STUDIES ON THE BIOSYNTHESIS OF ACUTE PHASE GLOBULINS OF RAT SERUM

by

F.E.ASHTON, B.Sc., M.Sc.

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

WINNIPEG, MANITOBA

AUGUST, 1972

TABLE OF CONTENTS

INTRODUCTION

Nomenclature. Carbohydrate-polypeptide complexes are widely distributed in nature and include such diverse substances as plasma proteins, proteins of mucous secretions, hormones, enzymes, cellular and extracellular membranes and both soluble and insoluble components of connective tissue. These substances vary widely in their content of carbohydrate and, in the past, a number of terms, such as mucoid, mucoprotein, chondroitin sulphuric acid and glycoprotein have been used to describe some of these complexes, different authors frequently using the same name to describe substances of different nature (1). Early classification of carbohydrate-polypeptide complexes divided these substances into groups according to the percentage of hexosamine that they contained (1-3) or the nature of the bond linking carbohydrate to polypeptide (4-6). In the latter classification Jeanloz (4) divided carbohydrate-polypeptide complexes into two distinct groups, namely those in which the linkage between carbohydrate and polypeptide is covalent, as is found in the carbohydrate-polypeptide complexes of serum, and those in which the carbohydrate is linked to polypeptide by easily split ionic forces. This latter

group was comprised of substances such as hyaluronic acid and chondroitin sulphates. However, further studies have shown that covalent linkages also exist in substances comprising the above group (5). It is therefore unlikely that the type of linkage joining carbohydrate to polypeptide provides a generally applicable criterion to distinguish between the two main groups of carbohydrate-polypeptide complexes present in animal tissues and body fluids.

In order to reduce confusion arising from the use of such nomenclature, Gottschalk (1), classified carbohydrate-polypeptide complexes according to characteristic structural features of their carbohydrate moieties. In the polysaccharide-protein complexes the carbohydrate is homo- or hetero-polysaccharide characterized by small repeating units and a high degree of polymerization; the carbohydrate may be linked to polypeptide by covalent or electrostatic bonds. Substances such as chondroitin sulphate and hyaluronic acid belong to this group. In the glycoproteins, on the other hand, the carbohydrate consists of a relatively small number of sugar residues, lacking a serially repeating unit and bound covalently to the polypeptide chain. Examples

of carbohydrate-polypeptide complexes belonging in this latter group are those found in serum and secretions from submaxillary glands together with substances such as ovomucoid and carbohydrate-containing polypeptide hormones. Spiro (6) has recently proposed a tentative classification scheme for the glycoproteins based on the amino acid involved in linkage with carbohydrate. Thus, four groups have been proposed in which the linkage involves asparagine (e.g. serum type of glycoprotein), threonine (e.g. mucins and blood group substances), hydroxylysine (e.g. plant cell walls) and hydroxyproline (e.g. collagens). According to the above classification the term glycoprotein can clearly be applied to describe a variety of substances having different properties and functions. However, since the work presented in this thesis is concerned with carbohydrate-polypeptide complexes found in serum, the term glycoprotein will be used to describe such complexes since their carbohydrate groups are relatively few in number, lack a repeating unit and are bound covalently to polypeptide which by virtue of size and amino acid composition would be referred to as a protein.

Serum glycoproteins: classification. Serum proteins are normally classified (7) according to their mobility on free moving boundary electrophoresis at pH 8.0 - 8.6, albumin being the fastest moving fraction followed by the α-, β- and γ-globulin fractions in order of decreasing mobility.

The occurrence of glycoproteins in mammalian tissues and body fluids was first recognized by Fruend in 1892 (8) in a fraction of serum not coagulated by heat. The name seromucoid was later applied to this substance by Zanetti (9) who isolated it by alcohol fractionation of serum. Material precipitated from human serum following addition of perchloric acid (final concentration of 0.6M), was found to be very similar to seromucoid (10). The seromucoid fraction of human serum contains a number of glycoprotein components, chief among these being α_1-acid glycoprotein (orosomucoid)* first isolated by Weimer, Mehl and Winzler (11). Mammalian serum has since been shown to contain a large number of distinct glycoproteins (1) indeed, it is becoming

* Orosomucoid is an α_1-acid glycoprotein which is the major component of the seromucoid fraction of serum. The seromucoid fraction consists of those serum proteins that are soluble in 0.6M-perchloric acid but precipitated by 5% phosphotungstic acid.

apparent that, with the possible exception of albumin, most other serum proteins contain carbohydrate, the α-globulins being particularly rich in glycoproteins. Over the past fifteen years interest in serum glycoproteins has been stimulated greatly as a result of the observation that there are noticeable changes in the levels of serum protein-bound carbohydrate in various pathological conditions where inflammation is involved (12,13). Consequently, with a view to explaining the significance of the response of serum glycoproteins to inflammation many workers have been studying the structure and biosynthesis of serum glycoproteins in normal animals and those suffering from a variety of inflammatory conditions. In order to describe this work clearly serum proteins are discussed below under the general headings of structure, biosynthesis and involvement in the acute inflammatory response.

Serum glycoproteins: general structure. The determination of the structure of serum glycoproteins is essential for an understanding of their mode of biosynthesis and their response to inflammation. Thus, several reviews have been published on the structure of serum glycoproteins (6,12,14-18).

Carbohydrate analysis of serum glycoproteins has shown that these compounds contain a characteristic group of sugars that include the neutral sugars D-galactose, D-mannose, L-fucose and, in a very few cases, D-glucose; the amino sugars D-glucosamine and D-galactosamine usually as their N-acetylated derivatives and the various derivatives of neuraminic acid (N-acetyl; N-glycolyl; N-acetyl-O-diacetyl and N, -O-diacetyl) collectively known as the sialic acids. The sialic acids or fucose have always been found to occupy a terminal position in the oligosaccharide chains of serum glycoproteins and, although the carbohydrate content of serum glycoproteins has been found to vary from a few percent to more than forty percent (6), the size of the carbohydrate groups is fairly constant ranging in molecular weight from about 2000 - 3500 (6). In the serum glycoproteins studied to date, the number of carbohydrate chains has ranged from two for transferrin (19) and human IgG immunoglobulin (20), three for fetuin (14) and calf throglobulin (21), five for human α_1-acid glycoprotein (22,23), nine or ten for human ceruloplasmin (24), thirteen for haptoglobin (25) and thirty-one for human α_2-macroglobulin (26). Despite the almost infinite variety

of structures that could be formed by sugars present in glycoproteins in terms of variations in sequence, linkages and branching, detailed structural studies of the carbohydrate groups of glycoproteins have indicated a relatively conservative utilization of sugar components, with certain preferred sequences recurring in many glycoproteins. A very common structural pattern is represented by the hetero-polysaccharide unit of serum glycoproteins such as bovine fetuin (27,28), human α_1-acid glycoprotein (22), α_2-macroglobulin (26), thyroglobulin (21) and IgG and IgA immunoglobulins (29,30). The carbo-hydrate units of these glycoproteins are extensively branched but, nevertheless, all have been found to contain terminal trisaccharides having the structure sialic acid (or fucose)-galactose-N-acetylgluco-samine (Fig. 1). This terminal triplet of sugars is linked to an inner core consisting mainly of mannose and N-acetylglucosamine.

The most extensively studied serum glycoprotein from a structural point of view has been human α_1-acid glycoprotein. It is generally believed that each oligosaccharide chain of human α_1-acid glyco-protein consists of fifteen to eighteen sugar residues (31) with 3-7 such chains per molecule of

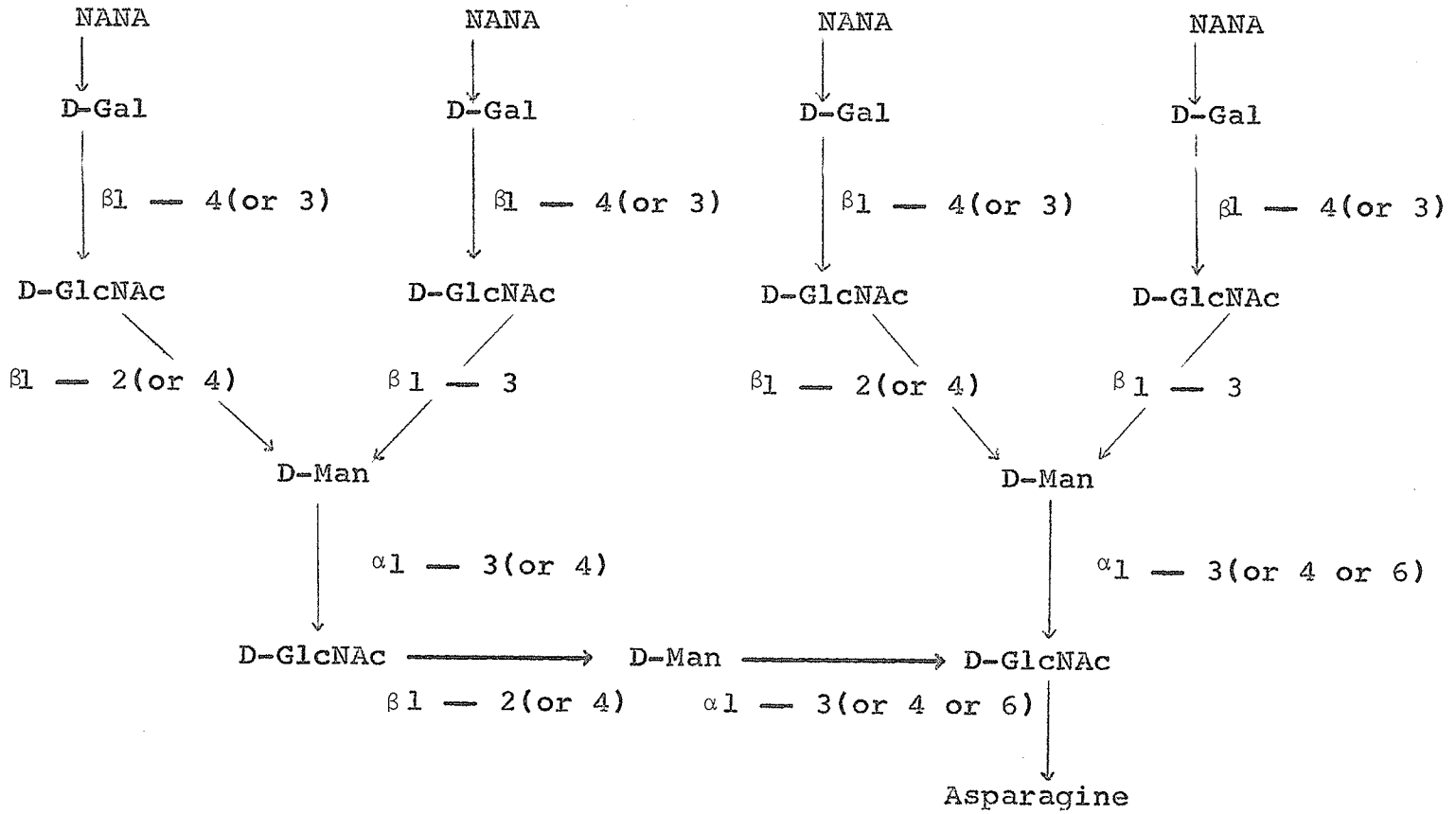


Fig.1 Proposed structure for a glycopeptide isolated from human α_1-acid glycoprotein (31).

glycoprotein (22,32). The structure of the oligosaccharide chains of human α_1-acid glycoprotein has recently been proposed by Wagh et al. (31), (Fig. 1). A similar structure has also been reported for one of the oligosaccharide chains of human transferrin (33). In the structure illustrated in Fig. 1, sialic acid occupies a terminal position and in the case of α_1-acid glycoprotein which contains about 10% sialic acid, is responsible for the low isoelectric point of that protein (13).

Studies on the structure of carbohydrate chains of serum glycoproteins have provided evidence that the carbohydrate units may appear in varying stages of completion or with minor modifications resulting in microheterogeneity of glycoproteins. This has been shown specifically with α_1-acid glycoprotein (22) and α_2-macroglobulin (26). Schmid (22) has reported that 5-9 polymorphic forms exist upon starch gel electrophoresis of α_1-acid glycoprotein. However, removal of the sialic acid reduces the number of forms to two. It has been suggested that this polymorphism is due to the positional isomerism displayed by the sialic acid which can be linked to either C-3, C-4 or C-6 of the galactose residues.

In serum glycoproteins the attachment of the

carbohydrate prosthetic group to the polypeptide chain is covalent and has been uniformly shown to involve the C-1 of N-acetylglucosamine and the amide nitrogen of asparagine. This type of linkage was originally described in ovalbumin (34,35) and was also found in IgG immunoglobulin (29), α_1-acid glycoprotein (32,36), fetuin (27) and thyroglobulin (21). The precise location of the carbohydrate units on the peptide chain depends on the complete elucidation of the amino acid sequence of specific glycoproteins. This has been accomplished for only a very limited number of serum glycoproteins including IgG myeloma immunoglobulin (37) and α_1-acid glycoprotein (38). Schmid et al. (38) determined the sequence of amino acids in two large glycopeptides from human α_1-acid glycoprotein. One glycopeptide was composed of 22 amino acid residues and one carbohydrate unit, and the other consisted of 65 amino acid residues and contained four carbohydrate units. All carbohydrate units were found to be attached to asparagine residues. In addition the carbohydrate units were linked to asparaginyl residues included in the general tripeptide -Asn-X-$\binom{\text{Ser}}{\text{Thr}}$, where -X- represents any amino acid. This supported earlier studies (23,31) in which partial

sequences of α_1-acid glycoprotein showed that threonine or serine residues were adjacent to, or near to, the asparagine-carbohydrate linkage. Similar serine and threonine residues have also been found in glycopeptides isolated from a variety of other serum glycoproteins of mammalian origin (39). The presence of the hydroxy-containing amino acids near to the asparagine residue involved in linkage with oligosaccharide has prompted the suggestion that these amino acids are recognized by the glycosyl-transferases responsible for attachment of the first carbohydrate to the asparagine residue in the polypeptide chain (39,40).

Biosynthesis of proteins in eucaryotic cells

Cell structure. Before describing a general scheme for the biosynthesis of proteins in eucaryotic cells it is necessary to first describe some of the morphological structures present in such cells. However, since eucaryotic cells can vary considerably in structure depending on species and tissue of origin the description below concerns the liver parenchymal cell in particular since this cell type is mainly responsible for the manufacture of most serum proteins (41).

The liver parenchymal cell (Fig. 2), consists of a nucleus which is separated from the cytoplasm by a double-layered nuclear membrane (42). The nuclear membrane possesses numerous aperatures and is believed to be connected to the endoplasmic reticulum which are the membranous components found in the cytoplasm. The nucleus has been shown to be the main site for transcription of ribonucleic acid (RNA) from deoxyribonucleic acid (DNA). Therefore, most of the cellular DNA is located in the nucleus although a small amount is located in the mitochondrion. The normal parenchymal cell contains about 800 mitochondria (42). A double membrane limits their structure, the inner membrane having folds called cristae which extend deep inside the structure. The mitochondria are the site of cellular respiration and intracellular energy production (42). About 20-25% of the total protein of the inner membrane consists of enzymatically active proteins functioning in electron transport and oxidative phosphorylation. The outer membrane contains the fatty acid thiokinases while the matrix contains enzymes involved in the Kreb's cycle (42). Isolated mitochondria from many types of eucaryotic cells have been found to incorporate labelled amino acids

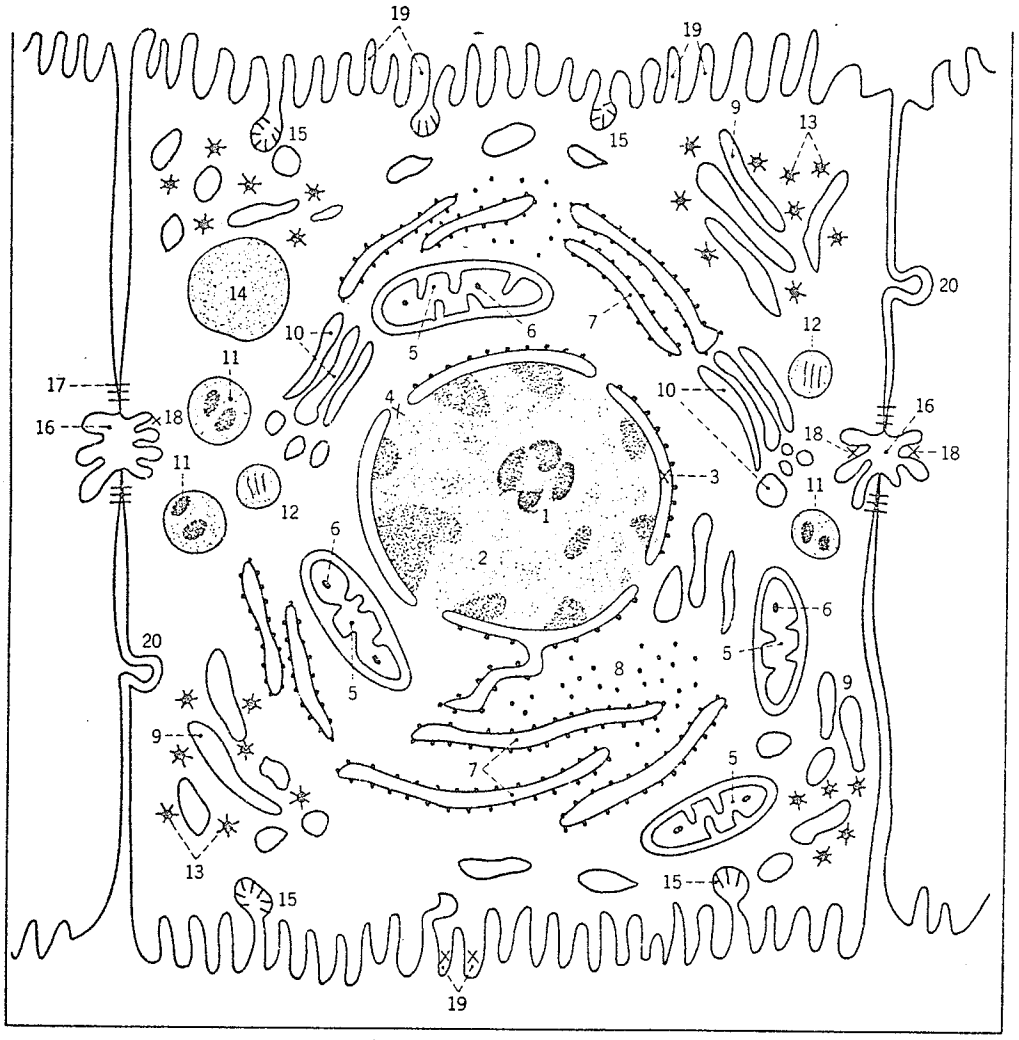


Fig. 2 Schematic view of hepatocyte

1. nucleolus. 2. nucleoplasm. 3. nuclear membrane. 4. nuclear pore. 5. mitochondrion. 6. mitochondrial granule. 7. rough surfaced endoplasmic reticulum. 8. free ribosomes. 9. smooth surfaced endoplasmic reticulum. 10. Golgi complex. 11. lysosome. 12. microbody. 13. glycogen particles. 14. fat droplet. 15. pinocytic invagination. 16. bile canaliculus. 17. junctional complex. 18. microvillus of the bile canaliculus. 19. microvillus of the sinusoidal border. 20. studlike projection of the cytoplasm.

into mitochondrial protein. That protein synthesis does occur in the mitochondrion is supported by the fact that the mitochondrion possesses a specific circular DNA, a DNA directed RNA polymerase, specific mitochondrial forms of t-RNA and activating enzymes, and ribosomes similar to those found in bacteria (42). In addition, it has been shown that mitochondria are capable of synthesizing their own glycoproteins (43). The nucleus and the mitochondrion, however, are not the intracellular sites concerned with the synthesis of most proteins produced by secretory cells such as the liver or pancreas. It is now well established that the biosynthesis of serum proteins in particular is carried out by ribosomes in association with endoplasmic reticulum present in the cytoplasm. The general structure of the endoplasmic reticulum is that of a network of parallel membranes making up tubules, and enclosing spaces or channels separated from the cytoplasm. These membranes are connected with one another to form a continuum (44-46). The endoplasmic reticulum is composed of rough and smooth membranes and Golgi apparatus. The rough membranes are studded with the protein synthesizing units, the ribosomes, (47) while the smooth membranes lack such particles. The smooth membranes are most clearly seen in certain

areas of the cytoplasm, particularly near the lateral plasma membrane or concentrated near areas of glycogen granules and around the Golgi apparatus. Another type of differentiated intracellular membrane is the Golgi apparatus (48), which is composed of a series of flattened or rounded smooth-surfaced cisternae, vesicles and vacuoles. In addition to being found attached to the endoplasmic reticulum, ribosomes are also found free in the cytoplasm. The isolation, structure and physical properties of ribosomes have been studied extensively in recent years (49,50). In eucaryotic cells ribosomes consist of two subunits having sedimentation coefficients of 60S and 45S giving a final particle with a sedimentation coefficient of about 80S. Magnesium is required to preserve the structural integrity of the ribosome (49,50). Each ribosome contains several protein molecules (50) which are thought to be responsible along with a close structural relationship with ribonucleic acid for the shape and compatability of the ribosome, allowing it to function in protein synthesis. Other cytoplasmic components include lysosomes (12), particles which contain a large number of hydrolytic enzymes, microbodies, glycogen particles, and fat droplets.

The recognition of various membranous components in the cytoplasm of the cell led to the establishment of methods to isolate and characterize these membranes. Claude (51-53) first isolated microsomes by the technique of differential centrifugation of postmitochondrial supernatant. The microsomal material consists of fragments of the endoplasmic reticulum, Golgi complex and disrupted plasma membranes. The first attempt to separate intact rough and smooth membranes from microsomes was made by differential centrifugation in 0.88M sucrose (54). Later, Rothschild (55) isolated rough and smooth surfaced membranes from rat liver by high-speed centrifugation on a discontinuous sucrose gradient. Dallner (56) showed that rough membranes could be more easily separated from smooth membranes by treatement with cesium ions. Cesium ions tend to bind specifically to rough membranes rendering them more dense, and consequently they are easier to separate from smooth membranous material. Smooth membrane fractions were subsequently prepared by treatment with magnesium ions which bound to some smooth membrane components, but not to others. In smooth membranes by zone centrifugation on stabalized

sucrose gradients. More recent techniques have led to the isolation of fractions containing mainly Golgi complex (58).

Thus the isolation and characterization of these various membranous components has led to more detailed studies on the biosynthesis of proteins at the subcellular level.

General scheme for biosynthesis of proteins. An outline of the mechanism of protein synthesis as it is thought to occur in eucaryotic cells is shown in Fig. 3. It is thought that the actual mechanism of protein synthesis is similar to that determined for prokaryotic cells. However, the overall process of protein synthesis in eucaryotic cells appears to be much more complicated than in prokaryotes. This complexity arises from the unique feature of eucaryotic cells, i.e., the spatial separation of transcriptional and translational processes afforded by the sequentering of the first of these into the nuclear structure.

The nucleus is the site of transcription of messanger RNA (mRNA) from DNA molecules. The mRNA chain is synthesized, directly utilizing the corresponding section of DNA as a template; therefore,

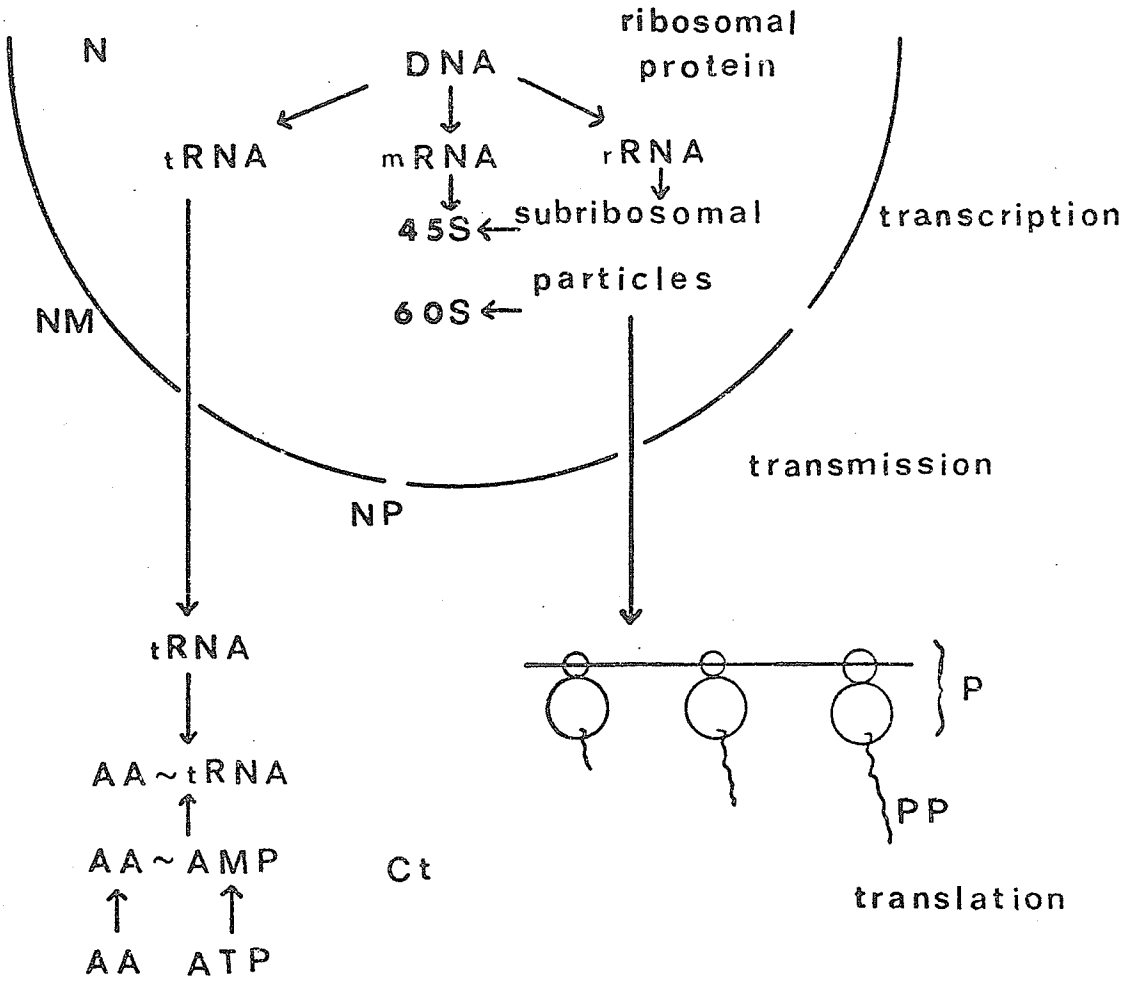


Fig. 3 Protein synthesis as it is thought to occur in eucaryotic cells. N, nucleus, NP, nuclear pore, Ct, cytoplasm, AA, amino acid, ANP, Adenosine monophosphate, ATP, Adenosine triphosphate, PP, polypeptide chain, P, membrane bound polysome, NM, nuclear membrane.

the DNA determines the nucleotide sequence in the mRNA chain being synthesized and the entire specificity of the RNA formed (59,60). The presence of RNA polymerase is essential for mRNA transcription (61,62). In addition, transfer RNA (tRNA) and ribosomal (rRNA) are synthesized in the nucleus. It has been shown that ribosomal RNA (rRNA) is a major product of gene transcription in nucleated cells (63). Of all the species of DNA-like RNA synthesized in the nucleus it seems that only a small number are transferred into the cytoplasm (64,65). The transfer of mRNA from the nucleus into the cytoplasm may be brought about by the formation of a complex with the smaller (45S) of the two ribosomal subunits. This process has been termed transmission (66). The 45S-mRNA complex when in the cytoplasm combines with the larger subunit (60S) to yield the polysome directly (67, 68). In the intact cell only polysomes bound to membranes are the major sites of protein synthesis (69). It is clear that pools of polysomes, monosomes, and native ribosomal subunits exist in eukaryotes (70-72). Studies have shown that the free subunits are in equilibrium with those in polysomes (70,73), and it is likely that cyclic

dissociation of ribosomes into subunits occurs between rounds of polypeptide chain initiation in mammalian cells as in bacterial cells (e.g. 74). The membranes of the endoplasmic reticulum, to which the ribosomes are attached, may undergo turnover rates comparable to those of the ribosomes (68,75).

The formation of the polypeptide chains on the membrane bound polysomes is believed to be similar to that in bacteria and occurs in four main stages. In the first stage of protein synthesis the amino acids are activated and then transferred to their corresponding tRNA molecules by the action of specific aminoacyl-tRNA synthetases (76). The aminoacyl-tRNA complex is the intermediate form from which activated amino acid residues are transferred into the growing polypeptide chain in the ribosome. The formation of aminoacyl-tRNA from the free amino acids and t-RNA molecules occurs outside the ribosome in the cytoplasm (77). The process is catalyzed by the enzyme aminoacyl-tRNA synthetase and occurs in two steps. In the first step the free amino acid reacts with ATP, resulting in the production of aminoacyl adenylate and pyrophosphate (78,79). The aminoacyl adenylate is then bound to the specific

synthetase and this complex then reacts with tRNA (80) as the carboxyl of the amino acid residue of aminoacyl adenylate is transferred to the 3'-OH group of the ribose of the terminal adenosine of tRNA with a release of AMP (81).

The second stage of protein synthesis, called initiation of polypeptide chain synthesis, is thought to occur as found in bacteria. The ribosome must be dissociated into its subparticles so that the mRNA can be bound to the 45S subunit before chain initiation will begin (72). Should there exist an AUG (or GUG) codon at the 5' end of the mRNA, then formylmethionyl-tRNA associates with its codon on the 45S subparticle forming a stable ternary complex of the 45S-AUG-formylmethionyl-tRNA. There is evidence that formylation of $tRNA^{Met}$ is not necessary for initation of protein synthesis in Hela cells (82). Three initation factors (M_1, M_2 and M_3) have been found to be required in this process. The function of these initation factors are uncertain; M_1 and M_2 are capable of stimulating the binding of Met-$tRNA_f$ to reticulocyte ribosomes (83) in the presence of AUG. Also, M_3 is required for the translation of natural mRNA (84), which indicates that its function may be related to

selecting or recognizing natural mRNA. The 60S subparticle then associates with the 45S subparticle to form the complete ribosome, thus prepared to perform the next stage of polypeptide synthesis.

Elongation of the polypeptide chain in eukaryotes is thought to be similar to that found in prokaryotes. As a result of the tRNA translocation and the corresponding drawing over of the template, the aminoacyl-tRNA binding site becomes vacant and a triplet is positioned on it. Then the aminoacyl-tRNA binding site of the small subparticle, with the positioned triplet adjacent to the initiating codon, selectively binds the aminoacyl-tRNA corresponding to this triplet. As a consequence the initial complex ribosome-mRNA-$(\text{aminoacyl-tRNA})_2$ arises (85). The peptide bond is formed by reaction of the amino group of the newly bound aminoacyl-tRNA with the esterified carboxyl group of the carboxyl terminal amino acid residue of the peptidyl-tRNA. This reaction occurs by a nucleophilic displacement in which the parting group is the tRNA. In bacteria an enzyme, peptidyl transferase, which is part of the 50S portion of the ribosome, is required to catalyze the reaction (86). This whole process is

repeated using the specific amino acid bearing tRNA molecules until termination of the polypeptide chain occurs.

Stage IV involves the termination of the polypeptide chain. The release of polypeptide chains from the ribosome has been shown in vitro (87,88) to be due to "nonsense" triplets, i.e., triplets that do not code for any of the amino acids. Similar evidence exists for the termination of protein synthesis in bacterial protein synthesis where the nonsense "amber" and "ochre" mutations show that the triplets UAA and UAG possess a terminating function (89). The final release of the polypeptidyl-tRNA from the ribosome, when a termination codon is reached, is promoted by a specific release factor which is bound to the ribosome and promotes the hydrolysis of the ester linkage between the polypeptide chain and tRNA molecule (90). The ribosome then runs off the mRNA in free form, dissociates into its subparticles and re-enters the cycle for protein synthesis.

Biosynthesis of Serum Proteins. It has been known for some time that the biosynthesis of proteins in mammalian cells occurs on the ribosomes present in

the cytoplasm of the liver parenchymal cell according to the general scheme mentioned above.

The first evidence that the liver was the main site of synthesis of serum proteins came from the studies of Peters and Anfinsen (91) who demonstrated that label was incorporated into chicken serum albumin from ^{14}C-labelled CO_2 in a chicken liver slice system. They also demonstrated a net increase in serum albumin during incubation (92). Miller and his coworkers (93, 94) perfused rat livers with blood to which ^{14}C-lysine had been added; radioactivity was incorporated into all electrophoretically separated fractions of serum with the exception of the γ-globulins. With liver perfusion or slice techniques or in tissue culture experiments, the liver has since been shown to synthesize a large number of specific serum proteins including rat serum albumin (95,96), a rat α-globulin fraction (97), rat (acute phase) α_2-glycoprotein (98), human and monkey α_1-acid glycoprotein (99), rabbit and human haptoglobin (99,100), rat and human transferrin (99,101), rat (93) and human fibrinogen (102) and mouse and rat ceruloplasmin (103,104). Although the above studies have clearly implicated the liver as the main site of synthesis of serum glycoproteins, they

did not indicate the subcellular site of synthesis of these proteins nor the manner in which they are secreted from the cell. Zamecnik and Keller (105) first demonstrated the incorporation of label into protein when the isolated microsome fraction from rat liver was incubated with labelled amino acids and a source of regenerating adenosintriphosphate (ATP). These workers further showed that the microsome fraction from rat liver isolated at various times after administration of L-leucine-^{14}C contained the most radioactive protein. In addition they found that when the microsome fractions were treated with deoxycholate which solubilizes the membranous components of the fractions but not the ribosomes, it was the ribosomes that were active in protein synthesis (106,107).

Among the proteins present in serum, albumin comprises about 40% of the total protein. This readily facilitates its isolation in a homogeneous state so that it may be used for studies on its biosynthesis. In addition, serum albumin is not a glycoprotein and consists of only one polypeptide chain. Consequently the biosynthesis of this protein has been studied extensively. Several workers have shown that albumin is synthesized preferentially

on ribosomes attached to membranes of the endoplasmic reticulum, rather than on free ribosomes (69, 108-111). Studies by Peters (112, 113) on the incorporation of L-leucine-^{14}C into subcellular fractions from rat liver have shown that the synthesis of albumin occurs on the rough endoplasmic reticulum. It appears that albumin is synthesized in association with ribosomes in the first few minutes (112, 114), and is transferred to the cisternae of the rough endoplasmic reticulum and is subsequently channeled into the smooth endoplasmic reticulum and then to the Golgi apparatus, with 15-20 minutes elapsing before the appearance of radioactivity in the blood. Similar studies by Glaumann and Ericsson (111) have confirmed the sequential passage of albumin through the membranes. However, an understanding of the biosynthesis of conjugated proteins, such as glycoproteins, require considerations beyond those encountered in the biosynthesis of simple proteins such as albumin. These arise from the fact that in addition to the synthesis of the polypeptide chain, the carbohydrate units must be assembled and attached in some manner to the polypeptide portion. Workers generally agree that the polypeptide backbone of glycoproteins is synthesized

according to the well established pathway of protein synthesis previously described. The attachment of sugars to the polypeptide chain has been studied with great fervor in the past few years. With D-glucose-^{14}C as precursor Spiro (115) was the first to show that the rat liver was the major site of addition of glucosamine residues to serum proteins. This was later confirmed by studies on the intact rat and rabbit with D-glucosamine-^{14}C as precursor (116,117). It was found in these studies that glucosamine was used efficiently for the biosynthesis of glycoproteins, with some conversion to sialic acids but negligible conversion to neutral hexoses and amino acids. Although these studies showed that the liver was the site of attachment of carbohydrate groups to polypeptide chains, they did not indicate the mechanism or subcellular site of attachment of sugars to polypeptide. Numerous studies have since been carried out to elucidate the mechanism of attachment of sugars to polypeptide chains. These studies have resulted in the postulation of two main theories as to the glycosylation of protein and completion of the oligosaccharide chains in glycoproteins. According to one hypothesis the carbohydrate chain is built up in the Golgi cisternae

of mammalian cells by a multienzyme complex of carbohydrate transferases which is localized in the membranes of the Golgi apparatus. This hypothesis has been termed the single-site hypothesis. This idea is based on the original observations of Neutra and Leblond (118) who injected ^{3}H-labelled glucose or galactose into rats, and found that macromolecules, other than glycogen, were labelled only in the Golgi zone of liver and other tissues. Supporting this interpretation, Wagner and Cynkin (119), and Caccam, Jackson and Eylar (120) have reported that incorporation of N-acetylglucosamine and mannose in endogenous proteins is carried out by the smooth microsomal fractions (rich in Golgi fragments), but not by the rough microsomal fractions of rat and rabbit liver. Similar observations have been reported by using Hela cells (121). According to the other hypothesis (multi-site hypothesis) glycosylation of proteins is initiated on the rough membranes and the remainder of the oligosaccharide chain is assembled within the smooth membranes and the Golgi apparatus. Early evidence that this type of glycosylation of glycoproteins occurred came from work by Robinson, Molnar and Winzler (116) and Molnar, Robinson and Winzler (122) who studied the incorpora-

tion of D-glucosamine-^{14}C into liver subcellular fractions *in vivo* and observed that there was incorporation of label into components of rough membrane subfractions in addition to incorporation into smooth membrane subfractions. Subcellular acquisition of carbohydrate groups by glycoprotein was confirmed and further extended by Lawford and Schachter (123) who studied the kinetics of incorporation *in vivo* of D-glucosamine-^{14}C into protein-bound hexosamine and sialic acid of rat subcellular fractions and of rat plasma proteins. These studies indicated that hexosamine was incorporated into glycoprotein in the channels of both rough and smooth endoplasmic reticulum whereas sialic acid was incorporated primarily within the smooth endoplasmic reticulum. In addition, it was found that D-glucosamine-^{14}C was incorporated into rat liver ribosomes *in vivo*, indicating that some glucosamine was incorporated into nascent ribosome-bound polypeptide. Other studies by Molnar and his coworkers (124,125) and Redman and Cherian (126) have supported the multi-hit theory. These workers investigated the incorporation of radioactive sugar nucleotides into subcellular fractions of liver from rats and rabbits. The rough microsomal fraction was more

active than the smooth microsomal fraction for the incorporation of hexosamine and mannose, but only one-third as active for the incorporation of D-galactose-^{14}C. On the other hand D-galactose-^{3}H was taken up 3-4 times faster by the smooth microsomes than by rough microsomes. Further studies by Tetas, Chao and Molnar (127) confirmed these results. In addition, it was suggested by these workers that a sugar acid labile lipid intermediate is involved in the biosynthesis of glycoproteins, rather than a direct transfer of the sugar to the acceptor molecule.

Wagner and Cynkin (128) studied the ability of the Golgi apparatus isolated from rat liver to mediate steps in the biosynthesis of glycoproteins. These workers studied five enzymatic activities: the transfer of glucosamine, galactose, glucuronic acid, mannose and N-acetylneuraminic acid from their sugar nucleotide precursors to endogenous trichloroacetic acid-precipitable proteins. Their results were consistant with the hypothesis that the N-acetylglucosamine, galactose and N-acetylneuraminic acid residues of the terminal sialic acid-galactose-N-acetylglucosamine sequence of many glycoproteins (12) are added to nascent glycoprotein within the Golgi

apparatus while the sugar residues in the inner positions of the oligosaccharide chains are inserted at other sites in the endoplasmic reticulum. Similar results have also been obtained by Schachter *et al.*(129) who suggested that enzymes present in the Golgi apparatus are involved in terminating the synthesis of α_1-acid glycoprotein by the liver during secretion from the cell.

Much of the evidence that has accumulated for the mechanism of biosynthesis of glycoproteins has come from studies by several workers on the biosynthesis of thyroglobulin in thyroid slices. Spiro(130) used puromycin to inhibit synthesis of the peptide portion of soluble thyroglobulin. Puromycin, however, did not appreciably interfere with the synthesis of the carbohydrate portion of the protein indicating that the peptide portion is synthesized before and independently of carbohydrate attachment. Examination of the particle-bound thyroglobulin precursors isolated after incubation of thyroid slices with puromycin again showed that the synthesis of the peptide portion was completely inhibited and indicated that the synthesis of the carbohydrate portion was partially impaired, with the sugars more internally located in the carbohy-

drate units being affected more than those in more peripheral locations. Since the material on the particles represents a more recently synthesized material than that present in the soluble fraction, these findings were consistant with a depletion of membrane-bound precursors, and suggested that the attachment of the carbohydrate groups to the polypeptide chain takes place in a stepwise manner by the sequential attachment of sugar residues to a series of membrane bound precursors (130). Attachment of carbohydrate groups to a polypeptide chain in this manner has also been suggested by other studies on the biosynthesis of thyroglobulin (131-134). Herscovics (131) found that labelled mannose is incorporated on subunits of thyroglobulin at about the time of their synthesis, whereas labelled galactose is incorporated over 30 minutes later near the time of aggregation of subunits to form non-iodinated thyroglobulin. Similarily, labelled fucose is incorporated into thyroglobulin at the terminal stage of synthesis of the protein (132-133). Radioautography studies (134) have indicated that mannose incorporation takes place within the rough endoplasmic reticulum of thyroid follicular cells, whereas galactose addition occurs within the Golgi

apparatus after migration of thyroglobulin precursors to that site.

Of the two theories existing on the biosynthesis of glycoproteins the multi-hit theory has gained acceptance from recent investigations. The concept that sugar molecules are added to the polypeptide chain while it passes through the endoplasmic reticulum has led to a search for glycosyltransferases which are capable of transferring the carbohydrate groups to the precursor protein molecule. Indeed, enzymes responsible for the attachment of several carbohydrate units have been described. Thyroid particles have been shown to contain the enzymes responsible for the assembly of the sialyl-galactosyl-N-acetylglucosamine sequences of the oligosaccharide chains present in thyroglobulin (130,135 136). Sialyltransferases were first described by Roseman et al. (137,138). A membrane-bound sialyltransferase has been demonstrated in liver and subsequently characterized (129,139). These sialyltransferases have been shown to incorporate sialic acid into partially deglycosylated α_1-acid glycoprotein (129) or fetuin preparations (139). Other enzymes found to incorporate sugars into glycoprotein are galactosyltransferases (129,140), N-acetylglucos-

aminyltransferases (129,141,142) and a fucosyltransferase (143). A series of studies by Hudgin and Schachter (144-146) have described the properties of a pork liver and blood serum sialytransferase (144), galactosyltransferase (145) and N-acetylglucosaminyltransferase (146). It was found that in both liver and serum a single sialytransferase is responsible for incorporation of sialic acid into α_1-acid glycoprotein, fetuin and N-acetyllactosamine and sialic acid incorporation occurs whenever a terminal galactose linked (1,4) to a penultimate N-acetylglucosamine is presented to the enzyme. Indication that similar glycosyltransferases exist in serum as were found in livers was first noticed by Mookerjea et al. (147). Studies by Hudgin and Schachter (144-146) have also indicated that similar enzymes exist in pork liver and serum. However, the function of serum glycosyltransferases have yet to be elucidated. From the numerous studies performed on the biosynthesis of glycoproteins it appears that such molecules are synthesized in the following manner. The polypeptide chain is synthesized on the ribosome (polysomes) by the usual machinery for protein synthesis. The polypeptide chain then moves through the channels of the endoplasmic reticulum

(148). As the protein moves through these channels, membrane bound glycosyltransferases, firmly attached to membranes, attach sugar molecules one at a time from an activated nucleotide sugar to the growing carbohydrate chain. The enzyme specificity is directed toward several properties of the acceptor, foremost among which is the nature of the terminal nonreducing sugar of the carbohydrate chain to which the new sugar is attached. The penultimate sugar, as well as the linkage of the terminal sugar to penultimate sugar may also have a determining role. Eventually the glycoprotein passes into the Golgi apparatus where terminal sugars are added and the glycoprotein is concentrated and packaged into granules which are secreted into serum.

Serum glycoproteins: Their involvement in the acute inflammatory response.

Inflammation in mammals, caused by chemical inflammatory agents, neoplastic diseases, bacterial infections, and a variety of other conditions results in an increase in the total protein-bound carbohydrate of serum arising from increases in the concentration of a variety of serum glycoproteins

(Table 1). However, in spite of the well documented response of serum glycoproteins to the inflammatory stimulus, little is known about the biochemical mechanisms that lead to the increased content of certain glycoproteins in serum as a result of inflammation. With a view to obtaining some information on the mechanism and significance of the response of serum glycoproteins to inflammation, several groups of workers have studied the events that take place in the body in response to inflammation, Glenn, Bowman and Koslowske (159) have separated the process of inflammation into two reactions, the local reaction and the systemic reaction (Fig. 4). The local reaction refers to the events occuring in the immediate area of tissue damage, whereas the systemic reaction describes events induced by the local reaction. Glenn et al. (159) suggested that the local events are "contributory", or events which initiate the overall process, whereas the systemic responses are "protective" or "inhibitory"; that is events which protect the body or slow down the overall process of inflammation.

