Littlefield (63). The microsomal fraction from 10 gms of wet liver was first suspended in 3 ml of freshly prepared 5% sodium deoxycholate in 0.03 M tris buffer, pH 7.4, with the use of glass homogenizer, then 0.03 M tris buffer, pH 7.4, containing 5 mM $MgCl_2$ was quickly added to reach the final volume of 30 ml. The resulting suspension was immediately centrifuged at 78,000 X g in a Spinco Model L ultracentrifuge (No. 30 rotor) for two hours. After centrifugation, the supernatant was mixed with an equal volume of

cold acetone added with constant stirring. The protein solubilized by sodium deoxycholate was completely precipitated by 50% acetone. The precipitate was then washed with 95% ethanol, 100% ethanol, chloroform-methanol (2:1), benzene and ether as described by Busch et al. (62).

Hepatectomy of Rats

The animal was first anesthetized with Nembutal (50 mg/kg body wt.) and a long midline incision was made on the ventral surface, starting from the xiphoid cartilage and ending just above the pubis. With a light pull on the stomach, the esophagus was visualized and ligated to prevent air going into the stomach when artificial respiration was used. After elevation of the intestine, the superior mesenteric vessels were seen and ligated. Then the portal vein was quickly ligated. Ligation of the inferior vena cava was done twice, the first ligation was made below the liver and above the renal veins. This could be done easily if the peritoneum on both sides was previously snipped by a pair of fine scissors. The other ligation of the inferior vena cava was done between the liver and the diaphragm. This was a difficult step to do without puncturing the very thin diaphragm. The safest way proved to be to clean the fascia along the liver and inferior vena cava first, then to place a thread around the inferior vena cava with the help of an aneurism ligature needle and thus ligate the inferior vena cava. After the ligation of inferior vena cava, the liver can be removed easily. Closure of the abdomen was done by two lines of continuous sutures after irrigating the peritoneum with two or three milliliters of normal saline. Two or three milliliters of 5% glucose solution was injected intraperitoneally every hour.

Administration of Radioactive Compound to the Rats

The radioactive compound was dissolved in saline to make a concentration of 10 μc per 0.5 ml. The animal was anesthetized with Nembutal. The saphenous vein was exposed by a short skin incision about half way down the medial surface of the thigh. One half milliliter of isotope material was injected into the saphenous vein by a tuberculin syringe fitted with a 27 gauge needle. After the injection, the skin was closed with a few sutures. In the case of hepatectomized rats, the isotope was injected into the jugular vein.

Measurement of Radioactivity

Each radioactive sample was prepared by suspending 5 to 10 mg of lipid free subcellular fraction in 2 ml of distilled water by means of a Potter-Elvehjem type homogenizer. One milliliter aliquots were plated on stainless steel planchets and counted in the gas-flow counting system (Nuclear Chicago). Proper corrections were made for self-absorption by means of a curve previously prepared with a known amount of radioactive glucosamine added to various amounts of a suspension of lyophilized rat liver. In the case of serum samples 0.05 to 0.5 ml aliquots were pipetted directly to the planchet and counted in the gas-flow counting system after being air dried.

Chemicals

Radioactive Compounds

D-Glucosamine-1-C-14 (3.1 μc/μmole) and L-leucine-1-C-14 (14.5 μc/μmole) were purchased from New England Nuclear Corp., Boston, Massachusetts. Both radioactive compounds were chromatographically pure as shown on the radiochromatograms which were sent along with the radio-

active compounds from the company.

Proteolytic Enzyme and Anti Serum

Pronase-P, Streptomyces griseus protease (64) was purchased from the California Corporation for Biochemical Research. Rabbit anti-rat serum was purchased from Mann Research Laboratories, Inc.

Analytical Methods

For the determination of sialic acid in subcellular fractions the protein powder was suspended in 0.1 N H_2SO_4 and dispersed by sonication with a Biosonic Ultrasonic Probe (Bronwill-Blackstone). The suspension was then subjected for one hour to hydrolysis at 80°C in an oven equipped with an automatic shaker; the liberated sialic acid was determined by the thiobarbituric acid method of Warren (65). It was found that hydrolysis of subcellular fractions with 0.1 N H_2SO_4 at 80°C for 1 hour resulted in the release of material that produced a chromophore with an absorption maximum at 532 mμ by Warren's periodate-thiobarbituric acid reagents. This was especially prominent in the case of the nuclear fraction. This chromophore could not be removed effectively by chromatography on Dowex 1 or by extraction with isoamyl alcohol. The absorption at 549 mμ due to sialic acid was consequently corrected for absorbency at 532 mμ as proposed by Warren (65).

Total hexosamine was determined according to the method of Boas (66). Qualitative identification of neutral sugars and amino sugars was made by paper chromatography with Whatman No. 1 filter paper and the solvent system containing n-butanol-pryidine-0.1 N HCl (5:3:2), and pyridine-ethylacetate-water (1:24:0.8). Carbohydrates were detected by dipping the chromatogram into 2-aminobiphenyl reagent (67). For the quanti-

tative determination of individual monosaccharides, the developed spots were cut from the paper and eluted with methanol for 15 minutes at room temperature. The optical density of the colored solutions was measured at 400 mμ. Standard sugar samples were always included with each chromatographic separation. Subcellular fractions were prepared for the chromatographic identification of neutral sugars by an initial hydrolysis of 200 mg of the sample with 5 ml of 1 N HCl in a tightly closed screw-capped tube in a water bath at 100°C for 6 hrs. The hydrolysate was evaporated to dryness in vacuo to remove all HCl. The residue was taken up with a minimal amount of water and passed through a Dowex 50 (hydrogen form) column (1 X 10 cm). The neutral sugars were eluted with water until the solution gave a negative anthrone test. The eluate was evaporated to dryness and analyzed for galactose and mannose according to the procedure described previously. Fucose was determined by the cysteine-H_2SO_4 method of Dische and Shettles (68). Quantitative analysis of glucosamine and galactosamine was performed by the Spinco amino acid analyzer with the short column technique. The glucosamine and galactosamine peaks appeared in about 40 minutes before the lysine peak. The glucosamine peak preceded the galactosamine peak by 10 minutes. For the determination of uronic acid, 100 mg aliquots of different fractions were placed in a 20 ml tightly screw-capped tube with 4 gm of Dowex 50-X8 (hydrogen form) and 10 ml 0.1 N H_2SO_4 and hydrolyzed in a shaking oven at 78°C for 72 hrs by the method of Shetlar and Shetlar (69). The hydrolysate was filtered through Whatman No. 1 filter paper. The clear supernatant was analyzed for uronic acid by Dische's carbazole reagent (70). The main advantage of using resin hydrolysis is to remove the interfering substances such as amino acids produced during the hydrolysis

by resin. This method works satisfactorily for the determination of the uronic acid content in cartilage and aorta tissues. Protein-bound neutral sugar was determined by the tryptophan-H_2SO_4 method of Shetlar et al. (71).

Amino Acid Analysis

The protein samples were hydrolyzed with 6.0 N HCl in sealed, thickwall tubes at 110°C for 22 hours. Quantitative determinations of individual amino acid were carried out by a Technicon amino acid analyzer according to the procedure described by Hamilton (72).

Determination of Total Protein

Total protein was determined by using Folin's phenol reagent as described by Lowry et al. (73). Lab-Trol of DADE Reagents Inc., Miami, Florida was used as a standard.

Determination of Deoxycholic Acid

Sodium deoxycholic acid was determined by the method of Kier (74). The ultraviolet absorption was measured with a Beckman DB Spectrophotometer.

Determination of α-Mannosidase Activity

α-Mannosidase activity was measured by running the assays in the presence of 0.1% (v/v) Triton X-100 (Rohm and Haas Co., Philadelphia). The reaction mixture contained 1.5 μmoles of p-nitrophenyl-α-D-mannoside, Triton X-100, and enzyme in 1.5 ml of acetate buffer, pH 5.0, $\Gamma/2=0.05$. This mixture was incubated at 37°C. At the end of the incubation, 1.5 ml of 0.2 M Na_2CO_3 were added to the incubation mixture, and the liberated nitrophenolate ion was determined with a Beckman B Spectro-

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photometer at 420 mμ (75). One unit of enzyme is defined as the amount of enzyme that will liberate 0.1 μmole of nitrophenol per 30 minutes under the conditions stipulated.

CHAPTER IV

EXPERIMENTAL RESULTS

Incorporation of D-Glucosamine-1-C-14 into the Serum of Normal and Hepatectomized Rats

As stated in Chapter II, when the specific activities of the bound glucosamine in liver and in serum were plotted as the function of time after administration of glucose-U-C-14 or glucosamine-1-C-14 into the rats, Spiro (28) and Shetlar et al. (25) found that liver and serum glucosamine meet the criteria for the precursor-product relationship as formulated by Zilversmit, Entenman and Fishler (76). Both Spiro and Shetlar's results suggested that the liver is the major site of serum glycoprotein biosynthesis. In order to obtain the further evidence that liver is the actual site for the biosynthesis of serum glycoprotein, observations were made of the incorporation of D-glucosamine-1-C-14 into serum protein of rats before and after complete hepatectomy. Figure 1 shows the specific activity of serum protein at various times after intravenous injection of D-glucosamine-1-C-14 into the normal and hepatectomized rats. All serum samples were dialyzed against running tap water for 48 hours to remove any free radioactive glucosamine. In the normal rats, very rapid and efficient incorporation of radioactivity into serum proteins was noticed. This radioactivity continued to increase up to 3 hours after injection. However, no appreciable incorporation of radioactivity into serum protein could be detected in the hepatectomized rats

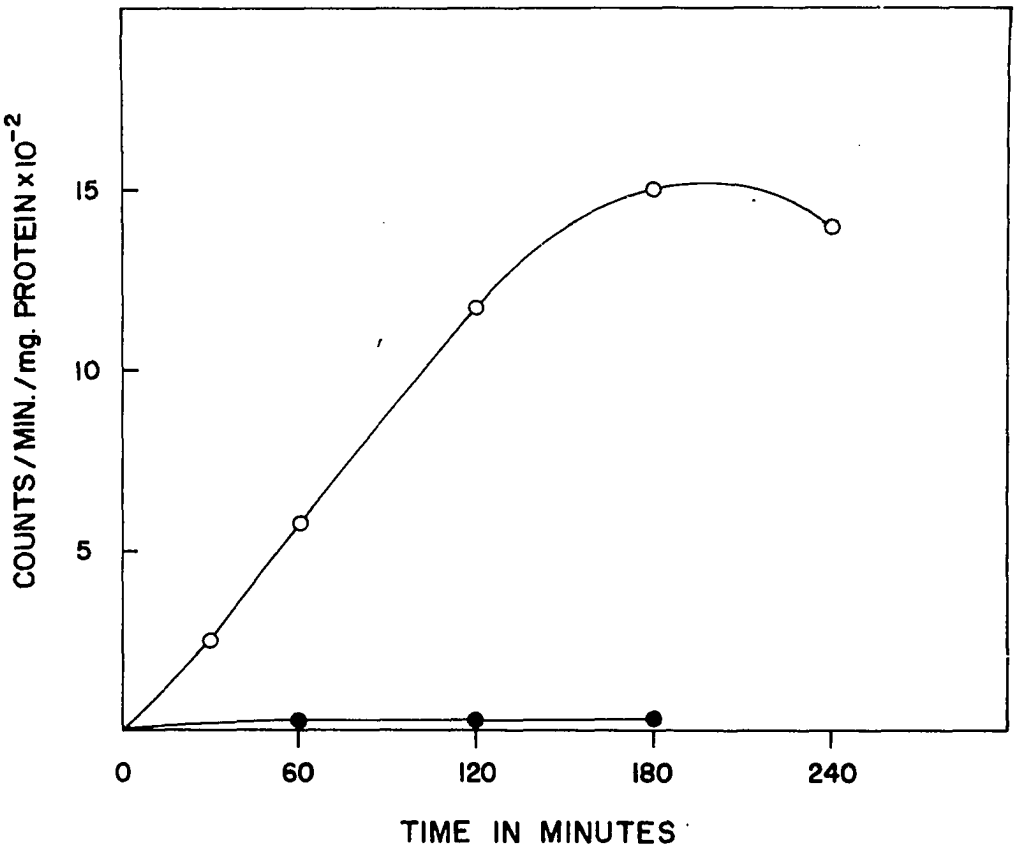

Figure 1.—Incorporation of D-glucosamine-1-C-14 into the serum of normal (O) and hepatectomized (●) rats. D-glucosamine-1-C-14 (10 μc) was administered intraveneously into each rat. Each point represents the mean value of three rats.

within three hours after injection of glucosamine-1-C-14. The results of this experiment clearly indicate that the incorporation of radioactive glucosamine into serum glycoprotein is largely dependent on the presence of the liver.

Fractionation of Rat Liver and Walker 256 Carcinosarcoma Tissue

Rat liver and Walker 256 carcinosarcoma tissue were fractionated according to the method described in Chapter III. The distribution of protein and enzyme activities in subcellular fractions are summarized in Table 1. The purity of mitochondrial and microsomal fraction was checked by a determination of cytochrome oxidase and glucose-6-phosphatase activities. α-Mannosidase was used as an enzyme marker for lysosomal fraction. The purity of the nuclear fraction was checked by microscopic examination. The protein from all the fractions was precipitated with 10% trichloroacetic acid; lipids were removed from all of the fractions by washing successively with a series of lipid solvents as described by Busch *et al*. (62).

