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An investigation of total and 35S-labeled mucopolysaccharides in intestinal wall kidney of cattle, sheep and swine

Robert Kerns Berry

To the Graduate Council:

I am submitting herewith a dissertation written by Robert Kerns Berry entitled "An investigation of total and 35S-labeled mucopolysaccharides in intestinal wall kidney of cattle, sheep and swine." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

M.C. Bell, Major Professor

We have read this dissertation and recommend its acceptance:

R.G. Cragle, K.J. Monty, S.R. Tipton

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

August 22, 1967

To the Graduate Council:

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M C Bell
Major Professor

We have read this dissertation
and recommend its acceptance:

Raymond G. Cragle

Samuel R. Tipton

Robert H. Temberg

Accepted for the Council:

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Vice President for
Graduate Studies and Research

AN INVESTIGATION OF TOTAL AND ^{35}S-LABELED MUCOPOLYSACCHARIDES IN INTESTINAL WALL AND KIDNEY OF CATTLE, SHEEP AND SWINE

A Dissertation

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

Robert Kerns Berry

December 1967

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CHAPTER I

INTRODUCTION

Mucopolysaccharides (MPS) occur in the ground substance of connective tissue and have been isolated and identified from many diverse types of tissue. Because MPS have recently been shown to be associated with numerous physiological and pathological processes, interest has tremendously increased in their metabolic synthesis, functions and degradation. As MPS have the ability to bind water and cations, they are essential for the maintenance of an extracellular homeostatic environment. Regulation of water reabsorption in the kidney, lubricant for joints, regulation of cell permeability and involvement in the calcification processes of bone formation are other metabolic processes in which MPS have been demonstrated to participate. Abnormal MPS tissue concentrations and/or excretory patterns have been reported for individuals exhibiting atherosclerosis, gastric ulcers, arthritis, Marfan's disease and Hurler's syndrome.

Previous studies by Bell *et al.* (1964) which revealed that yearling cattle absorb and excrete ^{99}Mo very differently from growing swine cultivated our interest in MPS. Because of the strong electronegative charge due to active sulfate and carboxyl radicals which exist on the acid-MPS molecule, it was speculated that they could facilitate absorption, excretion and reabsorption of cations such as molybdenum; and, thus, be involved in the mechanisms responsible for dissimilarity

in utilization of radioactive Mo among species.

Research by Boström (1952) strongly justifies the utilization of ^{35}S isotopic labeling techniques to investigate chondroitin sulfuric acid metabolism *in vivo*. His results indicated clearly that ^{35}S retained by rats was firmly incorporated by articular cartilage as a part of the chondroitin sulfate structure and that the greatest rate of labeled $SO_4^=$ fixation occurred in tissue most rapidly synthesizing MPS.

The principle objective of this investigation was to establish and compare the concentration of total and sulfated MPS in intestine and kidney of cattle, sheep and swine. In order to accomplish the primary goal, it was necessary to determine: (1) accurate hexosamine, protein, mineral and sulfur analysis procedures; (2) a reliable method for tissue homogenization; (3) a procedure for separation and isolation of MPS from protein; (4) an estimation of MPS biosynthesis rate; and (5) plasma $SO_4^=$ and ^{35}S levels.

CHAPTER II

LITERATURE REVIEW

I. BIOCHEMISTRY OF SULFUR

Occurrence and evolutionary role. Sulfur occurs everywhere in the living world in a variety of compounds in animals, plants and microorganisms. The sulfur compounds found in living organisms represents different oxidized and reduced states of inorganic and organic sulfur. Some sulfur containing vital substances in mammalian tissue are the vitamins thiamine, biotin, lipoic acid and pantothenic acid; the amino acids cysteine and methionine; and structural elements such as the sulfated MPS and sulpholipids. Inorganic forms of sulfur of various oxidized states are hydrogen sulfide, elemental sulfur, thiosulfate, sulfur dioxide, sulfuric acid, sulfite and sulfate. Although the sulfur oxides and other sulfur compounds have shown toxic effects when present in abnormally high concentrations (Greenwald, 1954), sulfur and numerous compounds containing sulfur as a constituent are of importance for the proper functioning and well-being of many living organisms.

From the standpoint of evolution, this element has played an important biological role since the beginning of life on earth (Florkin, 1960; Oparin, 1938). One of the major constituents of the initial abmosphere of the planet earth was hydrogen sulfide. Because of its high presence in volcanic gases, it is generally assumed that sulfur was

originally derived primarily from sulfides of iron and other heavy metals below the earth's surface. Once the oxygen content of the atmosphere had become appreciable, the various sulfur oxides were formed. Szabo et al. (1950) report that metal sulfides became transformed into sulfates about 700 to 800 million years ago.

Urey (1952) stated that before the presence of an oxygen atmosphere, the sparse oceans were a "concentrated soup" consisting of a 10 per cent solution of organic matter. Conditions which existed at that time such as intense ultraviolet rays, high temperatures, raging thunder storms and lightning and powerful radioactivity would have highly favored the chemical and photochemical synthesis of self-perpetuating organic substances, for example, sulfur-carbon and sulfur-sulfur bonds. There is evidence that anaerobic life of some kinds existed more than 2 billion years ago (Urey, 1952) and one could speculate that sulfur, being one of the most prevalent elements of that age, must have been essential for these primitive organisms. The so-called "sulfur bacteria" of present time are believed to be representative of the earliest forms of life which existed in an atmosphere rich in hydrogen sulfide. In a primarily anaerobic atmosphere, sulfur probably functioned as a terminal electron acceptor for its users. Subsequently as life processes developed still further and aerobic conditions became established, plants and microorganisms incorporated sulfur into sulfur amino acids and other sulfur compounds. With the evolvement of the higher heterotrophic forms of life, they developed much more sophisticated uses of sulfur in their metabolic processes.

Sulfur cycle. A cyclic process affecting the distribution of sulfur operates in nature and is presented diagrammatically in Figure 1 according to the simplified scheme proposed by Young and Maw (1958). Oxidation of reduced sulfur compounds can be accomplished by animals and microorganisms, with formation of inorganic sulfate which may then be returned to the environment or used further in production of originally bound sulfur. Following death, most of the sulfur is converted to hydrogen sulfide during putrefaction, however, it becomes available to plants and animals once more through re-oxidation by other micro-organisms.

Sulfate activation and its incorporation into sulfur-containing compounds. It has now become apparent that one or more of the activated forms of sulfate (adenosine 5'-phosphosulfate, generally abbreviated as APS and 3'phosphoadenosine 5'phosphosulfate, generally abbreviated as PAPS) is involved in a number of assimilatory pathways of sulfur metabolism in microbes, plants and animals. De Meio and his co-workers (1953) were among the pioneers investigating sulfate activation and from their studies using rat liver systems, it soon became evident that adenosine triphosphate (ATP) was an important component required for producing phenyl hydrogen sulfate from phenol and inorganic sulfate. Hilz and Lipmann (1955) and Robbins and Lipmann (1956, 1957) established that two "active" intermediates (Equations 1 and 2) were formed during the overall process:

$$ATP + SO_4^{=} \rightleftharpoons APS + PPi \qquad (1)$$

$$APS + ATP \longrightarrow PAPS + ADP. \qquad (2)$$

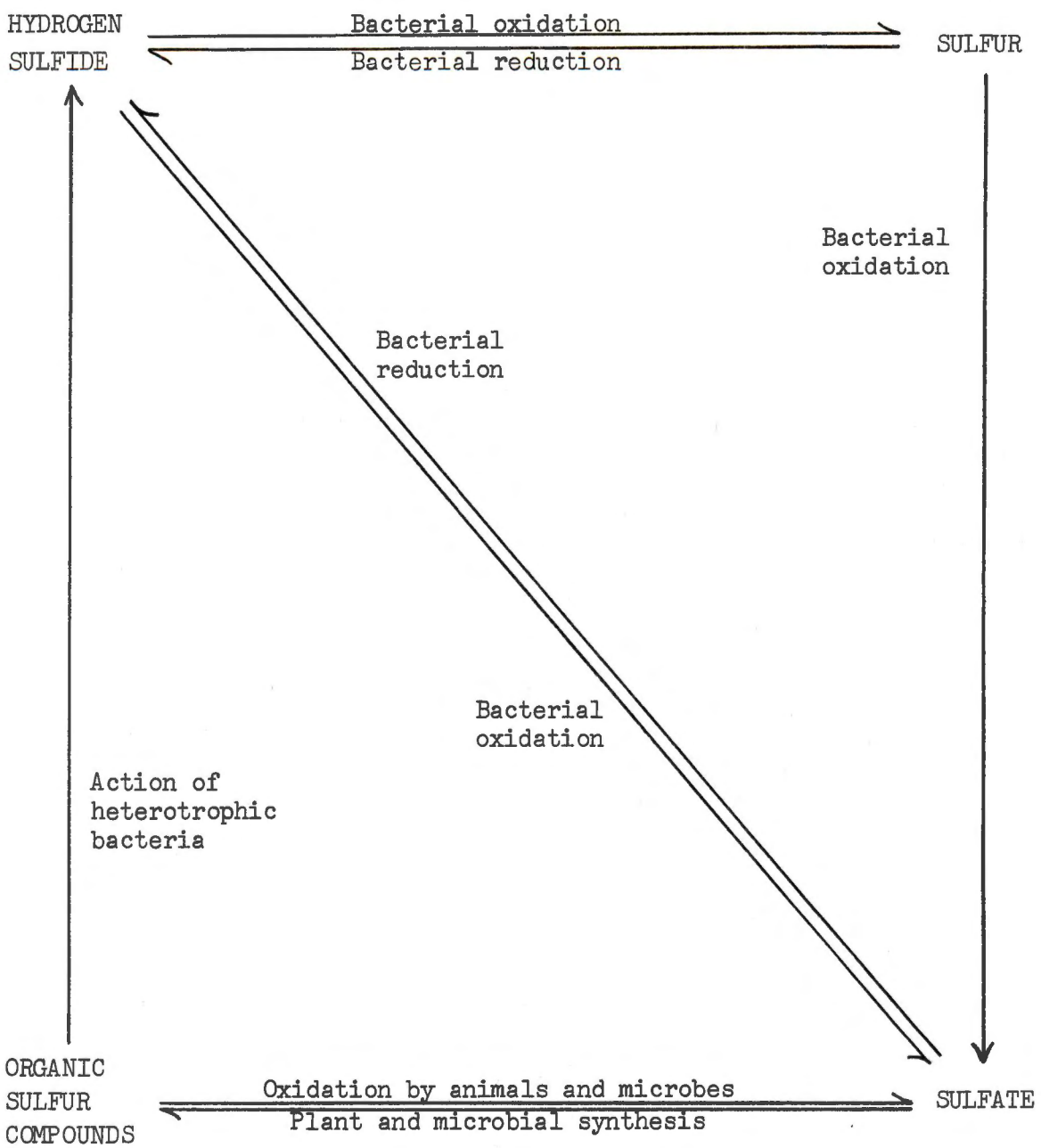

Figure 1. The sulfur cycle.