The local reaction. When injury occurs in a tissue blood flows into the wound and forms a clot as

TABLE 1

Total Hexose and Hexosamine of Glycoprotein in Normal and in Some Pathological Sera

Sera from normal and diseased individuals	Total glycoprotein hexose (mg/100ml serum)	Total glycoprotein hexosamine (mg/100ml serum)	References
Normal	100	66	(149)
Advanced cancer	195 ± 25	203 ± 12	(150)
	177 ± 8.8	131.9 ± 56	(151)
Diabetes	193	153	(152)
	174 ± 24.7	145.2 ± 23	(153)
Rheumatic fever	190 ± 1.9	100.4 ± 3.5	(154)
Rheumatoid arthritis	215	-	(155)
	153 ± 3	128 ± 3	(156)
Tuberculosis	204 ± 15.6	211 ± 9	(150)
Cholera	272 ± 7	-	(157)
Pregnant women	272 ± 4	-	(158)
Acute leukemia	157.5 ± 19.8	127.5 ± 12.3	(151)
Hodgkin's disease	189.3 ± 38.9	141 ± 30.1	(151)
Lymphosarcoma	218.2 ± 32.7	142.5 ± 23.4	(151)
Multiple myeloma	255 ± 76.1	165 ± 9.5	(151)

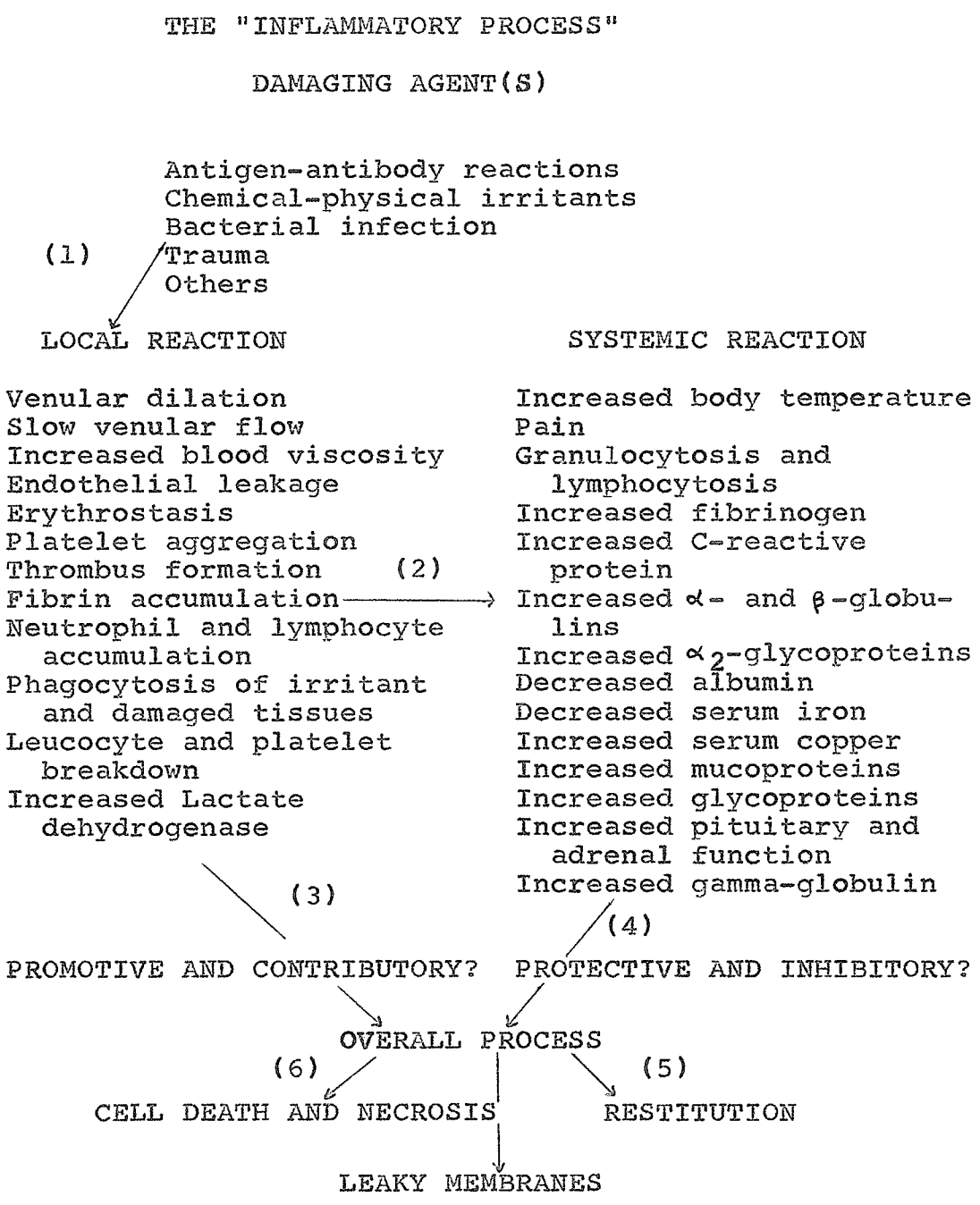


Fig. 4 Schematic representation of the inflammatory process. The numbers in parenthesis describe the order in which the events are believed to occur.

fibrinogen molecules from the blood quickly link up into interconnected strands of fibrin. After the clot has formed, inflammation begins as dying and broken tissue produce substances that cause blood vessels in the nearby uninjured area to leak (160). The resulting flow of serum into the injured area provides a maintaining environment for white blood cells that follow into the wound. These white blood cells are mainly neutrophils and macrophages which kill bacteria and remove debris from the injured area by a process of phagocytosis (160). The release of lysosomal enzymes during the inflammatory response is thought to be important in activating cutaneous collagenase which degrades collagen in structural components of the tissue into soluble breakdown products which drain away from the area of tissue damage (160). Eventually another kind of cell, the fibroblast, appears in the wound. The fibroblast then repairs the injured tissue by synthesizing and secreting collagen and protein polysaccharides that form scar tissue (160).

The systemic response. According to the scheme put forward by Glenn et al. (159), (Fig. 4), the local reaction by some unknown mechanism, is believed to

induce the systemic response to inflammation. The systemic response consists primarily of increases in the levels of a variety of serum glycoproteins (Fig. 4). The proteins that increase in content in serum as a result of inflammation are normally referred to as acute phase reactants (161). The most important acute phase reactants present in human serum are orosomucoid, ceruloplasmin, haptoglobin, transferrin, fibrinogen, α_2-macroglobulin, and the γ-globulins (162). In addition to the above acute phase reactants, new serum proteins not normally present in serum, make their appearance; these include the C-reactive protein in man (163) and the α_2-(acute phase) globulin in rats (149,164). The response of an α_1-acid glycoprotein corresponding to orosomucoid and the α_2-(acute phase) globulin to experimentally induced inflammation in rats has been studied extensively by several groups of workers (161,164-166). Darcy (164,165) found a 7-fold increase in the α_1-acid glycoprotein of rat serum in response to turpentine induced inflammation and a 20-fold increase due to growth of Walker tumor. It was originally suggested by Darcy (167) that the increase in serum α_1-acid glycoprotein was a response to substances liberated from damaged necrotic

cells. The presence of α_1-acid glycoprotein in the area of tissue damage prompted the suggestion that the serum glycoprotein response to inflammation resulted from the release of glycoproteins synthesized locally in the inflammed tissue (168). However, it has since been established that the liver is the site of synthesis of most of the acute phase reactants including rat α_1-acid glycoprotein and α_2-(acute phase) globulin (169-172). Thus, the increase in concentration of these proteins appears to be a direct consequence of increased hepatic synthesis, suggesting that the liver is involved in some aspects of the control of the inflammatory reaction. It is now generally believed that hormonal factors that may be released from the site of inflammation, stimulate the liver to increase the synthesis rates of acute phase glycoproteins that are normally present in serum (163,167,173). This may be accomplished by some "switching on" and "switching off" mechanism affecting the biosynthesis of acute phase globulins in mammalian liver (98). However, the precise mechanism of this process is not understood at the present time and is currently under investigation in many laboratories.

Introduction to the work presented in this thesis

Many studies have been performed on the effect of inflammation on serum proteins of various animal species. Thus, it is well documented that there is an increase in the content of certain glycoprotein fractions in the serum of animals in response to inflammation. However, very little is known about the biochemical mechanism behind the increase in production of serum glycoproteins. Therefore, the present studies have been designed to follow the biosynthesis of a specific glycoprotein at the subcellular level in response to inflammation in order to eventually determine what biochemical process is responsible for the increase in content of glycoproteins in serum as a result of inflammation.

In order to perform these studies it was necessary to isolate in a homogeneous state one or more serum glycoproteins that increase in content in response to inflammation. Therefore, crude protein fractions were obtained from serum from normal and experimental animals by ion-exchange chromatography on columns of diethylaminoethyl cellulose. These fractions were analyzed for protein-bound hexose and hexosamine and were examined by immunoelectrophoresis and electrophoresis on cellogel strips.

The fraction which showed the greatest increase in protein-bound carbohydrate was subsequently used as starting material for the isolation of specific acute phase reactants. Serum albumin, and α_1-acid glycoprotein and an α_2-macroglobulin were isolated from the starting material by a combination of ion-exchange chromatography, sephadex gel filtration and isoelectric focusing. The isolation and analyses of the α_1-acid glycoprotein and the α_2-macroglobulin were performed in collaboration with Mr. A.D. Friesen who reported some of these results for the degree of Master of Science. These results have also been reported briefly in this thesis in order to maintain the continuity of this presentation.

The quantitative precipitin technique was used to determine the content of albumin, α_1-acid glycoprotein and α_2-macroglobulin in serum samples obtained from control rats and rats suffering from inflammation for 5-72h. The α_1-acid glycoprotein and α_2-macroglobulin were found to be acute phase reactants increasing about 6-fold as a result of inflammation; the greatest increase occurred at 24-48h after induction of inflammation; albumin did not change in content as a result of inflammation. Studies on the incorporation of L-leucine-^{3}H and

D-glucosamine-^{14}C into heterogeneous serum fractions and into the three specific proteins were performed to study the rates of synthesis of these proteins in response to inflammation. Labelled leucine was used as a precursor for polypeptide synthesis, while labelled glucosamine was used as a precursor for carbohydrate synthesis, since it is used efficiently for the synthesis of serum proteins and also because N-acetylglucosamine residues participate both in linkage with polypeptide in serum glycoproteins and occur in more peripheral positions of the carbohydrate chains. It was found that there was an increase in incorporation of labelled precursors into the two acute phase proteins under study when isolated from the serum of experimental animals when compared with corresponding proteins isolated from control animals. There was negligible change in incorporation of labelled leucine into serum albumin. These results suggested that there may be an increase in the rate of synthesis of the acute phase globulins under study in response to inflammation.

The increase in content and the increase in the incorporation of labelled leucine and glucosamine into α_1-acid glycoprotein and α_2-macroglobulin in response to inflammation indicated that it

was important to study the biosynthesis of specific acute phase globulins at their cellular and subcellular sites of synthesis in the organ responsible for their manufacture. However, before detailed studies could be undertaken, the cellular and subcellular site of synthesis of the proteins under examination in the present work had to be located. Studies on the site of synthesis were restricted to the α_1-acid glycoprotein, an acute phase reactant, and albumin, a protein which does not respond to inflammation. In experiments in vivo, tissues were removed at appropriate times after administration of L-leucine-^{3}H and D-glucosamine-^{14}C and subjected to subcellular fractionation. The non-ionic detergent Lubrol-W* was used to extract tissues or subcellular fractions. Immunodiffusion studies, involving the use of antisera to albumin or α_1-acid glycoprotein, followed by radioautography were performed to determine the site of synthesis of the two proteins. Since the microsome fraction of liver proved to be the main or sole site of synthesis of the α_1-acid glycoprotein and albumin, the quantitative precipitin technique was used to determine the

* Lubrol-W is a polyoxyethylene ether of general formula $R.(O.CH_2.CH_2)_n.OH$ where R is cetyl or a mixture of cetyl radicles oleyl radicles and n is approximately 16.

content of α_1-acid glycoprotein in microsome material from livers from normal rats and rats suffering from inflammation. A five to six-fold increase in content of α_1-acid glycoprotein was found in extracts of microsome material from experimental animals when compared with controls; the greatest increase occurring at 8-12h following inflammation; there was little change in the content of microsomal serum albumin as a result of inflammation. Because of the large increase in content of acute phase glycoproteins in livers from experimental animals an electron microscope study was performed in order to determine if there were any changes in appearance of intracellular structures in liver cells from experimental animals as a result of the increased content of α_1-acid glycoprotein found in liver microsome material isolated from such animals. Changes were observed, particularly in the membranous components of the cytoplasm, which were consistent with an increase in the storage capacity of liver for proteins destined for secretion from the cell in livers from experimental animals.

Incorporation of labelled leucine and glucosamine into α_1-acid glycoprotein and albumin isolated from

rough and smooth membranes and the Golgi apparatus was studied in order to determine the pathway of secretion of these two proteins. It was found that there was no difference in the pathway of secretion or rates of secretion of the proteins under study in experimental animals when compared to controls. These results suggested that the increase in content of α_1-acid glycoprotein found in serum following induction of inflammation was likely to result from increased production or rate of synthesis rather than increased secretion or changes in the pathway of secretion from the liver into serum.

EXPERIMENTAL

Materials

Radioactive compounds. n-hexadecane-1,2-^{3}H (2.46μCi/g), n-hexadecane-1-^{14}C (1.06μCi/g), L-leucine-4,5-^{3}H (1000mCi/mM), L-leucine-^{14}C (10mCi/mM), D-glucosamine-1-^{14}C hydrochloride (3.1mCi/mM) and D-glucosamine-1-^{14}C hydrochloride (55mCi/mM), promethium-147 hydrochloride, Amersham Searle, Toronto, Ontario.

Chromatographic and electrophoresis media. Carboxymethylcellulose (CM-cellulose) and diethylaminoethylcellulose (DEAE-cellulose) were prepared as described by Peterson and Sober (174) from Whatman cellulose powder, CF11 (W & R. Balston, Ltd., London, England) seived to 200 mesh before use; Sephadex and Sepharose 4B, Pharmacia (Canada) Ltd., Montreal; starch hydrolyzed for gel electrophoresis, Connaught Medical Research Laboratories, Toronto, Canada; Nobel agar, Difco Laboratories, Detroit, Michigan; Cellogel strips and Cellogel blocks, Consolidated Laboratories (Canada) Ltd., Weston, Ontario and Colab Laboratories, Inc., Chicago, Illinois; ampholine carrier ampholytes, LKB-Producter AB,

Stockholm-Bromma, Sweden; Dowex 50 and Dowex 2, Sigma Chemical Co., St. Louis, Mo.; acrylamide and N,N' methylenebisacrylamide, Eastman Organic Chemicals, New York; Cyanogum 41 (b-dimethylamino-propionitrile), Fisher Scientific Co., Toronto.

Detergents. Lubrol-W flakes, Imperial Chemical Industries Ltd., Blackley, Manchester, England; sodium deoxycholate, Sigma Chemical Co., St. Louis, Mo.

Proteins and Sugars. Bovine serum albumin (crystalline), human serum albumin, D (+) galactose (crystalline), D (+) mannose (crystalline), D (+) glucosamine HCl, D (+) galactosamine HCl, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, N-acetyl-neuraminic acid (type IV) and N-glycolylneuraminic acid, Sigma Chemical Co., St. Louis, Mo.; human glycoprotein fraction VI, Pentex Inc., Kankakee, Illinois; human plasma, Red Cross Blood Bank, Winnipeg, Manitoba; human α-globulins and horse apo-ferritin, Mann Research Laboratories, Orangeburg, New York.

Chemicals for scintillation counting. 2,5-Diphenyloxazole (PPO) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), Packard Instrument Co., Inc., Illinois; Bio-solv solubilizer (BBS-3), Beckman Instruments Inc., Toronto, Canada.

Other chemicals and materials were obtained as follows: Dextran T70, Pharmacia (Canada) Ltd., Montreal; 1-dimethylaminonaphthalene-5-sulphonyl chloride (DANS), glucose-6-phosphate, ribonucleic acid (type IV), Sigma Chemical Co., St. Louis, Missouri; Freund's complete adjuvent, Difco Laboratories, Detroit, Michigan; X-ray film, Ilford Ltd., Ilford, Essex, England; turpentine oil, double rectified, Fisher Scientific Co., Toronto, Canada.

Methods

Animals

Male hooded rats of 300-350g body weight were purchased from the Quebec Breeding Farm Inc., St. Eustache, Quebec. Rats were maintained on a diet of Purina laboratory chow and tap water and were starved for 16h prior to sacrifice. Albino rabbits of about 2.4Kg body weight were obtained from Western Laboratory Animal Breeders, Springfield,

Manitoba. Rabbits were maintained on Purina rabbit chow and an automatic water supply.

Physical measurements

Extinctions in the visible region of the spectrum were measured with a Unicam SP600 spectrophotometer. Extinctions at 280mμ were measured with a Beckman model DB spectrophotometer. Fluoresence was measured with a Turner model 110 Fluorometer with a 360mμ primary filter and a 510mμ secondary filter. Measurements of pH were made with a radiometer model 28b pH meter. Measurement of radioactivity was with a Packard Tri-carb scintillation spectrometer (model 3320). Aqueous solutions of protein (up to 0.4ml solution and 3mg protein) were added to 10ml scintillation cocktail containing 0.7% PPO, 0.036% POPOP, 10ml BBS-3, and 90ml toluene. Mixtures of ^{3}H and ^{14}C were counted concurrently with red and green channels at pulse height settings of 10-1000 divisions (60% gain) and 100-1000 divisions (4.5% gain), respectively. Efficiences, as determined with standard ^{3}H-hexadecane and ^{14}C-hexadecane, were 37% and 33% for ^{3}H and ^{14}C, respectively, in the red channel and 0.05% and 53% for ^{3}H and ^{14}C, respectively, in the green channel.

When ^{14}C was counted alone a pulse height setting of 10-1000 divisions (4.5% gain) was used giving an efficiency of 85%. Quenching was tested for using the machine automatic external standard or with ^{3}H- and ^{14}C-labelled n-hexadecane as internal standards. With few exceptions, the standard deviation of the net count rate was not greater than $\pm$ 5%.

Ultrafiltration

The procedure used was based on that of Sober et al. (175). Samples were reduced to small volumes by ultrafiltration through dialysis tubing immersed in an appropriate dialysis medium in an evacuated chamber.

Dowex ion-exchange resins

Dowex 50 and Dowex 2 were washed successively with organic solvents as follows: acetone-water (1:1, v/v), acetone, petroleum ether (40^{o}-60^{o}); acetone and acetone-water (176). Dowex 50 was converted to hydrogen form by washing successively with 2N-NaOH, water, 2N-HCl and water. The procedure was repeated. Dowex 2 was first converted to hydroxyl form by washing with 6N-NaOH and was then washed with water. It was converted to the formate

form by washing successively with 2N-HCl, water and 2M-sodium formate until the effluent gave a negative test for chloride (177) and was finally washed with water.

Chemical analyses

Protein. Protein was determined by the method of Lowry et al. (178), but with modified reagents and volumes as described by Miller (179). Crystalline bovine serum albumin was used as standard.

Protein-bound hexose. For direct determination of total protein-bound hexose the method of Winzler (150) was used scaled down to a total volume of 4.75ml. An equimolar mixture of galactose and mannose was used as standard; extinctions were read at 540mμ.

Qualitative and quantitative analyses for sugars. Proteins were analyzed for sialic acid, hexose and hexosamine using a method based on that described by Simkin et al. (180).

For qualitative and quantitative analyses of sialic acid a solution containing 1-2mg glycoprotein in 2.0ml 0.05N-H_2SO_4 was heated in a glass stoppered

tube at 90° for 60 minutes. The hydrolysate was applied to a 7.0cm x 1.0cm column of Dowex 2 (formate form). The tube was washed with 4 x 0.5ml water, the washings being applied to the column, and then the column was eluted with a further 2 x 2.5ml water. The eluate was discarded. The sialic acid was eluted with 15ml 0.3N formic acid; the eluate was collected in a pear shaped flask. Formic acid was removed from the eluate by evaporation *in vacuo* at 35° to one-third the original volume. The solution was diluted to the original volume with water and again reduced to one-third the original volume. The process was repeated. Finally, the eluate was evaporated to dryness *in vacuo* at 35°. The residue obtained is referred to as the sialic acid fraction.

For qualitative and quantitative analyses of hexose and hexosamine, glycoproteins were first hydrolyzed with Dowex 50 and HCl (181). Dowex 50 (H^+ form) was washed with five volumes 0.46N HCl and then suspended in an equal volume of 0.46N HCl. The glycoprotein (1-2mg protein) dissolved in 0.25ml water was mixed with 0.6ml of the Dowex 50, 0.46N HCl resin suspension and placed in a 14cm x 1cm bomb tube. The tubes were sealed and heated in an

oven at 100° for 30-36h. The tubes were positioned at an angle of 45° and rotated through 180° about twenty times during hydrolysis to mix the contents (180). For fractionation of hydrolysates an arrangement was made such that the filtrate from a small funnel fitted with a glass wool plug could pass through a 7.0cm x 0.5cm column of Dowex 50 (H^+ form) and the effluent from the latter could pass directly through a similar column of Dowex 2 (formate form). The hydrolysis mixture was transferred to the small funnel and the bomb tube washed with 5 x 0.5ml water and the washings transferred to the funnel. The filtrates were allowed to pass successively through the Dowex 50 and Dowex 2 columns. The resin in the funnel was then washed with a total of 12.5ml water applied in 1-2ml volumes, each wash being allowed to flow through both columns before the next wash was applied. The effluent from the Dowex 2 column was collected and evaporated to dryness *in vacuo* at 35°. The residue is referred to as the neutral sugar fraction. When the flow of liquid through the Dowex 50 column had stopped the column was clipped off and 1.0ml water applied to the top of the column. The resin in the funnel containing the glass wool plug was then washed successively with 0.25ml 2N HCl,

2 x 0.25ml water and 0.25ml 2N HCl and the eluate allowed to pass into the water on the top of the Dowex 50 column. When all the washings had passed through the funnel the clip on the Dowex 50 column was removed and the liquid allowed to pass through the column; the eluate was discarded. The Dowex 50 column was then eluted with 3ml 2N HCl (182) to elute hexosamine; the eluate was collected and evaporated to dryness *in vacuo* at 35°. The residue is referred to as the hexosamine fraction. In some experiments a uronic acid fraction was prepared after elution of the neutral sugar fraction from the Dowex 2 column. This was accomplished by passing 8ml 1.2N formic acid through the column (180). The eluate was evaporated to dryness as directed for the preparation of the sialic acid fraction.

For qualitative analysis the neutral sugar, sialic acid, hexosamine and uronic acid fractions were examined by paper chromatography. Downward development at room temperature was employed for all chromatographic procedures. Whatman No 1 was used for examination of the sialic acid and hexosamine fractions, whereas Whatman 3MM was used for examination of the neutral sugar fraction. Butan-1-ol: acetic acid: water (4:1:5 v/v) was employed as

solvent in all cases. In addition, butan-1-ol: pyridine; water (6:4:3 v/v) was employed for examination of the sialic acid fraction and butan-1-ol: ethanol; water (10:1:2 v/v) was employed for examination of the neutral sugar fraction. Sialic acid was detected by spraying with the periodate-thiobarbituric acid system of Warren (183). Hexoses were detected by spraying with aniline phthalate reagent (184). Hexosamines were detected by spraying with Ehrlichs reagent as described by Partridge (185) and uronic acids were detected by spraying with benzidine-trichloroacetic acid-HCl (186).

For quantitative analysis of sialic acid the periodate-thiobarbituric acid method of Aminoff (187) was employed. N-Acetylneuraminic acid was used as standard and the method was scaled down to a maximum of 25μg N-acetylneuraminic acid. Hexose was determined by the orcinol method of Winzler (150) with an equimolar mixture of galactose and mannose as standard; the method was scaled down to a total volume of 4.75ml. Hexosamine was determined by the method of Rondle and Morgan (188) with glucosamine HCl as standard; the method was scaled down to a total volume of 5.0ml.

In the above quantitative and qualitative

experiments, two kinds of control were included. In the case of hydrolysis with Dowex 50 and HCl for the preparation of neutral sugar and hexosamine fractions, the assay solution for hydrolysis was replaced with 0.25ml of water or 0.25ml of a solution containing 2mg bovine serum albumin, 50μg each of galactose and mannose and 50μg or 100μg of glucosamine HCl. In the case of hydrolysis with 0.05N H_2SO_4 for the preparation of a sialic acid fraction a blank containing sulphuric acid alone and a solution containing 2mg bovine serum albumin and 50μg N-acetylneuraminic acid were prepared.

Determination of ribonucleic acid. Ribonucleic acid was recovered from liver subcellular fractions according to the method of Munroe and Fleck (189). To 3ml of subcellular fraction of liver was added 1.5ml ice cold 0.6N-perchloric acid. After 10 minutes in ice the precipitate was collected by centrifuging at 1000 r.p.m. for 5 minutes. The supernatent was discarded and the sediment washed by resuspension followed by centrifuging with iced 0.2N-perchloric acid. To the residue was added 2.4ml 0.3N-potassium hydroxide and incubation allowed to take place at 37^{o} for 60 minutes. After incubation,

1.5ml 1.2N-perchloric acid was added to precipitate the protein and deoxyribonucleic acid. After 10 minutes in ice the precipitate was separated by centrifugation and washed twice with 3ml 0.2N-perchloric acid. The original supernatent was combined with the washings and made to 25ml with water. The amount of ribonucleic acid was determined by the orcinol method (190) using ribonucleic acid as standard.

Assay for glucose-6-phosphatase activity. The method of Hubscher and West (191) was used to assay subcellular fractions for glucose-6-phosphatase activity. Sodium fluoride was added to samples of subcellular fractions to inhibit acid phosphatase, while ethylenediaminetetraacetic acid (EDTA) was added to inhibit alkaline phosphatases. Incubation mixtures (1.0ml total volume) contained 40mM-maleic acid-36mM-NaOH, pH 6.5, 4mM-NaF, 4mM-EDTA, 30mM-glucose-6-phosphate and enzyme in the form of a suspension of a subfraction of liver containing 1-3mg protein. Incubation was allowed to proceed for 15 minutes at 37° and the reaction was terminated by the addition of 5% (w/v) trichloroacetic acid. After centrifuging at 2° samples of supernatant were assayed for

phosphate by a scaled-down modification of the method of Allen (192).

Electrophoretic methods

Cellogel. Cellogel electrophoresis was performed with 20cm x 5cm strips or 12cm x 3.5cm x 0.3cm blocks of gelatinized cellulose acetate by the procedure and with apparatus described by Kohn (193). The buffer contained 8.3mM diethylbarbituric acid and 41.6mM sodium diethylbarbiturate, pH 8.6. For analytical electrophoresis on Cellogel strips, 3-5µl of rat serum or a 5% solution of protein were applied per centimeter width, and a potential of 140 volts was applied for 5.5h at room temperature. Strips were stained for protein by immersing in a solution containing 0.5% naphthalene black (194) in water-methyl alcohol-acetic acid (5:4:1 by volume) for 5 minutes. Strips were decolorized by washing in water-methyl alcohol-acetic acid (50:35:7 by volume) and were stored in wash solution. Strips were stained for carbohydrate by the periodic acid Schiff technique (193). For preparative electrophoresis on Cellogel strips 1-1.5mg protein were applied per centimeter width and electrophoresis was for 4.5h. Protein was detected by staining narrow strips cut

from each edge of the strip. The section containing the protein to be isolated was removed, cut into small pieces, and eluted by shaking with 3 x 1ml water over about 5-6h. Solid material was removed by centrifuging at 2000 r.p.m. for 5 minutes; the supernatant was dialyzed against water and freeze-dried. When Cellogel blocks were used for preparative electrophoresis 10-12mg protein were applied per centimeter width and electrophoresis was for 1.5h at 4°. Protein was recovered as described above.

Starch gel. For starch gel electrophoresis the horizontal procedure of Smithies (194) was followed. The gels were formed from a mixture of 11g of starch-hydrolyzed and 100ml of a buffer containing 76mM Tris-5mM citric acid, pH 9.0. The gels were allowed to form in troughs measuring 18.5cm x 5.0cm x 0.6cm. Samples (0.5-1.0mg protein) were applied on Whatman 3MM filter paper inserted into a slit in the gel located 5cm from the cathode. The buffer in the electrode and bridge compartments of the tank contained 300mM boric acid-50mM sodium hydroxide, pH 8.2. Whatman 3MM filter paper was used as a bridge between the electrode compartments

and the gel. A potential of 170 volts was applied for 5h at 2°. Gels were stained for protein with naphthalene black (194).

Polyacrylamide gel. Electrophoresis on polyacrylamide gels was performed in two ways. For use as an analytical technique disc electrophoresis was performed by a modification of the method of Ornstein (195) and Davis (196). The separating gel was formed by mixing equal volumes of solutions A and B; a suitable volume of the mixture was then added to an equal volume of solution C before use. Solution A contained 1.5M-tris, 0.24N-HCl and 0.12ml cyanogum 41 catalyst (b-dimethylaminopropionitrile) per 100ml, pH 8.9; solution B contained 28g acrylamide and 0.735g N,N' methylenebisacrylamide per 100ml and solution C contained 0.14g ammonium persulphate per 100ml. The gel columns measured 6.0cm x 0.5cm and contained a final buffer concentration of 0.375M tris, 0.06N HCl, pH 8.9, and a final acrylamide concentration of 7%. The electrode compartments of the tank contained 0.38M glycine, 0.05M tris, pH 8.2. Solutions of protein (10-50μg) dissolved in 25μl 10% sucrose containing 5% bromophenol blue were layered on top of the gel columns under a 0.5cm

column of tank buffer. A current of 4ma per gel column was applied for 1-2h until the bromophenol blue had migrated to the bottom of the gel. Gels were stained for protein with naphthalene black as described by Smithies (194) and for carbohydrate by the periodic acid Schiff method described by Clarke (197). For determination of molecular weights dodecyl sulphate-polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborne (198).

Isoelectric focusing

Isoelectric focusing was performed by the method of Vesterberg and Svensson (199). Experiments were performed with pH 4-6, pH 4-5 ampholine carrier ampholytes or with a pH 1-3 gradient system. A 110ml isoelectric focusing column (LKB 8100-10) and a gradient mixing device (LKB 8121) obtained from LKB Producter AB, Stockholm-Bromma-1, Sweden was used in all experiments. The following method was used with ampholine carrier ampholytes. A dense electrode solution containing 0.2ml concentrated sulphuric acid, 12g sucrose and 14ml distilled water was added to the anode at the bottom of the column. A sucrose gradient containing the carrier

ampholytes was slowly introduced into the column using the LKB gradient device. The dense gradient solution contained 28g sucrose, 1.9ml ampholine carrier ampholytes and 42ml water. The light gradient solution contained 0.6ml ampholine carrier ampholytes in 60ml water. The final concentration of carrier ampholytes in the gradient was 1% (w/v). After about 50ml of the gradient had entered the column, the sample (10-20mg protein), dissolved in 1ml water was added to the light gradient solution. When the column had filled the light electrode solution consisting of 10ml NaOH (1% w/v) was added to the cathode at the top of the column. About 3h were required to fill the column. All procedures were performed at 2° with water at 2° circulating through the cooling jacket of the column. When isoelectric focusing was completed the valve at the bottom of the column was closed and the column emptied by the lower exit at a flow rate of about 1-2ml/min. Fractions of 1ml were collected by hand. The pH and extinction at 280mμ of each sample was determined. Appropriate fractions were pooled and concentrated to about 5ml by ultrafiltration with concurrent dialysis against water as described by Sober et al. (175). Samples were finally freeze

dried. When isoelectric focusing in pH 4-5 gradients was required, pH 4-6 ampholytes were first focused alone and the pH 4-5 region of the gradient recovered for subsequent use.

For isoelectric focusing in pH 1-3 gradients a method based on the modification of Pettersson (200) was used (Vesterberg, personal communication). The method used was as described above, but with the following changes. The dense electrode solution contained 0.15ml concentrated sulphuric acid, 16ml glycerol and 4ml water. The dense gradient solution contained 0.1g monochloroacetic acid, 0.1g orthophosphoric acid, 0.1g dichloroacetic acid, 35ml glycerol and 20ml water. The light gradient solution contained 0.1g acetic acid, 0.1g formic acid, 0.05g glutamic acid, 0.05g aspartic acid, 0.30ml ampholine carrier ampholytes, pH 5-8, and 60ml water.

For isoelectric focusing in pH 4-5 and pH 4-6 gradients a potential of 400 volts was applied for 72h; during isoelectric focusing the current dropped from 8ma to 1ma. For isoelectric focusing in pH 1-3 gradients the potential was slowly increased from 150V to 400V and isoelectric focusing was carried out for 120-144h; the current slowly dropped from 15ma to 1ma.

Preparation of serum

Inflammation was induced in rats by subcutaneous injection of 0.5ml of oil of turpentine per 100g body weight into the dorsolumbar region (201). Rats used as controls received injections of sterile 0.15ml M NaCl. After injection, rats were lightly anesthetized with ether, and blood was collected from individual animals be severing the juglar veins 5-96h after injection of turpentine or NaCl. Blood was collected for 2 minutes and allowed to stand for a further 45 minutes at room temperature to clot. Serum was prepared by centrifuging at 2500 r.p.m. for 10 minutes and was stored at -20^{o} until required for use.

Fractionation of serum by stepwise elution from columns of DEAE-cellulose.

Seven protein fractions were prepared from serum by stepwise elution from columns of DEAE-cellulose by elution with buffers of decreasing pH and increasing molarity. Before use the exchanger was washed twice with 0.5M NaOH containing 0.5M NaCl and then with water until neutral, and suspended in 0.02M sodium phosphate buffer, pH 8.0. Columns containing a bed of exchanger, 30cm x 1.5cm,

were prepared using columns of constant-bore tubing provided by Pharmacia (Canada) Ltd. Columns were packed and run under a hydrostatic pressure of 30cm; phosphate buffer, pH 8.0, was allowed to pass through the columns for 30h at 2° before use. All subsequent procedures were carried out at 2°.

For each experiment 3ml rat serum was dialyzed against three changes of 1 liter 0.02M phosphate buffer, pH 8.0, over a total period of 48h. When serum was isolated from a rat which had been injected with radioactive compounds (see later), 10mM L-leucine and 10mM D-glucosamine-HCl were included in the initial dialysis solution. Serum was removed from the dialysis sac which was washed with small volumes of buffer. The serum and washings were pooled and made to 4.5ml with 0.02M sodium phosphate buffer, pH 8.0. Samples were removed for determination of radioactivity, protein, and protein-bound hexose and hexosamine. A 4ml sample was applied to a column of DEAE-cellulose and the column was eluted with 0.02M sodium phosphate buffer, pH 8.0, and then with a series of 0.02M sodium phosphate buffers containing 30mM NaCl (pH 7.6), 45mM NaCl (pH 7.0), 70mM NaCl (pH 6.6), 100mM NaCl (pH 6.0), 150mM NaCl (pH 5.5), and 300mM NaCl

(pH 5.0); 120-140ml of each buffer were passed through the column except for those containing 30mM NaCl (pH 7.6) and 150mM NaCl (pH 5.5), when 80ml were passed through. The flow rate was 15ml/h and fractions of 3.0ml were collected. The protein content of fractions was determined by measurement of extinction at 280mμ. Fractions eluting with each buffer were pooled and concentrated to about 10ml by ultrafiltration with concurrent dialysis against water (175). Samples were finally freeze-dried.

Isolation of α_1-acid glycoprotein, α_2-macroglobulin and albumin from rat serum.

α_1-acid glycoprotein. Fraction 5, the fraction which eluted from the DEAE-cellulose column with 0.02M sodium phosphate buffer containing 100mM NaCl (pH 6.0) was used as starting material for the isolation of acute phase proteins since this fraction contained a large part of the increase in total protein-bound carbohydrate found in rat serum as a result of induced inflammation (see Results section).

For preparation of large quantities of fraction 5 a scaled up modification of the method previously

described was used. Serum, (30-35ml), obtained from rats suffering from induced inflammation for 48h, was dialyzed against three changes of 2 liters of 0.02M sodium phosphate, pH 8.0, over a total period of 48h. Columns containing a bed of DEAE-cellulose exchanger, 45cmX2.5cm, were prepared. Serum was applied to the column and eluted with 0.02M sodium phosphate buffer, pH 8.0, and then with a series of buffers as described above except that the sodium phosphate buffer containing 150mM NaCl (pH 5.5) was omitted and 350-400ml of each buffer was passed through the column. Fraction 5 was recovered and concentrated to about 10ml by ultrafiltration with concurrent dialysis against water, and finally freeze-dried. Fraction 5 was further fractionated by stepwise elution from columns of CM-cellulose. CM-cellulose was first regenerated by washing twice with 0.25M NaOH containing 0.25M NaCl and then with water until neutral. The exchanger was suspended in 0.05M sodium acetate buffer, pH 4.9, for 24h and columns packed and run as described for DEAE-cellulose but with 0.05M sodium acetate buffer, pH 4.9. Fraction 5 (250-300mg) was dissolved in 5ml 0.05M sodium acetate buffer, pH 4.9, and applied to the CM-

cellulose column. The column was eluted with 300ml 0.05M sodium acetate buffer, pH 4.9, and then with 300ml 0.40M sodium acetate buffer, pH 5.1. The flow rate was 10ml/h and fractions of 3.0ml were collected. Protein was detected by measurement of extinction at 280mμ. Appropriate fractions were pooled, concentrated to about 10ml by ultrafiltration with concurrent dialysis against water and freeze-dried.

Fraction 5A, which eluted from the CM-cellulose column with 0.05M sodium acetate, pH 4.9, was subjected to isoelectric focusing in pH 1-3 gradients to prepare an α_1-acid glycoprotein (see Results section).

Fraction 5B, which eluted from the CM-cellulose column with 0.4M sodium acetate, pH 5.1, was used as the starting material for the isolation of albumin and α_2-macroglobulin (see below).

α_2-macroglobulin and albumin. Fraction 5B was subjected to preparative electrophoresis on Cellogel blocks as previously described. The α_2-macroglobulin fraction was eluted from Cellogel blocks as previously described, dialyzed against water and freeze-dried. The fraction (10-15mg protein)

dissolved in 1-2ml 0.02M phosphate buffer, pH 7.0, was applied to a 90cm x 1.5cm column of Sephadex G-200 previously equilibrated with the same buffer. The column was eluted with buffer at a flow rate of 4ml/h and fractions of 2ml were collected. A peak of E_{280}-positive material eluted near the void volume. This material was recovered, concentrated by ultrafiltration and subjected to isoelectric focusing in pH 4-6 or pH 4-5 gradients to prepare an α_2-macroglobulin. Albumin was also eluted from the Cellogel block and was further purified by isoelectric focusing in pH 4-6 gradients. A narrow fraction was collected from the main peak, dialyzed against water and freeze-dried. Albumin prepared in this way was found to be immunologically homogeneous.

Gel filtration on Sepharose

Gel filtration on Sepharose 4B was employed to obtain information on the molecular weight of the α_2-macroglobulin. A sample of α_2-macroglobulin (5mg protein) was dissolved in 1ml 0.02M sodium phosphate buffer, pH 7.0, and applied to a 45cm x 2.5cm column of Sepharose 4B equilibrated with the same buffer. Elution was by upward flow at a flow

rate of 8ml/h; fractions of 1ml were collected and protein was detected by measurement of extinction at 280m μ. The elution volumes of 5mg samples of human α-globulins (M.Wt., 160,000); horse apoferritin (M.Wt., 480,000); bovine thyroglobulin (M.Wt., 670,000) and blue dextran (mean M.Wt., 2,000,000) were determined before and after gel filtration of α_2-macroglobulin on Sepharose 4B. The molecular weight of the α_2-macroglobulin was estimated by referring to a graph plotting log molecular weight of marker proteins against their elution volumes as described by Andrews (202).

Preparation of DANS-labelled α_1-acid glycoprotein α_2-macroglobulin and albumin

DANS-labelled proteins were prepared by a method based on that described by Fothergill (203). Proteins (5-10mg) were dissolved in 0.4ml 0.15M NaCl, 0.75ml of a buffer containing 0.43M $NaHCO_3$ and 0.056M Na_2CO_3, pH 9.0, were then added. A solution of 1mg dansyl chloride in 25μl acetone was added over 15 minutes. The mixture was stirred at 2^{o} for 6h. Insoluble material was removed by centrifuging and the supernatant applied to a 25cm x 2cm column of Sephadex G-25 equilibrated with a

buffer containing 0.15M NaCl, 7mM Na_2HPO_4 and 2.5mM NaH_2PO_4, pH 7.1. The column was eluted with the same buffer. The DANS-labelled proteins emerged rapidly from the column whereas the free dansyl was retarded. The DANS-labelled proteins were dialyzed against water and freeze-dried.

Isotope dilution technique

Rat serum albumin labelled with L-leucine-^{14}C was isolated by the method of Campbell et al. (204) from the serum of a rat killed 90 minutes after intraperitoneal injection of 25μCi L-leucine-^{14}C. The crude material was purified by electrophoresis on Cellogel blocks. Samples of 0.40ml of a solution of ^{14}C-labelled albumin (0.5mg protein) were added to 0.2-0.4ml rat serum and allowed to stand at 2^{o} for 1h. Albumin was recovered from serum by the method of Campbell et al. (204) followed by preparative electrophoresis on Cellogel strips. The content of rat albumin in serum was calculated from the decrease in specific radioactivity of the recovered material.

Immunological methods

Preparation of antisera. Antisera were prepared by a modification of the method described by Simkin et al. (180). For preparation of antisera to α_1-acid glycoprotein, α_2-macroglobulin and albumin, an emulsion of 0.75mg protein, 0.25ml 0.15M NaCl and 0.4ml Freund's complete adjuvent was prepared. Rabbits were given intramuscular injections of the emulsion, one half of the dose being injected into each thigh. After six days, a second injection of emulsion containing 1.25mg antigen with Freund's adjuvent was injected intramuscularly. After a further 22 days, a series of injections was given of a suspension of coprecipitate of antigen with $Al(OH)_3$. This was prepared by adding 0.4ml 10% (w/v) $Al_2(SO_4)_3$, K_2SO_4. $24H_2O$ to a mixture of 3.0mg antigen in 0.2ml water to which 0.08ml M $NaHCO_3$ was added and the mixture stood at 2^o for 16h. After centrifuging, the sediment was resuspended in 0.4ml 122mM-Na_2HPO_4-28mM-NaH_2PO_4-0.01% thiomersal, pH 7.4. Samples of 0.05, 0.10, 0.25 and 0.40ml of this suspension were injected intravenously on alternate days. The rabbits were bled five days after the final injection. For preparation of antisera against proteins by further immunization of a

previously used rabbit only one injection of emulsion in Freund's adjuvent containing 2.5mg antigen was given, and samples of 0.10, 0.25 and 0.40ml of alum suspension were administered. Rabbits were bled as described above. For preparation of antisera to rat and human serum the same procedure as described above was used except that Freund's emulsions were made with 25µl and 50µl of serum. Alum precipitates were made using 200µl of serum.

Double diffusion analysis. The procedure used was based on the technique of Ouchterlony (205) as described by Simkin et al. (180). The medium used contained 1.25% (w/v) Nobel agar, 0.15M NaCl and 0.01% thiomersal. Diffusion was allowed to proceed at room temperature.

Immunoelectrophoresis. Immunoelectrophoresis was carried out on 3 in. x 1 in. glass slides together with equipment supplied by Shandon Scientific Co. Ltd. The buffer added to the buffer compartments contained 49mM sodium diethylbarbiturate 55mM sodium acetate 65mM HCl, pH 8.6. The gel used consisted of 1% (w/v) Ionagar No. 2 in buffer at one-third of the concentration of that used in the buffer compartments.

A potential of 60V was applied for 30-100 minutes at room temperature.

Quantitative precipitin technique. The quantitative precipitin technique was a modification of the method described by Simkin and Jamieson (206). For preparation of quantitative precipitin curves mixtures were prepared (total volume 0.45ml) containing up to 100µg albumin and α_2-macroglobulin or 40µg α_1-acid glycoprotein or the corresponding DANS derivatives, 0.15M NaCl, 1mM-sodium azide and 4.7% Dextran T70; 0.05ml antiserum to α_2-macroglobulin and albumin or 0.15ml antiserum to α_1-acid glycoprotein were then added to solutions containing their corresponding antigens. Mixtures were incubated at 37° for 45 minutes and then allowed to stand for 48h at 2°. The precipitates which formed were collected by centrifuging at 2000g for 10 minutes at 2° and washed with 0.3ml 0.15M NaCl containing 4% Dextran T70 and then with 3 x 0.3ml 0.15M NaCl. Precipitates were dissolved in 1ml 0.1N NaOH and suitable volumes removed for determination of protein, fluoresence and radioactivity. When the technique was applied to serum from control and experimental rats, 0.05-0.35ml volumes of 80-120-fold

dilutions of serum were reacted with antisera as described above. In some experiments DANS labelled proteins were added to serum samples prior to addition of antiserum to test for quantitative precipitation of antigens.

For isolation of specific proteins from Lubrol extracts of subcellular fractions of liver, Lubrol extracts were concentrated six-fold by ultrafiltration with concurrent dialysis against 0.15M NaCl-1% Lubrol containing 10mM of appropriate unlabelled precursor. Prior to precipitation with antisera to specific rat proteins, precipitation with a heterologous immune system was carried out to remove any nonspecific radioactivity. To Lubrol extracts (3ml) were added 500µg human albumin and 1.2ml antiserum to human serum and the extracts incubated as described above. The precipitates were removed by centrifuging at 2000 r.p.m. for 5 minutes. To the supernatants were added 360µg human glycoprotein fraction VI and the supernatants were incubated as previously described. After centrifuging the precipitation process was repeated on the supernatants. All precipitates were washed with 3.0ml 0.15M NaCl and were dissolved in 0.1N NaOH for determination of protein and radioactivity. Suitable volumes of the

final supernatants were precipitated with antisera to specific rat proteins as described above.

Radioautography

The procedure used was based on that of Morgan et al. (207). Gels were washed at room temperature for two days with 0.15M NaCl, two days with tap water and two days with distilled water; the wash liquids were continuously changed. The gels were dried at 25° and numbered with marking ink containing ^{147}PmCl (208) and then placed on Ilford X-ray film, for 3-8 weeks before development. Gels were later stained for protein with Nigrosin(209).

Incorporation studies in vivo

When experiments involved incorporation of labelled compounds into serum proteins isolated by stepwise elution from columns of DEAE-cellulose, rats received intraperitoneal injections of 0.4ml of an isotope mixture 90 minutes before sacrifice. The mixture contained 150µCi L-leucine-4,5-^{3}H (1000mCi/mM) and 5 µCi D-glucosamine-1-^{14}C hydrochloride (3.1mCi/mM) dissolved in 0.4ml 0.15M NaCl. Blood was collected and serum prepared as previously described.

For studies on the site of synthesis of albumin and α_1-acid glycoprotein rats were lightly anaesthetized with ether and given an intravenous injection into the femoral vein of 25μCi L-leucine-^{14}C (10mCi/mM) or 10μCi D-glucosamine-1-^{14}C hydrochloride (3.1mCi/mM). After 12-15 minutes animals were killed by a blow on the head, a sample of blood was collected and appropriate tissue removed and transferred to iced 0.25M sucrose prior to subcellular fractionation (see below).

When experiments involved incorporation of labelled compounds into microsome material or rough and smooth subfractions of microsome material rats were lightly anaesthetized with ether and given an intravenous injection into the femoral vein of an isotope mixture at 5, 10, 15, 30 and 60 minutes prior to sacrifice. The mixture contained 150μCi L-leucine-4,5-^{3}H (1000mCi/mM) and 15μCi D-glucosamine-1-^{14}C (55mCi/mM), dissolved in 0.15ml of 0.15M NaCl. Rats were killed 1 minute before the times chosen for sacrifice, blood was collected over a 1 minute period and livers rapidly excised and transferred to iced 0.25M sucrose or 0.5M sucrose in Solution A prior to subcellular fractionation (see below).