Mechanism of the Biosynthesis of Serum Glycoprotein in Rat Liver

Incorporation of D-Glucosamine-1-C-14 into the Subcellular Fractions of Rat Liver

As described in the previous section, the liverless rats failed to incorporate detectable amounts of D-glucosamine-1-C-14 into serum glycoprotein. It can be concluded that liver is the major site responsible for incorporation of radioactive glucosamine into serum glycoprotein. In order to investigate the site of subcellular incorporation, the radioactivity associated with subcellular fractions after intravenous

TABLE 1

DISTRIBUTION OF PROTEIN AND ENZYME ACTIVITIES IN SUBCELLULAR FRACTIONS OF RAT LIVER AND WALKER 256 CARCINOSARCOMA TISSUE[a]

Fractions	Protein mgm/10 gm wet tissue				Relative Enzyme Activities					
					Cytochrome Oxidase		Glucose-6-Phosphatase		α-Mannosidase	
	Liver	%	Walker 256 Tumor	%	Liver	Walker 256 Tumor	Liver	Walker 256 Tumor	Liver	256 Tumor
Whole Homogenate	1462±110	100	937±86	100	100	100	100	100	100	100
Mitochondria	138±27	9.4	71±13	7.6	47.5±14.2	41.2±16.4	3.2±1.2	9.7±5.5	10.3±4.1	8.6±4.5
Microsome	292±18	19.9	127±16	13.6	4.1±1.5	5.2±2.2	48.4±11.4	44.8±9.5	8.2±3.7	6.4±3.1
Lysosome	55±9	3.8	34±6	3.6	16.4±9.4	18.2±8.7	9.0±6.5	11.2±4.4	60.1±12	45.3±9.7
Nucleus[b]	95±14	6.5	81±12	8.6	—	—	—	—	—	—
Soluble	415±60	28.4	260±58	27.7	0	0	0	0	0	0
Debris[b]	408±70	27.9	375±66	40.0	—	—	—	—	—	—

a) Number of experiments = 15. Values following ± signs are standard error of mean = $\sqrt{\frac{(X_1-\bar{x})^2}{n(n-1)}}$

b) Since 2.2M sucrose was used for the separation of nuclei and debris, it was found that enzyme activities were heavily inhibited under this condition.

injection of D-glucosamine-1-C-14 was examined. The specific activity (counts per minute per mg protein) of various subcellular fractions as well as serum protein at time intervals from 5 minutes to four hours are summarized in Figure 2. A rapid incorporation of radioactivity into lysosomal and microsomal fractions was noticed. The radioactivities in these two fractions continued to increase up to 2 hours after injection and gradually decreased thereafter. The specific radioactivity of the dialyzed serum increased somewhat slower and reached a maximum at 3 hours. Considerable amounts of radioactivity were also found associated with the mitochondrial fraction. Very little activity was associated with nuclear and soluble fractions. The specific activity of the lysosomal fraction, in terms of count per minute per mg protein, was the highest among those fractions observed. However, this fraction represents only 3.8% of the dried weight of liver (See Table 1). On the other hand, the specific activity of the microsomal fraction was very close to that of the lysosomal fraction, but this fraction constitutes about 20% of the liver (See Talbe 1). On the basis of the total radioactivity, the microsomal fraction has much more radioactivity than any other fraction. Figure 3 shows the specific radioactivity per μmole of hexosamine in microsome, lysosome and serum. The microsomal fraction has the highest specific radioactivity. From both the specific radioactivity and the total radioactivity, it appears that the microsomal fraction is most active in the incorporation of D-glucosamine-1-C-14 into serum glycoprotein.

Incorporation of L-Leucine-1-C-14 into Subcellular Fractions of Rat Liver

In order to compare the mechanism of the incorporation of D-glucosamine-1-C-14 into subcellular fractions of rat liver with that of

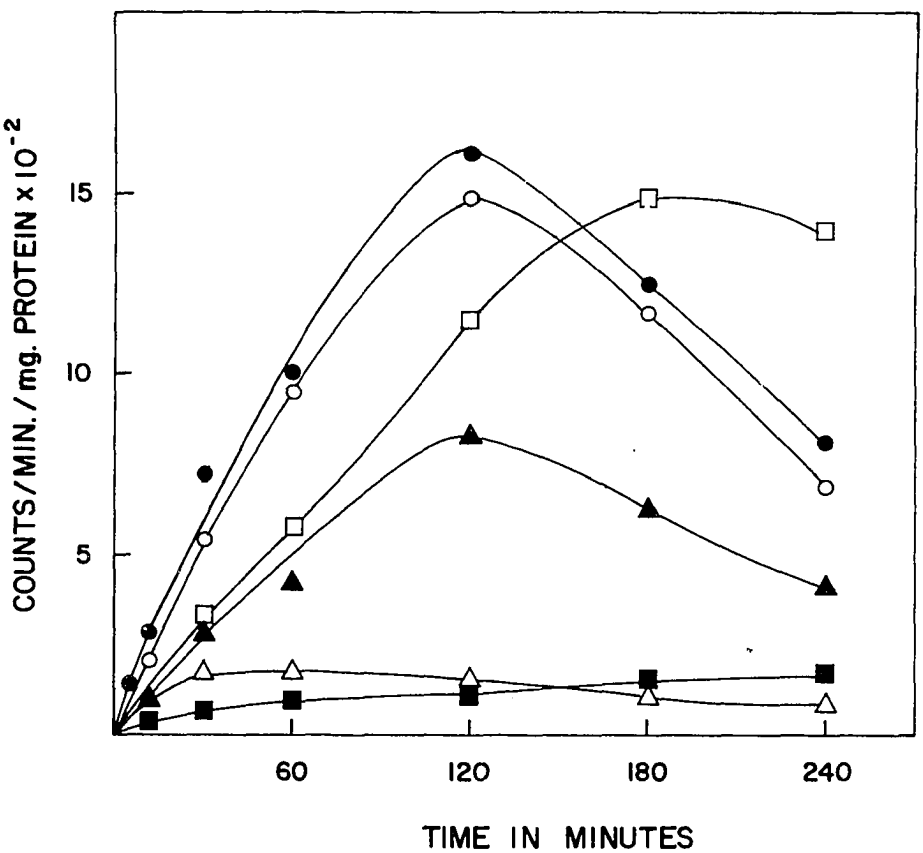

Figure 2.—Incorporation of D-glucosamine-1-C-14 into the subcellular fractions of rat liver after injection with 10 μc of D-glucosamine-1-C-14. Each point represents the mean value of three rats.
—□—□—, dialyzed serum; —■—■—, nucleus;
—○—○—, microsome; —●—●—, lysosome;
—Δ—Δ—, supernatant; —▲—▲—, mitochondria.

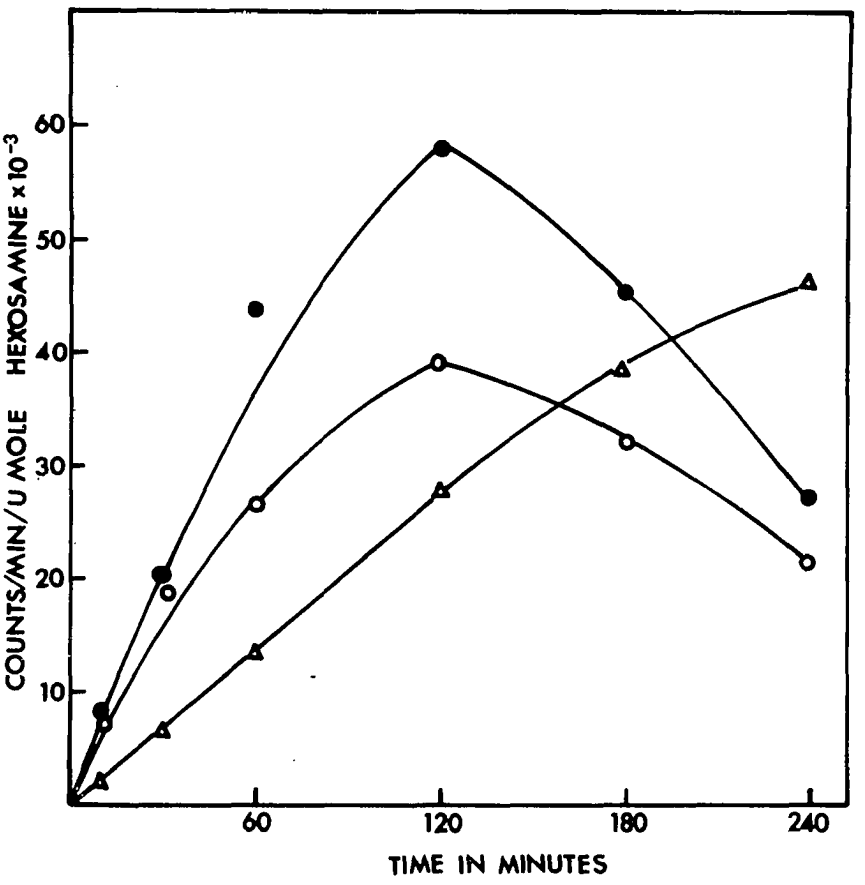

Figure 3.—Incorporation of D-glucosamine-1-C-14 into the subcellular fractions of rat liver after injection with 10 μc of D-glucosamine-1-C-14. The specific radioactivity expressed as counts per minute per μmole hexosamine in microsome, lysosome and dialyzed serum. Each point represents the mean value of three rats.
—●—●—, microsome; —○—○—, lysosome;
—Δ—Δ—, dialyzed serum.

an amino acid; L-leucine-1-C-14 was administered to the rats as previously done with D-glucosamine-1-C-14. The radioactivity associated with subcellular fractions was followed at various time intervals after the injection. Figure 4 shows the general pattern of L-leucine-1-C-14 incorporation. Among the fractions examined, the microsomal fraction had the highest radioactivity. Considerable amounts of radioactivity were also associated with lysosomal and mitochondrial fractions. Very little radioactivity was associated with nuclear and soluble fractions. The specific radioactivity of the microsomal fraction reached a maximum at 10 minutes after the injection. The over all pattern was compatible with the well established fact that the microsomal fraction is the site of cytoplasmic protein synthesis (77, 78). When Figures 2 and 4 are compared, the pattern of incorporation of D-glucosamine-1-C-14 is obviously different from that of L-leucine-1-C-14. The rate, in terms of time, of incorporation of L-leucine-1-C-14 into the microsomal fraction is much faster than the incorporation of D-glucosamine-1-C-14. The incorporation of L-leucine-1-C-14 reached a plateau at 10 to 30 minutes, while the incorporation of D-glucosamine-1-C-14 took 1 to 2 hours to reach the maximum activity.

Incorporation of D-Glucosamine-1-C-14 and L-Leucine-1-C-14 into the Sodium Deoxycholate Soluble and Insoluble Fractions of Rat Liver Microsome

As indicated previously D-glucosamine-1-C-14 was incorporated into the microsomal fraction of rat liver, but the average rate of incorporation of D-glucosamine-1-C-14 was much slower than that of L-leucine-1-C-14. An attempt was therefore made to study the incorporation of D-glucosamine-1-C-14 and L-leucine-1-C-14 into the sodium deoxycholate soluble and insoluble fractions of the microsomes. For direct comparison,

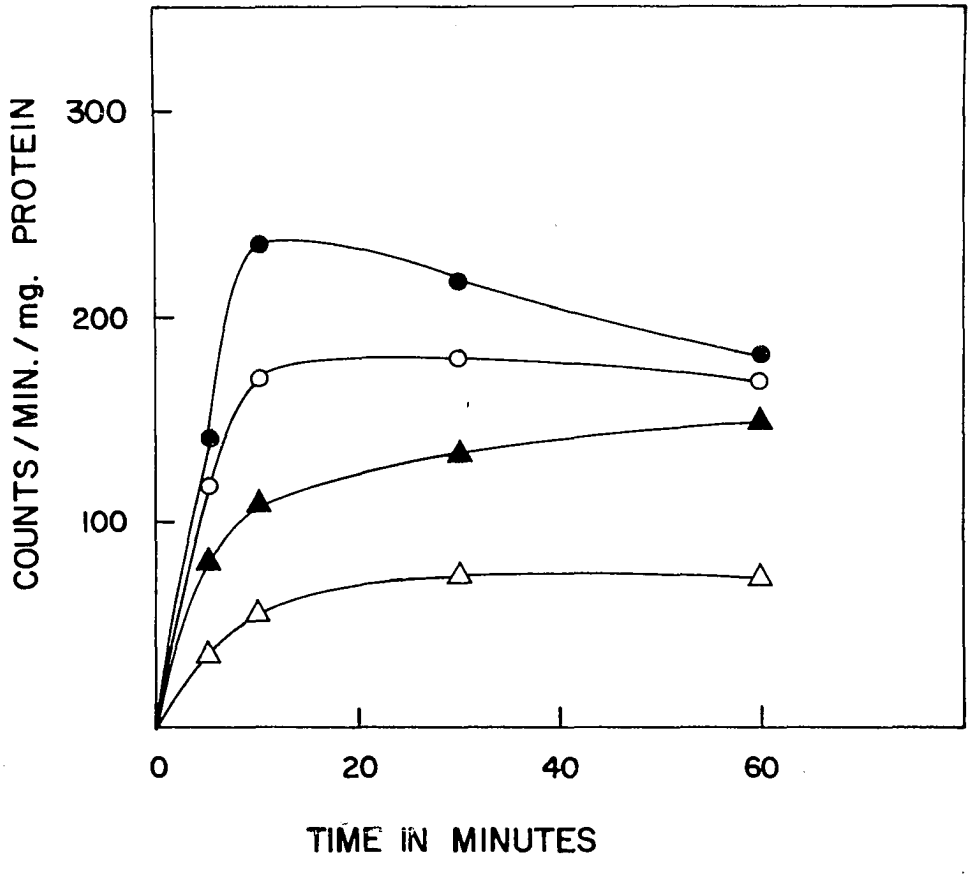

Figure 4.—Incorporation of L-leucine-1-C-14 into the subcellular fractions of rat liver after injection with 2.43 μc of L-leucine-1-C-14. Each point represents the mean value of three rats.
—○—○—, lysosome; —●—●—, microsome;
—Δ—Δ—, supernatant; —▲—▲—, mitochondria.

the individual rat received 10 μc of either D-glucosamine-1-C-14 or L-leucine-1-C-14 intravenously and the rats were sacrificed at various time intervals. After separation of the microsomal fraction by differential centrifugation, microsomes were further treated with freshly prepared 0.5% sodium deoxycholate to obtain deoxycholate soluble (membrane and its contents), and insoluble (ribosomes) fractions. The radioactivity in these fractions was determined and is presented in Figures 5 and 6. Figure 5 shows the incorporation pattern in which L-leucine-1-C-14 was administered to the rats. Very quick labelling and disappearance of the radioactivity in the deoxycholate insoluble fraction was noticed. In the deoxycholate soluble fraction, radioactivity increased progressively, reached a plateau at 10 minutes after injection, and then increased slowly up to 30 minutes. The results were very similar to those reported by Littlefield, Keller and Zamecnik (63) who employed radioactive leucine and valine to demonstrate that the cytoplasmic ribonucleoprotein particles were the site of initial incorporation of amino acids into proteins.