The equilibrium of reaction 1 is much in favor of the reverse reaction, but the destruction of pyrophosphate (PPi) and the removal of APS by subsequent transformations in reaction 2 makes the entire system slightly exergonic for formation of PAPS. Once PAPS has been formed, sulfate can be transferred from it to acceptors under the influence of the appropriate transferring enzymes.

The ability of microorganisms to use inorganic sulfate for the formation of cysteine and methionine is fairly widespread (Peck, 1962). Early indications that sulfate activation was an obligatory first step in the process came from studies on bacterial mutants which could grow on sulfide and thiosulfate but not on sulfate (Ragland and Liverman, 1958; Spencer and Harada, 1959). The sulfate $\longrightarrow$ cysteine pathway has been examined in a number of fungi and bacteria and studies have revealed that Salmonella typhimurium and Neurospora crassa convert inorganic sulfate to cysteine by the following pathway:

$$SO_4^{=} \longrightarrow \text{APS} \longrightarrow \text{PAPS} \longrightarrow SO_3^{=} \longrightarrow H_2S \xrightarrow{(\text{serine})} \text{cysteine}$$

(Leinweber and Monty, 1963; Siegel, Leinweber and Monty, 1965). Bacteria can form methionine from cysteine in the following stepwise reactions:

$$\text{cysteine} + \text{homoserine} \rightleftharpoons \text{cystathionine}$$

$$\text{cystathionine} \rightleftharpoons \text{homocysteine} + \text{serine}$$

$$\text{homocysteine} \xrightleftharpoons{(CH_3\text{-cobamide})} \text{methionine.}$$

Animals lack the ability to synthesize homoserine and, hence, are dependent on dietary provision of methionine. On the other hand, animals can form homocysteine from methionine and do not have to rely on a dietary supply of cysteine, provided there is available an excess of

methionine great enough to compensate for the lack of cysteine. Animals can readily reduce cystine to cysteine in a reversible process.

Sulfate represents the principal form of sulfur available to plants and at present, though the evidence is limited, there are indications that assimilatory sulfate reduction in higher plants resembles that in bacteria (Dodgson and Rose, 1966). Investigations by Pate (1965) show that the root, though a non-photosynthesizing tissue, is able to incorporate sulfate sulfur absorbed from the soil directly into sulfur-containing amino acids.

Through evolutionary development, the higher organisms (mammals) have lost the ability to incorporate inorganic sulfur into sulfated amino acids. On those occasions when incorporation has apparently been observed, it has generally been possible to trace it to microorganisms living symbiotically in the gastrointestinal tract. Symbiotic production of sulfur-containing amino acids is very important in the nutrition of ruminants.

One must look elsewhere other than amino acid synthesis for the importance of PAPS to animals, and at least two are known. The better known and more clearly defined one is to provide activated sulfate for the formation of sulfate-ester compounds. Their synthesis by transfer of sulfate from PAPS to the proper acceptors is governed by sulphotransferase enzymes which in many instances shows a high degree of acceptor specificity. Progress is being made in the study of the biosynthesis of the MPS, but there still exists a large gap in understanding the participating reactions and particularly the sequence in which they occur

during formation of the MPS molecule. Several sulphotransferases appear to be involved in chondroitin sulfate synthesis, each evidently showing specificity towards the macromolecule being synthesized. There is much doubt as to whether sulfate is transferred to preformed polysaccharide macromolecules or to some simpler building unit. The sharp specificity of the transferase enzyme in the synthesis of chondroitin 4 and 6 sulfates would tend to support the view that sulfation takes place at an early stage during biosynthesis.

Kent (1961) diagramed the metabolic steps necessary for MPS biosynthesis and the scheme he proposes indicates that PAPS supplies activated $SO_4^{=}$ to an oligosaccharide for the formation of the sulfated compound. As can be observed from his scheme, interconversion pathways exist for the conversion of sulfated and nonsulfated MPS within animal tissue and can probably be used by the animal to meet its specific need. The polymerization mechanism involved to form long alternating chains of uronic acid and N-acetyl-hexosamine is not presently understood. Also, from a review by Kent and Whitehouse (1955), it is evident that much knowledge needs to be acquired about the enzymatic degradation of MPS. Recent evidence reveals that PAPS may play a functional role in the transport of $SO_4^{=}$ through the intestinal wall of the rabbit (Dodgson and Rose, 1966).

II. MUCOPOLYSACCHARIDE METABOLISM

Chemistry. Acid MPS are of two types, sulfated and nonsulfated. The most prominent nonsulfated MPS is hyaluronic acid, whereas, the

chondroitin sulfates distinguish themselves as the most important of the sulfated polysaccharides. Chondroitin sulfate was first isolated in 1861 by Fischer and Boedeker. Meyer and co-workers (1956) have identified and characterized six distinct MPS with distinguishable properties. They were shown to differ in optical rotation, susceptibility to hyaluronidase and solubility of their salts in aqueous ethanol (Meyer and Rapport, 1951; Meyer et al., 1956). Hyaluronic acid and chondroitin sulfates A and C are attacked by testicular hyaluronidase, whereas, chondroitin sulfate B is not degradated by the enzyme (Meyer et al., 1956). Mucopolysaccharide molecules consist of long nonbranched polymers and most have alternating units of N-acetyl-hexosamine and uronic acid connected by a β 1,3 uronidic bond linkage. The linkage between repeating oligosaccharides is believed to be a β 1,4 glycosidic linkage. The structural components of MPS known to exist in mammalian tissue are presented in Table I (Clark and Grant, 1961). Available evidence suggests that in cartilage the typical unit consists of a single protein core to which are affixed 25 to 50 chains of chondroitin sulfate A, each of molecular weight 30,000 to 50,000. These chains appear bound to the protein core by covalent linkage resulting in a macromolecule complex having a minimum molecular weight of 4,000,000. Practically all MPS are found in combination with proteins, but neither the proteins nor the binding linkages have been entirely characterized. Levine (1925) suggested that chondroitin sulfate is bound to protein by ester linkage because it is so easily released by alkali. Meyer, Palmer and Smyth (1937), on the other hand, feel that chondroitin sulfate is combined in

TABLE I

STRUCTURAL COMPONENTS OF MUCOPOLYSACCHARIDES WHICH HAVE BEEN ISOLATED FROM MAMMALIAN TISSUE

Mucopolysaccharide	Hexosamine	Uronic acid	$SO_4^=$ on hexosamine carbon
Hyaluronic acid	Glucosamine	Glucuronic	None
Chondroitin sulfate A	Galactosamine	Glucuronic	C-4
Chondroitin sulfate B	Galactosamine	Iduronic	C-4
Chondroitin sulfate C	Galactosamine	Glucuronic	C-6
Heparin	Glucosamine	Glucuronic	Amino group of C-2
Heparitin sulfate	Glucosamine	Glucuronic	?
Chondroitin	Galactosamine	Glucuronic	None
Keratosulfate	Glucosamine, galactose	None	?

salt-like complexes with protein as it forms precipitates with a variety of proteins which are soluble in salt solutions. Muir (1958) postulates an ester linkage of the β-hydroxyl group of serine as a possible point of peptide attachment.

Distribution in mammalian tissue. The MPS are contained in the "ground substance" of connective tissue. "Ground substance" is loosely defined as an amphoteric material which form a matrix to bind the insoluble protein fibers, collagen or elastin of connective tissue into bundles. Its occurrence in tissue is widespread and the relative concentration of MPS varies greatly among tissues. Mucopolysaccharides have been isolated from skin, cartilage, tendons, ligaments, umbilical cord, vitreous humor, synovial fluid, heart valves, arterial wall, spinal disks, bone, cornea, liver, kidney, intestinal wall and embryonic cartilage. Tissues practically always contain a mixture of MPS, with an exception being vitreous humor which contains only hyaluronic aicd. Moreover, all MPS so far examined are found in combination with protein except hyaluronic acid of vitreous and synovial fluid. Meyer et al. (1956) classified tissues into seven groups according to the MPS isolated from them.

Biosynthesis and degradation. MPS synthesis occurs locally and chiefly in connective tissue cells, fibroblasts or chondrocytes, and it was well-documented in a recent review by Schiller (1966) that a normal concentration of acid-MPS is somewhat dependent upon adequate supplies of hormones to the connective tissue. According to Dorfman and Schiller

(1958), hypophysectomy reduces the turnover rates of both hyaluronate and chondroitin sulfate in the rat, but the administration of growth hormone only restores the turnover time of chondroitin sulfate. Schiller's studies in 1963 revealed that skin from young hypophysectomized or hypothyroid rats was characterized by increased concentrations of hyaluronic acid and decreased quantities of chondroitin sulfate. Thyroxine corrected the errors in MPS metabolism observed in the hypothyroid animal and reversed only the elevated level of hyaluronic acid found in the rats with removed pituitary glands. From these studies, it would appear that sulfated-MPS metabolism is influenced to a greater extent by somatotrophin, whereas, thyroid hormones play the greater role in the metabolism of hyaluronic acid.

