THE UNIVERSITY OF MANITOBA

BIOSYNTHETIC STUDIES ON ACUTE PHASE PROTEINS OF RAT SERUM IN RATS SUFFERING FROM INDUCED INFLAMMATION AND IN RESPONSE TO HORMONES USING A LIVER SLICE SYSTEM.

by

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ABBREVIATIONS

RNA	Ribonucleic Acid
ASN	Asparagine
SER	Serine
THR	Threonine
DNA	Deoxyribonucleic Acid
Cyclic AMP	Adenosine 3'5'-cyclic Monophosphate
ACTH	Adrenocorticotropin Hormone
CDP	Cytidine 5'-diphosphate
Dibutryl cAMP	Dibutryl Cyclic Adenosine 3'5'-monophosphate

ABSTRACT

Liver slices from normal rats and those suffering from inflammation for 8-72 h were incubated with L-leucine-^{14}C or D-glucosamine-^{14}C. The contents of albumin and α_1-acid glycoprotein in medium and in extracts of liver were determined using the quantitative precipitin technique. There was a net increase in synthesis of both proteins when slices from control and experimental animals were used, the increase showing up in medium proteins. Slices from livers from experimental rats had a greater capacity for synthesis of α_1-acid glycoprotein and a lower capacity for synthesis of albumin than slices from control rats, the greatest changes occurring with slices from 24 h experimental rats. Changes in synthetic capacities of liver slices from experimental rats for albumin and α_1-acid glycoprotein were always accompanied by large increases in specific radioactivies of total medium proteins when liver slices were incubated with L-leucine-^{3}H and D-glucosamine-^{14}C. It is suggested that the increase in specific radioactivies of medium proteins following incubation of experimental liver slices with labelled precursors is characteristic of the response of liver to inflammation and reflects changes in the synthetic capacity of liver for α_1-acid glycoprotein and other acute phase serum proteins. Liver slices from normal rats were challenged with various compounds in an attempt to induce the acute phase response of liver as seen by the rise in specific radioactivies of total medium proteins. When significant changes in specific radioactivies in total medium proteins were observed, medium and total liver extracts were examined by the quantitative precipitin technique or radial diffusion.

Bradykinin at 8.0×10^{-5} M yielded the most significant increase in specific radioactivies of total medium proteins which was accompanied by a significant increase in medium and tissue-bound α_1-acid glycoprotein; however the content of albumin also increased. Hormones at any concentration did not show the induced changes in α_1-acid glycoprotein and albumin synthesis which are known to accompany the acute phase response and which were observed with experimental liver slices.

INTRODUCTION

Structure of Serum Glycoproteins

Glycoproteins are by definition proteins that contain carbohydrate groups with a relatively low number of sugar residues covalently bound to the polypeptide chain. Glycoproteins constitute approximately 43% of plasma proteins by weight (1); serum albumin is the only major serum protein which is free of carbohydrate (TAPIE 1) (2). Serum glycoproteins vary in carbohydrate content and in relative amounts of the sugars present. Common sugar units are N-acetylglucosamine, N-acetylgalactosamine, galactose, mannose, fucose, xylose, and N-acetylneuraminic acid. Carbohydrates are bonded to the peptide chain in the form of large oligosaccharide units usually *via* the reducing group of N-acetylglucosamine to the amide group of asparagine. Sequence of the sugar units has been determined in some cases by the enzymatic degradation of the glycopeptide with glycosidases. In human α_1-acid glycoprotein, terminal N-acetylneuraminic acid is bonded to galactose or N-acetylglucosamine and carbohydrate branches occur at N-acetylglucosamine or mannose residues. Minor variations occur with serum glycoproteins as to the sequence and location of the carbohydrate in the peptide chain. Human α_1-acid glycoprotein contains about 180 amino acids (3) and five oligosaccharide units; all of which are situated in the N-terminal third of the molecule and attached to asparagine.

TABLE 1

PROPERTIES OF SOME HUMAN PLASMA GLYCOPROTEINS

PROTEIN	MOLECULAR WEIGHT	ISOELECTRIC POINT	SIALIC ACID (g/100g)	TOTAL CARBOHYDRATE (g/100g)	PLASMA CONCENTRATION (mg/100ml)	BIOLOGICAL FUNCTION
PREALBUMIN	61,000	4.7	0	0.5	28-35	BINDS THYROXINE
OROSOMUCOID (α_1-acid glycoprotein)	44,100	2.7	12.1	41.4	75-100	BINDS PROGESTERONE; OTHER FUNCTIONS?
α_1- LIPOPROTEIN (HDL_3)	195,000	5.2	0.3	1.5	217-270	TRANSPORT LIPIDS
α_1-ANTITRYPSIN	45,000	4.0	3.6	12.4	210-500	INHIBITS TRYPSIN AND CHYMOTRYPSIN
TRANSCORTIN			3.2	14.1	7	BINDS CORTISOL
THYROXINE-BINDING GLOBULIN	40-50,000	4.0			1-2	BINDS THYROXINE
HAPTOGLOBIN	100,000	4.1	5.3	19.3	30-190	BINDS HAEMOGLOBIN
CERULOPLASMIN	160,000	4.4	2.4	8.0	27-39	ROLE IN METABOLISM OF COPPER
α_2-MACROGLOBIN	820,000	5.4	1.8	8.4	220-380	ANTI-TRYPSIN ACTIVITY BINDS INSULIN

Biosynthesis of Serum Glycoproteins

Most serum glycoproteins with the exception of the γ-globulins are synthesized exclusively in the liver (4); the microsome fraction of the liver is the subcellular site of synthesis of all serum glycoproteins that have been studied to date (5). Possible mechanisms include: (A) synthesis of the protein and carbohydrate units separately and then combination at the site of formation of the peptide chain i.e. the ribosomes, (B) incorporation of the carbohydrate groups after the peptide chain has left the ribosome and is in the endoplasmic reticulum, (C) combination of the above two possibilities, with part of the carbohydrate incorporated in the ribosome phase and the remainder after release from the ribosome. Serum glycoproteins seem to follow the third possibility as suggested by the "pipeline" scheme of Redman (6) Fig. 1. Sugars are added stepwise to the protein part of the molecule beginning at the ribosome and continuing as the protein passes through its intracellular route. The first sugar, usually N-acetylglucosamine, is added to the incomplete peptide chain during assembly on rough endoplasmic reticulum, subsequent sugars are added during secretion via the channels of the endoplasmic reticulum, and terminal N-acetylneuraminic acid is attached mainly in the Golgi complex. Although albumin is not a glycoprotein it seems to travel the same secretory route and can be located in the rough and smooth endoplasmic reticulum. The synthesis of the oligosaccharide units is controlled by the presence of the appropriate glycosyl transferases which transer sugar

molecules from nucleotide sugar derivatives to the suitable glycoprotein acceptor molecules. The sugar nucleotides can all be derived from glucose; the pathway of their metabolism is shown in figures 2 and 3 (7). The incorporation of carbohydrate onto the peptide chain is not believed to be coded by RNA but seems to be fixed by the amino acid sequence of the polypeptide backbone of the glycoprotein. The amino acid sequence which codes for the attachment of carbohydrate to polypeptide has recently been referred to as the sequon (8). The sequon for the serum type of glycoprotein is almost certainly the sequence ASN - X - SER (THR), a sequence which has been found in the linkage region of many glycoproteins of the serum type. It is likely that other sequons will soon be recognized for glycoproteins containing other types of carbohydrate chains.

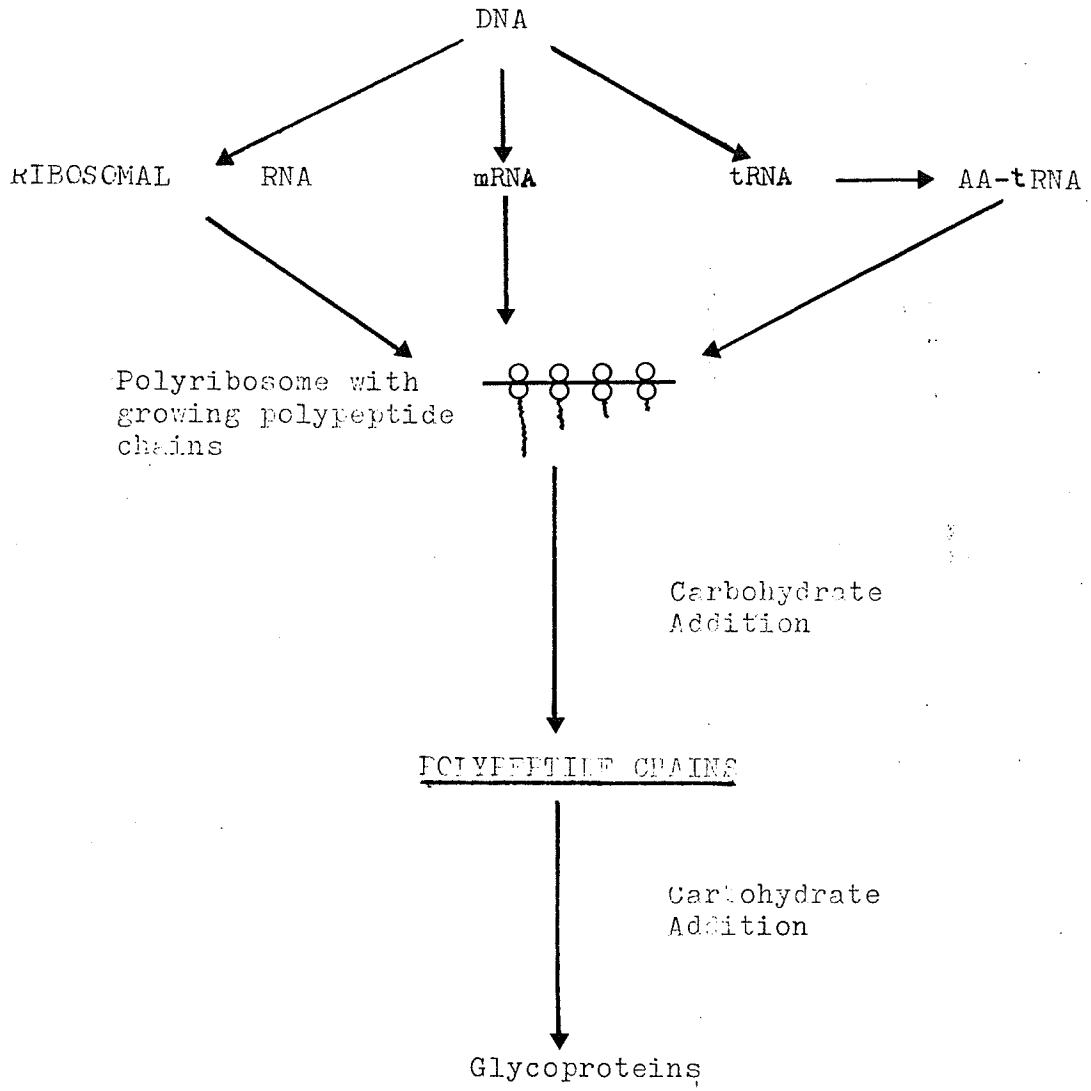
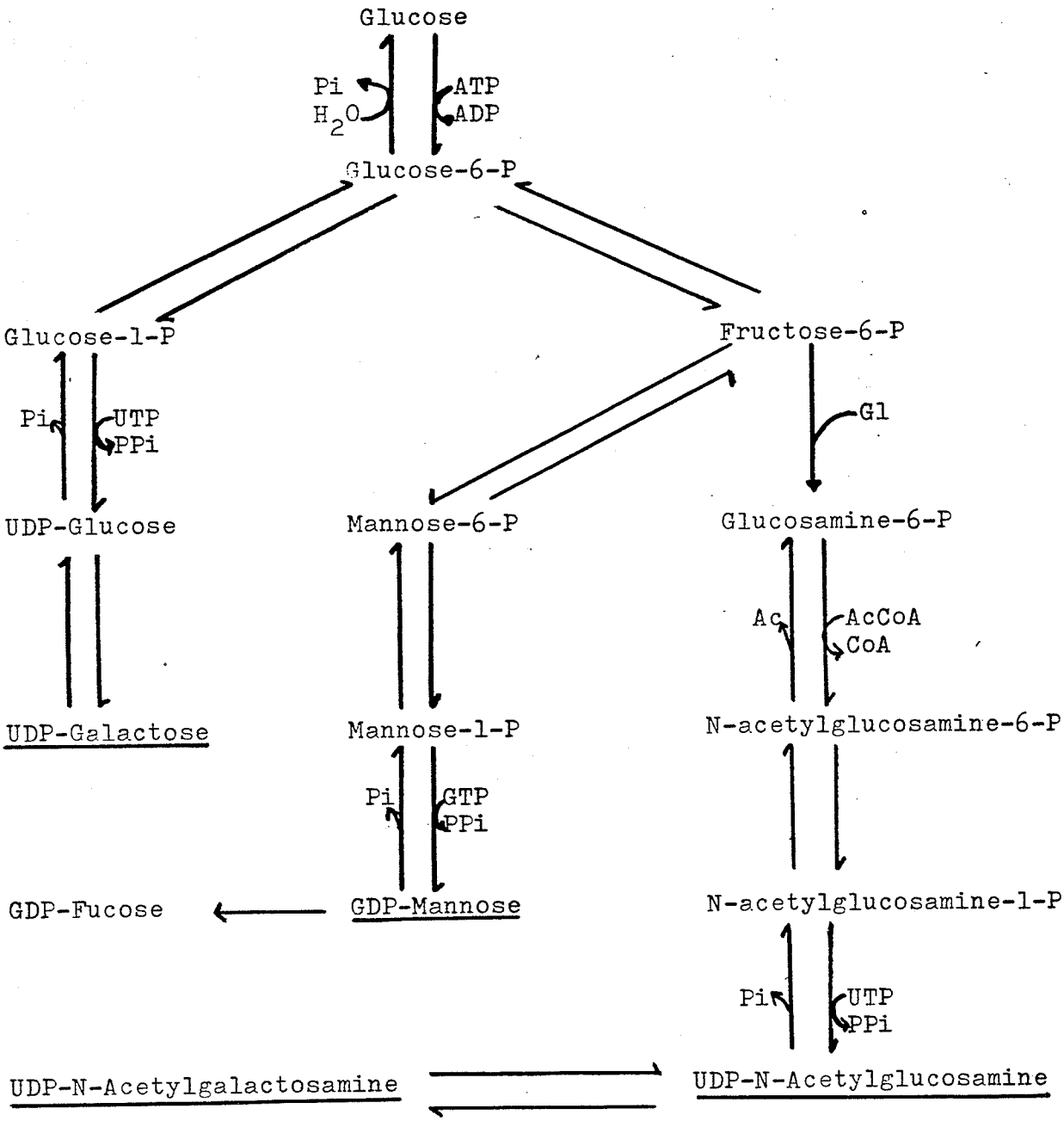


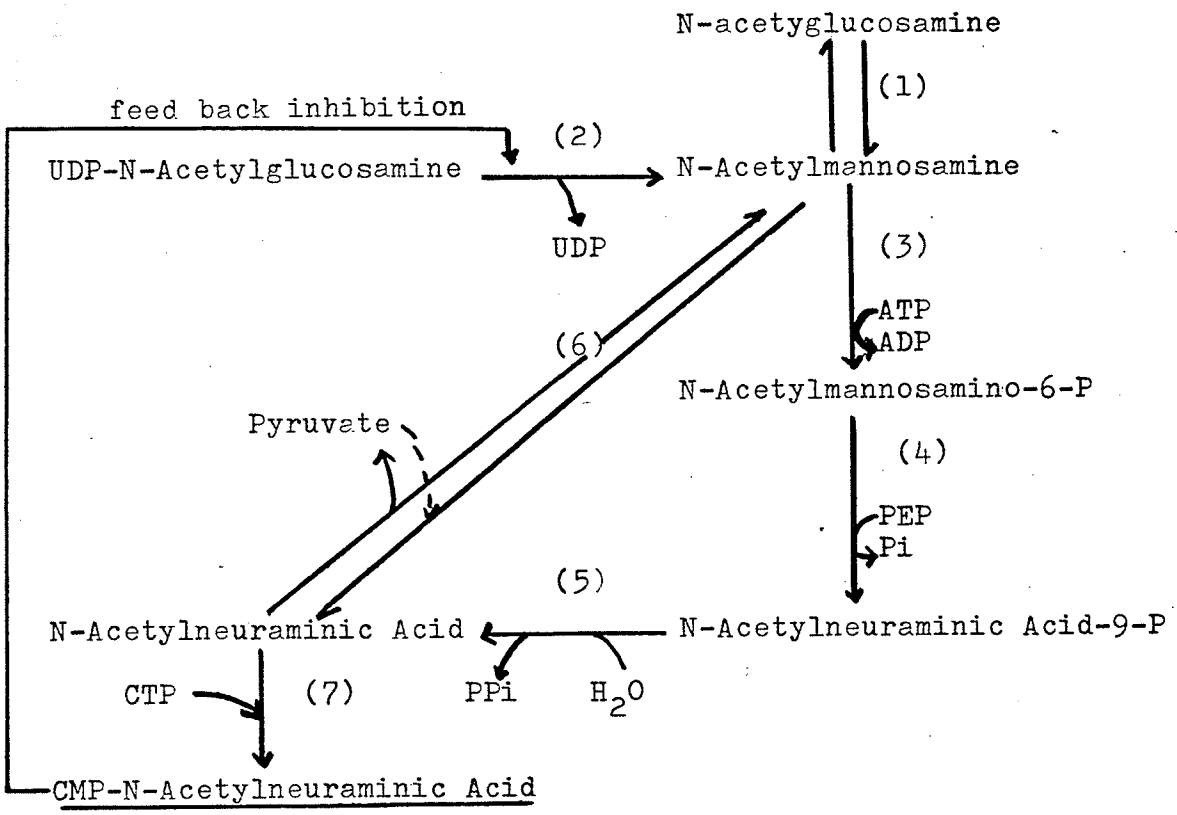
Fig. 1 General sequence for glycoprotein biosynthesis

Fig. 2. Pathways for biosynthesis of nucleotide derivatives of sugars found in glycoproteins



AcCoA	acetylcoenzyme A
CoA	coenzyme A
Pi	inorganic phosphate
PPi	inorganic pyrophosphate
Gl	glutamine
PeP	phospholnolpyruvate

Fig. 3.



Enzymes:

(1) N-acetylglucosamine-2-epimerase
(2) UDP-N-acelylglucosamine -2-epimerase
(3) N-acetylmannosamine kinase
(4) N-acelylneuraminate-9-phosphate synthetase
(5) N-acetylneuraminic acid-9-phosphatase
(6) N-acetylneuraminate pyruvate-lyase
(7) CMP-sialic acid synthetase

The Acute Inflammatory Response

Characteristic changes in plasma protein concentrations are associated with various infectious and inflammatory processes (TABLE 2) (9). Proteins which increase in concentration during the acute inflammatory process are referred to as acute phase reactants. Most of the acute phase reactants contain a significant amount of carbohydrate and are synthesized by liver parenchymal cells. Conditions under which acute phase reactants have been studied include local inflammation (10-12), thermal or mechanical injury (13-14), major surgery (15-16) and bacterial infection (11). Other factors found to influence the acute phase response as well as the difference in stimuli are age, nutritional factors, genetic differences due to sex or strain, and previous history of the subject, i.e. a second stimulation will bring a greater increase than the first. Variations in response of individual acute phase reactants to inflammation is believed to occur in the liver due to differences in the rate of formation or stability of specific m-RNA or indirectly by hormonal control. However, there are conflicting reports concerning the importance of hormones and chemical mediators which may be released at the site of injury, in the induction and regulation of acute phase protein synthesis.