As presented in Figure 6, the incorporation pattern of D-glucosamine-1-C-14 into deoxycholate soluble and insoluble fractions of rat liver microsome was very different from that of L-leucine-1-C-14. In contrast to L-leucine-1-C-14 incorporation, very little D-glucosamine-1-C-14 was incorporated into the deoxycholate insoluble fraction during the experimental period (5 minutes to 3 hours). In the deoxycholate soluble fraction, the incorporation of D-glucosamine-1-C-14 reached a peak approximately 2 hours after the administration of radioactive tracer. In comparison, the rate of incorporation of D-glucosamine-1-C-14 into the deoxycholate soluble fraction was slower than that of L-leucine-1-C-14. Table 2 summarizes the distribution of dried weight and radio-

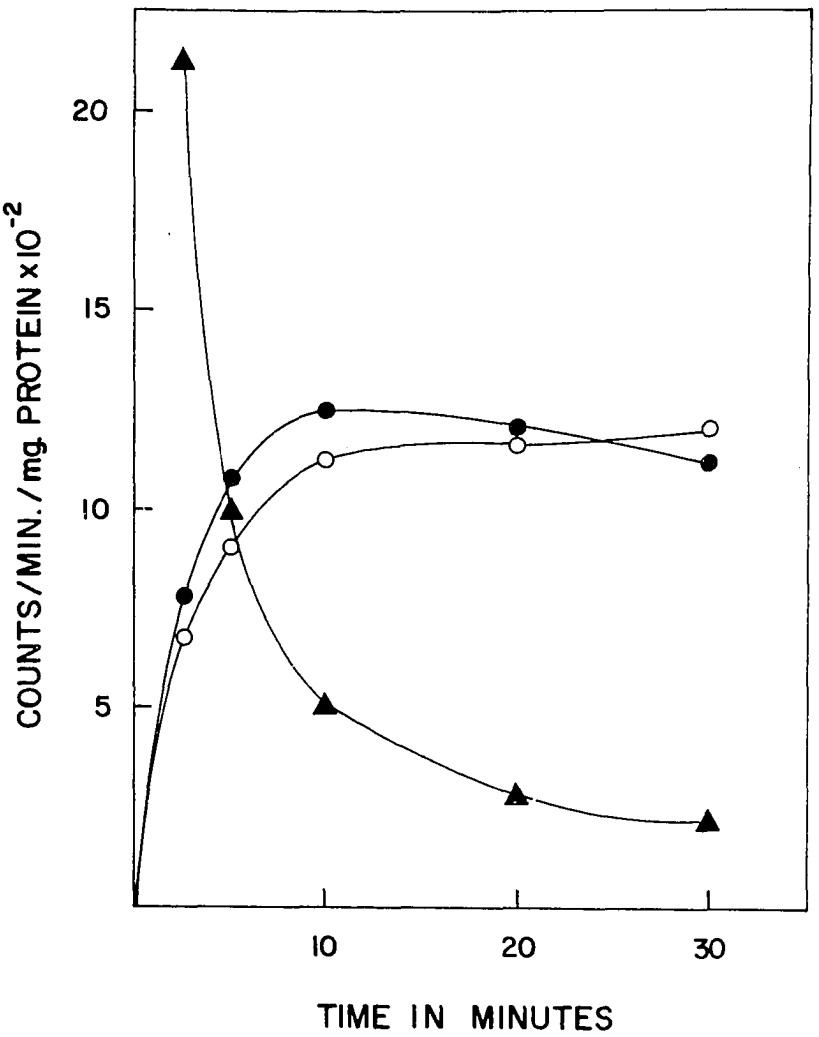

Figure 5.—Incorporation of L-leucine-1-C-14 into the deoxycholate soluble and insoluble fractions of rat liver microsome after injection with 10 μc of L-leucine-1-C-14. Each point represents the mean value of three rats.

—○—○—, deoxycholate soluble fraction;
—●—●—, microsome;
—▲—▲—, deoxycholate insoluble fraction.

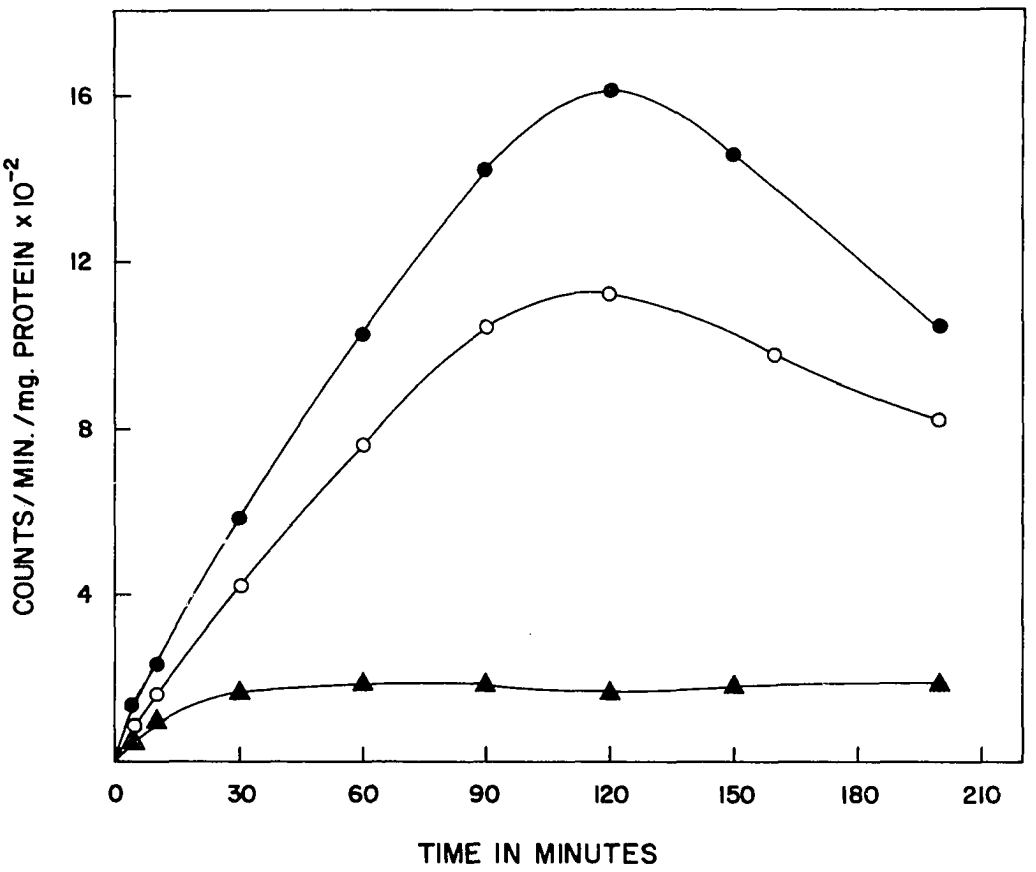

Figure 6.—Incorporation of D-glucosamine-1-C-14 into the deoxycholate soluble and insoluble fractions of rat liver microsome after injection with 10 μc of glucosamine-1-C-14. Each point represents the mean value of three rats.

—●—●—, deoxycholate soluble fraction;
—○—○—, microsome;
—▲—▲—, deoxycholate insoluble fraction.

TABLE 2

DISTRIBUTION OF DRY WEIGHT AND RADIOACTIVITY IN DEOXYCHOLATE SOLUBLE AND INSOLUBLE FRACTIONS OF RAT LIVER MICROSOME 1.5 HOURS AFTER INJECTION OF D-GLUCOSAMINE-1-C-14

Fractions	Dry Weight[a]		Radioactivity	
	Expt. 1 %	Expt. 2 %	Expt. 1 %	Expt. 2 %
Whole Homogenate	100	100	100	100
Microsomes	6.1	7.8	19.5	17.2
Deoxycholate Soluble Fraction	3.8	5.6	15.6	16.2
Deoxycholate Insoluble Fraction	2.0	2.2	1.1	1.0

[a] The weight after treatment of the fractions with lipid solvents as described in the test.

One rat was used in each experiment.

activity in the deoxycholate soluble and insoluble fractions of rat liver microsomes. Ninety minutes after injection of D-glucosamine-1-C-14, more than 80% of the total radioactivity associated with the microsomal fraction was found to be located on the deoxycholate soluble fraction. About 6% of the total radioactivity in the microsomes was found in the deoxycholate insoluble fraction.

In accordance with these results, it may be suggested that during the course of biosynthesis of glycoprotein, the protein moiety is first synthesized in ribosome and then released to the membranous portion of the microsome where D-glucosamine-1-C-14 is attached to the protein moeity.

Incorporation of D-Glucosamine-1-C-14 into Walker 256 Carcinosarcoma

A consistent increase of serum glycoproteins in patients with cancer has been described by numerous investigators (5, 25, 79, 80). Elevation of the protein-bound hexose level in rats bearing Walker 256 tumor was first reported in 1950 by Shetlar and his associates (81). The significance of the elevated serum glycoproteins in tumor bearing animals is still unknown. The possibility that tumor cells may liberate protein-bound carbohydrate into the circulation has been proposed (8, 82, 83).

If Walker 256 tumor does really liberate protein bound carbohydrate into the blood, one should be able to detect the biosynthesis of glycoprotein in the tumor tissue.

The present study was designed to investigate the biosynthesis of glycoprotein by studies of the incorporation of D-glucosamine-1-C-14 into tumor tissue after intravenous injection of this compound to the

rat bearing Walker 256 tumor. As shown in Figure 7, D-glucosamine-1-C-14 was readily incorporated into subcellular fractions of Walker tumor. Generally the subcellular fractions of Walker tumor take up the radioactivity slower than those of the liver (See Figure 2). Microsomal and mitochondrial fractions were most active in incorporating radioactive glucosamine. The nuclear fraction has relatively low radioactivity. In contrast to the incorporation pattern in the liver subcellular fractions, the soluble fraction of Walker tumor had rather high radioactivity. However, this may be due to the contamination by plasma proteins, since the solid tumor is difficult to perfuse.

In order to compare the rate of incorporation of amino sugar and amino acid into the subcellular fractions of Walker tumor, L-leucine-1-C-14 was also used to study the time course of the labelling of various subcellular fractions in tumor tissue. As presented in Figure 8, the incorporation of L-leucine-1-C-14 into subcellular fractions of Walker 256 tumor is much faster than that of D-glucosamine-1-C-14. The microsomal fraction was rather quickly labeled and reached the plateau at 30 minutes after the injection. The radioactivity in cytoplasmic sap (soluble fraction) continued to increase after the microsomal fraction reached its maximum activity.

The results of the above experiment suggest that a tumor is able to perform independent synthesis of glycoprotein. The synthetic pattern, however, was not similar to that of protein biosynthesis, as indicated by leucine incorporation. For more evidence that Walker tumor is able to synthesize glycoprotein independently, D-glucosamine-1-C-14 was injected into the hepatectomized rats bearing Walker 256 tumors.

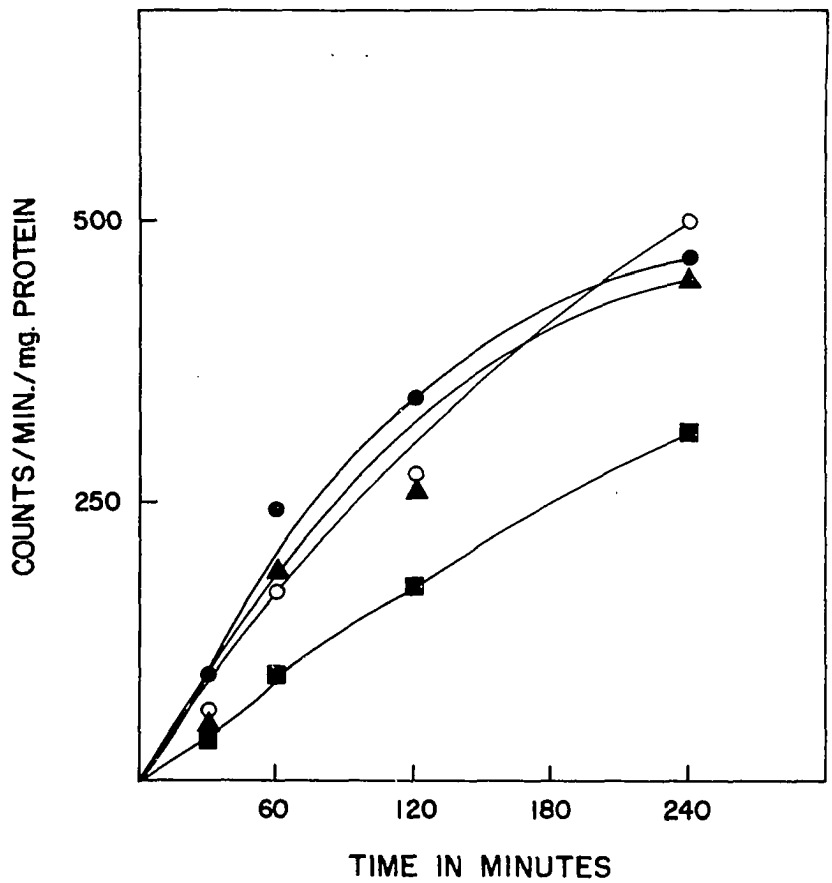

Figure 7.—Incorporation of D-glucosamine-1-C-14 into the subcellular fractions of Walker 256 tumor after injection with 10 μc of D-glucosamine-1-C-14. Each point represents the mean value of three rats.
—○—○—, supernatant; —●—●—, microsome;
—▲—▲—, mitochondria; —■—■—, nucleus.