Dorfman (1958), using ^{14}C and ^{35}S labeling techniques, showed a marked reduction in the incorporation of isotope in both hyaluronic acid and chondroitinsulfuric acid in the skin of alloxan-diabetic as compared to normal rats. Insulin administration restored the rate of MPS synthesis to normal, thus revealing conclusive evidence that the level of insulin is important in regulating the rate of formation of this heteropolysaccharide. Cortisol inhibits the synthesis of sulfated polysaccharides in chick embryos (Ebert and Prockop, 1967) and in cartilaginous bone rudiments of chick embryos <u>in vitro</u> (Barrett, Clement and Dingle, 1966). Whitehouse and Lash (1961) have described inhibition of sulfation without interruption of polysaccharide synthesis by chick somites when cortisol was added to the incubation mixture, whereas, Whitehouse and Borström (1962) found cortisol to inhibit polysaccharide

synthesis and sulfation equally in slices of bovine cartilage and cornea. Nakamura and Mosuda (1966) demonstrated that daily subcutaneous injections of synthetic estrogen reduced acid-MPS levels and incorporation of sulfate-^{35}S into MPS of eyeball, cartilage and aorta of rats. Investigations with rabbit nucleus pulposus by Davidson and Small (1963a) revealed that the ratio of keratosulfate to chondroitin sulfate increases uniformly as a function of time and that growth hormones, estrogen and androgen alter the MPS composition of mature tissue toward that represented by a younger age. The studies by Davidson and Small (1963b) indicate that testosterone causes a marked increase in hyaluronic acid content of skin.

Experiments have been performed to show the effect of the enzyme, papain, on protein-polysaccharide metabolism. Small doses of papain, administered in vivo, caused a marked increase in ^{35}S-MPS incorporation in rat epiphyseal cartilage 6 and 24 hours after injection (Guri and Bernstein, 1967). Intravenous injections of papain in young rabbits have consistently resulted in marked collapse of the ears, which was shown to be due to massive loss of chondroitin sulfate from the cartilage (Bryant, Leader and Stetten, 1958; Jasinski and Weigel, 1965).

Several investigations indicate that the concentration and relative proportion of the different types of acid polysaccharides vary with age. Foley and Boas (1955) report that the concentration of hexosamine in the carcass of growing rats decrease between 10 and 80 days of age. Loewi and Meyer (1958) have determined that hyaluronate is the chief constituent of embryonic pig skin, which also contain 20 per cent

of chondroitin sulfate C, whereas, in adult pig skin the chief MPS is chondroitin sulfate B, with only a trace of C being present. In contrast, the rib cartilage of the newborn contains chondroitin sulfate A, compared to a predominance of C in adult life (Meyer et al., 1956). Kaplan and Meyer (1959) state that the total chondroitin sulfate content of human rib cartilage decreases progressively from birth but reaches a plateau between 20 and 30 years.

There is ample evidence indicating that certain dietary constituents influence MPS rate of synthesis in the vertebrate. Brown, Button and Smith (1965) have obtained results revealing that the level of inorganic sulfur in the diet of the monogastric animal may be important for sulfation of ester-sulfate compounds. Significantly less neutral salt-soluble and total collagen was produced in the skins of rats fed diets which contain 0.0002 per cent sulfate compared with those fed diets containing 0.02 per cent sulfate. Studies by Leach and Muenster (1962) demonstrated that manganese deficiency causes a severe reduction in the MPS content of epiphyseal cartilage along with the organic matrix of other portions of the chick bone. A decrease in MPS which contain galactosamine accounted for most of the changes observed. Linker, Coulson and Carnes (1964) obtained approximately three times as much MPS from the aortas of copper-deficient swine as from normal controls.

As the fundamental tissue change observed in ascorbic acid deficiency occur in the intercellular matrix, it was felt that vitamin C might be concerned with synthesis of the glycosaminoglycans (MPS). However, the effect of the vitamin on MPS production has received

contradictory answers and remains obscure. The works of Reddi (1954) and Slack (1957) revealed that vitamin C deficient guinea pigs synthesize less ^{35}S into chondroitin sulfate than do those receiving normal levels of ascorbic acid. Increased concentrations of total acid MPS in tissue of scorbutic guinea pigs have been reported by Slack (1957) and Robertson and Hinds (1956) and evidence was obtained in both studies that most of this increased polysaccharide level was due to an elevated quantity of hyaluronic acid. Kofoed and Robertson (1966) investigated the influence of vitamin C on sulfated-MPS metabolism under conditions of enhanced synthesis. Guinea pigs fed an ascorbic acid deficient diet had similar quantities of sulfated-MPS in their trachael, nasal and auricular cartilage as did animals receiving diets adequate in vitamin C. The rate of resynthesis measured as net synthesis during 7 days following depletion of the tissue by papain injection was significantly decreased in vitamin C deficient animals. They report, however, that when other animals were restricted to the amount of feed eaten by the scorbutic animals, but were given adequate ascorbic acid, the resynthesis was even less than in the scorbutic animals.

Studies have implicated vitamin A to be involved in MPS formation. The <u>in vitro</u> incorporation of ^{35}S sulfate into chondroitin sulfate of the cartilage matrix (Dziewiatkowsky, 1954) and colon (Wolf and Johnson, 1960) of rats deficient in vitamin A was much less than the control animals. Levi and Wolf (1965) and Subba Rao, Sastry and Ganguly (1963) found reduced ATP: sulfate adenylyl transferase in many tissues of rats fed rations deficient in vitamin A. Platelets from vitamin A-treated

and vitamin A-deficient rats had a greater uptake of ^{35}S than did smaller control platelet (McDonald, 1966). His studies also revealed that the hexosamine content of platelets from vitamin A-excess rats was 26 per cent greater and that the hexosamine content of platelets from rats deficient in vitamin A was 26 per cent less than platelets from control animals.

Metabolic functions. MPS are found widely distributed throughout the animal's body and has many diverse physiologic and pathologic functions. Because they are a compound of large molecular weight and thus unable to pass through cell walls and as they have the capacity to bind water and cations, such as Na (Dorfman, 1958), they may be important factors in maintaining homoestasis of extracellular space. The presence of both a carboxyl and sulfate radical on each repeating unit of chondroitin sulfate confers a negative charge, thereby, making it possible for them to attach to cations. Unfortunately, very little knowledge exists concerning the role of MPS in this phenomenon. Fessler (1957) hypothesized that as MPS are viscous, highly hydrated polyanions, showing a marked degree of interaction even in dilute solution, they may impede the flow of interstitial water and thus contribute to the resistance of compression of connective tissue. Mucopolysaccharides occur in the connecting tubules of the nephron and in collecting tubules of the kidney of all land vertebrate and this is the area where facultative reabsorption of water occurs in response to antidiuretic hormone. According to Ginetsinskii (1959), hyaluronic acid is the substrate of

hyaluronidase, which is secreted under the influence of antidiuretic hormone. The studies of Rienits (1966) also suggest that MPS may be involved in water retention as a significant positive relationship was found to exist between the total hyaluronic acid and water content of monkey and baboon skin. Day's (1952) findings, which indicate that MPS reduce cell permeability, are in agreement with those of Duran-Reynals (1942). The addition of hyaluronidase showed a ten-fold increase in the flow of saline through mouse connective tissue membrane. A known effect of radiation injury is the depolymerization of MPS and connective tissue ground substance (Edgerly, 1952). Investigations of Detrick (1963) have shown that the decrease in these substances brought about by irradiation leads to increased capillary permeability.

Chondroitin sulfate has been suggested as a necessary factor in bone calcification. Sobel (1955) proposed that it, together with collagen, was responsible for initiating the process of crystal formation, whereas, Glimcher (1959) proposed inhibition of calcification as the main function. Bowness and Lee (1967) showed that soluble sodium chondroitin sulfate, from bovine ribs or puppy epiphyseal plate, at a concentration of 80 mM. in terms of glucuronate, decreased the amounts of calcium and phosphate precipitated from solutions of 6.9 mM. phosphate and 6.9 or 13.8 mM. calcium during a 2-hour incubation.

Doyle (1967) has recently isolated an up to then undistinguished MPS which he named "lorenzan sulphate" from the glands of Lorenzini of Squalus acanthias and he speculated that the function of this MPS might be to bind pharmacologically active bases and release them to nerve

endings on changes of temperature or salinity.

An accumulation of acid-MPS has been reported by Gore and Larkey (1960) to be an early manifestation of atherosclerosis. Sinitsyna (1966) reports that a rather high MPS content is characteristic of a completely "mature," fully developed atherosclerotic plaque. Changes in MPS organization of the human aorta which occur constantly throughout life with or without any pathological abnormality have been described by Velican and Velican (1964) as "the evolution potential soil" for the development of atherosclerosis. To the contrary, Kirk and Dyrbye (1957) did not observe any variation with age in the total hexosamine, uronic acid, sulfate and protein content of 30 samples of MPS isolated from human aorta tissue which were obtained from individuals ranging in age between 1 and 76 years. However, results obtained from their chemical and enzyme analyses did support the findings of Velican and Velican (1964), in that samples derived from aortic tissue of 60- to 70-year-old subjects contained a greater proportion of chondroitin sulfate to total MPS than samples obtained from children and young adults.

Faulty MPS metabolism has been connected to several other pathological conditions. The knees of patients with arthritic joint diseases have synovial fluid of lower viscosity, and Ogston and Stanier (1953) have speculated that this reduction is due to a lowered concentration of hyaluronic acid. The data of Schrager (1964) indicated that gastric secretions of patients with duodenal ulcers or gastric carcinoma contain a decreased quantity and proportion of sulfated-MPS to nonsulfated-MPS. Cartilage from individuals having Marfan's syndrome have more

keratosulfate than in normal and Berenson and Serra (1959) report that the urinary excretion of chondroitin sulfate A or C is considerably increased above normal in this disease. In patients with Hurler's syndrome, tissue and urine contain unusually high amounts of chondroitin sulfate B and heparitin sulfate, however, it was determined by Friman (1967) that the spinal fluid of individuals with this abnormality had a very low concentration of acid-MPS compared to other tissue and body fluid.

III. COPPER, MOLYBDENUM AND SULFATE INTERRELATIONSHIP

As the sulfate ion is a component of chondroitin sulfate and because it was postulated that the quantity of this compound in certain tissue of the animal's body is concerned with influencing the excretory pattern of molybdenum, a brief review of the relationship between Cu, Mo and $SO_4^=$ will be presented. Numerous investigations have amply substantiated that the metabolism of copper and molybdenum is influenced by their dietary levels along with the concentration of inorganic sulfate in the diet and this relationship can be either synergistic or antagonistic depending upon the concentration of the ions and the elements involved (Dick and Bull, 1945; Ferguson, Lewis and Watson, 1938; Comar, Singer and Davis, 1949; Gray and Daniel, 1954; Wynee and McClymont, 1955; Miller, Price and Engel, 1956; Davies, Reid and Couch, 1959). The copper and molybdenum interrelationship in sheep and cattle was reviewed by Cunningham in 1950, by Dick in 1956, and in both the ruminant and monogastric animal by Miller and Engel in 1960 and by Underwood in 1962.