While various methods have been used in the study of the biosynthesis of acute phase proteins, isotope labelling techniques have shown the changes in plasma concentrations of acute phase reactants reflect enhanced synthesis. Measurement of incorporation of precursors labelled with

TABLE 2.

PROTEINS OF HUMAN PLASMA SHOWING ALTERED CONCENTRATION AFTER TRAUMA

CONCENTRATION	PROTEINS	CONCENTRATIONS IN PLASMA (% of Preoperative Values)
Increased	Fibrinogen	> 200
	Haptoglobin	206
	Orosomucoid	> 200
	C-Reactive Protein	> 200
	α_1-Antitrypsin	> 200
	Slow β-globulin	173
	Inter α-globulin	189
	Complement C'3	122
	Caeruloplasmin	124
	Easily precipitable glycoprotein	140
Unchanged	More than 30 other proteins	
Decreased	Thyroxine binding prealbumin	69
	α-Lipoprotein	-
	β-Lipoprotein	77
	Transferrin	78
	Albumin	80

^{3}H or ^{14}C has yielded proof of net synthesis of acute phase reactants *in vivo* in response to experimentally induced inflammation. *In vitro* systems, such as the liver perfusion technique of Miller (17-19) or the use of cell free liver suspensions, have also been effective in investigations of net synthesis of acute phase reactants, however limited work has been done with liver slices, a whole cell *in vitro* system.

The trauma-induced synthesis of plasma glycoproteins in the liver is only part of the acute phase response of an animal to injury. Glenn *et al* (11) proposed a general scheme (Fig. 4) with the "local" events being primarily "destructive" yet necessary for the induction of the "systemic" events which were "protective" and eventually lead to tissue repair. Vasodilation and alterations in blood flow occur initally and are followed by local increases in vascular permeability and the local migration of neutrophil and other leucocyte cells into the area of tissue damage. Contributing to cellular destruction at the site of tissue damage are low molecular weight mediators, such as histamine and lysosomal enzymes. The release of lysosomal enzymes is referred to by Weissman (20) as the "final common pathway" in inflammation and may represent a "trigger" mechanism linking a variety of injuries with increased acute phase reactant synthesis. According to Glenn *et al* the local reaction, by an unknown mechanism, stimulates the systemic response. The approximate time sequence of events in the acute phase protein response in rats is seen in Fig. 5 (21). An intact nervous system

THE "INFLAMMATORY PROCESS"

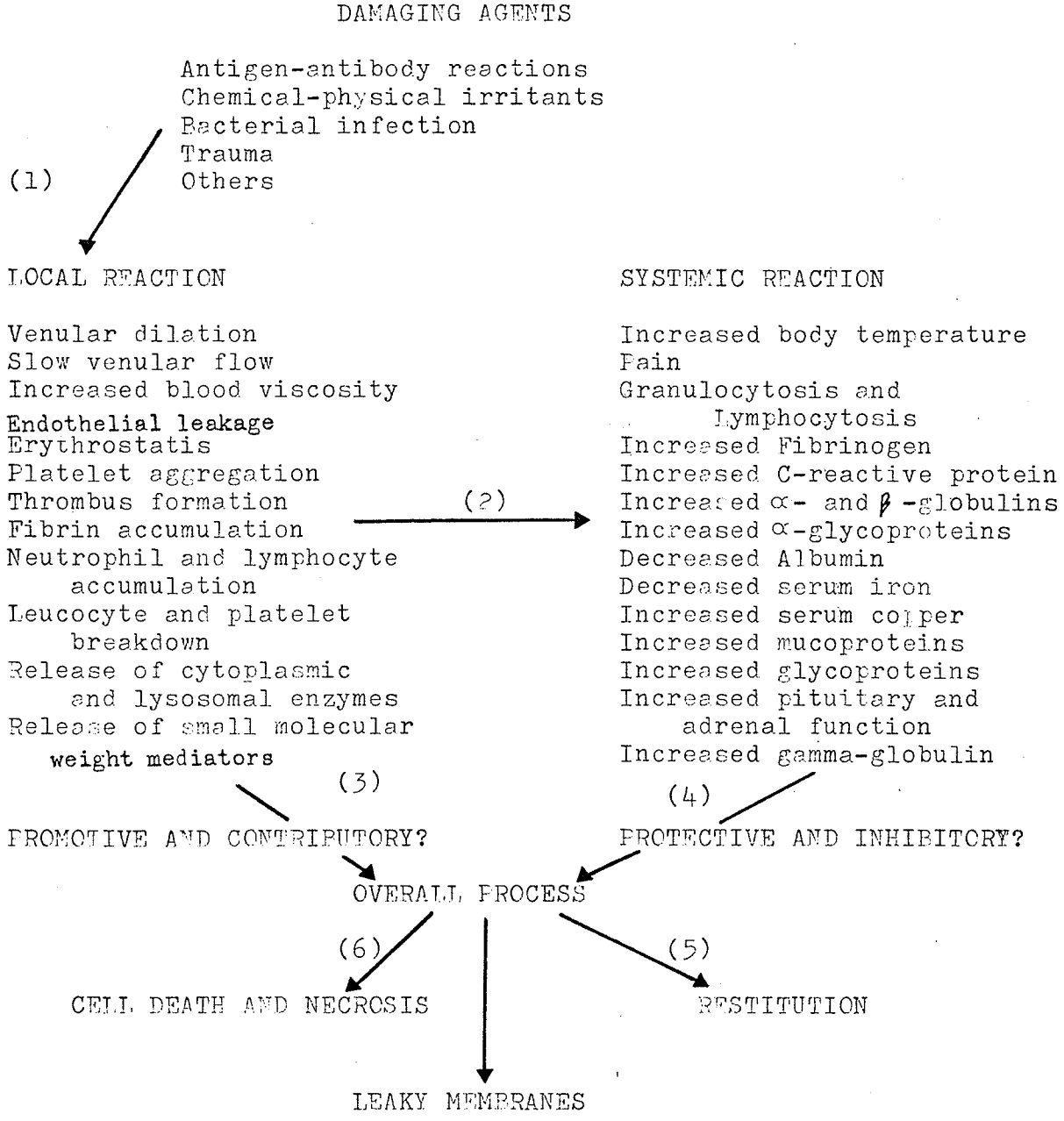


Fig. 4. Schematic representation of the inflammatory process The numbers in parenthesis describe the order in which the events are believed to occur

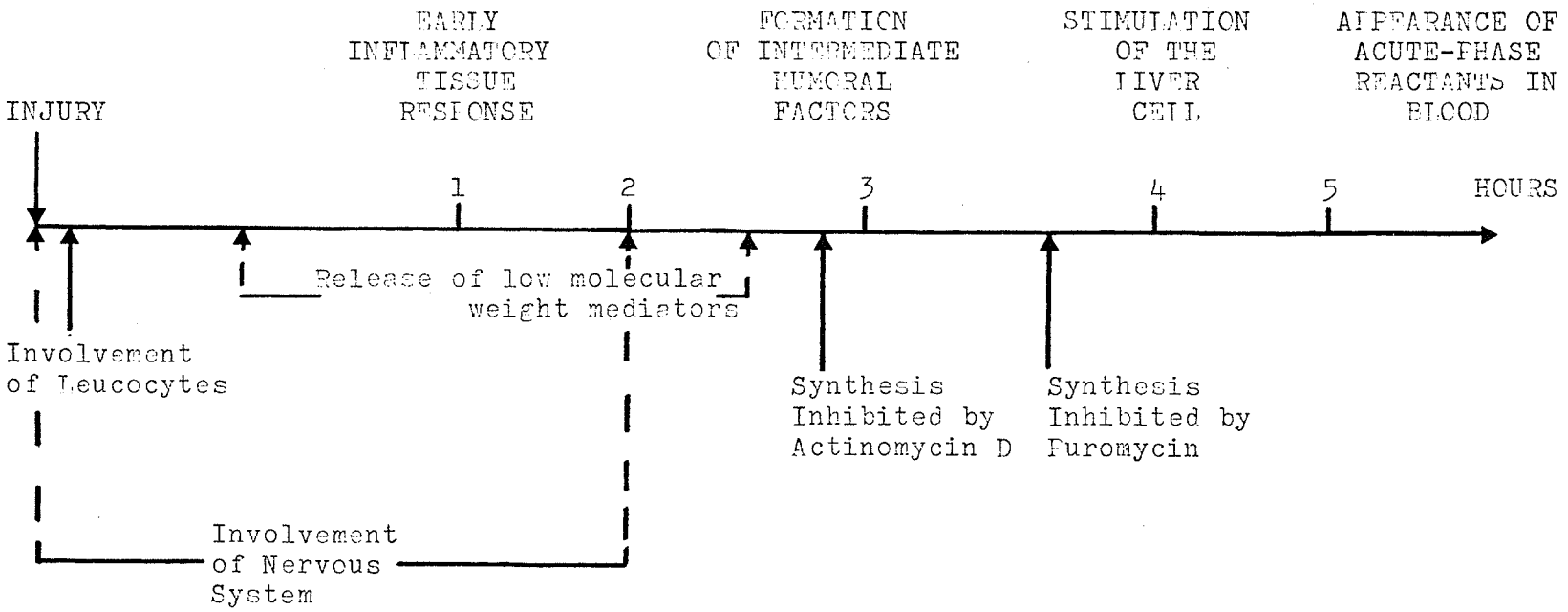


Fig. 5. Time Sequence of Events in the Acute Phase Response in rats.

is necessary for the full development of the early stages of inflammation as the first phase,i.e. (0-2h) can be abolished by denervation or spinal section in rats (22). The importance of intermediate humoral factors linking the site of injury and the liver cell was suggested initially by Homburger (23) and still remains to be elucidated. Stimulative factors have been reported in connective tissue (24), blood of injured animals (18) and several tissue extracts; however, subsequent investigators have failed to confirm the presence of such substances in blood (25-26), and their involvement in the induction of the systemic response either directly or indirectly via hormone release is speculative.

Primarily the systemic response consists of the increased synthesis of fibrinogen, haptoglobin, ceruloplasmin, and α_1-acid glycoprotein and decreased synthesis of albumin and transferrin. New proteins appear in response to trauma as well; the α_2 acute phase globulin in rats, the C-reactive proteins in man, and the C-x protein in rabbits. Stimulation of the liver cell is thought to occur in three steps (27); the initial increase in cellular permeability to amino acids, increased synthesis of m-RNA, and finally the increase in the proportion of polyribosomes which corresponds to the increased incorporation of labelled amino acids into hepatic proteins. The hypothesis that increased biosynthesis of plasma proteins may be due to "recruitment of previously inactive cells" as well as additional stimulation of cells already engaged in the formation of the acute phase proteins has received some support from immunofluorescent studies

(28), but is not widely accepted.

Response of serum glycoproteins to inflammation has been studied by numerous workers. Darcy (29-30) investigated an α_1-acid glycoprotein in rat serum which increased 7 fold during turpentine-induced inflammation and 15-20 fold in Walker tumour-bearing rats. Other workers have isolated a number of acid glycoproteins from rat plasma, which differ in amino acid composition and isoelectric points from human orosomucoid and Darcy's α_1-globulin. Neuhaus et al (27) showed a five fold increase in ^{14}C-glycine incorporation into the seromucoid fraction and decreased incorporation into albumin in response to trauma. The seromucoid fraction contains at least six glycoproteins including haptoglobin and the α_1 globulin of Darcy. Further incorporation studies with Actinomycin D and puromycin demonstrated the de novo synthesis of the α_1 globulin. Actinomycin D acts on protein synthesis indirectly by inhibiting synthesis of new m-RNA at the level of the DNA dependant RNA polymerase, while puromycin inhibits protein synthesis directly at the formation of the polypeptide chain. Injections of Actinomycin D up to 4 h after injury suppressed α_1 globulin synthesis with no effect on albumin synthesis while puromycin inhibited synthesis of both proteins. Control of synthesis by gene activation or derepression with a different half-life for the specific m-RNA associated with each hepatic protein was suggested.

Chemical Mediators of Acute Phase Response

Chemical mediators of acute inflammation satisfy three main criteria;

1) induction by the mediator of some or all signs of inflammation (Fig. 4).

2) release of the substance which is capable of inducing inflammation during an inflammatory reaction.

3) reduction of release by known anti-inflammatory drugs.

The relative importance of chemical mediators in inducing the systemic response is unknown. DiRosa et al (31) proposed, the appearance of chemical mediators in three distinct phases (Fig. 6) in response to carrageenin and turpentine inflammation. Complement was thought to be involved throughout the response.

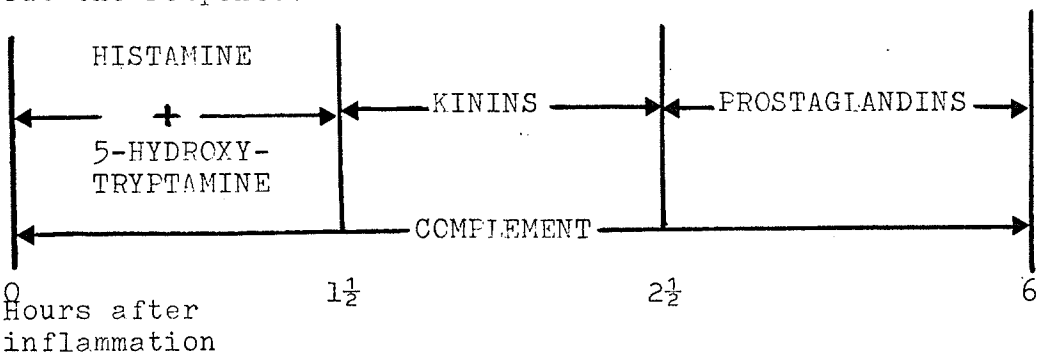
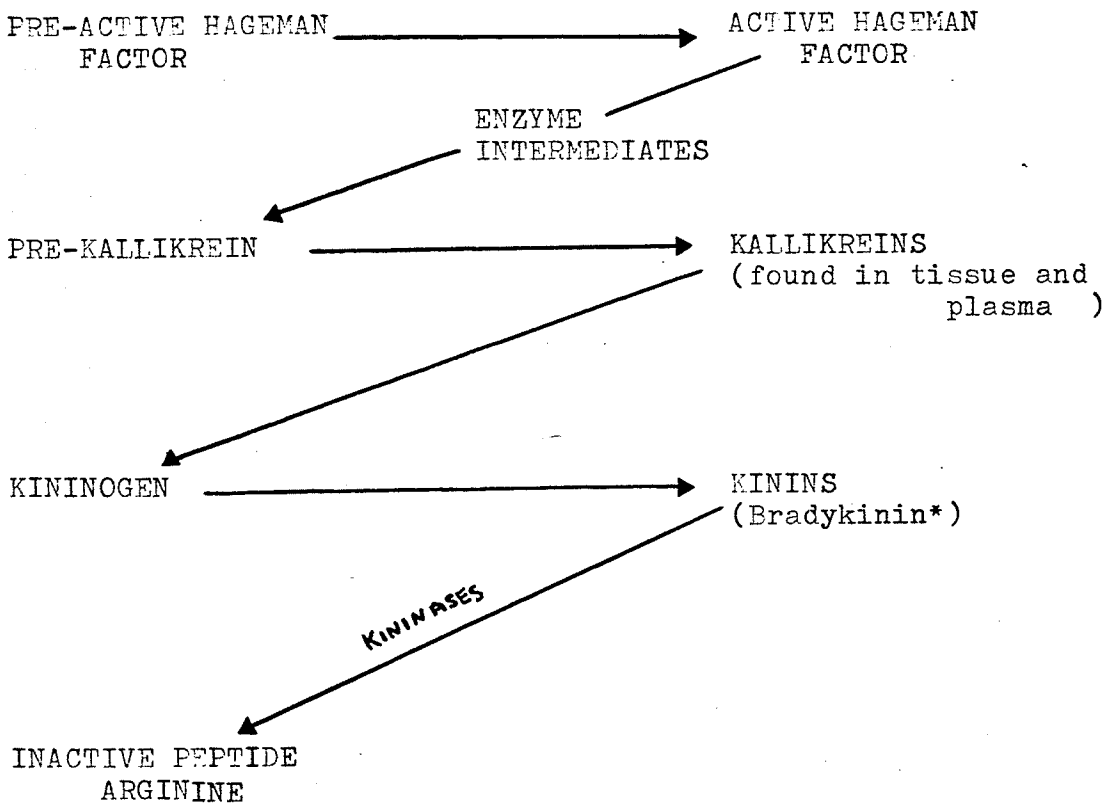


Fig. 6

Initial release of histamine and 5-hydroxytryptamine is simultaneous which could explain the failure of other workers to implicate histamine and 5-hydroxytryptamine as mediators. Histamine may be released from mast cells by polypeptides present in lysosomes or formed by the action of histidine carboxylase. Increased enzyme activity has been shown to accompany local irritation, infection, and trauma (32). Histamine is known to play a major role in allergic type

reactions but it seems to be of minor importance in the early stage of acute inflammation. 5-hydroxytryptamine , an important mediator in mice and rats , can cause release of histamine , and can potentiate bradykinin action in some cases.

Kinins are released from plasma by an entirely endogenous mechanism closely linked to the initiation of blood clotting (Fig. 7). Kinins are a group of straight chain polypeptides with all members of the group exhibiting the nonapeptide sequence of bradykinin (Fig. 7) and differing only in additional residues at the N- or C-terminal. Bradykinin is the most promising kinin to satisfy the criteria suggested above for mediators of the acute inflammatory response. It can produce increased local blood flow, vasodilation, increased vascular permeability, and pain (33). Normal levels in the region of 0.07 ng/ml of bradykinin (34) can be detected in human blood and elevated levels have been reported due to trauma or disease (33,35,36). Kininases which normally inactivate plasma kinins within 15 sec may be inhibited locally by the acid environment of inflamed tissue resulting in variations in normal bradykinin levels. In guinea pigs, administration of alum-precipited antigens caused the level of bradykinin in blood to rise with the peak occurring 1 h after injection and then to return to normal levels (37). Anti-inflammatory drugs can block effects of bradykinin in guinea pigs. Cortisol at physiological levels 4-8 times that in normal animals can inhibit the release of kinins from its substrate (38), but antiserotonin or antihistaminic drugs do not diminish bradykinin



*Amino Acid Sequence of Bradykinin

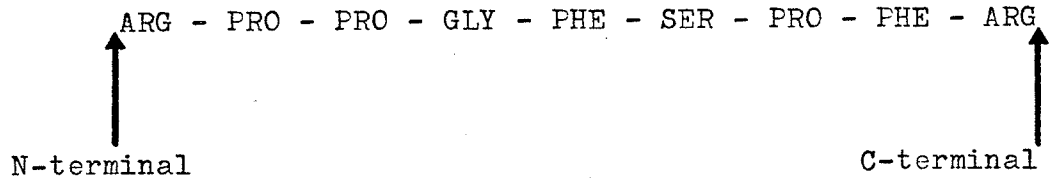
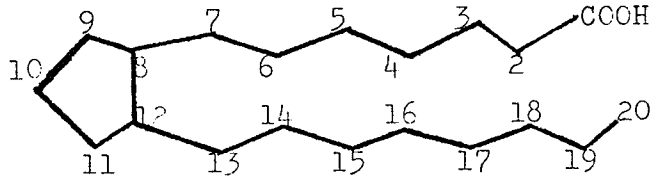


Fig. 7 Scheme for endogenous release of Bradykinin

action. Bradykinin or its metabolities may be taken up by tissues since after infusion of tritiated bradykinin, large amounts of radioactivity were found in kidney and liver (39).