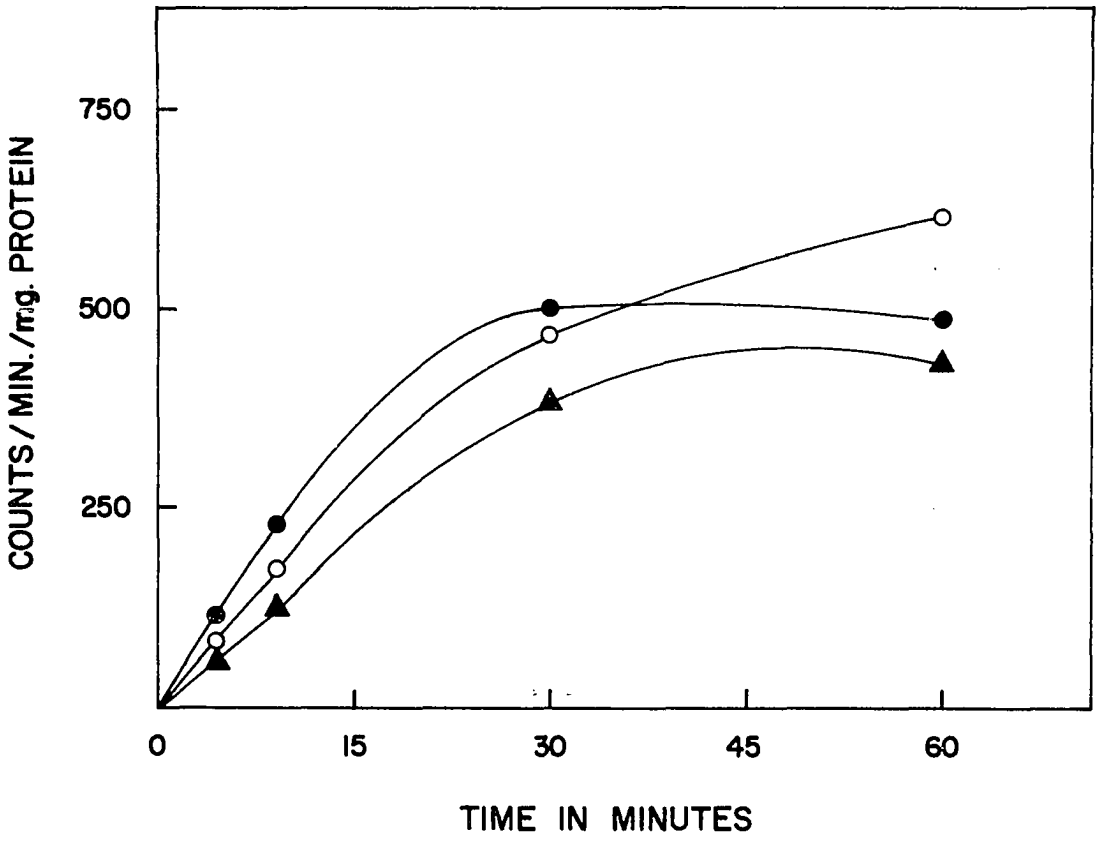

Figure 8.—Incorporation of L-leucine-1-C-14 into the subcellular fractions of Walker 256 carcinosarcoma after injection with 10 μc of L-leucine-1-C-14. Each point represents the mean value of three rats.

—○—○—, supernatant; —●—●—, microsome;
—▲—▲—, mitochondria.

As shown in Table 3, Walker 256 tumor tissue was still able to incorporate D-glucosamine-1-C-14 into its subcellular fraction after complete hepatectomy. The sera from the same rats were subjected to paper electrophoresis using a Spinco Model R electrophoretic system. Thirty microliters of whole serum were applied on the paper strips. After electrophoresis, the paper strips were examined for the radioactivity by using a Baird-Atomic 4π strip scanner. Each fraction on the paper strips was also separated by cutting and examined for radioactivity with a Nuclear Chicago liquid scintillation counter (720 series). No detectable radioactivity was found in any of the serum protein fractions. Similar results were obtained with a low background counter. These results may be due to the short duration of the experiment or the poor circulation in the hepatectomized animal. It was very difficult to keep hepatectomized rats alive more than 3 hours. When tumor slices were incubated *in vitro*, with the serum from the same animal, in the presence of D-glucosamine-1-C-14, a definite up take of radioactivity was also observed (See Table 3). When the microsomal fraction of Walker tumor, which was labeled with glucosamine-1-C-14, was further separated into deoxycholate soluble and insoluble fractions (Table 4), most of the radioactivity was associated with the deoxycholate soluble fraction. Very little radioactivity was presented in the deoxycholate insoluble fraction.

Nature of the Radioactive Compound in the Microsomal Fraction

The previous studies on the incorporation of D-glucosamine-1-C-14 into various tissue were mainly based on the measurement of the radioactivity present in the tissues. It is therefore important to establish the nature of the radioactive compound in that tissue. Since

TABLE 3

INCORPORATION OF GLUCOSAMINE-1-C-14 INTO WALKER TUMOR TISSUE OF HEPATECTOMIZED RATS, INTO TUMOR TISSUE IN VITRO AND INTO LIVER TISSUE IN VITRO

Fractions	Walker Tumor cpm per mg Protein		Liver cpm per mg Protein
	Hepatectomized 90 min.	Slice[a] 180 min.	Slice[a] 180 min.
Mitochondria	58	112	60
Lysosome	57	139	59
Microsome	47	120	107
Nucleus	44	177	51
Supernatant	39	136	24

[a]Twenty grams of tissue slices were incubated with 5 μc of glucosamine-1-C-14 in the presence of 10 ml of rat serum.

TABLE 4

DISTRIBUTION OF RADIOACTIVITY IN DEOXYCHOLATE SOLUBLE AND INSOLUBLE FRACTIONS OF WALKER TUMOR MICROSOME 1.5 HOURS AFTER INJECTION OF D-GLUCOSAMINE-1-C-14

Fractions	Dry Weight %	Radioactivity %
Whole Homogenate	100	100
Microsomes	10.9	21.9
Deoxycholate Soluble Fraction	4.5	14.2
Deoxycholate Insoluble Fraction	6.4	7.7

the liver microsomes were the subcellular particles most actively incorporating D-glucosamine-1-C-14 into glycoprotein, the nature of the radioactivity associated with neutral sugars, amino sugars and amino acids in this fraction was carefully examined.

For the identification of sialic acid, 10 mg of the microsomes were treated with 0.1 N H_2SO_4 at 80° for 1 hour. This was reported to cleave the majority of sialic acid from glycoproteins (84). Quantitative measurement of sialic acid was made by Warren's thiobarbituric acid method (65). The chromophore developed in this reaction was extracted with cyclohexanone. The intensity of ruby red color was measured by colorimetric determination. An aliquot of the cyclohexanone extract was plated out on a stainless steel planchet for determination of radioactivity (31). An authentic sialic acid sample was also carried through all the procedures. The absorption spectra of the chromophores obtained from microsome and authentic sialic acid are presented in Figure 9. Very good correspondence of these two absorption spectra was observed. It is known that all of the free 2-keto-3-deoxy sugars will give the same chromophore using Warren's thiobarbituric acid procedure. To be certain that the chromophore obtained from the microsomal fraction was really derived from sialic acid, a sample of microsomal hydrolysate was passed through anion exchange resin (AG 2 x 8) and sialic acid was isolated from the column by the procedure described by Svennerholm (84). The isolated sialic acid was then determined by Warren's thiobarbituric acid procedure. The amounts and specific radioactivities of the microsomal sialic acid preparations, whether determined after 0.1N H_2SO_4 hydrolysis or after isolation by Svennerholm's method, were essentially the same. This proves that the chromophore obtained by Warren's method from microsomal hydroly-

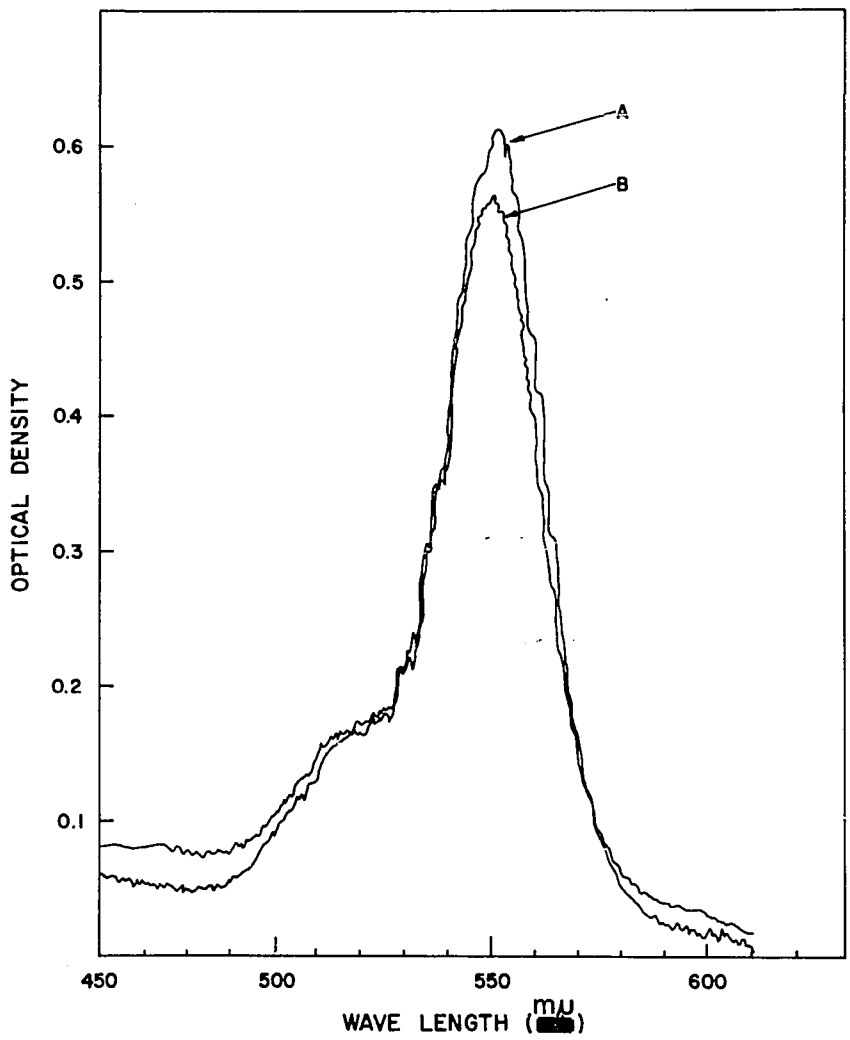

Figure 9.—The absorption spectra of the chromopores obtained from sialic acids by Warren's thiobarbituric acid method. (A) authentic sialic acid (B) sialic acid from the rat liver microsomes. The spectra was determined by Cary Model 14 recording spectrophotometer.

sate was from sialic acid, therefore the direct application of Warren's method to microsomal preparation was sufficient to estimate the sialic acid content. Table 5 summarizes the amount of protein bound radioactivity contributed by sialic acid and by hexosamine in mitochondrial, microsomal and supernatant fractions.

The radioactivities associated with sialic acid in mitochondrial, microsomal and supernatant fractions were 5.5%, 8.4% and 13.0% respectively. Since the specific radioactivities of sialic acid and the total radioactivities in these three fractions were very close, the higher percentage of radioactivity associated with sialic acid in supernatant fraction indicated that the soluble fraction had a higher content of sialic acid relative to hexosamine; also part of this was due to the lower specific radioactivity of hexosamine in supernatant fraction.

For the determination of radioactivity associated with neutral sugars, amino acids and hexosamine, 50 mg of dried protein powder were suspended in 2 ml of 4 N HCl in a tightly closed screw cap tube and hydrolyzed in a boiling-water bath for 6 hours. The resulting hydrolysate was taken to dryness in a vacuum oven to remove HCl. The residue was then suspended in distilled water and passed through a 0.9 x 15 cm Dowex-50 column according to a method described by Boas (66). Neutral sugars were washed from the column until the eluent showed a negative anthrone test. The hexosamine was eluted from the column with 2 N HCl. The first 10 ml of the eluent were collected as hexosamine fraction and the following 20 ml of the eluent were collected as amino acid fraction. After drying these fractions, an aliquot of each fraction was taken to measure the radioactivity using a Nuclear Chicago gas flow counter. No radioactivity was detectable in either the neutral sugar or the amino acid

TABLE 5

PROTEIN-BOUND RADIOACTIVITY ASSOCIATED WITH SIALIC ACID AND HEXOSAMINE IN MITOCHONDRIAL, MICROSOMAL AND SUPERNATANT FRACTIONS OF RAT LIVER TISSUE TWO HOURS AFTER ADMINISTRATION OF ISOTOPE

	Mitochondria	Microsome	Supernatant
[a]cpm per μmole sialic acid	9,460	12,144	11,441
μmole sialic acid per g protein	8.4	11.1	1.3
cpm associated with sialic acid per g protein	79,464	134,794	14,873
cpm per μmole hexosamine	40,418	45,972	15,269
μmole hexosamine per g protein	32.9	37.5	5.9
cpm associated with hexosamine per g protein	1,329,752	1,723,950	90,087
Sum of cpm from sialic acid and hexosamine per g protein	1,409,216	1,858,744	104,960
cpm per g protein measured	1,446,000	1,600,000	114,000
% cpm associated with sialic acid	5.5	8.4	13.0
% cpm associated with hexosamine	92.0	107.7	79.0
% Total Radioactivity associated with sialic acid and hexosamine	97.5	116.1	92.0

[a]cpm: Counts per minute

fractions. The hexosamine fraction was highly radioactive. Hexosamine was also determined quantitatively by Boas' method (66) and specific radioactivity of hexosamine was thus obtained. All three fractions were also examined for radioactive spots by paper chromatography and autoradiographic technique. For neutral sugars and hexosamine, the chromatogram was developed in a solvent system consisting n-butanol:pyridine:0.1 N HCl (5:3:2). For amino acids, the chromatogram was developed in the top layer of n-butanol:acetic acid: water (4:1:5).

The autoradiograms of each chromatograph were made by direct contact for 3 weeks of the paper with blue brand medical X-ray film from Eastman Kodak Company. In the neutral sugar and amino acid fractions, no radioactive spot was found, but in the hexosamine fraction, a major and a minor spot was identified as glucosamine and galactosamine, respectively. Also a trace of radioactivity showed as a tailing strip. This result suggests that most of the radioactivity remained as hexosamine after administration of D-glucosamine-1-C-14. No significant amount of radioactivity was converted into neutral sugars and amino acids under this experimental condition. The fact was also supported by the analytical data shown in Table 5. The radioactivity associated with hexosamine in mitochondrial, microsomal and supernatant fractions were 92%, 107.7%, and 79%, respectively. The total radioactivity obtained from sialic acid and hexosamine in each fraction were calculated by the following formula:

$$\% \text{ Total radioactivity associated with sialic acid and hexosamine} = 100 \times \left[\frac{\text{cpm/}\mu\text{mole sialic acid}}{\text{cpm/g protein measured}} \times \mu\text{mole sialic acid/g protein} + \frac{\text{cpm/}\mu\text{mole hexosamine}}{\text{cpm/g protein measured}} \times \mu\text{mole hexosamine/g protein} \right]$$

The values obtained were 116.1% in microsomes, 97.5% in mitochondria and 92% in supernatant fraction. These data strongly suggested that no appreciable alteration of D-glucosamine-1-C-14 occurred under these experimental conditions. Only a small percentage of the radioactivity was converted into sialic acid. No detectable amount of radioactivity was converted into protein-bound neutral sugars and amino acids. Therefore, D-glucosamine-1-C-14 is an excellent tracer for the studies on glycoprotein biosynthesis.