Subcellular fractionation

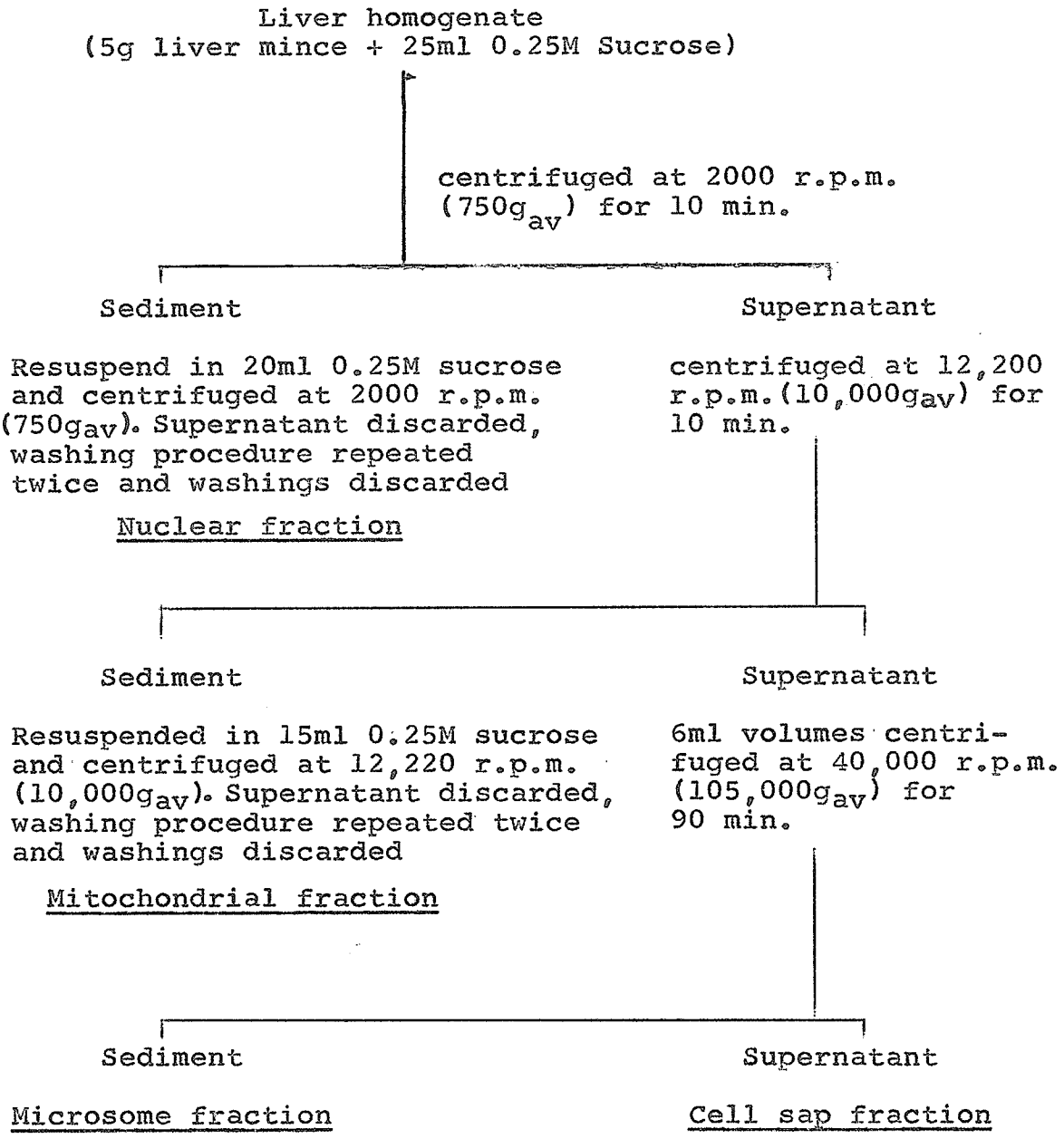
A Sorvall model SS1 centrifuge located in a cold room at 4^{o} was used for the preparation of nuclear and mitochondrial fractions of rat liver. A Spinco model L-1 ultracentrifuge with a No. 50 angle head was used for all other subcellular fractionations except for the preparation of the Golgi fraction of liver when a Beckman model ultracentrifuge and a SW 25.2 swing out head was used.

Preparation of liver homogenates. Liver was first minced and freed of connective tissue by forcing through a stainless steel tissue mincer with holes of 1mm diameter (210). A Potter-Elvehjem type homogenizer with a polytetrafluorethylene pestle operated by a GT21 laboratory stirrer and GT21 motor controller was used to homogenize tissues. Unless otherwise stated, liver mince was homogenized with 5 vol. of 0.25M sucrose for 30 seconds; the pestle was rotated at 2000 r.p.m. and about seventeen up and down strokes of the pestle were completed over 20 seconds.

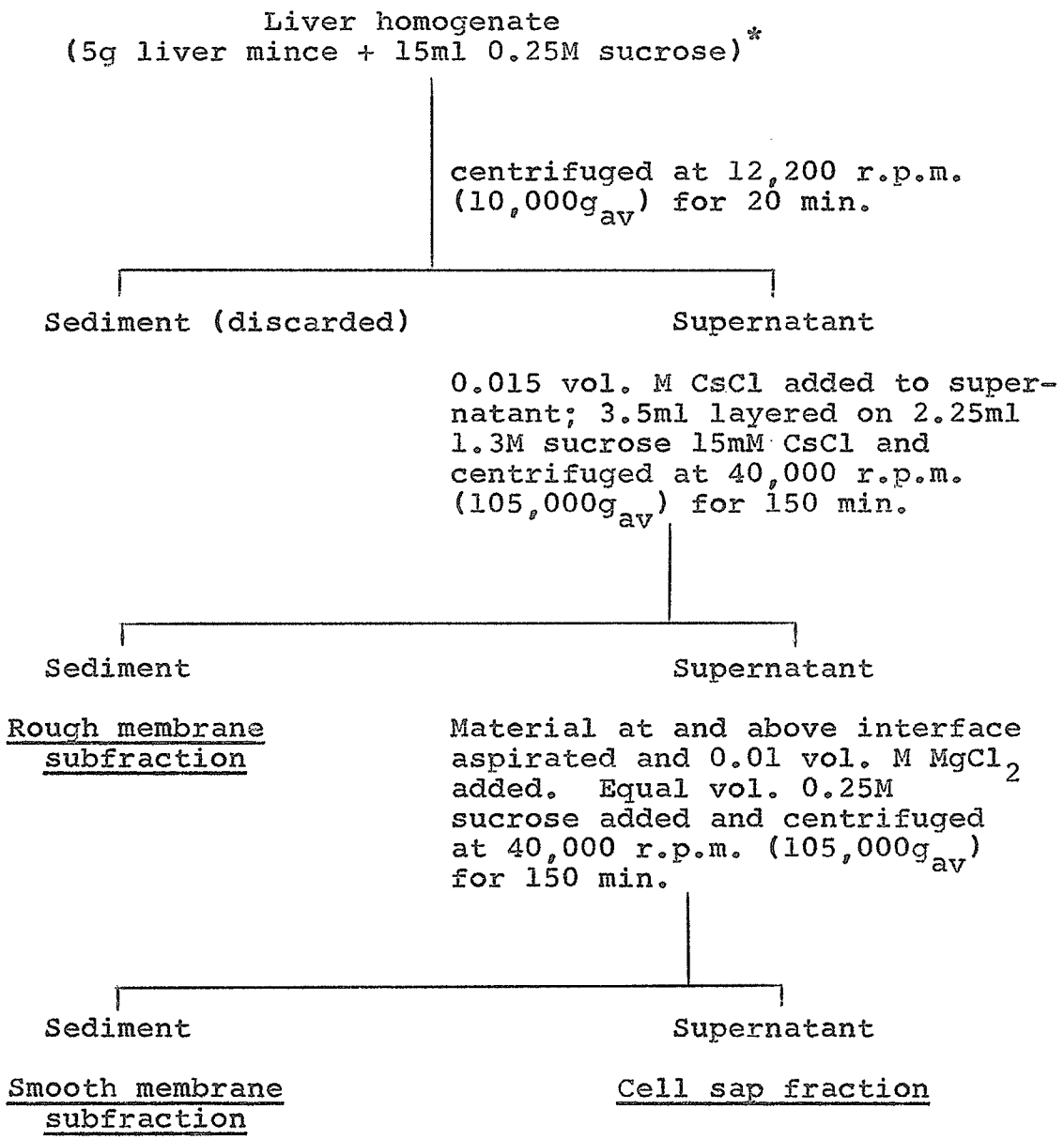
Preparation of subcellular fractions of liver. Nuclear, mitochondrial, microsomal and cell sap fractions were prepared from liver by fractionation according to scheme (1) below (211). For preparation of rough and smooth membrane subfractions of liver microsomal material the method of Dallner (56) was used as indicated in scheme (2).

Golgi fraction. Golgi fractions were prepared according to the method described by Schachter et al. (129). Liver was minced and homogenized as described above except that liver mince was homogenized with 4 vol. of 0.5M sucrose in medium A (0.1M tris-HCl, pH 7.6, -0.01M $MgCl^2$-1% dextran) per g wet weight of tissue with five up and down strokes of the Potter homogenizer. The homogenate was filtered through surgical gauze and 8ml of the filtered homogenate were layered over a discontinuous sucrose density gradient consisting of 20ml 1.7M sucrose in medium A, 16ml 1.3M sucrose in medium A and 16ml 0.7M sucrose in medium A. The tube was centrifuged at 24,000 rev/min (70,000 g_{av}) in a Spinco SW 25.2 rotor for 45 minutes. The Golgi enriched fraction at the interface between the 0.7M and 1.3M sucrose layers was collected, diluted four-

Scheme (1)


Liver homogenate
(5g liver mince + 25ml 0.25M Sucrose)
centrifuged at 2000 r.p.m.
(750g_{av}) for 10 min.
Sediment
Supernatant
Resuspend in 20ml 0.25M sucrose and centrifuged at 2000 r.p.m. (750g_{av}). Supernatant discarded, washing procedure repeated twice and washings discarded
Nuclear fraction
centrifuged at 12,200 r.p.m.(10,000g_{av}) for 10 min.
Sediment
Supernatant
Resuspended in 15ml 0.25M sucrose and centrifuged at 12,220 r.p.m. (10,000g_{av}). Supernatant discarded, washing procedure repeated twice and washings discarded
Mitochondrial fraction
6ml volumes centrifuged at 40,000 r.p.m. (105,000g_{av}) for 90 min.
Sediment
Supernatant
Microsome fraction
Cell sap fraction

Scheme (2)



* A microsome fraction corresponding to the material fractionated was prepared by centrifuging a sample of 10,000g$_{av}$ supernatant at 40,000 r.p.m. (105,000g$_{av}$) for 150 min.

fold with 0.25M sucrose and centrifuged at 40,000 r.p.m. ($105,000g_{av}$) to obtain a pellet of Golgi enriched fraction.

Preparation of spleen and kidney fractions. Homogenates were prepared in 0.25M sucrose as described above for liver. A large granule fraction was prepared for each tissue by centrifuging at 12,200 r.p.m. ($10,000g_{av}$) and microsome and cell sap fractions prepared from the supernatants according to scheme (1).

Extraction of subcellular fractions. Subcellular fractions were normally dispersed in extractant by homogenization. After extraction, insoluble material was removed by centrifuging for 120 minutes at 40,000 r.p.m. ($105,000g_{av}$) in a Spinco model L ultracentrifuge.

Subcellular fractions were extracted with 1% Lubrol at 2^{o}, 1ml extractant being used per 7-10mg protein (206). For the extraction of spleen and kidney *in toto*, 0.7g minced tissue was homogenized with 6ml 1% Lubrol. Lubrol extracts were concentrated six-fold by ultrafiltration with concurrent dialysis against 1% Lubrol; the appropriate

unlabelled precursor (10mM) was added to the Lubrol used for the first 6h of dialysis. When sodium deoxycholate was used as extractant a 2.5% solution in 0.25M sucrose was used at 2°; 1ml extractant was employed per 14-20mg protein.

Isolation of protein for the measurement of radioactivity

In order to determine whether administered doses of L-leucine-^{3}H or D-glucosamine-^{14}C given to rats represented proper pulse labels, serum and liver protein were isolated from samples at various times after administration of isotope according to the method of Simkin and Work (210) and analyzed for radioactivity. After labelling with L-leucine-^{3}H, protein was precipitated from serum and liver samples by addition of an equal volume of iced 10% (w/v) trichloroacetic acid containing 10mM L-leucine. Precipitates were collected by centrifuging and dissolved in warm N NaOH containing 20mM unlabelled L-leucine. Protein was reprecipitated with 20% trichloroacetic acid and washed at room temperature with the following reagents: once with 5% (w/v) trichloroacetic; once with 5% trichloroacetic acid at 90°

for 15 minutes; once with 5% trichloroacetic acid; twice with acetone; twice with ethanol:ether (3:1 v/v) at 60° for 1 minute, and finally twice with ether. After labelling with D-glucosamine-^{14}C, an equal volume of iced 10% trichloroacetic acid was added to iced serum and liver samples containing about 5mg/ml protein and 20mM D-glucosamine. Precipitates were collected by centrifuging and washed at room temperature with volumes of the following reagents such that the protein concentration was maintained at about 5mg/ml; once with 5% trichloroacetic acid; once with acetone 0.1M NaCl (4:1, v/v) (212); twice with ethanol:ether:chloroform (2:2:1, by volume) for 15 minutes, the first wash being at 50°; and twice with ether. All samples were dried in air and dissolved in 0.1N NaOH for determination of protein and radioactivity.

Preparation of sections for examination in the electron microscope.

Livers were cut into cubes measuring 0.5cm x 0.5cm x 0.5cm and placed in a solution containing 0.4% acrolein and 4% gluteraldehyde in 0.1M cacodylate buffer, pH 7.4 for 2h at room temperature and then washed with four changes of 0.1M cacodylate

buffer over 1h. Cubes measuring 1mm x 1mm x 1mm were removed from the outer surface of the larger cubes and placed in 1.0% osmic acid in 0.1M cacodylate buffer, pH 7.4 at 0° for 2h and were then washed with 0.1M cacodylate pH 7.4 over 1h. The sections were allowed to stand for 12h in 0.5% uranyl acetate and then placed in the following solutions: distilled water for 15 minutes; 50% ethyl alcohol for 15 minutes; 70% ethyl alcohol for 15 minutes; 90% ethyl alcohol for 15 minutes and finally absolute ethyl alcohol for 60 minutes. The cubes were placed in gelatin capsules containing 5 drops unpolymerized cross-linked methacrylate solution and allowed to stand for 3 days, the plastic solution being changed daily. Polymerization was performed at $48\text{-}50^{\circ}$ for 12h and sections were prepared with an LKB ultrotome, post-stained with lead citrate, and examined with an AEI EMGB electron microscope. The sections and electron micrographs were kindly prepared by Dr. L. VanCasaele in the Botany Department, University of Manitoba.

RESULTS

Effect of inflammation on protein-bound hexose and hexosamine of rat serum proteins.

Serum, isolated from groups of rats 5-96 h after administration of turpentine (experimental animals) or 0.15 M NaCl (control animals), was analyzed for protein-bound hexose and hexosamine. There was an increase in the content of protein-bound hexose (Fig. 5) and hexosamine (Fig. 6) of serum from experimental animals reaching a maximum at 48 h after injection. There was little change in the content of protein-bound hexose and hexosamine in serum from control animals.

In order to isolate those proteins which contribute to the elevated content of protein-bound hexose and hexosamine found in the serum of experimental animals, samples of serum from individual animals were fractionated on columns of DEAE-cellulose. A typical elution profile of serum isolated from an animal 48 h after administration of turpentine is shown in Fig 7. Serum isolated from control animals gave similar elution patterns

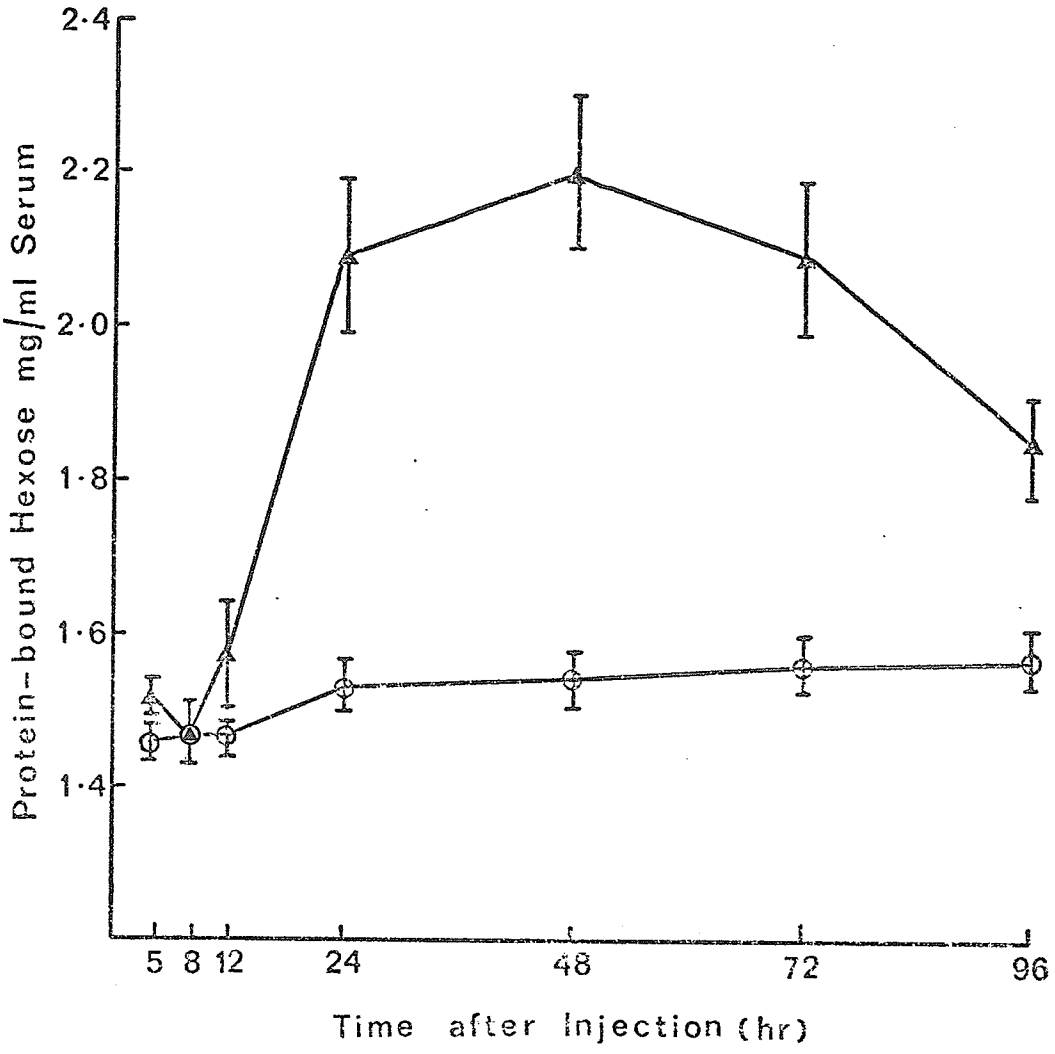


Fig. 5

Effect of turpentine - induced inflammation on total protein - bound hexose of rat serum; -▲-, experimental animals, -O- control animals. Each bar represents results from four to eight animals.

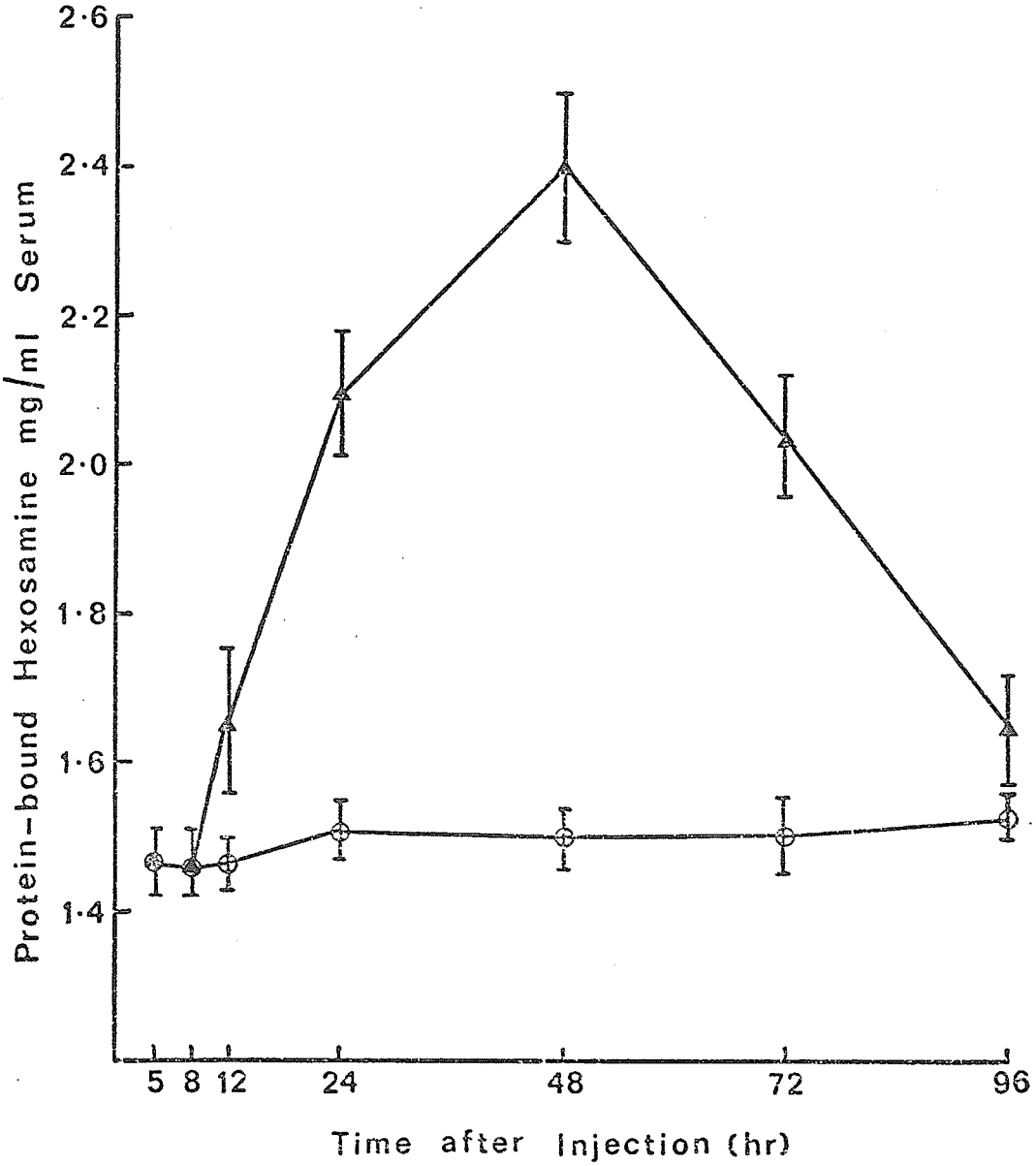


Fig. 6

Effect of turpentine-induced inflammation on total protein-bound hexosamine of rat serum; -▲- experimental animals, -O- control animals. Each bar represents results from four to eight animals.

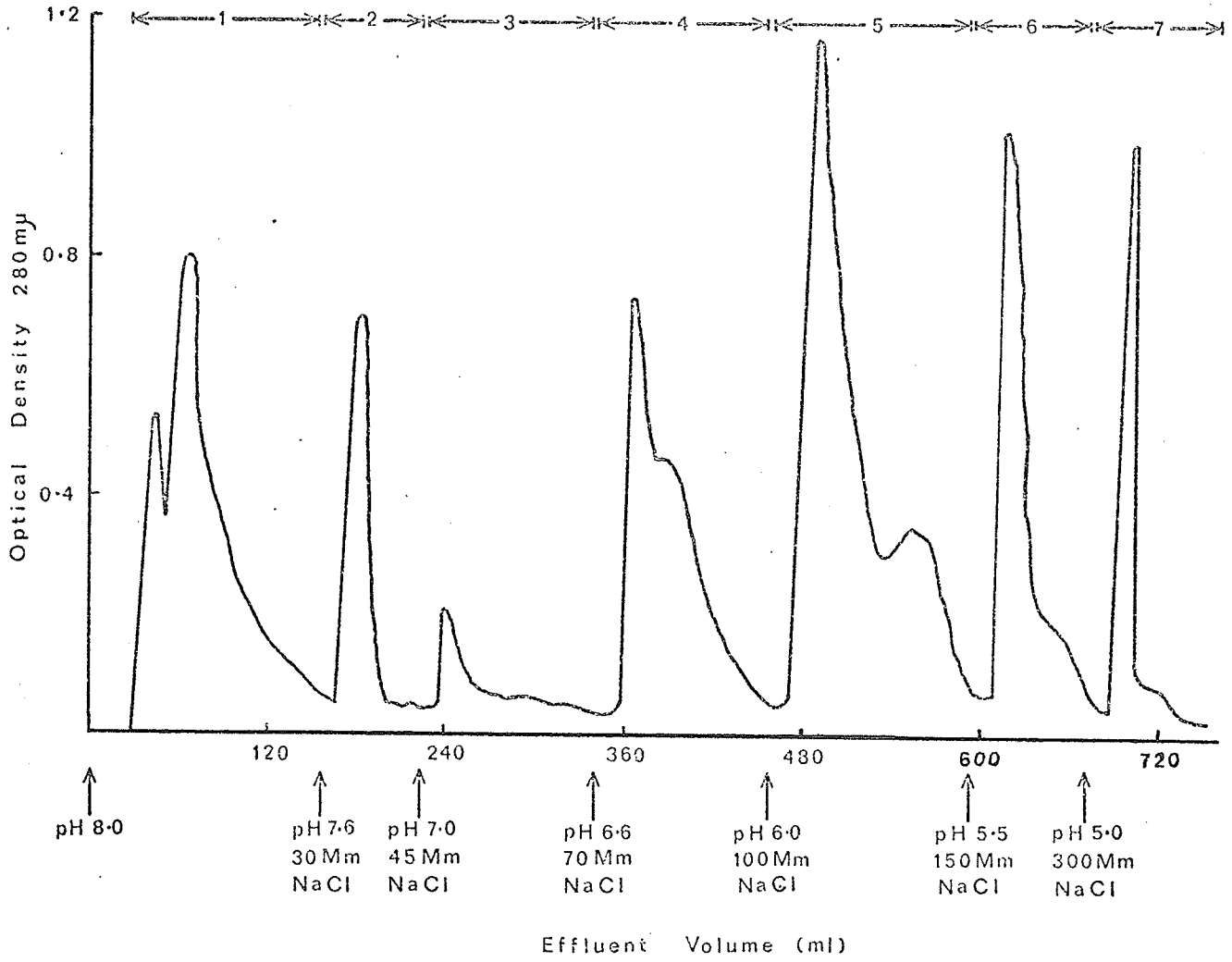


Fig. 7

Stepwise elution chromatography on a 30cm x 1.5cm column of DEAE-cellulose of serum isolated from a rat 48 h after injection of turpentine. The column was eluted with 0.02M sodium phosphate buffers of decreasing pH and increasing concentrations of NaCl. Arrows indicate the positions at which the buffers were applied. The fractions were pooled as indicated.

to that shown in Fig. 7 except that there was a decrease in the amount of E_{280}-positive material eluting in fractions 1, 4 and 5. Fraction 5 was further fractionated by preparative electrophoresis on Cellogel strips into two subfractions, 5a and 5b, fraction 5a contained serum albumin and 5b contained the carbohydrate staining proteins present in fraction 5 (see below).

Protein fractions obtained after fractionation of serum from control and experimental animals on DEAE-cellulose were analyzed for protein-bound hexose (Table 2) and protein-bound hexosamine (Table 3). Most of the increase in protein-bound hexose (78-86%) and protein-bound hexosamine (77-85%), found in the serum of experimental animals killed 24-72 h after injection of turpentine, was associated with proteins present in fractions 1, 4 and 5b (Tables 2 and 3). The protein-bound hexose and hexosamine contents of fractions isolated from the serum of rats killed at 5, 8 and 12 h after injection of turpentine were not significantly different from those of control fractions. There was only a slight increase over control values in the protein-bound hexose and hexosamine contents of fractions 1, 4 and 5b prepared from the serum of rats

Table 2

Distribution of protein-bound Hexose in fractions of rat serum from control and experimental animals*

	Total protein-bound Hexose			mg Hexose/fraction			
Fraction No.	Controls (10)	Experimental 24 Hr. (5)	Increase %+	Experimental 48 Hr. (5)	Increase %+	Experimental 72 Hr. (5)	Increase %+
1	0.72 ± 0.05	1.03 ± 0.06	22.0	1.09 ± 0.07	18.0	0.99 ± 0.06	15.0
2	0.45 ± 0.03	0.46 ± 0.04	1.0	0.61 ± 0.05	7.0	0.56 ± 0.03	6.0
3	0.38 ± 0.03	0.45 ± 0.03	5.0	0.40 ± 0.03	1.0	0.45 ± 0.03	4.0
4	0.68 ± 0.04	1.02 ± 0.05	25.0	1.25 ± 0.07	26.0	1.23 ± 0.07	30.0
5a.	-	-		-		-	
5b.	1.20 ± 0.06	1.75 ± 0.03	39.0	2.10 ± 0.04	41.0	1.80 ± 0.07	33.0
6	0.89 ± 0.06	0.99 ± 0.06	7.0	1.00 ± 0.07	5.0	1.08 ± 0.06	10.0
7	0.41 ± 0.03	0.42 ± 0.03	-	0.44 ± 0.03	-	0.45 ± 0.03	-
Total Hexose	4.73	6.12		6.89		6.56	

* Results are given as the content of protein-bound hexose in 3.0 ml undiluted serum and are expressed as means ± standard error of the mean; numbers in parentheses represent the number of experiments performed.

\+ Increase in protein-bound hexose per fraction expressed as a percentage of the increase in total protein-bound hexose of serum; values are given to the nearest whole number.

Table 3

Distribution of protein-bound Hexosamine in fractions of rat serum from control and experimental animals*

	Total protein-bound Hexosamine mg Hexosamine/fraction						
Fraction No.	Controls (9)	Experimental 24 Hr. (4)	Increase %+	Experimental 48 Hr. (4)	Increase %+	Experimental 72 Hr. (4)	Increase %+
1	0.83 ± 0.06	1.09 ± 0.06	18.0	1.25 ± 0.06	18.0	1.15 ± 0.08	18.0
2	0.45 ± 0.03	0.49 ± 0.04	3.0	0.56 ± 0.02	5.0	0.54 ± 0.04	5.0
3	0.27 ± 0.02	0.40 ± 0.02	8.0	0.31 ± 0.01	2.0	0.40 ± 0.02	7.0
4	0.78 ± 0.05	1.12 ± 0.06	23.0	1.38 ± 0.07	26.0	1.26 ± 0.08	27.0
5a.	-	-		-		-	
5b.	1.03 ± 0.08	1.67 ± 0.09	42.0	1.98 ± 0.08	41.0	1.58 ± 0.09	32.0
6	0.99 ± 0.06	1.03 ± 0.07	3.0	1.09 ± 0.06	4.0	1.11 ± 0.10	7.0
7	0.34 ± 0.02	0.39 ± 0.04	3.0	0.42 ± 0.03	3.0	0.39 ± 0.04	3.0
Total Hexosamine	4.69	6.19		6.99		6.43	

* Results are given as the content of protein-bound hexosamine in 3.0 ml undiluted serum and are expressed as means ± standard error of the mean numbers in parentheses represent the number of experiments performed.

\+ Increase in protein-bound hexosamine per fraction expressed as a percentage of the increase in total protein-bound hexosamine of serum; values are given to the nearest whole number.

killed 96 h after injection of turpentine. There was little change in the content of protein-bound hexose and hexosamine of fractions prepared from serum from control animals at different times after injection of sodium chloride. Therefore, the results in Tables 2 and 3 for analyses of fractions from control animals represent mean values from rats killed at 5-96 h after injection of sodium chloride. In all experiments not less than 90% of protein, protein-bound hexose, and protein-bound hexosamine applied to columns of DEAE-cellulose were recovered.

Electrophoretic analysis of serum fractions

Electrophoresis on Cellogel strips (Fig. 8 of fractions prepared by chromatography of serum on DEAE-cellulose showed that all fractions were heterogeneous, except fraction 5a which contained electrophoretically homogeneous serum albumin. Fraction 1 contained γ- and β-globulins and fractions 2 and 3 contained β-globulins together with some α-globulins of slower electrophoretic mobilities. Fractions 4, 5b, 6 and 7 contained mainly α-globulins. In some experiments a trace of material corresponding in electrophoretic mobility to serum albumin was detected in fractions 4 and 6.

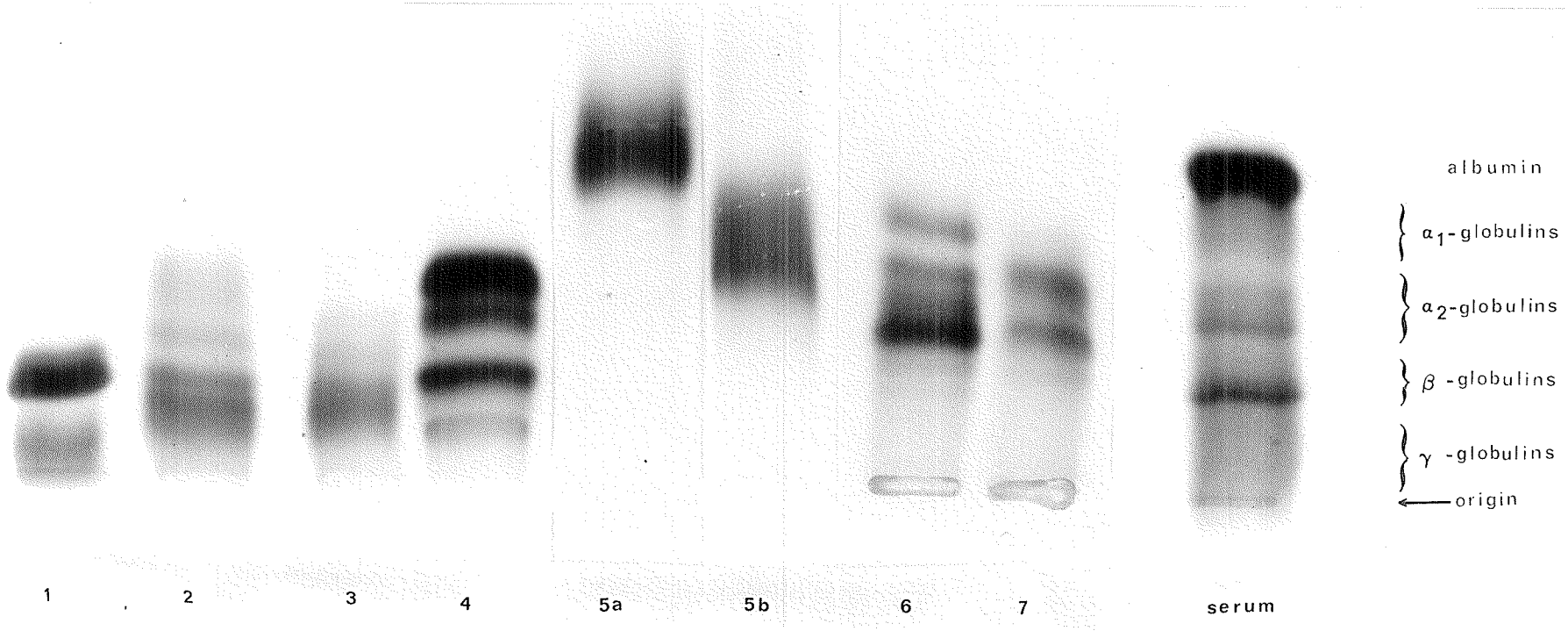


Fig. 8

Electrophoresis on Cellogel strips at pH 8.6, of normal rat serum and fractions obtained from rat serum by chromatography on DEAE-cellulose and preparative electrophoresis on Cellogel strips. The arrow shows the point of application of samples.

Fractions 2 and 3 stained weakly for carbohydrate, but all other fractions stained strongly for carbohydrate except for fraction 5a which did not stain for carbohydrate. There was no obvious difference in electrophoretic patterns of fractions obtained from control serum when compared with corresponding fractions obtained from the serum of experimental animals.

Isolation of α_1-acid glycoprotein, α_2-macroglobulin and albumin from rat serum

Since fraction 5 contains those proteins responsible for the increase in total protein-bound carbohydrate of serum (about 40% of the increase in total protein-bound carbohydrate of serum was located in this fraction; see e.g. Tables 2 and 3), this fraction was used as the starting material for the isolation of specific acute phase globulins for use in subsequent studies on the biosynthesis of glycoproteins that respond markedly to inflammation. For preparative purposes, fraction 5 was isolated by a scaled-up version of the fractionation procedure previously described in which serum was eluted from columns of DEAE-cellulose. In this way about

30-35ml volumes of serum were fractionated yielding 250-300mg fraction 5. In order to isolate specific carbohydrate-containing proteins present in fraction 5 a stepwise elution procedure from columns of CM-cellulose was then employed. Two fractions were obtained (Fig. 9). Fraction 5A, which eluted with the 0.05M acetate buffer, pH 4.9, contained carbohydrate staining proteins with electrophoretic mobilities just less than that of serum albumin (Fig. 10). Fraction 5B, which eluted with the 0.40 M acetate buffer, pH 5.1, contained serum albumin together with the second band of carbohydrate-staining proteins (Fig.10). Fraction 5A was further fractionated by the technique of isoelectric focusing in pH 1-3 gradients using a modification of the method of Petterson(200). A sharp peak of E_{280}-positive material was obtained at an isoelectric point of pH 2.95 (Fig. 11). This fraction was collected and is referred to as the α_1-acid glycoprotein. The α_1-acid glycoprotein moved as a single band when examined by electrophoresis on Cellogel strips at pH 8.6 (Fig. 10), stained strongly for carbohydrate and corresponded to the carbohydrate-staining band of greatest electrophoretic mobility present in fraction 5A (Fig. 10). Fraction 5B was fractionated

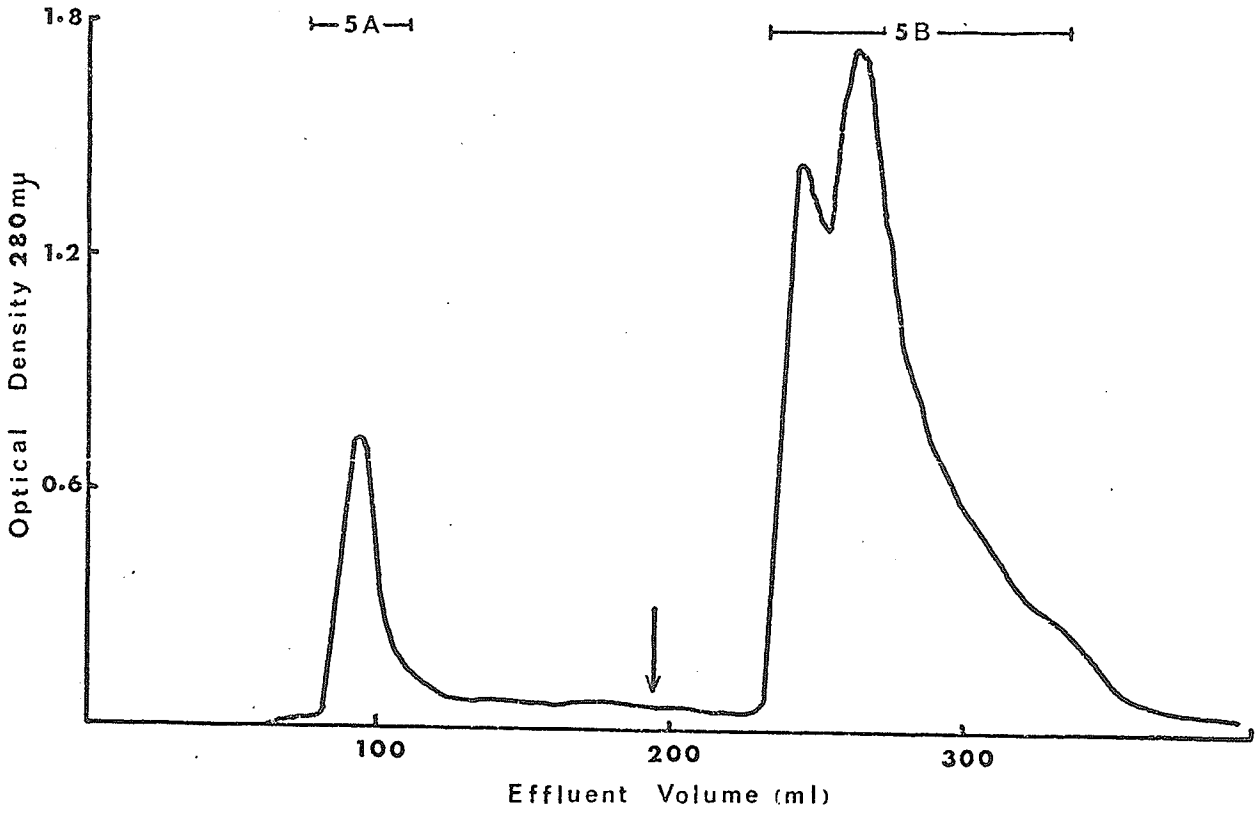


Fig. 9

Stepwise elution chromatography of 100mg of fraction 5 on a 45cm x 2.5cm column of CM-cellulose. The column was eluted initially with 0.05M acetate buffer, pH 4.9. The arrow indicates the position at which a 0.40M acetate buffer, pH 5.1 was applied. The fractions were pooled as indicated.

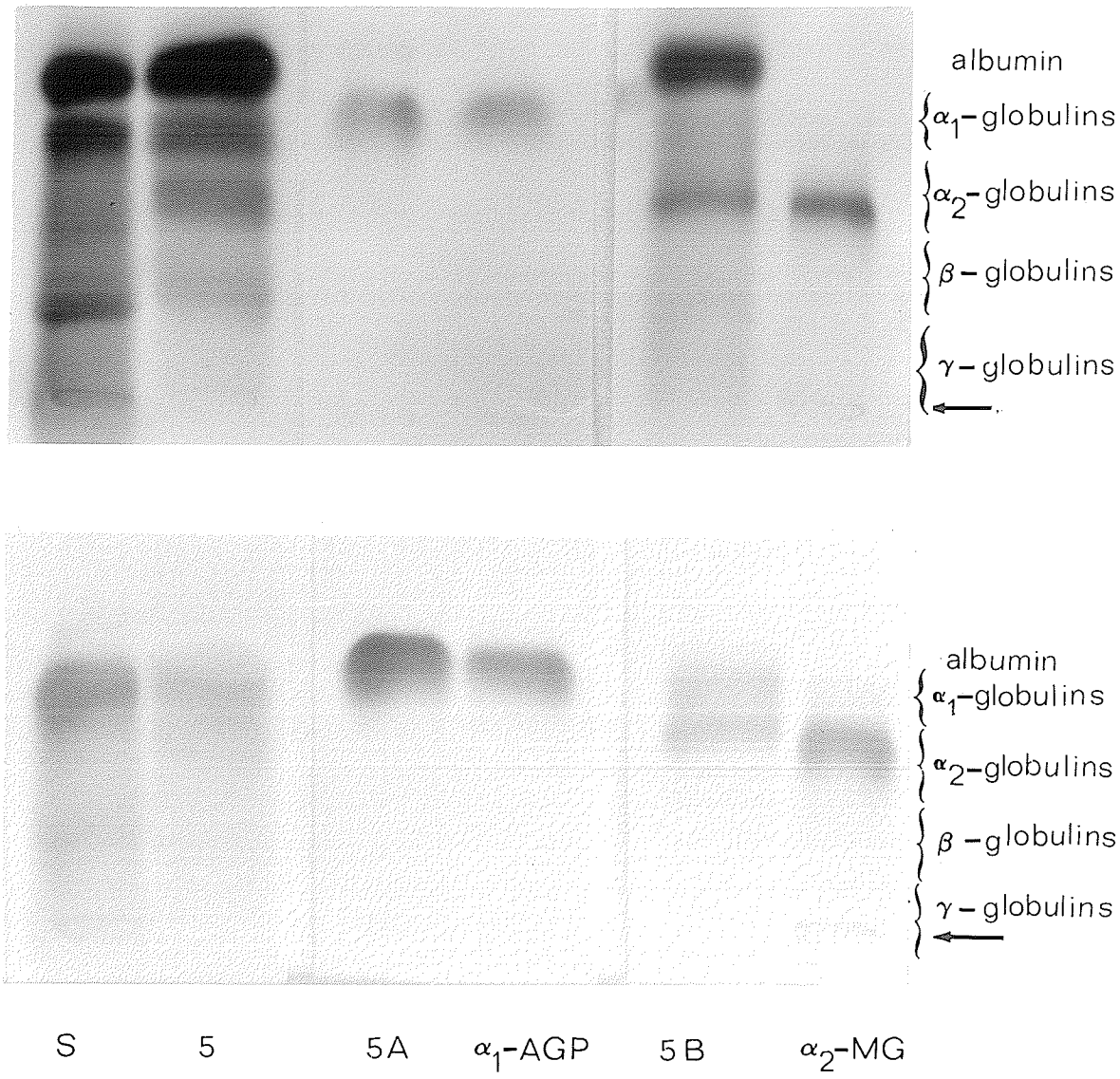


Fig. 10

Electrophoresis on Cellogel strips at pH 8.6 of: S, serum from rats 48 h after injection of turpentine; 5, fraction 5; 5A fraction 5A; α_1-AGP, α_1-acid glycoprotein; 5B, fraction 5B; α_2-MG, α_2-macroglobulin. Bottom, Cellogel strips stained for carbohydrate; top, Cellogel strips stained for protein. The arrows show the points of application of samples.