Prostaglandins can be defined as nitrogen-free fatty acids derived from prostanoic acid. The six primary prostaglandins E's and F's (PGE and PGF) differ from each other by the number of double bonds and the substituents in the 9, 11, and 15 positions in the chain.



PROSTANOIC ACID

Fig. 8

Prostaglandins can induce vascular permeability, vasodilation and pain sensitivity associated with inflammation (40). Local concentrations of PGE and PGF compounds are in a precise balance as PGE's are potent vasodilators and PGF's are vasoconstrictors. Tissues do not appear to store prostaglandins therefore release is equivalent to synthesis. Prostaglandin synthesis is inhibited by indomethacin and aspirin in both cell free and single cell systems at the level of prostaglandin synthetase (41, 42). Hydrocortisone is ineffective in inhibition of prostaglandin biosynthesis. Prostaglandins have been implicated in variations in Cyclic AMP levels (43), in regulation of histamine release from mast cells and with increased pain sensitivity to bradykinin (44). PGE_1 increases synthesis and release of corticosteroids and pituitary ACTH in rats

and can reduce release of lysosomal enzymes from human leucocytes. The action of prostaglandins form a paradox ; promoting local immune response and initiation of cell proliferation needed for repair of injured tissue while contributing to vascular permeability and pain production .

HORMONAL REGULATION OF ACUTE PHASE PROTEIN SYNTHESIS

The inflammatory process is self-limiting and its control mechanism may involve a series of responses regulated by the pituitary and the glands controlled by it. Absolute requirements of certain hormones for the expression of the acute phase response were suggested by liver perfusion experiments of Miller and John (19). Normal rat livers were perfused with medium enriched with insulin, cortisol, bovine growth hormone, and an amino acid mixture. Net synthesis of the plasma proteins, fibrinogen, haptoglobin, α_1-acid glycoprotein and α_2 AP globulin increased significantly with up to 10 h of perfusion. Cortisol was found essential for enhanced synthesis. However short-term perfusion studies by Gordon (45) suggested cortisol significantly reduced the synthesis of albumin, fibrinogen, and transferrin. Weimer and Coggshall (45) had shown cortisol plus tissue damage were necessary for α_2 AP globulin synthesis in rats. Cortisol administration in rabbits (47) increased fibrinogen synthesis, but large increases observed may have been due the chronic levels of cortisol used.

Miller and John (19) found the presence of insulin yielded maximal induction of the acute phase proteins. In agreement Neuhaus (48) found reduced incorporation into seromucoid fraction by diabetic rats in response to injury. Insulin was reported to facilitate the association of ribosomes and m-RNA and to stabilize polyribosomes in perfused liver (49). Growth hormone has been reported to

regulate the ^{14}C amino acid incorporation into protein and serum albumin by Korner (50) and to stimulate fibrinogen synthesis 2-3 h after administration (51). Miller's work showed only a minimal effect of growth hormone on albumin synthesis and no effect on the synthesis of the acute phase proteins studied.

Recently Koj (52) using a liver perfusion system and conditions similiar to Miller reported the additional hormones had insignificant effects on lysine incorporation in plasma proteins in normal rats while in turpentine-injected rats, hormones enhanced synthesis. Koj maintains increases observed by Miller after prolonged perfusions were due to effects of the operation procedure; the employed hormones only exerted a moderating influence on an already stimulated liver. Further work with hypophysectomized rats by Griffin and Miller (53) confirmed most acute phase proteins are sensitive to hormones. The exception was α_1 acid glycoprotein which was maximal in liver from hypophysectomized rats and which was not enhanced by hormones. After hypophysectomy α_1 acid glycoprotein levels were three times higher than normal from 2 weeks to 3 months after the operation. Therefore, pituitary hormones and endocrine secretions dependant upon them are not an absolute requirement for α_1 acid glycoprotein synthesis.

While a hormone is a chemical messenger produced in one place and distributed in the blood to distant sites of action, a local hormone can be produced in many locations and acts at the site of synthesis. This arrangement implies a local hormone is at a concentration which would be toxic

if widely distributed. Studies with histamine and bradykinin "local hormones" on regulation of acute phase protein synthesis are limited. Additions of histamine (0.016mM) or bradykinin (.035mM) to rat liver microsomal suspensions significantly decreased incorporation of labelled leucine (54) into seromucoid fraction but results were found to be erratic, presumably due to variable contamination with albumin and perhaps other proteins (54). Histamine did not significantly affect incorporation of ^{14}C glycine into seromucoid using a perfused rat liver system (55).

Other substances found to influence glycoprotein synthesis include phosphoryl choline, CDP-choline, dibutyrl cyclic AMP and Vitamin A. Phosphoryl choline stimulates glycoprotein synthesis in vivo and in a liver slice system (56-57) with the effect being pH and temperature dependant. However stimulation is thought to occur at the level of transfer of sugars on the carbohydrate portion of the glycoprotein due to inhibition of pyrophosphatases which inactivates the UDP-galactose complex. Cyclic AMP can mediate a number of rat liver enzymes and in the form of dibutyrl cyclic AMP has been reported to enhance incorporation of ^{14}C-glucosamine into mucins and intestinal glycoproteins (58). Cyclic AMP levels have been found to influence prostaglandin production but the importance of this relationship is not known (43).

INTRODUCTION TO PRESENT WORK

The initial purpose of biosynthetic work done in this laboratory was to isolate a specific acute phase rat serum protein which could account for a large part of the observed increase in protein-bound carbohydrate in serum resulting from an inflammatory condition. This was accomplished by a process involving quantitative fractionation of serum by stepwise elution from columns of diethylaminoethyl cellulose. The α_1-acid glycoprotein isolated was used in subsequent studies on the effect of experimentally induced inflammation on the mechanism of biosynthesis of a specific rat protein. It contained 34.1% carbohydrate, had a molecular weight of 43000, an isoelectric point of 2.95 and a sedimentation coefficient ($S_{20,W}$) of 3.3. Although the human α_1-acid glycoprotein has a slightly higher carbohydrate content (34-40%) and a lower molecular weight (33000 - 43000) than the rat protein, it was not unreasonable to assume that both proteins would function in a similiar manner during the inflammatory process, thus making a study of the rat α_1-acid glycoprotein particularly important.

The application of a quantitative precipitin technique to serum from control and experimental animals showed that the protein under examination was indeed an acute phase reactant, increasing significantly in content as a result of inflammation. Results involving incorporation of labelled precursors of glycoprotein biosynthesis into α_1-acid glycoprotein *in vivo* suggested the increased content in serum found following inflammation was most likely explained

by an increased rate of synthesis in experimental animals, rather than a decreased rate of catabolism. Serum albumin was also studied since it represented a protein which did not respond to inflammation. The content of α_1-acid glycoprotein in serum increased about six fold at 48-72 h after induced inflammation, whereas the albumin content remained fairly constant, but did show a slight decrease at 24 h .

The acute phase response to induced inflammation is thought to be a complex process which may involve the release of active substances from the site of inflammation. The substances may act directly on the liver cell or indirectly via a series of intermediates released from other tissues or organs causing an increase in the production of acute phase reactants at their site of synthesis, the microsomal fraction of liver. The active substances must gain access to the liver cell to stimulate protein synthesis and synthesis of the oligosaccharide side chains of the α_1-acid glycoprotein. The precise mechanism of this process and the identity of the active substances are unknown and further studies were directed towards gaining this information.

It appeared that about 5 h were required before a significant increase in α_1-acid glycoprotein was detected in microsome material, thus stimulation probably commences within 4-5 h after inflammation is induced. The α_1-acid glycoprotein reached a maximal content in the liver about 12 h after inflammation and was secreted from the liver by way of the rough and smooth endoplasmic reticulum and Golgi complex and showed up as an increased blood content, reaching a maximum at 48 h following

inflammation. No significant changes in the pathway of secretion of α_1-acid glycoprotein were observed in normal rats as compared to inflamed rats. In vivo studies had provided considerable information on the biosynthesis of rat α_1-acid glycoprotein, but they were not suitable for studies on the control factors of biosynthesis. Other organs could be involved which would complicate interpretation of results. Therefore an in vitro liver slice system was developed to study α_1-acid glycoprotein and albumin biosynthesis in livers from normal rats and those suffering from induced inflammation.

It was hoped that the liver slice system would behave in a similiar manner to the liver in vivo with respect to synthesis and secretion of albumin and α_1-acid glycoprotein, namely, livers from experimental animals would have a greater capacity for the synthesis of α_1-acid glycoprotein which is passed into extracellular medium in greater amounts than in controls, and a lower capacity for synthesis of albumin which is passed into the medium in lower amounts than in controls. Utilizing labelled precursors of polypeptide and carbohydrate synthesis, changes in α_1-acid glycoprotein and albumin synthesis in vitro could be determined quantitatively by examination of the content and specific radioactivities of these proteins in intracellular and extracellular form as well as qualitatively by immunodiffusion studies.

The stimulation of synthesis of acute phase reactants such as α_1-acid glycoprotein in response to induced inflammation is thought to be mediated directly by humoral substances released from the site of tissue damage or indirectly by the

release of secondary effectors or hormones from other sites. In an attempt to determine the factors which can influence glycoprotein synthesis *in vitro* and thus, which may be responsible for stimulation of acute phase reactants *in vivo*, the effects of various compounds including hormones, peptides, and possible chemical mediators of the acute phase response were studied on rat liver slices from normal animals. A liver slice system was chosen for these biosynthetic studies as it allowed the observation of more conditions on the same tissue than would be possible with the perfused liver system and without the various complicating factors accompanying the interpretation of *in vivo* studies. Attempts to determine the nature of the factors which are responsible for induction of α_1-acid glycoprotein synthesis and reduction of albumin synthesis in response to injury by examination of possible candidates proved unsuccessful, however information was gained on the effects of various hormones on an *in vitro* whole cell system.

The alternate approach to the problem was to isolate substances from experimental blood, i.e. 2-5 h after inflammation which were not present in normal blood or present at different levels and add these substances to normal slices. This work was attempted by Ellen Morrison and her results are reported in her Master's thesis.

EXPERIMENTAL

Materials

Radioactive Compounds

L-leucine-4,5-^{3}H (1000mCi/m mole), D-glucosamine-1-^{14}C hydrochloride (55 mCi/m mole), Amersham Searle, Toronto, Ontario; Bradykinin-2 [L-Proline-3,4-H^{3}(N)] triacetate (34.2 C/mM), New England Nuclear, Boston, Mass.

Chemicals for liquid 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, Ontario.

Proteins, Peptides and Hormones

Human fraction V1, Pentex Inc., Kankakee, Illinois; Heparin (sodium salt), Nutritional Biochemicals Corp., Cleveland, Ohio; Bradykinin, Peptide Institute Protein Research Foundation, Osaka, Japan. Prostaglandins E_1(PGE_1), E_2(PGE_2) and $F_{2\alpha}$($PGF_{2\alpha}$) were gifts from Upjohn Co., Kalamazoo, Michigan. Crystalline bovine serum albumin, human serum albumin, N^{6},O^{21}-dibutyryl adenosine 3'5'-cyclic monophosphoric acid (monosodium salt), insulin (bovine pancreas crystalline), acetylcholine chloride (crystalline), cytidine 5' diphosphocholine (crystalline monosodium salt), somatatropin (STH), growth hormone, hydrocortisone-21-acetate, phosphoryl choline chloride (calcium salt), histamine (free base) thyroxine (sodium salt) and vasopressin (antidiuretic hormone - synthetic); Sigma Chemical Co., St. Louis, Mo.

Other chemicals were obtained as follows: Agarose, Hoechst Pharmaceuticals Montreal, Quebec; Dextran T70, Pharmacia (Canada) Ltd., Montreal; Lubrol-Wflakes were a gift from Imperial Chemicals

Industries Ltd., Blackley, Manchester, England. Unless otherwise stated other chemicals and reagents were of analytical reagent grade obtained from local suppliers.

Rat albumin and α_1-acid glycoprotein and their corresponding antisera were prepared as previously described (59-61). Human serum was supplied by the Winnipeg Rh Institute; antiserum was prepared as before (59-61).

Physical Methods

Extinctions in the visible region of the spectrum were measured with a Unicam SP 600 spectrophotometer. Measurements of pH were made with a Radiometer Model 286 p H-meter. Measurements of radioactivity were determined with a Packard-Tri-Carb scintillation spectrometer (model 3320). Aqueous solutions of protein (up to .4 ml solution containing 1 mg protein) were added to 10 ml scintillation cocktail containing 0.7% PPO, 0.036% POPOP, 10% BBS-3 and 90% toluene. Mixtures of ^{3}H and ^{14}C were counted simultaneously with red and green channels at pulse height settings of 10-1000 divisions (60% gain) and 100-1000 divisions (4.5% gain) respectively.

Efficiencies 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 55% for ^{3}H and ^{14}C respectively, in the green channel. Quenching was tested for using the machine automatic external standard. With few exceptions the standard deviation of the net count rate was not greater than $\pm$ 5%.

Preparation of pure bradykinin

Bradykinin triacetate (10 mg) from Sigma was dissolved in about 0.5 ml water and streaked on a 40 cm origin on a 46 cm x 57 cm sheet of Whatman 3MM chromatography paper. Chromatography was for 18 h by downward development in freshly prepared n-butanol: acetic acid: water (7:1:2 by vol) at room temperature. Guide strips (2cm) were cut from each side, air-dried, and developed with ninhydrin (.2% in acetone w/v)(62) or with Sakaguchi sprays (63) in order to locate the bradykinin. The region of the chromatogram containing the bradykinin was cut out and eluted with 40 ml water; bradykinin was recovered by freeze-drying and dissolved in a suitable volume of water for use. In some experiments ^{3}H-labelled bradykinin was used as a marker.

Chemical Methods

Protein was determined by the method of Lowry et al (64) but with modified reagents and volumes as described by Miller (65). Crystalline bovine serum albumin was used as standard .

Bradykinin was determined by the Sakaguchi reaction (66) as described by Tomlinson (67) but with modified reagents. The modified reagents were 0.1% α-naphthol in 95% ethanol, 10% NaOH, and sodium hypobromite (0.64ml Br_2 in 100ml 5% NaOH). The following relationship was used to calculate the bradykinin concentration of an unknown solution using arginine (25-200 ug/ml) as a standard.

i.e. ug/ml arginine = x O.D. units

ug/ml bradykinin = y.x O.D. units

The value of y determined with pure bradykinin was y = 2.02.

Incorporation studies with a liver slice system

Male hooded rats of 300-320g body weight were obtained from

North American Laboratory Supplies, Gunton, Manitoba. Rats were maintained on a diet of Purina Laboratory Chow and tap water and were starved for 16 h prior to sacrifice. Inflammation was induced by subcutaneous injection of 0.5 ml oil of turpentine per 100g body weight into the dorso lumbar region (68) 24 h prior to sacrifice. Rats used as controls received injections of sterile 0.15 M NaCl. At the appropriate times the rats were killed by a blow on the head and unless otherwise stated livers were perfused with cold 0.15 M NaCl *via* the portal vein and were then transferred to beakers containing cold 0.15 M NaCl.

Slices were cut by hand by a method similiar to that of Hultin *et al* (69); the pre-cooled aluminium template had a groove 7mm wide and 0.36 mm deep (69,70). Iced 0.15M NaCl served as the medium during preparation of the slices. Slices were transferred to beakers containing a volume of 0.15 M NaCl equal to ten times the wet weight of the slices; the slices were washed at $4^{o}C$ with gentle agitation for 30 min. The NaCl solution was then replaced with an equal volume of iced incubation medium and washed for 15 min; the washing procedure with iced incubation medium was repeated. The incubation medium was as described by Marsh and Drabkin (71) and contained 77mM KCl, 39mM NaCl, 32.5mM $NaHCO_3$, 3.1mM $MgSO_4$, 1.3mM $CaCl_2$, 0.6mM KH_2PO_4, and 25mM glucose; 25mg of penicillin G and streptomycin sulphate were added per liter of medium. Prior to use the medium was saturated with $O_2 + CO_2$ (95:5) and samples of 1-5g wet weight slices were incubated under a constant flow of $O_2 + CO_2$ for 1-10 h at $37^{o}C$ with gentle shaking. Each flask contained 5ml incubation medium per g of slices and 0.1ml of an L-amino acid solution such that the final concentration of each amino acid was approximately twice that present in blood

(72); these concentrations (in μ moles/100ml) were: arginine, 44; aspartic acid, 7.6; threonine, 58; serine, 58; glutamic acid, 38; glycine, 86; alanine, 96; valine, 40; methionine, 14; isoleucine, 18; leucine, 34; tyrosine, 18; phenylalanine, 16; proline, 48; cysteine, 7.6; glutamine, 38; tryptophan, 13.8; histidine, 18; lysine, 96. For qualitative studies in which medium proteins or extracts of subcellular fractions from slices were examined by immunodiffusion, 4μCi L-[^{14}C] leucine or 4μCi D-[^{14}C]-glucosamine were added per gram of slices, and the livers were not perfused prior to preparation of the slices. For quantitative work 10μCi L-leucine -^{3}H and 1μCi D-glucosamine-^{14}C were added per g slices, together with up to 0.2 ml of aqueous solutions of bradykinin or other compounds as described in the Results section. After incubation, samples were chilled and 5 ml of iced 0.15M NaCl containing 20 mM unlabelled leucine and glucosamine were added per g slices. The medium was aspirated and the insoluble material removed by centrifugation at 10000 rpm (13500 gav) for 10 min in a RC2-B centrifuge and stored at -20°C until required for use. For qualitative work liver was subjected to subcellular fractionation (see below) or for quantitative work liver was extracted <u>in toto</u> with Lubrol-W by homogenization as described below.