The Linkage of D-Glucosamine in Deoxycholate Soluble Fraction of Rat Liver Microsome

In recent years, more and more evidence has been accumulated that aspartic acid and D-glucosamine are responsible for linking the polypeptide and oligosaccharide chains of several glycoproteins isolated from serum and other sources (85).

Our experimental results presented above suggested that deoxycholate soluble proteins of the microsomal fraction are involved in the biosynthesis of glycoprotein. If serum glycoproteins are synthesized in the deoxycholate soluble fraction of microsome and then by some unknown mechanism liberated to the serum, one should be able to find a linkage of D-glucosamine to the protein moiety similar to that found in serum glycoprotein (85). The following study was undertaken to investigate the linkage between the carbohydrate and peptide moieties of the deoxycholate soluble protein isolated from the rat liver microsomes.

Two grams of the deoxycholate soluble fraction were suspended in 20 ml of 0.01 M $CaCl_2$ and adjusted to pH 7.8 with 1 N NaOH. Three milligrams of Pronase was added. The reaction mixture was then left at 37° for

48 hours. The progress of the reaction was followed by the decrease of pH in the reaction mixture. During those 48 hours, the pH of the reaction mixture was examined from time to time, and adjusted to pH 7.8 by adding a few drops of 1 N NaOH. At subsequent 8 to 9 hour intervals, additional 3 mg quantities of Pronase were added to this mixture. After the digestion, an insoluble precipitate was removed by centrifugation, and the clear supernatant was brought to 80% ethanol concentration. After being allowed to stand overnight in the cold, the solution was centrifuged, and the precipitate was dissolved in 5 ml of water. This solution was brought to 3% trichloroacetic acid concentration and centrifuged to remove the insoluble precipitate, and the clear brownish supernatant was applied to a column (3 x 40 cm) of Sephadex G-25 equilibrated with distilled water. Three milliliter fractions were collected at the flow rate of 0.44 ml per minute. The fractions were examined for radioactivity and for the substances positive in the ninhydrin reaction. Figure 10 shows the pattern of Sephadex G-25 gel filtration. The radioactivity was eluted at the void volume of the column and the ninhydrin positive substances come after the radioactive peak.

A small amount of radioactivity was also found in the 80% ethanol solution of the Pronase digest. The ethanol solution was concentrated to about 10 ml with a vacuum evaporator. The radioactive compound in this solution could be separated from the ninhydrin positive substances by Sephadex G-25 gel filtration in the same manner as the trichloroacetic acid soluble fraction. Essentially, the pattern of gel filtration was similar to Figure 10, except the radioactive peak was smaller and the ninhydrin positive peak was larger.

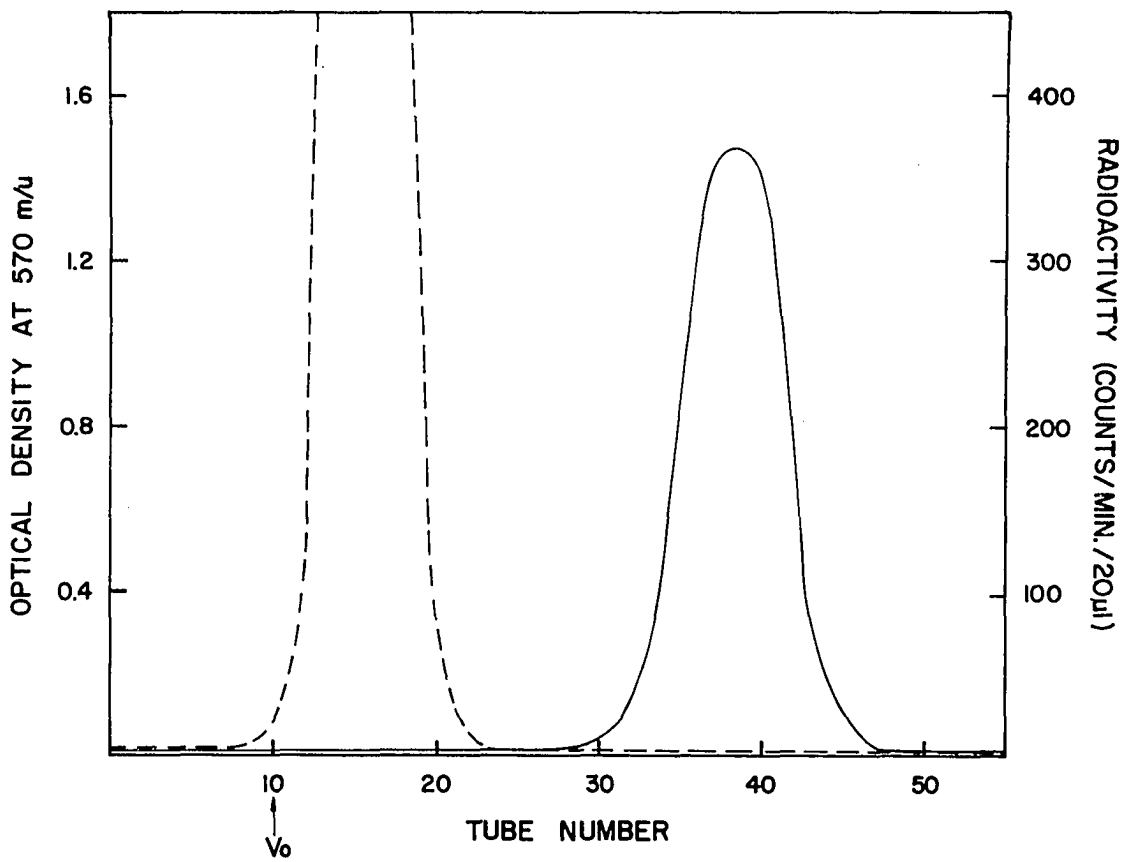

Figure 10.—Gel filtration of Pronase digest of deoxycholate soluble fraction from rat liver microsome on Sephadex G-25. ———, ninhydrin reaction; — — —, radioactivity. Vo, the void volume of the column.

The radioactive peaks from gel filtrations were pooled and dried by freezing. The glycopeptide fraction thus obtained was dissolved in 2 ml of 0.01 M $CaCl_2$ and again subjected to enzymic digestion with 1 mg of Pronase for 24 hours. After the second digestion, the solution was treated by the same procedure described above. More amino acids were removed from the glycopeptide during the second digestion. The radioactive glycopeptide obtained from the second digestion was designated glycopeptide I. The distribution and the recovery of radioactivity are summarized in Table 6, where it is shown that 78.3% of the radioactivity from microsomal membrane was recovered in glycopeptide I. Only 6.6% of the radioactivity was left as undigested residue.

Chemical analysis of glycopeptide 1 showed that it was comprised of 12.83% sialic acid, 38.1% hexoses, 8.66% glucosamine and 40.24% amino acids. The high content of amino acids in glycopeptide 1 may be due to the fact that the considerable amount of sialic acid presenting in glycopeptide 1 interferes with the enzymic digestion by Pronase. To eliminate the interference by sialic acid, glycopeptide 1 was further subjected to mild acid hydrolysis by means of 0.1 N HCl at 80^{o} for one hour. This procedure hydrolyzes the bound sialic acid from glycoprotein (84). After hydrolysis, the glycopeptide was separated from sialic acid by a Sephadex G-25 column chromatography. The glycopeptide fraction was pooled and freeze-dried. The dried material, free from sialic acid, was subjected to a third Pronase digestion as described before. The glycopeptide obtained from final digestion was designated as glycopeptide II. Figure 11 shows the scheme of Pronase digestion and Table 7 summarizes the chemical analysis of deoxycholate soluble material from rat liver microsome, glycopeptide I and glycopeptide II.

TABLE 6

DISTRIBUTION AND RECOVERY OF DRY WEIGHT AND RADIOACTIVITY IN THE PRONASE DIGEST OF DEOXYCHOLATE SOLUBLE FRACTION OF RAT LIVER MICROSOME

Fractions	Specific Radioactivity cpm[a] /mg dried Substance	Dry Weight		Total Radioactivity	
		g	%	cpm[a]	%
DOC[b] Soluble Fraction	560	2.000	100.0	1.12×10^6	100.0
Glycopeptide I	31280	0.028	1.4	8.77×10^5	78.3
Undigested Residue	187	0.363	18.1	6.79×10^4	6.6
Alcohol Soluble Fraction	116	1.500	75.0	1.75×10^5	15.0

[a] cpm; Count per minute

[b] DOC; Deoxycholate

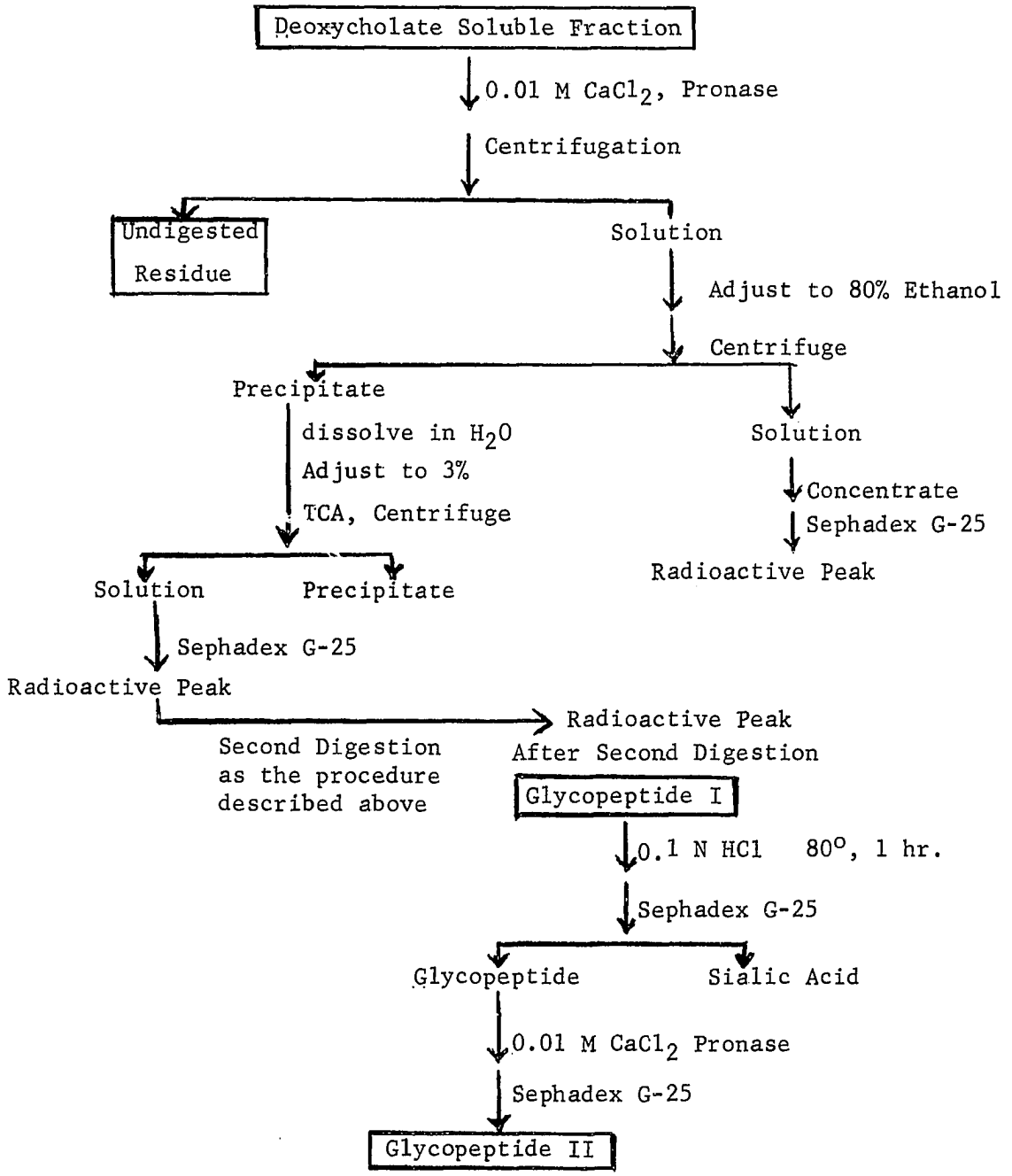

Figure 11.—The scheme of Pronase digestion and separation of glycopeptide from deoxycholate soluble fraction of rat liver microsome.

TABLE 7

CHEMICAL ANALYSIS OF DEOXYCHOLATE SOLUBLE MATERIAL FROM RAT LIVER MICROSOME AND ITS GLYCOPEPTIDE FROM PRONASE DIGEST

	Deoxycholate Soluble Fraction		Glycopeptide I		Glycopeptide II	
	μ mole/g[b]	%	μ mole/g[b]	%	μ mole/g[b]	%
Sialic Acid	8.4	0.26	415	12.8	--	--
Hexoses	101	1.8	2,117	38.1	3,368	60.6
Glucosamine[a]	5.6	0.1	483	8.7	829	14.9
Total Amino Acid	6,555	90	2,768	40.2	1,743	21.3
Radioactivity (cpm/mg)[b]	560		31,280		44,903	
Weight (g)	2.000		0.028		0.0165	
Yield (%)	100		1.4		0.8	
Recovery of total Radioactivity (%)	100		78.3		65.9	
$\frac{\text{Hexoses}}{\text{Glucosamine}}$	--		$\frac{4.3}{1}$		$\frac{4.1}{1}$	

[a]As determined by the amino acid analyzer.

[b]The values are all based on gram or milligram of dry weight.