A series of studies by Hart and associates (1928) at the University of Wisconsin revealed that a small amount of copper is necessary for a normal rate of hematopoiesis. It has been shown that copper is not a component of the hemoglobin molecule and it is believed to prevent anemia by facilitating the utilization of iron by the blood-forming organs and mobilization of iron from tissue. Clinical disorders which have been associated with a dietary deficiency of copper are anemia, depressed growth, bone disorders, depigmentation of hair or wool, abnormal wool growth, neonatal ataxia, impaired reproductive performance, heart failure and gastrointestinal disturbances. Particularly in cattle and sheep, continued injection of copper in excess of that needed can lead to chronic copper poisoning which is characterized by an accumulation of copper in the tissues, especially the liver, followed by catastrophic liberation of the liver copper into the blood stream. Copper toxicity usually results in extensive hemolysis and jaundice followed by death. Low intakes of molybdenum increase the animal's susceptability to the disease, whereas molybdenum supplement is of value in controlling the condition.

Evidence has appeared implicating dietary copper as being important in preventing and controlling pathological disorders, "peat scours" and "teart." These diseases occur frequently in cattle grazing pastures containing abnormally high concentrations of molybdenum and are found to be most prevalent in Nevada, California, England and New Zealand. Cummingham (1950) states that in forages which contain 3, 5, 7 and 10 p.p.m. copper; 3, 7, 15 and 20 p.p.m. molybdenum, respectively,

must be present to produce molybdenum toxicity in ruminants. However, Kirchgessner, Weser and Friesecke (1964) reported that 4 millimoles of supplemental copper per kilogram feed increased molybdenum retention in growing swine.

In the past, molybdenum has been viewed primarily from its toxic standpoint. Underwood (1962) stated that all cattle are susceptible to molybdenum toxicity from "teart" pasture, sheep are less susceptible and horses are not affected. Davis (1950) reported no toxicity from feeding 1,000 p.p.m. Mo to pigs for 3 months. Evidence accumulated by Bell et al. (1964), which indicate that cattle lack renal mechanisms necessary to eliminate excess molybdenum from the body proper, reveal evidence as to why cattle and sheep are less tolerant and thus more susceptible to conditions involving elevated levels of molybdenum than swine. Twenty barrows and six steers fed similar dietary levels of copper, molybdenum and sulfate were administered oral and intravenous ^{99}Mo which contained two levels of stable carrier molybdenum. Molybdenum-99 from an oral dose entered the blood of cattle at a much slower rate and to a lesser degree than swine, however, once it was absorbed into the blood, it was released considerably slower by steers than pigs. In swine, both oral and intravenously given ^{99}Mo were rapidly removed primarily by way of urinary excretion. To the contrary, cattle eliminated most of their radioactive Mo through fecal excretion, regardless of the type of dosing employed. Added carrier molybdenum increased ^{99}Mo excretion more in intravenously than orally dosed swine, suggesting the existance of renal processes in swine that rapidly eliminate excess molybdenum.

Recently, evidence has been emerging which indicate that molybdenum is an essential element for animals. A nutritional role for molybdenum for growth by chicks (Reid, Davies and Couch, 1958) and by lambs (Ellis et al., 1958) has been reported. Molybdenum was shown to be necessary for xanthine oxidase and aldehyde oxidase, and the need for its presence to maintain normal activity of this enzyme in the rat intestine has been clearly demonstrated. Roussos and Morrow (1966) reported the isolation of a bovine intestinal xanthine oxidase, a metalloprotein enzyme which contains iron, copper and flavin adenine dinucleotide, but not molybdenum.

The effect of sulfate upon molybdenum-fed rats has been reported by a number of investigators (Miller, Price and Engel, 1956; Van Reen, 1959). Sulfate overcame the rat growth depression caused by levels of molybdenum fed up to 1,200 p.p.m. The inclusion of molybdenum in the diet caused an increase in the liver and blood levels of molybdenum, which could be reduced when inorganic sulfate was added to the diet containing molybdenum. Feaster and Davis (1959) postulated that as lower levels of ^{35}S were found in the liver and bones of rats receiving elevated amounts of molybdenum, the beneficial effect of sulfate lies simply in its replacement value. In studies by Goodrich and Tillman (1966), increasing the level of dietary sulfur from 0.10 per cent to 0.40 per cent significantly elevated molybdenum concentration in the liver.

During the 1940 decade, research workers discovered there was an interrelationship between copper and molybdenum (Dick and Bull, 1945;

Kulwich et al., 1951). Investigations by Dick and Bull showed that the concentration of copper in the liver of sheep and cattle could be drastically reduced by increased intakes of molybdenum. It was not until 1952 that sulfate was implicated as a factor in complicated copper deficiency (Dick, 1952; 1953a; 1953b; 1953c; 1954a; and 1954b). The first indication that elements other than copper and molybdenum were involved was obtained by Dick when he was able to develop copper deficiency in sheep fed chaffed lucerne hay and unable to produce the condition by feeding chaffed oaten hay even though both rations contained equal amounts of copper and molybdenum. The reason for the different response to the two forages was established to be the higher content of sulfate in the lucerne hay. Through further studies, Dick demonstrated that neither molybdenum nor sulfate alone interferes with copper retention and that the effectiveness of either is increased to a maximum as the intake of the other is increased.

The mechanisms involved in this complex mineral relationship have not been clearly defined. Dick has formulated the hypothesis that permeability of membranes is decreased by elevated Mo and $SO_4^=$ and that a membrane whose permeability to molybdenum is impeded or blocked by sulfate impedes or blocks copper transport. He believes that under the influence of Mo and sulfate in the diet, the absorption of copper will be decreased and consequently the rate of accumulation of copper in the liver will be reduced. As the molybdenum and sulfate intakes are increased, the amount of copper will be insufficient to make good the normal wastage of copper by excretion, the animal's copper reserves will

be depleted and a state of copper-depletion deficiency will be established if the animal is exposed to these conditions for a sufficiently long period.

Mills et al. (1958), Halverson et al. (1960), Monty and Click (1961) and Siegel and Monty (1961) using rats as the experimental animal have developed a mechanism for the copper-molybdenum interrelationship. They have presented evidence that during molybdenum toxicity, there was a reduction in the activity of liver sulfide oxidase. From these studies they postulated that elevated molybdenum exerted its effect on enzymes involved in the normal metabolic transformation of sulfur in the body and that a decrease in the level of sulfide oxidase would cause an accumulation of highly-insoluble copper sulfide. The formation of this compound would remove metabolically available copper and eventually place the animal in a state of copper deficiency. This information would also reveal why the effect of molybdenum is greatest on reducing copper utilization when the sulfur content of the diet is elevated.

From this brief discussion of the copper, molybdenum and sulfate interrelationships, it is apparent that the animal's ability to utilize any of these ions is determined by the concentration of all three elements in its diet.

In reviewing various facets of literature associated with this problem, it has become evident that practically all investigations concerning MPS used humans or the small laboratory animal as the experimental subject, whereas, the majority of studies dealing with the Cu, Mo and $SO_4^=$ relationship were executed with large animals, particularly ruminants.

This study should aid in bridging the gap between data from small laboratory animals and farm livestock in these areas of research.

CHAPTER III

MATERIALS AND METHODS

I. EXPERIMENTAL ANIMALS AND RATIONS

Seven Hereford steers, eight pigs of mixed breeding and seven Hampshire-Suffolk crossbred sheep, weighing 168 kg., 19 kg. and 39 kg., respectively, were fed ad libitum practical rations (Tables II, III and IV) which contained equivalent amounts of 8.3 p.p.m. copper, 2.1 p.p.m. molybdenum and 0.16 per cent sulfur throughout the 30-day experiment. The rations were formulated to provide the necessary requirements for growth as listed by N.R.C.'s standards for cattle (1963), for sheep (1964) and for swine (1964). The required feed for the entire study was obtained and separately stored with aliquots taken for copper, molybdenum and sulfur analyses. Feed copper and molybdenum were determined on triplicate samples by the procedures outlined in the Perkin-Elmer Manual (Analytical Methods for Atomic Absorption Spectrophotometry: 1964). The total sulfur content of the feed was assayed in triplicates according to procedures outlined by Snell and Snell (1949).

All experimental animals were obtained from UT-AEC Agricultural Research Farm and approximately 1 week prior to being placed on experiment, the swine were castrated and treated for external and internal parasites. For convenience, the cattle trials were conducted during October-November, 1966; the swine trials during December-January, 1966-67; and the sheep trials during February-March, 1967. As no evidence was

TABLE II

COMPOSITION OF RATIONS[a]

Cattle		Sheep		Swine	
Ingredient	Per cent	Ingredient	Per cent	Ingredient	Per cent
Corn	35.40	Corn	36.40	Corn	74.50
Ground alfalfa hay	64.00	Ground alfalfa hay	62.90	Soybean oil meal	18.00
Vitamin A and D premix[b]	0.01	Vitamin A and D premix[b]	0.01	Dehyd. alfalfa meal	3.00
NaCl	0.43	NaCl	0.69	Fish meal	2.62
$CaHPO_4 \cdot 2H_2O$	0.16			NaCl	0.50
				$CaCO_3$	0.95
				$CaHPO_4 \cdot 2H_2O$	0.17
				$CuCO_3$	0.00028
				MoO_3	0.00014
				Terramycin TM-10[c]	0.10
				Vitamin premix	0.09
				Trace mineral mix	0.07

[a]Rations contained the following according to calculated values:

	Cattle	Sheep	Swine
Total digestible nutrients	60.5%	60.8%	74.8%
Crude protein	9.1%	9.1%	16.8%

[b]Vitamin A and D premix contained 4,000,000 I.U. of vitamin A and 800,000 I.U. of vitamin D per 454 gm. premix.

[c]Terramycin TM-10, manufactured by Chas. Pfizer and Co., New York, N. Y., contains 10 gm. oxytetracycline hydrochloride/454 gm., thus 45.4 gm. TM-10 supplied 10 mg./454 gm. of ration.