Extraction of liver slices

A motor driven Potter Elvehjem type homogenizer with a polytetrafluoroethylene pestle was used for all homogenization procedures. A Sorvall RC2-B was used for the preparation of nuclear and mitochondrial fractions from liver slices. A Beckman L5 - 50 with a no. 60Ti angle head was used for all other subcellular fractionation procedures. For the preparation of extracts of subcellular fractions of liver in qualitative work the liver slices were homogenized with 5 volumes 0.25M sucrose for 30s; the pestle was rotated at 2000 rpm and about 17 up and down strokes were accomplished (60). Nuclear and mitochondrial fractions were prepared by centrifuging at 2500 rpm (750gav) for 10 min and 8400 rpm (10000gav) for 10 min, respectively. The fractions were resuspended and gently homogenized in 5ml 0.25M sucrose per g slices used initially, followed by recentrifugation as above. The washing procedure was repeated. Microsome and cell sap fractions were prepared from the 10 000 gav supernatant by centrifuging at 40 000 rpm (113700gav) for 90 min. The soluble cell sap fraction was removed and the microsomal pellet was washed three times with 1.0ml volumes of 0.25M sucrose prior to extraction with Lubrol-W as described below. For direct extraction of liver for quantitative work, slices were suspended in ten times their volume of 1% Lubrol-W and homogenized as described above. The Lubrol-W extracts were centrifuged at 40000 rpm (113700gav) for 90 min to remove any insoluble material.

Isolation of Protein for Measurement of Radioactivity

Following labelling with L-leucine-H^3 and D-glucosamine-1-C^{14}, protein was precipitated from medium and total liver homogenates by the addition of an equal volume of iced 10%(W/V) trichloroacetic acid containing 10 mM L-leucine (73) and 20mM D-glucosamine (70) hydrochloride. After standing for 10 min at room temperature precipitates were collected by centrifuging at 2000 rpm and then washed with 5%(W/V) trichloroacetic acid. Protein was then washed with 2.0ml of the following reagents; once with acetone 0.1M NaCl (4:1) (74), twice with ethanol/ether/chloroform (2:2:1) by volume, the first at 50°C for 15 min and the second at room temperature for 15 min and finally twice with ether. All protein samples were dried in air and dissolved in 0.2N NaOH and suitable aliquots were removed for determination of protein and radioactivity.

Immunological Methods

For qualitative studies Lubrol extracts of subcellular fractions were concentrated twelve-fold with concurrent dialysis (68) against two changes of 0.15M NaCl containing 1% Lubrol; 10mM carrier leucine or glucosamine, as appropriate, were present in the initial dialysis solution, medium samples were treated in an identical way except that samples were concentrated twenty-fold and Lubrol-W was omitted from the dialysis solution. For quantitative studies the medium samples were adjusted to 1% with respect to Lubrol-W and were concentrated ten-fold by ultrafiltration against 0.15M NaCl containing 1% Lubrol-W, 10mM carrier leucine and glucosamine, as appropriate, and then against two changes of

0.15M NaCl containing 1% Lubrol-W. The Lubrol extracts of total liver were concentrated ten fold as the medium samples.

Medium samples and Lubrol-W extracts of subcellular fractions were examined qualitatively by double diffusion analysis coupled with radioautography as previously described (75); medium samples were also examined by immunoelectrophoresis (59) followed by radioautography.

For quantitative work, albumin and α_1-acid glycoprotein in medium and extracts of total liver were determined by radial immunodiffusion in antibody containing gels or by the quantitative precipitin technique. The quantitative precipitin technique was a modification of the original method of Simkin and Jamieson (70). Mixtures were prepared (total volume 0.45ml) containing 0.05-0.2ml medium samples, 0.2-0.35ml extracts of liver slices or up to 100 ug albumin or 40ug α_1-acid glycoprotein; 0.15M NaCl, 1mM sodium azide and 4.7% Dextran T70.

For medium and total liver extracts human albumin or human fraction VI and antihuman serum were added and the mixtures were incubated at 37°C for 45 min and allowed to stand for 24 h at 2°C. The precipitates were removed by centrifuging at 2000 rpm for 10 min and the above precipitation with the human systems was repeated. The supernatants were incubated with the appropriate antiserum to albumin or α_1-acid glycoprotein and allowed to stand 48 h at 2°C. The precipitates which formed were collected as mentioned above and washed with 0.3ml 0.15M NaCl containing 4% Dextran T70 and then three times with 0.3ml 0.15M NaCl. Precipitates were dissolved in 0.5ml of 0.1N NaOH and suitable volumes were removed for determination

of protein and radioactivity (73).

When a rapid quantitative method for albumin was required without information on specific radioactivities, radial immunodiffusion was employed. The method was based on that of Mancini et al (76). Gels were 1.0-1.5mm thick and contained 1% antiserum to albumin, 1% agarose (W/V) in barbiturate buffer pH 8.6 (16mM sodium diethylbarbiturate, 18mM sodium acetate, 2.2mM H Cl). Wells of 4mm diameter were cut in the gel and 10 μl volumes of standard solutions of rat albumin (25-150ug/ml) or concentrated medium or total liver extracts were added and diffusion allowed to take place for 18 hours. The diameter of the precipitin rings was measured and the albumin in the unknown was determined from the plot relating the diameter of the ring to the logarithm of the albumin concentration in the standard solution (77). α_1-acid glycoprotein could not be effectively determined as most samples contained less than 25 ug/ml.

RESULTS

Immunodiffusion Studies on the Biosynthesis of Albumin and α_1-acid Glycoprotein

Immunological studies were done mainly by Ellen Morrison in connection with her work on quantitative albumin and α_1-acid glycoprotein synthesized by liver slices and are reported briefly below for sake of completeness . Previous *in vivo* studies have shown that the components of the microsome fraction of liver were the site of synthesis of the polypeptide chains of rat serum albumin and the polypeptide and carbohydrate chains of α_1-acid glycoprotein (75). These studies involved reaction of extracts of various liver subcellular fractions with appropriate antiserum after labelling *in vivo* with ^{14}C leucine or glucosamine; radioautography was subsequently employed to detect radioactivity in precipitin lines. This procedure was applied by Ellen Morrison to medium proteins and to subcellular fractions from liver in experiments in which liver slices from normal rats and those suffering from inflammation for 24-48 h were incubated for 2-4 h with ^{14}C leucine, a precursor of polypeptide synthesis or ^{14}C glucosamine, a precursor of carbohydrate synthesis. Immunodiffusion in agar gels with monospecific antisera to rat albumin and rat α_1-acid glycoprotein was used to detect albumin and α_1-acid glycoprotein, respectively, and coupled with radioautography the observed labelled precipitin lines indicated the presence of newly synthesized proteins.

Following incubation with labelled amino acid and glucosamine, the Lubrol-W extracts of liver microsome material yielded strongly labelled precipitin lines on reaction with antiserum to

α_1-acid glycoprotein. Lubrol extracts of microsome material also yielded strongly labelled precipitin lines on reaction with antiserum to serum albumin after amino acid incorporation, with little or no labelling following incubation with glucosamine confirming earlier in vivo studies. These immunodiffusion studies indicated that liver slices from normal rats and those suffering from inflammation were capable of incorporating labelled precursors into α_1-acid glycoprotein and albumin synthesized in the microsome fraction of the liver. Further studies were carried out to determine if labelled albumin and α_1-acid glycoprotein could be secreted into the extracellular medium as occurs in vivo (75,78). Following labelling with ^{14}C leucine or glucosamine medium proteins were examined by immunodiffusion coupled with radioautography. Immunoelectrophoresis was used to check that the albumin and α_1-acid glycoprotein present in medium were native serum proteins and not intracellular percursors leached from the liver cell during the incubation. In all cases labelled proteins had identical electrophoretic properties as compared to the appropriate serum proteins. The presence of puromycin at a concentration of $10^{-4}M$ in the incubation of liver slices abolished labelling of precipitin lines in all cases except for faint labelling found in Lubrol extracts of microsome material upon reaction with antiserum to α_1-acid glycoprotein following ^{14}C glucosamine incorporation.

Determination of Contents of Albumin and α_1-acid Glycoprotein in Total Liver and Medium Proteins following Incubation of Liver Slices from Control and Experimental Animals.

Immunodiffusion studies described above indicated that liver slices from control and experimental animals were capable of secreting native labelled serum albumin and α_1-acid glycoprotein into the incubation medium. These studies could not confirm if increased net synthesis of both proteins occurred or if liver slices from experimental animals could manufacture a greater amount of α_1-acid glycoprotein than liver slices from control animals as occurs in vivo (75). The quantitative precipitin technique was used in all quantitative assays and was applied to medium and Lubrol extracts of total liver; the latter were examined because immunodiffusion studies had shown the presence of some labelled albumin and α_1-acid glycoprotein in subcellular fractions of liver other than the microsome fraction.

The contents of albumin (Fig. 9) and α_1-acid glycoprotein (Fig. 10) in medium increased with the length of incubation with control and experimental slices. In experiments with livers from 8,12, and 24 h experimental rats, the content of α_1-acid glycoprotein in medium was significantly greater when compared to control slices. Experimental liver slices from animals 48 and 72 h after induced inflammation yielded results similiar to or slightly greater than the control values. In contrast, control slices yielded the highest content of serum albumin in medium with decreasing amounts found with slices from 8,12, and 24 h experimental animals. Results from 48-72 h experimental animals were again similiar to control values.

Experimental slices from rats up to 24 h after inflammation appeared to secrete greater amounts of α_1-acid glycoprotein and lesser amounts of albumin into medium than control slices. Since previous in vivo studies had shown that livers from experimental animals contained a higher content of α_1-acid glycoprotein and a lower content of albumin (75), the possibility exists that the observed changes in medium albumin and α_1-acid glycoprotein were due to the leaching out of proteins already present in differing amounts in tissue-bound form at the start of the experiment rather than changes in the new synthesis of the proteins in question. In order to show that increased medium albumin and α_1-acid glycoprotein was due to increased net synthesis of these two proteins, the content of albumin and α_1-acid glycoprotein in tissue-bound form in liver and soluble form in medium were determined following various times of incubation. The results are presented in detail in TABLES 3 and 4 for slices from experiments with control and 24 h experimental rats. There was a definite increase in medium albumin and α_1-acid glycoprotein as seen in Fig. 9 and 10 while there was little or no change in the content of the tissue-bound proteins present in the liver resulting in a new increase of the proteins isolated. Rates of synthesis of both proteins are also shown in TABLES 3 and 4; the decrease observed with increasing times of incubation indicates a gradual loss in the synthetic capacity of the liver slices for both proteins. Contents of tissue-bound albumin and α_1-acid glycoprotein in control and 8,12,24,48, and 72 h experimental slices and the rates of synthesis of these proteins are seen in Fig. 11. The rates of synthesis presented

here are the means of individual rates of synthesis calculated for the 1,2,3,4 and 6 h incubations. From Fig. 11 it can be seen that the slices have the greatest capacity for synthesis of α_1-acid glycoprotein and the lowest capacity for synthesis of albumin when prepared from livers from rats suffering from inflammation for 24 h.

In all the above studies the quantitative precipitin technique was used to isolate albumin and α_1-acid glycoprotein from liver extracts or medium; samples were pre-precipitated twice with a heterologous immune system to remove any non-specific precipitating material which could precipitate with immune complexes formed by the rat proteins. The use of two heterlogous systems has been discussed earlier (75,78). It was shown in control experiments using dansyl labelled albumin or α_1-acid glycoprotein that there was about a 15% loss of both proteins from total liver extracts, but a negligible loss from medium proteins when pre-precipitation was used. The values in TABLES 3 and 4 are corrected for this 15% loss. Radioactivity loss due to pre-precipitation was less than 10% of the values obtained with rat immune systems.

Fig. 9.

Effect of time of incubation on content of albumin in medium from experiments with liver slices from - O -, control rats and - ▲ -, 8 h; - □ -, 12 h; and - △ -, 24 h; experimental rats. Each point represents the mean from 3-6 experiments.

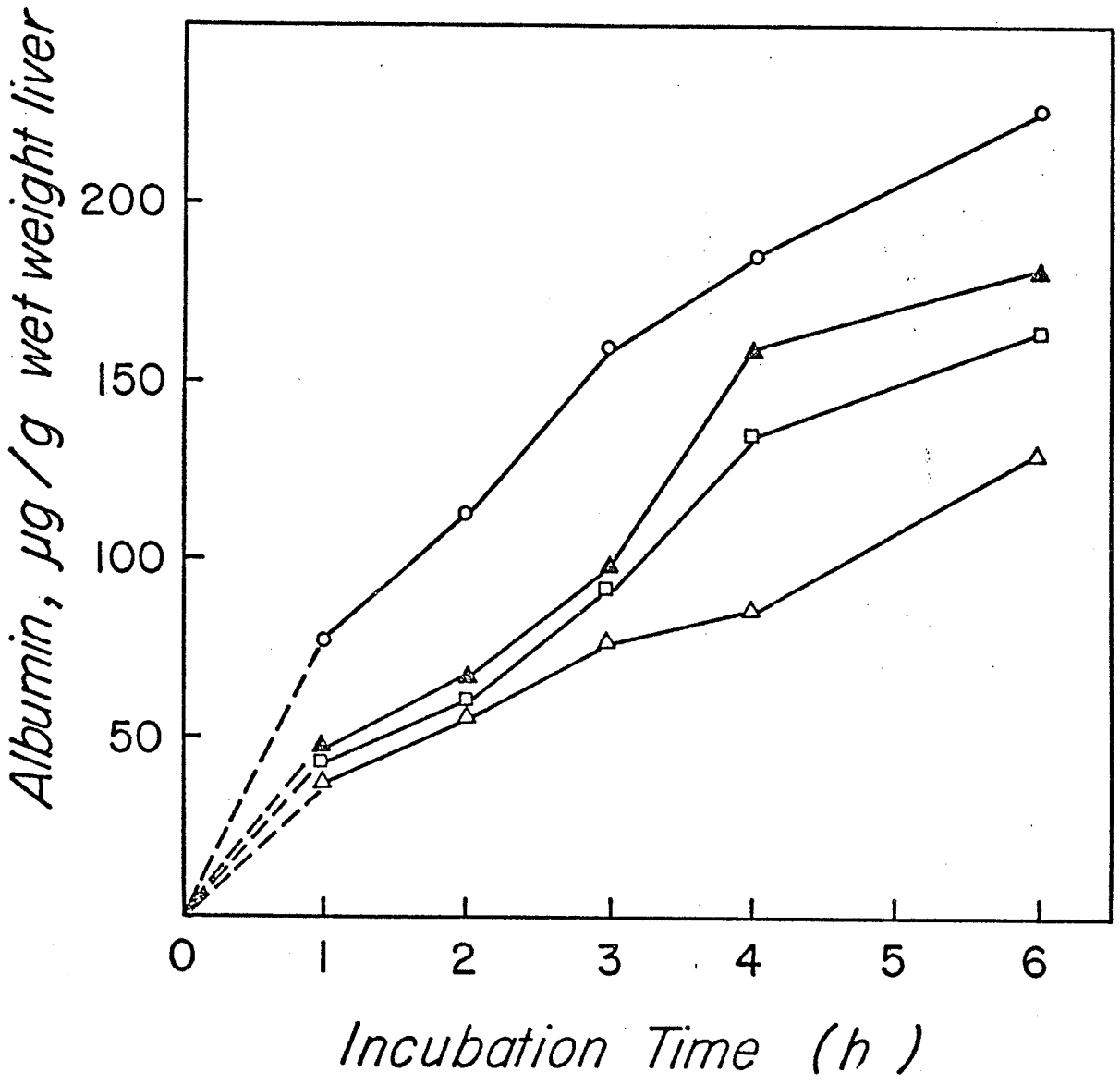

Albumin, µg/g wet weight liver
200
150
100
50
0
1
2
3
4
5
6
Incubation Time (h)

Fig. 10

Effect of time of incubation on content of α_1-acid glycoprotein in medium from experiments with liver slices from - O - control rats and - ▲ -, 8 h ; - □ -, 12 h ; and - △ -, 24 h experimental rats. Each point represents the mean from 3-6 experiments.

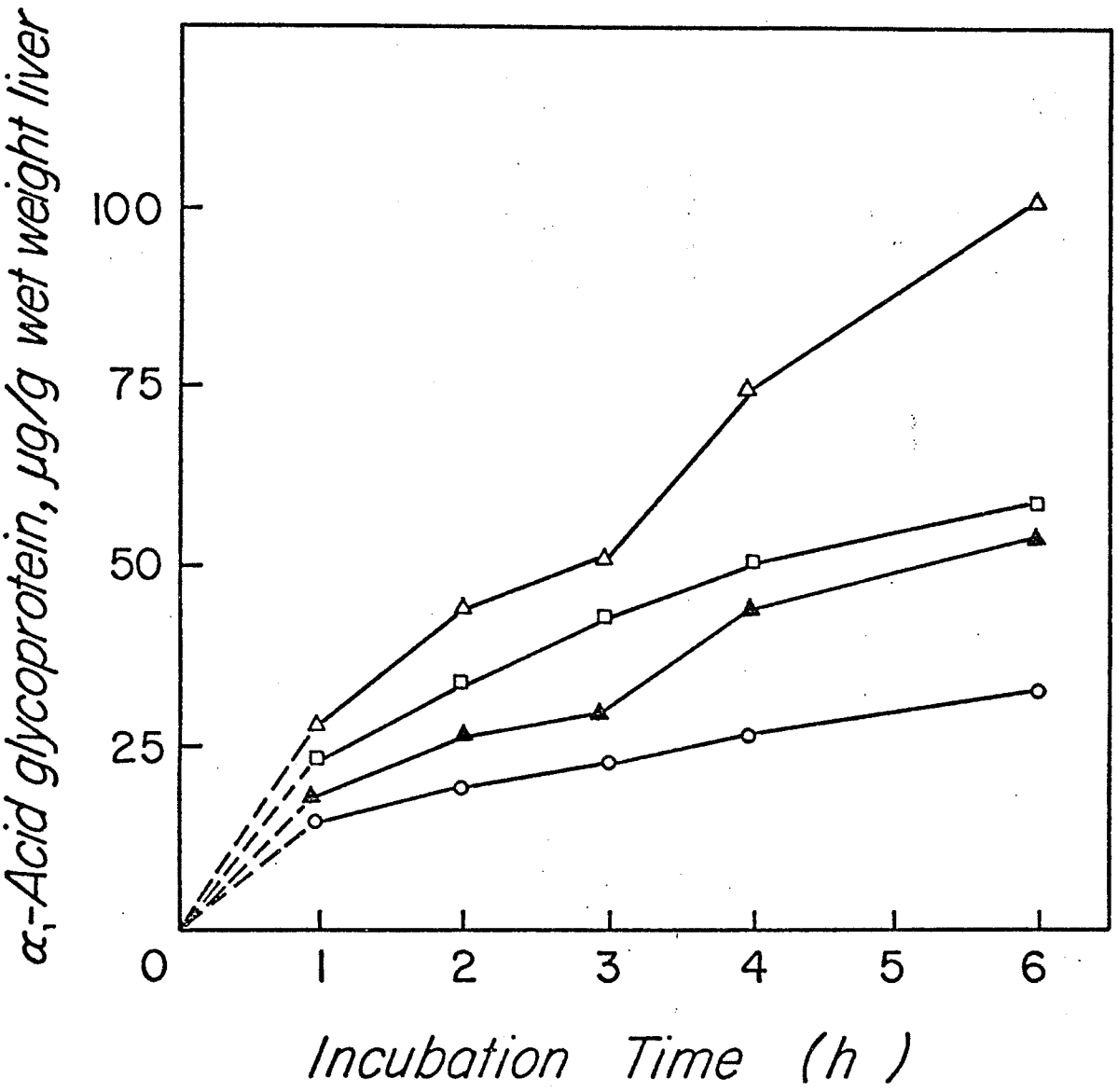

α1-Acid glycoprotein, μg/g wet weight liver
100
75
50
25
0
1
2
3
4
5
6
Incubation Time (h)

TABLE 3

Albumin in Liver and Medium from Control and 24 h Experimental Rats

	CONTROL RATS (6)*					24 H. EXPERIMENTAL RATS (4)*				
Incubation Time h	Liver µg	Medium µg	Total µg	Net Increase µg	Rate of Synthesis µg/g liver/h	Liver µg	Medium µg	Total µg	Net Increase µg	Rate of Synthesis µg/g liver/h
0	323±11	-	323	-	-	244±15	-	244	-	-
1	311±12	78±6	389	66	66	241±12	38±4	279	35	35
2	315±19	112±14	427	104	52	246±17	55±6	301	57	29
3	313±18	170±12	483	160	53	256±16	80±8	336	92	31
4	318±16	184±12	502	179	45	250±19	88±8	338	94	24
6	319±15	225±19	544	221	36	248±16	130±11	378	134	22

* Results are expressed as mean values ± standard errors of the mean; 2 g liver slices were used per flask, but results are given on the basis of 1 g slices; the number of experiments performed is given in parentheses.

