hydrolysis or utilization of the energy of complex W. The Mg-stimulated ATPase of aged mitochondria presumably is mediated via W. Also W probably participates in ion transport and in mitochondrial swelling and contraction phenomena.

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The Subcellular Site of Hexosamine Incorporation into Liver Protein*

Edward J. Sarcione, Mary Bohne, and Marian Leahy

From the Division of Medicine, Roswell Park Memorial Institute, New York State Department of Health, Buffalo

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The incorporation of hexosamine into subcellular components known to be implicated in protein synthesis was investigated by adding [^{14}C]glucosamine to blood perfusing the isolated rat liver *in vitro*. The deoxycholate-soluble (membranous) protein fraction of microsomes proved to be a major site of hexosamine incorporation. Relatively little incorporation of hexosamine into either ribosomal or soluble cytoplasmic protein was observed. The isolation of radioactive glucosamine from glycopeptides derived from the membranous fraction of microsomes provided evidence that at least part of the hexosamine radioactivity was in fact incorporated into polypeptide chains of glycoproteins. These data support the conclusion that hexosamine or hexosamine-containing oligosaccharides are incorporated into completed peptide chains in the membranes of the endoplasmic reticulum, after their release from the ribosomes.

Considerable evidence is currently available to indicate that the ribosomes are the subcellular site of incorporation of amino acids into a growing peptide chain; this is followed by release of the completed polypeptide into the membranous portion of the microsomes (Littlefield and Keller, 1957; Kirsch *et al.*, 1960; Dintzis, 1961). However, relatively little is known about the subcellular sites and mechanisms involved in the completion of the tertiary structure of proteins such as folding, the formation of disulfide bonds, or the attachment of prosthetic groups such as carbohydrates.

Recent evidence that the liver is a primary site for the covalent incorporation of carbohydrates into serum glycoproteins has been obtained in several laboratories (Spiro, 1959; Shetlar, 1961; Sarcione, 1962, 1963; Robinson *et al.*, 1964). In a previous study (Sarcione, 1964), evidence was presented that galactose and mannose are incorporated into peptide chains within the membranous fraction of liver microsomes subsequent to completion of polypeptide synthesis by the ribosomes. Since an aspartyl-glucosamine linkage has been established in several different glycoproteins (Johansen *et al.*, 1961; Eylar, 1962; Izumi *et al.*, 1962), while galactose has been shown to occupy a penultimate position (Eylar and Jeanloz, 1962), it is possible that the subcellular site and mechanisms for the insertion of hexosamine into polypeptide chains may be different from that of hexoses.

In the currently accepted scheme for protein synthesis, a number of alternative pathways for the incorporation of hexosamine into polypeptide chains can be envisaged. For example, hexosamine could be first linked to aspartic acid and the activated complex incorporated into the growing peptide chain on the ribosomal template. Or, hexosamine could be incorporated into completed peptide chains while they remain bound to the ribosomal template. Finally, hexosamine could

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be inserted at specific sites along the completed polypeptide chain subsequent to its release from the ribosome. These possibilities have been discussed by Roseman (1962).

The present experiments were designed to differentiate among these alternative pathways for hexosamine incorporation into liver protein. The kinetics of incorporation of [^{14}C]glucosamine into liver subcellular components were examined after the addition of a tracer dose of [1-^{14}C]glucosamine to blood perfusing the isolated rat liver *in vitro*.

Experimental Methods

Liver Perfusion.—Liver donors were fasted male Sprague-Dawley rats weighing 300–400 g. Livers were excised and perfused with approximately 80 ml of diluted heparinized whole rat blood to which antibiotics (penicillin, 4 units/ml; streptomycin, 5 μg/ml; polymyxin, 6 μg/ml) had been added. The details of the perfusion technique and perfusion mixture have been described (Sarcione, 1962). Blood entered the liver by free flow through a cannula in the portal vein, at a pressure of 13–15 cm of blood. A cannula in the inferior vena cava returned blood to the reservoir, from which it was continuously recirculated through the liver. In all experiments, an equilibration period of at least 30 minutes was allowed after initiation of the perfusion. A tracer dose of 50 μc of D-[1-^{14}C]glucosamine (8.2 mc/mmole, obtained from the New England Nuclear Corp.) was injected through the rubber tubing into the perfusing blood just proximal to its entry into the liver. At intervals of from 3 to 15 minutes, minor and major liver lobes were ligated with previously positioned ligatures and removed rapidly. It was determined that two liver samples weighing 3–4 g each could be removed in this manner without impairing the perfusion of the remaining major lobe, which was used as the terminal liver sample. For the double labeling experiment, combined tracer doses of 50 μc [1-^{14}C]glucosamine and 250 μc of DL-[4,5-^{3}H]leucine (5.45 curies/mmole, obtained as the hydrochloride from New England Nuclear Corp.) were injected into blood entering the liver, and liver samples were removed 1.5, 3, and 7 minutes thereafter. For one group of experiments, whole livers were used after perfusion with [1-^{14}C]glucosamine for varying time intervals.