Glycopeptide II (Table 7) is a carbohydrate enriched fraction. It is comprised of 75.5% sugars, of which 60.6% are hexoses and 14.9% is glucosamine. The molar ratio of hexoses to glucosamine is the same in both glycopeptide I and glycopeptide II. This constant ratio suggests that no appreciable amount of hexose was lost in the process of Pronase digestion. The yield of glycopeptide II as indicated by dry weight was only 0.8% of the original deoxycholate soluble substance, however, the recovery of the total radioactivity in glycopeptide II was 65.9% of the original material. This strongly indicates that most of the glucosamine, if not all, is linked to the carbohydrate portion or the core portion of the glycopeptide rather than being randomly distributed throughout the glycoprotein molecule. Aspartic acid is the most abundant amino acid in glycopeptide II (Table 8). Aspartic acid content was almost four times greater than any other amino acids. There were about equal amounts of threonine, serine, glutamic acid, proline and glycine in glycopeptide II while alanine and valine were present in minor amounts. From the analysis of amino acid content of glycopeptide I and II, it is clear that generally the relative content of amino acids was decreased in these glycopeptides except for aspartic acid which was slightly increased. There are about equimolar amounts of glucosamine and aspartic acid in glycopeptide II (Tables 8 and 9). These results suggest that the carbohydrate moiety is very likely linked to the peptide by a glucosamine-aspartic acid linkage. The absence of, or low content of some amino acids such as histidine, arginine, isoleucine, leucine, alanine, valine, phenylalanine, tyrosine, methionine in glycopeptide I and II suggests that these amino acids are more readily removed by Pronase digestion. Threonine, serine, glutamic acid, proline, glycine residues were more resistant to the digestion by

TABLE 8

AMINO ACID ANALYSIS OF DEOXYCHOLATE SOLUBLE MATERIAL FROM RAT LIVER MICROSOME AND ITS GLYCOPEPTIDE AFTER PRONASE DIGEST

Amino Acids	Deoxycholate Soluble Fraction		Glycopeptide I		Glycopeptide II	
	μmole/g[a]	%	μmole/g[a]	%	μmole/g[a]	%
Asp.	649	8.64	693	11.69	719	9.6
Thr.	342	4.07	235	3.42	187	2.2
Ser.	408	4.29	217	2.82	185	2.0
Glu.	728	12.02	435	8.78	181	2.9
Pro.	317	3.65	258	3.62	182	2.1
Gly.	492	3.70	280	2.59	179	1.3
Ala.	497	4.43	110	1.20	68	0.5
Val.	448	5.25	65	1.03	42	0.4
Cyst.	- -	- -	- -	- -	- -	- -
Meth.	126	1.88	- -	- -	- -	- -
Ileu.	314	4.12	31	0.48	- -	- -
Leu.	667	8.75	193	0.80	- -	- -
Tyr.	180	3.26	- -	- -	- -	- -
Phe	316	5.22	- -	- -	- -	- -
Lys.	618	9.10	143	2.59	- -	- -
His.	185	2.89	45	0.83	- -	- -
Arg.	268	4.70	63	1.31	- -	- -

[a]The values are all based on gram of dry weight

Pronase. The resistance of these amino acid residues to Pronase digestion may be due to the presence of proline as it has been reported that L-glycyl-L-proline is not hydrolyzed by Pronase and that L-glycyl-L-prolyl-L-leucine is poorly hydrolyzed at the linkage between proline and leucine (64).

Carbohydrate Content in Subcellular Fractions of Rat Liver and of Walker 256 Carcinosarcoma

Studies of the incorporation of D-glucosamine-1-C-14 into subcellular fractions of rat liver and Walker 256 carcinosarcoma suggest that D-glucosamine-1-C-14 is distributed throughout all of the subcellular components. The results also indicate that the microsomal fraction is most active in the glycoprotein biosynthesis. Subcellular fractions, therefore, ought to contain bound carbohydrate. Although many studies of the distribution of enzymes in subcellular fractions exist, the carbohydrate content in these fractions is relatively unexplored. A study of the carbohydrate content of the subcellular fractions of liver tissue and Walker 256 tumor tissue should consequently add valuable information to the studies of glycoprotein biosynthesis. Yamashina et al. (59) have reported that in the rabbit liver the microsomal fraction is richest in hexosamine and sialic acid among the fractions. Wallach and Eyler (57), Patterson and Touster (58) have reported preliminary observations on the distribution of sialic acid in the subcellular fractions of rat liver and Ehrlich ascites carcinoma cells. No detailed examination of amino sugars and neutral sugars in subcellular fractions of either liver or other tissue could be found in the literature. It is the purpose of this section to report studies of the distribution of neutral sugars, amino sugars and sialic acid in the subcellular fractions of Walker 256 tumor tissue and in those of the liver of the same animals.

Sialic Acid Content in Subcellular Fractions

Table 9 shows the sialic acid content in subcellular fractions of rat liver and of Walker 256 carcinosarcoma. The figures for the whole homogenate are of the same order of magnitude as those reported by Macbeth and Bekesi (56) for acetone extracted tissue. They reported that the value for sialic acid as N-acetylneuraminic acid in liver was 78 mg/100 g acetone dried tissue; in the Walker 256 tumor, it was 240 mg/100 g tissue protein. Our result calculated on the same basis as theirs showed that sialic acid content in liver was 198 mg/100 g defatted dried tissue; the Walker tumor, it was 302 mg/100 g defatted dried tissue. It is noted that our liver sialic acid is higher than theirs. This may be due to the difference in methods of preparing the samples before analysis. In general, sialic acid is higher in the subcellular fractions of the Walker tumor. In both liver and the Walker tumor, the lysosomal fraction contains the highest concentration, based on protein, of sialic acid. Mitochondrial and microsomal fractions are next highest in this sugar. In liver, the sialic acid content of the soluble fractions is relatively low. In the Walker tumor, the nuclear fraction is also low in sialic acid, but the soluble fraction contains relatively large amounts of this sugar.

The presence of sialic acid in the subcellular particles was further established by using pneumococcal neuraminidase (87,88). More than 90% of the sialic acid in the subcellular particles was released by this enzyme. The released sialic acid was isolated by the column chromatographic technic of Svennerholm (84), and exhibited the mobility in paper chromatography of N-acetylneuraminic acid.

TABLE 9

DISTRIBUTION OF SIALIC ACID IN SUBCELLULAR FRACTIONS OF RAT LIVER AND WALKER 256 CARCINOSARCOMA TISSUE

Fractions	No. of Animals	Sialic Acid μmole per g Protein		Sialic Acid / Hexosamine	
		Liver	Tumor	Liver	Tumor
Whole Homogenate	15	6.5 ± 0.4[a]	9.9 ± 0.3	1:3.7	1:3.4
Nucleus	12	0.5 ± 0.0	5.6 ± 0.4	1:22.4	1:4.1
Mitochondria	15	8.4 ± 0.5	15.2 ± 0.5	1:3.9	1:3.2
Microsome	15	7.5 ± 0.0	12.5 ± 0.6	1:3.4	1:3.0
Lysosome	15	11.1 ± 0.4	18.8 ± 0.5	1:3.8	1:2.8
Soluble	13	1.3 ± 0.2	11.8 ± 0.6	1:4.5	1:2.7

[a]Values following ± signs are standard error of mean.

Hexosamine Content in Subcellular Fractions

The hexosamine content of the subcellular fractions of rat liver and of Walker 256 carcinosarcoma are summarized in Table 10. Macbeth and Bekesi (86) reported their figures of hexosamine content in acetone extracted tissue. Liver contained 525 mg/100 g acetone dried tissue; Walker 256 tumor contained 701 mg/100 g tissue protein. Our data on the whole homogenate showed that hexosamine content of liver was 422 mg/100 g defatted dried tissue; in Walker 256 tumor it was 542 mg/100 g defatted dried tissue. Our results agree well with theirs. In both Walker 256 tumor and liver, hexosamine is about 3 to 4 times higher than sialic acid on a molar basis. The ratio of hexosamine to sialic acid is higher in liver fractions than in those of Walker 256 carcinosarcoma (Table 9). Among the subcellular fractions of both tissues, the lysosomal fraction contains the greatest amount of hexosamine. Qualitative identification of the hexosamine fraction of 3 N HCl hydrolysates, by paper chromatography with N-butanol-pyridine-0.1 N HCl (5:3:2) as the solvent system, revealed that glucosamine is the major amino sugar in all of the fractions. However, a small amount of galactosamine was also detected. Quantitative analysis of glucosamine and galactosamine was carried out with a Spinco amino acid analyzer; the molar ratio of galactosamine to glucosamine is smaller in the subcellular fractions of the liver, except nuclear fraction, than in those of the Walker tumor.

Neutral Sugar Content of Subcellular Fractions

Galactose, mannose, and fucose are the major neutral sugars identified in most glycoproteins. The presence of these sugars was qualitatively demonstrated by the paper chromatography in the solvent

TABLE 10

DISTRIBUTION OF HEXOSAMINE IN SUBCELLULAR FRACTIONS OF RAT LIVER AND WALKER 256 CARCINOSARCOMA TISSUE

Fractions	No. of Animals	Hexosamine μmoles per g Protein		$\frac{\text{Gal } NH_2}{\text{Glu } NH_2}$[a]	
		Liver	Tumor	Liver	Tumor
Whole Homogenate	15	23.6 ± 1.2[b]	30.3 ± 1.0	1:13.8	1:10.1
Nucleus	12	11.2 ± 1.4	23.3 ± 0.8	1: 5.6	1: 9.4
Mitochondria	15	32.9 ± 1.7	48.8 ± 1.6	1:20.8	1:13.1
Microsome	15	25.7 ± 2.0	37.9 ± 1.9	1: 9.3	1: 7.6
Lysosome	15	37.5 ± 2.0	53.1 ± 1.5	1:16.7	1: 9.4
Soluble Fraction	13	5.9 ± 0.4	32.4 ± 1.9	- -[c]	1:10.8

[a]Gal NH_2: Galactosamine: Glu NH_2: Glucosamine as determined by Spinco amino acid analyzer.

[b]Value following ± signs are standard error of mean.

[c]The values are too small to be determined accurately.

system consisting n-butanol—pyridine—0.1 N HCl (5:3:2). As shown in Figure 12, galactose and mannose are distributed throughout the various fractions. A very small amount of fucose is also found in most of the fractions. A larger amount of glucose was found in most of the liver subcellular fractions (Figure 12B). This is probably due to the presence of glycogen. In the Walker tumor (Figure 12A), the glucose content is relatively low, except for the soluble and nuclear fractions. The high quantities of glucose found in the soluble fractions may also be due to the glycogen; however, this problem requires further investigation.

A careful examination of Figure 12B shows that mitochondrial, lysosomal, and microsomal fractions of the rat liver contain greater amounts of mannose than galactose, whereas this difference is not as prominent in the subcellular fractions of the Walker tumor (Figure 12A).

Since the difference in the paper chromatograms of the subcellular fractions of the rat liver and the Walker tumor was so striking, a quantitative analysis of mannose and galactose was made as the method described in page 17 and shown in Table 11. The galactose content in the subcellular fractions of the Walker tumor is definitely higher than in the corresponding fractions of liver. In the liver, the lysosomal fraction is highest in both galactose and mannose content. The galactose contents of the mitochondrial, microsomal, and nuclear fractions are not strikingly different; however, the soluble fraction is low in this sugar. The mitochondria and microsomes contain relatively large amounts of mannose, while the nuclear and soluble fractions have smaller amounts of this sugar. The mannose content of mitochondrial, microsomal, and lysosomal fractions of the liver is more than 3 times

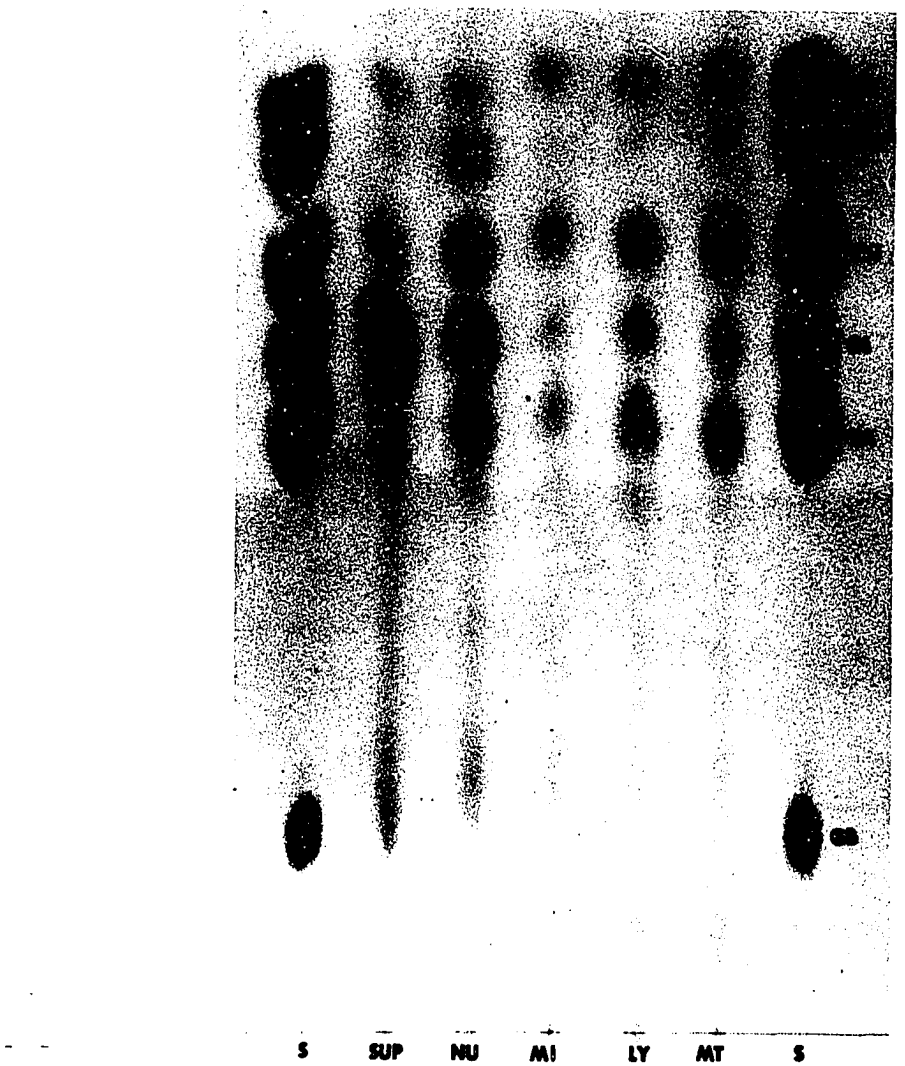

Figure 12A.—Paper chromatogram of neutral sugars found in the subcellular fractions of Walker 256 tumor after hydrolysis with 1 N HCl at 100° for 6 hrs.