TABLE 4

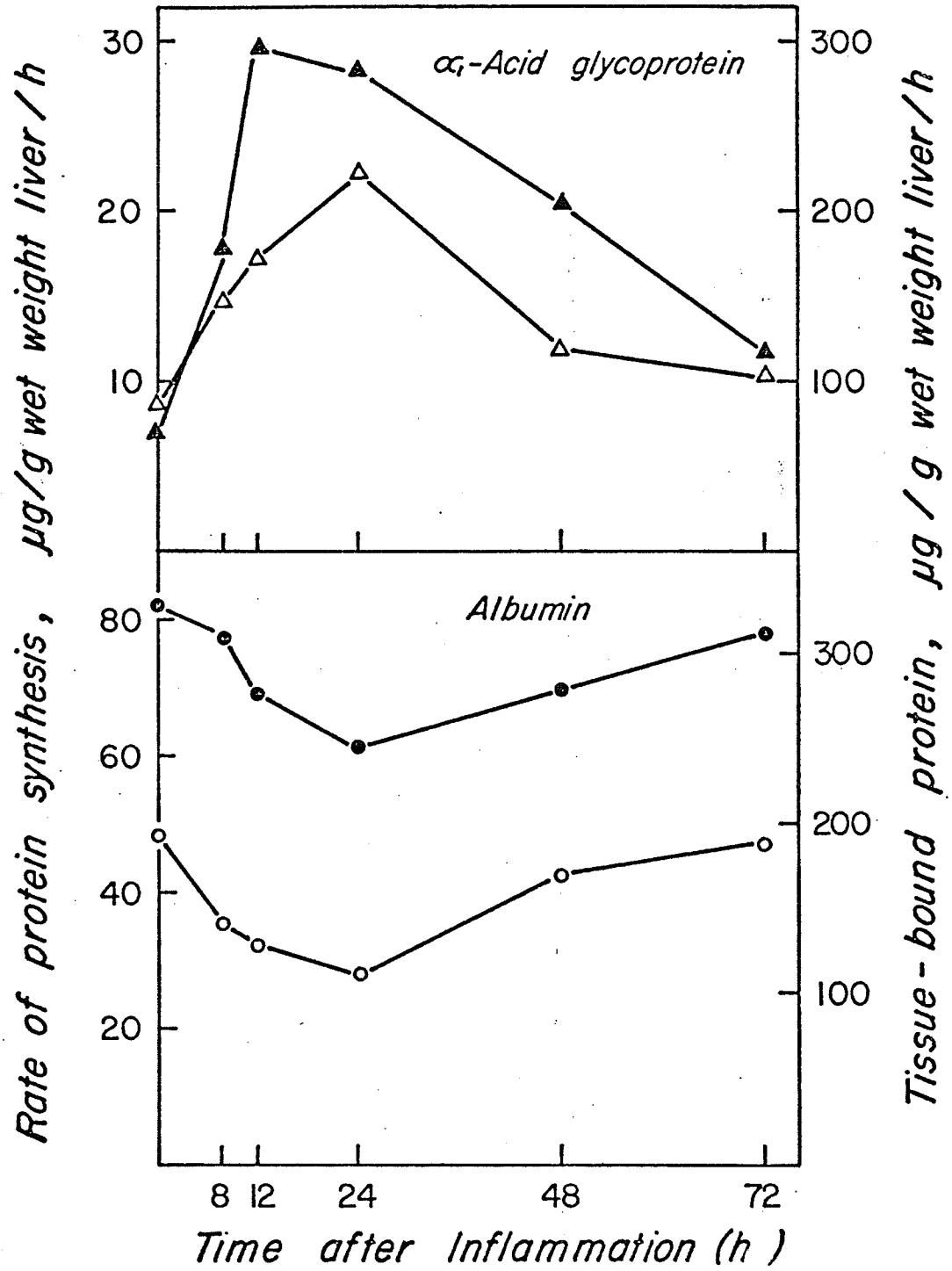
α_1-Acid Glycoprotein in Liver and Medium from Control and 24 h Experimental Rats*.

	CONTROL RATS (6)*					24 H EXPERIMENTAL RATS (4)*				
Incubation Time H	Liver µg	Medium µg	Total µg	Net Increase µg	Rate of synthesis µg/g liver/H	Liver µg	Medium µg	Total µg	Net Increase µg	Rate of synthesi µg/g liver H
0	78±8	-	78	-	-	290±18	-	290	-	-
1	77±6	15±2	92	14	14	287±15	28±3	315	25	25
2	80±7	20±2	100	22	11	289±14	41±4	330	40	20
3	81±7	24±3	105	27	9	308±19	56±4	363	73	25
4	79±6	27±3	106	28	7	305±15	75±7	380	90	22
6	77±7	35±4	112	34	6	300±20	100±8	400	110	18

*Results are expressed as mean values ± standard errors of the mean; 2 g liver slices were used per flask, but results are given on the basis of 1 g slices; the number of experiments performed is given in parentheses.

Fig. 11

Effect of time after induction of inflammation on content of - ● - albumin and - ▲ - α_1-acid glycoprotein in tissue-bound form in liver and the mean rates of synthesis of - O - albumin and - Δ - α_1-acid glycoprotein by liver slices.


α1-Acid glycoprotein
Albumin
Rate of protein synthesis, µg/g wet weight liver/h
Tissue-bound protein, µg/g wet weight liver/h
30
20
10
300
200
100
80
60
40
20
300
200
100
8
12
24
48
72
Time after Inflammation (h)

Incorporation of labelled precursors into Total Liver and Medium Proteins and into Albumin and α_1-Acid Glycoprotein from Medium and Total Liver from Control and Experimental Animals

Previous in vivo studies had shown a two fold increase in specific radioactivities of ^3H leucine and ^{14}C glucosamine in α_1-acid glycoprotein isolated from liver microsome material from experimental animals as compared to controls. To determine if any changes in specific radioactivities occurred during synthesis of α_1-acid glycoprotein and albumin with liver slices, ^3H leucine and ^{14}C glucosamine were added to the incubation flasks at the start of the experiment. Tissue-bound and soluble albumin and α_1-acid glycoprotein and total medium and liver proteins were isolated at various times of incubation and specific radioactivities of ^3H and ^{14}C were determined. An increase in the specific radioactivities of ^3H leucine and ^{14}C glucosamine in α_1-acid glycoprotein isolated from medium and total liver extracts was observed with increasing incubation time (Fig. 12). Increased specific radioactivities were also observed in α_1-acid glycoprotein isolated from medium and total liver from experimental animals when compared to controls; liver slices from 24 h experimental animals showed the largest increases in specific radioactivities. The specific radioactivity of ^3H leucine in albumin isolated from medium and total liver extracts also increased with incubation (Fig. 13), but values for tissue-bound and medium albumin were lower in experimental slices when compared to controls. Specific radioactivities of ^3H and ^{14}C in α_1-acid

glycoprotein and ^{3}H in albumin isolated from total liver extracts were lower than specific radioactivities of the labelled proteins isolated from medium (Fig. 12 and 13).

These results indicate that there are significant changes in the incorporation of labelled precursors into α_1-acid glycoprotein and albumin in liver slices from experimental animals when compared to controls. However, there were large differences in specific radioactivities of ^{3}H and ^{14}C incorporated into total medium proteins. With 6 h incubation there was about a three fold increase in specific radioactivity of ^{3}H leucine and ^{14}C glucosamine with 24 h experimental slices as compared to controls (TABLE 5). Intermediate increases in specific radioactivity of medium proteins were also found with liver slices 8 and 12 h, while 48 and 72 h experimental slices (not shown) were only slightly greater than controls. Increases in specific radioactivities of ^{3}H and ^{14}C in total liver proteins from experimental slices were not as significant as those found with medium proteins. These results are also shown in TABLE 5 in terms of a ratio of specific radioactivities of medium and total liver proteins isolated from experimental liver slices to specific radioactivities of the corresponding proteins isolated from control liver slices. This ratio represents the change in the amount of labelled precursor incorporated into proteins isolated from experimental liver slices when compared with the amount of the same labelled precursor incorporated with control slices for the same time of incubation. An increase in this ratio indicates increased incorporation, while a value less than one indicates decreased incorporation as compared to controls. Increased

synthesis of α_1-acid glycoprotein was shown to be accompanied by large increases in specific radioactivities of labelled precursors in total medium proteins, therefore an increase in specific radioactivity of total medium proteins or a ratio value greater than one indicates the increase in highly labelled secretable proteins present. This observation is considered to be a characteristic response of liver during the acute phase reaction and is used in subsequent studies as a crude indication that liver is synthesizing more α_1-acid glycoprotein when challenged with various hormones.

Fig. 12

Effect of incubation on specific radioactivities of L-leucine-^{3}H and D-glucosamine-^{14}C in medium and liver α_1-acid glycoprotein from experiments with liver slices from control and 24 h experimental rats: -O- medium and - ● - liver α_1-acid glycoprotein from control rats; - Δ - medium and - ▲ - liver α_1-acid glycoprotein from 24 h experimental rats. Each point represents the mean from three experiments.

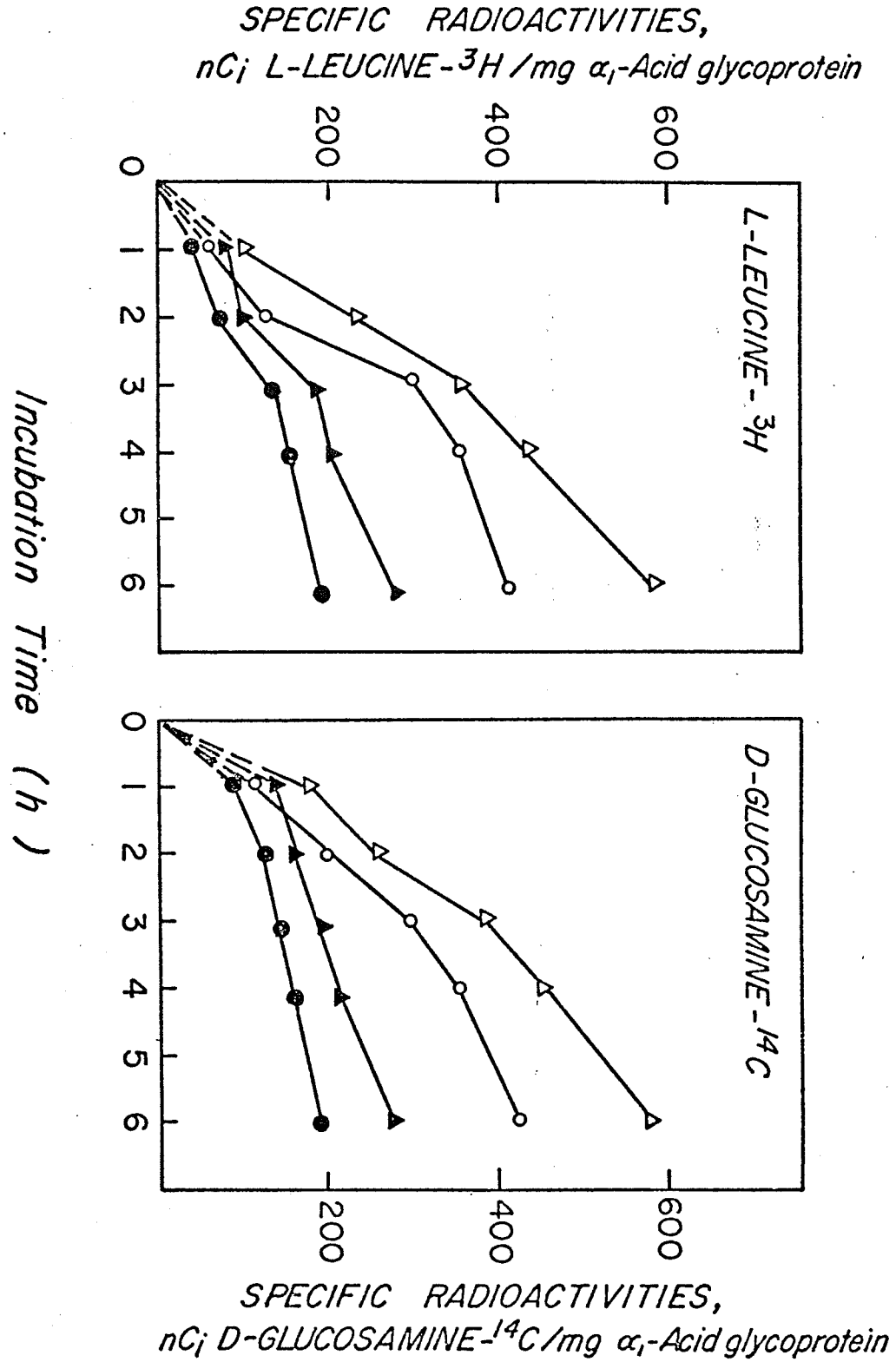

SPECIFIC RADIOACTIVITIES,
nCi L-LEUCINE-3H /mg α1-Acid glycoprotein
200
400
600
0
1
2
3
4
5
6
L-LEUCINE-3H
D-GLUCOSAMINE-14C
Incubation Time (h)
SPECIFIC RADIOACTIVITIES,
nCi D-GLUCOSAMINE-14C /mg α1-Acid glycoprotein

Fig. 13

Effect of time of incubation on specific radioactivities of L-leucine-H^3 in medium and liver albumin from experiments with liver slices from control and 24 h experimental rats; - O - medium and - ● - liver albumin from control rats; - Δ - medium and - ▲ - liver albumin from 24 h experimental rats. Each point represents the mean from three experiments.

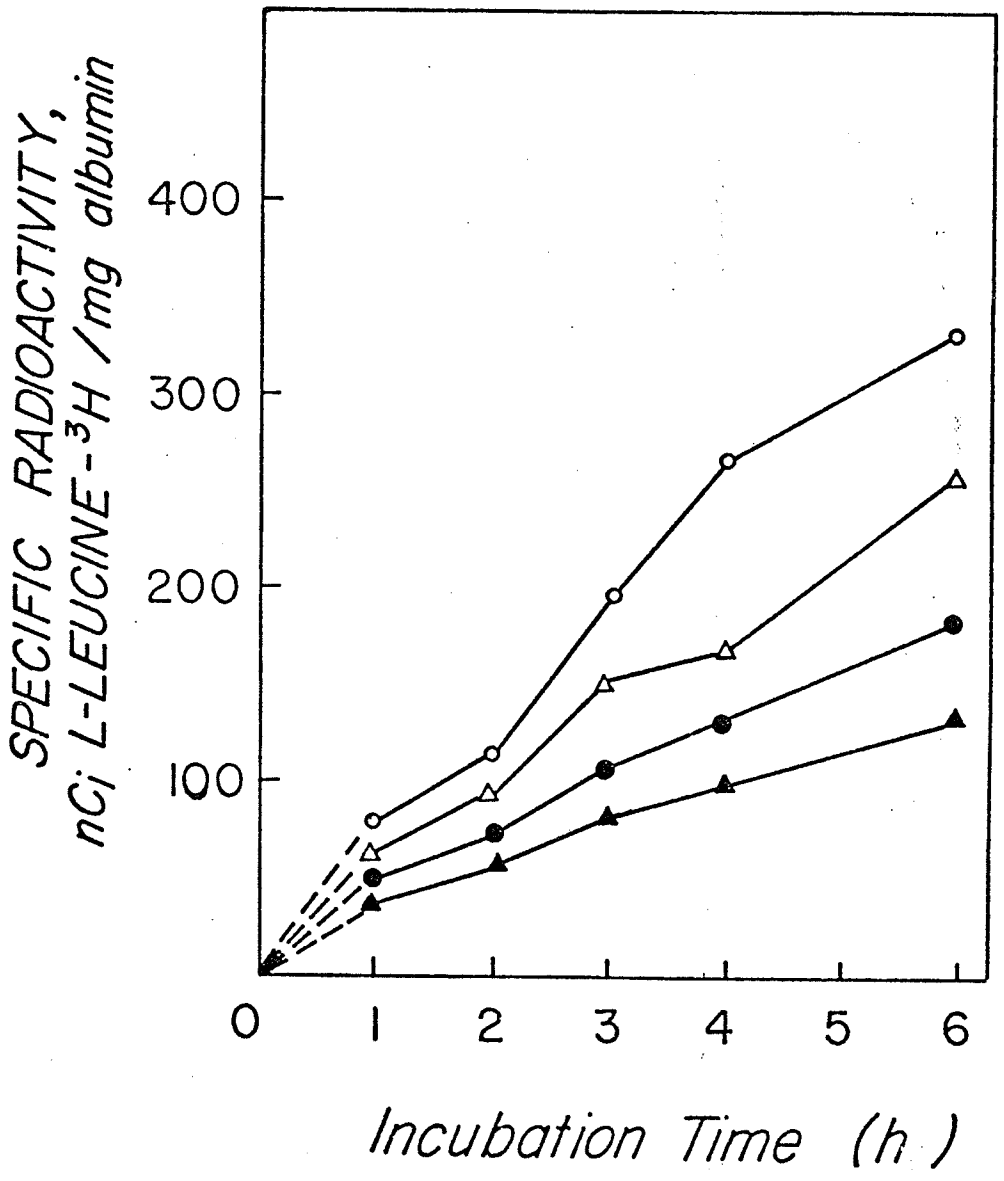

SPECIFIC RADIOACTIVITY,
nCi L-LEUCINE-^{3}H /mg albumin
400
300
200
100
0
1
2
3
4
5
6
Incubation Time (h)

TABLE 5

Incorporation of [^{3}H] Leucine and [^{14}C] Glucosamine into Total Medium and Liver Proteins from Experiments with Control and Experimental Rats after 6 h of Incubation

Time After Induction of Inflammation (h)	Specific Radioactivity, nCi/mg Protein				EXPERIMENTAL/CONTROL †			
	Medium Protein*		Liver Protein*		Medium Protein		Liver Protein	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
CONTROL	11.5±1.0	9.5±1.0	6.5±0.5	1.0±0.1	1.00	1.00	1.00	1.00
8	23.0±2.3	14.5±1.5	7.1±0.8	1.2±0.1	2.00	1.52	1.09	1.20
12	30.5±3.1	18.0±2.0	7.8±0.8	1.3±0.15	2.65	1.90	1.20	1.30
24	40.5±3.5	29.5±3.0	9.1±1.2	1.8±0.15	3.52	3.11	1.40	1.80

*Results are expressed as mean specific radioactivities ± standard errors of the means, from four to six experiments.