TABLE III

TRACE MINERAL MIX

Ingredient	Gm./100 kg.	Provides in ration		
		Mg./454 gm. feed	P.P.M.	Element
$CoCO_3$	0.16	0.37	0.81	Co
KI	0.03	0.11	0.24	I
$MnCl_2 \cdot 4H_2O$	17.65	22.24	49.00	Mn
Fe_2O_3	12.14	38.50	85.00	Fe
$ZnCO_3$	9.60	22.70	50.00	Zn

TABLE IV

VITAMIN PREMIX[a]

Vitamin	Gm./454 kg.		Provides/kg. feed
Vitamin A and D premix	70.85	(8,810 I.U. Vit. A/gm.)	1,373.32 I.U.
		(1,762 I.U. Vit. D/gm.)	274.65 I.U.
Riboflavin	1.25		2.75 mg.
Calcium panthothenate	5.00		11.00 mg.
Niacin	6.25		13.75 mg.
Pyridoxine	0.63		1.39 mg.
Choline chloride (25%)	37.52		20.64 mg.
Vitamin B_{12}	5.00	(1 mcg./mg.)	11.00 mcg.
Antioxidant BHT	62.50		137.50 mg.
Methionine hydroxy analogue[b]	227.00		499.40 mg.
Soybean oil meal	719.00		
TOTAL	1,135.00		

[a]1,135 gm. of vitamin premix equals 0.25 per cent of ration of which 0.09 per cent was vitamins, antioxidant and methionine. The remainder of the premix was 0.16 per cent soybean oil meal which has been deducted from the total SBOM content of the ration.

[b]Courtesy of Monsanto Chemical Company, St. Louis, Mo. Methionine content of the swine ration was calculated to be low, therefore, it was added to the ration by way of the vitamin premix.

found in the literature indicating a seasonal affect on MPS metabolism and as the animals were maintained in closed housing with regulated temperature, it was believed that the seasons would have no influence on the results obtained from this investigation. Also because of lack of manual help and facilities, it would not have been possible to study all three species at one time.

Three weeks following initiation of the trial, the animals were placed in metabolism crates for a 7-day adjustment period. Depending on their performance during the feeding and adjustment period, six animals of each species were selected for use in the metabolic phase of the study.

II. DOSING PROCEDURE AND COLLECTION OF SAMPLES

The animals were intraperitoneally injected twice with 0.23 mCi. of $Na_2{}^{35}SO_4$/kg. body weight/dose at 24-hour intervals. Boström (1952) reported that in adult rats intraperitoneally injected with ^{35}S-labeled Na_2SO_4, maximum uptake by chondroitinsulfuric acid of the cartilage occurred 24 hours after injection. Radiosulfur was obtained from Nuclear Science and Engineering Corporation, Pittsburgh, Pennsylvania, as carrier free $Na_2{}^{35}SO_4$ in 0.1 N. HCl. The $Na_2{}^{35}SO_4$ dosing solution was adjusted to approximately pH 7 with 1 N. NaOH using phenolphthalein as an indicator. The final concentration of the dosing solution was 2 mCi. $Na_2{}^{35}SO_4$/ml. for the calves and 1 mCi. $Na_2{}^{35}SO_4$/ml. for the pigs and sheep.

To obtain plasma the animals were bled 2, 4, 6, 8, 12, 18 and 24 hours after each dosing. Sodium heparin was applied to syringes and

bleeding tubes prior to use. Approximately 20 ml. of blood were taken from the jugular vein at each bleeding period with a California bleeding needle (cattle), 2 inch-18 gauge bleeding needle (swine) and 1 inch-19 gauge bleeding needle (sheep). A 200 µl. sample of plasma from each bleeding period was analyzed for sulfur-35 activity. Samples of plasma were composited for stable inorganic sulfate assays. The animals were sacrificed 24 hours following the second administration of ^{35}S and sections of duodenum (3 to 30 cm. caudal to the omasul-duodenal orifice), jejunum (equidistance from the ends of the small intestine), ileum (3 to 30 cm. cranial to the ileal-cecal orifice), cecum, middle of colon and kidney were washed with cold autoclaved physiological saline and frozen in plastic bags for future laboratory analyses. A section 5 cm. in length from the middle of the small intestine of each animal was fixed in formaldehyde for future preparation of autoradiograms.

III. LABORATORY ANALYSIS

In order to assure that $^{35}SO_4^=$ labeling was indicative or representative of the quantity of sulfated MPS in these tissues, certain laboratory procedures were established. As a considerable proportion of the time and energy employed in this investigation was concerned with establishing these laboratory procedures, some of this preliminary work will be discussed.

One of the primary laboratory problems was the development of a homogenization procedure which would produce repeatable concentrations of hexosamine/unit protein for the same tissue. Homogenization of tissue

with a Waring blendor, scraping the mucosal cells from intestinal wall followed by homogenization of the cells for 1 minute and homogenization of the tissue with a Potter-Elvehjem homogenizer were all tested on triplicate samples of swine tissue and the results obtained with each technique summarized in Appendix Tables XI, XII and XIII, respectively. Analysis of hexosamine following dialysis revealed good recovery for all procedures which indicated that the MPS molecule was not degraded during sample preparation to the extent that any of it was able to pass through the dialysis tubing. Sulfur-35 activity per micromole hexosamine was less after dialysis and was probably due to removal of free $^{35}SO_4^=$ that was not incorporated into the MPS molecule. Use of a Potter-Elvehjem homogenizer on small pieces of tissue (Procedure III) was shown to be the most suitable method. Within 2 days after sacrifice of the animal, approximately 2 gm. of tissue/10 ml. ice-cold single-distilled water was homogenized for about 1 minute. It was generally necessary to perform four separate homogenizations of each tissue to provide sufficient sample for future analyses. These homogenates were then stored at 4° C.

Protein and hexosamine (a component of MPS) analyses were performed in duplicate on the tissue homogenates. Protein was assayed according to the procedure described by Lowry et al. (1951) and optical density readings of unknowns compared to that of known concentrations of bovine serum albumin (Figure 2). The procedure of Boas (1953) with modifications was used for hexosamine determinations. One ml. of homogenate or enzyme purified sample and 1 ml. of 2 N. hydrochloric acid were placed in a 10 ml. volumetric flask and hydrolyzed for 4 hours.

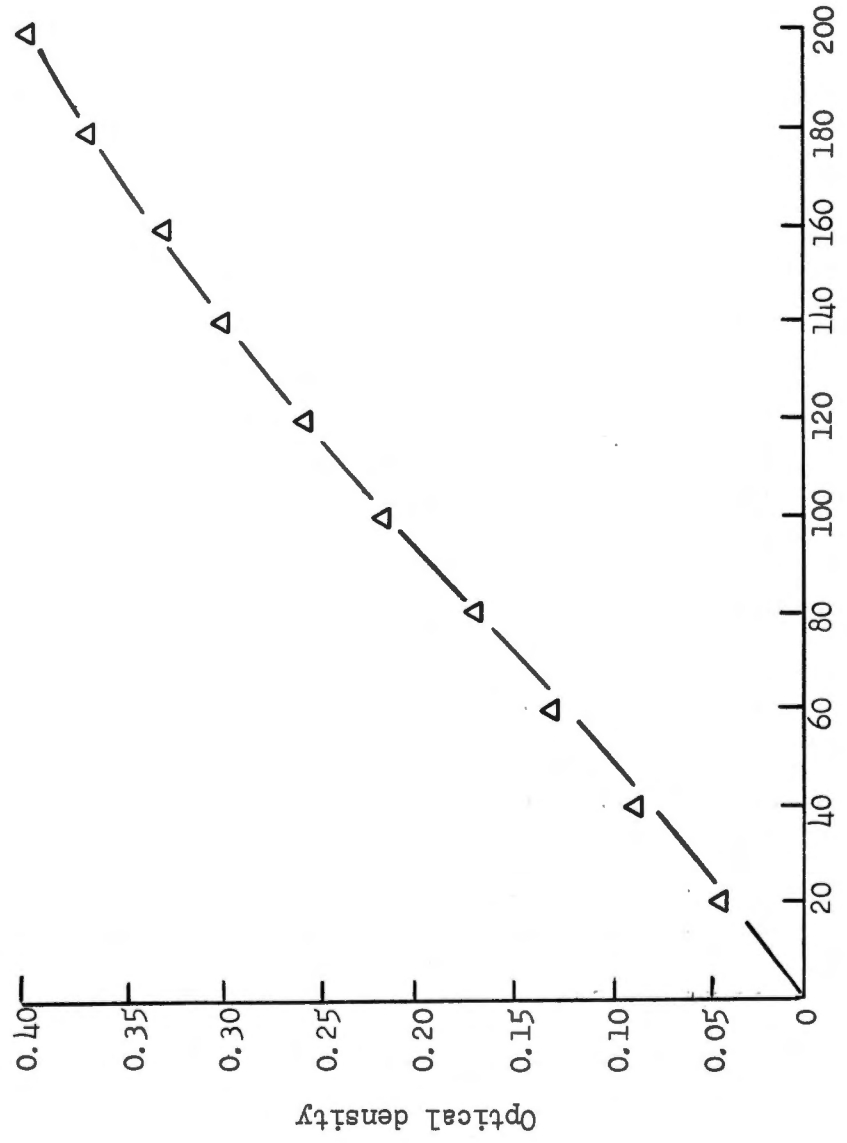

Figure 2. Protein (bovine serum albumin) standard curve.

After cooling, the samples were taken to volume with single-distilled water and filtered through No. 1 Whatman filter paper. For each group of sample determinations, a blank and a glucosamine HCl[1] and galactosamine HCl[1] standard of known quantity were prepared to an acid concentration equal to that of the samples from which identical volumes were taken for color development. Two ml. of the filtered hydrolyzed sample, blank and standard were pipetted into a 10 ml. volumetric flask to which 1 ml. of 2 per cent acetylacetone (2,4-pentanedione) in 1 M. Na_2CO_3 was added. The samples were placed in a thermostatically controlled water bath (89-92° C.) for 45 minutes. After cooling the samples in a water bath at room temperature, 2.5 ml. of 95 per cent ethanol was placed into each volumetric flask and mixed. To this solution, 1 ml. Ehrlich's reagent (2.67 per cent solution of p-dimethylaminobenzaldehyde in a 1:1 mixture of ethyl alcohol and concentrated HCl) was added and carefully mixed. The flasks were made to volume with 95 per cent ethanol, mixed and after 1 to 2 hours, optical density readings were made at 530 mμ. on a Bausch and Lomb Spectronic 20 Spectrophotometer. A series of glucosamine HCl and galactosamine HCl optical density values of known concentration are presented in Figure 3.