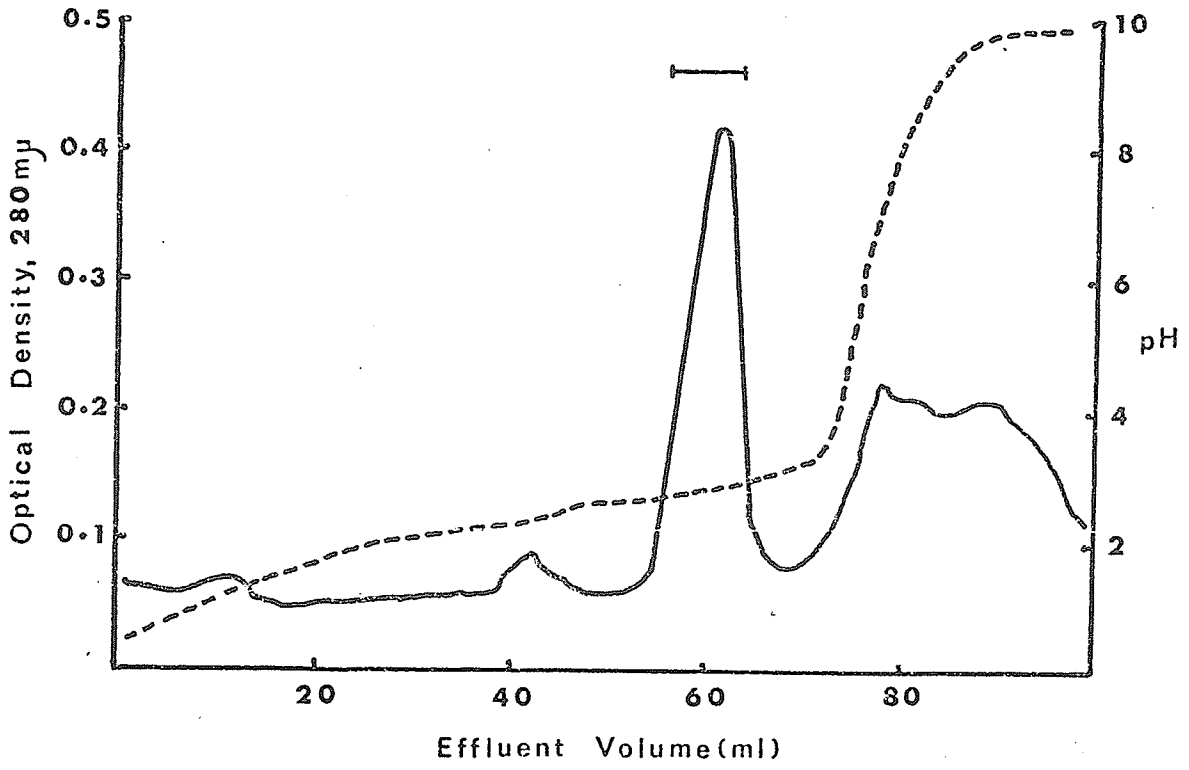


Fig. 11

Fractionation of 15mg of fraction 5A by isoelectric focusing in a pH 1-3 gradient; solid line, optical density; broken line, pH. The fraction was pooled as indicated.

by preparative electrophoresis on Cellogel blocks at pH 8.6. Two fractions were prepared, one containing serum albumin, and the other containing the carbohydrate-containing proteins present in fraction 5B. The carbohydrate-containing fraction was applied to a column of Sephadex G-200 and eluted with 0.02 M phosphate buffer, pH 7.0. A large peak of E_{280}-positive material eluted near the void volume of the column (Fig. 12). This material, called fraction $5B_1$ was collected and further fractionated by isoelectric focusing in pH 4-6 gradients (Fig. 13). The material eluted as a series of three overlapping peaks; the major peak corresponding to an isoelectric point of pH 4.6. A narrow fraction was collected from the major peak (Fig. 13) and is referred to as the α_2-macroglobulin. In some experiments isoelectric focusing was repeated on pH 4-5 gradients to remove small amounts of contaminating proteins. The α_2-macroglobulin moved as a single band when examined by electrophoresis on Cellogel strips at pH 8.6 (Fig. 10), stained strongly for carbohydrate (Fig. 10), and corresponded in electrophoretic mobility to the main carbohydrate staining band present in fraction 5B (Fig. 10).

Since serum albumin was required in subsequent

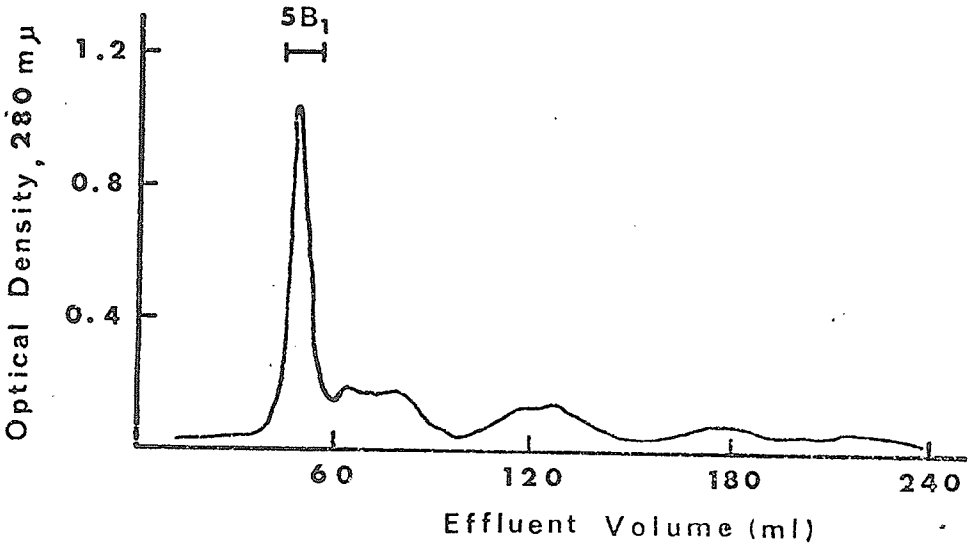


Fig. 12

Gel filtration of the carbohydrate staining band of fraction 5B on a 90cm x 1.5cm column of Sephadex G-200. The column was eluted with 0.02M phosphate buffer, pH 7.0. The fraction was pooled as indicated.

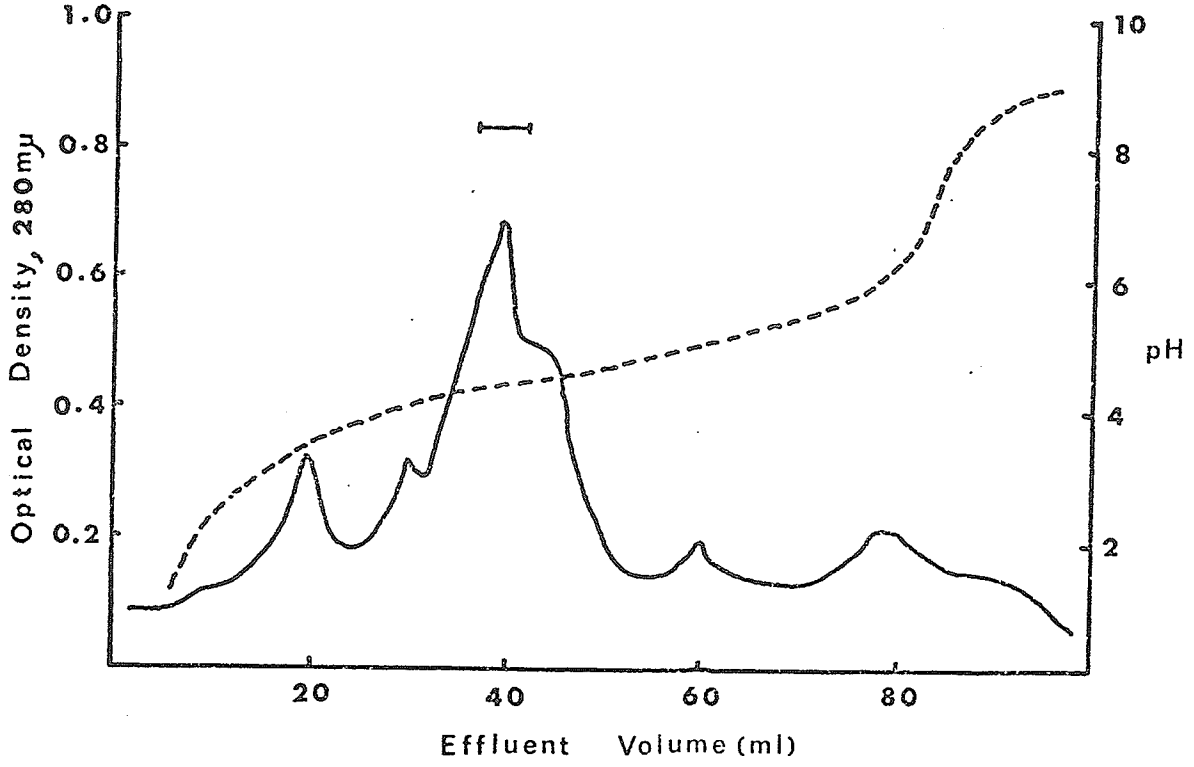


Fig. 13

Fractionation of 20mg of fraction 5B by isoelectric focusing in a pH 4-6 gradient; solid line, optical density; broken line, pH. The fraction was pooled as indicated.

biosynthetic studies (see later) this protein was routinely recovered following preparative electrophoresis on Cellogel blocks at pH 8.6 (see above) and was further fractionated by isolectric focusing in pH 4-6 gradients (Fig. 14). The crude material gave a sharp peak at pH 4.6 which proved to be homogeneous on subsequent examination by electrophoresis on starch and polyacrylamide gels.

Electrophoretic analysis of α_1-acid glycoprotein and α_2-macroglobulin on starch and polyacrylamide gels

Examination of the α_1-acid glycoprotein by electrophoresis on starch gel using the discontinuous buffer system of Smithies(194) gave only one band that stained for protein (Fig. 15). The α_1-acid glycoprotein had an electrophoretic mobility slightly greater than that of serum albumin and corresponded in electrophoretic mobility to the main protein staining band present in fraction 5A (Fig. 15). Fraction 5A contained some minor components of slower electrophoretic mobilities than the α_1-acid glycoprotein. Disc electrophoresis of the α_1-acid glycoprotein on polyacrylamide gels at pH 8.9 gave similar results to those found on starch gels. One

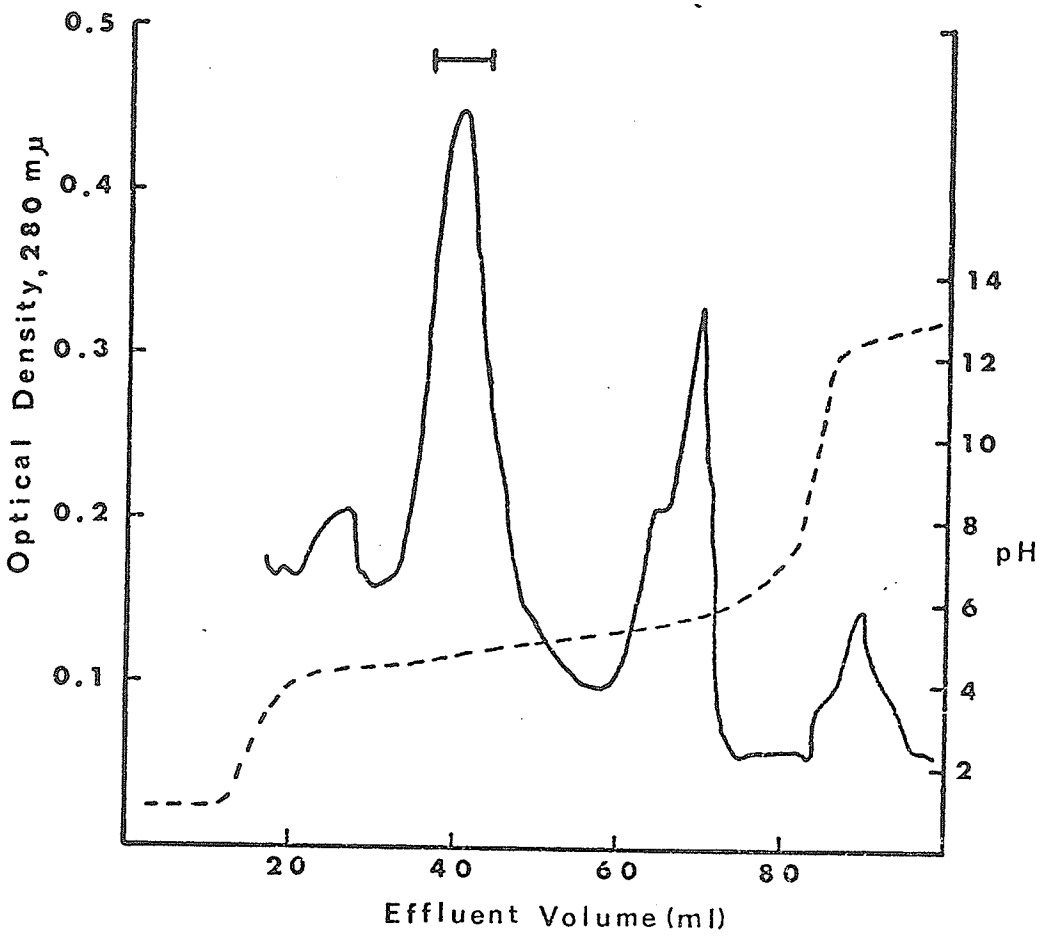


Fig. 14

Fractionation of 25mg of crude albumin obtained by preparative electrophoresis on cellogel blocks by isoelectric focusing in a pH 4-6 gradient; solid line, optical density, broken line, pH. The fraction was pooled as indicated.

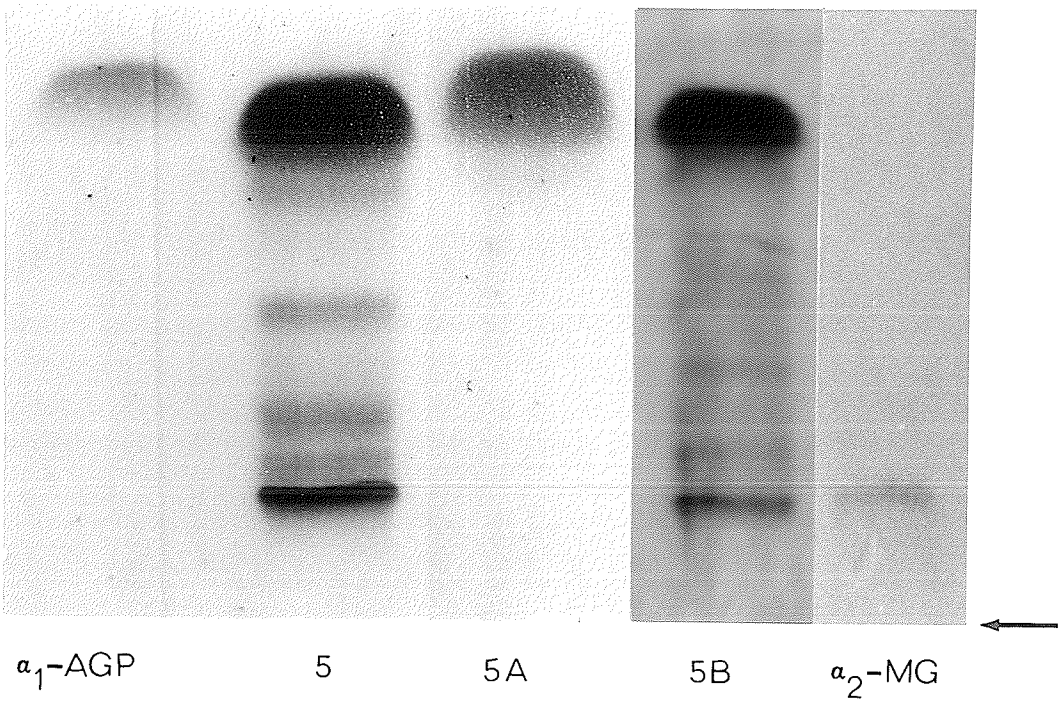


Fig. 15

Electrophoresis on starch gels of: α_1-AGP, α_1-acid glycoprotein; 5, fraction 5; 5A, fraction 5A; 5B, fraction 5B; α_2-MG, α_2-macroglobulin. The arrow shows the point of application of the samples.

band was obtained from the α_1-acid glycoprotein which stained for protein and for carbohydrate and which corresponded in electrophoretic mobility to a band given by fraction 5A (Fig. 16). Fraction 5A contained some minor components of slower electrophoretic mobilities than the α_1-acid glycoprotein.

The α_2-macroglobulin was examined in a similar manner to the α_1-acid glycoprotein described above. Electrophoresis of the α_2-macroglobulin on starch gel gave one band that stained for protein (Fig. 15) and which had an electrophoretic mobility similar to that of a strongly staining macroglobulin present in fractions 5 and 5B (Fig. 15); the α_2-macroglobulin also gave one band that stained for protein and carbohydrate when examined by disc electrophoresis on polyacrylamide gels (Fig. 16). However, an additional band of lower electrophoretic mobility was occasionally observed with the α_2-macroglobulin when examined by disc electrophoresis, but this band did not readily stain for carbohydrate.

Immunological analysis of α_1-acid glycoprotein and α_2-macroglobulin

The α_1-acid glycoprotein was examined by double diffusion analysis and immunoelectrophoresis

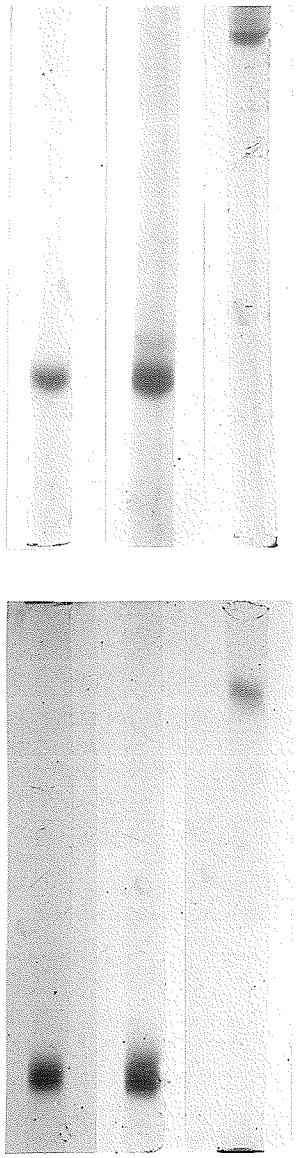


Fig. 16

Electrophoresis on polyacrylamide gels of: α_1-AGP, α_1-acid glycoprotein; 5A, fraction 5A; α_2-MG, α_2-macroglobulin; top, gels stained for protein; bottom, gels stained for carbohydrate. In the above photographs electrophoresis was run for 90 minutes in the case of those gels stained for protein, but 120 minutes in the case of those gels stained for carbohydrate.

employing antiserum to α_1-acid glycoprotein and fraction 5A. Only one precipitin line was obtained on double diffusion analysis of α_1-acid glycoprotein with both antisera, a reaction of immunological identity being observed with lines obtained from rat serum, fraction 5 and fraction 5A (Fig. 17). The α_1-acid glycoprotein gave one precipitin arc in a region of high electrophoretic mobility when examined by immunoelectrophoresis (Fig. 18). The electrophoretic mobility of the α_1-acid glycoprotein was slightly less than that of rat serum albumin which formed a precipitin arc just ahead of the α_1-acid glycoprotein when allowed to react with antiserum to rat serum albumin (Fig. 18).

The α_2-macroglobulin was also examined immunologically in a similar manner to the α_1-acid glycoprotein except that antisera to α_2-macroglobulin and fraction 5 were employed. In most cases only one major precipitin line was obtained on double diffusion analysis of α_2-macroglobulin with both antisera, a reaction of immunological identity being observed with lines obtained from rat serum, fraction 5 and fraction 5B (Fig. 19). However, a faint additional line was frequently observed which may be due to a minor contaminating

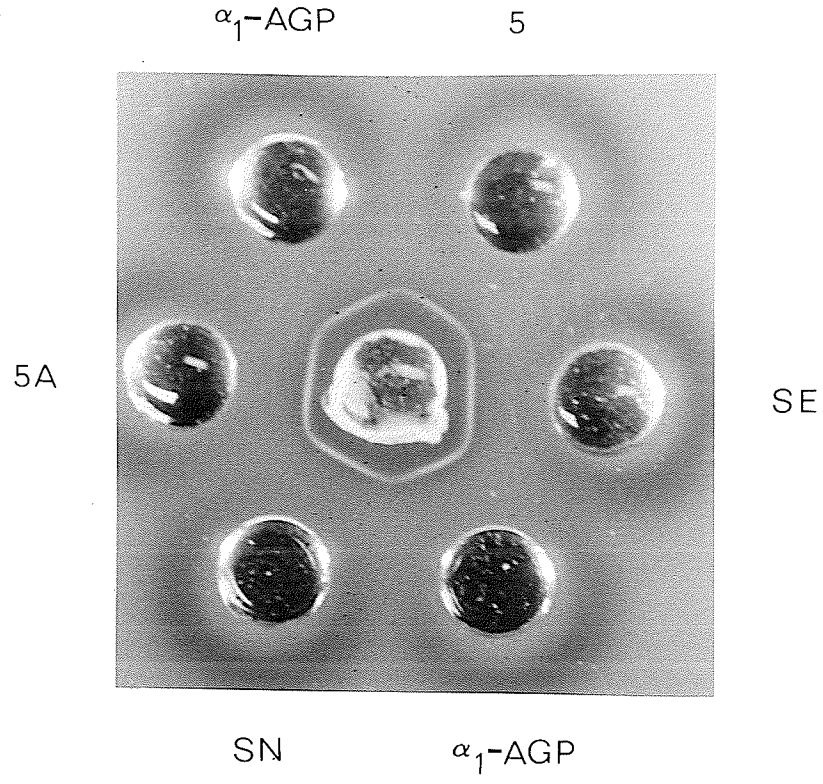


Fig. 17

Double diffusion analysis with antiserum to α_1-acid glycoprotein (central reservoir) of α_1-AGP, α_1-acid glycoprotein (5mg/ml); 5, fraction 5 (7.5mg/ml); 5A, fraction 5A (5mg/ml); SN, normal rat serum and SE, serum from a rat suffering from inflammation for 48h (diluted 1:4 v/v). Diffusion was at room temperature for 30h. All protein solutions were in 0.15M-NaCl.

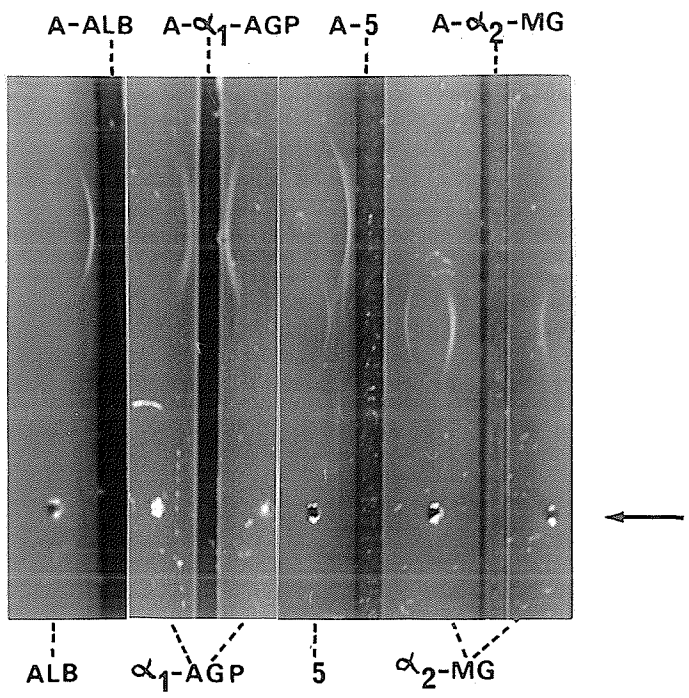


Fig. 18

Immunoelectrophoresis of ALB, rat serum albumin (2mg/ml); α_1-AGP, α_1-acid glycoprotein (3mg/ml); 5, fraction 5 (3mg/ml) and α_2-MG, α_2-macroglobulin (3mg/ml). The antisera used were A-Alb, anti-rat serum albumin; A-α_1-AGP, anti-α_1-acid glycoprotein; A-5 anti-fraction 5 and A-α_2-MG, anti-α_2-macroglobulin. All protein solutions were in 0.15M-NaCl. The arrow shows the point of application of samples.

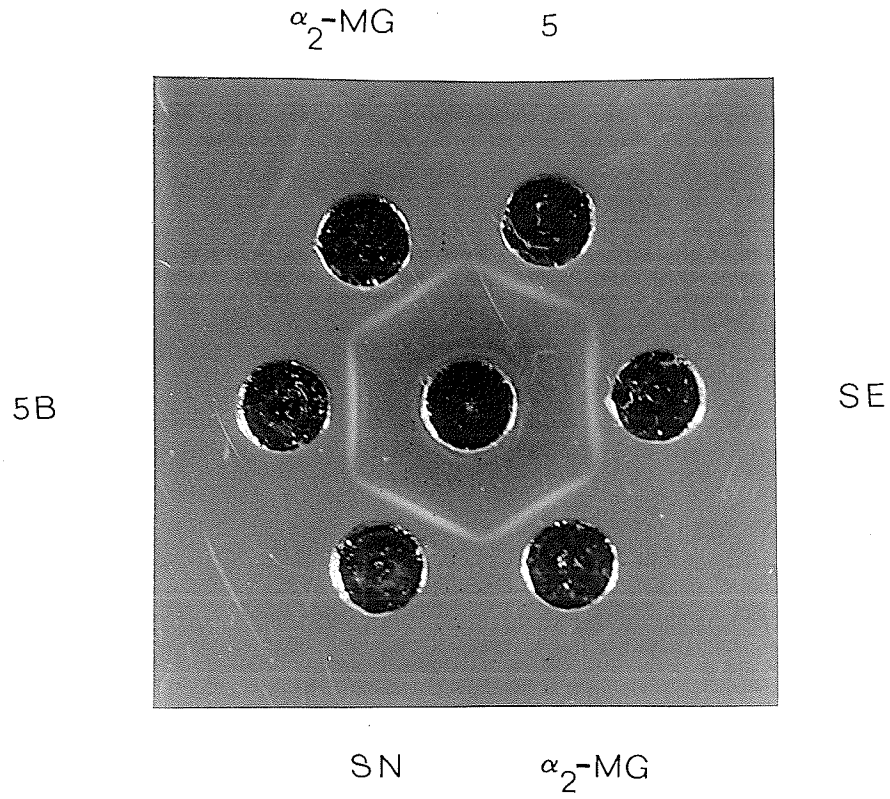


Fig. 19

Double diffusion analysis with antiserum to α_2-macroglobulin (central reservoir) of α_2-MG, α_2-macroglobulin (5mg/ml); 5, fraction 5 (7.5mg/ml); 5B, fraction 5B (7.5mg/ml); SN, normal rat serum, and SE, serum from a rat suffering from inflammation for 48h (diluted 1:2, v/v). Diffusion was at room temperature for 30h. All protein solutions were in 0.15M-NaCl.

protein or some degradation product of the α_2-macroglobulin. Although the minor component mentioned above gave a precipitin line on double diffusion analysis in agar gels only one precipitin arc was obtained from the α_2-macroglobulin when examined by immunoelectrophoresis (Fig. 18) employing antiserum to α_2-macroglobulin. The precipitin arc formed by the α_2-macroglobulin corresponded to a distinct precipitin arc formed on reaction of fraction 5 with antiserum to fraction 5 following immunoelectrophoresis (Fig. 18). The precipitin arc corresponding to serum albumin was also clearly observed on reaction of fraction 5 with antiserum to fraction 5 following immunoelectrophoresis (Fig. 18); however the precipitin arc corresponding to α_1-acid glycoprotein was not distinct on reaction of fraction 5 with antiserum to fraction 5 probably because it formed very close to the precipitin arc formed by serum albumin.

Chemical analyses and physical properties of α_1-acid glycoprotein and α_2-macroglobulin

As previously mentioned in the introduction the chemical analyses and examination of the physical

properties of α_1-acid glycoprotein and α_2-macroglobulin were performed by Mr. A. D. Friesen who presented this work for the degree of Master of Science. However, since some information on the composition and properties of the two proteins in question is important for an understanding of subsequent biosynthetic studies presented in this thesis, and also in order to maintain the continuity of this presentation, a brief summary of the results obtained is presented in this section.

The α_1-acid glycoprotein and α_2-macroglobulin were analyzed qualitatively and quantitatively for neutral sugars, hexosamines and sialic acids. Paper chromatography of sugar fractions from the α_1-acid glycoprotein and α_2-macroglobulin showed the presence of galactose, mannose, glucosamine and N-acetylneuraminic acid. Fucose, uronic acid and galactosamine were not detected in either glycoprotein. Results for the quantitative analyses of sugars in the α_1-acid glycoprotein and α_2-macroglobulin, together with data on molecular weights, sedimentation coefficients and isoelectric points are presented in Table 4.

Table 4

Carbohydrate composition and physical properties of α_1-acid glycoprotein and α_2-macroglobulin*

	α_1-acid glycoprotein	α_2-macroglobulin
Hexose	14.4 ± 0.3 (5)	9.3 ± 0.3 (3)
Hexosamine	9.5 ± 0.2 (5)	4.0 ± 0.2 (3)
Sialic acid	10.2 ± 0.2 (6)	2.6 ± 0.1 (4)
Total carbohydrate	34.1	15.9
Molecular weight	43,000+	800,000‡
Isoelectric point‡‡	2.95	4.60
Partial Specific volume	0.685	0.698
$S_{20,w}$	3.30	19.0

* Results for carbohydrate analyses expressed as g. carbohydrate per 100g glycoprotein (dry wt.); results are expressed as means ± standard error of the mean with the number of analyses performed given in parenthesis.

\+ Determined by electrophoresis on polyacrylamide gels in presence of sodium dodecyl sulphate (198).

‡ Determined from elution volume on Sepharose 4B.

‡‡ Determined from elution profile on isoelectric focusing.

Determination of the contents of α_1-acid glycoprotein, α_2-macroglobulin and albumin in rat serum

In order to determine if the α_1-acid glycoprotein and α_2-macroglobulin isolated in the present work were acute phase reactants increasing in content in serum as a result of induced inflammation, a quantitative precipitin technique was applied to serum samples from control rats and rats suffering from inflammation for 5-96 h. Quantitative precipitin curves for α_1-acid glycoprotein and α_2-macroglobulin when titrated with their corresponding antisera are shown in Figs. 20, and 21, respectively. The contents of these two proteins in serum from control and experimental animals was therefore determined by titrating serum samples with antiserum in a region of antibody excess. The amount of each protein in serum samples was calculated from the amount of protein recovered in the precipitates using the quantitative precipitin curves in Figs. 20 and 21 as standard curves. The results obtained for the contents of α_1-acid glycoprotein and α_2-macroglobulin in serum from control and experimental animals are shown in Figs. 22 and 23, respectively. Both proteins increased significantly in content in

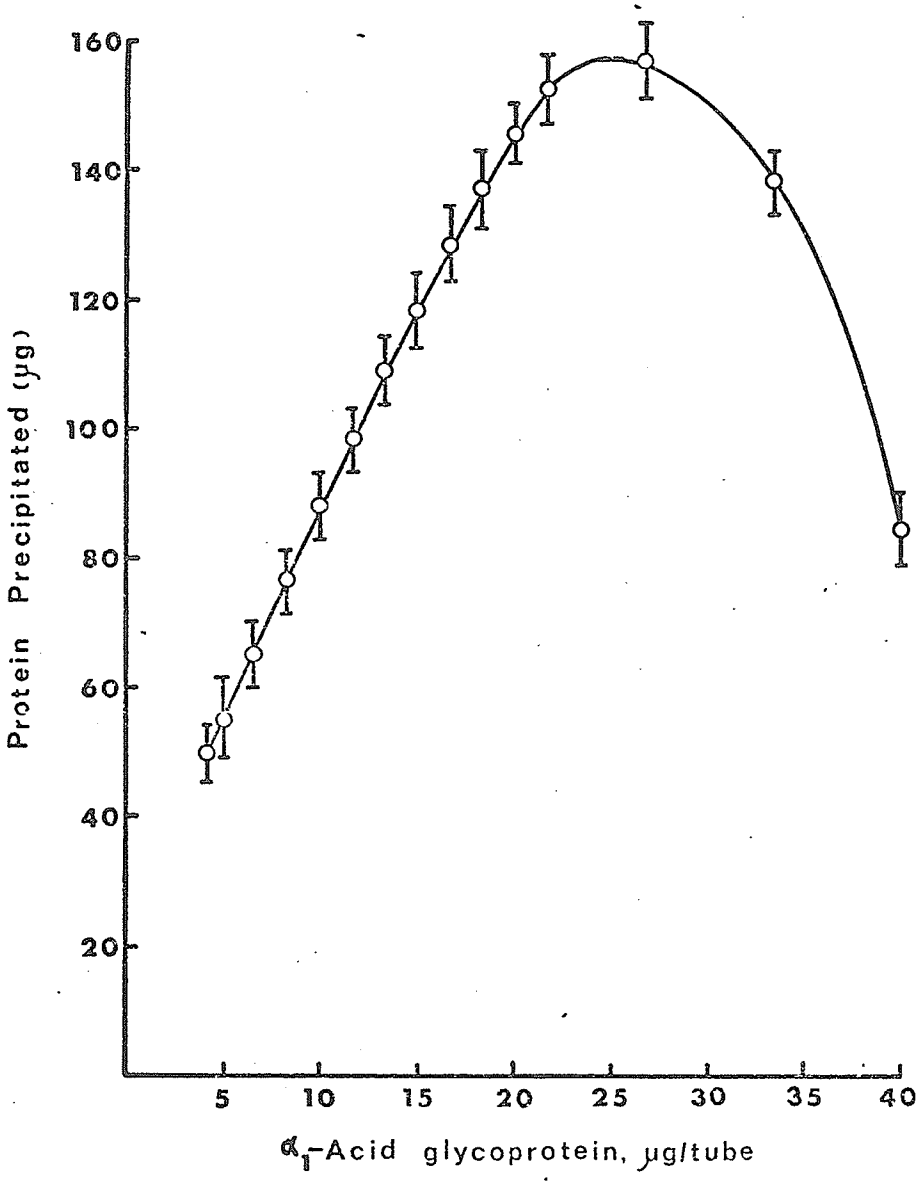


Fig. 20

Quantitative precipitin curve for α_1-acid glycoprotein. Each bar represents results from 4-8 experiments.

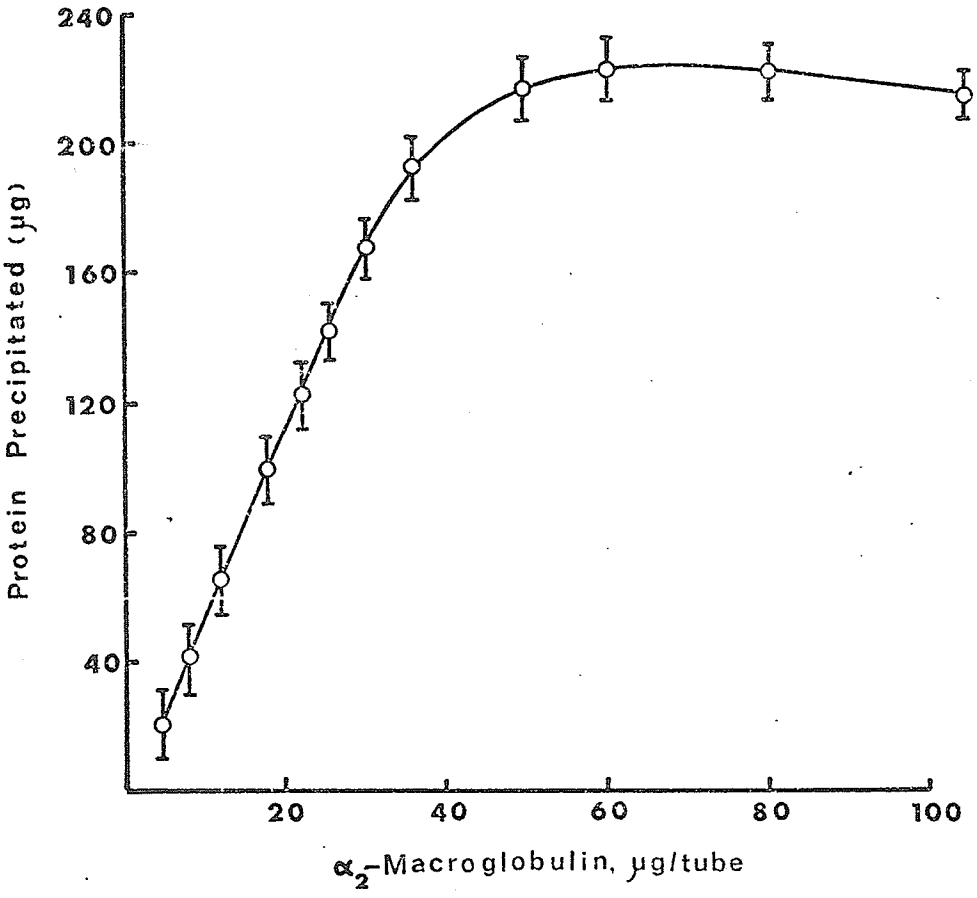


Fig. 21

Quantitative precipitin curve for α_2-macroglobulin. Each bar represents results from 4-8 experiments.

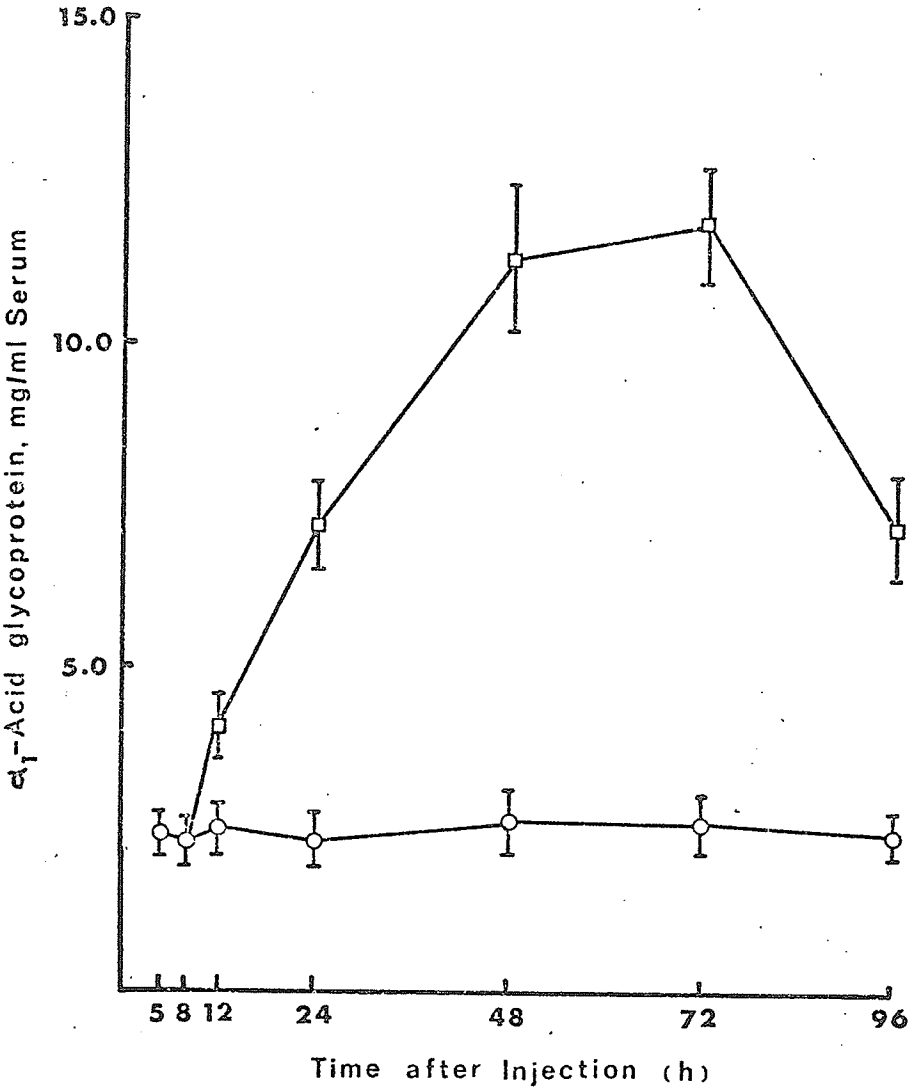


Fig. 22

The effect of turpentine induced inflammation on the content of α_1-acid glycoprotein of rat serum. -□-; results from experimental animals, -O-; results from control animals. Each bar represents results from 4-8 separate serum samples.

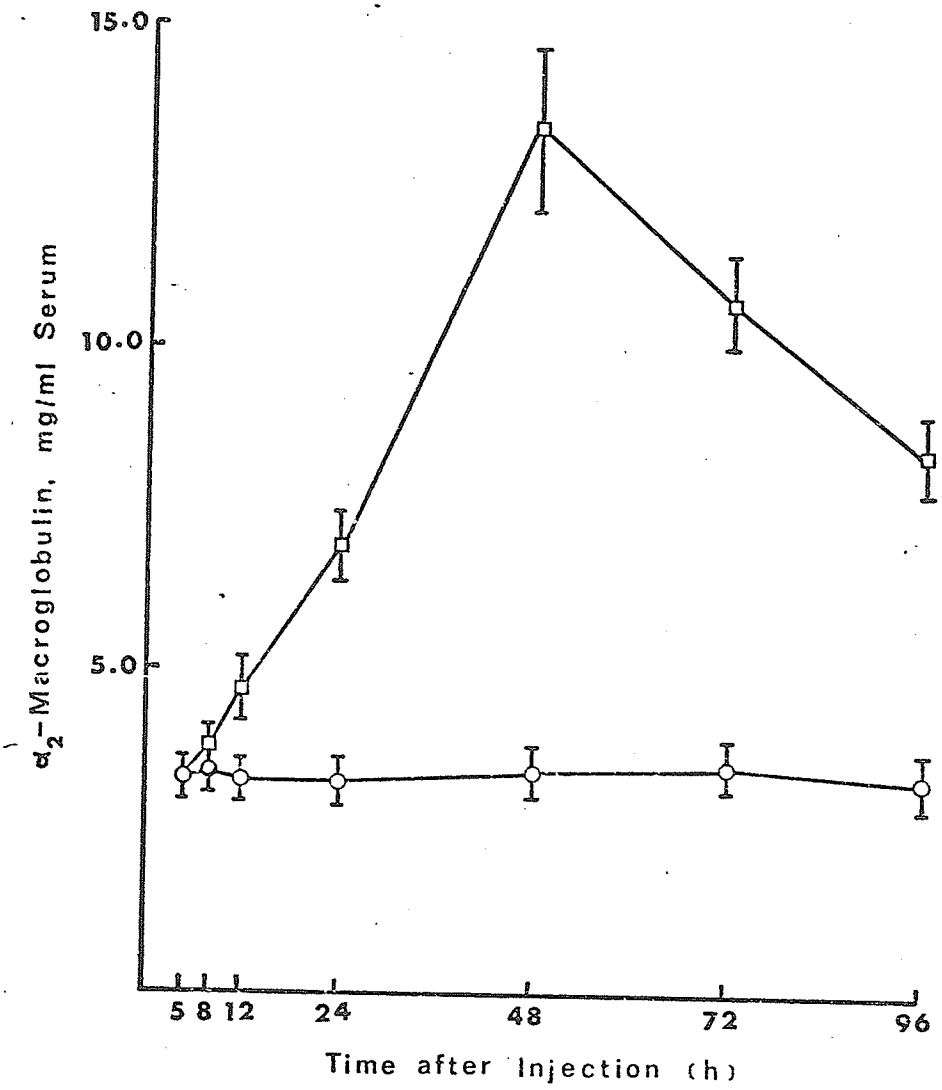


Fig. 23

The effect of turpentine induced inflammation on the content of α_2-macroglobulin of rat serum. -□-; results from experimental animals, -O-; results from control animals. Each bar represents results from 4-8 separate serum samples.

serum from experimental animals reaching a maximum at 48-72 h after injection of inflammatory agent indicating that both are clearly acute phase reactants.

Previous workers have indicated that serum albumin is not an acute phase globulin (213-215). Since it was the intention to use this protein as a reference protein in subsequent biosynthetic studies (see below), the content of albumin in serum from control and experimental animals was determined in order to confirm that albumin does not change in content in serum as a result of inflammation. Two methods were used to determine the content of albumin in rat serum. In the first method the quantitative precipitin technique described above was applied to serum samples from control and experimental animals employing antiserum to rat serum albumin. Fig. 24 shows a quantitative precipitin curve obtained when albumin was titrated with its corresponding antiserum and Table 5 shows the results obtained for the content of albumin in serum from control and experimental rats. Also shown in Table 5 are results obtained using the second method in which an isotope dilution technique was employed to determine the content of albumin in serum from

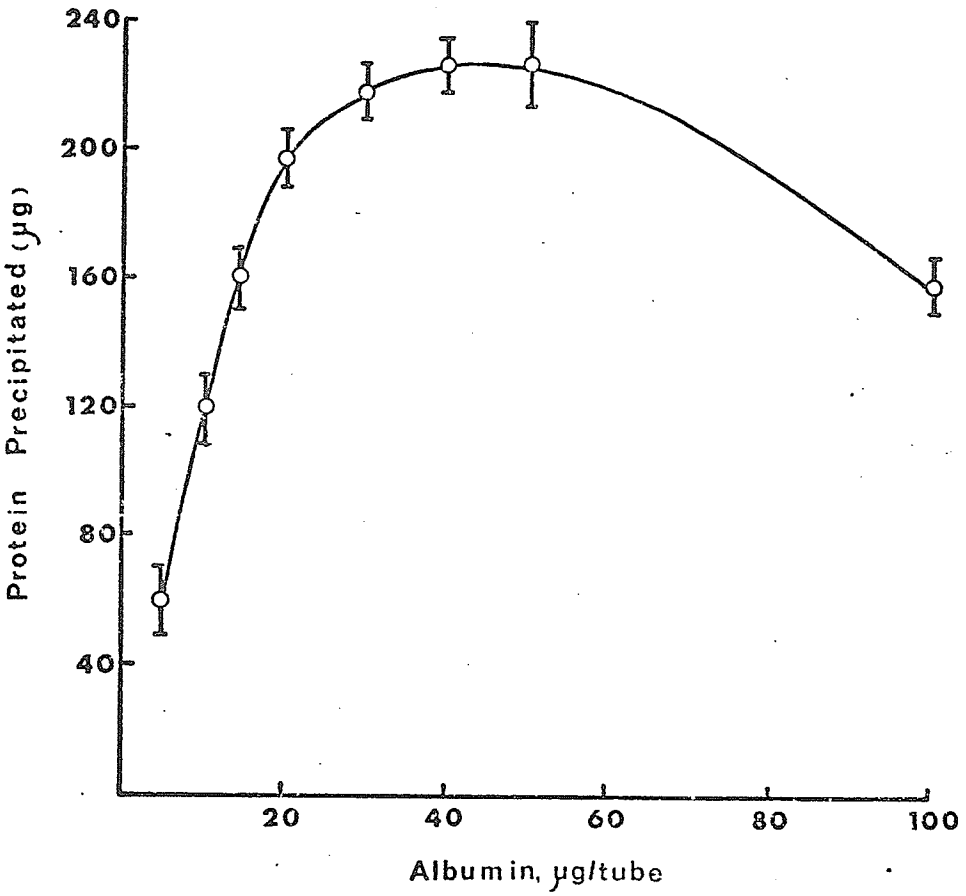


Fig. 24

Quantitative precipitin curve for albumin. Each bar represents results from 4-8 experiments.