Isolation of Subcellular Components and Protein Fractions.—The microsomal and supernatant fractions of liver samples were obtained by homogenization in cold 0.35 M sucrose buffer A, followed by differential centrifugation in a Spinco preparative centrifuge, as described by Keller and Zamecnik (1956). Ribosomes were obtained from microsomes by extraction with deoxycholate as described by Kirsch *et al.* (1960). The proteins were precipitated from the supernatant, deoxycholate-soluble, and ribosomal fractions by the addition of two volumes of cold 1.8 M perchloric acid. After standing for 20 minutes, the protein precipitates were collected by centrifugation, washed twice by suspending in cold 0.6 N perchloric acid, dialyzed for 24 hours against distilled water at 4°, then lyophilized to dryness. The proteins were washed twice with 95% ethanol and defatted by extraction with ethyl ether–ethanol–chloroform (2:2:1) mixture at 50° for 30 minutes. The extraction was repeated once for 10 minutes, and the proteins were washed with ethyl ether and air dried. To avoid the possibility of removal of protein-bound hexoses by hydrolysis, RNA was removed by incubation with RNAase rather than by extraction with hot trichloroacetic acid. The defatted proteins were dissolved in water and adjusted to *p*H 7.4

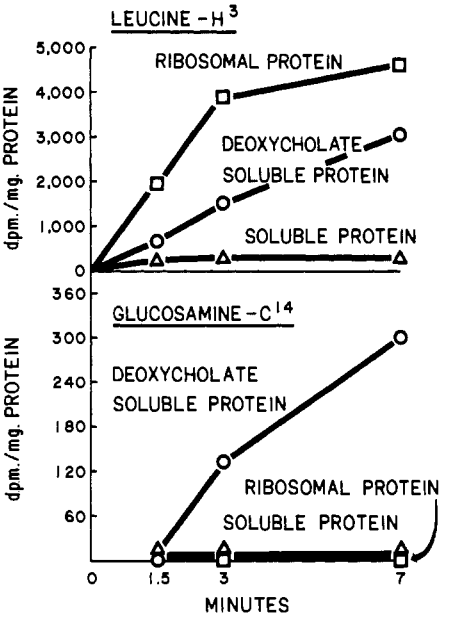

FIG. 1.—A comparison of incorporation of radioactivity from [^{3}H]leucine and [^{14}C]glucosamine into perchloric acid–insoluble protein fractions of subcellular components of sequential lobes of rat liver after addition of a combined tracer dose of DL-[4,5-^{3}H]leucine (250 μc) and D-[1-^{14}C]-glucosamine (50 μc) to blood perfusing the isolated rat liver *in vitro*. The radioactivity was added at zero time. The microsomes were fractionated with sodium deoxycholate.

with base, and 1.0 ml of phosphate buffer (*p*H 7.4) containing 100 μg of ribonuclease (Sigma) was added to each sample. The samples were incubated at 37° for 1 hour, dialyzed for 24 hours against distilled water at 4°, and lyophilized to dryness.

Isolation and Analysis of Glucosamine.—Glucosamine was isolated from the dried protein fractions and its specific activity determined by methods previously described (Sarcione, 1962). Preliminary experiments indicated that some subcellular protein fractions obtained from individual liver lobes occasionally yielded insufficient glucosamine for isolation; hence, a standard amount of unlabeled carrier glucosamine (5 μg/mg protein) was added to each protein fraction subsequent to acid hydrolysis and prior to chromatography on Dowex 50. When the entire perfused liver was used, no carrier glucosamine was added. Protein-bound hexosamine was determined by Rimington's modification (1940) of the Elson-Morgan method.