†Results are expressed in terms of the ratio of the mean of specific radioactivities of medium and liver proteins from experimental rats to the mean of specific radioactivities of medium and liver proteins from control rats.

Effect of Various Hormones on the Incorporation of labelled precursors into Total medium and liver proteins

Significant differences between experimental and control animals were obtained with the liver slice system using rats suffering from induced inflammation. A lower capacity of the experimental slices for albumin synthesis and a greater capacity for α_1-acid glycoprotein synthesis was accompanied by a large increase in specific radioactivities of labelled precursors into total medium proteins. The increase in the specific radioactivities of total medium proteins could be attributed to increased amounts of highly labelled secretable proteins such as α_1-acid glycoprotein. Biosynthesis of a variety of acute phase proteins studied with a liver perfusion system by other workers (19,52,79) has indicated that steroid or peptide hormones may be involved in the regulation of acute phase protein synthesis. Therefore, liver slices from normal rats were challenged with various compounds in an attempt to induce the acute phase response as seen by the rise in specific radioactivities of total medium proteins. Possible mediators of the acute phase response were added individually or in combinations to the incubation flasks. An increase in specific radioactivities over control values would be a crude indication that the liver might be functioning in a similiar manner as that observed in response to experimental inflammation. Initial studies consisted of the incubation of non-perfused liver slices in the presence and absence of additional compounds and the determination of the specific radioactivities of medium and total liver proteins as presented in TABLE 6. Specific radio-

activities are expressed in terms of the ratio of the mean of specific radioactivities of normal slices plus additions to the mean of specific radioactivities of normal slices. Slices from control rats and 24 h stressed animals without additions are also presented as controls. Significant increases in specific radioactivities of medium proteins were observed with bradykinin, thyroxine, cortisol and prostaglandin $F_{2\alpha}$ and significant decreases in specific radioactivities accompanied additions of prostaglandins E_1 and E_2 and dibutryl cAMP at the concentration levels indicated.

TABLE 6

Effect of Various Compounds on the Incorporation of ^{3}H Leucine and ^{14}C Glucosamine into Total Medium and Liver Proteins from Experiments with Normal Non-perfused Liver Slices after 6 h of Incubation

	SPECIFIC RADIOACTIVITIES			
	MEDIUM PROTEIN*		TOTAL LIVER PROTEIN*	
	^{3}H	^{14}C	^{3}H	^{14}C
CONTROL (14)	11.2±1.5	7.6 ± 1.0	7.5 ± 0.8	1.2 ± 0.2
EXPERIMENTAL ,24h (14)	34.2±3.0	18.2 ± 2.2	12.5 ± 1.2	1.4 ± 0.3

	EXPERIMENTAL/CONTROL ‡			
	MEDIUM PROTEIN		TOTAL LIVER PROTEIN	
ADDED COMPOUND †	^{3}H	^{14}C	^{3}H	^{14}C
BRADYKININ				
2.4×10^{-5}M(2)	1.05	1.03	0.92	0.91
1.2×10^{-4}M(6)	1.62	1.56	1.27	1.17
2.4×10^{-4}M(2)	1.03	1.00	1.52	1.47
PROSTAGLANDIN $F_{2\alpha}$				
2.0×10^{-5}M(2)	1.08	1.05	1.10	1.05
4.0×10^{-5}M(2)	1.38	1.31	1.18	1.16
6.0×10^{-5}M(2)	0.38	0.19	0.65	0.40
THYROXINE				
1.0×10^{-5}M(2)	1.25	1.16	1.16	1.07
CORTISOL				
2.7×10^{-5}M(2)	1.45	1.11	1.11	0.92
CORTISOL 2.7×10^{-5}M plus THYROXINE 1.0×10^{-5}M(2)	1.34	1.16	1.08	1.00

ANGIOTENSIN II				
1.0×10^{-5}M(2)	1.06	1.06	0.77	0.70
BOVINE GROWTH HORMONE				
2.3×10^{-7}M(2)	0.90	0.90	0.90	0.86
ACTH - 4 units (2)	0.67	0.67	1.34	1.16
PROSTAGLANDIN E_1				
5.0×10^{-5}M(2)	0.48	0.45	0.77	0.24
PROSTAGLANDIN E_2				
5.0×10^{-5}M(2)	0.40	0.39	0.96	0.80
DIBUTRYL cAMP				
1×10^{-5}M(2)	0.54	0.35	0.58	0.54
5×10^{-6}M(2)	0.93	0.89	1.26	1.13
PHOSPHORYL-CHOLINE				
3.9×10^{-6}M(2)	3.30	2.43	1.23	1.08
CDP-CHOLINE				
7.6×10^{-5}M(2)	5.11	4.00	1.32	1.09
PHOSPHORYL-CHOLINE				
3.9×10^{-6}M plus				
CDP-CHOLINE 7.6×10^{-5}M(2)	2.48	1.85	0.91	0.82

* Results are expressed as mean specific radioactivities ± Standard errors of the means. The number of experiments performed is in parentheses.

† final concentration of added compound in the incubation flask

‡ Results are expressed in terms of the ratio of the mean of the specific radioactivities with normal slices plus additions to the mean of the specific radioactivities with normal liver slices.

Effect of Various Hormones on Contents of Albumin and α_1-Acid Glycoprotein in Total Liver and Medium Proteins following Incubation of Liver Slices from Control Animals

The differences between normal slices with and without additional compounds were not expected to be as great as the observed differences between normal and 24 h experimental slices but at an intermediate level between normal and experimental slices. If a significant increase or decrease in specific radioactivities in total medium protein was observed, experiments were repeated with perfused livers and medium and total liver extracts were examined by the quantitative precipitin technique or radial diffusion to determine the content of α_1-acid glycoprotein and albumin (TABLE 7). The values in TABLE 7 are ratios of the α_1-acid glycoprotein or albumin content with control slices with additions to the α_1-acid glycoprotein or albumin content with control slices without additions. Bradykinin at 8.0×10^{-5}M yielded a significant increase in specific radioactivities of total medium protein which was accompanied by a significant increase in medium and tissue-bound α_1-acid glycoprotein. However, the content of albumin in medium and total liver extracts also increased, but not as much as the increases in α_1-acid glycoprotein. A combination of cortisol, insulin, and somatropin at concentration levels similiar to those employed by Miller (79) yielded an increase in α_1-acid glycoprotein content in medium and in albumin content in total liver extracts. Decreases in specific radioactivities of total medium proteins observed with dibutyryl ^{c}AMP and prostaglandin E_2 corresponded to decreased amounts of α_1-acid glycoprotein in

medium and total liver extracts. Cortisol and histamine which had increased specific radioactivies of medium proteins , yielded results that showed no significant change in α_1-acid glycoprotein or albumin content in medium. Th combination of bradykinin and cortisol provided an increased level of α_1-acid glycoprotein in medium and total liver proteins with little or no increase in the content of albumin.

The results observed with bradykinin appeared to vary with the commercial preparation used, therefore an attempt was made to purify Sigma bradykinin triacetate using preparative paper chromatography. Modified Sakaguchi assay was used to determine the amount of purified bradykinin added to the incubation flasks. The effect of bradykinin was shown to be concentration dependant as seen in Fig. 14; and all further experiments employed the purified bradykinin preparation. Bradykinin levels found to yield the greatest increases in specific radioactivities of medium protein (Fig. 14) and corresponding increases in medium and tissue-bound α_1-acid glycoprotein (Fig 15) were one fifth of the levels of commercial bradykinin employed originally. The ratios of specific radioactivities of ^{3}H-leucine and ^{14}C glucosamine in α_1-acid glycoprotein isolated from medium and total liver extracts showed increases over controls with specific radioactivity of ^{3}H leucine in albumin showing a decrease especially in tissue-bound form (Fig. 16). These changes parallel the changes observed in specific radioactivities with experimental slices, however the magnitude of change is not as large as that observed with the 24 h experimental slices. Further studies with bradykinin were done at various times of incubation in

order to determine the time interval between addition of bradykinin and the observed increase in specific radioactivity of medium proteins and the corresponding increase in medium and tissue-bound α_1-acid glycoprotein. Control slices with and without bradykinin and experimental 24 h slices were incubated at various times between 2-10 h and results are presented in Figs. 17 and 18. Increases in specific radioactivities in medium proteins can be detected after 4 h of incubation with 1.44×10^{-5} M bradykinin with the largest increases in specific radioactivities occurring after 6 h. An increase in α_1-acid glycoprotein isolated from total liver extracts appears maximal after 3 h and the corresponding increase in α_1-acid glycoprotein in medium occurs after at least 4 h. Results are expressed as the ratio of the mean from two experiments and specific radioactivities of albumin and α_1-acid glycoprotein isolated from medium and total liver extracts showed corresponding changes.

TABLE 7

Effect of Various Compounds on the Incorporation of [^{3}H] Leucine and [^{14}C] Glucosamine into Total Medium Protein and the Content of α_1-Acid Glycoprotein and Albumin isolated from Medium and Total Liver Extracts from Experiments with Control Liver Slices after 6 h of Incubation.

	Specific Radioactivities*		Contents †			
			Albumin		α_1-acid Glycoprotein	
	Medium Proteins		Medium	Total Liver	Medium	Total Liver
ADDED COMPOUND	^{3}H	^{14}C				
BRADYKININ 8.0×10^{-5}M(6)	1.58	1.49	1.60	1.19	1.74	1.65
1.2×10^{-4}M(6)	1.24	1.24	1.12	1.32	1.22	1.37
CORTISOL 2.7×10^{-5}M(2)	1.31	1.22	1.13	1.02	0.98	1.00
BRADYKININ 8.0×10^{-5}M + CORTISOL 2.7×10^{-5}M(2)	1.39	1.28	1.01	1.06	1.22	1.30
HISTAMINE 5.0×10^{-6}M(2)	1.22	1.09	1.04	1.48	0.89	1.13
CORTISOL 2.7×10^{-5}M INSULIN 0.2 units/ml Bovine Growth Hormone 2.3×10^{-7}M(2)	1.23	1.16	1.00	1.85	1.33	1.02
PROSTAGLANDIN E_2 3.0×10^{-5}M(2)	0.77	0.79	1.42	1.20	0.80	0.80
DIBUTRYL cAMP 1.0×10^{-5}M(2)	0.81	0.69	0.81	1.06	0.78	1.00
EXPERIMENTAL 24h (10)	3.17	2.25	0.62	0.52	2.59	4.50

* Specific radioactivities are expressed in terms of the ratio of the mean of specific radioactivities of normal slices plus additions to the mean of the specific radioactivities of normal slices.

† Contents of albumin and α_1-acid glycoprotein are expressed in terms of the ratio of the contents of these proteins isolated from medium and total liver with normal slices plus additions to the contents of these proteins isolated from medium and total liver with normal slices.

The number of experiments performed is in parentheses.

Fig. 14

Effect of bradykinin concentration on the specific radioactivities of L-leucine-^{3}H and D-glucosamine-^{14}C in total medium protein with liver slices from control rats; -●- ^{3}H leucine and -△- ^{14}C glucosamine incorporation is expressed as the ratio of the mean of specific radioactivities of medium proteins with normal slices plus bradykinin to the mean of the specific radioactivities of medium proteins with normal slices, averaged from two experiments.

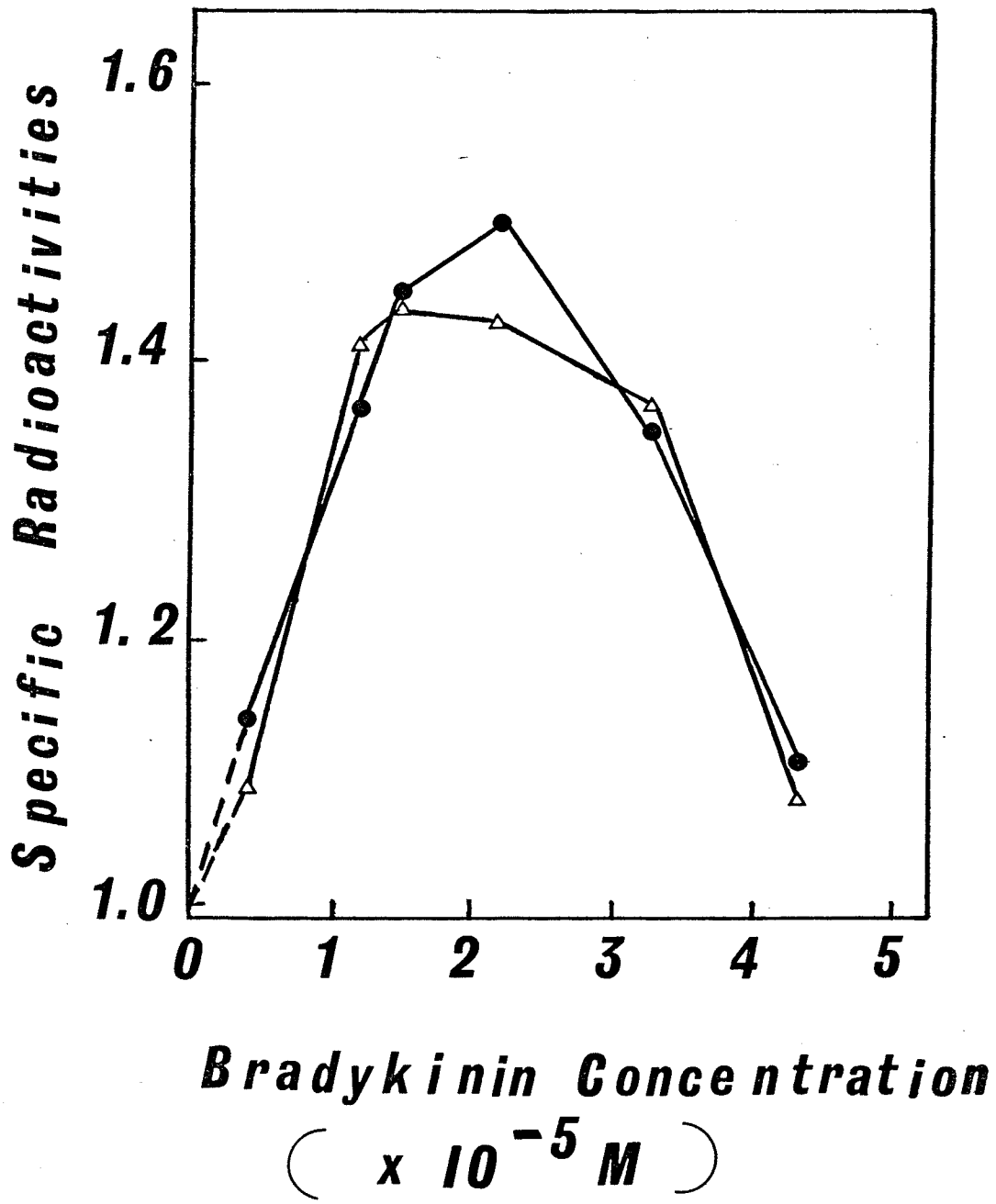

Specific Radioactivities
1.6
1.4
1.2
1.0
0
1
2
3
4
5
Bradykinin Concentration
(x 10⁻⁵ M)

Fig. 15

Effect of Bradykinin concentration on the contents of albumin and α_1-acid glycoprotein isolated from medium and total liver with normal liver slices; -□- medium and -■- total liver albumin and -○- medium and -●- total liver α_1-acid glycoprotein. Results are expressed in terms of the ratio of the contents of these proteins isolated from medium and total liver with normal slices plus bradykinin to the contents of these proteins isolated from medium and total liver with normal slices from two experiments.

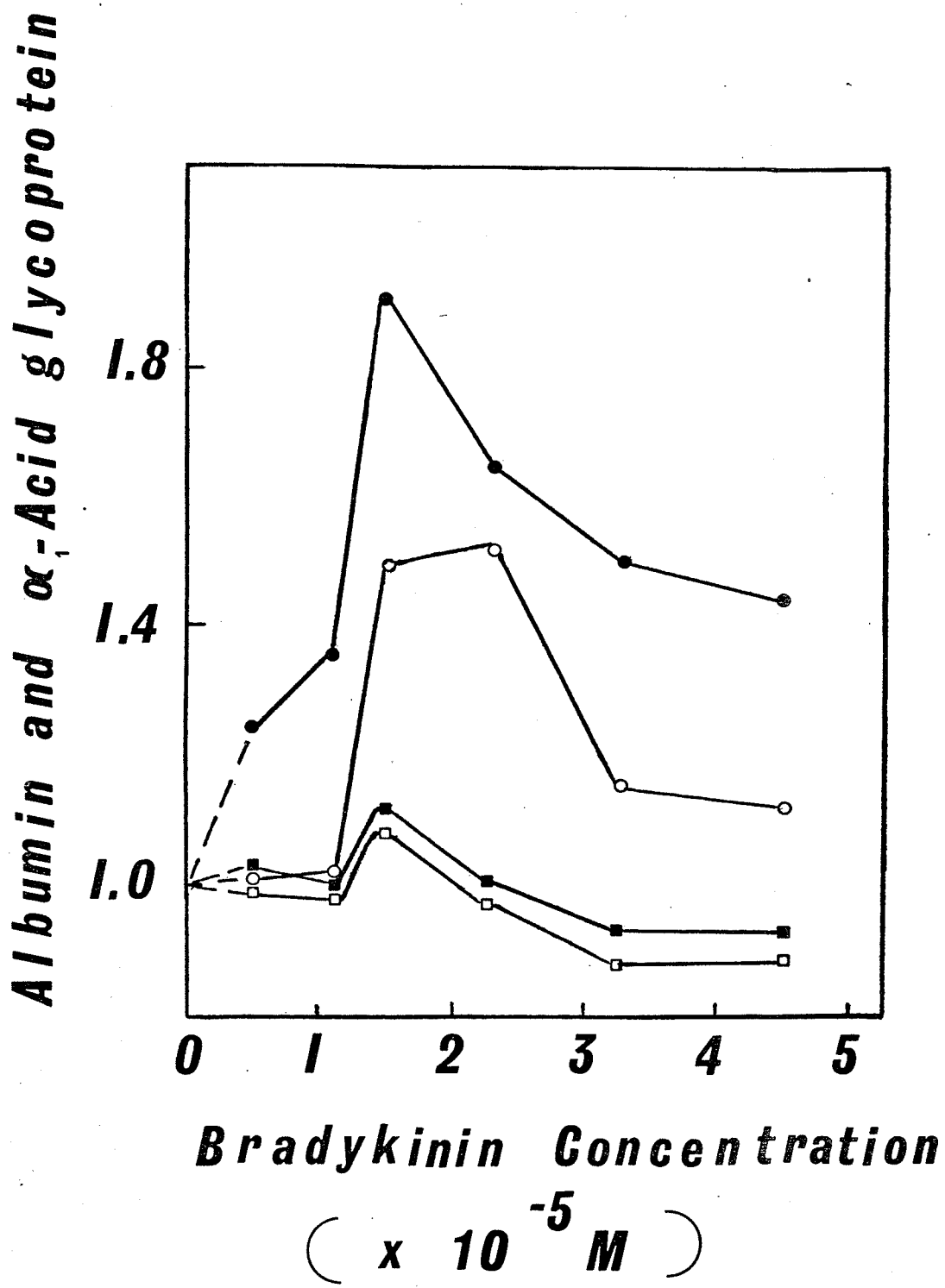

Albumin and α1-Acid glycoprotein
1.8
1.4
1.0
0
1
2
3
4
5
Bradykinin Concentration
(x 10 -5 M)

Fig. 16

Effect of bradykinin concentration on specific radioactivities of L-leucine-^{3}H in medium and total liver albumin and of D-glucosamine-^{14}C in medium and total liver α_1-acid glycoprotein with normal liver slices: - O - medium and - ● - total liver α_1-acid glycoprotein; - □ - medium and - ■ - total liver albumin. Results are expressed in terms of the ratio of the specific radioactivities of these proteins isolated from medium and total liver with normal slices plus bradykinin to the specific radioactivities of these proteins isolated from medium and total liver with normal slices.