It was believed essential to determine or eliminate the amount of ^{35}S in these tissue that was incorporated into sulfated amino acids by microorganisms located in the gastrointestinal tract. Today, most investigators seem to agree that the best way of extracting all MPS from

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[1]Purchased from Nutritional Biochemicals Corporation, Cleveland 28, Ohio.

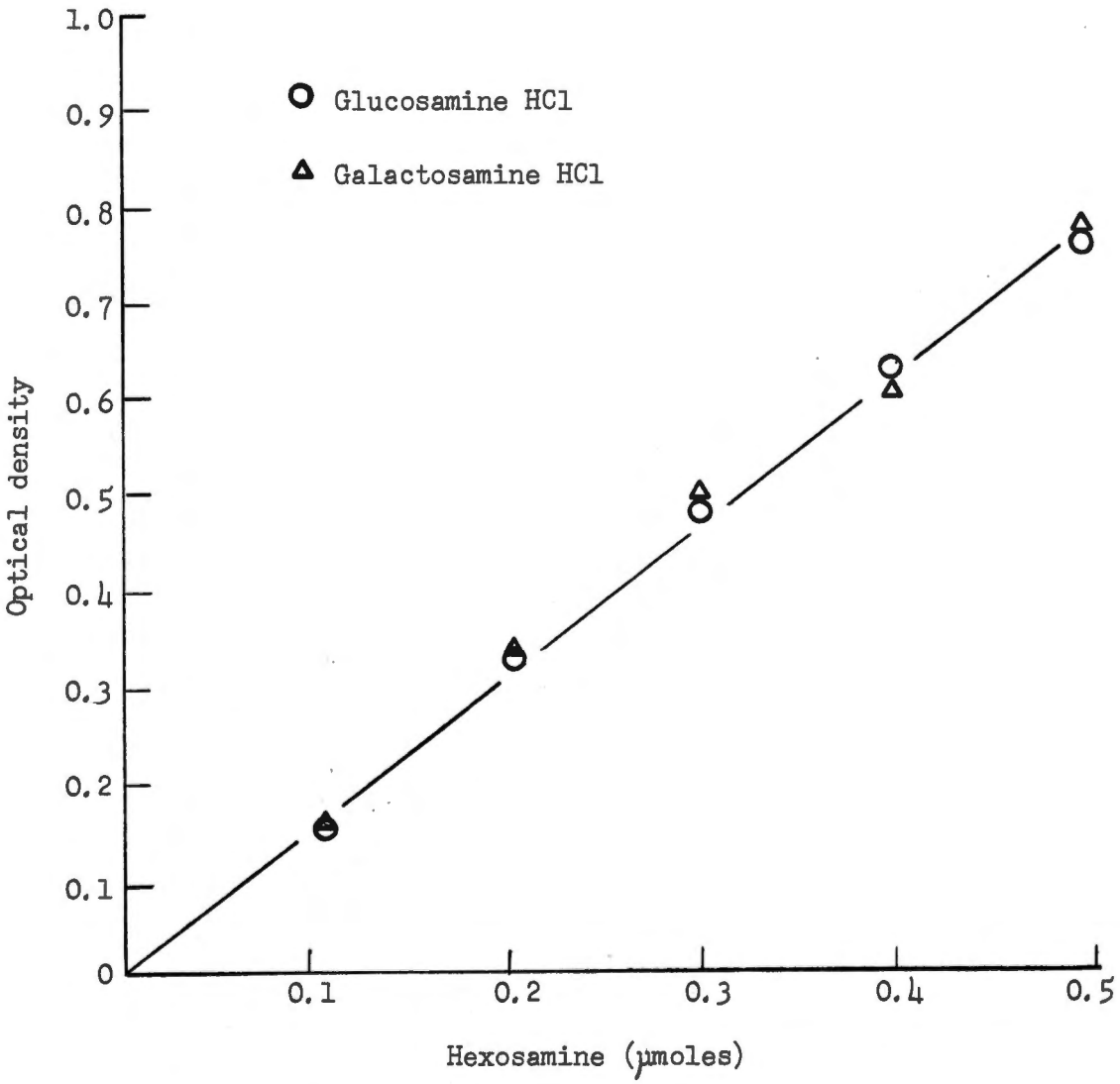

Figure 3. Hexosamine standard curve.

tissue is to break down the protein by proteolytic enzymes and follow this treatment by some further purification procedures in which the products of protein splitting are removed (Scott, 1960). The macro-procedure of Meyer et al. (1956) for protein-MPS separation by pepsin and trypsin enzymes was, comparatively speaking, adjusted to one of micro dimension for use in this study. Ten ml. samples of the tissue homogenates were placed in 40 ml. centrifuge tubes and digested with pepsin and trypsin enzymes at 37° C. in a temperature regulated water bath. Initially, the pH of the homogenate was adjusted to 1.5 according to alkacid tester pH paper with 1 or 2 N. HCl and NaOH and the homogenate suspension was covered with 1 ml. toluene. Fifty mg. pepsin[2] (3 X crystalline) was added to each tube and the mixture allowed to incubate for 72 hours, which was followed by readjustment of the pH to 7 and incubation for 24 hours with 70 mg. trypsin[2] (2 X crystalline-salt free). Upon completion of the digestion period, the undigested residue was removed by centrifugation in the cold (3° C.) at 1,500 X G. Two hundred and fifty mg. of solid calcium acetate dehydrate and 0.15 ml. glacial acetic acid were added to the supernate, and the MPS were precipitated with 3 volumes of 95 per cent ethanol during 24 hours in a cold room (4° C.). The ethanol-MPS precipitate was washed with approximately 20 ml. of 80 per cent ethanol and taken into solution with 5 ml. of 0.5 N. acetic acid and 5 per cent sodium acetate mixture. Sevag reagent (0.67 ml. of amyl alcohol and 2.0 ml. of chloroform per 10 ml. of sample) was

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[2]Nutritional Biochemical Corporation, Cleveland 28, Ohio.

added and the solution placed on a rotor for 2 hours to further remove protein impurities (Sevag, Lackman and Smolens, 1938). The protein-organic complex was centrifuged at 1,800 X G. from the collodial suspension, leaving a solution containing a much higher purified quantity of MPS than the original homogenate. Five ml. of the hexosamine-isolated fraction was dialyzed (3/8 inch flat width size dialyzer tubing of 48 Ångstrom unit average pore diameter; Arthur H. Thomas Co., Philadelphia 5, Pa.) for 40 hours at 4° C. against four 1,000 ml. portions of single-distilled water to remove amino acids and ions which would interfere with counting of the radioactive sulfate. Hexosamine, protein and ^{35}S determinations were performed on the final-dialyzed purified hexosamine fraction.

In preliminary work six other procedures for removal and isolation of protein from MPS were investigated. The use of the proteolytic enzyme, papain, for protein decomposition was examined and results indicated it was as efficient as pepsin-trypsin in digestion of the samples (Appendix Tables XIV and XV). Woods and Anastassiadis (1965) examined the comparative effectiveness of pepsin and trypsin against papain for hydrolysis of pig skin and their results indicated that purified fractions obtained from digestion with the former enzymes contained a greater quantity of hexosamine, sugars and hexuronic acid than those fractions digested with papain. It was decided that pepsin-trypsin would be employed because some difficulty arose in taking the ethanol-precipitated MPS into solution when the tissues were digested with papain. Through trial and error, it was determined that this procedure could be accomplished with 1 ml. 6 N. HCl, however, fear developed that some

ester sulfate might be degraded from the MPS molecule under these very acid conditions. Mild alkaline hydrolysis, followed by complex formation with organic solvents was also tested. One ml. of 1 N. NaOH was added to 4 ml. tissue homogenate and placed on a rotator for 24 hours in a room at 4° C. The hydrolysis was followed by neutralization with 1 ml. of 4 M. acetic acid and four extractions with amyl alcohol and chloroform reagent to remove hydrolyzed protein. It was possible to remove 90 to 95 per cent of the original protein with this method, however, analysis for hexosamine revealed that none were present in the nonprotein fraction. In another attempt to isolate MPS, the protein could not be sufficiently removed by trypsin digestion followed by trichloroacetic acid (40 per cent) protein precipitation. Acid hydrolysis followed by ester sulfate precipitation with $BaCl_2$, mild alkaline hydrolysis succeeded by hexosamine precipitation with cetyl pyridinium chloride, and HCl hydrolysis followed by filtration or centrifugation of undegraded particles were other methods examined for separation of MPS from protein which gave very limited success.

Five ml. of scintillation solution (125 gm. napthalene, 7 gm. of 2,5-Diphenyloxazole and 0.375 gm. 1,4-bis-2-(5-phenyloxazolyl)-benzene brought to 1 liter volume with 1,4-dioxane) were added to each 200 µl. sample or standard in a glass counting vial and counted for 2 minutes at 10° C. in a multi channel liquid scintillation counter (Mark I Nuclear-Chicago). Radioactivity of the 200 µl. samples was compared with a recently pipetted aliquot of diluted dosing solution after subtraction of the background as determined from a scintillation mixture blank.

During preliminary studies, neither internal standardization nor channel ratio methods were completely satisfactory in determining per cent quenching, therefore, dialysis of the final purified MPS fraction was performed to guarantee a minimum amount of quenching during sample counting.

The total ^{35}S activity in the plasma was assayed by the previously described counting procedure. Two hundred and fifty µl. of $BaCl_2$ was added to a 0.5 ml. aliquot of plasma to precipitate free sulfate. From these data the quantity of free and bound $^{35}SO_4^=$ in the plasma was determined. The stable inorganic sulfate content of the plasma was measured, except for slight modification, according to the procedure of Snell and Snell (1949). To 25 ml. of plasma, 55 ml. of distilled water and 20 ml. of 20 per cent trichloroacetic acid were added and the mixture shaken on a vortex mixer. After 15 minutes, the precipitate was removed by first centrifugation and then filtration through Whatman No. 42 ashless filter paper. To 10 ml. of the deproteinized, neutrally adjusted pH filtrate and sulfate standards, 1 ml. of 10 per cent barium chloride solution was added in the cold (4° C.). The samples were shaken and after 15 minutes, their optical densities were read at 400 mµ. in the cold room (4° C.) on a Bausch and Lomb Spectronic 20.

Tissue micro-autoradiograms were prepared by the histology section at UT-AEC-ARL for the purpose of estimating the biosynthesis rate of MPS formation within each species.