Isolation of Glycopeptides.—Glycopeptides were obtained from the 15-minute deoxycholate-soluble protein fraction of microsomes by a combination of pronase digestion followed by gel-filtration on Sephadex G-25 as described by Marks *et al.* (1962). The glycopeptides were isolated by preparative high-voltage electrophoresis carried out on sheets of Whatman 3 MM paper in 1 M formic acid at 40 V/cm for 1 hour. Guide strips were cut from edges of the sheets and stained with ninhydrin (0.25% in acetone) to locate the peptides. The central portion of the sheets was sprayed lightly with ninhydrin and heated at 90° for 5 minutes to locate the peptide bands precisely. These bands were cut from the paper and the peptides were eluted with 1 M NH_4OH by descending chromatography. The collected eluates were combined and lyophilized to dryness, and glucosamine was isolated by methods identical to those described above. Unlabeled carrier glucosamine (5 μg/mg original protein) was added to the pooled glycopeptide fraction subsequent to acid hydrolysis and prior to chromatography on Dowex 50.

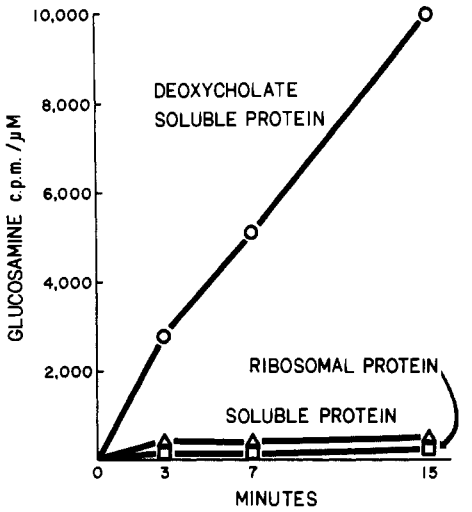

FIG. 2.—Incorporation of [^{14}C]glucosamine into perchloric acid–insoluble protein fractions of subcellular components of sequential lobes of rat liver after addition of a tracer dose of D-[1-^{14}C]glucosamine (50 μc) to blood perfusing the isolated rat liver *in vitro*. [^{14}C]Glucosamine was added at zero time. The microsomes were fractionated with sodium deoxycholate.

Radioactivity Measurements.—In the double-labeling experiment, 10 to 20 mg of finely ground protein fractions was suspended in 20 ml of scintillator gel solution containing 0.5% 2,5-diphenyloxazole, 0.01% *p*-bis-2′-(5′-phenyloxazolyl)benzene, and 400 mg Cab-O-Sil in toluene. The resulting suspensions were measured for ^{14}C and ^{3}H activity simultaneously with a Packard Tri-Carb liquid scintillation counter equipped with split channel operation as described by Rosenthal and Anger (1954).

RESULTS

A comparison of the kinetics of tritium and carbon-14 incorporation into liver subcellular protein fractions, usiug [^{3}H]leucine and [^{14}C]glucosamine to measure amino acid and hexosamine incorporation in a double labeling experiment, is shown in Figure 1. Initial incorporation of [^{3}H]leucine was into ribosomal protein. The specific activity of this fraction increased rapidly during the first 3 minutes of the experiment and then more slowly. The deoxycholate-soluble protein fraction of microsomes showed a slower but more prolonged increase in specific activity, while that of the soluble cytoplasmic protein fraction showed little or no change during this 7-minute interval.

In contrast, the pattern of [^{14}C]glucosamine incorporation into these protein fractions was markedly different from that of [^{3}H]leucine. A distinct delay of 1.5 minutes was observed before appreciable ^{14}C incorporation into the deoxycholate-soluble protein fraction of microsomes took place; this was followed by a rapid and progressive increase in specific activity during the remaining 5.5 minutes. Essentially no ^{14}C incorporation into either the soluble cytoplasmic protein or the ribosomal protein fraction was observed.