Table 5

The effect of inflammation on the content of albumin in rat serum*

Time h	Content of albumin in rat serum mg albumin/ml	
	1	2
Controls+	39.5 ± 2.9(5)	38.9 ± 1.7(8)
5	36.0 ± 2.5(4)	38.3 ± 2.0(4)
8	36.0 ± 2.0(3)	39.2 ± 1.7(4)
12	37.9 ± 1.9(6)	37.3 ± 2.8(5)
24	34.2 ± 1.0(3)	34.0 ± 1.5(5)
48	33.5 ± 1.5(4)	34.5 ± 1.9(5)
72	33.0 ± 2.5(4)	35.0 ± 3.0(4)
96	36.0 ± 0.9(4)	36.2 ± 2.0(4)

* Results are expressed as mean values plus or minus standard errors of the mean with the number of experiments performed in brackets

\+ Controls represent results from serum from control animals 5-96h after injection of NaCl

1 Results obtained using quantitative precipitin technique

2 Results obtained using isotope dilution technique

control and experimental animals. The content of albumin in serum remained fairly constant in rats suffering from inflammation for 5-12 h although there was a slight decrease in the content of albumin in serum of experimental rats at 48-96 h after injection of turpentine. Thus the content of albumin in serum does not appear to change significantly in response to inflammation indicating that it is probably not an acute phase reactant.

In order to determine whether complete precipitation of antigens was obtained using the quantitative precipitin technique the procedure was applied to dansyl labelled proteins. The amount of each antigen precipitated was determined by measuring the amount of fluoresence in the precipitated material. The quantitative precipitin curves obtained using dansyl labelled antigens were indistinguishable from curves obtained using unlabelled antigens (c.f. Figs.20,21,24). Measurement of fluoresence in the precipitates indicated that not less than 90% of the three antigens were recovered, negligible fluoresence was present in the supernatants following precipitation with antiserum. In order to determine whether quantitative precipitation of antigens was obtained with serum samples,

small amounts of fluorescent antigens were added to several serum samples prior to addition of the three antisera used in the present studies. Fluoresence was again measured in the precipitates which indicated that not less than 90% of albumin, α_1-acid glycoprotein and α_2-macroglobulin were recovered by application of the quantitative precipitin technique to serum samples.

Studies on the effect of inflammation on the incorporation of L-leucine-^{3}H and D-glucosamine-^{14}C into rat serum proteins *in vivo*

The results of analyses of serum samples from control and experimental animals for protein-bound hexose and hexosamine (Figs. 5 and 6) indicate that there is an increase in content of carbohydrate-containing proteins of serum as a result of inflammation. The carbohydrate-containing proteins which respond to inflammation appear in large part in fractions 1, 4 and 5b upon fractionation of serum on DEAE-cellulose coupled with preparative electrophoresis on Cellogel strips (Tables 2 and 3). Two carbohydrate-containing proteins were subsequently isolated from fraction 5 as starting material and

were shown to be acute phase globulins (Figs. 22 and 23). One explanation for the increase in content of acute phase glycoproteins such as the α_1-acid glycoprotein and α_2-macroglobulin in experimental animals is that there may be an increase in the rate of synthesis of certain carbohydrate-containing proteins as a result of experimentally induced inflammation. The increase in content of acute phase proteins in serum from experimental animals could also arise from a decreased rate of catabolism of such proteins. At present, however, the only accurate method for measuring synthesis rates of plasma proteins is the ^{14}C-carbonate method (213,216), a procedure which is technically difficult to perform and cannot easily be applied to several proteins concurrently. An alternative method of obtaining information on the rates of synthesis of plasma proteins is to use another protein synthesized by liver parenchymal cells as a reference protein for the incorporation of labelled compounds. In this way errors due to differences in intracellular pool sizes of amino acid, corresponding to the amino acid used as labelled precursor, between individual animals can be largely overcome. Serum albumin has been used as a reference

protein in studies on acute phase reactants by other workers, mainly because it was not apparently affected by experimental inflammation (213-215). Also, the present work has shown that the content of albumin in serum did not change significantly at 5-12 h after exposure to inflammatory agent, although there was a slight reduction in content at longer times after administration of inflammatory agent (Table 5). In order to determine if there was any change in the incorporation of labelled precursors into rat serum albumin as a result of inflammation control and experimental animals were given injections of L-leucine-^{3}H and killed after 90 minutes incorporation. Albumin was isolated from serum using two methods. In the first method the specific radioactivity of serum albumin was calculated from the specific radioactivity of precipitates recovered by application of the quantitative precipitin technique to serum samples. In the second method albumin was recovered by precipitation of serum samples with ethanolic trichloroacetic acid followed by preparative electrophoresis on Cellogel strips and the specific radioactivity determined. Table 6 gives the results for the specific radioactivities of albumin isolated from serum from control and

Table 6

Effect of inflammation on the incorporation of L-leucine-^{3}H into rat serum albumin*

Time h	Specific radioactivity of albumin from rat serum mμCi/mg	
	1	2
Controls+	6.3 ± 0.3(5)	6.2 ± 0.4(12)
5	6.5 ± 0.3(4)	6.8 ± 0.5(4)
8	6.7 ± 0.6(3)	6.1 ± 0.4(4)
12	6.5 ± 0.4(6)	6.5 ± 0.6(6)
24	5.3 ± 0.2(3)	6.3 ± 0.5(8)
48	5.5 ± 0.2(4)	6.2 ± 0.4(6)
72	6.0 ± 0.3(4)	6.3 ± 0.7(6)
96	6.0 ± 0.4(4)	6.4 ± 0.5(6)

* Results are expressed as mean values plus or minus standard errors of the mean with the number of experiments performed in parenthesis

\+ Controls represent results from serum from control animals 5-96h after injection of NaCl

1 For measurement of specific radioactivity, albumin was isolated from serum by the quantitative precipitin technique

2 For measurement of specific radioactivity, albumin was isolated from serum by precipitation with ethanolic trichloroacetic acid followed by preparative electrophoresis on Cellogel blocks

experimental animals by application of the above procedures. It can be seen from Table 6 that there is little change in specific radioactivities of serum albumin, particularly at 5-12 h following inflammation, but there may have been a slight decrease in specific radioactivities at longer times of exposure to inflammatory agent.

Since the above results for specific radioactivities of albumin and results presented earlier for the contents of albumin in serum from control and experimental animals indicate that albumin does not change significantly as a result of inflammation, particularly at short times of exposure to inflammatory agent, this protein was employed as a reference protein or internal standard for measurement of the incorporation of L-leucine-^{3}H into other serum proteins.

Control animals and animals suffering from inflammation for 5, 8 and 12 h were used in studies designed to provide information on changes in the rates of synthesis of serum proteins as a result of inflammation. Short times of exposure to turpentine were chosen since there was no large increase in protein-bound carbohydrate (Tables 2 and 3) or α_1-acid glycoprotein (Fig. 22) and α_2-macroglobulin

(Fig. 23) of serum from experimental animals 5-12 h after administration of turpentine. The pool sizes of proteins in the serum of these animals should, therefore, be similar to controls. A mixture of L-leucine-^{3}H and D-glucosamine-^{14}C was administered to rats and incorporation allowed to proceed for 90 minutes and serum prepared. L-Leucine-^{3}H was used as a precursor for polypeptide synthesis, and D-glucosamine-^{14}C was used as a precursor for the synthesis of the carbohydrate chains of serum glycoproteins. Glucosamine was used in these studies since it is incorporated only into N-acetylglucosamine and sialic acid residues of glycoproteins, but not into amino acids (116). Two types of experiment were performed. In the first type, serum samples were fractionated on DEAE-cellulose using the stepwise elution technique previously described (see Experimental, also Fig. 7). Table 7 gives the specific radioactivities of the fractions so obtained. In the case of labelling with L-leucine-^{3}H specific radioactivities of fractions are expressed in terms of a specific radioactivity of 1.0 mμCi/mg for serum albumin isolated from the same serum sample. The serum albumin has, therefore, been used as a reference protein in calculating the

Table 7

Effect of inflammation on the incorporation of L-leucine ^{3}H and D-glucosamine - ^{14}C into fraction of rat serum*

	Specific radioactivities m μC ^{3}H/mg protein+						
		Experimental 5 Hr (4)		Experimental 8 Hr (4)		Experimental 12 Hr (4)	
	Controls (10)		Ratio Exp./control		Ratio Exp./control		Ratio Exp./control
1	0.74 ± 0.07	0.73 ± 0.06	0.99	0.97 ± 0.10	1.31	1.80 ± 0.14	2.44
2	0.69 ± 0.07	0.75 ± 0.06	1.09	1.10 ± 0.08	1.59	1.20 ± 0.10	1.74
3	0.87 ± 0.07	0.95 ± 0.09	1.09	1.27 ± 0.11	1.46	1.50 ± 0.10	1.73
4	1.16 ± 0.11	2.00 ± 0.07	1.72	2.65 ± 0.17	2.29	3.50 ± 0.21	3.02
5a	1.00	1.00	1.00	1.00	1.00	1.00	1.00
5b	1.20 ± 0.10	2.40 ± 0.19	2.00	2.40 ± 0.10	2.00	3.35 ± 0.19	2.80
6	1.09 ± 0.08	1.08 ± 0.06	0.99	1.25 ± 0.11	1.14	1.41 ± 0.11	1.29
7	2.01 ± 0.12	2.15 ± 0.15	1.07	2.10 ± 0.14	1.04	2.71 ± 0.21	1.35

	Specific radioactivities m μC ^{14}C/mg protein						
		Experimental 5 Hr (4)		Experimental 8 Hr (4)		Experimental 12 Hr (4)	
	Controls (10)		Ratio Exp./control		Ratio Exp./control		Ratio Exp./control
1	1.40 ± 0.12	1.43 ± 0.05	1.02	1.95 ± 0.15	1.39	1.99 ± 0.17	1.42
2	1.21 ± 0.11	1.61 ± 0.15	1.33	1.65 ± 0.06	1.36	1.55 ± 0.14	1.28
3	1.08 ± 0.07	1.36 ± 0.13	1.26	1.47 ± 0.11	1.36	1.37 ± 0.11	1.27
4	1.45 ± 0.13	2.80 ± 0.22	1.93	2.95 ± 0.20	2.04	2.90 ± 0.08	2.00
5a	-	-	-	-	-	-	-
5b	1.72 ± 0.13	3.33 ± 0.25	1.94	3.65 ± 0.27	2.12	2.95 ± 0.25	1.72
6	1.43 ± 0.11	1.88 ± 0.15	1.31	2.10 ± 0.19	1.47	1.80 ± 0.11	1.26
7	1.98 ± 0.16	2.20 ± 0.20	1.11	2.30 ± 0.20	1.16	2.02 ± 0.09	1.02

* Results are expressed as mean specific radioactivities ± standard error of the mean with the number of experiments performed in parenthesis.

\+ Specific radioactivities of ^{3}H are expressed in terms of the ratio of the specific radioactivity of a given fraction to the specific radioactivity of fraction 5a (serum albumin) isolated from the same serum sample.

results in Table 7 for labelling with L-leucine-^{3}H. Since serum albumin contains little or no glucosamine a similar calculation could not be performed with D-glucosamine-^{14}C. The results in Table 7 are also expressed in terms of a ratio of specific radioactivities of fractions from experimental animals to specific radioactivities of corresponding fractions from controls. Therefore, this ratio represents a change in the amount of labelled compound incorporated into protein fractions isolated from serum from experimental animals when compared to the amount of the same labelled compound incorporation into corresponding fractions from control animals for the same period of incorporation. An increase in this ratio probably indicates that there is an increase in the amount of these proteins synthesized and perhaps also in the rate of synthesis of proteins present in the fraction in question. The ratio was increased in most fractions following labelling with ^{3}H and ^{14}C, but the ratio was greatest in fractions 1, 4 and 5b at 8-12 h after administration of turpentine. The increase in protein-bound carbohydrate found in fractions 1, 4 and 5b isolated from the serum of experimental animals (Tables 2 and 3) is, therefore, most likely explained

by an increase in the production of the carbohydrate-containing proteins present in these fractions probably as a result of an increase in their rates of synthesis.

In the second type of experiment the above procedure was applied to α_1-acid glycoprotein, α_2-macroglobulin and albumin isolated from serum samples by application of the quantitative precipitin technique previously described. In this way information was obtained on the incorporation of precursors of glycoprotein biosynthesis into specific proteins which are known to be acute phase globulins present in serum of control and experimental animals. In the case of labelling with L-leucine-^3H, albumin was again employed as a reference protein as described above. The results shown in Table 8 indicate that there is an increase in the ratio of specific radioactivities of both proteins isolated from serum of experimental animals when compared to specific radioactivities of proteins isolated from serum of control animals. This suggests that the increased content of the two acute phase globulins in question in serum from experimental animals is probably due to an increased production or rate of synthesis of the proteins under examination.

Table 8

Effect of Inflammation on the incorporation of L-leucine-^{3}H and D-glucosamine-^{14}C into α_1-acid glycoprotein and α_2-macroglobulin*

Specific radioactivities, mμCi/mg protein+

Protein	Controls(5)	Exptl. 5h(4)	Exptl. / Control	Exptl. 8h(3)	Exptl. / Control	Exptl. 12h(4)	Exptl. / Control
Incorporation of ^{3}H							
α_1-acid glycoprotein	2.05 $\pm$ 0.14	3.15 $\pm$ 0.10	1.55	3.50 $\pm$ 0.10	1.71	4.15 $\pm$ 0.15	2.02
α_2-macroglobulin	1.33 $\pm$ 0.04	1.40 $\pm$ 0.05	1.05	1.81 $\pm$ 0.08	1.35	2.43 $\pm$ 0.19	1.83
Incorporation of ^{14}C							
α_1-acid glycoprotein	5.90 $\pm$ 0.20	11.0 $\pm$ 0.90	1.85	11.8 $\pm$ 1.05	2.00	12.2 $\pm$ 1.10	2.10
α_2-macroglobulin	1.12 $\pm$ 0.04	1.29 $\pm$ 0.10	1.15	1.32 $\pm$ 0.06	1.18	1.90 $\pm$ 0.14	1.70

* Results are expressed as mean specific radioactivities plus or minus standard errors of the mean with the number of experiments performed in parenthesis.

+ Specific radioactivities of ^{3}H are expressed in terms of the ratio of the specific radioactivity of α_1-acid glycoprotein or α_2-macroglobulin to the specific radioactivity of serum albumin isolated from the same serum sample.

Studies on the effect of inflammation on the biosynthesis of α_1-acid glycoprotein and and albumin *in vivo*

The previous results indicated that the increase in content of α_1-acid glycoprotein and α_2-macroglobulin in serum from experimental animals may be due to an increased synthesis or rate of synthesis of the two proteins in question. Albumin was apparently not affected as a result of induced inflammation. The work presented hereafter describes some aspects of the biosynthesis of albumin and α_1-acid glycoprotein in control and experimental animals with the object of obtaining some information on the mechanism of response of an acute phase glycoprotein to induced inflammation and, at the same time, studying the synthesis of albumin which is not an acute phase globulin. The α_1-acid glycoprotein was chosen for all subsequent studies instead of the α_2-macroglobulin for three main reasons: 1. the α_1-acid glycoprotein has a higher carbohydrate content than the α_2-macroglobulin (Table 4) and is probably making a greater contribution to the increase in total protein-bound carbohydrate of serum as a result of inflammation; 2. the

α_1-acid glycoprotein increases about six-fold in content in serum from experimental animals whereas the α_2-macroglobulin increases only three-fold (Figs. 22,23); 3. the α_1-acid glycoprotein closely resembles human α_1-acid glycoprotein (orosomucoid) in its carbohydrate content, molecular weight and isoelectric point (see Introduction). As previously mentioned orosomucoid has been implicated in the inflammatory response in humans and as a consequence has been studied in some detail by other workers (e.g. 162) (see Introduction).

Time course of incorporation of L-leucine-^{3}H and D-glucosamine-^{14}C into albumin and α_1-acid glycoprotein in serum

Subsequent biosynthetic studies on albumin and α_1-acid glycoprotein will be meaningful only if incorporation studies are performed at relatively short times of exposure of animals to labelled compounds. Therefore, the time course of incorporation of labelled precursors of protein and glycoprotein biosynthesis into albumin and α_1-acid glycoprotein was studied in order to determine the time required for the appearance of labelled proteins in serum.

In these experiments, experimental animals suffering from inflammation for 12 h were chosen since it was at this time after induction of inflammation that the highest specific radioactivity of α_1-acid glycoprotein was observed (Table 8). Also, the time chosen corresponded to a time prior to the appearance of a significant increase in α_1-acid glycoprotein in serum from experimental animals (Fig. 22).

Rats were given injections of L-leucine-^{3}H and D-glucosamine-^{14}C, killed at various times thereafter, and albumin and α_1-acid glycoprotein isolated from serum by application of the quantitative precipitin technique previously described. Fig. 25 shows that little label from L-leucine-^{3}H appeared in albumin isolated from serum for about 15 minutes after injection; thereafter there was a rapid rise in specific radioactivity. There was little difference between the time course of incorporation of L-leucine-^{3}H into albumin isolated from control and experimental animals. A similar lag period was observed in the appearance of label from L-leucine-^{3}H (Fig. 26) and D-glucosamine-^{14}C (Fig. 27) into serum α_1-acid glycoprotein, however, there was a more rapid increase in specific

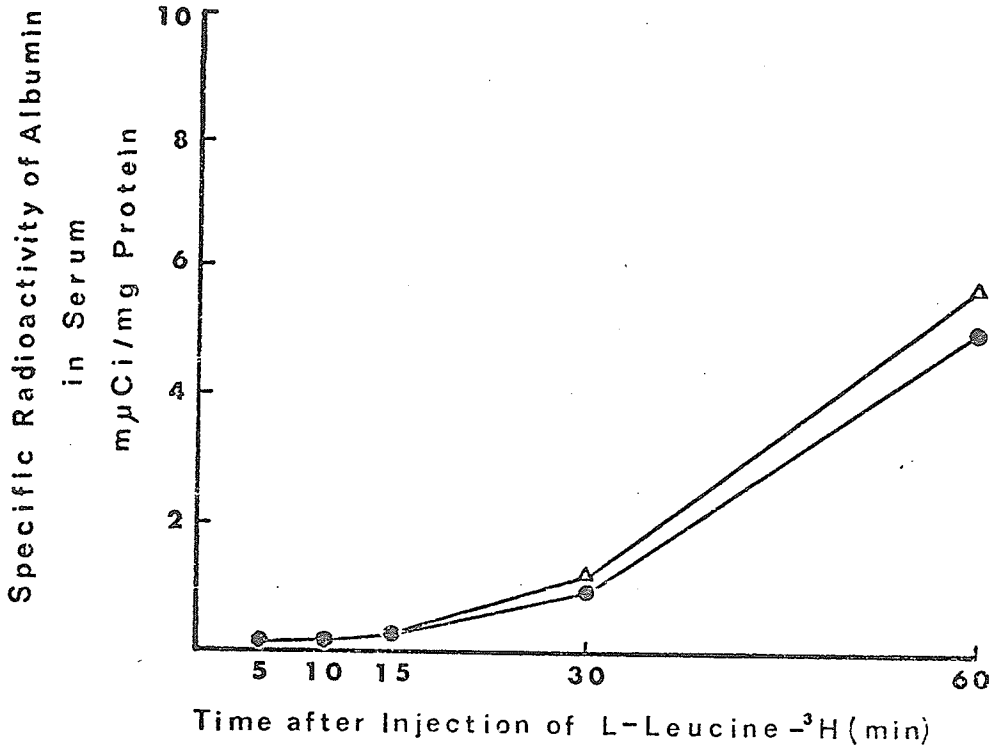


Fig. 25

Time course of incorporation of radioactivity from L-leucine-^{3}H into serum albumin from experimental animals (-△-) and control animals (-●-). Each point represents the mean of three to six experiments; animals were killed one minute before the times indicated and blood was collected for 1 minute.

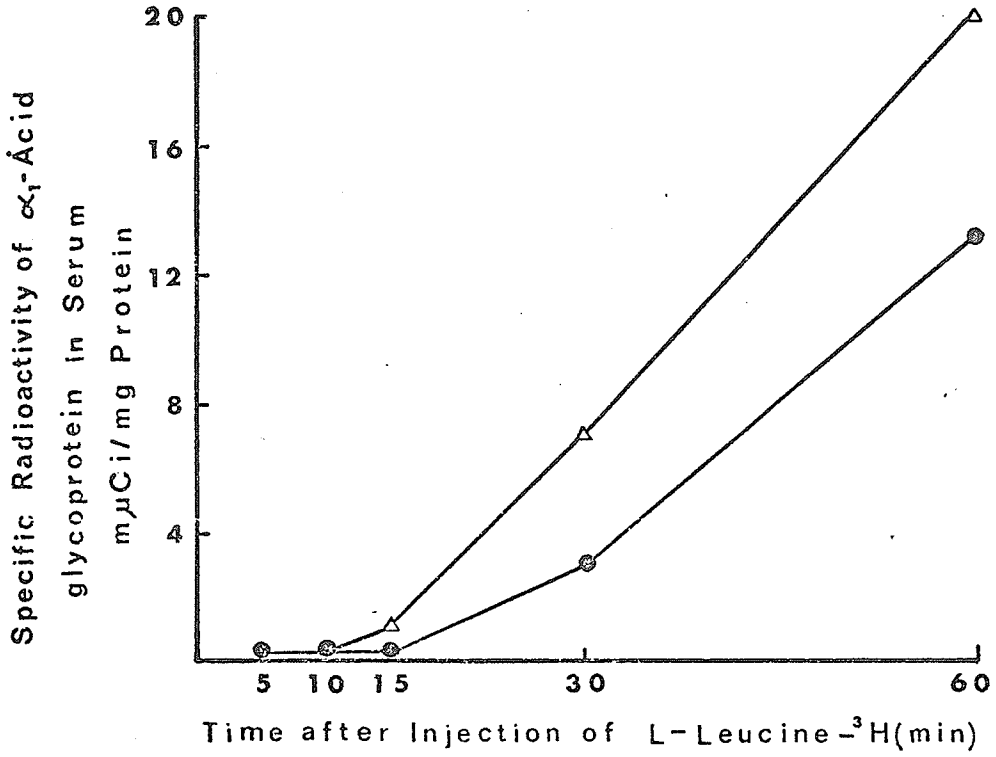


Fig. 26

Time course of incorporation of radioactivity from L-leucine-^{3}H into serum α_1-acid glycoprotein from experimental animals (-△-) and control animals (-●-). Each point represents the mean of three to six experiments; animals were killed one minute before the times indicated and blood was collected for 1 minute.

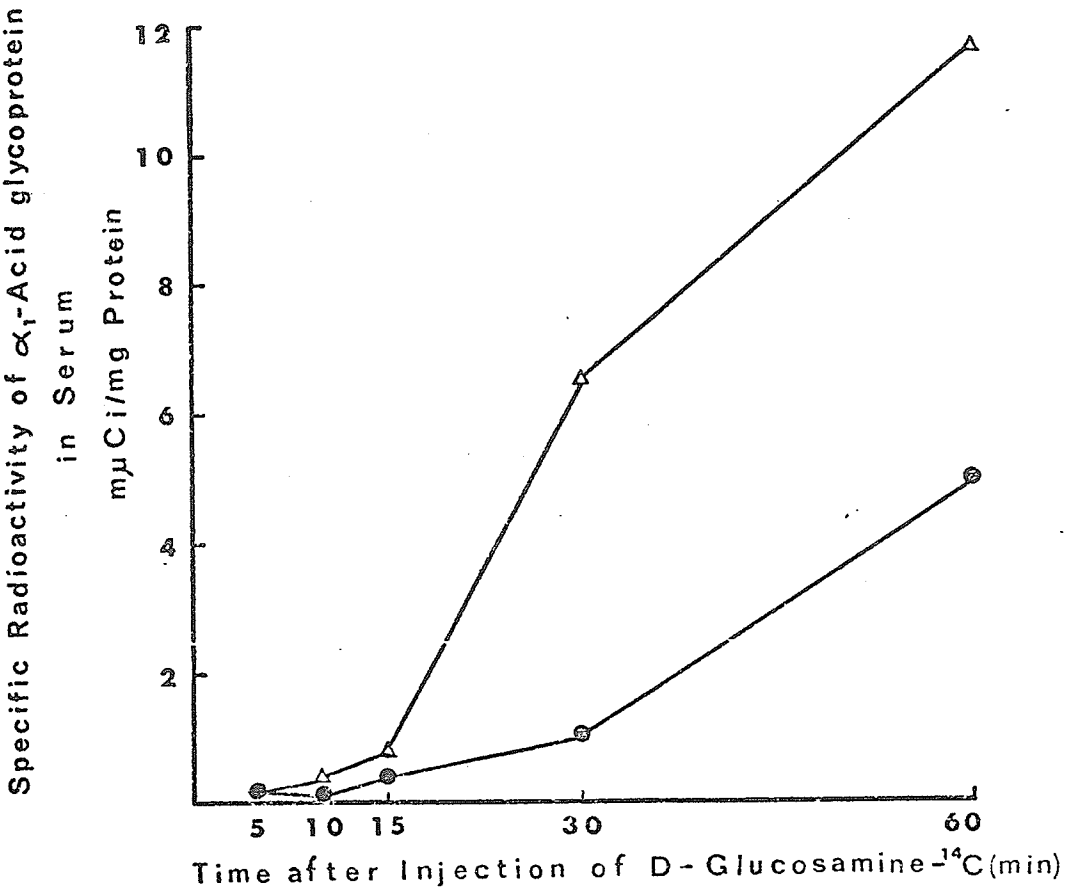


Fig. 27

Time course of incorporation of radioactivity from D-glucosamine-^{14}C into serum α_1-acid glycoprotein from experimental animals (-△-) and control animals (-●-). Each point represents the mean of three to six experiments; animals were killed one minute before the times indicated and blood was collected for 1 minute.

radioactivity of both labelled compounds in α_1-acid glycoprotein isolated from the serum of experimental animals.

Immunodiffusion studies on the biosynthesis of albumin and α_1-acid glycoprotein: determination of the cellular and subcellular site of synthesis

In studies designed to determine the cellular and subcellular site of synthesis of α_1-acid glycoprotein and also to confirm results reported by other workers that components of the liver microsome fraction are the site of synthesis of serum albumin (111-113), tissues were removed from animals killed at 12-15 minutes after intravenous injection of L-leucine-^{14}C or D-glucosamine-^{14}C. As previously indicated negligible label from radioactive leucine or glucosamine as precursors is incorporated into α_1-acid glycoprotein or albumin of serum at these short time intervals after injection (e.g. Figs. 25-27). Thus, the presence in intracellular structures of labelled material capable of reaction with antisera to the two proteins under examination should provide information on the site of synthesis of these proteins. The tissues investigated on the

basis of previous work (e.g. 94,99,103,217) were liver, the site of synthesis of many serum proteins; spleen, which can synthesize some serum proteins; and as a control kidney, which has little or no capacity for the synthesis of serum proteins.

Livers were fractionated into nuclear, mitochondrial and microsome fractions and the fractions extracted with the nonionic detergent Lubrol-W. Spleen and kidney were normally extracted with Lubrol-W *in toto*. Lubrol-W is known to dissolve lipoprotein membranes and has previously been shown to solubilize most or all of the nascent serum proteins normally associated with particulate fractions following subcellular fractionation of animal tissues (206). Unless otherwise stated Lubrol extracts were concentrated about six-fold and the concentrated extracts together with the soluble cell sap fraction from the fractionation of liver examined by double diffusion analysis in agar gels employing antiserum to albumin or α_1-acid glycoprotein. After reaction with antisera the gels were washed, dried and a radioautograph prepared in order to detect radioactivity. With amino acid as precursor, extracts of microsome material gave a precipitin line that was strongly labelled on reaction with antiserum to

α_1-acid glycoprotein (Fig. 28). A precipitin line which formed on reaction of Lubrol extracts of the nuclear fraction with antiserum to α_1-acid glycoprotein also showed faint labelling. With glucosamine as precursor, only extracts of microsome material gave a precipitin line that was labelled on reaction with antiserum to α_1-acid glycoprotein (Fig. 29). In most cases lines formed by Lubrol extracts of microsome material gave reactions of immunological identity with lines formed by serum and α_1-acid glycoprotein. With amino acid as precursor extracts of microsome material were the main source of precipitin lines that were labelled following reaction with antiserum to serum albumin (Figs. 30 and 31). Occasionally, however, a precipitin line formed by extracts of the nuclear fraction was faintly labelled, possibly because of slight contamination of the nuclear fraction by components of the microsome fraction. Since albumin does not contain glucosamine, precipitin lines formed by reacting extracts of the microsome fraction with antiserum to serum albumin, following labelling with glucosamine, were not radioactive. In most cases immunological correspondence was demonstrated between lines given by extracts of the

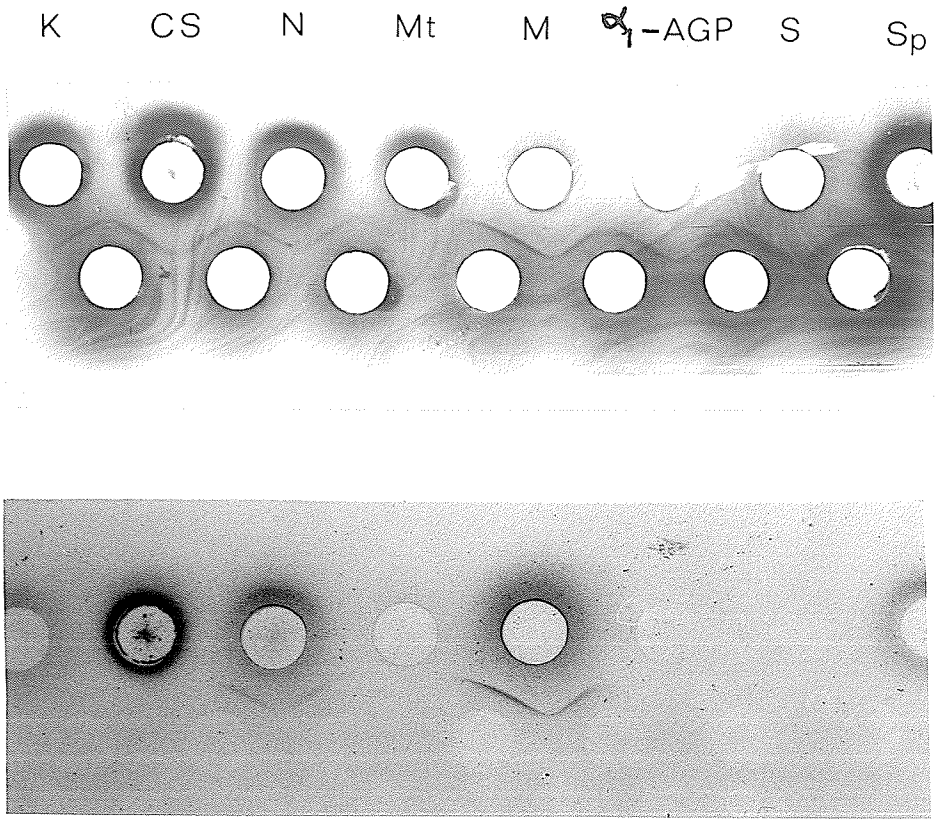


Fig. 28

Results of double diffusion analysis with antiserum of α_1-acid glycoprotein in the lower wells of Lubrol extracts of M, microsome, Mt, mitochondria, N, nuclear fractions of rat liver, K, kidney and Sp, spleen; also reacted were CS, liver cell sap, S, serum isolated from the animal at death (diluted 1:4 with 0.15M NaCl) and α_1-AGP, α_1-acid glycoprotein (5mg/ml). Labelling was for 13 minutes with L-leucine-^{14}C and immunodiffusion was for 30h. Top photograph: precipitin bands stained for protein; bottom photograph: autoradiograph.

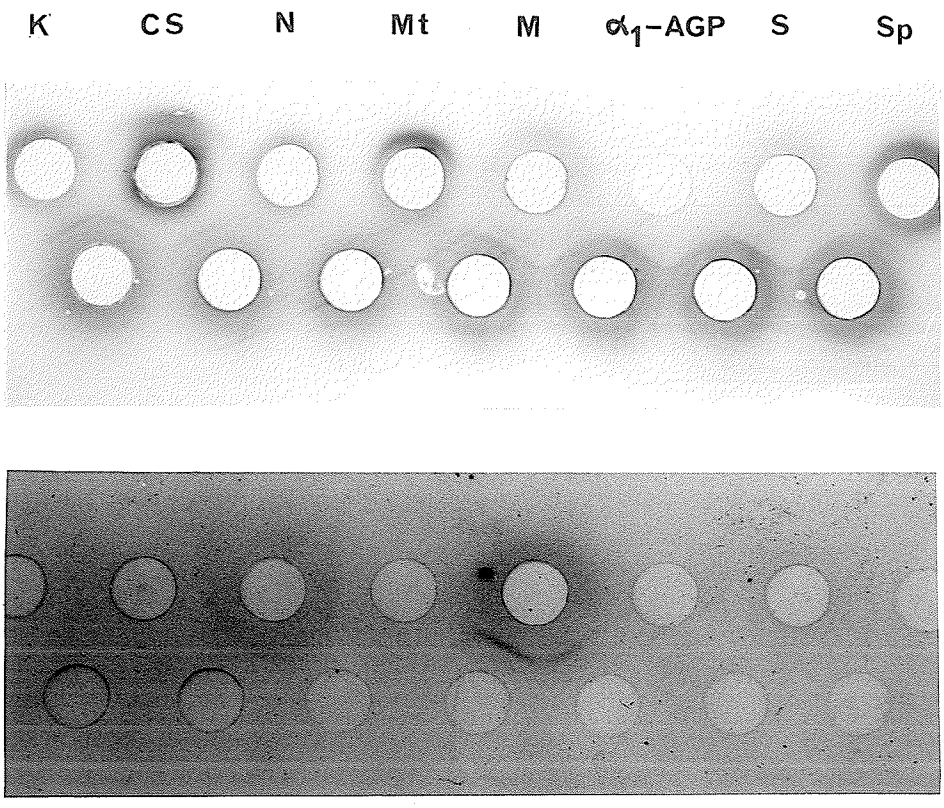


Fig. 29

Results of double diffusion analysis with antiserum to α_1-acid glycoprotein in the lower wells of Lubrol extracts of M, microsome, Mt, mitochondria, N, nuclear fractions of rat liver, K, kidney and Sp, spleen; also reacted were CS, liver cell sap, S, serum isolated from the animal at death (diluted 1:4 with 0.15M NaCl) and α_1-AGP, α_1-acid glycoprotein (5mg/ml). Labelling was for 13 minutes with D-glucosamine-^{14}C and immunodiffusion was for 30h. Top photograph: precipitin bands stained for protein; bottom photograph: autoradiograph.

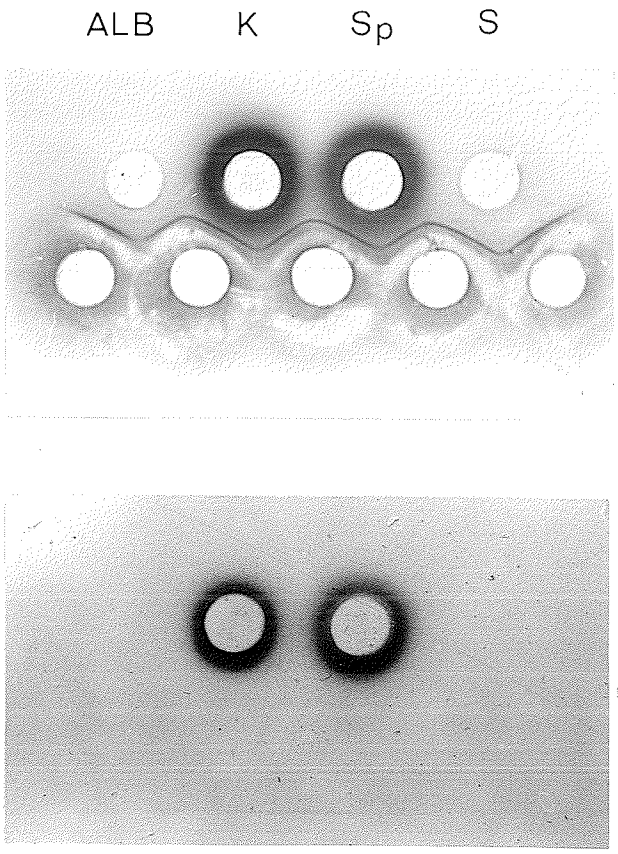


Fig. 30

Results of double diffusion with antiserum to albumin in the lower wells of Lubrol extracts of K, kidney and Sp, spleen; also reacted were ALB, albumin (2mg/ml) and S, serum isolated from the animal at death (diluted 1:20 with 0.15M NaCl). Labelling was for 13 minutes with L-leucine-^{14}C and immunodiffusion was for 30h. Top photograph: precipitin bands stained for protein; bottom photograph: autoradiograph.

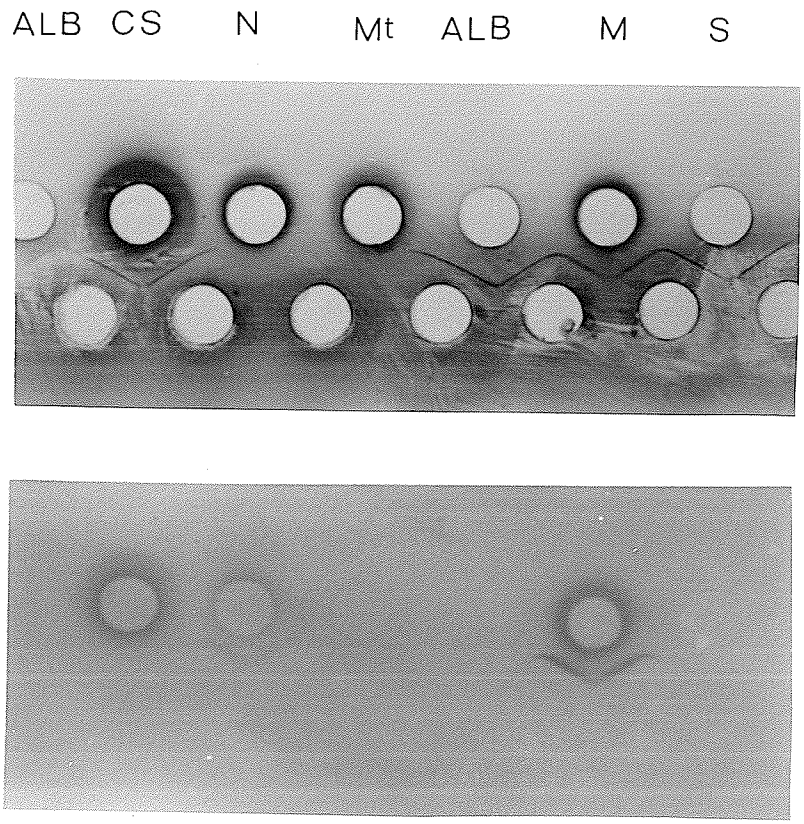


Fig. 31

Results of double diffusion with antiserum to albumin in the lower wells of Lubrol extracts of M, microsome, Mt, mitochondria, and N, nuclear fractions of rat liver; also reacted were CS, liver cell sap, S, serum isolated from the animal at death (diluted 1:20 with 0.15M NaCl) and ALB, albumin (2mg/ml). Labelling was for 13 minutes with L-leucine-^{14}C and immunodiffusion was for 30h. Top photograph: precipitin bands stained for protein; bottom photograph: autoradiograph.

microsome fraction and those formed by serum and albumin on reaction with antiserum to albumin. In all experiments, lines given by serum isolated from the animals at death were not radioactive (Figs. 28-31), confirming the delay in labelling of α_1-acid glycoprotein and albumin in serum found in studies on the time course of incorporation of labelled amino acid and glucosamine into the two proteins under examination. The results obtained from control animals and experimental animals at 12-48 h following induction of inflammation did not differ significantly when extracts of liver fractions were examined by double diffusion analysis as described above. In most experiments involving immunodiffusion studies employing antiserum to α_1-acid glycoprotein and albumin only one precipitin line was obtained from the microsome fraction, serum and the corresponding antigens, further demonstrating the homogeneity of the two proteins under study.

In order to show that labelling of precipitin lines was not due to non-specific absorption or precipitation of labelled material, Lubrol extracts of microsome material from experiments involving incorporation of L-leucine-^{14}C and D-glucosamine-^{14}C were reacted with anti-human serum either directly

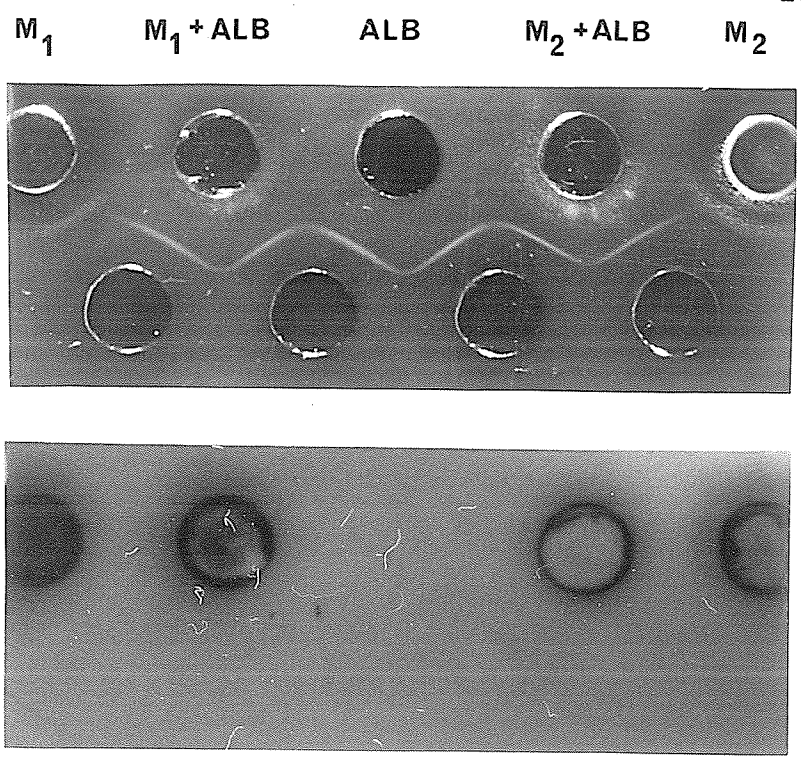


Fig. 32

Results of double diffusion analysis with antiserum to human albumin in the lower wells of M_1, Lubrol extract of rat liver microsome material isolated from a rat 13 minutes after injection of L-leucine-^{14}C; M_1 + ALB, mixture of equal volumes of M_1 and human albumin (4mg/ml); ALB, human albumin (2mg/ml); M_2, Lubrol extract of rat liver microsome material isolated from a rat 13 minutes after injection of D-glucosamine-^{14}C; M_2 + ALB, mixture of equal volumes of M_2 and human albumin (4mg/ml). Immunodiffusion was for 30h. Top photograph: developing precipitin lines; bottom photograph: autoradiograph.

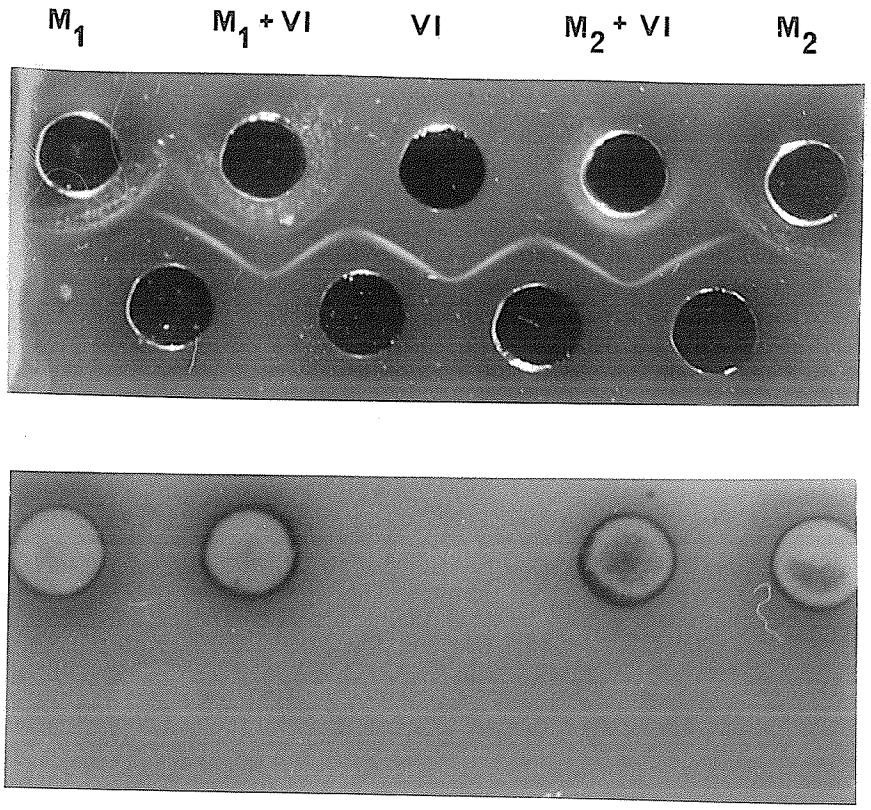


Fig. 33

Results of double diffusion analysis with antiserum to human glycoprotein fraction VI in the lower wells of M_1, Lubrol extract of rat liver microsome material isolated from a rat 13 minutes after injection of L-leucine-^{14}C; M_1 + VI, mixture of equal volumes of M_1 and human fraction VI (6mg/ml); VI, human glycoprotein fraction VI (3mg/ml); M_2, Lubrol extract of rat liver microsome material isolated from a rat 13 minutes after injection of D-glucosamine-^{14}C; M_2 + VI, mixture of equal volumes of M_2 and human glycoprotein fraction VI (6mg/ml). Immunodiffusion was for 30h. Top photograph: developing precipitin lines; bottom photograph: autoradiograph.

or as mixtures with human fraction VI or human albumin. Precipitin lines were not formed on reaction of the extracts in the absence of human fraction VI or human albumin and lines formed by the mixtures were not radioactive (Figs. 32 and 33).

Determination of the contents of α_1-acid glycoprotein and albumin in liver microsome material from control and experimental rats

Results reported above clearly indicate that the microsome fraction of liver is the subcellular site of synthesis of α_1-acid glycoprotein and albumin in control and experimental animals. However, since there is an increase in the content of α_1-acid glycoprotein in the serum of experimental animals which may result from a greater production or rate of synthesis of this protein, changes might be expected in the content of α_1-acid glycoprotein in microsome fractions from livers from experimental animals. On the other hand little change in content of serum albumin in microsome material from experimental animals would be expected since this protein does not apparently respond to induced inflammation.