IV. EXPRESSION OF DATA

Hexosamine concentration is expressed on the basis of μmoles hexosamine per gram of protein. From the initial tissue homogenate, the μmoles of hexosamine per milligram of protein was determined and from the purified hexosamine fraction, it was possible to ascertain the ^{35}S/μmole hexosamine. From these data, the total quantity of ^{35}S-labeled-MPS/gram tissue protein was calculated.

All radiochemical values are expressed either as per cent of dose administered to the animal or as per cent of radioactivity in the plasma. Data were statistically analyzed by analysis of variance and when applicable the means were subjected to Duncan's Multiple Range test for determination of significant differences (Steele and Torrie, 1960). Standard deviations were calculated to illustrate data variability.

CHAPTER IV

RESULTS AND DISCUSSION

I. HEXOSAMINE CONCENTRATION

Hexosamine contents of intestine and kidney from cattle, sheep and swine are listed in Table V. There were no detectable differences among species in the amounts of total MPS within the mucosal layer of the duodenal wall, kidney medulla and cortex. The pig's jejunum and ileum contained significantly ($P<0.01$) more MPS than the same tissue from calves and sheep. This was not true for the cecum and colon as the cecum of the sheep contained greater ($P<0.01$) quantities of MPS than that of either cattle or swine, whereas, the sheep's colon had more ($P<0.01$) total MPS than cattle. From these data it is shown that in the areas of the intestine where the greatest absorption of molybdenum is believed to occur the swine had more total MPS (sulfated and nonsulfated) than the other two species investigated in this study. These findings support the hypothesis that a greater quantity of MPS in swine small intestine could be responsible for the greater uptake of racioactive Mo by this species as compared to cattle or sheep. As previously discussed, MPS are found widespread throughout the animal's body, particularly in connective tissue, however, no data could be located which indicated the amount of this complex tissue component in the intestine. Thus, no comparison between intestinal MPS concentration in the animals investigated in these studies could be made to that of other species or

TABLE V

HEXOSAMINE CONCENTRATION IN TISSUE OF CATTLE, SHEEP AND SWINE (μMOLES HEXOSAMINE/GM. PROTEIN)[a]

Tissue	Species		
	Cattle	Sheep	Swine
Duodenum	111±15[b]	106±35[b]	117±15[b]
Jejunum	77± 7[b]	62± 8[b]	105±13[c]
Ileum	78±11[b]	64± 8[b]	121±21[c]
Cecum	150±20[b]	184±20[c]	137±16[b]
Colon	170±41[b]	200±28[c]	184±17[b,c]
Kidney			
Medulla	68±10[b]	70±10[b]	76± 5[b]
Cortex	52± 3[b]	54± 5[b]	56± 2[b]

[a]Mean ± standard deviation.

[b,c]Means across the same line with same superscript are not significantly different (P<0.01).

other findings. The majority of the investigations pertaining to MPS metabolism have been performed with humans or small laboratory animals with only a limited amount of research having been performed in this area with large farm animals.

The kidney medulla of the three species studied contained more total MPS than the cortex. According to Krestinskaya (1965), the distribution of MPS in the kidney of mammals is fairly characteristic. His findings indicate that no MPS exist in the kidney cortex nor the external zone of the medullary substance. All of the basement membranes of the malpighan corpuscles, the nephron tubules and the initial parts of the collecting tubules are made up of neutral MPS. On the contrary, the internal zone of the medullary substance in the region of the papilla, particularly its middle third, contain mainly acid MPS. Individual data for MPS concentration of intestinal wall and kidney are presented in Appendix Table XVI and significant differences in quantity of MPS in segments of intestine and sections of kidney as determined by Duncan's Multiple Range Test are shown in Appendix Table XVII. The small intestine contained lesser amounts of MPS than the large intestine and larger amounts than either sections of the kidney. In sheep and cattle, the duodenum contained more MPS than the other two segments of the small intestine. The kidney cortex had the smallest concentration of MPS of the tissues analyzed but by those procedures it was detectable in contrast to data presented by Krestinskaya (1965).

II. MUCOPOLYSACCHARIDE SEPARATION AND PURIFICATION

Approximately 95 to 98 per cent of the protein was removed and 20 to 50 per cent of the MPS were recovered from the original homogenate of these tissues (Table VI). Protein was most difficult to remove from the cecum and colon and MPS were hardest to recover from the kidney cortex. The MPS were purified almost ten-fold from protein according to computations derived from the following equation:

$$\frac{\mu\text{mole hexosamine/mg. protein in the hexosamine purified fraction}}{\mu\text{mole hexosamine/mg. protein in the initial tissue homogenate}}.$$

Separation of MPS from protein was necessary to eliminate the radioactive sulfur synthesized into sulfur containing amino acid and, therefore, establish only that quantity specifically incorporated into sulfated MPS. Slightly greater purification of MPS was obtained for samples from calves and sheep than swine. This was desirable as protein from these species would have a greater quantity of ^{35}S incorporated into their amino acids than protein of the monogastric animal. As previously discussed in the materials and methods chapter, several laboratory procedures have been investigated to determine the most efficient procedure for separation of MPS from protein. These preliminary laboratory findings are in agreement with a majority of other investigators results as the use of proteolytic enzymes produced the largest purification of MPS. Woods and Anastassiadis (1965) became concerned when they were only able to recover a portion of the total hexosamine from the tissue and, therefore, they investigated the degree of purification upon the quality of hexosamine yielded. The patterns of distribution of hexosamine and hexuronic acid among the

TABLE VI

SEPARATION AND PURIFICATION OF MUCOPOLYSACCHARIDES FROM PROTEIN

Tissue	Per cent protein removed			Per cent hexosamine recovered			Purification factor[a]		
	Cattle	Sheep	Swine	Cattle	Sheep	Swine	Cattle	Sheep	Swine
Duodenum	95	96	96	43	43	30	8.2	11.4	8.3
Jejunum	95	97	97	46	28	25	8.4	9.0	7.8
Ileum	95	97	96	37	28	28	7.7	8.8	6.4
Cecum	94	95	93	46	49	34	7.8	10.7	4.7
Colon	93	96	92	50	50	44	7.2	11.6	6.3
Kidney									
Medulla	96	98	96	30	23	21	9.5	11.4	4.8
Cortex	98	98	98	17	12	9	9.2	7.7	5.6

[a] $\frac{\mu\text{moles hexosamine/mg. protein in mucopolysaccharide fraction}}{\mu\text{moles hexosamine/mg. protein in tissue homogenate}}$.

fractions obtained during MPS fractional precipitation were not affected by the degree of recovery of the MPS or the purity of the proteolytic enzymes utilized.

III. PLASMA $SO_4^=$ AND ^{35}S ACTIVITY

The concentration of radioactive ^{35}S in the plasma of the three species was found in the following proportion: cattle > sheep > swine, whereas, the amount of stable sulfate in their plasma was present in the following order: cattle > swine > sheep (Figure 4). The inorganic sulfate analysis indicates that no significant differences existed in the quantity of stable sulfate in the plasma of these cattle, sheep or swine. The quantity of ^{35}S was considerably higher in the plasma of cattle and sheep compared to swine and the radioactive sulfur disappeared more rapidly from the plasma of swine than from the ruminants.

Following the determination of total ^{35}S in the plasma, $BaCl_2$ was added to a separate aliquot of plasma to remove free inorganic sulfate. Previous laboratory studies revealed that greater than 98 per cent of $Na_2{}^{35}SO_4$ could be removed from a "spiked" plasma sample by this procedure. All of the ^{35}S could not be eliminated from the plasma of cattle, sheep or swine and variable quantities of activity (cattle, 11.8; sheep, 23.6; swine 17.1 $\bar{x}$ per cent) remained for each species after $BaSO_4$ precipitation. Richmond (1959) reports that a portion of the sulfate is transported bound to plasma protein, thus, it was assumed that radioactive sulfur remaining after barium precipitation was not necessarily incorporated into sulfated amino acids.

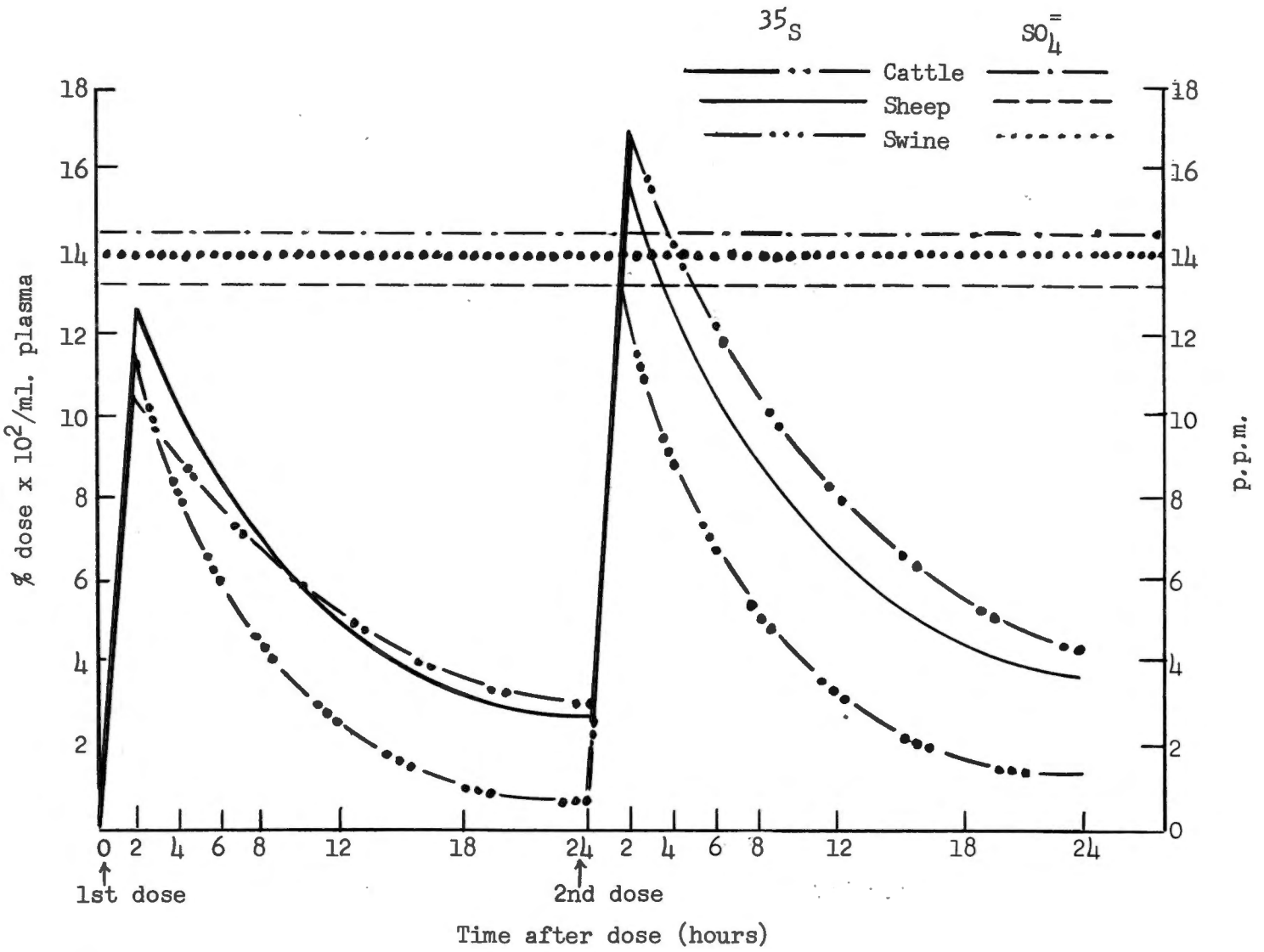

Figure 4. Plasma inorganic $SO_4^=$ and ^{35}S activity.