S, mixture of standards;	RI, D-ribose;	FU, L-fucose;
MN, D-mannose;	GL, D-glucose;	GA, D-galactose;
GB, galactobiose;	NU, nucleus;	MI, microsome;
SUP, Soluble fraction;	LY, lysosome;	MT, mitochondria.

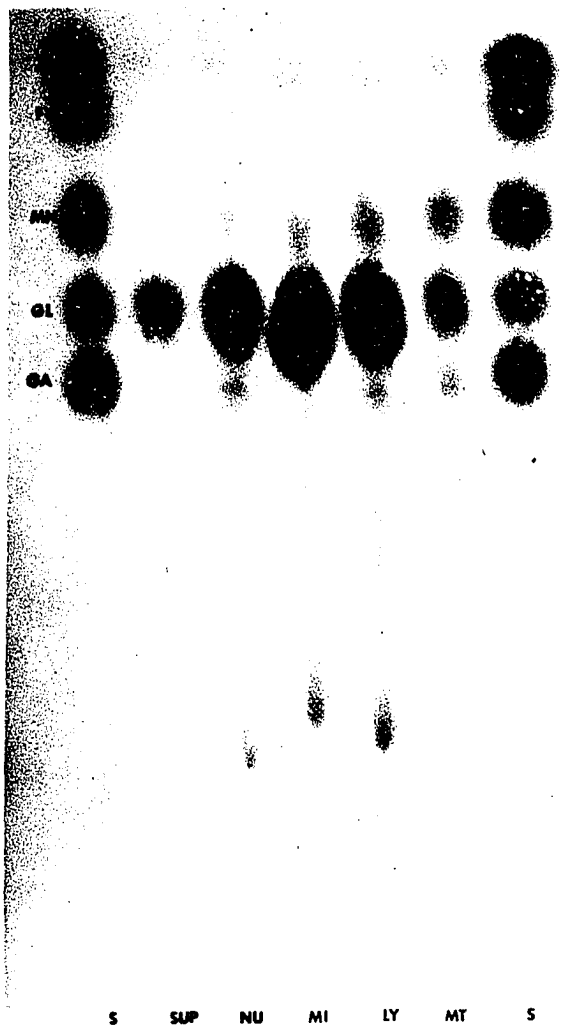

Figure 12B.—Paper chromatogram of neutral sugars found in the subcellular fractions of rat liver after hydrolysis with 1 N HCl at 100° for 6 hrs.

S, Mixture of standards;	RI, D-ribose;	FU, L-fucose;
MN, D-mannose;	GL, D-glucose;	GA, D-galactose;
GB, galactobiose;	NU, nucleus;	MI, microsome;
SUP, soluble fraction;	LY, lysosome;	MT, mitochondria.

TABLE 11

DISTRIBUTION OF NEUTRAL SUGARS IN THE SUBCELLULAR FRACTIONS OF NORMAL RAT LIVER AND WALKER 256 CARCINOSARCOMA TISSUE[a]

Fractions	D-galactose (μmoles/g Protein)	D-Mannose (μmoles/g Protein)	Ratio D-Galactose: D-Mannose	Fucose (μg/g Protein)
Liver				
Whole homogenate	8.4	28.4	1:3:4	- -[b]
Mitochondria	6.0	23.1	1:3.8	1.7
Microsome	9.8	30.4	1:3.1	2.3
Lysosome	13.7	44.1	1:3.2	2.4
Nucleus	4.4	6.7	1:1.5	1.2
Soluble	2.9	4.8	1:1.7	0.7
Walker Tumor				
Whole homogenate	16.9	27.4	1:1.6	- -[b]
Mitochondria	12.9	16.7	1:1.3	4.8
Microsome	19.3	34.4	1:1.8	3.6
Lysosome	20.3	46.3	1:2.3	2.7
Nucleus	7.9	14.5	1:1.8	3.2
Soluble	18.0	21.2	1:1.2	2.5

[a]Mean of 3 determinations.

[b]Not determined.

greater than that of galactose. In the Walker tumor, only the nucleus is low in galactose. There are only a little difference in the distribution of galactose in the remainder of the fractions. Although the mannose content is also higher than that of galactose throughout all of the fractions, the difference is less striking than that found in the liver. As the detection of more mannose than galactose in the subcellular fractions might be due to the differences in destruction rate during the acid hydrolysis, this matter was carefully evaluated. When 20 mg each of mannose and galactose were hydrolyzed with 200 mg of crystalline bovine albumin under the conditions described above for the hydrolysis of subcellular fractions, the recovery of both sugars was 75-80%. The fact that there was little difference between the recovery of mannose and galactose suggests that the presence of more mannose than galactose is not due to an artifact. The fucose content in both the Walker tumor and the rat liver is low compared with other neutral sugars; however, the Walker tumor fractions contain more fucose than those of the liver. In the liver, the microsomal and lysosomal fractions are highest in fucose; however, in the Walker tumor, the mitochondrial and lysosomal fractions are richest in this sugar. As noted in Table 10, subcellular fractions of both liver and tumor are relatively high in hexosamine. Whether hexosamine is present along with uronic acid, as in the case of acid mucopolysaccharides, was considered. The result of uronic acid analysis indicated that the subcellular fractions of both liver and Walker tumor did contain a very small amount of uronic acid. However, its concentration was too small to be determined accurately with available techniques.

Immunoelectrophoresis of Deoxycholate Soluble Fraction of Rat Liver Microsomes

Immunoelectrophoresis is an analytic procedure originally described by Grabar and Williams (89) in 1953. Since then modifications have been introduced by many investigators. Most immunoelectrophoretic tests employ the double diffusion technique in conjunction with electrophoretic separation of one of the reactants which precedes the antigen-antibody fraction-indicating reaction. Antigen-antibody precipitation is the result of specific and firm, but partially or totally reversible combination of the two reactants in which entire reactant molecules or fragments of them may combine, apparently at their respective surfaces without chemical change of antigen or antibody. This reaction is considered to be the most sensitive and specific reaction for the presence of a protein. Studies mentioned in the previous sections, revealed that the deoxycholate soluble fraction of rat liver microsomes is apparently very active in incorporation of D-glucosamine-1-C-14 into glycoprotein. If the synthesized glycoprotein is related to serum glycoprotein, it should be possible to find a precipitate reaction between the specific anti-rat serum antibodies and the newly synthesized serum protein or its precursor in the deoxycholate soluble fraction prepared from rat liver microsomes. In order to identify whether the precipitated or any other band in immunoelectrophoresis is a glycoprotein, the most sensitive and accurate method is to label the deoxycholate soluble fraction with D-glucosamine-1-C-14 and detect the radioactivity in the precipitated band by autoradiographic technique. For this purpose, a microsomal deoxycholate soluble fraction was prepared at 90 minutes after intravenous injection of 20 μc of D-glucosamine-1-C-14 according to the pro-

cedure described on page 13. After the microsomes were treated with 0.5% freshly prepared sodium deoxycholate solution, the centrifuged supernatant was concentrated 20 times with glass suction ultrafiltration apparatus from the Carl Schleicher & Schuell Co. The concentrated material was then applied on agar slides made from Difco Special Agar Noble for electrophoresis. Electrophoresis of this substance was accomplished in an LKB Electrophoresis Apparatus 3276BN with Veronal buffer at pH 8.6, ionic strength 0.1, at 250 volts for one hour. After electrophoresis, the slides were then removed from the electrophoresis apparatus and a central trough was made on the slide by means of a gel knife. One hundred microliters of anti-rat serum from a rabbit was deposited in the central trough and the slides were left for incubation at room temperature for 48 hours. At the end of incubation, the unprecipitated protein was rinsed out with three washes: first, with 1% NaCl for 6 hours; second, with 1% NaCl for 16 hours; and third, with distilled water for one hour. The slides after being washed and air-dried were stained with amido black 10B. The result of immunoelectrophoresis is presented in Figure 13A. The deoxycholate soluble fraction from rat liver microsome, at the left hand side, has two to three precipitated bands. The serum from the same animal, at the right hand side, shows many precipitated bands. The autoradiograms of those slides were made by fixing the slides on Kodak Royal Ortho sheet-film with direct contact of emulsion side and agar surface for 8 weeks. The resulting autoradiogram is shown in Figure 13B. When Figures 13A and B are compared it is found that, in the deoxycholate soluble fraction, band 1 which migrated at the rate of serum albumin in electrophoresis has no detectable radioactivity. Therefore, band 1 may be albumin.

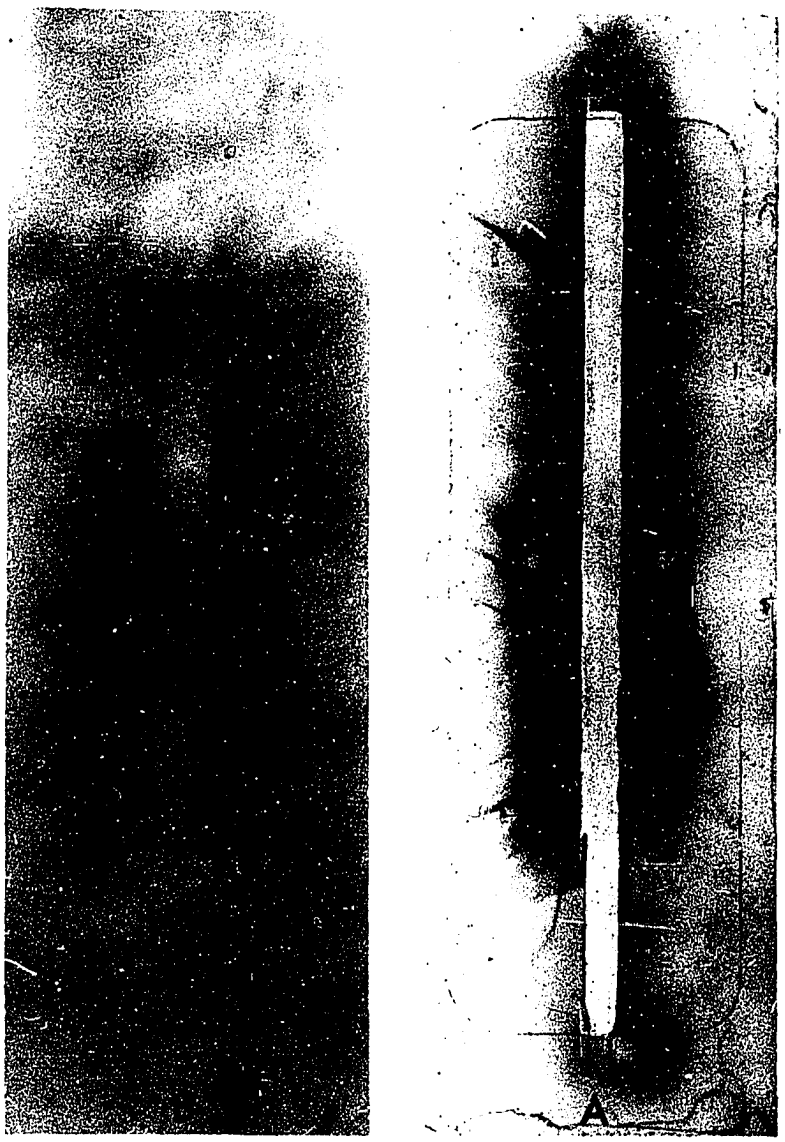

Figure 13—(A) Immunoelectrophoresis:
Right, deoxycholate soluble material; Left, rat whole serum.
(B) Autoradiogram of (A):
Right, rat whole serum; Left, deoxycholate soluble material.
D-Glucosamine-1-C-14 was used as a tracer.

Band 2 was a very diffuse band with certain amounts of radioactivity. Because of the diffused picture, it was difficult to conclude that band 2 was an antigen-antibody reacting band. Band 3 was a rather sharp and definite band with certain amount of radioactivity. With careful examination of both the electrophoretic pattern and the autoradiogram of whole serum, it was not possible to find any band derived from the whole serum corresponding to the band 3 in deoxycholate soluble fraction. On the autoradiogram of whole serum, it appeared that most of the radioactive bands were in the α-globulin region although it was impossible to name them individually from this picture. Only a few radioactive bands were in the β and γ globulin region. Albumin and prealbumin had no detectable radioactivity under this experimental condition. In a previous section it was concluded that the deoxycholate soluble fraction of liver microsomes is the site of incorporation of D-glucosamine-1-C-14 into glycoportein, but in the immunological study, the deoxycholate soluble fraction showed only two or three bands without any correspondence to the bands from whole serum under the same condition. This result suggests that the deoxycholate soluble fraction of liver microsome may synthesize the glycoprotein which is not the direct precursor of the serum glycoprotein. In other words, biosynthesis of serum glycoprotein may involve a more complicated procedure in which part of the serum glycoprotein molecules is synthesized in the deoxycholate soluble fraction of liver microsomes and as these molecules are released to the serum more reactions occur which convert the molecules to the circulating glycoprotein.