Microsome fractions were prepared from livers from control and experimental animals at various

times following administration of inflammatory agent, extracted with Lubrol and the contents of α_1-acid glycoprotein and albumin in the extracts determined by application of the quantitative precipitin technique previously described employing antiserum to albumin or α_1-acid glycoprotein. Fig. 34 shows that there was about a five-fold increase in content of α_1-acid glycoprotein in microsome material of livers isolated from experimental animals at 8-12 h following induction of inflammation. Although there was no significant change in the content of albumin in liver microsome fractions from experimental and control animals (Table 9) at short times of exposure to inflammatory agent there was a slight decrease in the content of albumin at longer times of exposure (Table 9).

When the precipitin technique is applied to extracts of particulate material, in this case Lubrol extracts of liver microsome fractions, the extracts must first be precipitated with a heterologous immune system to reduce the possibility of non-specific precipitation when antiserum to α_1-acid glycoprotein and albumin are subsequently added to the system. In the present work two heterologous immune systems were employed. Lubrol extracts were,

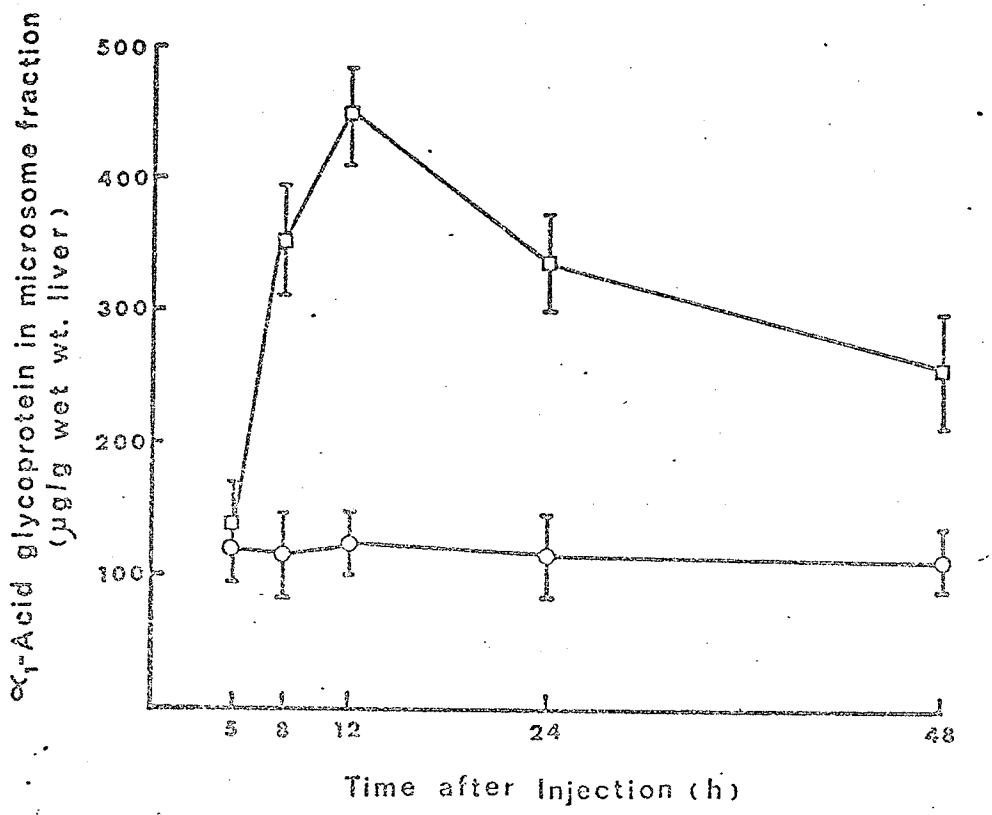


Fig. 34

Effect of turpentine-induced inflammation on the content of α_1-acid glycoprotein in microsome fractions from rat livers. -□-; results from experimental animals, -o-; results from control animals. Each bar represents results from 4-8 animals.

Table 9

Effect of turpentine-induced inflammation on the content of albumin in microsome fractions from rat livers*

Time after administration of turpentine h	Content of albumin in microsome fractions µg/g wet weight liver
Controls+	398 ± 12(10)
5	385 ± 15(6)
8	357 ± 13(4)
12	379 ± 11(10)
24	310 ± 10(4)
48	350 ± 14(3)

* Results are expressed as mean values ± standard errors of the mean with the number of experiments performed in parenthesis

\+ Controls represent results from control animals 5-48h after injection of NaCl

therefore, incubated twice with the system, human albumin-anti-human serum and twice with the system, human glycoprotein fraction VI-anti-human serum. The precipitates were discarded and the supernatants further incubated in order to check that there was no additional precipitation of material. Extracts were then divided into two parts for precipitation with antiserum to rat albumin and antiserum to α_1-acid glycoprotein.

There are three possible sources of error using the above procedure. Firstly, there may be incomplete release of α_1-acid glycoprotein and albumin from microsome material by the extraction procedure employed; this would tend to give low recoveries of α_1-acid glycoprotein and albumin from extracts of the microsome fraction. In order to determine if there was incomplete release of α_1-acid glycoprotein and albumin from microsome material on extraction with Lubrol, microsome fractions prepared from the same sample of liver were extracted directly with 2.5% deoxycholate and Lubrol; in other cases the Lubrol insoluble residues were then extracted with 2.5% deoxycholate*. The

* Deoxycholate is believed to result in complete solubilization of lipoprotein membranes (204), with subsequent release of most or all nascent proteins present within the intravesicular space of the endoplasmic reticulum and Golgi complex.

deoxycholate extracts were precipitated with antisera as described above and were also examined by double diffusion analysis in agar gels employing antiserum to rat serum albumin and α_1-acid glycoprotein. Lubrol extracts of microsome material contained about 95% of the albumin and α_1-acid glycoprotein found in the corresponding deoxycholate extracts when assayed with the quantitative precipitin technique. Deoxycholate extracts of Lubrol insoluble residues contained only a trace of albumin and α_1-acid glycoprotein. The results from immunodiffusion studies confirmed the above observations. The Lubrol and deoxycholate extracts gave precipitin lines on reaction with antiserum to albumin and to α_1-acid glycoprotein, but no lines were observed on reaction of deoxycholate extracts of Lubrol-insoluble residues.

The second source of error in applying the precipitin technique to extracts of microsome material can arise if there is precipitation of rat proteins with the heterologous immune system used in the present work; this would also tend to give low recoveries of albumin and α_1-acid glycoprotein from extracts of the microsome fraction. In order to determine if precipitation of rat proteins was

occuring with the heterologous immune system, albumin and α_1-acid glycoprotein were prepared from the serum of a rat following labelling with L-valine-^{14}C for 90 minutes. The labelled albumin and α_1-acid glycoprotein were then reacted with dansyl chloride to prepare the corresponding DANS derivatives. The DANS labelled albumin and α_1-acid glycoprotein were then employed in an experiment designed to determine whether there were any losses in recovery of albumin and α_1-acid glycoprotein from Lubrol extracts of microsomal material by application of the precipitin technique. Samples of DANS albumin and α_1-acid glycoprotein were added to separate microsome pellets prior to homogenization with Lubrol. After centrifuging, the Lubrol extracts were precipitated with the heterologous immune systems followed by the rat immune systems as previously described. Immune precipitates obtained at all stages in the precipitation procedure and the Lubrol extracts remaining after precipitation with antiserum to rat albumin or α_1-acid glycoprotein were assayed for fluoresence and radioactivity in order to determine at what stages losses of material had occured. Table 10 shows the results obtained in a typical experiment where it can be seen that about

TABLE IO

Determination of the recovery of DANS and ^{14}C in rat albumin and α_1-acid glycoprotein recovered from Lubrol extract of microsome material by application of the quantitative precipitin technique (see text for details).*

	rat albumin added to microsome material +		rat α_1-acid glycoprotein added to microsome material ‡	
Antiserum	recovery DANS %	recovery ^{14}C %	recovery DANS %	recovery ^{14}C %
Human albumin (1)	7	6	1	2
Human VI (1)	8	6	4	1
Human albumin (2)	3	2	1	2
Human VI (2)	2	0	5	5
Rat albumin	76	79	3	2
Rat α_1-acid glycoprotein	1	2	81	76
Residual Lubrol extract ‡‡	3	5	5	7

+ 2.85 optical density units DANS albumin containing 6,550 d.p.m. ^{14}C (1.05mg albumin) added to Lubrol extract of microsome material prior to precipitation with heterologous immune systems and anti-rat systems.

‡ 3.10 optical density units DANS α_1-acid glycoprotein containing 7,450 d.p.m. ^{14}C (0.905mg α_1-acid glycoprotein) added to Lubrol extract microsome material prior to precipitation with heterologous immune systems and anti-rat systems.

‡‡ Lubrol extract remaining after precipitation with all antisera.

* The precipitation was scaled-up three-fold employing 9ml Lubrol extract (see Experimental).

75-80% of the dansyl label and radioactivity were recovered in precipitates obtained from Lubrol extracts following addition of antiserum to rat albumin and α_1-acid glycoprotein. Therefore, in calculating the results for the contents of α_1-acid glycoprotein (Fig. 34) and albumin (Table 9) in Lubrol extracts of liver microsome fractions it was assumed that there was a 20% loss of albumin and α_1-acid glycoprotein when the quantitative precipitin technique was applied to extracts of microsome material.

Another source of error in experiments designed to determine the quantities of serum proteins in extracts of liver microsome material is the possibility of contamination of subcellular fractions by residual serum proteins present in liver. Therefore, in some experiments livers were perfused with physiological saline prior to subcellular fractionation. There was little difference between the contents of albumin and α_1-acid glycoprotein in Lubrol extracts prepared from perfused livers from control and experimental animals when compared with corresponding values from livers which had not been perfused prior to fractionation. However double diffusion analysis of cell sap and Lubrol extracts

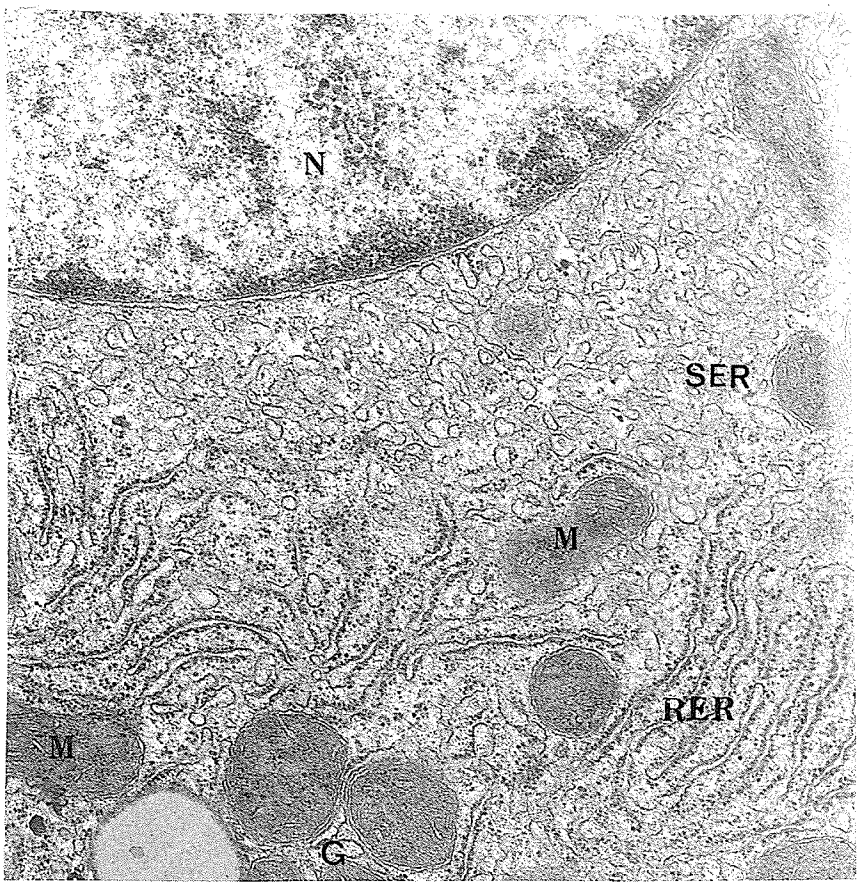
of nuclear and mitochondrial fractions isolated from perfused livers indicated that there was a decrease in material capable of reacting with antiserum to albumin and α_1-acid glycoprotein in cell sap and to a lesser extent in the nuclear fraction, thus suggesting that any residual serum proteins are more likely to be found in cell sap and nuclear material rather than extracts of microsome or mitochondrial fractions. Therefore, it was assumed that residual serum protein did not make a significant contribution to the contents of albumin and $\alpha\alpha_1$-acid glycoprotein found in Lubrol extracts of microsome material.

Ultrastructural studies on livers from control and experimental rats

As indicated above liver microsome material isolated from rats suffering from inflammation for 8-48 h contained increased amounts of α_1-acid glycoprotein, but not of albumin. If this result represents a general response of acute phase globulins to induced inflammation then increased amounts of other acute phase globulins, such as α_2-macroglobulin and haptoglobin, might be expected to occur as a result of inflammation thus leading to a

considerable increase in content of acute phase globulins in liver microsome material. Therefore, livers from control and experimental rats were examined under the electron microscope in order to determine if there were any changes in the appearance of the endoplasmic reticulum and Golgi apparatus in livers from experimental animals as a result of the increased content of α_1-acid glycoprotein and possibly other acute phase globulins associated with microsome fractions prepared from livers from experimental animals.

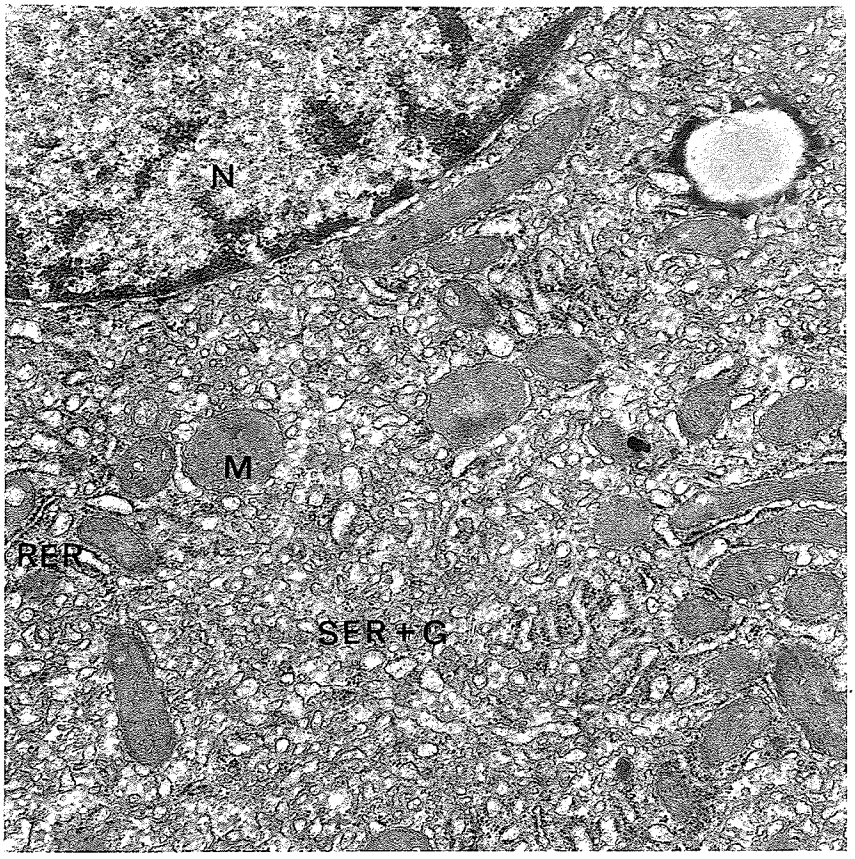
Typical electron micrographs of livers from normal and experimental animals are shown in Figs. 35-36 . The greatest changes in the appearance of cytoplasmic components was observed in livers from rats suffering from inflammation for 12 h, the time after induction of inflammation when the content of α_1-acid glycoprotein, and possibly other acute phase globulins, in liver microsome material is at its greatest (Fig. 34). The changes observed were characterized by a general decrease in the amount of rough endoplasmic reticulum with an increase in the amount of smooth endoplasmic reticulum and, perhaps, also of Golgi complex. The rough endoplasmic reticulum was much less organized in livers



x 20,000

Fig. 35

Electron micrograph of liver cell from normal rat. N, nucleus, M, mitochondria, RER, rough endoplasmic reticulum, SER, smooth endoplasmic reticulum, G, Golgi apparatus.



x 20,000

Fig. 36

Electron micrograph of liver cell from an experimental rat 12h after administration of turpentine. N, nucleus; M, mitochondria; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum, G, Golgi apparatus.

from experimental animals and all three types of membranous components appeared to be dilated (Fig. 36). There was little obvious change in the appearance of the cell nucleus or other components of the cytoplasm. As previously mentioned (see Introduction), serum proteins, destined for secretion from the cell, are believed to be synthesized on ribosomes attached to the rough endoplasmic reticulum and are then chanelled out of the cell by passage through the intracisternal space of the rough and smooth endoplasmic reticulum and are found in the Golgi complex prior to their appearance in serum. Thus the dilation of the endoplasmic reticulum and Golgi complex observed in the electron micrographs of liver cells from experimental animals may be associated with the increased capacity of livers from experimental rats for storage of acute phase globulins.

Studies on the secretion of albumin and α_1-acid glycoprotein in control and experimental animals

The studies described above indicate that there is an increased production and, perhaps, rate of synthesis of α_1-acid glycoprotein, but not albumin as a result of induced inflammation.

Experiments were, therefore, designed to examine the pathway of secretion of α_1-acid glycoprotein and albumin in livers from control and experimental animals in order to determine if there was an increase in the rate of secretion of acute phase globulins as a result of inflammation.

In experiments in which secretion of α_1-acid glycoprotein and albumin from liver was examined, experimental animals suffering from inflammation for 12 h were employed. This time period following inflammation was chosen since it was thought that changes in the secretion patterns of α_1-acid glycoprotein and albumin would be more noticeable because the content of α_1-acid glycoprotein in microsome material is highest at 12 h following administration of inflammatory agent.

As previously mentioned serum proteins are believed to be synthesized on ribosomes associated with the rough endoplasmic reticulum of liver. The polypeptide chain appears first in the intracisternal space of the rough endoplasmic reticulum and the pathway of secretion of the protein from the cell is by way of smooth endoplasmic reticulum and then the Golgi complex. The latter complex is believed to concentrate material prior to secretion from the cell.

It is believed that the carbohydrate groups of serum glycoproteins are added to polypeptide chains during secretion from the cell by sequential addition of sugars by specific transferases located in the membranes of the endoplasmic reticulum and Golgi complex (e.g.129). In order to trace the pathway of secretion of α_1-acid glycoprotein and albumin from liver cells, control and experimental rats were given injections of L-leucine-^{3}H and D-glucosamine-^{14}C, killed at various times thereafter and fractions prepared from liver containing mainly rough endoplasmic reticulum, smooth endoplasmic reticulum and Golgi complex. Figs.37 and 38 show that both types of labelled compounds are rapidly taken up from the plasma and appear in liver protein, over 90% of the L-leucine-^{3}H and D-glucosamine-^{14}C cleared from the serum within the first few minutes following injection. A pulse injection label of both labelled compounds has, therefore, effectively been given.

Lubrol extracts of microsome subfractions were prepared and α_1-acid glycoprotein and albumin recovered by application of the quantitative precipitin technique and their specific radioactivities determined. Figs. 39 and 40 show the results obtained for the specific radioactivity of ^{3}H in

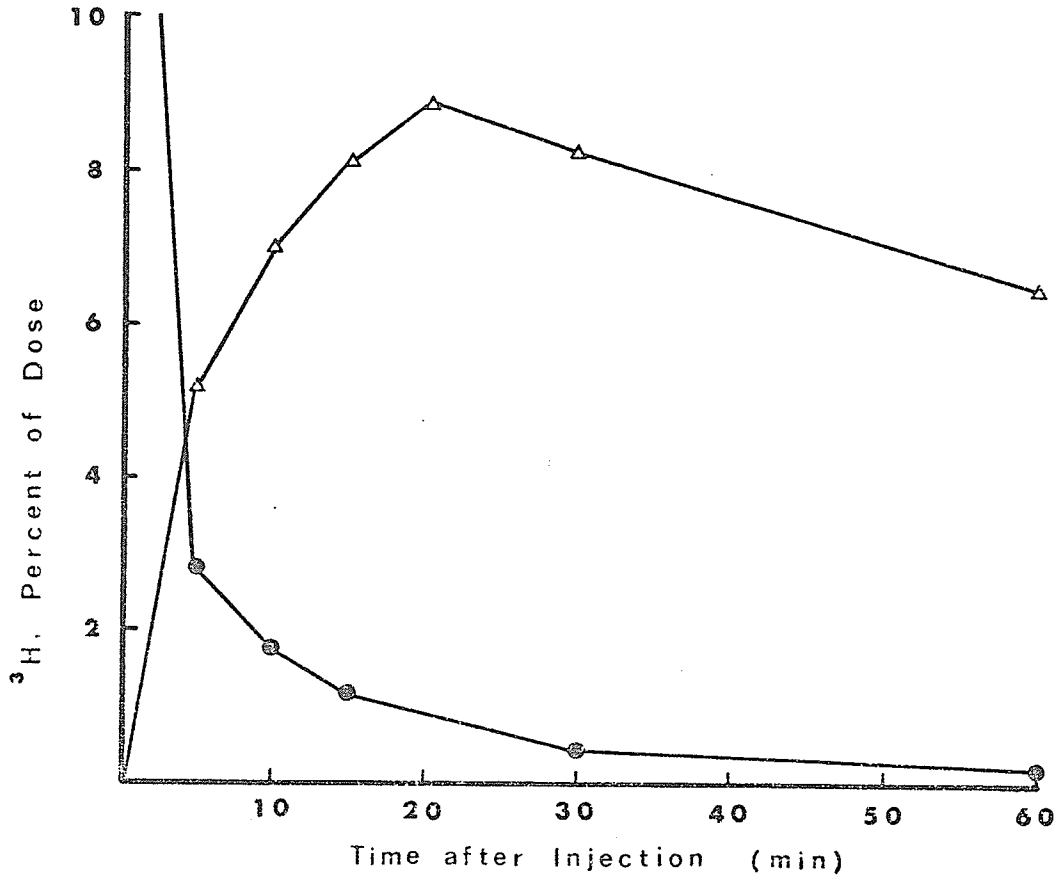


Fig. 37

Time course of incorporation of radioactivity from L-leucine-^{3}H into total liver protein (-△-). Disappearance of free L-leucine-^{3}H from serum is represented by solid circles (-●-). Animals received an intravenous injection of 50μCi L-leucine-^{3}H/100g body weight. Each point represents the mean of three to five experiments. In order to construct the above figure it was assumed that serum represented 3.5% and liver 4% of body weight.

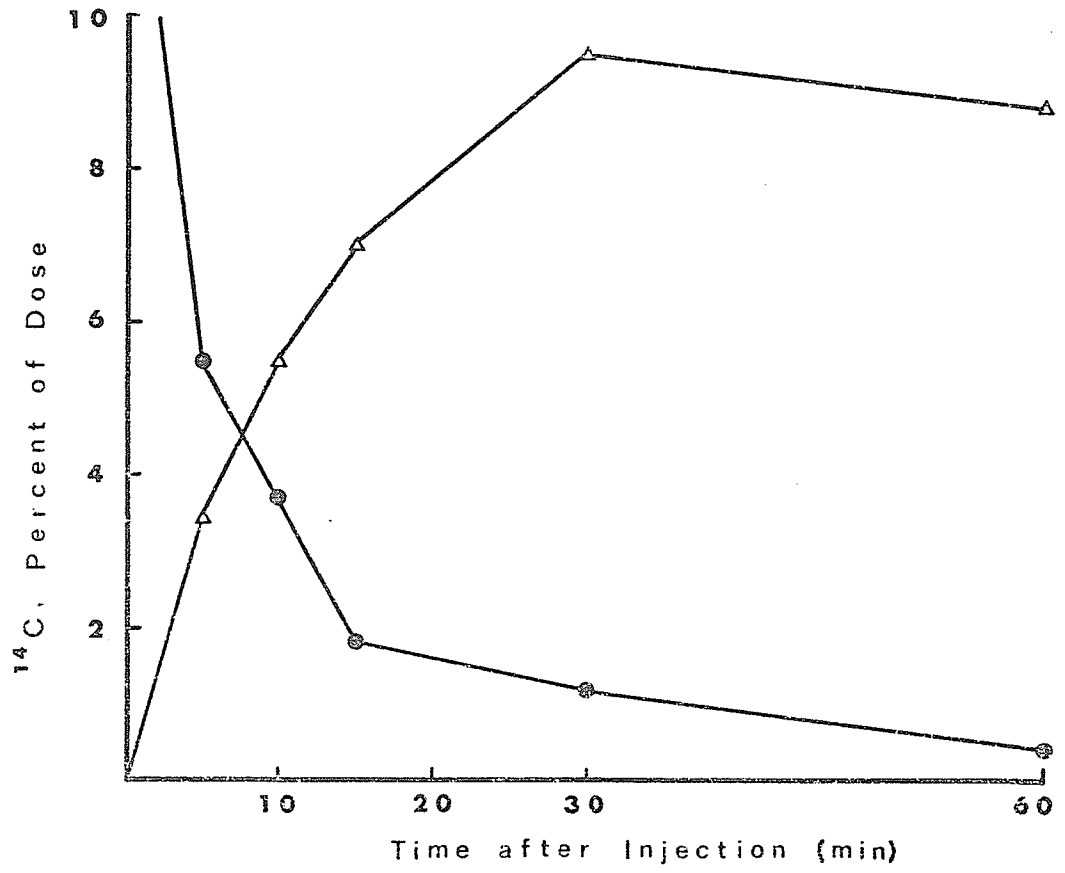


Fig. 38

Time course of incorporation of radioactivity from D-glucosamine-^{14}C into total liver protein (-△-). Disappearance of free D-glucosamine-^{14}C from serum is represented by solid circles (-●-). Animals received an intravenous injection of 5 μCi D-glucosamine/100g body weight. Each point represents the mean of three to five experiments. In order to construct the above figure it was assumed that serum represented 3.5% and liver 4% of body weight.

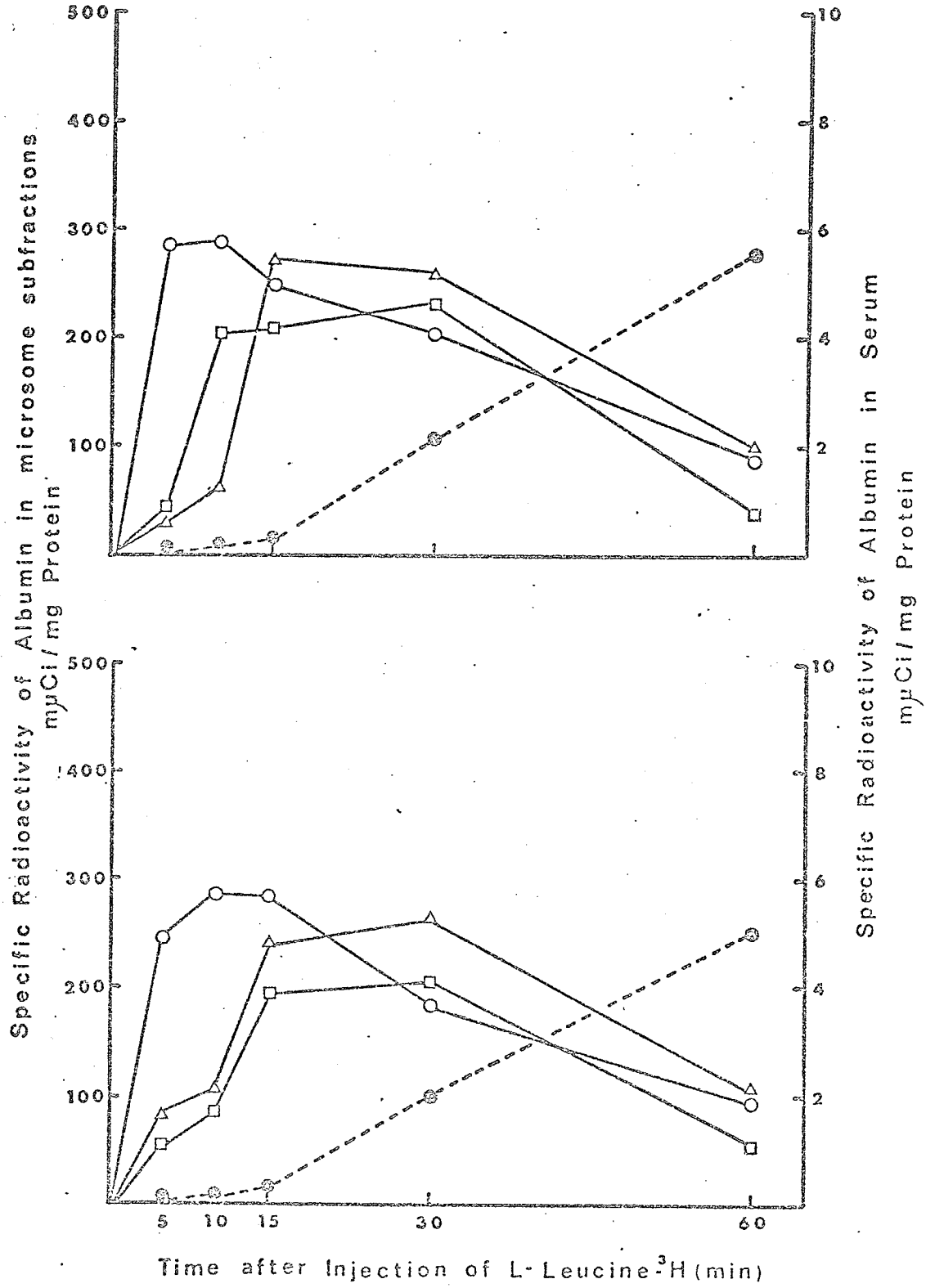


Fig. 39

Specific radioactivites of albumin isolated from rough membrane subfraction of microsome material-O-, smooth membrane subfraction of microsome material-□-, Golgi complex -△- and serum -⊙- following intravenous injection of L-leucine-^{3}H. Top, rats suffering from inflammation for 12h; bottom, control rats.

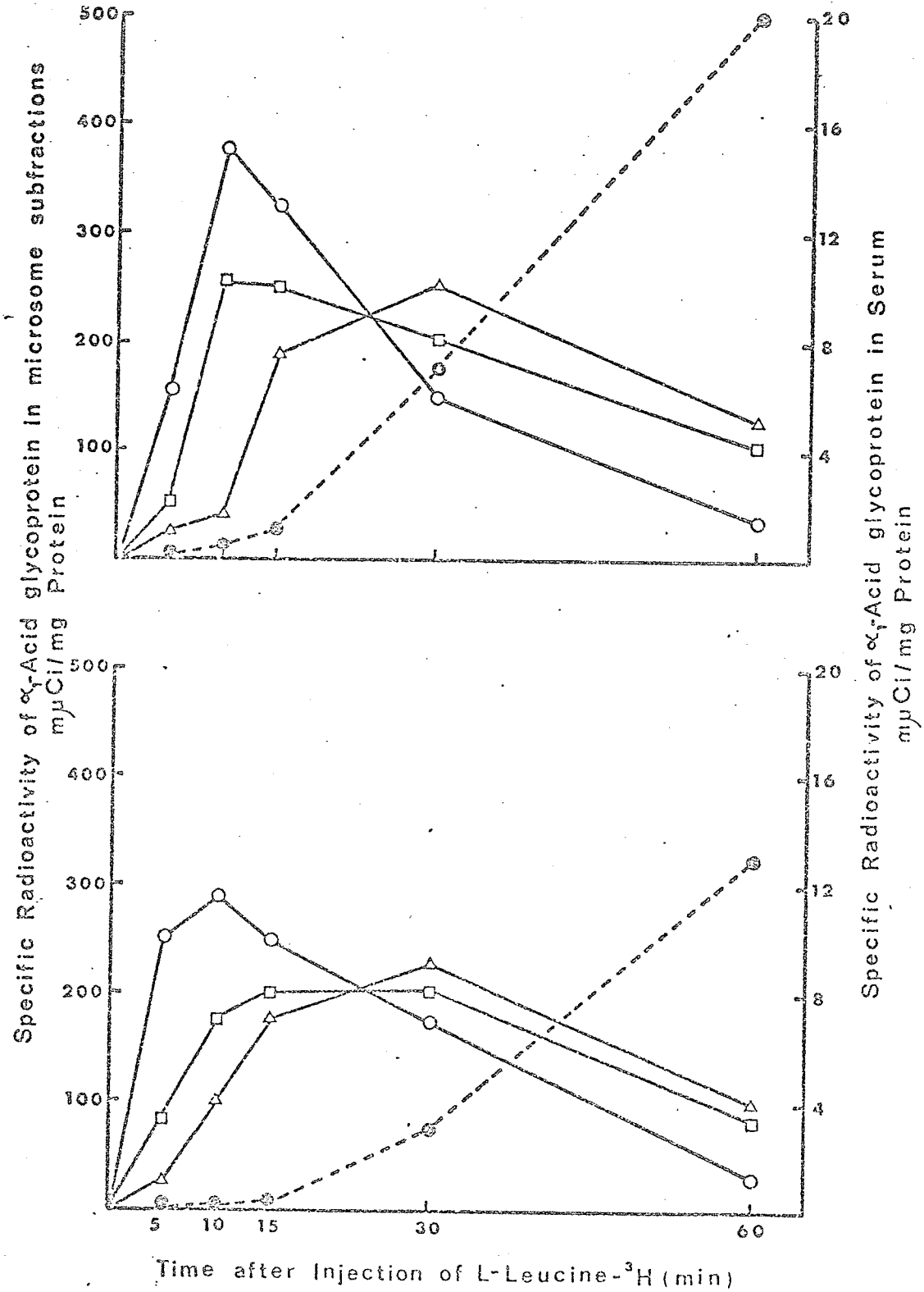


Fig. 40

Specific radioactivities of α_1-acid glycoprotein isolated from rough membrane subfraction of microsome material -O-, smooth membrane subfraction of microsome material -□-, Golgi complex -△- and serum -◎- following intravenous injection of L-leucine-^{3}H. Top, rats suffering from inflammation for 12h; bottom, control rats.

albumin and α_1-acid glycoprotein, respectively, in microsome subfractions prepared from livers from control and experimental animals as a function of time after administration of L-leucine-^{3}H; Fig. 41 shows results obtained in similar experiments following labelling with D-glucosamine. Also shown on the graphs (Figs. 39-41) are specific radioactivities of albumin and α_1-acid glycoprotein isolated by application of the quantitative precipitin technique to serum collected from the animals at death. In the case of labelling of albumin or α_1-acid glycoprotein with L-leucine-^{3}H there was negligible change in the secretion patterns of both proteins in experimental animals when compared to controls, maximum labelling occuring in albumin and α_1-acid glycoprotein at 5 to 10 minutes in rough microsomes, about 15 minutes in smooth microsomes and 30 minutes in Golgi fractions. Release of albumin and α_1-acid glycoprotein into blood occured after a lag period of about 15 minutes. Thereafter, there was a fairly linear increase in the specific radioactivity of albumin and α_1-acid glycoprotein in blood with a concurrent decrease in the specific radioactivity of albumin and α_1-acid glycoprotein in the Golgi fraction.

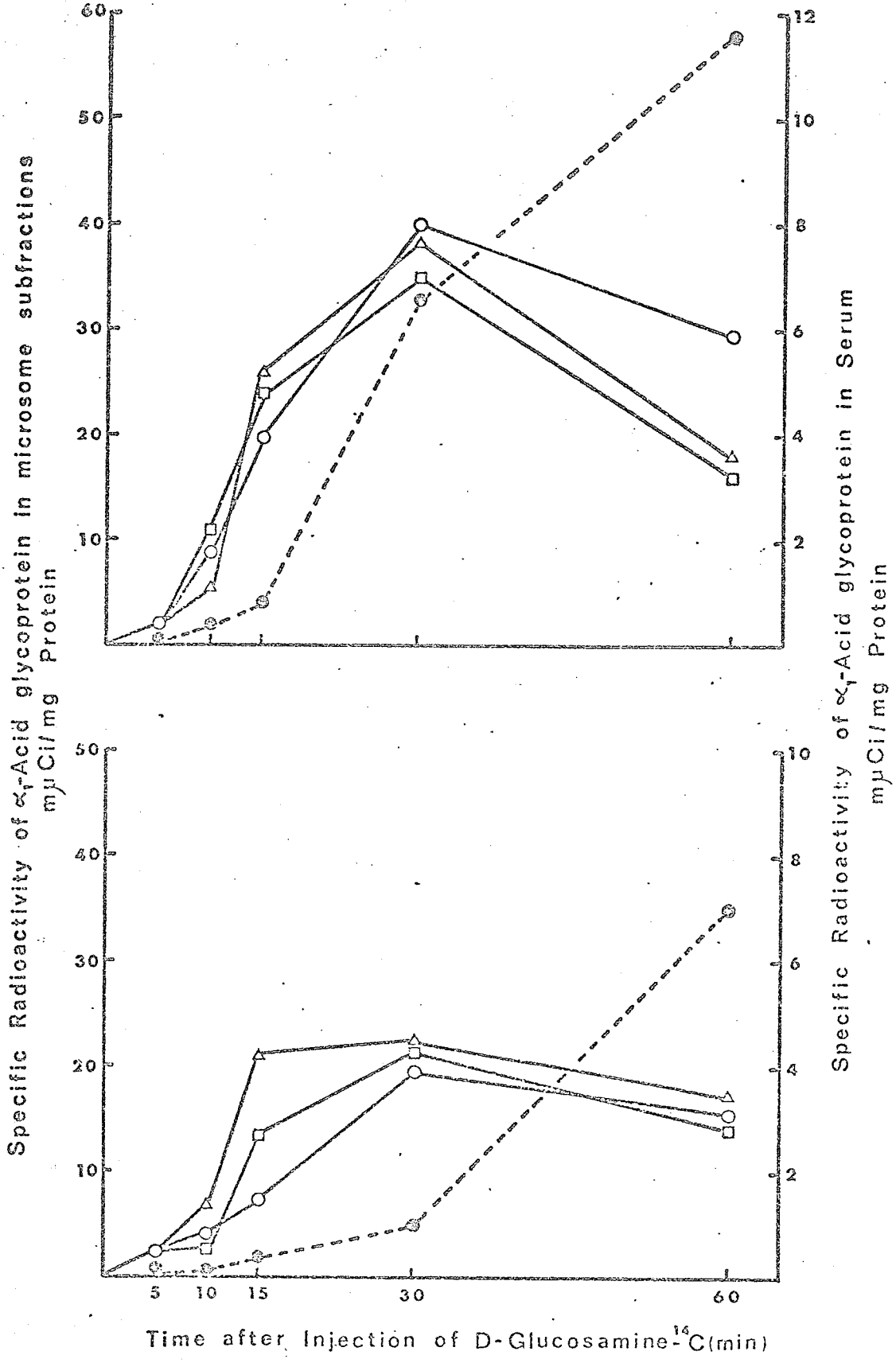


Fig. 41

Specific radioactivities of α 1-acid glycoprotein isolated from rough membrane subfraction of microsome material -○-, smooth membrane subfraction of microsome material -□-, Golgi complex -△- and serum -●- following intravenous injection of D-glucosamine-^{14}C. Top, rats suffering from inflammation for 12h; bottom, control rats.

In the case of labelling with D-glucosamine-^{14}C there was no one fraction which contained α_1-acid glycoprotein of greatly increased specific radioactivity. The maximum labelling of α_1-acid glycoprotein occured after about 30 minutes in all fractions from both control and experimental animals. However, the specific radioactivity of α_1-acid glycoprotein isolated from experimental animals after 30 minutes labelling was about twice that observed in controls. As was found after labelling of α_1-acid glycoprotein with L-leucine-^{3}H in the above experiments and as was previously reported there was a lag period of about 15 minutes before the appearance of label from D-glucosamine-^{14}C in α_1-acid glycoprotein in serum, thereafter, there was a rapid rise in specific radioactivity corresponding with a decline in the specific radioactivity of α_1-acid glycoprotein in the liver subfractions.

In accord with results reported earlier there was a more rapid increase in specific radioactivity of both L-leucine-^{3}H and D-glucosamine-^{14}C in α_1-acid glycoprotein isolated from serum from experimental animals when compared with controls; moreover, there was negligible change in the specific

radioactivity of L-leucine-^{3}H in albumin isolated from serum from control and experimental animals.

In the above experiments precipitation of albumin and α_1-acid glycoprotein from Lubrol extracts of microsome subfractions with their corresponding antisera was preceded by precipitation with the heterologous immune systems previously described. When Lubrol extracts are prepared from microsome fractions following administration of labelled precursors any non-specific protein which is capable of precipitation with immune precipitates will be labelled and will cause miscalculations in the determination of the specific radioactivity of rat proteins. In order to test for non-specific precipitation of radioactivity from Lubrol extracts isolated following administration of L-leucine-^{3}H and D-glucosamine-^{14}C the precipitates obtained following precipitation with the heterologous immune systems were collected for measurement of radioactivity. The results shown in Table 11, indicate that significant radioactivity was removed in the first and second precipitates obtained by the addition of the human albumin-anti-human serum system and human fraction VI - anti-human serum system, respectively. However, subsequent precipitates

TABLE II

Incorporation of L-leucine-^{3}H and D-glucosamine-^{14}C into material precipitated from Lubrol extracts of microsome fractions and subcellular fractions of microsome material isolated from rat livers.*

Cell fraction	Rat albumin		Rat α_1-AGP		Human albumin (1)		Human VI (1)		Human albumin (2)		Human VI (2)	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Total Microsomes ‡	275	-	360	29	34	0.6	34	4	4.1	-	6.1	2.0
+	20,625	-	9,500	700	3,000	160	1,050	95	500	-	110	25
Rough Membranes ‡	280	-	388	20	21	0.5	33	2.5	2.9	-	4.3	1.0
+	22,500	-	10,599	680	2,500	125	1,300	90	450	-	145	20
Smooth Membranes ‡	200	-	250	24	35	0.3	30	3.0	1.0	-	5.2	0.6
+	18,040	-	7,500	600	2,250	215	1,710	120	320	-	150	15
Golgi Apparatus ‡	270	-	200	26	17	0.4	14	3.5	1.5	-	4.2	0.7
+	15,900	-	6,000	450	2,750	220	1,250	75	280	-	130	19

α_1-AGP (α_1-acid glycoprotein), Human VI (Human glycoprotein fraction VI). The numbers (1) and (2) refer to the first and second human preprecipitates.

* Results are expressed in two ways; the top row of figures corresponding to each subcellular fraction expresses results as specific radioactivities mμCi/mg protein i.e. indicated by ‡ ; the bottom row of figures expresses results in terms of total radioactivity i.e. disintegrations per minute i.e. indicated by +.

obtained by the addition of the heterologous immune systems contained little radioactivity. Although the first precipitates obtained with the heterologous immune systems contained significant radioactivity (10-15%), the second precipitates represented only about 4% of the radioactivity recovered in the subsequent rat immune precipitates.

Characterization of liver subcellular fractions

Although the subcellular fractionation procedures employed in the present studies were well documented procedures applied to rat liver (see methods section) some characterization studies were performed to check that subcellular fractionation was satisfactory. Assays for glucose-6-phosphatase and ratios of RNA/protein were determined to check the purity of nuclear, mitochondrial and microsome fractions. The results of RNA/protein ratios and assays for glucose-6-phosphatase activity are shown in Table 12. The RNA/protein ratios indicate that satisfactory separation of rough and smooth microsomes was obtained by the fractionation procedure used. In addition, the Golgi complex appeared to be isolated without significant contamination by rough microsomes. The assays for

Table 12

Characterization of subcellular fractions of rat liver cells.

Cell fraction	RNA/protein ratio	Glucose-6-phosphotase*
Total microsomes	0.20	6.10
Rough microsomes	0.30	6.40
Smooth microsomes	0.08	2.50
Golgi apparatus	0.09	1.70
Nuclear fraction	-	0.45
Mitochondrial fraction	-	0.35

* µmoles Pi/20 min per mg protein

glucose-6-phosphatase indicate that nuclear and mitochondrial fractions were devoid of significant glucose-6-phosphatase activity while the rough microsomes contained more enzyme activity than either the smooth microsomes or the Golgi complex. The results of RNA/protein ratios and glucose-6-phosphatase activity are similar to those reported by Glaumann *et al.* (111) for the characterization of subcellular fractions of liver.

DISCUSSION

Effect of inflammation on serum and serum protein fractions

There was an increase in protein-bound carbohydrate of rat serum reaching a maximum at 48-72h after administration of turpentine. A similar response has been reported in rats and other species for total protein-bound carbohydrate of serum and for specific proteins or protein fractions isolated from serum (163,214,218-220). Most of the increase in protein-bound carbohydrate of serum resulting from an inflammatory condition was located in fractions 1,4 and 5b upon fractionation of serum by chromatography on DEAE-cellulose coupled with preparative electrophoresis on cellogel strips. The fractions eluted from the DEAE-cellulose column were examined immunologically by Dr. J.C. Jamieson using immunoelectrophoresis and double diffusion analysis. Although these latter studies are not reported in the results section of this thesis they are worthy of discussion since they were performed in order to determine which fractions contained glycoproteins which are known to be acute phase reactants and also to determine if any new proteins were present in serum from

experimental animals. It was found that the acute phase reactant fibrinogen (213,216,219) was present in fraction 1 and may have made a contribution to the increased content of protein-bound carbohydrate found in fraction 1 when isolated from the serum of experimental animals. Fibrinogen, however, is probably present in fraction 1 in relatively low concentration since this protein is largely removed during the clotting process. Haptoglobin, an acute phase reactant which increases markedly as a result of inflammation (218,221-223), was detected mainly in fraction 4 and probably makes a significant contribution to the increase in protein bound carbohydrate found in this fraction when isolated from the serum of experimental animals. Proteins found in the perchloric acid soluble and seromucoid fractions were detected mainly in fractions 4 and 5b, and to a lesser extent in fractions 5a, 6 and 7. Since fraction 5a is serum albumin and fractions 6 and 7 do not contribute significantly to the increase in protein-bound carbohydrate found in the serum of experimental animals studies on the effects of inflammation on the perchloric acid soluble and seromucoid fractions of serum may therefore give mis-

leading results, since not all the proteins present in these fractions appear to be acute phase reactants. Immunological studies on serum and serum fractions from control and experimental animals did not reveal the presence of any new proteins in serum from experimental animals which were not present in serum from control animals. Weimer et al.(219) have reported a similar result in rats suffering from adjuvant-induced arthritis when serum was examined at times after inflammation similar to those employed in these studies. However, a new protein was detected by Weimer et al.(219) at longer time periods of exposure to inflammatory agent. Also, in an earlier study, Weimer and Benjamin(163) detected a new protein in rat serum as early as 8h after administration of turpentine. Several other workers have described an α_2-(acute phase) globulin that can be detected in fetal, neonatal, pregnant and tumor-bearing rats, but which cannot be detected in normal adult rats (163,224-226). Therefore, it is possible that a new protein is present in serum from experimental animals in the present studies, but this protein may have escaped detection by the immunological procedures employed. Failure to detect

a new protein may be explained by a low content of such a protein in serum from experimental animals used for the production of antiserum.