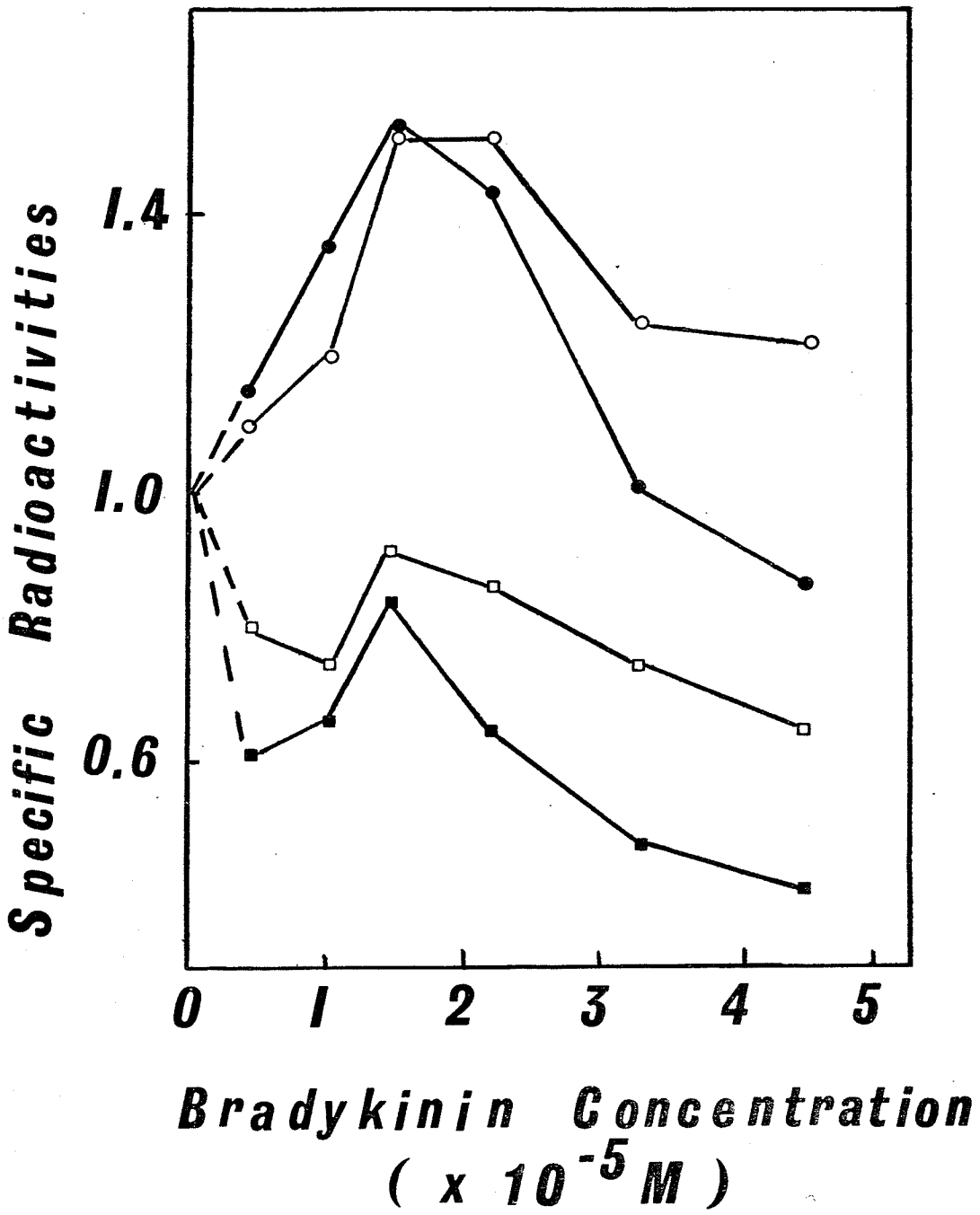

Specific Radioactivities
1.4
1.0
0.6
0
1
2
3
4
5
Bradykinin Concentration
(x 10⁻⁵ M)

Fig. 17

Effect of time of incubation on the specific radioactivities of L-leucine-^{3}H and D-glucosamine-^{14}C in total medium protein with normal liver slices plus bradykinin 1.44×10^{-5}M; -●- ^{3}H leucine and -Δ- ^{14}C glucosamine incorporation is expressed as the ratio of the mean of specific radioactivities of medium proteins with normal slices plus bradykinin to the mean of the specific radioactivities of medium proteins with normal slices from two experiments.

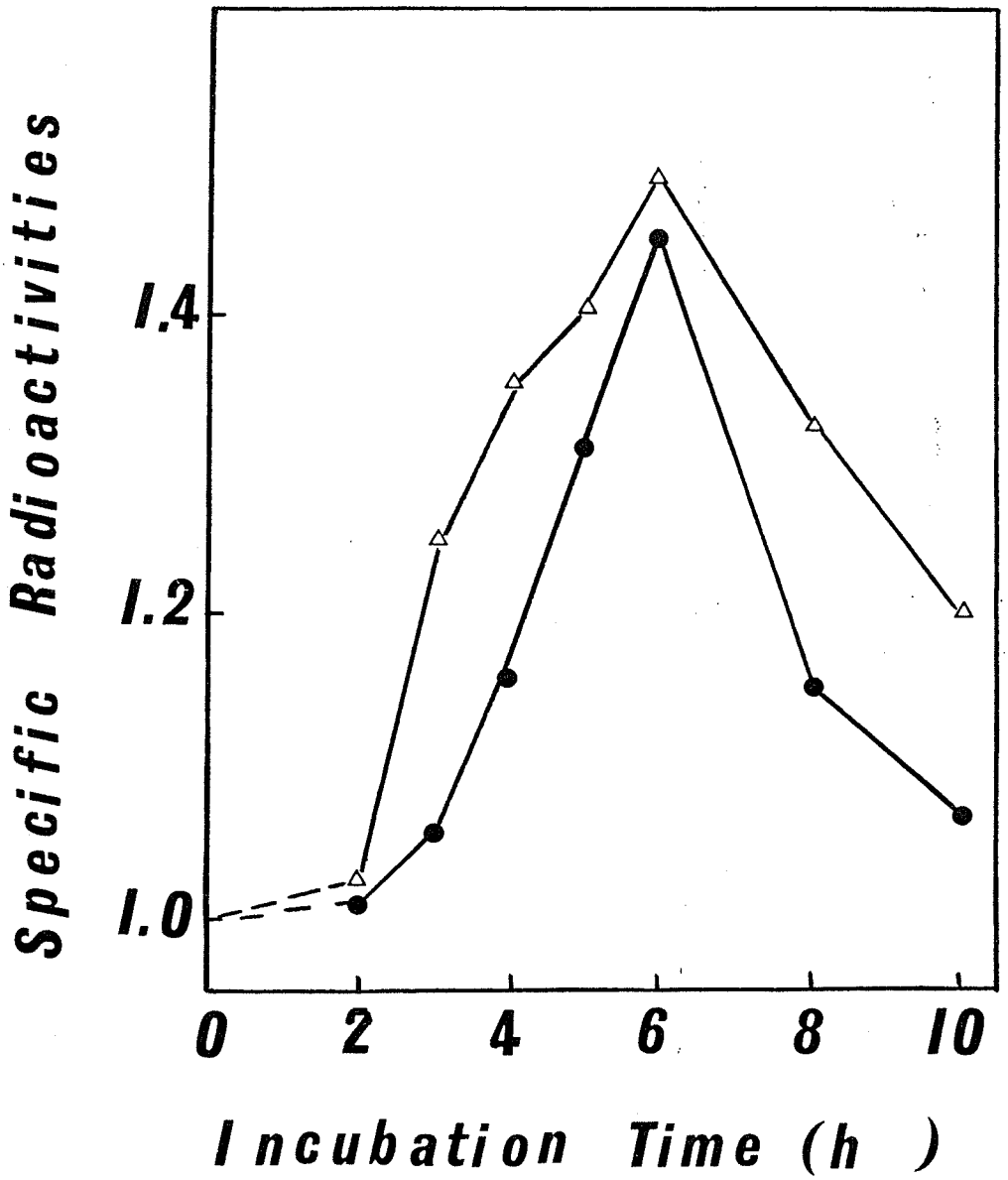
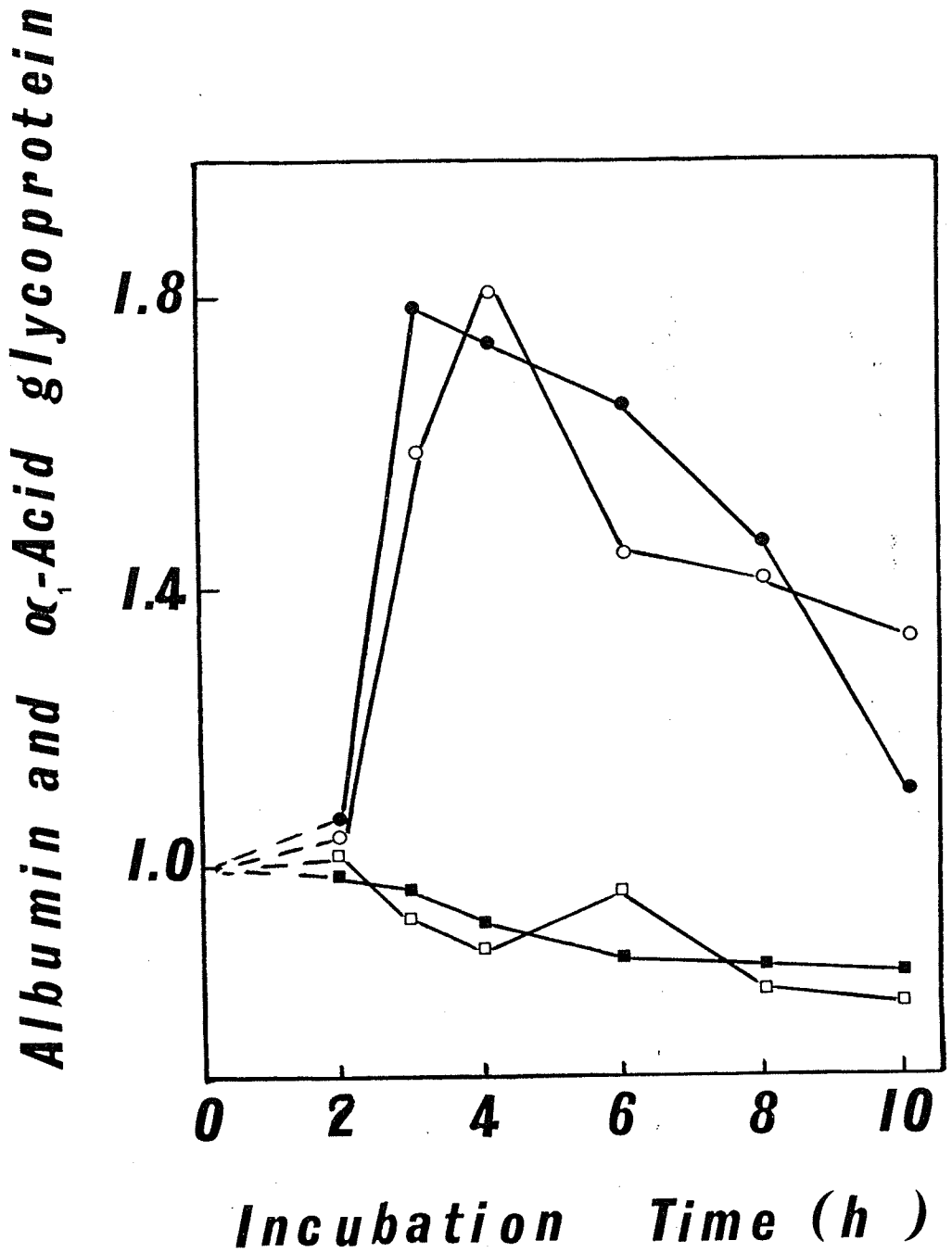

Specific Radioactivities
1.4
1.2
1.0
0
2
4
6
8
10
Incubation Time (h)

Fig. 18

Effect of time of incubation on the contents of albumin and α_1-acid glycoprotein isolated from medium and total with normal liver slices plus bradykinin 1.44×10^{-5}M; -□- medium and -■- total liver albumin and -○- medium and -●- total liver α_1-acid glycoprotein. Results are expressed in terms of the ratio of the contents in terms of the ratio of the contents of these proteins isolated from medium and total liver with normal slices plus bradykinin to the contents of these proteins isolated from medium and total liver with normal slices from two experiments.


Albumin and α1-Acid glycoprotein
1.8
1.4
1.0
0
2
4
6
8
10
Incubation Time (h)

DISCUSSION

Immunodiffusion studies showed that the components of the microsomal fraction of liver are the main site of synthesis of polypeptide and carbohydrate moieties of α_1-acid glycoprotein and of polypeptide moieties of serum albumin. These results confirm earlier in vivo studies (75) as well as reports by Peters (80) and other workers (81) on the biosynthesis of rat serum albumin. Present work with liver slices did show significant labelling with fractions other than the microsome fraction on reaction with monospecific antiserum to albumin and α_1-acid glycoprotein. These observations may be explained by some disruption of the normal morphology of the liver cell during the slicing and incubation procedure. Subsequent fractionation may have yielded a poorer separation of microsomal material from other subcellular fractions, and the possibility also exists that the proteins under study may have bound to other subcellular fractions during the isolation procedure. Immunodiffusion and immunoelectrophoresis clearly demonstrated that liver slices are capable of secreting both proteins into medium, since both albumin and α_1-acid glycoprotein isolated from medium have identical electrophoretic mobilities to corresponding native serum proteins. It is known that tissue-bound forms of both proteins have different electrophoretic mobilities from those of corresponding serum proteins. (Jamieson J.C. personal communication). Quantitative studies (TABLE 3+4, Fig. 9+10) indicate the net increase of both proteins in medium as a function of the time of incubation with no significant change in the content of tissue-bound α_1-acid glycoprotein

and albumin present in liver slices. Therefore α_1-acid glycoprotein can be classified as a serum protein which is known to be synthesized and secreted by liver slices. Other serum proteins which have been reported in this category include albumin (82,83) β-lipoprotein (84), transferrin (85) and total globular proteins, but in the case of transferrin, medium proteins were not examined separately. The present work included studies on liver slices from normal rats and those suffering from turpentine-induced inflammation for various times. Results from quantitative studies showed significant differences between control and experimental animals while immunodiffusion studies had shown little qualitative differences due to induced inflammation. Liver slices from experimental animals contained greater amounts of α_1 acid-glycoprotein and lower amounts of albumin than liver slices from control animals at the start of the experiment as reported *in vivo* (75). Liver slices from experimental animals were capable of secreting larger amounts of α_1-acid glycoprotein and lower amounts of albumin into medium as compared to control slices, thus resulting in an increased net synthesis of α_1-acid glycoprotein and a decreased net synthesis of albumin. The greatest differences between experimental and control animals were observed after 24 h of inflammation. The liver slice system appears to function in a similiar manner to liver *in vivo* (75,78) with respect to synthesis and secretion of the two serum proteins, albumin and α_1-acid glycoprotein. Similiar changes in synthetic capacities of liver for other acute phase glycoproteins and serum albumin have been reported with studies using the

isolated perfusion technique of Miller (86). However rat liver slices offer a system that allows the observation of more conditions on the same tissue than is possible with the perfused liver.

In the present study, the amount of albumin synthesized by normal liver slices was 36-66 ug per g wet weight of tissue per hour, depending on the time of incubation (TABLE 3). These values are lower than values reported by Peters (83); in the presence of glucose and an amino acid supplement, a value of 107 ug per g wet weight per hour was reported for livers from 5-8 week old Wistar rats fasted for 18 h. In adult rats, the formation of albumin was about 30% of that in 5-8 week control rats (83), and it should be noted adult hooded rats were used in the present study. The content of albumin found in liver in control rats was about 320 ug per g wet weight of tissue (TABLE 3). This value is lower than values reported in in vivo (87,88) and in vitro (89) studies. The lower values reported in the present work could be due to loss of intracellular albumin from damaged cells on the surface of slices during the exhaustive washing procedure. However, higher values reported by others may include residual albumin from blood present in the liver at the time of death which could remain bound to tissue material. The amount of albumin synthesized by 24 h experimental animals was 60% of control values (TABLE 3) and this decrease was more significant than the change observed in in vivo work (60,75,78). However in in vivo experiments the large pool of albumin in blood and other body fluids makes it difficult to assess changes in albumin synthesis quantitatively.

The amount of α_1-acid glycoprotein synthesized by normal slices was 6-14 ug per g wet weight of tissue per hour and slices from 24 h experimental rats yielded values with about a three-fold increase over control values (TABLE 4). With 24 h experimental rats, there was an increase in specific radioactivities of labelled leucine and glucosamine in α_1-acid glycoprotein isolated from medium and total liver, and a decrease in specific radioactivity of labelled leucine in albumin isolated from medium and total liver. If it is assumed that the polypeptide chains of albumin and α_1-acid glycoprotein utilize the same intracellular pool of leucine molecules, differences in pool sizes, particularly in the case of leucine, could not account for the differences in specific radioactivities found in α_1-acid glycoprotein and albumin. Therefore, stimulation in incorporation of labelled compounds into α_1-acid glycoprotein and reduction in incorporation into albumin can be correlated with increased synthesis of α_1-acid glycoprotein and decreased synthesis of serum albumin.