Figure 2 demonstrates that the specific activity of glucosamine isolated from the deoxycholate-soluble (DOC) protein fraction of liver microsomes, obtained from sequential lobes of the same liver, increased rapidly and progressively and was considerably higher than that of glucosamine found in either the soluble cytoplasmic protein or the ribosomal protein fraction at each interval studied. Since no determination was made on the amount of glucosamine present before the addition of carrier, the specific activities reported represent relative values for the protein-bound glucosamine, but give no indication of their true specific activities.

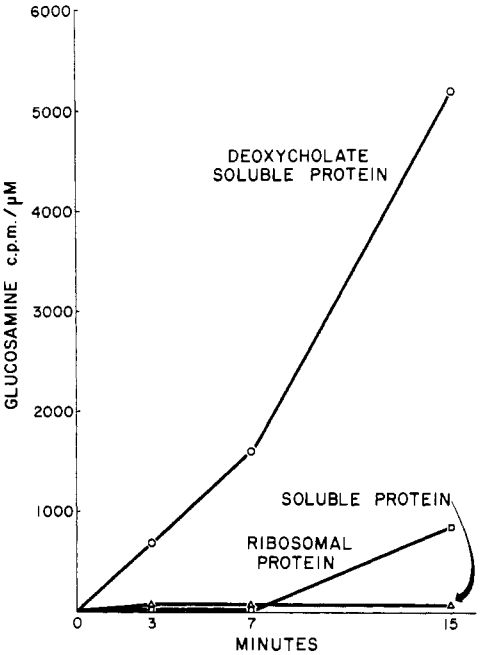

FIG. 3.—Incorporation of [^{14}C]glucosamine into perchloric acid–insoluble protein fractions of subcellular components of individual rat livers after addition of a tracer dose of D-[1-^{14}C]glucosamine (50 μc) to blood perfusing the isolated rat liver *in vitro*. [^{14}C]Glucosamine was added at zero time. The microsomes were fractionated with sodium deoxycholate.

TABLE I

DISTRIBUTION OF PROTEIN-BOUND HEXOSAMINE IN PERCHLORIC ACID–INSOLUBLE PROTEIN OF SUBCELLULAR COMPONENTS OF RAT LIVER

Liver Cell Fractions	Hexosamine (mg/100 mg of dry protein)
Soluble cytoplasmic protein	0.36
Deoxycholate-soluble protein of microsomes	0.76
Ribosomal protein	0.41

In attempts to assess the maximum dilution effect produced by added carrier glucosamine on the protein-bound glucosamine present, the distribution of protein-bound hexosamine in defatted perchloric acid–insoluble fractions of the liver subcellular components was determined (Table I).

To eliminate the possibility that in the previous kinetic experiment the addition of carrier glucosamine produced unequal dilution effects on the protein-bound [^{14}C]glucosamine, individual livers were perfused with [1-^{14}C]glucosamine for various time intervals and the glucosamine was isolated from the subcellular protein fractions without the addition of carrier glucosamine. Figure 3 demonstrates that the specific activity of the glucosamine derived from the deoxycholate-soluble (DOC) protein fraction of liver microsomes was again consistently higher than that isolated from either of the two remaining subcellular components. A small but definite increase in the specific activity of ribosomal protein-bound glucosamine is evident during the 7–15 minute interval. However, the pattern of [^{14}C]glucosamine incorporation is essentially similar to that shown in Figure 2.

To provide further evidence that the [^{14}C]glucosamine isolated from the 15-minute deoxycholate-soluble protein fraction of microsomes shown in Figure 1 was in fact incorporated into the protein, glucosamine was isolated from glycopeptides obtained by a combination of pronase digestion and gel-filtration on Sephadex G-25. The orcinol-positive, ninhydrin-positive glycopeptide fraction obtained with Sephadex G-25, when subjected to high-voltage electrophoresis in formic acid, was resolved into five ninhydrin-positive components (Fig. 4). These were isolated by preparative high-voltage electrophoresis and pooled, and the glucosamine was isolated after the addition of carrier glucosamine. The isolated glucosamine was radioactive and its specific activity was 11,500 cpm/μmole. This specific activity is quite comparable to that found (Fig. 2) in the glucosamine isolated from the deoxycholate-soluble protein fraction from which the glycopeptides were derived. A total of 4–6 amino acids were observed in each of the individual glycopeptides by hydrolysis in 6 N HCl for 18 hours at 105° followed by a combination of paper chromatography in 1-butanol–acetic acid–water (4:1:3) and high voltage electrophoresis in formic acid.