The fraction with the greatest increase in protein-bound carbohydrate, when isolated from the serum of experimental animals, was fraction 5b which contained mainly α_1- and α_2-globulins and was responsible for about 40% of the increase in protein-bound carbohydrate found in the serum of experimental animals at 24-72h following inflammation. This fraction was therefore used as the starting material for the isolation of homogeneous acute phase globulins for use in studies on the biosynthesis of serum proteins that respond to inflammation.

Isolation and characterization of α_1-acid glycoprotein and α_2-macroglobulin. An α_1-acid glycoprotein and an α_2-macroglobulin were isolated from fraction 5 by a combination of ion-exchange chromatography, gel filtration and isoelectric focusing. The latter technique was also employed to prepare serum albumin which is not believed to be an acute phase globulin.

The α_1-acid glycoprotein appeared to be homogeneous when examined immunologically and by electrophoresis on starch and polyacrylamide gels. The

α_1-acid glycoprotein contained 34% carbohydrate, had a molecular weight of 43,000, an isoelectric point of 2.95 and a sedimentation coefficient ($S_{20,w}$) of 3.3. Kawasaki et al.(227) have reported the isolation of an α_1-acid glycoprotein from rat serum which resembles that isolated in the present studies. The total carbohydrate (34%) and composition of carbohydrate of the α_1-acid glycoprotein isolated by Kawasaki et al.(227) was almost identical to that found in the α_1-acid glycoprotein isolated in the present studies, although Kawasaki et al.(227) suggested the presence of a trace of fucose in their protein and reported a slightly higher hexose content (15.3%), but a lower hexosamine content (8.3%). However, the greatest difference between the two proteins appears to be with their molecular weights. Kawasaki et al.(227) reported a molecular weight of 35,000 for their α_1-acid glycoprotein, considerably lower than the molecular weight of the α_1-acid glycoprotein isolated in the present study (43,000); the isoelectric point of the protein isolated by Kawasaki et al.(227) was not reported. An α_1-acid glycoprotein was also isolated from rat serum by Zito et al.(228), but this protein was not characterized electrophoretically or immunologically. However,

although the protein isolated by Zito et al.(228), had a similar molecular weight (45,000) and sedimentation coefficient ($S_{20,w}$) 3.15 to the α_1-acid glycoprotein isolated in the present work, its carbohydrate content was considerably lower (25%) suggesting that it may not be the same protein. Gordon and Louis(229) have also reported the isolation of α_1-acute phase globulins from rat serum. However, the proteins isolated by these authors had higher isoelectric points (4.4-4.8) and lower carbohydrate contents (approx. 14%) and are, therefore, clearly different from the α_1-acid glycoprotein isolated in the present work.

The α_1-acid glycoprotein that has been studied in most detail in recent years is that isolated from human serum. Human α_1-acid glycoprotein is a well characterized glycoprotein which is known to be an acute phase protein present in human serum (e.g.161). Although the carbohydrate content of human α_1-acid glycoprotein is a little higher than the rat protein (Winzler(230) reports 40% carbohydrate; Yamashina (231) reports 41.3% carbohydrate) and the molecular weight a little lower (Kawasaki et al.(227) reports 33,000; Bezkorovainy(232) reports 41,600 although Winzler's group(233) reports 44,100) it appears that

the two proteins are not too dissimilar both having a relatively high carbohydrate content, a low molecular weight and a low isoelectric point. Therefore, it is not unreasonable to assume that both proteins may function in a similar manner during the inflammatory process, thus making a study of the biosynthesis and structure of the rat α_1-acid glycoprotein isolated in the present work of particular importance.

The α_2-macroglobulin was examined in a similar manner to the α_1-acid glycoprotein described above. The α_2-macroglobulin had a molecular weight of about 800,000, a sedimentation coefficient ($S_{20,w}$) of 19S, an isoelectric point of 4.6 and a carbohydrate content of 15.9%. Although the α_2-macroglobulin appeared to be fairly pure when examined immunologically and by electrophoresis on starch and polyacrylamide gels, it was not apparently homogeneous since a faint precipitin line, in addition to the main precipitin line, was usually observed on double diffusion analysis in agar gels and a faint second band of protein was often observed on disc electrophoresis. It is possible, however, that the contaminating material may be a degradation product of the α_2-macroglobulin since a similar protein isolated from human serum is known to be easily degraded during

isolation (234). Although the isolation of α_2-macroglobulins from rat serum is reported in the literature (235,236), little information is available concerning physical properties and carbohydrate contents of such proteins. However, similar proteins have been isolated from human serum and characterized (e.g. 230,234,237). Although the molecular weights and sedimentation coefficients of human α_2-macroglobulin (M.Wt., 850,000; $S_{20,w}$, 19S) are similar to those found for the $_2$-macroglobulin isolated in the present work, the carbohydrate content reported for the human protein (i.e. 7.8 - 9.8% carbohydrate) is somewhat lower than that found for rat α_2-macroglobulin (15.9% carbohydrate).

Determination of the contents of α_1-acid glycoprotein, α_2-macroglobulin and albumin in serum from normal and experimental rats. The $_1$-acid glycoprotein and α_2-macroglobulin isolated in the present work have been recovered in a fairly pure form and have been well characterized with respect to physical properties and carbohydrate composition. These proteins would be suitable for study of structure and biosynthesis of proteins that respond to inflammation. These studies would be meaningful only if the proteins

in question are first shown to be acute phase globulins increasing significantly in content as a result of inflammation. Therefore the contents of α_1-acid glycoprotein, α_2-macroglobulin and albumin in serum was measured using the quantitative precipitin technique. The content of albumin in serum was also determined using an isotope dilution technique. The content of α_1-acid glycoprotein in normal rat serum was found to be 2.36 $\pm$ 0.08mg/ml (mean of 10 values $\pm$ standard error of the mean). This result is similar to values reported by John and Miller(238) for the content of an α_1-acid glycoprotein isolated from rat serum by further fractionation of the seromucoid fraction. The content of α_1-acid glycoprotein in normal rat serum, however, is about twice that reported by Gordon and Koj(239) and Weimer *et al.*(219) for two α_1-globulins isolated from rat serum. There was about a six-fold increase in the content of α_1-acid glycoprotein in the serum of rats suffering from induced inflammation when compared with controls reaching a maximum at 48-72h after administration of inflammatory agent. A similar increase in the content of an α_1-acid glycoprotein was reported by John and Miller(238) in serum from rats exposed to

whole body irradiation. Darcy(240) reported a seven-fold increase in content of an α_1-globulin in rat serum as a result of turpentine induced inflammation.

The content of α_2-macroglobulin in rat serum was found to be 3.31 $\pm$ 0.15mg/ml (mean of 6 values $\pm$ standard error of the mean). This value is approximately twice that reported by others for the content of α_2-globulins in rat serum(219). The α_2-macroglobulin increased about four-fold in content as a result of inflammation reaching a maximum at 48h after administration of inflammatory agent. Both α_1-acid glycoprotein and α_2-macroglobulin studied in the present work are clearly acute phase globulins contributing to the increase in content of protein-bound carbohydrate in serum as a result of inflammation. However, the contribution made by the α_1-acid glycoprotein to the increase in protein-bound carbohydrate is probably greater than that of the α_2-macroglobulin in view of the higher carbohydrate content of the α_1-acid glycoprotein (see Table 4).

The content of albumin in serum, determined either by the quantitative precipitin technique or an isotope dilution technique was found to be approximately 39mg/ml serum isolated from normal rats.

This value generally agrees with that reported by Peters(241) and Howe(242); however, it is higher than that reported by other workers (214,219,243). The content of albumin in serum from experimental animals did not change appreciably at short times (4-12h) of exposure to inflammatory agent. However, there was a slight decrease in content of albumin in serum from experimental animals at 24-72h after administration of inflammatory agent. This observation is in agreement with results reported by Neuhaus et al.(214) and by Koj (213). The latter author has suggested that the reduction in content of albumin in serum at 24-72h following inflammation may be due to a redistribution of albumin between plasma and other body fluids.

Studies on the incorporation of L-leucine-^{3}H and D-glucosamine-^{14}C into serum proteins in control and experimental animals

The increase in protein-bound carbohydrate found in serum from experimental animals may be explained by an increase in the rate of synthesis of certain serum glycoproteins, or by a decrease in their rates of catabolism. Therefore, an attempt was made to detect changes in synthesis rates of

proteins in experimental animals. The procedure used involved measuring the specific radioactivities of ^{3}H-leucine and ^{14}C-glucosamine in α_1-acid glycoprotein, α_2-macroglobulin and DEAE-cellulose fractions isolated from serum from control and experimental rats. In order to minimize errors due to differences in pool sizes of amino acids between individual animals serum albumin was used as a reference protein or internal standard in these studies. The illustration that the content of albumin did not change significantly in serum isolated from experimental animals as compared to control animals suggested that this protein was ideal as a reference protein for the incorporation of labelled compounds into other serum proteins. Use of albumin as a reference protein was further supported by the fact that there was little change in the specific radioactivity of albumin in control and experimental animals following incorporation of leucine, especially at short times of exposure to inflammatory agent. Since serum albumin is synthesized by the liver (95, 96), the use of this protein as a reference protein will be valid only for those proteins synthesized by this organ. With the exception of γ-globulins, all

serum proteins, including several acute phase reactants are synthesized by the liver. The results obtained in the labelling studies for fraction 1 will therefore be subject to error since this fraction contains γ-globulins. In addition, results obtained following labelling with glucosamine are not corrected for differences in pool sizes between individual animals, hence some fluctuation would be expected from these studies.

In order to determine if there was an increase in the synthesis rates of certain glycoproteins in response to inflammation, serum samples obtained from control and experimental rats which had been injected with L-leucine-^{3}H and D-glucosamine-^{14}C 90 minutes prior to sacrifice were examined for the incorporation of these labelled precursors into fractions 1-7 obtained by chromatography of serum on DEAE-cellulose and into α_1-acid glycoprotein and α_2-macroglobulin. The results presented for incorporation of labelled precursors into serum fractions, α_1-acid glycoprotein and α_2-macroglobulin from control and experimental animals suggest that the increased content of protein-bound carbohydrate in the serum of experimental animals is likely to result from an increase in the rate of synthesis of glycoproteins such as the specific

glycoproteins under discussion in this thesis. This conclusion is in agreement with results obtained by other workers using more accurate methods to study the rates of synthesis of acute phase reactants under *in vivo* (213,216,244) and *in vitro* (239) conditions. A similar result has been reported by Nauhaus *et al.* (214) for the incorporation of D-glycine-^{14}C into the seromucoid fraction of serum isolated from rats following sham operations. It is unlikely that decreased catabolism of serum glycoproteins is the main explanation for the increased content of protein-bound carbohydrate in serum from experimental animals, since one would not expect an increase in the amount of labelled precursors incorporated into serum glycoproteins from experimental animals. Moreover, preliminary studies by Robinson (245) suggest that there may be an increased rate of catabolism of serum glycoproteins as a result of inflammation. The exact mechanisms which result in the increased rate of synthesis of serum glycoproteins in response to inflammation is not known. However, it may be assumed that the initial response to inflammation is the release of some active substance from the site of inflammation as suggested by Koj (213). The active substance must operate at the site of

synthesis of the acute phase reactants resulting in an increase in their synthesis rates. Therefore, it is important to study the biosynthesis of specific acute phase globulins at their cellular and subcellular sites of synthesis in the organ responsible for their manufacture. However, before detailed studies can be undertaken, the cellular and subcellular sites of synthesis of the proteins under examination in the present work must be located. Unfortunately, for technical reasons (see also Results) it was found that it was impractical to perform biosynthetic studies on all three proteins isolated in the present work i.e. α_1-acid glycoprotein, α_2-macroglobulin and albumin. Therefore, all subsequent studies were restricted to the α_1-acid glycoprotein, an acute phase globulin which contains a large amount of carbohydrate and which increases significantly in content in serum, as a result of inflammation and serum albumin, which is not an acute phase globulin and, therefore, represents a protein which does not apparently change as a result of induced inflammation.

Immunodiffusion studies on the biosynthesis of albumin and α_1-acid glycoprotein following incorporation of L-leucine-^{3}H and D-glucosamine-^{14}C: determination of cellular and subcellular site of synthesis.

In order to determine the cellular and subcellular site of synthesis of α_1-acid glycoprotein and albumin, immunodiffusion studies were performed in which Lubrol extracts of various organs and subcellular fractions prepared from these organs were reacted with antiserum to α_1-acid glycoprotein or albumin and the immunodiffusion plates were subsequently dried and radioautographs prepared to determine which precipitin lines were radioactive. Lubrol extracts prepared from the microsome fraction of rat liver gave precipitin lines with antiserum to α_1-acid glycoprotein that were labelled following administration of ^{14}C-amino acid or glucosamine as precursor. Similar Lubrol extracts gave precipitin lines with antiserum to albumin that were labelled after administration of amino acid as precursor, but not when glucosamine was used as precursor. This latter observation probably suggests that albumin does not contain carbohydrate at any time while passing through the

endoplasmic reticulum as was once suggested by Schachter et al. (129). Precipitin lines formed by extracts of the nuclear fraction were slightly radioactive in some experiments, but not in others. This labelling probably arose from slight contamination of the nuclear fraction by microsomal material. Precipitin lines formed by mitochondrial and cell sap fractions from liver and lines formed by extracts of spleen and kidney contained no radioactivity. These results indicate that the microsome fraction of liver is the main or sole site of synthesis of both polypeptide and carbohydrate moieties of α_1-acid glycoprotein. These results are in agreement with studies on the site of synthesis of the polypeptide chain and carbohydrate groups of an acidic glycoprotein fraction studied by Simkin and Jamieson (206) in the liver of the guinea pig. The results are also in agreement with those of other workers studying more heterogenous serum protein fractions (97,98,170,246-248).

Although differences occured in relative concentrations between albumin or α_1-acid glycoprotein present in tissue extracts and serum, the precipitin lines given by the tissue extracts formed reactions of immunological identity with precipitin lines formed by serum suggesting that proteins present in the

extracts were similar or identical to those present in serum. Experiments with heterologous human immune systems indicated that labelling of precipitin lines and immune precipitates represents the occurrence of biosynthesis of α_1-acid glycoprotein and albumin rather than the non-specific binding of radioactive material to precipitin lines. The main procedure used to extract microsomal material involved treatment with Lubrol. Addition of 2.5% sodium deoxycholate which is believed to result in complete solubalization of serum proteins from microsomal material (204)to the residue remaining after extraction of microsome material with Lubrol released little further material that reacted with antisera to α_1-acid glycoprotein and albumin. These results suggest that Lubrol is a satisfactory extractant for solubalization of microsomal bound serum proteins.

Determination of the contents of albumin and α_1-acid glycoprotein in rat liver microsomes isolated from normal and experimental animals

The content of albumin in the microsome fraction isolated from livers from control rats was 0.39 ± 0.012 mg/g wet weight liver (mean of 10 values ± standard error of the mean). This value is similar to that

reported by Peters (241) and Morgan and Peters (249). It is, however, considerably lower than that reported by Glaumann and Ericsson (0.9 mg/g wet weight liver) (111) and Marsh and Drabkin (0.59 mg/g wet weight liver) (96) in liver slices. These discrepancies could possibly be explained by the use of different procedures to determine the content of albumin in the fractions in question. The content of albumin in microsome fractions of livers isolated from experimental rats did not change appreciably up to 12h after the administration of turpentine. At 24-72h a slight reduction was apparent. These results generally agree with the observation that the content of albumin in serum was reduced at approximately the same time period of 24-72h after administration of turpentine. The four to five-fold increase in content of α_1-acid glycoprotein in microsomes of livers isolated from 8-12h experimental rats compared to livers isolated from control rats was also similar to the increase subsequently observed in serum at 24-72h after induction of inflammation. This observation suggests the existence a precursor product type of relationship between increased microsomal α_1-acid glycoprotein and increased serum α_1-acid glycoprotein as a result of inflammation.

Ultrastructural studies on livers from control and experimental rats

The observed increase in the content of α_1-acid glycoprotein in the microsome fraction of livers isolated from experimental rats led to the examination of liver cells under the electron microscope. The α_1-acid glycoprotein is probably only one of several glycoproteins (e.g. haptoglobin, α_2-macroglobulin) that respond by an increase in their content in serum when inflammation occurs. Therefore, it was thought that it would be of interest to compare the appearance of liver cells from normal and experimental animals. A surprising difference was found between normal and experimental liver cells in that there appeared to be a great proliferation of endoplasmic reticulum in experimental liver cells, with less rough endoplasmic reticulum and more smooth endoplasmic reticulum and Golgi apparatus. These observations appear to be similar to those of Claude (250) who examined regenerating livers. The apparent increase in endoplasmic reticulum in experimental liver cells is almost certain to be a reflection of increased hepatic protein synthesis leading to an increased capacity for storage of proteins by the liver cell. In studies on the effect of growth

hormone and tri-iodothyronine on protein synthesis, Tata (251,252) has shown that in response to increased hepatic synthesis of proteins induced by hormones there is an increase in the production of ribosomes, with more ribosomes being membrane-bound. In addition, an increased rate of synthesis or proliferation of phospholipids into microsomal membranes was detected. Although it remains to be seen whether changes in liver structure that occur as a result of inflammation represents a hormonally controlled process or not it is nevertheless clear from the present work that increased hepatic production of glycoproteins as a result of inflammation is accompanied by significant changes in the cytoplasmic components of the liver cell.

Studies on the secretion of albumin and α_1-acid glycoprotein in normal rats and rats suffering from inflammation

Examination of the content of α_1-acid glycoprotein and albumin in serum and liver microsomes isolated from normal rats and rats suffering from inflammation suggested that there was an increased production and perhaps increased rate of secretion of the α_1-acid glycoprotein, but not albumin, in

response to inflammation. In order to obtain some information about the pathway of secretion of α_1-acid glycoprotein and albumin in response to inflammation, rats suffering from inflammation for 12h were injected with L-leucine-^{3}H and D-glucosamine-^{14}C and sacrificed at 5,10,15,30 and 60 minutes after administration of isotope. Lubrol extracts of subcellular fractions of liver microsome material were prepared and the specific radioactivities of α_1-acid glycoprotein and albumin determined. The pathway of secretion of albumin did not appear to differ in normal rats and rats suffering from inflammation. Maximum labelling of albumin first occurred in the rough membrane subfraction of microsome material. This suggests that the albumin molecules are synthesized on the ribosomes associated with the rough endoplasmic reticulum. This has been previously suggested by the demonstration that isolated rough membrane subfractions of microsome material (204,253) and also isolated ribosomes (254-256) can effect the incorporation of labelled amino acid into rat serum albumin. The curve for the smooth membrane subfraction of microsome material rises more slowly to its maximum, than that of the rough membrane subfraction. These observations suggest that the

labelled albumin which appears in the rough membrane subfraction after 5-10 minutes subsequently appears in components of the smooth membrane subfraction. Also, since no labelled albumin was detected outside the cell for 15-20 minutes albumin was apparently not secreted directly into serum from the rough endoplasmic reticulum. The steeply rising portions of the curves for the rough and smooth membrane subfractions (Fig. 39) are about 4-5 minutes apart, implying that the average albumin molecule spends about 5 minutes in the rough endoplasmic reticulum. The results of these studies are very similar to those described by Peters (112,113) and also by Glaumann and Ericsson (111). The Golgi fraction exhibited a higher specific radioactivity than the smooth membrane subfraction at the times examined. Generally, the curve for the smooth membrane subfraction and the Golgi fraction paralled one another initially. However, the specific radioactivity of albumin in the Golgi apparatus was higher in some cases than that in the smooth membrane subfraction. Similar results were obtained by Peters (113) and Glaumann and Ericsson (111), with the exception that the specific radioactivity of albumin in the Golgi fraction was also considerably

higher at 20-30 minutes than that in the smooth membrane subfraction. Similar results may have been obtained in the present studies if experiments had been carried out at 20 minutes after administration of labelled compounds. The early appearance of labelled albumin in the Golgi fraction as compared to the smooth membrane subfraction is difficult to explain. As suggested by Peters (113) the early appearance of labelled albumin in the Golgi-rich fraction when compared with the albumin in the rough and smooth membrane subfractions is not in accord with a simple precursor, product relationship (257). One reason could be the presence of previously formed albumin in some components of the rough and smooth endoplasmic reticulum which is temporarily inactive insofar as secretion is concerned. This albumin would not have incorporated the radioactive tracer and would depress the measured specific radioactivity per ug albumin present in the fractions. In addition there could be differences in the contents of albumin in the three fractions thus creating a differential pool size effect. Another source of unlabelled albumin in the rough and smooth membrane subfractions might be attributed to albumin taken up from the serum by pinocytosis (258) into vesicles

which cosediment with the fractions. The early appearance of labelled albumin in the Golgi fraction may be explained by contamination of the Golgi fraction with components of rough and smooth endoplasmic reticulum. However, the absence of RNA and the low glucose-6-phosphatase activity found in the Golgi fraction does not favor such an explanation. In addition, both Peters (113) and Glaumann and Ericsson (111) who have done more exhaustive enzymatic purity checks on Golgi fractions, have presented the same results as in this study. Judging from the curves obtained, the pathway of secretion of albumin follows the sequence: rough surface endoplasmic reticulum ⟶ smooth surface endoplasmic reticulum ⟶ Golgi apparatus ⟶ blood. These results are in agreement with the sequence originally proposed by Bruni and Porter for albumin (259) and confirmed by Peters (113) and Glaumann and Ericsson (111). There was no real apparent difference for the pathway of secretion of albumin in subcellular fractions obtained from livers of control rats and rats suffering from inflammation for 12h. These results support the previous findings that there is little difference in the content of albumin in serum and microsome fractions obtained from control rats

and rats suffering from inflammation for 12h. Eylar (40) and Schachter et al. (129) have suggested that addition of carbohydrate components is obligatory during secretion of proteins and that albumin may carry carbohydrate groups at some time during secretion and subsequently lose them. However, analysis of albumin, isolated from the subcellular fractions, for the presence of ^{14}C (after injection of D-glucosamine-^{14}C into rats) failed to indicate that at any time during the biosynthesis of albumin were any carbohydrate groups present. As previously discussed a similar conclusion was arrived at on examination of autoradiographs following double diffusion analysis on studies on the site of synthesis of albumin. Lo and Marsh (260) were also unable to find any labelling of plasma albumin by the Golgi apparatus, when incubated with labelled UDP-N-acetyl-glucosamine.

Investigation into the incorporation of L-leucine-^{3}H into α_1-acid glycoprotein present in subcellular fractions of microsome material from control rats indicated that labelling of the polypeptide chain of α_1-acid glycoprotein first occurs in the rough surface endoplasmic reticulum followed by the smooth surface endoplasmic reticulum

and the Golgi apparatus. The steeply rising portions of the curves for α_1-acid glycoprotein in the rough and smooth membrane subfractions are about 5 minutes apart, implying that the average polypeptide chain of the α_1-acid glycoprotein spends about 5 minutes in the rough surface endoplasmic reticulum. This appears to be approximately the same time as was found with the albumin molecule discussed above. Somewhat similar results to those described above were obtained in studies on the pathway of secretion of α_1-acid glycoprotein in subfractions of microsome material from rats suffering from inflammation. There was, however, a slightly higher specific radioactivity of α_1-acid glycoprotein isolated from rough and smooth membrane subfractions, but not in the Golgi fraction of experimental rats. However, this difference in specific radioactivity is not significant enough to suggest that there might be a slightly greater synthesis of the polypeptide chain of α_1-acid glycoprotein in livers of experimental rats in response to inflammation. Moreover, the results are difficult to interpret since the pool size of α_1-acid glycoprotein in microsome material at 12h following inflammation is significantly higher than that in control animals.

In the case of labelling with D-glucosamine-^{14}C there was no one subcellular fraction obtained from livers of control rats which contained α_1-acid glycoprotein of greatly increased specific radioactivity with the possible exception of the Golgi fraction isolated 15 minutes after administration of isotope. The maximum labelling of α_1-acid glycoprotein occured after 30 minutes in all fractions from both control and experimental animals. However, the specific radioactivity of α_1-acid glycoprotein isolated from subcellular fractions of livers of experimental animals, 30 minutes after administration of isotope was about twice that observed in controls. This suggests that there is an increased rate of attachment of carbohydrate to precursor molecules of α_1-acid glycoprotein in response to inflammation. It is more apparent from the examination of the specific radioactivity of ^{14}C in α_1-acid glycoprotein isolated from serum samples from control and experimental rats that there is an increased attachment of carbohydrate groups to α_1-acid glycoprotein in response to inflammation. For example, comparison of the amount of labelled glucosamine incorporated into α_1-acid glycoprotein in serum of normal and experimental rats

after 30 minutes, indicates that about 7 times more radioactivity is present in α_1-acid glycoprotein in serum of experimental rats. The difference in specific radioactivity of ^{14}C in α_1-acid glycoprotein isolated from serum and subcellular fractions of livers of control and experimental rats is noticeably greater than the difference in specific radioactivity of ^{3}H in α_1-acid glycoprotein isolated from the same samples. This might tend to suggest that the attachment of carbohydrate groups to polypeptide chains of α_1-acid glycoprotein is more important than polypeptide synthesis and may represent an important rate limiting step in the biosynthesis of molecules of the type under study especially in response to inflammation.

The time required for the appearance of α_1-acid glycoprotein in serum of normal rats was about 20 minutes. Lo and Marsh (260) also found a characteristic lag of 20 minutes before appreciable label appeared in serum lipoproteins. The appearance of albumin in serum of normal and experimental rats was also first noticeable at about 20 minutes, a result in accordance with Peters (113). Peters (113), in comparing the secretion times of albumin and the glycoprotein transferrin, found that

secretion of transferrin required 80 minutes before appreciable transferrin appeared in serum. This is considerably longer than the time required for the secretion of α_1-acid glycoprotein into serum found in the present study. Transferrin molecules, however, appear to have a high affinity for membranes (261,262), which might result in a slower rate of secretion of the transferrin molecule. Redman and Cherian (126) and Morgan and Peters (263) have also discussed other reasons for the long period of time required for the secretion of transferrin into serum. It was suggested that different proteins (e.g. albumin and transferrin) might be secreted via pathways or channels which are anatomically distinct from each other, with the molecules of transferrin moving at a slower rate. Anatomical segregation of pathways could mean that synthesis of two proteins takes place in different liver cells. However, it has been shown that under conditions of increased transferrin synthesis, 70% or more of the liver cells react strongly with fluorescent anti-transferrin serum, presumably because of transferrin synthesis within them (264). It would seem unlikely that the quantitatively greater synthesis of albumin was confined to 30% of cells not containing demonstrable

transferrin. Therefore, it is probable, that both albumin and transferrin are synthesized within the same cell and share the same pool of amino acids and tRNA molecules. Similarily, it is probable that albumin and α_1-acid glycoprotein are synthesized in the same liver cells, especially since these two proteins are synthesized and secreted into serum at similar rates.

The pathway of secretion of α_1-acid glycoprotein in normal and experimental rat livers as judged from the results of incorporation of L-leucine-^{3}H into polypeptide appears to be rough endoplasmic reticulum ⟶ smooth endoplasmic reticulum ⟶ Golgi apparatus ⟶ blood. This is in agreement with the secretion pathway suggested for plasma proteins by radioautography (265,266) immunological examination of an acidic glycoprotein fraction (206) and data from other studies (123-126). Examination of the incorporation of D-glucosamine-^{14}C into α_1-acid glycoprotein did not reveal the pathway of secretion since the specific radioactivity of the various fractions reached their maximum at the same time (30 minutes) after injection of isotope into the rat. This probably indicates that glucosamine is being added to the incomplete precursor α_1-acid glycoprotein

molecules as they pass from the rough to smooth surface endoplasmic reticulum then to the Golgi apparatus. Since there is some conversion of glucosamine to sialic acids in rats and rabbits (116,117), it is probable that some of the ^{14}C found in α_1-acid glycoprotein isolated from the Golgi apparatus resides in molecules of sialic acid being attached at the terminal positions of the α_1-acid glycoprotein molecule.

Possible mechanisms for the increased production of α_1-acid glycoprotein in response to inflammation.

The biochemical mechanism(s) responsible for the increased production or synthesis of α_1-acid glycoprotein or other acute phase glycoproteins in response to inflammation is generally unknown at the present time. However, increasing evidence is accumulating to indicate that certain hormones are capable of controlling the synthesis of hepatic enzymes and also plasma proteins synthesized by liver. Studies on the effect of hormones on the synthesis and regulation of hepatic tyrosine α-ketoglutarate transaminase (66, 267) have led to several hypotheses on the mechanism of hormonal regulation of this enzyme. An interesting hypothesis has been suggested

by Tompkins (267) (Fig. 42) with regard to enzyme induction by steroid hormones in hepatoma cells grown in culture (HTC cells). Steroids induce tyrosine transaminase synthesis in these cells very rapidly. The enzyme level rises from a basal level for some hours and then reaches a plateau at a new level. When the hormone is removed, the enzyme activity falls back to a basal level. Actinomycin administered at the same time as the hormone abolishes the induction effect but given at the plateau level causes superinduction of the enzyme. These facts are explained by suggesting that message for the enzyme is made at a constant rate and is inactivated and destroyed by a repressor substance (Fig. 44). The repressor is synthesized at a constant rate on a message with shorter half-life than that of the message for the enzyme. Addition of the hormone interferes with the repressor action so that less message is destroyed, more accumulates and consequently more enzyme is synthesized. Addition of actinomycin at the same time as the hormone abolishes the hormone effect but does not inhibit the basal production of the enzyme. Hence, further synthesis of the enzyme (superinduction) is seen in the presence of actinomycin. This hypothesis

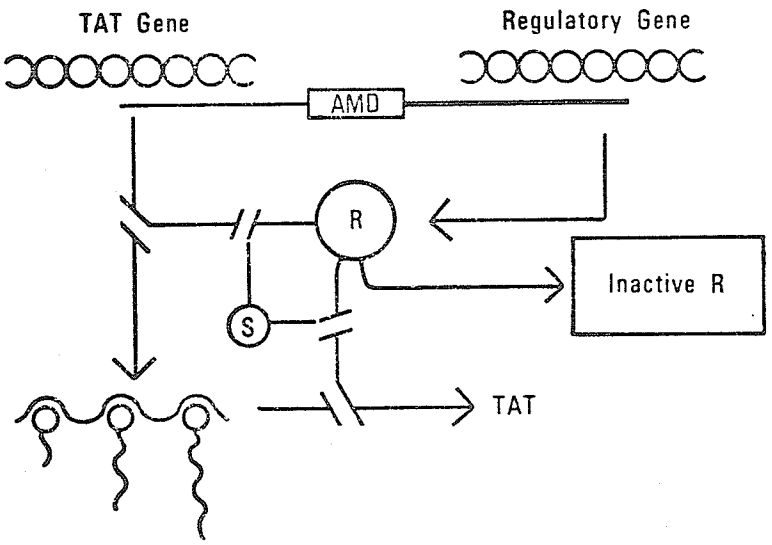


Fig. 42

Scheme for Regulation of Tyrosine Aminotransferase Synthesis in HTC Cells.

This model depicts two genes which are concerned with TAT biosynthesis and its control. The structural gene is shown acting as a template for the synthesis of a stable messenger RNA which in turn codes for the enzyme. The regulatory gene is shown acting as a template for a labile substance, R, which interferes with both the appearance of messenger RNA and with its translation. The steroid inducer, S, is shown antagonizing the action of R. Actinomycin D, depicted as "AMD", blocks the production of both R and TAT messenger. However, since the messenger is stable, its concentration does not immediately fall, whereas that of R decreases rapidly after the inhibition of RNA synthesis.

differs considerably from the Jacob-Monod hypothesis, placing control more at the translational rather than the transcriptional stage in protein synthesis.

A second hypothesis for the biosynthesis and regulation of tyrosine α-ketoglutarate transaminase has been proposed by Kenny et al. (66) and is shown in Fig. 43. These workers have studied the effect of the hormones hydrocortisone, insulin and glucagon on the biosynthesis of the enzyme. Hydrocortisone is shown to increase the rate of production of message allowing enzyme synthesis to occur. Similar to the findings of Tompkins (267) hydrocortisone induces the enzyme as long as the hormone is administered, then enzyme activity falls to the basal level when the hormone is removed. Insulin and glucagon, however, elevate the synthesis of the enzyme for only 2-3h, then the synthesis rate falls to the basal level. Kenny has suggested that these polypeptide hormones might be responsible for the passage of pre-existing mRNA into the cytoplasm. Thus, enzyme synthesis would be elevated quickly but would continue only as long as the excess mRNA persits and the induction could not be reinitiated until the pool is reformed at a constant low rate of RNA synthesis. An alternative suggestion has

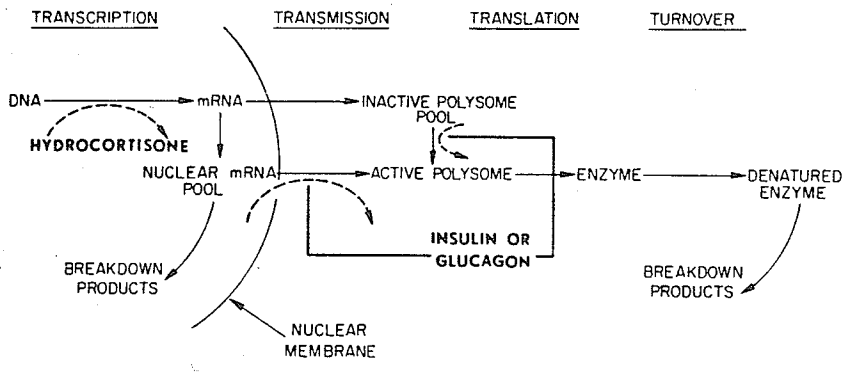


Fig. 43

Suggested mechanisms of hormonal induction of tyrosine α-ketoglutarate transaminase (66). Hydrocortisone increases the production of mRNA for enzyme synthesis. Insulin and glucagon are postulated to act by increasing the rate of transmission of mRNA from nucleus to cytoplasm or to activate an inactive polysome pool precoded for enzyme synthesis.

come from the work of Pitot and his coworkers (268). These workers postulate that there exists a cytoplasmic pool of inactive or partially active polysomes encoded for the enzyme which is being induced. The effect of the hormone is to activate the polysomes so that the enzyme is synthesized. This postulation also assumes that an inactive pool of mRNA becomes activated to induce enzyme synthesis. There are also other supporting data that suggests that ribosomes are susceptible to hormonal control. Tyrosine α -ketoglutarate transaminase induction by hydrocortisone in vivo is accompanied by a large burst in RNA synthesis (269,270) which virtually encompasses all types of hepatic RNA, including rRNA (271,272). Also, treatment of rats with insulin, growth hormones, corticosteroids, thyroxine, testerone and oestrogens is followed by enhanced activity of the ribosomes for the incorporation of amino acids in a cell free system (273). Martin and Wool (274) have shown that insulin affects the large ribosomal subunit, and, possibly the binding of tRNA molecules to it. Barden and Korner (275) have found a change in the small subunit of liver ribosomes as a result of hypophysectomy. As previously mentioned actinomycin blocks induction of transaminase by

insulin and glucagon. Furthermore, there is some evidence that the passage of RNA from nucleus to cytoplasm is also blocked by this antibiotic (276). Thus, Kenny favours the idea of a nuclear pool over a "polysome pool". However, both hypotheses are open to further experimentation.

There are a few lines of evidence from past studies on the biosynthesis of glycoproteins that similar mechanisms for the increased production of glycoproteins occur as that described for hepatic enzymes. For example, hormones are also thought to affect the synthesis of serum proteins. It has been shown that, in vivo, the synthesis of α_2-globulin occurs only in the presence of the adrenal glands (277). On the other hand, high blood concentration of α_1-acid glycoprotein with enhanced rate of incorporation of L-leucine-^{14}C into α_1-acid glycoprotein (164,165,167,239) has been observed in adrenalectomized rats. It has been possible to demonstrate an absolute requirement for the steroid hormone cortisol, for the synthesis of the α_2-(acute phase) globulin to occur (166,277). However, in addition to cortisol for the synthesis of α_2-globulin to occur, the rat must be injured. Therefore, some

substance, as yet unknown, must be present for the synthesis of this particular serum protein. Increased plasma concentrations of haptoglobin have been found to occur after repeated injections of testosterone, parathyroid hormone and cortisone (278). The effect of insulin on the rate of synthesis of certain plasma proteins has been investigated by Neuhaus (243). In these experiments incorporation of L-leucine-^{14}C into seromucoid and albumin was measured in rats rendered diabetic by means of alloxan. The response to injury was found to be less in these experimental animals than in normal animals. John and Miller (249) have studied the effects of hormones on the regulation of acute phase plasma protein synthesis in the perfused liver. These workers found that addition of a combination of insulin, cortisol, growth hormone and an amino acid mixture resulted in increased synthesis of fibrinogen, haptoglobin, α_2-(acute phase)globulin and α_1-acid glycoprotein. It was found that increased synthesis of these four proteins was critically dependent upon the presence of cortisol. Studies involving the use of known inhibitors of protein synthesis, to prevent incorporation of labelled amino acids into glycoprotein have also suggested some possible

mechanisms by which the synthesis of glycoproteins can be controlled. For example, Neuhaus et al. (214) has suggested that the increased rate of synthesis of the seromucoid fraction of serum of rats is a result of induced transcriptional processes, probably resulting from increased messanger RNA production. These workers found, however, that increased incorporation of D-glycine-^{14}C into the seromucoid fraction of actinomycin D-treated but uninjured rats took place 4h after the injection of actinomycin D. Such a mechanism appears similar to the "super-induction" of tyrosine transaminase by actinomycin found by Tompkins (267). Inhibition of incorporation of D-glycine-^{14}C into seromucoid after treatment with puromycin also suggests that the translational level of protein synthesis is affected.

The mechanism of the increased production or synthesis of α_1-acid glycoprotein in response to inflammation in the present work may be somewhat similar to the schemes postulated from the data of Tompkins (267) (Fig. 44) and Kenny (66) (Fig. 43) for the induction of synthesis of tyrosine α -keto-glutarate transaminase. As suggested by some workers (163,213,216) there may be the release of some factor from the site of tissue damage that is

responsible either directly or indirectly for the increased production or synthesis of acute phase reactants. For example, this factor may be released from the damaged cells, circulate in the blood, and then act on the liver to cause an increase in protein synthesis itself or in turn stimulate the release of hormones capable of increasing the production of proteins. In the present study it would appear that the liver responds to inflammation as early as 4h after subcutaneous injection of turpentine. Thus, a relatively short time is required before sufficient messanger substance or substances pass from the site of injury to the liver to alter the rates of synthesis of α_1-acid glycoprotein. Gordon and Koj (239) have observed a similar time period (i.e. 5h) for the onset of increased synthesis of α_1-globulins and transferrin in perfusion studies on livers isolated from rats at various times after subcutaneous injections of talc.

It is tempting to suggest that steroid hormones may be involved in the stimulation of the synthesis of acute phase globulins since several workers performing studies *in vivo* and *in vitro*, have illustrated the need for the presence of

corticosteroids for induction of hepatic enzymes (e.g. tyrosine α-ketoglutarate transaminase and tryptophan pyrrolase) (280-283) or the increased synthesis of acute phase reactants (166,277). Also, because actinomycin D blocks the induction of the hepatic enzymes (66, 267) and has been reported to block the acute phase response to injury in rats (214) it has been suggested (214,283) that cortisol acts somehow by enhancing transcription of DNA to produce more functional messanger RNA for the synthesis of the specific proteins in question. In the present study the increase in specific radioactivity of α_1-acid glycoprotein in serum from experimental rats compared to normal rats after administration of L-leucine-^{3}H suggests that there is some mechanism operating which speeds up the production or synthesis of the polypeptide chain. Thus the hypothetical substance responsible for stimulating the synthesis of α_1-acid glycoprotein could be a steroid hormone and the mechanism behind the increased production of the polypeptide chain of α_1-acid glycoprotein in response to inflammation may be taking place at the transcriptional level with an increase in the rate of production of messanger RNA resulting in an increased production of polypeptide chains of α_1-acid

glycoprotein. Such a hormone could also act according to the scheme put forward by Tompkins (Fig. 42) for the induction of tyrosine α-ketoglutarate transaminase in hepatoma cells. Sarcione (284) has proposed that there is a derepression of the genetic information coding for the α_2-(acute phase) globulin when injury occurs in the rat. This derepression allows production of the specific messanger RNA and thus α_2-(acute phase) globulin synthesis. However, increased synthesis of messanger RNA by a hormone could only affect those proteins where the turnover of messanger RNA is rapid or those proteins (e.g. α_2-(acute phase) globulin) which were not previously synthesized by the cell. Thus, transcriptional stimulation is probably not the only mechanism for the increased production of α_1-acid glycoprotein in response to inflammation. In the present study the electron micrographs indicate a tremendous proliferation of membranous material in experimental rat liver cells compared to control rat liver cells. This would tend to suggest that increased translational activity may also be responsible for an increased production of α_1-acid glycoprotein. Thus, there may be some mechanism operating at the ribosomal level which provides a greater number of

membrane bound polysomes for the purpose of increased production of polypeptide chains of α_1-acid glycoprotein. Such a mechanism could involve hormonal control of protein synthesis on cytoplasmic ribosomes (Fig. 45). Tata (251,252) has indicated that hormones increase the rate of incorporation of labelled phospholipids into membranous components. This effect may be a consequence of a specific action of the hormone or it may reflect a direct assembly of the whole protein synthesizing unit. Whatever the reason for the increased membrane proliferation it is almost certain that the membranous components play a vital role in the biosynthesis of α_1-acid glycoprotein and other acute phase reactants. For example, studies by Tetas et al. (127) and Caccam et al. (120) have shown that glucosamine and mannose are involved with a lipid intermediate prior to attachment to the polypeptide chain of trichloroacetic acid precipitable protein. Since nucleotide sugars are known to be added to the polypeptide chain while it is being sequestered through the endoplasmic reticulum it is possible that lipids in the membranes act as carriers of the sugars to the enzyme reaction site for attachment to polypeptide as has been described for bacterial cell wall synthesis (285,286).

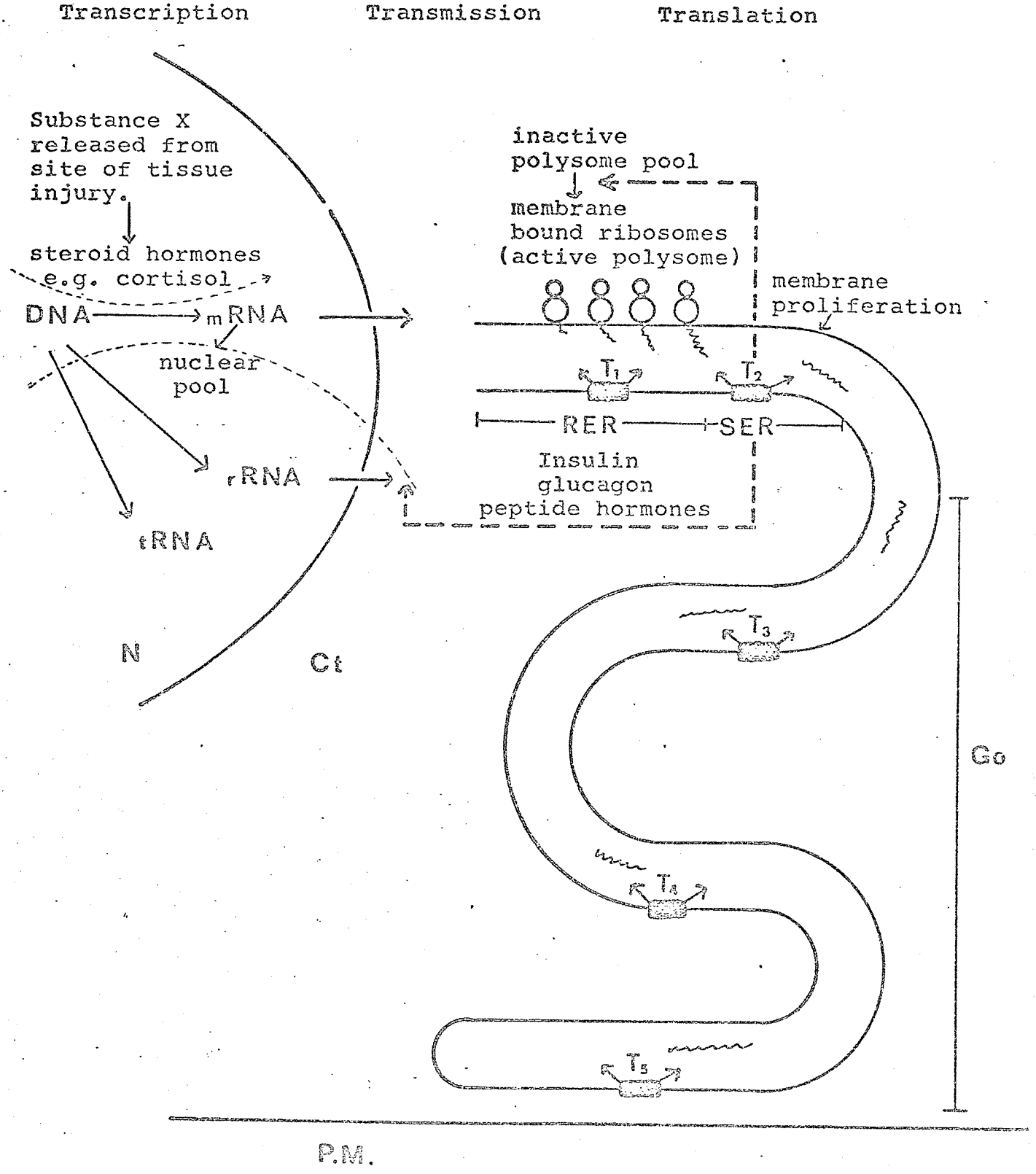
In addition, Mookerjea (287) has shown that there is an impairment of incorporation of radioactive glucosamine into the trichloroacetic-acid-insoluble fraction of rat plasma during early choline deficiency. It also appears that the synthesis of specific proteins that are to be complexed with carbohydrate in the smooth endoplasmic reticulum and Golgi apparatus are affected in choline deficiency and that there is little defect of ribosomal polypeptide synthesis. Thus choline deficiency could conceivably result in an impairment of membrane regeneration and as a consequence impair the stimulation of sugar transferases responsible for the attachment of sugars to polypeptide chain.