Specific radioactivities of labelled precursors in α_1-acid glycoprotein and albumin isolated from total liver extracts were always lower than those of the corresponding medium proteins at all times of incubation (Fig. 12 and 13). These results are in agreement with reports by others (82), in the case of albumin; that after 2 h of incubation of non-perfused or perfused liver slices there was, on the average, an eight-fold difference between specific radioactivities of medium albumin and albumin present in the liver cell. Lower specific radioactivity of ^{14}C-glucosamine labelling of α_1-acid glyco-

protein in total liver extracts can easily be explained as intracellular α_1-acid glycoprotein molecules are known to be incomplete with respect to carbohydrate (Jamieson J.C. unpublished work), therefore fewer glucosamine residues are present than in the completed extracellular protein. It is more difficult to explain the lower specific radioactivities of ^{3}H-leucine in albumin and α_1-acid glycoprotein when isolated from total liver as compared to medium. If it is assumed that the intracellular proteins are simple precursors of extracellular proteins, specific radioactivities of medium albumin would be expected to be lower at short periods of incubation and to increase to the same values after longer periods of incubation as labelled intracellular proteins are secreted into extracellular medium. However, the results discussed above do not fit this pattern, thus implying intracellular proteins are not the direct precursors of the extracellular proteins in the system studied.

There are a number of possible explanations for the observations discussed above. As suggested by others (89), there may be two pools of albumin and presumably α_1-acid glycoprotein in the liver cell; one may be turning over rapidly, every 4-5 minutes; this pool would consist of proteins to be secreted from the cell and would be mainly in the microsomal fraction; the other pool may turn over slowly and would consist of proteins found in nuclear, mitochondrial, or soluble fractions upon subfractionation of liver. If it is assumed that serum albumin and α_1-acid glycoprotein secreted from the liver cell are mainly derived from the pool with a short turnover time associated

with the microsomal fraction, it would be expected that medium proteins would have higher specific radioactivities than intracellular proteins which would be the product of both pools in the cell. Secondly, precursors of albumin have been isolated from liver (89-91) and it is thought the precursor molecules are formed slowly and then converted into albumin by the removal of a peptide. The site of conversion of precursor into albumin is suggested to be the microsomal fraction of the liver already known to be the site of synthesis of newly formed albumin. The molecular weight of the peptide relative to the albumin molecule would be insignificant but it should be noted that the peptide is not labelled with leucine (91), the labelled precursor used in present work. Therefore slight differences in specific radioactivities of albumin and its precursor would be expected; the degree of difference would depend on their relative molecular weights. The nature of the immune reaction of precursor albumin with anti-albumin is not known and it may differ from albumin - anti-albumin reaction which was used to construct the quantitative precipitin curve. Whether the above argument may be used to explain observed decreases in ^{3}H-leucine in α_1-acid glycoprotein in total liver as compared to medium is dependant upon the assumption that extracellular α_1-acid glycoprotein is formed by the removal of a peptide from a precursor molecule; there is no evidence for this assumption at the present time. A third possible explanation for the observations discussed above is that livers contained residual albumin and α_1-acid glycoprotein from blood present in livers at death, even after perfusion of the whole organ followed

by washing of slices. It is known that albumin has the capacity to bind to cells and other macromolecules (92) and it is possible albumin may have bound to tissue material. However other studies (82) with non-perfused and perfused liver slices showed little differences in specific radioactivities of albumin in medium and liver slices with non-perfused slices which would have contained a larger amount of residual blood as compared to perfused liver slices. Specific radioactivities of medium albumin were slightly higher with perfused slices, specific radioactivities of intracellular albumin were slightly higher with perfused slices, and total specific radioactivities were slightly lower in perfused slices. Therefore a change, if any, in specific radioactivities of albumin due to the presence of residual albumin would be minimal. Also it should be noted that the radiochemical purity of serum albumin separated by antibody precipitation from whole liver has been questioned by others (93). A highly labelled impurity localized only in tissues with the ability to bind to anti-albumin complexes was suggested since specific radioactivities of albumin isolated by immunoprecipitation are always higher than those of albumin isolated by methods which yield radiochemically pure proteins. Therefore specific radioactivities of intracellular albumin and α_1-acid glycoprotein may be even lower than values presented (Fig. 12 and 13). Whatever the explanation for the lower specific radioactivities of intracellular proteins when compared to extracellular proteins, it does not detract from the valuable information supplied by the present work.

From quantitative and incorporation studies discussed above it was shown that liver slices from experimental rats were capable of secreting larger amounts of α_1-acid glycoprotein and lower amounts of albumin which corresponded to changes in the synthetic capacities of liver for these proteins. Similiar changes in the synthetic capacities of liver for α_1-acid glycoprotein, an acute phase reactant, and serum albumin have been reported by Miller (79) to be induced by the addition of a hormone supplement to the perfusate of livers isolated from normal rats. The effects of various hormones on regulation of protein synthesis, as mentioned in the introduction, are dependant upon the quality and quantity of hormone used and the experimental conditions employed, making confirmation of results from present work with liver slices difficult in some cases. Studies on the effects of various hormones in vivo by removal of glands and hormone treatment are difficult to interpret because observed changes may be secondary to altered nutrition, radioactive precursor specific activity or other factors, not due to specific effects of hormone studied. The direct effects of hormones on plasma protein synthesis by rat liver are few in number (19, 53, 79). Results from the present study indicate that the synthesis of specific plasma proteins by liver can be influenced by a number of factors including hormones and chemical mediators released from site of injury.

In the present study, thyroxine at a level 1.0×10^{-5}M showed increased incorporation of labelled precursors into medium and total liver proteins; these results are in agreement with

liver perfusion studies at similiar levels of hormone (53). Synthesis of a number of acute phase proteins including α_1-acid glycoprotein have been shown to be responsive to the thyroid status of the liver donor and the addition of thyroxine to the perfusate. Thyroxine is metabolized and excreted rapidly by liver and can bind to rat plasma proteins, therefore greater than physiological levels (10^{-7}-10^{-9}M) were employed in present work. Thyroxine also showed a modifying effect on cortisol action which is in agreement with perfusion studies (53) that added thyroxine partially inhibits the effectiveness of cortisol stimulation. Bovine growth hormone at a level of 2.3×10^{-7}M (5µg/ml) failed to show any significant effects on incorporation of ^{3}H-leucine and ^{14}C-glucosamine into medium or total liver proteins. In vivo experiments by Korner (50) had reported growth hormones could activate ^{14}C-amino acid incorporation into protein in general, and serum albumin in particular. However perfusion studies (19) showed little effect of bovine growth hormone on incorporation of ^{14}C-lysine into liver protein or synthesis of four plasma proteins studies. Plasma concentration of growth hormone in male rats is 0.055 ug/ml, however the lowest concentrations found to exert effects in vitro are in the range 0.7-60ug/ml (94). Additions of ACTH also showed no significant change in incorporation of labelled percursors into medium and total liver proteins. Other studies (95) in vivo had shown stimulation of fibrinogen synthesis with repeated large doses of ACTH, however small doses had shown little effects. The proposed stimulatory effect was not thought to be mediated by the adrenal gland but it is

not known whether the hormone can act on the hepatic cell directly or through an indirect extra-adrenal mechanism. The effect of prostaglandin $F_{2\alpha}$ on incorporation into medium and total liver proteins appeared to be concentration dependent. Low levels of prostaglandin $F_{2\alpha}$ showed no significant effects; stimulation of incorporation of labelled precursors into medium proteins was maximal at 4.0×10^{-5}M; and higher concentrations caused decreases in incorporation of labelled precursors into medium and total liver proteins. At comparable levels prostaglandins E_1 and E_2 caused significant decrease in incorporation of ^{3}H-leucine and ^{14}C-glucosamine into medium and total liver proteins. The levels of prostaglandins employed in the present work are comparable to levels used with two other in vitro systems, liver cell homogenates (96) and rat mast cell suspensions (97). Opposite effects of prostaglandin E_1 and E_2, and prostaglandin $F_{2\alpha}$ have been seen in many systems (96,97), and there seems to be a prescribed balance in local concentrations of E and F compounds (40). The observed effects of prostaglandins may have been mediated by cyclic AMP as it has been reported (96) that prostaglandins E_1 and E_2 have the ability to increase adenyl cyclase activity and thus cyclic AMP levels in liver cells, but prostaglandin $F_{2\alpha}$ does not effect cyclic AMP levels. Cyclic AMP at levels of 1.0×10^{-5}M produced significant decreases in the incorporation of ^{3}H-leucine and ^{14}C-glucosamine into medium and total liver protein. This result fails to confirm results that cyclic AMP (10^{-3}M) can stimulate incorporation of ^{14}C-glucosamine into glycoprotein which was found in intestinal slices from

rats (58). Cyclic AMP has been found to influence protein synthesis in a variety of systems, but effects are dependant upon the tissue and the concentration level used. CDP-choline and phosphoryl choline produced significant increases in incorporation of labelled precursors into medium and total liver proteins, as reported *in vivo* and *in vitro* (55,57). Overall stimulation of plasma glycoprotein synthesis and reduction of liver triglycerides were reported for CDP-choline and CDP-choline plus phosphoryl choline additions. However liver slices were obtained from choline deficient rats in which the tissue would be more sensitive for stimulation by phosphoryl choline. CDP-choline is known to stimulate glycosyl transferases by inhibition of UDP-galactose pyrophosphatase activity (98) and it is unlikely to be the physiological mechanism involved in the stimulation of α_1-acid glycoprotein synthesis in response to inflammation.

The observed changes in incorporation of ^{3}H-leucine and ^{14}C-glucosamine into medium and total liver proteins were only a crude indication that the liver was responding as it would during the acute inflammatory response. When the content of albumin and α_1-acid glycoprotein in medium and total liver extracts were examined, increases in specific radioactivities of medium proteins need not necessarily correspond to the increase in α_1-acid glycoprotein content and the decrease in albumin content observed with the liver slices from experimental animals. The levels of cortisol which yielded moderate increases in specific radioactivities of medium proteins in perfused and non-perfused liver slices were com-

parable to levels of cortisol investigated in liver perfusion studies (79,99). The present work showed no significant changes in albumin and α_1-acid glycoprotein content in medium or total liver, and failed to confirm reports by Gordon (99) that cortisol significantly reduces synthesis of albumin, transferrin, and other plasma proteins, or reports by Miller (79) that cortisol was essential for the enhanced synthesis of acute phase proteins studies. In vivo (47) cortisol administration depressed albumin synthesis and stimulated acute phase protein synthesis. A possible explanation for the difference between the present work and other reports, may be that the length of the perfusion experiments and the method of administration of the cortisol determine the effects observed. With perfused liver slices bradykinin 8.0×10^{-5}M produced increases in specific radioactivities of medium proteins and a corresponding increase in amounts of α_1-acid glycoprotein and albumin. However bradykinin plus cortisol at the same concentrations as discussed above yielded about a 30% increase in specific radioactivities of medium proteins with a corresponding increase in the α_1-acid glycoprotein content in medium and total liver extracts and with no significant change in albumin content. Cortisol when added with bradykinin partially suppressed the increase in α_1-acid glycoprotein content and effectively suppressed the increase in albumin content in medium seen with addition of bradykinin alone. Cortisol is capable of preventing the release of active kinin from its substrate in vitro (38) at concentration levels comparable to those employed in the present study and it has been suggested that cortisol and similiar

steroids exert their anti-inflammatory effect in part by preventing the formation of vasoactive kinin or by antagonizing their effects. However, the mechanism by which cortisol could influence the action of bradykinin already present in the liver slice system has yet to be proposed. The results with a combination of cortisol, insulin, and growth hormone showed a 20% increase in specific radioactivities of medium proteins which corresponded to a 30% increase in medium α_1-acid glycoprotein and no change in medium albumin. In perfusion studies by Miller (79) this combination of hormones at similiar concentration levels produced increased levels of albumin and α_1-acid glycoprotein after 12 h of perfusion. However after 6 h of perfusion with liver donors fasted for 18 h, only marginal increases in albumin and about a 50% increase in α_1-acid glycoprotein were reported in the perfusate. Other work with perfused liver by Koj (52) reported that after 4 h of perfusion, this combination of hormones had insignificant effects on relative lysine incorporation into albumin, ceruloplasmin and seromucoid fraction. Results after long periods of incubation are believed to show the effects of injury inflicted during the operation and isolation procedure of the liver. Present work supports the reports that hormones may influence the expression of the liver response to induced inflammation, however, the primary factor stimulating the liver cell to produce more acute phase reactants is still unknown and may be released from the site of injury.

Histamine, which is believed to be involved in the initial stages of the acute inflammatory response, produced marginal

increases in specific radioactivities of medium proteins but no significant changes were observed in the levels of α_1-acid glycoprotein and in medium. These results disagree with other reports (54) which had shown a significant decrease in leucine incorporation into albumin and globulin fractions with 1.6×10^{-5}M histamine. However others (55) had reported that with perfused rat liver, no significant effects on albumin were observed with infusion of histamine at greater concentrations than those used in present work. Prostaglandins are thought to be chemical mediators of the period $2\frac{1}{2}$-6 h after inflammation (31). Decreased specific radioactivities of medium proteins observed with addition of prostaglandin E_2 corresponded to decreased amounts of α_1-acid glycoprotein in medium and about a 40% increase in albumin content in medium. The effect of prostaglandin E_2 seemed dependant upon the protein studied and therefore could not be directly correlated with a mediated increase or decrease in cyclic AMP levels. With additions of dibutyrl cyclic AMP, the decrease in specific radioactivities of medium proteins corresponded to decreased amounts in medium of the two proteins studies and possibly to an overall decrease in protein synthesis. Histamine and prostaglandins, directly or indirectly by influencing cyclic AMP levels, failed to induce the increase in α_1 acid-glycoprotein content in medium and total liver proteins associated with the acute phase response.

Initial work with non-perfused liver slices showed bradykinin at a concentration of 1.2×10^{-4}M stimulated increases in incorporation of labelled precursors into total

medium and liver proteins in contrast with reports (54) that at a similiar level with rat liver microsomal fraction, incorporation into the isolated seromucoid fraction was significantly decreased. With perfused liver slices bradykinin at a concentration of 8.0×10^{-5}M showed stimulation of incorporation into medium proteins by 50-60% over controls, at levels of bradykinin significantly less than those employed with non-perfused slices. This result may be attributed to the removal of residual blood which contains kininases with the ability to inactivate bradykinin added, by perfusion of the whole organ in situ. The commercial preparation of bradykinin used in these studies was chemically synthesized and not the naturally occurring peptide, and other workers (100) have found indications of poor synthesis or decomposition in some commercial preparations of bradykinin. In agreement, preparatory paper chromatography did show a number of other ninhydrin positive spots which did not correspond to bradykinin by R_f comparison. Therefore, possible contaminating material or decomposition products could have decreased the effective level of bradykinin present in the incubation flasks. Previous work was repeated with the purified bradykinin preparation, in order to find optimum concentrations for maximum stimulation of α_1-acid glycoprotein synthesis. Bradykinin effects were shown to be concentration dependant with optimum levels between $1.5\text{-}2.0 \times 10^{-5}$M; levels almost one-fifth of those used originally with perfused slices. Lower than optimum levels had little effect on specific radioactivities of medium proteins and α_1-acid glycoprotein and albumin

synthesis. Higher levels produced less stimulation of α_1-acid glycoprotein and slight decreases in albumin synthesis; which may be due to toxic effects at high concentrations of bradykinin. Physiological levels of bradykinin in rats are about 0.5 ng/ml or 10^{-9}M in circulating blood, however elevated levels have been found under certain conditions and variations in normal levels are thought to exert regulatory effects. The levels of bradykinin used in the present study are greater than normal physiological levels, however these elevated levels appeared to be necessary to maintain the effect of bradykinin in the liver slice system. The actual level of biologically active bradykinin present during the incubation of liver slices may have been considerably less than the value determined from chemical assays because of possible rapid degradation during incubation. However, bradykinin at any concentration studied did not show the induced changes in α_1-acid glycoprotein and albumin synthesis which are known to accompany the acute phase response and which are observed with experimental liver slices. Changes in albumin synthesis seemed to parallel changes in α_1-acid glycoprotein synthesis, although to a lesser degree, however the synthetic capacity of liver for these two proteins was definitely modified by the presence of bradykinin. Results of specific radioactivities of ^{3}H-leucine and ^{14}C-glucosamine in α_1-acid glycoprotein and of ^{3}H-leucine in albumin isolated from medium and total liver extracts parallel the changes observed with quantitative results discussed above. The specific radioactivity of ^{3}H-leucine in medium and total liver albumin reflects a much greater decrease in albumin synthesis than

was evident from quantitative studies, however at the bradykinin concentration which yielded maximum α_1-acid glycoprotein incorporation of labelled precursors, albumin incorporation of ^{3}H-leucine increased to a value only slightly lower than controls. There was the possibility that the changes induced by the addition of bradykinin were not fully expressed due to the time required to initiate these changes and for increased levels of α_1-acid glycoprotein and for decreased levels of albumin to show up in medium proteins. Time course experiments of bradykinin action showed 3-4 h were required before increased specific radioactivities of medium proteins and increased content of α_1-acid glycoprotein in medium and total liver were significant. This finding is in agreement with Miller (79) that at least 3 h is required for enhancement of acute phase protein synthesis by his combination of hormones. However, with extended periods of incubation, there was a decrease in the ratio of specific radioactivities of medium proteins isolated from normal slices plus bradykinin to specific radioactivities of medium proteins isolated from normal slices. Actual specific radioactivities of medium proteins isolated from liver slices plus bradykinin were greater than control values in all cases, as would be expected. A possible explanation for this finding is that bradykinin may stimulate the rate of catabolism of highly labelled proteins, specifically α_1-acid glycoprotein; this effect would be more important after long periods of incubation due to the decrease in the rate of synthesis of both albumin and α_1-acid glycoprotein with time of incubation, discussed earlier (TABLES 3 and 4).

The mechanism for stimulation of α_1-acid glycoprotein synthesis and possibly for α_1-acid glycoprotein catabolism by bradykinin is unknown, and whether these results can be duplicated in vivo is doubtful as kinins have a half-life in circulating blood of 15 seconds, being hydrolzed by kininases to inactive peptides. Others (33) have suggested that the activation of Hageman factor by injured tissue results in the release of kinin which is chemotactic for granulocytes, these cells in turn release more kinin and the tempo of the inflammatory response is accelerated. When accumulated granulocytes begin to disintegrate, destruction of kinin exceeds generation and inflammatory response subsides. These changes in bradykinin levels correspond roughly to the changes in the acute phase reactants in the inflammatory response but there is no evidence that bradykinin directly or indirectly induces these changes. The primary factor stimulating the liver cell to produce more acute phase reactants and less albumin is yet to be identified and may still originate from the site of the injury.

Although the factors which stimulate the synthesis of α_1-acid glycoprotein in response to inflammation were not identified, clearly, the approach used in this study was worthwhile and produced some interesting results. It is hoped that this initial study will provide others with the stimulus to further investigate the acute phase response of α_1-acid glycoprotein and eventually lead to the determination of the factors responsible.

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