Discussion

Assuming that the polypeptide backbone of a glycoprotein is synthesized on the ribosomes and not by a separate protein-synthesizing system, then carbohydrates could be covalently incorporated into these polypeptide chains at several points in the known sequence of protein synthesis. If glucosamine of glucosamine-containing oligosaccharides were incorporated into the growing peptide chain at any step up to the completion of polypeptide synthesis at the ribosomes, but prior to peptide release, then the specific activity of ribosomal protein-bound glucosamine would be expected to be higher and would reach its maximum earlier than that in other subcellular protein fractions. If, on the other hand, hexosamine is inserted at specific sites along the completed peptide chain after release from the ribosomes, then the specific activity of protein-bound glucosamine would be expected to be minimal in the ribosomal protein. In the latter case, it would increase most rapidly at the actual site of glucosamine attachment.

In a recent study *in vivo*, Robinson *et al.* (1964) have demonstrated that [^{14}C]glucosamine is incorporated into protein in a liver particulate fraction which is then released into the plasma without appreciable accumulation in the soluble proteins of the liver. The present kinetic studies of the incorporation of [^{14}C]glucosamine are in accord with the view that the microsomes play a predominant role in its incorporation into protein. More precisely, the site important for this function was demonstrated to be the deoxycholate-soluble, or membranous, fraction of the microsomes, and not the ribosomes. In the double-labeling experiment, the patterns of [^{3}H]leucine and [^{14}C]glucosamine incorporation into protein were strikingly different. Initial incorporation of [^{3}H]leucine was into the ribosomal protein; this was followed by its progressive appearance in the deoxycholate-soluble protein of microsomes. In contrast, incorporation of [^{14}C]glucosamine was most rapid in the deoxycholate-soluble fraction, and virtually no incorporation was observed into the ribosomal protein.

The kinetics of [^{3}H]leucine incorporation into liver protein fractions observed in the present experiments are similar to those described by Littlefield and Keller (1957) for intact animals. These data strengthen the inference that the isolated perfused rat liver satisfactorily duplicates normal metabolic processes.

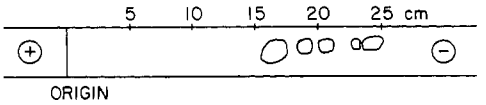

FIG. 4.—Paper electrophoretic pattern of the orcinol-positive, ninhydrin-positive effluent, obtained from Sephadex G-25, of a pronase digest of the deoxycholate-soluble protein fraction of liver microsomes (Whatman 3 MM paper, 40 V/cm, 60 minutes, 1 M formic acid, *p*H 1.87, strip stained with 0.2% ninhydrin in acetone).

It is well known that *N*-acetylglucosamine is an integral part of both glycoproteins and the acid mucopolysaccharides of mucoproteins. The existence of covalent linkages between the protein and carbohydrates of acid mucopolysaccharide-protein complexes are also known (Muir, 1958; Roden *et al.*, 1963). The isolation of radioactive glucosamine from glycopeptides derived from the deoxycholate-soluble protein fraction of microsomes provides evidence that at least part of the glucosamine radioactivity of this fraction was in fact incorporated into polypeptide chains.

The strikingly different patterns obtained for amino acid and glucosamine incorporation are in accord with the conclusion that hexosamine or hexosamine-containing oligosaccharides are incorporated into completed polypeptide chains after their release from the ribosomes, and not during synthesis of the growing peptide chain.

Since the most rapid turnover rate of protein-bound glucosamine was in the membranous component of microsomes, it is further postulated that the membranes of the endoplasmic reticulum are the actual site of covalent linkage of hexosamine to completed polypeptide chains. Thus, polypeptide synthesis and covalent incorporation of carbohydrates into polypeptide chains are considered to take place in two spatially separated cellular components.

The similarity of the data for hexosamine incorporation with those previously obtained for hexoses (Sarcione, 1964) suggests the generalization that the membranous portion of the microsomes is the site of incorporation of all the carbohydrate moieties covalently linked in glycoproteins.

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