In evaluating the possible sites for regulation of the synthesis of glycoproteins, it is important to appreciate that attachment of the carbohydrate is mainly a postribosomal event and is not under direct genetic control. Roseman and coworkers (137) have isolated and purified a series of monosaccharide glycosyltransferases each of which transfers one type of sugar residue from a sugar nucleotide either to the functional group of the side chain of a peptide-bonded amino acid residue or to a monosaccharide unit already present. In the present studies

the greatly observed incorporation of D-glucosamine-^{14}C into α_1-acid glycoprotein isolated from livers from experimental rats compared to normal rats would tend to suggest that the attachment of the carbohydrate groups may well be the main limiting factor in the production of α_1-acid glycoprotein. In turn, a reason for the increased rate of attachment of carbohydrate groups would be the availability of various glycosyltransferases responsible for the attachment of individual sugars. If this were the case, then certain hormones may increase the rate of synthesis of α_1-acid glycoprotein by stimulating the production of enzymes, possibly according to the scheme put forward by Tompkins (Fig. 42). The increased availability of these enzymes would also allow for the glycosylation of the increased amount of α_1-acid glycoprotein present in the endoplasmic reticulum. A study on the rate of synthesis of purified glycosyltransferases in liver of control rats and rats suffering from inflammation would provide an answer to such a question.

A scheme postulating the various possible mechanisms behind the increased rate of production of α_1-acid glycoprotein in response to inflammation is shown in Fig. 44. This scheme is an intregation

of the postulation of Kenny et al. (66) and Tompkins et al. (267) for hormonal induction of tyrosine α-ketoglutarate transaminase, the postulation of Schachter et al. (129) for the role of glucosaminyltransferases in attachment of carbohydrate to glycoprotein, the limited evidence existing on the biosynthesis of acute phase reactants in response to injury, and evidence presented in this thesis for the biosynthesis of α_1-acid glycoprotein in response to inflammation


Transcription
Transmission
Translation
Substance X released from site of tissue injury.
steroid hormones e.g. cortisol
DNA
mRNA
nuclear pool
rRNA
tRNA
N
Ct
inactive polysome pool
membrane bound ribosomes (active polysome)
membrane proliferation
T1
T2
RER
SER
Insulin glucagon peptide hormones
T3
T4
T5
Go
P.M.

Proposals for future work on the biosynthesis of α_1-acid glycoprotein in response to inflammation

In the scheme put forward in Fig.44, illustrating the possible mechanisms for the increased production of α_1-acid glycoprotein in response to inflammation, most of the control levels have been suggested on the basis of studies on hormonal induction of hepatic enzymes, supplemented by some additional information on the biosynthesis of acute phase reactants and evidence presented in this thesis. However, there is very little known about the actual controls involved in the increased production of α_1-acid glycoprotein or other acute phase reactants in response to inflammation.

The isolation and characterization of a homogeneous α_1-acid glycoprotein reported in this study will now allow for a detailed study of the factors controlling the biosynthesis of this protein in response to inflammation. Thus, further *in vivo* studies employing the effect of various hormones on the biosynthesis of α_1-acid glycoprotein, coupled with data obtained from studies on protein synthesizing inhibitors should help to pinpoint the important control positions that influence the

biosynthesis of acute phase reactants such as the α_1-acid glycoprotein. Furthermore, *in vitro* studies using cell free systems, liver cell suspensions liver slice systems, isolated liver perfusion technique should help to confirm the *in vivo* studies. As well, the use of these *in vitro* systems would help to avoid confusion in interpretation of *in vivo* results because of metabolic breakdown of hormones administered *in vivo*.

The present study seems to indicate that there is an increase in the rate of attachment of sugar molecules (i.e. glucosamine and probably sialic acid) to the polypeptide chain of α_1-acid glycoprotein. Therefore, it is possible that the attachment of sugars to polypeptide may be a controlling factor in the regulation of the biosynthesis of α_1-acid glycoprotein. This suggestion thus provides an interesting approach to a study on the glycosaminyl transferases responsible for the attachment of sugar molecules to the polypeptide chain of α_1-acid glycoprotein. Therefore, an important study lies in the effect of inflammation on the activities, contents and biosynthesis of the enzymes themselves to determine if there is an increased production of the transferases as a result of inflammation which might

result in increased attachment of sugars to polypeptide chains.

Studies on the biosynthesis of carbohydrate prosthetic groups of α_1-acid glycoprotein should also be studied in a cell free system which is capable of incorporating labelled sugars into α_1-acid glycoprotein. It is possible to study the effect on the biosynthesis of carbohydrate prosthetic groups in a cell-free system is that the pool size of the labelled precursor used can be reduced resulting in the preparation of material of higher specific activity than could otherwise be obtained by studies in vivo.

The nature of the hypothetical substance X (see Fig. 46) is not known. An increase in rates of synthesis of acute phase plasma proteins has been reported when livers isolated from normal rats were perfused with blood from acutely injured rats (173, 288); however, subsequent studies have not confirmed these observations (220, 239). Further studies of this nature are required in order to locate the hypothetical substance that causes the increase in synthesis of acute phase reactants. Studies are required to isolate and characterize such a substance. Such an accomplishment is difficult, but would be a

major advance in determining the mechanism behind the acute phase response to injury.

Finally, little is known about the function of serum glycoproteins. It may be possible by studying the catabolism of α_1-acid glycoprotein, to reveal the function of this protein which might shed some light on the reasons behind the increased content of this protein in serum found as a result of inflammation.

SUMMARY

Studies have been carried out to determine the effect of turpentine-induced inflammation on the biosynthesis of rat serum proteins. The effect of inflammation on rat serum proteins has been studied at short time periods of exposure to inflammatory agent (5-96h). There was an increase in protein-bound hexose and hexosamine of serum reaching a maximum at 48h after administration of turpentine. Eight fractions were prepared from serum by a combination of chromatography on DEAE-cellulose and preparative electrophoresis on strips of gelatinized cellulose acetate. Most of the increase in protein-bound carbohydrate (77-86%) found in serum from experimental animals was located in three fractions following the fractionation procedure; immunological studies revealed the presence of fibrinogen and haptoglobin in two of these fractions. There was no evidence for the presence of a new protein in serum at short times of exposure to inflammatory agent. Proteins present in the perchloric acid soluble and seromucoid fractions of serum were found in five fractions, only two of which contributed to the increase in protein-bound carbohydrate of serum

found as a result of inflammation.

Rat serum albumin, an α_1-acid glycoprotein and an α_2-macroglobulin were isolated from serum from rats suffering from inflammation for 48h by a combination of ion-exchange chromatography, Sephadex gel filtration and isoelectric focusing. Immunological characterization of the proteins indicated that albumin and α_1-acid glycoprotein were homogeneous proteins while the α_2-macroglobulin possibly contained a minor contaminant. The carbohydrate compositions and some physical properties of the α_1-acid glycoprotein and α_2-macroglobulin were determined. The α_1-acid glycoprotein contained 34% carbohydrate, had an isoelectric point of 2.95 and a molecular weight of 43,000. The α_2-macroglobulin contained 16% carbohydrate, had an isoelectric point of 4.60 and a molecular weight of about 800,000.

A quantitative precipitin technique has been employed to determine the contents of α_1-acid glycoprotein, α_2-macroglobulin and albumin in serum from control rats and rats suffering from induced inflammation for 5-96h. There was an increase in the content of α_1-acid glycoprotein and α_2-macroglobulin in serum from experimental animals reaching

a maximum at 48-72h after administration of inflammatory agent indicating that both proteins are acute phase globulins. There was only a slight change in the content of albumin in serum from experimental animals when compared with controls.

Studies involving incorporation of L-leucine-^{3}H and D-glucosamine-^{14}C as precursors of glycoprotein biosynthesis into heterogeneous serum fractions and into α_1-acid glycoprotein and α_2-macroglobulin indicated that the most likely explanation for the increase in protein-bound carbohydrate in serum fractions and for the increase in content of α_1-acid glycoprotein and α_2-macroglobulin in serum from experimental animals was an increase in the rates of synthesis of these proteins.

The cellular and subcellular site of synthesis of albumin and α_1-acid glycoprotein was investigated by studies <u>in vivo</u> up to 15 minutes after the injection of labelled precursors into rats. Subcellular fractions isolated from liver, spleen and kidney were extracted with Lubrol-W. Extracts were reacted with antisera by double diffusion analysis and gels were subsequently subjected to radioautography. With amino acid as precursor, only extracts of the microsome fraction of liver formed lines to antiserum to

albumin and to antiserum to α_1-acid glycoprotein. Most precipitin lines formed by Lubrol extracts of liver microsomes interacted with lines given by rat serum or α_1-acid glycoprotein or albumin, reactions of identity being apparent in most cases.

A quantitative precipitin technique was employed to determine the content of α_1-acid glycoprotein and albumin in microsomes isolated from livers from control rats and rats suffering from inflammation for 5-48h. There was an increase in the content of α_1-acid glycoprotein in microsomes isolated from livers from experimental rats reaching a maximum at 12h after administration of inflammatory agent. There was only a slight change in the content of albumin in microsomes isolated from livers from experimental animals when compared with controls.

Incorporation studies with L-leucine-^{3}H and D-glucosamine-^{14}C to determine the pathway of secretion of albumin and α_1-acid glycoprotein in components of microsome material indicated there was little difference in the rates of secretion of these proteins in normal and 12h experimental rats. The pathway of secretion for both proteins proved to be rough endoplasmic reticulum$\longrightarrow$smooth endoplasmic reticulum$\longrightarrow$Golgi complex$\longrightarrow$serum. There was an

increase in specific radioactivity of ^{3}H in α_1-acid glycoprotein in serum of experimental rats compared to normal rats. Thus, there may be some mechanism operating at the transcriptional or translational level of protein synthesis in response to inflammation to effect an increase in the production of the polypeptide chain of α_1-acid glycoprotein. There was a two-fold increase in specific radioactivity of ^{14}C in α_1-acid glycoprotein isolated from the Golgi complex of livers from experimental rats compared to control rats. There was a seven-fold increase in specific radioactivity of ^{14}C in α_1-acid glycoprotein in serum from experimental rats compared to control rats. This suggests that there is an increased incorporation of sugars into α_1-acid glycoprotein in response to inflammation. Thus, the rate of incorporation of carbohydrate into incomplete molecules of α_1-acid glycoprotein as they are sequestered through the rough to smooth endoplasmic reticulum to the Golgi complex may be a regulating factor in the biosynthesis of α_1-acid glycoprotein and possibly other acute phase reactants.

Since there was an increase in microsomal α_1-acid glycoprotein in livers from experimental rats and there was no apparent change in the rate of

secretion of this protein, liver must be accomadating increased amount of α_1-acid glycoprotein and perhaps other acute phase globulins as a result of inflammation. An electron microscope study revealed changes in the membranous components of the cytoplasm in livers from experimental rats which may be related to an increased capacity for storage of acute phase globulins.

The results are discussed in terms of the current views on the biosynthesis of glycoproteins and the effect of hormones on the regulation of hepatic enzymes and acute phase globulins.

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to Dr. J.C. Jamieson to whom I am indebted for his valuable guidance during the course of these studies and for his helpful advice and suggestions throughout the preparation of this thesis. I wish to thank Mr. A.D. Friesen for his help in preparing the α_1-acid glycoprotein used in these studies. Thanks are extended to Dr. L. VanCaseele, Department of Botany, University of Manitoba, for carrying out the electron micrograph studies and to Dr. D. Burton of the Department of Microbiology, University of Manitoba, who performed the sedimentation velocity experiments. I would also like to extend my thanks to the Department of Microbiology for the liberal use of some items of equipment and for facilities for storing experimental animals.

I am indebted to the Faculty of Graduate Studies for a Manitoba Fellowship held during the performance of this work and also the Department of Chemistry for providing me with the opportunity to carry out this project.

APPENDIX

During termination of the studies on the pathway of secretion of albumin and α_1-acid glycoprotein, a report appeared in the literature criticizing the use of antiserum to precipitate homologous radioactive protein from tissue extracts for purposes of calculating specific radioactivities. Judah and Nicholls (289) have suggested that the antigen precipitated is not of radiochemical purity, thus leading to large errors in the calculation of the specific radioactivities of proteins. It has been shown that a good proportion of the radioactivity precipitated is associated with nonspecific bound proteins or free amino acids associated with the precipitate. Consequently, proteins (e.g. serum albumin) have been purified to radiochemical purity by ion-exchange chromatography (289). However, the work presented in the above studies did not include a preliminary series of preprecipitations with, for example, a heterologous immune system (e.g. human or chicken albumin) as used in the present study and by other workers (95,113). Therefore, one would expect nonspecific radioactivity to be associated with homologous precipitates obtained without prior preprecipitation of the extracts to remove "free"

radioactivity. Nevertheless, an experiment was carried out to ensure that the radioactivity of the albumin and α_1-acid glycoprotein precipitates isolated in the present work was in actual fact due to radiochemically pure antigens. After preprecipitation of tissue extracts, antiserum to albumin or α_1-acid glycoprotein was added and the precipitates so obtained were dissociated by dissolving in 0.5M-glycine buffer, pH 1.5. The antigen released from the precipitate was recovered by gel filtration on Sephadex G-200. The radioactivity of the recovered antigen was then compared to that of the precipitate before dissociation in glycine buffer. Not less than 90% of the specific radioactivity was found to be associated with the antigen. In addition, the total number of counts was the same after separation of the antigen on Sephadex G-200 as compared to the total counts in the precipitate before dissociation in glycine buffer. Therefore, on the basis of these preliminary experiments it was concluded that the antigens precipitated in the studies on the pathway of secretion of albumin and α_1-acid glycoprotein were of radiochemical purity and that the specific radioactivities calculated were valid.

BIBLIOGRAPHY

1. Gottschalk, A., In Glycoproteins: Their Composition, Structure and Function, Ed. by Gottschalk, A., Amsterdam: Elsevier Publishing Co., p. 5, (1966).
2. Meyer, K., Adv., Protein Chem., 2, 177, (1945).
3. Stacey, M., Adv., Carbohydrate Chem., 2, 161, (1946).
4. Jeanloz, R.W., Arthritis Rheumat., 3, 233, (1960).
5. Grant, P.T., and Simkin, J.L., Rep. Progs. Chem., 61, 491, (1965).
6. Spiro, R.G., Ann. Rev. Biochem., 39, 599, (1970).
7. Tiselius, A., Trans. Faraday Soc., 33, 524, (1937).
8. Freund, E., Zbl. Physiol., 6, 345, (1892). Cited by Bettelheim-Jevons, F.R., Adv. Prot. Chem., 13, 35, (1958).
9. Zanetti, C., Ann. Chim. Farm., 12, 1, (1897). Cited by Bettelheim-Jevons, F.R., Adv. Prot. Chem., 13, 35, (1958).
10. Winzler, R.J., Devor, A.W., Mehl, J.W., and Smyth, J.M., J. Clin. Invest., 27, 609, (1948).
11. Weimer, H.E., Mehl, J.W., and Winzler, R.J., J. Biol. Chem., 185, 561, (1950).

12. Spiro, R.G., New Eng. J. Med., 281, 1043, (1969).

13. Jeanloz, R.W., Medicine, 43, 363, (1964).

14. Spiro, R.G., New Eng. J. Med., 269, 566, (1963).

15. Schultz, H.E., Proc. 11th Colloq. Proteides Biol. Fluids, Ed. by Peeters, H., Amsterdam: Elsevier Publishing Co., p.288, (1964).

16. Gottschalk, A., and Graham, E.R.B., In The Proteins. Vol. IV, Ed. by Neurath, H., New York, Academic Press Inc., p.95, (1966).

17. Gottschalk, A., In Glycoproteins: Their Composition, Structure and Function, Ed. by Gottschalk, A., Amsterdam: Elsevier Publishing Co., (1966).

18. Apffel, C.A., and Peters, J.H., Progr. Exp. Tumor Res., 12, 1, (1969).

19. Jamieson, G.A., J. Biol. Chem., 240, 2914, (1965).

20. Clamp, J.R., and Putman, F.H., J. Biol. Chem., 239, 3233, (1964).

21. Spiro, R.G., J. Biol. Chem., 240, 1603, (1965).

22. Schmid, K., International Conference on Cystic Fibrosis of the Pancreas. 4th, Bern and Grindelwald. Cystic Fibrosis: Proceedings: Part 2: Biochemistry of Glycoproteins and Related Substances. Edited by Rossi, E., and Basel, E. Stoll., Karger, S., (1968).

23. Sataki, M., Okuyama, T., Ishihara, K., Schmid, K., Biochem. J., 95, 749, (1965).

24. Jamieson, G.A., J. Biol. Chem., 240, 2019, (1965).

25. Gerback, C.M., Bezkorovainy, A., Rafelson, M.E., Biochemistry, 6, 403, (1967).

26. Dunn, J.T., and Spiro, R.G., J. Biol. Chem., 242, 5556, (1967).

27. Spiro, R.G., J. Biol. Chem., 237, 646, (1962).

28. Spiro, R.G., J. Biol. Chem., 239, 567, (1964).

29. Rothfus, J.A., and Smith, E.L., J. Biol. Chem., 238, 1402, (1963).

30. Dawson, G., and Clamp, J.R., Biochem. J., 107, 341, (1968).

31. Wagh, P.V., Bornstein, I., and Winzler, R.J., J. Biol. Chem., 244, 685, (1969).

32. Kamiyama, S., and Schmid, K., Biochim. Biophys. Acta, 58, 80, (1962).

33. Jamieson, G.A., Jett, M., and DeBernardo, S.L., J. Biol. Chem., 246, 3686,(1971).

34. Johansen, P.G., Marshall, R.D., and Neuberger, A., Biochem. J., 78, 518, (1961).

35. Plummer, J.H., and Hirs, C.W.H., J. Biol. Chem., 239, 2530, (1964).

36. Yamashina, I., Makino, M., Ban-i, K., and Kojima, T., J. Biochem.(Tokyo), 58, 168, (1965).

37. Anderson, B., Seno, M., and Sampson, P., J. Biol. Chem., 239, P.C. 2716, (1964).

38. Schmid, K., Ishiguro, M., Emura, J., Isemure, S., Kaufmann, H., and Toshiko, M., Biochem. Biophys. Res. Communs., 42, 280, (1971).

39. Hunt, L.T., and Dayhoff, M.O., Biochim. Biophys. Res. Commun., 39, 757, (1970).

40. Eylar, E.H., J. Theor. Biol., 10, 89, (1965).

41. Miller, L.L., and Bale, W.F., J. Exp. Med., 99, 125, (1954).

42. Lehninger, A.L., In Biochemistry, Worth Publishers Inc., New York, (1970).

43. Bosmann, H.B., and Martin, S.S., Science, 164, 190, (1969).

44. Porter, K.R., and Blum, J., Anat. Record., 4, 685, (1953).

45. Palade, G.E., J. Biophys. Biochem. Cytol. Suppl., 2, 85, (1956).

46. Palade, G.E., and Siekevitz, P., J. Biophys. Biochem. Cytol., 2, 171, (1956).

47. Roberts, R.B., In Microsomal Particles and Protein Synthesis, Ed. by Roberts, R.B., Pergamon Press, London, (1958).

48. Neutra, M., and Leblond, C.P., Sci. Amer., 220, 100, (1969).

49. Peterson, In The Physical and Chemical Properties of Ribosomes, Elsevier Publishing Co., Amsterdam, (1964).

50. Spirin, A.S., and Gavrilova, L.P., In The Ribosome, Ed. by Kleinzeller, A., Springer, G.F., and Wittman, H.G., Springer-Verlag New York Inc., (1969).

51. Claude, A., Science, 87, 467, (1938).

52. Claude, A., Science, 90, 213, (1939).

53. Claude, A., Science, 97, 451, (1943).

54. Moulte, Y., Rouiller, C.H., and Chauveau, J., J. Biophys. Biochem. Cytol., 7, 547, (1960).

55. Rothschild, J., Biochem. Soc. Symp. (Cambridge, England), 22, 4, (1963).

56. Dallner, G., Acta. Path. Microbiol. Scand. Suppl., 166, (1963).

57. Glaumann, H., and Dallner, G., J. Cell Biol., 47, 34, (1970).

58. Morre, D.J., Hamilton, R.L., Mollenhauer, H.H., Mahley, R.W., Cunningham, W.P., Cheetham, R.D., and Lequire, V.S., J. Cell Biol., 44, 484, (1970).

59. Watson, J.D., and Crick, F.H.C., Nature, Lond., 171, 737, (1953).

60. Watson, J.D., and Crick, F.H.C., Nature, Lond., 171, 964, (1953).

61. Hurwitz, J., Bresler, A., and Diringer, R., Biochem. Biophys. Res. Communs., 3, 15, (1960).

62. Weiss, S.B., Proc. Nat. Acad. Sci., U.S., 46, 1020, (1960).

63. Penman, S., Smith, I., Holtzman, E., and Greenberg, H., National Cancer Institute Monograph, No. 21, p.317, (1966).

64. Attardi, G., Parnas, H., Hwang, M., and Attardi, B., J. Mol. Biol., 20, 145, (1966).

65. Shearer, R.W., and McCarthy, B.J., Biochemistry, 6, 283, (1967).

66. Kenny, F.T., In Regalutory Mechanisms for Protein Synthesis in Mammalian Cells, Ed. by San Pietro, A., Lamborg, M.R., and Kenny, F.T., Academic Press, New York, p.119, (1968).

67. Henshaw, E.C., Revel, M., and Hiatt, H.H., J. Mol. Biol., 14, 241, (1965).

68. McConkey, E.H., and Hopkins, J.W., J. Mol. Biol., 14, 257, (1965).

69. Redman, C.M., J. Biol. Chem., 144, 4308, (1969).

70. Girard, M., Latham, H., Penman, S., and Darnell, J.E., J. Mol. Biol., 11, 187,(1965).

71. Ristow, H., and Kohler, K., Biochim. Biophys. Acta, 123, 265, (1966).

72. Bishop, J.O., Biochim. Biophys. Acta, 119, 130, (1966).

73. Hogan, B.L.M., and Korner, A., Biochim. Biophys. Acta, 169, 139, (1968).

74. Vesco, C., and Colombo, B., J. Mol. Biol., 47, 335, (1970).

75. Loeb, J.N., Howell, R.R., and Tompkins, G.M., Science, 149, 1093, (1965).

76. Berg, P., Ann. Rev. Biochem., 30, 293, (1961).

77. Hoagland, M.B., In The Nucleic Acids, Vol. 3, New York, Academic Press, p.349, (1960).

78. Hoagland, M.B., Keller, E.B., and Zamecnik, P.C., J. Biol. Chem., 218, 345, (1956).

79. Hoagland, M.B., Zamecnik, P.C., and Stevenson, M.L., Biochim. Biophys. Acta, 24, 215, (1957).

80. Hoagland, M.B., Stevenson, M.L., Scott, J.F., Hecht, L.I., and Zamecnick, P.C., J. Biol. Chem., 231, 241, (1958).

81. Sonnenbichler, J., Feldman, H., and Zachau, H.G., Hoppe-Seyl, Z., 334, 283, (1963).

82. Galper, J.B., and Darnell, J.E., Bichem. Biophys. Res. Communs., 34, 205, (1963).

83. Shafritz, D.A., and Anderson, W.F., Nature, Lond., 227, 918, (1970).

84. Prichard, P.M., Gilbert, J.M., Shafritz, D.A., and Anderson, W.F., Nature, Lond., 226, 511, (1970).

85. Spirin, A.S., and Gavrivola, L.P., In The Ribosome, Ed. by Kleinzeller, A., Springer, G.F., and Wittman, H.G., Springer-Verlag, New York Inc., (1969)

86. Monro, R.E., J. Mol. Biol., 26, 147, (1967).

87. Khorana, H.G., Buchi, H., Ghosh, H., Gupta, N., Jacob, T.M., Kossel, H., Morgan, R., Narang, S.A., Ohtsulta, E., and Wells, R.D., Cold Spring Harbor Symposia Quant. Biol., 31, 39, (1966).

88. Last, J.A., Stanley, W.M., Salas, M., Hille, M.B., Wahba, A.J., and Ochoa, S., Proc. Nat. Acad. Sci. U.S., 57, 1062, (1967).

89. Brenner, S., Stretton, A.O.W., and Kaplan, S., Nature, Lond., 206, 994, (1965).

90. Capecchi, M.R., Proc. Nat. Acad. Sci. U.S., 58, 1144, (1967).

91. Peters, T., and Anfinsen, C.B., J. Biol. Chem., 182, 171, (1950).

92. Peters, T., and Anfinsen, C.B., J. Biol. Chem., 186, 805, (1950).

93. Miller, L.L., Bly, G.G., Watson, M.L., and Bale, W.F., J. Exp. Med., 94, 431, (1951).

94. Miller, L.L., Bly, G.G., and Bale, W.F., J. Exp. Med., 99, 133, (1954).

95. Campbell, P.N., and Stone, N.E., Biochem. J., 66, 19, (1957).

96. Marsh, J.B., and Drabkin, D.L., J. Biol. Chem., 230, 1073, (1958).

97. Sarcione, E.J., Biochemistry, 1, 1132, (1962).

98. Sarcione, E.J., Biochemistry, 9, 3059, (1970).

99. Asofsky, R., and Thorbecke, G.J., J. Exp. Med., 114, 471, (1961).

100. Krauss, S., and Sarcione, E.J., Biochim. Biophys. Acta, 90, 301, (1964).

101. Saddi, R., and Decken, A., von der Experentia, 21, 227, (1965).

102. Straub, P.W., J. Clin. Invest., 42, 130, (1963).

103. Hochwald, G.M., Thorbecke, G.J., and Asofsky, R., J. Exp. Med., 114, 459, (1961).

104. Miller, L.L., Hanavan, H.R., Titthasiri, N., and Chowdhury, A., Adv. Chem. Ser., 44, 17, (1964).

105. Zamecnik, P.C., and Keller, E.B., J. Biol. Chem., 209, 337, (1954).

106. Keller, E.B., Zamecnik, P.C., and Loftfield, R.B., J. Histochem. Cytochem., 2, 378, (1954).

107. Littlefield, J.W., Keller, E.B., Gross, J., and Zamecnik, P.C., J. Biol. Chem., 217, 111, (1955).

108. Redman, C.M., Biochem. Biophys. Res. Communs., 31, 845, (1968).

109. Ganoza, M.C., Williams, C.A., and Lippmann, F., Proc. Nat. Acad. Sci. U.S., 53, 619, (1965).

110. Takagi, M., Tanaka, T., and Ogata, K., J. Biochem., 65, 651, (1969).

111. Glaumann, H., and Ericsson, J.L.E., J. Cell Biol., 47, 555, (1970).

112. Peters, Jr., T., J. Biol. Chem., 237, 1186, (1962).

113. Peters, Jr., T., J. Biol. Chem., 246, 240, (1971).

114. Hirokawa, R., and Ogata, K., J. Biochem., Tokyo, 52, 377, (1962).

115. Spiro, R.G., J. Biol. Chem., 234, 742, (1959).

116. Robinson, G.B., Molnar, J., and Winzler, R.J., J. Biol. Chem., 239, 1134, (1964).

117. Macbeth, R.A.L., Bekesi, J.G., Sugden, E., and Rice, S., J. Biol. Chem., 240, 3707, (1965).

118. Neutra, M., and Leblonde, C.P., J. Cell Biol., 30, 137, (1966).

119. Wagner, R.R., and Cynkin, M.A., Biochem. Biophys. Res. Communs., 35, 139, (1969).

120. Caccam, J.F., Jackson, J.J., and Eylar, E.H., Biochem. Biophys. Res. Communs., 35, 505, (1969).

121. Bosmann, H.B., Hagopian, A., and Eylar, E.H., Arch. Biochem. Biophys., 128, 51, (1968).

122. Molnar, J., Robinson, G.B., and Winzler, R.J., J. Biol. Chem., 240, 1882, (1965).

123. Lawford, G.R., and Schachter, H., J. Biol. Chem., 241, 5408, (1966).

124. Molnar, J., and Sy, D., Biochemistry, 6, 1941, (1967).

125. Molnar, J., Tetas, M., and Chao, H., Biochem. Biophys. Res. Commun., 37, 684, (1969).

126. Redman, C.M., and Cherian, M.G., J. Cell Biol., 52, 231, (1972).

127. Tetas, M., Chao, H., and Molnar, J., Arch. Biochem. Biophys., 138, 135, (1970).

128. Wagner, R.R., and Cynkin, M.A., J. Biol. Chem., 246, 143, (1971).

129. Schachter, H., Jabbal, I., Hudgin, R.L., and Pinteric, L., J. Biol. Chem., 245, 1090, (1970).

130. Spiro, R.G., and Spiro, M.J., J. Biol. Chem., 241, 1271, (1966).

131. Herscovics, A., Biochem. J., 112, 709, (1969).

132. Herscovics, A., Biochem. J., 117, 411, (1970).

133. Trujillo, J.L., and Gan, J.C., Biochim. Biophys. Acta, 230, 610, (1971).

134. Whur, P., Herscovics, A., and Leblond, C.P., J. Cell Biol., 43, 289, (1969).

135. Spiro, M.J., and Spiro, R.G., J. Biol. Chem., 243, 6520, (1968).

136. Spiro, M.J., and Spiro, R.G., J. Biol. Chem., 243, 6529, (1968).

137. Roseman, S., In Biochemistry of glycoproteins and related substances, proceedings of the fourth international conference on cystic fibrosis of the pancreas. Ed. by Rossi, E., and Stoll, E.S., Karger, Basel, p. 244, (1968).

138. Roseman, S., Carlson, D.M., Jourdian, G.W., McGuire, E.J., Kaufman, B., Basu, S., and Bartholomew, B., In Methods In Enzynology. Vol. VIII. Ed. Neufeld, E.F., and Ginsburg, V., Academic Press, New York, p.354, (1966).

139. Hickman, J., Ashwell, G., Morell, A.G., Van Den Hamer, C.J.A., and Scheinberg, I.H., J. Biol. Chem., 245, 759, (1970).

140. McGuire, E.J., Jourdian, G.W., Carlson, D.M., and Roseman, S., J. Biol. Chem., 240, P.C. 4112, (1965).

141. Johnston, I.R., McGuire, E.J., Jourdian, G.W., and Roseman, S., J. Biol. Chem., 241, 5735, (1966).

142. Bosmann, H.B., Eur. J. Biochem., 14, 33, (1970).

143. Grollman, A.P., and Marcus, D.M., Biochem. Biophys. Res. Commun., 25, 542, (1966).

144. Hudgin, R.L., and Schachter, H., Can. J. Biochem., 49, 829, (1971).

145. Hudgin, R.L., and Schachter, H., Can. J. Biochem., 49, 838, (1971).

146. Hudgin, R.L., and Schachter, H., Can. J. Biochem., 49, 847, (1971).

147. Mookerjea, S., Chow, A., and Hudgin, R.L., Can. J. Biochem., 49, 297, (1971).

148. Nadler, N.J., Young, B.A., and Leblond, C.P., Endocrinology, 74, 333, (1964).

149. Shetlar, M.R., Foster, J.V., Kelly, K.H., and Everett, M.R., Proc. Soc. Exptl. Biol. Med., 69, 507, (1948).

150. Winzler, R.J., Methods of Biochemical Analysis, 2, 279, (1955), edited by Glick, D., Interscience Publishers Inc., New York.

151. Moschides, E., Stefanini, M., Magalini, S.I., Kistner, S.A., and Mele, R., J. Clin. Invest., 37, 127 (1958).

152. Berkman, J., Rifkin, H., and Ross, G., J. Clin. Invest., 32, 415, (1953).

153. Andreani, D.V., and Gray, C.H., Clin. Chim. Acta., 1, 7, (1956).

154. Kelly, V.C., J. Pediat., 40, 405, (1952).

155. Shetlar, M.R., Payne, R.W., Bullock, J.A., Patrick, D.R., Hellbaum, A.A., and Ishmael, W.K., J. Clin. Invest., 32, 1208, (1953).

156. Böttiger, L.E., Malmquist, E., and Olhagen, B., Annals of the Rheumatic Diseases, 23, 489, (1964).

157. Banerjee, S., and Bhaduri, J.N., Proc. Soc. Exptl. Biol. Med., 101, 340, (1959).

158. Shetlar, M.R., Bullock, J.A., Shetlar, C.L., and Payne, R.W., Proc. Soc. Exptl. Biol. Med., 88, 107, (1955).

159. Glenn, E.M., Bowman, B.J., Koslowske, T.C., Biochem. Pharmac., Supplement, p.27, (1968), Pergamon Press, Great Britin.

160. Ross, R., Sci. Amer., 220, 40, (1969).

161. Winzler, R.J., The Amino Sugars, IIA, Edited by Balazas, E.A., and Jeanloz, R.W., (1965), Academic Press.

162. Petermann, M.L., The Plasma Proteins II, Edited by Putnam, F.W., (1960), Academic Press.

163. Weimer, H.E., and Benjamin, D.C., Amer. J. Physiol., 209, 736, (1965).

164. Darcy, D.A., Brit. J. Cancer, 14, 524, (1960).

165. Darcy, D.A., Brit. J. Cancer, 14, 534, (1960).

166. Weimer, H.E., and Coggshall, V., Can. J. Physiol. Pharmacol., 45, 767, (1967).

167. Darcy, D.A., Brit. J. Exptl. Pathhol., 45, 281, (1964).

168. Catchpole, H.R., Proc. Soc. Exptl. Biol. Med., 75, 221, (1950).

169. Darcy, D.A., Brit. J. Exptl. Pathol., 46, 155, (1965).

170. Sarcione, E.J., Arch. Biochem., Biophys., 100, 516, (1963).

171. Neuhaus, O.W., Balegno, H.F., and Chandler, A.M., Proc. Soc. Exptl. Biol. Med., 107, 960, (1961).

172. Hochwald, G.M., Thorbecke, C.J., and Asofsky, R.J., J. Exptl. Med., 114, 459, (1961).

173. Sarcione, E.J., Bohne, M., and Krauss, S., Fed. Proc., 24, 230, (1965).

174. Peterson, E.A., and Sober, H.A., J. Amer. Chem. Soc., 78, 751, (1956).

175. Sober, H.A., Gutter, F.J., Wycoff, M.M., and Peterson, E.A., J. Amer. Chem. Soc., 78, 756, (1956).

176. Montreuil, J., and Scheppler, N., Bull. Soc. Chim. Biol., Paris, 41, 13, (1959).

177. Svennerholm, L., Acta Chem. Scand., 12, 547, (1958).

178. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. Biol. Chem., 193, 265, (1951).

179. Miller, G.L., Anal. Chem., 31, 964, (1959).

180. Simkin, J.L., Skinner, E.R., and Seshadri, H.S., Biochem. J., 90, 316, (1964).

181. Haab, W., and Anastassiadis, P.A., Can. J. Biochem. Physiol., 39, 671, (1961).

182. Boas, N.F., J. Biol. Chem., 204, 553, (1953).

183. Warren, L., Nature, 186, 237, (1960).

184. Partridge, S.M., Nature, 164, 443, (1949).

185. Partridge, S.M., Biochem. J., 42, 238, (1948).

186. Chan, B.G., and Cain, J.C., Nature, 192, 69, (1961).

187. Aminoff, D., Biochem. J., 81, 384, (1961).

188. Rondle, C.J.M., and Morgan, W.T.J., Biochem. J., 61, 586, (1955).

189. Munroe, H.N., and Fleck, A., The Analyst, 91, 78, (1966).

190. Methods in Biochemical Analysis I., p.287, Ed. D. Glick, Interscience, New York, (1954).

191. Hubscher, G., and West, G.R., Nature, Lond., 205, 799, (1965).

192. Allen, R.T.L., Biochem. J., 34, 858, (1940).

193. Kohn, J., In Chromatographic and Electrophoretic Techniques, Vol. 2, Ed. by Smithh, I., William Heinemann Ltd., London, p.56, (1960).

194. Smithies, O., Biochem. J., 61, 629, (1955).

195. Ornstein, L., Ann. N.Y. Acad. Sci., 121, 321, (1964).

196. Davis, B.J., Ann. N.Y. Acad. Sci., 121, 404, (1964).

197. Clarke, J.T., Ann. N.Y. Acad. Sci., 121, 428, (1964).

198. Weber, K., and Osborn, M., J. Biol. Chem., 244, 4406, (1969).

199. Vesterberg, O., and Svensson, H., Acta Chem. Scand., 20, 820, (1966).

200. Petterssen, E., Acta Chem. Scand., 23, 2631, (1969).

201. Maung, M., Baker, D.G., and Murry, R.K., Life Sci., 3, 1349, (1964).

202. Andrews, P., Biochem. J., 96, 595, (1965).

203. Fothergill, J.E., In Fluorescent Protein Tracing, Ed. by Nairn, R.C., Edinburgh: E. and S. Livingston Ltd., p.4, (1962).

204. Campbell, P.N., Greengard, O., and Kernot, B.A., Biochem. J., 74, 107, (1960).

205. Ouchterlony, O., Acta Pathol. Microbiol. Scand., 32, 231, (1953).

206. Simkin, J.L., and Jamieson, J.C., Biochem. J., 103, 153, (1967).

207. Morgan, W.S., Perlmann, P., and Hultin, T., J. Biophys. Biochem. Cytol., 10, 411, (1961).

208. Gardiner, J.E., Nature, Lond., 197, 414, (1963).

209. Bodman, J., In Chromatographic and Electrophoretic Techniques, Vol. 2, Ed. by Smithh, I., William Heinemann Ltd., London, p.91, (1960).

210. Simkin, J.L., and Work, T.S., Biochem. J., 65, 307, (1957).

211. Jamieson, J.C., Ph.D. Thesis, University of Aberdeen, Aberdeen, Scotland.

212. Weinfeld, H., and Tunis, M., J. Biol. Chem., 235, 1668, (1960).

213. Koj, A., Biochim. Biophys. Acta, 165, 97, (1968).

214. Neuhaus, O.W., Balegno, H.F., and Chandler, A.M., Amer. J. Physiol., 211, 151, (1966).

215. Mouridsen, H.T., Clin. Sci., 33, 345, (1967).

216. Koj, A., and McFarlane, A.S., Biochem. J., 108, 137, (1968).

217. Espinosa, E., Int. J. Appl. Radiat. Isotopes, 12, 122, (1961).

218. Murray, R.K., and Connell, G.E., Nature, 186, 86, (1960).

219. Weimer, H.E., Wood, F.D., and Pearson, C.M., Can. J. Biochem., 46, 743, (1968).

220. Schumer, W., Molnar, J., Dowling, J.N., and Winzler, R.J., Amer. J. Physiol., 212, 184, (1967).

221. Robert, L., Mombelloni, P., and Crosti, P., Proc. Soc. Exp. Biol. Med., 107, 499, (1961).

222. Miale, J.B., and Kent, J.W., Proc. Soc. Exp. Biol. Med., 111, 589, (1962).

223. Krauss, S., Proc. Soc. Exp. Biol. Med., 112, 552, (1962).

224. Boffa, G.A., Nadal, C., Zajeela, F., and Fine, J.M., Nature, 203, 1182, (1964).

225. Heim, W.G., and Lane, P.H., Nature, 203, 1077, (1964).

226. Bodgen, A.E., Neville, G.A., Woodward, W.E., and Gray, M., Proc. Amer. Ass. Cancer Res., 5, 6, (1964).

227. Kawasaki, T., Koyama, J., and Yamashina, I., J. Biochem. (Tokyo), 60, 554, (1966).

228. Zito, R., Marcaute, M.L., and Caputo, A., Acta Un. Int. Cancer, 20, Part 2, 1146, (1964).

229. Gordon, A.H., and Louis, L.N., Biochem. J., 113, 481, (1969).

230. Winzler, R.J., In The Plasma Proteins, Vol. 1, Ed. by Putman, F.W., Academic Press, New York, p.309, (1960).

231. Yamashina, I., Acta Chem. Scand., 10, 1666, (1965).

232. Bezkorovainy, A., Biochim. Biophys. Acta., 101, 336, (1965).

233. Smith, E.L., Brown, D.M., Weimer, H.E., and Winzler, R.J., J. Biol. Chem., 185, 569, (1950).

234. Norberg, R., Birke, G., Hedfors, E., and Plantin, L.O., In Plasma Protein Metabolism, Ed. by Rothschild, M.A., and Waldmann, T., Academic Press Inc., New York, p.427, (1970).

235. Boffa, G.A., Jacquot-Armand, Y., Gaudin-Harding, F., Susbielle, H., and Fine, J.M., Compt. Rendus, 159, 1307, (1965).

236. Boffa, G.A., Fine, J.M., Jacquot-Armand, Y., Gaudin-Harding, F., and Susbielle, H., Compt. Rendus, 159, 1324, (1965).

237. Demaille, J., Broussal, J., Colette, C., Guilleux, F., and Magnan de Bornier, B., C.R. Acad. Sc. Paris, t., 270, 2133, (1970).

238. John, D.W., and Miller, L.L., J. Biol. Chem., 243, 268, (1968).

239. Gordon, A.H., and Koj, A., Brit. J. Exp. Pathol., 49, 436, (1968).

240. Darcy, D.A., In Protides of the Biological Fluids. Proceedings of the 10th Colloquim Bruges, Ed. by Peeters, H., Elsevier, Amsterdam, p.131, (1962).

241. Peters, Jr., T., J. Biol. Chem., 237, 1181, (1962).

242. Howe, P.E., J. Biol. Chem., 49, 93, (1921).

243. Balengo, H.F., and Neuhaus, O.W., Life Sciences, 9, 1039, (1970).

244. Maung, M., Baker, D.G., and Murry, R.K., Can. J. Biochem., 46, 477, (1968).

245. Robinson, G.B., Biochem. J., 114, 635, (1969).

246. Richmond, J.E., Biochemistry, 2, 676, (1963).

247. Krauss, S., and Sarcione, E.J., Biochim. Biophys. Acta, 90, 301, (1964).

248. Athineos, E., Thorton, M., and Winzler, R.J., Arch. Biochem. Biophys., 106, 338, (1964).

249. Morgan, E.H., and Peters, Jr., T., J. Biol. Chem., 246, 3500, (1971).

250. Claude, A., In Microsomes and Drug Oxidations, Ed. by Gillette, J.R., Conney, A.H., Cosmides, G.J., Estabrook, R.W., Fouts, J.R., and Mammering, G.J., Academic Press Inc., New York, p.3, (1969).

251. Tata, J.R., Biochem. J., 104, 1, (1967).

252. Tata, J.R., In Regulatory Mechanisms for Protein Synthesis in Mammalian Cells, Ed. by San Pietro, A., Lamborg, M.R., and Kenny, F.T., Academic Press Inc., New York, p.99, (1968).

253. Campbell, P.N., In Protein Biosynthesis, Ed. by Harris, R.J.D., Academic Press Inc., New York, p.19, (1961).

254. Lingrel, J.B., and Webster, G., Biochim. Biophys. Res. Communs., 5, 57, (1961).

255. Korner, A., Biochem. J., 76, p.59, (1960).

256. Von der Decken, A., and Campbell, P.N., Biochem. J., 80, p.38, (1961).

257. Zilversmit, D.B., Entenman, C., and Fishler, M.C., J. Gen. Physiol., 26, 325, (1943).

258. Hoffenberg, R., Gordon, A.H., Black, E.G., and Louis, L.N., Biochem. J., 118, 401, (1970).

259. Bruni, C., and Porter, K.R., Amer. J. Pathol. 46, 691, (1965).

260. Lo, C., and Marsh, J.B., J. Biol. Chem., 245, 5001, (1970).

261. Jandl, J.H., and Katz, J.H., J. Clin. Invest., 42, 314, (1963).

262. Baker, E., and Morgan, E.H., Quart. J. Exp. Physiol., 54, 173, (1969).

263. Morgan, E.H., and Peters, Jr., T., J. Biol. Chem., 246, 3508, (1971).

264. Lane, R.S., Brit. J. Haematol., 15, 355, (1968).

265. Droz, B., and Lacassagne, M.A., C.R. Hebd. Seances Acad. Sci. Paris, 262, 1654, (1966).

266. Ashley, C.A., and Peters, Jr., T., J. Cell Biol., 43, 237, (1969).

267. Tompkins, G.M., In Regulatory Mechainisms for Protein Synthesis in Mammalian Cells. Ed. by San Pietro, A., Lamborg, M.R., and Kenny, F.T., Academic Press, New York, p.268, (1968).

268. Pitot, H.C., Cited as personal communication by Kenny,F.T., In Regulatory Mechanisms for Protein Synthesis in Mammailan Cells. Ed. by San Pietro, A., Lamborg, M.R., and Kenny, F.T., Academic Press, New York, p.99, (1968).

269. Feigelson, M., Gross, P.R., and Feigelson, P., Biochim. Biophys. Acta., 55, 495, (1962).

270. Kenny, F.T., and Kull, F.J., Proc. Nat. Acad. Sci. U.S., 50, 493, (1963).

271. Wicks, W.D., Kenny, F.T., and Greenman, D.L., J. Biol. Chem., 240, 4420, (1965).

272. Greenman, D.L., Wicks, W.D., and Kenny, F.T., J. Biol. Chem., 240, 4420, (1965).

273. Korner, A., Proc. Roy. Soc. Lond. B., 176, 287, (1970).

274. Martin, T.E., and Wool, I.G., Proc. Nat. Acad. Sci., U.S., 60, 569, (1968).

275. Barden, N., and Korner, A., Biochem. J., 114, p.30, (1969).

276. Georgiev, G.P., In Progress in Nucleic Acid Research and Molecular Biology. Ed. by Davidson, J.N., and Cohn, W.E., Vol. 6, Academic Press, New York, p.259, (1967).

277. Heim, W.G., and Ellenson, S.R., Nature, Lond. 213, 1260, (1967).

278. Krauss, S., Fed. Proc. Fed. Amer. Soc. Exp. Biol., 27, 254, (1968).

279. John, D.W., and Miller, L.L., J. Biol. Chem., 244, 6134, (1969).

280. Knox, W.E., Brit. J. Exp. Pathol., 32, 462, (1967).

281. Lin, E.C.C., and Knox, W.E., Biochim. Biophys. Acta, 26, 85, (1957).

282. Goldstein, L., Stella, E.J., and Knox, W.E., J. Biol. Chem., 237, 1723, (1962).

283. Barnabei, O., and Sereni, F., Biochim. Biophys. Acta, 91, 239, (1964).

284. Sarcione, E.J., In Plasma Protein Metabolism. Ed. by Rothschild, M.A., and Waldman, T., Academic Press, New York, p.369, (1970).

285. Struve, W.G., Sinha, R.V., and Neuhaus, F.C., Biochemistry, 5, 82, (1966).

286. Higashi, V., Strominger, J.L., and Sweeley, C.C., Proc. Nat. Acad. Sci. U.S., 57, 1878, (1967).

287. Mookerjea, S., Can. J. Biochem., 47, 125, (1969).

288. Gordon, A.H., and Darcy, D.A., Brit. J. Exp. Pathol., 48, 81, (1967).

289. Judah, J.D., and Nicholls, M.R., Biochem. J., 123, 643, (1971).