CHAPTER V

DISCUSSION

As indicated earlier, it is well established that the level of serum glycoprotein is greatly increased in a variety of pathological conditions (3,4,5). However, the significance and cause of this elevation are still unknown. Considerable indirect evidence has been accummulated to implicate that the liver is the major site for the biosynthesis of serum glycoproteins. Werner (16) and Greenspan et al. (17,18,19,20,21) have provided indirect evidence that the liver is the site of serum glycoprotein synthesis by demonstrating decreased levels of serum glycoprotein in patients with liver disease or in subjects having chemically damaged livers. However, this sort of evidence will not explain whether the decrease in serum glycoprotein in either liver disease or chemically poisoned liver is due to a decreased rate of protein synthesis, which diminishes the synthesis of serum glycoprotein or whether it is due to the decrease in the synthesis of oligosaccharide chains or due to a decrease in the synthesis of peptide moiety as well as oligosaccharide moiety. Moreover, whether the biosynthesis of polypeptide and oligosaccharide moieties takes place simultaneously in the same place or not is still unknown. Spiro (23) and Shetlar et al. (25, 26) presented evidence that specific-activity-time-curves for liver and serum glucosamine meet the criteria for the precursor-product relationship as formulated by Zilversmit et al. (76) and suggested that liver is the site

of synthesis of serum glycoprotein. The results presented in Figure 1, show that hepatectomized rats failed to incorporate D-glucosamine-1-C-14 into serum protein while intact rats incorporate the radioactivity rather rapidly into serum protein. These results provide a further evidence that the liver is the major site for the biosynthesis of serum glycoprotein. Similar results have been observed by Macbeth et al. (38). Athineos, Kukral and Winzler (37) reported that when D-glucosamine-1-C-14 was administered to the intact and hepatectomized dogs, the α_1-acid glycoprotein of the intact dog serum was highly labeled with almost all of the radioactivity present in glucosamine. However, the identical glycoprotein isolated from hepatectomized dogs showed no radioactivity. They concluded that the liver is the sole site of biosynthesis of this glycoprotein. The results obtained from hepatectomized rats also agree with those of Sarcione (30) who demonstrated the production of α_1-acid glycoprotein by the perfused rat liver.

When radioactivity associated with subcellular fractions of rat liver was measured at various time intervals after injection of D-glucosamine-1-C-14, radioactivity was found to be rapidly incorporated into the microsomal fraction of rat liver (Figure 2). The radioactivity in the microsomal fraction reached its peak at two hours after the injection of radioactive glucosamine. Robinson, Molnar and Winzler (32) and Macbeth et al. (38) also reported similar findings, but their results showed that the radioactivity associated with the microsomal fraction reached its peak at one hour after the administration of radioactive glucosamine. The difference could be due to two reasons: (1) the animals used in this experiment were fasted for 24 hours before the experiments to reduce the contamination of the microsomal fraction by liver glycogen.

Winzler's group and Macbeth's group all gave food and water to the animals ad libitum before and after the administration of the radioactive tracer. (2) The animals in this experiment were anesthetized with Nembutal before the injection of the isotopes. The anesthesia usually lasted one to two hours. Winzler's group used ether anesthesia which only lasted for 10 minutes and Macbethe's group injected the isotope intraperitoneally into the animal without anesthesia. It is conceivable that both fasting and anesthesia would retard the metabolic processes, but the overall figure of the incorporation of D-glucosamin-1-C-14 would not be altered.

When the time courses of the incorporation of D-glucosamine-1-C-14 into subcellular fractions were compared with that of L-leucine-1-C-14, it was found that L-leucine-1-C-14 was incorporated much faster into the microsomal fraction than D-glucosamine-1-C-14. This suggests that the polypeptide and oligosaccharide synthesis do not occur at the same time in the microsomal fraction, although the synthesis of α_1-acid glycoprotein by perfused rat liver suggested that liver cells were able to synthesize both carbohydrate and polypeptide moieties of that particular glycoprotein. Further fractionation of the microsomal fraction led to the conclusion that the site of incorporation of D-glucosamine-1-C-14 into the protein moiety is the deoxycholate soluble fraction. In contrast to protein synthesis, which takes place on the ribosome (Figure 4), very little radioactivity could be found in the ribosomal fraction (deoxycholate insoluble fraction) after the administration of D-glucosamine-1-C-14 (Figure 6). The initial incorporation of L-leucine-1-C-14 took place in ribosomal protein and then progressively appeared in the deoxycholate soluble protein fraction. In the case of incorporation of D-glucosamine-1-C-14, the rate of incorporation, was slower than that of L-leucine-1-C-14 and almost all of the radioactivity

was associated with the deoxycholate soluble protein. It is generally believed that ribosomes are the initial subcellular site for incorporation of amino acids into growing peptide chains and that the completed peptide chain is then transferred to the deoxycholate soluble fraction of the microsomes (63,90,91). The delayed incorporation of D-glucosamine-1-C-14 may indicate that the oligosaccharide chain of glycoprotein is synthesized after the completion of polypeptide synthesis in the ribosomes. The present results, which indicate that deoxycholate soluble fraction as the site of incorporation of the glucosamine moiety agreed very well with Sarcione's results. Their results indicated that the subcellular site of hexose incorporation by utilizing the isolated rat liver (35) and the isolated deoxycholate soluble fraction (92) was also the deoxycholate soluble fraction of the microsomes. Molnar, Robinson, and Winzler (36), however, found that besides the labelling of deoxycholate soluble fraction there was also a definite labeling of the ribosomes when D-glucosamine-1-C-14 was used as the tracer. The incorporation of glucosamine on ribosome was reported to reach a maximum specific radioactivity somewhat earlier than the microsomal membrane. They also reported that specific activity of hexosamine was higher in the ribosomes than in the membranes. Therefore, it was concluded that both the ribosomes and the deoxycholate soluble fraction have the independent incorporation of glucosamine into glycoprotein. Cook, Laico and Eyler (41) studied the incorporation of amino acids and glucosamine into the smooth and rough endoplasmic reticulum of Ehrlich ascites carcinoma cell. They concluded that the biosynthesis of glycoprotein took place at the microsomes in two steps: First, the polypeptide was synthesized at the polysome, and then the polypeptide was linked by carbohydrate within the membranous complex. Sinohara and

Sky-Peak (40) attempted to isolate s-RNA capable of transferring glucosamine-amino acid *in vitro* or *in vivo* but were unsuccessful. They also found that glucosamine was incorporated into the deoxycholate soluble fraction of the microsomes and suggested that the carbohydrate moiety of glycoprotein was attached to the peptide after the completion of the polypeptide chain but before it was released to the cytoplasm.

The fact that glycoprotein is synthesized in the subcellular particle indicates that subcellular particles might contain glycoprotein. Generally, lysosomal, microsomal and mitochondrial fractions have a higher content of bound carbohydrate, including hexosamine, sialic acid, mannose, galactose and fucose. Nuclear and soluble fractions have a relatively low sugar content. Among those fractions examined, the lysosomal fraction is richest in carbohydrate. This finding is in agreement with the results of Novikoff's histochemical study (54). He demonstrated that the lysosomal particle gave the strongest periodate-schiff stain, and suggested that the substances responding to this stain are glycoprotein in nature. When the carbohydrate content of Walker tumor and that of rat liver are compared, the subcellular fractions of Walker tumor are generally richer in carbohydrate. The Walker 256 tumor might be the source of the extra glycoprotein increased in the rats bearing this tumor. In regard to the release of glycoprotein by tissues, the *in vitro* incubated medium from the experiment reported in Table 3 was also analyzed for protein bound radioactivity. The medium was subjected to paper electrophoresis and the protein-bound radioactivity measured with a low background counter. Only a trace of radioactivity was found in the α_1-globulin fraction. The experiment concerned with release of glycoprotein by the tissues is inconclusive because

only a trace of radioactivity was found. The tissue itself under these conditions, incorporated only one tenth to one twentieth of the radioactivity incorporated by the in vivo system in the same length of time, it is not surprising that very little protein-bound radioactivity was found in the medium.

After establishing that the major site of the incorporation of D-glucosamine-1-C-14 is the deoxycholate soluble fraction of liver microsomes the question arises as to how the glucosamine is linked to the protein in the microsomal membranes. The most direct approach to this question would appear to be to isolate the glycopeptide from the deoxycholate soluble fraction of the liver microsome, and to observe the nature of the linkage between the carbohydrate and residual amino acid. The sequential arrangement of sugars along this chain could also be determined. The work reported in this dissertation describes only a preliminary experiments concerned with this question. Pronase was chosen as a proteolytic enzyme, because this enzyme has a very broad substrate specificity and is capable of hydrolyzing most of the peptide-bonds in protein. The extent of hydrolysis of proteins by pepsin, trypsin, chymotrypsin and any other proteinase is limited to 10 to 30 per cent and then almost all the resulting digestion products are polypeptide or oligopeptides. The extent of hydrolysis of proteins by Pronase was estimated as 60 to 90 per cent by using casein, ovalbumin and wheat gluten as the substrate (64). After extensive digestion of the deoxycholate soluble fraction, about 80% of the material was digested. During the proteolysis, no cleavage of the sugar moeity from the glycopeptide was noticed as indicated by a constant ratio of neutral sugars to hexosamine in glycopeptide I and glycopeptide II. The presence of sialic acid in the glycopeptides interfered to some extent with proteolytic digestion

by Pronase. This interference by sialic acid also occures with other enzymes (35). The carbohydrate analysis of glycopeptide I or glycopeptide II showed that the ratio of bound hexoses to glucosamine was 4.3:1. In normal rat serum the ratio of neutral sugars to hexosamine is usually between 0.9:1 to 1.3:1 (93). Thus, glycopeptide II has less glucosamine relative to the hexoses than does serum protein. This may suggest that the glycoprotein synthesized in the deoxycholate soluble fraction of the liver microsomes is not a final product. There could be more reactions to complete the glycoprotein molecule before the partially completed glycoprotein is transferred from the liver microsomes to the blood stream. This concept is also suggested by the immunological studies on antigenicity of the deoxycholate soluble protein to the anti-rat serum antibody. If the deoxycholate soluble fraction of the liver microsomes contained the final product of some serum protein, one would logically expect to find the same precipitating bands produced by the deoxycholate soluble substances and the whole serum. This was not found in the immunoelectrophoretic experiment. It is possible that the sodium deoxycholate might cause the protein molecule extracted from liver microsome to lose its antigenicity. No work could be found in the literature about the effect of sodium deoxycholate on the antigenicity of proteins, but many investigations have been published to indicate that enzymes may be prepared from the liver microsomes by deoxycholate without an appreciable change in enzyme activity. In considering the point that both the enzyme activity and antigenicity of the protein are determined by the special conformation of the protein molecule, it would be hard to explain how sodium deoxycholate could destroy the antigenicity of some proteins but not decrease the enzyme activity of other proteins.

CHAPTER VI

SUMMARY

Incorporation of D-glucosamine-1-C-14 into the subcellular fractions of the rat liver showed a rapid incorporation of radioactivity into lysosomal and microsomal fractions. The radioactivity in these two fractions reached a plateau at 2 hours after the injection of isotope. The specific radioactivity of serum protein from the same series of animals increased somewhat more slowly and reached the maximum activity at 3 to 4 hours. On the basis of both the specific radioactivity and the total radioactivity, it is suggested that the liver microsomes are most active in incorporation of D-glucosamine-1-C-14 into glycoprotein. The experiment on hepatectomized rats showed that no appreciable radioactivity was incorporated into serum protein in the absence of the liver. This is a further evidence that liver is the major site for the biosynthesis of serum glycoprotein.

The general pattern of incorporation of L-leucine-1-C-14 into the subcellular fractions of rat liver was different from that of D-glucosamine-1-C-14 in two ways. (A) The average rate, in terms of time, of incorporation of L-leucine-1-C-14 into the microsomal fraction was much more rapid than that of D-glucosamine-1-C-14. (B) Incorporation of L-leucine-1-C-14 into the submicrosomal fraction showed a very quick labeling and disappearance of the radioactivity in the deoxycholate insoluble frac-

tion (ribosomal fraction) with a latter increase of radioactivity in the deoxycholate soluble fraction. In the case of incorporation of D-glucosamine-1-C-14, most of the radioactivity associated with the microsomes was found in the deoxycholate soluble fraction. Very little radioactivity was detected in the ribosomal fraction. This may suggest two steps in the biosynthesis of glycoprotein: (1) the synthesis of the protein moiety by ribosomes and (2) the attachment of D-glucosamine and other sugar moiety into the protein by the microsomal membrane.

Incorporation of amino acid and amino sugar into subcellular fractions of the Walker 256 tumor was indicated by time courses with tumor bearing rats. Slow but progressive incorporation of radioactivity into microsomal, mitochondrial and supernatant fractions was observed. The independent incorporation of the radioactive compound by the tumor tissue was shown in the hepatectomized rat bearing Walker tumor in vivo and tumor slices in vitro. When the microsomal fraction of Walker tumor, after labelling with D-glucosamine-1-C-14, was further separated into deoxycholate soluble and insoluble fractions, it was found that most of the radioactivity presented in the microsomes was associated with the deoxycholate soluble fraction.

The nature of the radioactivity after administration of D-glucosamine-1-C-14 was examined in microsomal, mitochondrial and supernatant fractions. Except for a small percentage of radioactivity converted into sialic acid, essentially no alteration of D-glucosamine-1-C-14 had occurred under these experimental conditions. The protein-bound radioactivity expressed as a percentage of total radioactivity associated with sialic acid at two hours after the administration of isotope was

5.5% in mitochondrial, 8.4% in microsomal and 13.0% in supernatant fractions. The radioactivity associated with hexosamine, expressed similarly, was 97.5% in mitochondrial, 116.1% in microsomal and 92.0% in supernatant fractions.

By means of Pronase digestion, a glycopeptide preparation was isolated from the deoxycholate soluble fraction of the rat liver microsomes. The yield of this glycopeptide was 0.8% by weight. However, 65.9% of the radioactivity associated with the deoxycholate soluble fraction was recovered in this glycopeptide. Chemical analysis of this glycopeptide showed that it was comprised of 60.6% hexoses, 14.9% glucosamine and 21.3% amino acids. Among the amino acids, aspartic acid occurred in an equimolar amount with glucosamine. Threonine, serine, glutamic acid, proline and glycine were present in equal molar quantities; each was present at about one fourth of the amount of aspartic acid. These results suggest that aspartic acid is very likely linked to glucosamine which is then attached to the rest of the sugar moieties in this glycopeptide preparation.

The distribution of protein-bound neutral sugars, hexosamine, and sialic acid in the subcellular fractions of the Walker 256 tumor was compared with those in the rat liver. The subcellular fractions of the Walker 256 tumor contained more bound carbohydrates than did those of the liver. The molar ratio of sialic acid to hexosamine and the molar ratio of galactosamine to glucosamine were higher in the subcellular fractions of the Walker tumor. Mitochondrial, lysosomal, and microsomal fractions of the rat liver contained strikingly greater amounts of mannose than of galactose; this difference was less marked in the subcellular fractions

of the Walker tumor. Lysosomal, microsomal, and mitochondrial fractions of both tissues were higher in bound carbohydrate constituents, including hexosamine, sialic acid, mannose, galactose, and fucose, than were the nuclear and soluble fractions.

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