As the ^{35}S available for MPS labeling should be rather dependent on the plasma concentration of ^{35}S and the proportion of radiochemical to chemical $SO_4^{=}$; the quantity of each animal's sulfur-labeled MPS will be expressed in relation to its plasma activity. The initial plan was to represent the quantity of ^{35}S-labeled MPS as a per cent of the activity administered per kilogram body weight. Since the large differences in plasma radioactivity gave questionable results, the data will be expressed in relationship to both the amount of activity injected into the animal and its plasma activity.

IV. MUCOPOLYSACCHARIDE BIOSYNTHESIS RATE

Tissue micro-autoradiograms of cross sections from the jejunum indicated that the label was distributed uniformly throughout the intestinal wall of all three species. No pictures of the autoradiograms will be presented because the entire cross section of the intestinal wall could not be photographed under high microscopic power and the resolution was unsatisfactory for detection of the radioactivity from stained cell nuclei when the tissue were viewed at low magnification. These autoradiograms indicate that biosynthesis and labeling rate of MPS was comparable for each species. Experiments carried out by Schiller et al. (1956) demonstrated the half-life time for hyaluronic acid to vary between 2.4 and 4.5 days and chondroitin sulfuric acid to fluctuate from 7.6 to 10.7 days in the skin of normal rats and rabbits. Keratosulfate, compared to the other MPS, has a very long half-life in excess of 120 days (Davidson and Small, 1963a).

V. PROPORTION OF MUCOPOLYSACCHARIDES LABELED WITH RADIOACTIVE SULFUR

The proportion of MPS labeled with ^{35}S in various intestinal segments and kidney medulla and cortex of cattle, sheep and swine are shown in Table VII. Sulfur-35 activity per micromole hexosamine is expressed as a per cent of the radioactive sulfur administered per kilogram body weight. Generally, the small intestine of swine and sheep contained more ^{35}S/μmole hexosamine than similar tissue from cattle. Jejunum from pigs had a larger ($P<0.05$) part of its MPS affixed with ^{35}S than this area of intestine from calves. The ileum of cattle incorporated less ($P<0.05$) ^{35}S into its MPS than either sheep or swine. The cecum of sheep had significantly ($P<0.05$) more MPS marked with ^{35}S than the cecum of swine. Swine kidney cortex contained approximately two-fold the amount of ^{35}S in its MPS as compared to this section of the kidney in sheep and cattle. The proportion of MPS labeled in the duodenum, colon and kidney medulla was similar for these calves, lambs and pigs.

Data for the proportion of MPS labeled with ^{35}S in intestinal wall and kidney of individual animals when expressed as either per cent of administered dose or as per cent of activity in the plasma are listed in Appendix Tables XVIII and XIX, respectively. Appendix Table XX reveals significant differences ($P<0.05$) for the proportion of MPS labeled with ^{35}S among the various tissues as tested by Duncan's Multiple Range Analysis. For all species, the large intestine had the greatest quantity of MPS labeled with radioactive sulfur. The duodenum, kidney medulla

TABLE VII

PROPORTION OF MUCOPOLYSACCHARIDES LABELED WITH ^{35}S IN INTESTINAL WALL AND KIDNEY OF CATTLE, SHEEP AND SWINE (% DOSE X 10^3/μMOLE HEXOSAMINE)[a,b]

Tissue	Species		
	Cattle	Sheep	Swine
Duodenum	3.70±1.73[c]	3.90±1.05[c]	3.38±1.08[c]
Jejunum	3.50±1.79[c]	4.99±1.08[c,d]	5.77±1.69[d]
Ileum	2.76±1.27[c]	6.13±1.05[d]	5.25±1.73[d]
Cecum	7.31±1.94[c,d]	8.09±1.11[c]	6.00±1.80[d]
Colon	7.61±2.13[c]	6.27±1.58[c]	6.65±2.59[c]
Kidney			
Medulla	3.31±0.91[c]	3.09±0.95[c]	2.80±0.82[c]
Cortex	3.87±0.69[c]	3.94±1.67[c]	6.24±2.19[d]

[a]Per cent dose = sulfur-35 activity in relation to the amount of radioactive sulfate administered per kilogram of body weight.

[b]Mean ± standard deviation.

[c,d]Means across the same line with same superscript are not significantly different ($P<0.05$).

and kidney cortex, with the exception of swine, had the smallest amount of ^{35}S incorporated per unit hexosamine.

The proportion of MPS labeled with ^{35}S when the activity is expressed in relation to the quantity of radioactive sulfur in the plasma is listed in Table VIII. When expressing the proportion of MPS labeled with ^{35}S in this manner, all intestinal and kidney tissue from swine demonstrated much greater labeling per unit of hexosamine than did the same tissue from calves and sheep. The proportion of MPS labeled with ^{35}S in the small intestinal wall of the three species investigated occurred in the following descending order: swine, sheep and cattle, which correlates positively with the quantity of ^{99}Mo absorbed by each species as shown in previous investigations of Bell et al. (1964) and Bell, Sneed and Hall (1967).

VI. CONCENTRATION OF ^{35}S-LABELED MUCOPOLYSACCHARIDES

The quantity of ^{35}S-labeled MPS in intestinal wall and kidney of cattle, sheep and swine when expressing the activity per milligram of protein in comparison to amount of radioactive sodium sulfate administered per kilogram body weight is displayed in Table IX. A detectable difference was not found in the concentration of ^{35}S-labeled MPS in duodenum, colon, kidney medulla and cortex among these animals. The jejunum and ileum of cattle contained less ($P<0.05$) radiosulfur-labeled MPS than swine. The jejunal and ileal portions of sheep small intestine contained intermediate amounts of sulfated-labeled MPS compared to swine and cattle. The cecum of each species contained diverse ($P<0.05$) quantities of

TABLE VIII

PROPORTION OF MUCOPOLYSACCHARIDES LABELED WITH ^{35}S IN INTESTINAL WALL AND KIDNEY OF CATTLE, SHEEP AND SWINE (% OF ACTIVITY IN 1 ML. PLASMA/μMOLE HEXOSAMINE)[a]

Tissue	Species		
	Cattle	Sheep	Swine
Duodenum	6.82±4.13[b]	7.22±2.43[b]	12.53±3.28[c]
Jejunum	6.59±4.24[b]	9.18±2.02[b]	21.40±5.17[c]
Ileum	4.96±2.72[b]	11.44±3.36[c]	18.92±3.26[d]
Cecum	13.36±5.96[b]	14.90±2.76[b]	22.20±4.89[c]
Colon	13.22±3.58[b]	11.57±3.32[b]	24.24±6.37[c]
Kidney			
Medulla	5.77±1.71[b]	5.59±1.31[b]	10.12±1.24[c]
Cortex	7.07±2.61[b]	7.10±2.50[b]	22.30±3.78[c]

[a]Mean ± standard deviation.

[b,c,d]Means on the same line with the same superscript are not significantly different ($P<0.05$).

TABLE IX

CONCENTRATION OF ^{35}S-LABELED MUCOPOLYSACCHARIDES IN INTESTINAL WALL AND KIDNEY OF CATTLE, SHEEP AND SWINE (% DOSE X 10^4/MG. PROTEIN)[a,b]

Tissue	Species		
	Cattle	Sheep	Swine
Duodenum	3.91±1.56[c]	3.90±0.67[c]	3.88±0.99[c]
Jejunum	2.62±1.21[c]	3.06±0.54[c]	6.00±1.88[d]
Ileum	2.20±1.08[c]	3.94±0.72[c,d]	6.14±1.65[d]
Cecum	10.73±2.05[c]	14.81±1.65[d]	8.03±1.83[e]
Colon	13.30±6.05[c]	12.86±4.60[c]	12.16±4.44[c]
Kidney			
Medulla	2.32±0.81[c]	2.13±0.49[c]	2.11±0.53[c]
Cortex	2.01±0.40[c]	2.16±1.03[c]	3.48±1.55[c]

[a]Per cent dose = sulfur-35 activity in relation to the amount of radioactive sulfate administered per kilogram body weight.

[b]Mean ± standard deviation.

[c,d,e]Means across same line with same superscript are not significantly different at the ($P<0.05$) level.

^{35}S-labeled chondroitinsulfuric acid. The relationship of the concentration of ^{35}S-MPS in the cecum among these species was as follows: sheep > cattle > swine. Individual data for the concentration of ^{35}S-labeled chondroitin sulfate in intestinal segments and kidney are listed in Appendix Tables XXI and XXII.

If the concentration of ^{35}S-labeled MPS is expressed in reference to the amount of plasma radioactive sulfate (Table X), practically all swine tissue exhibited considerably greater quantities of MPS labeled with radioactive sulfur. The most striking differences occurred in the small intestine and kidney cortex, as these tissues from swine had a two- to four-fold increase in ^{35}S-labeled MPS over that present in sheep and cattle. These results indicate that the small intestine of swine does contain a greater quantity of sulfated MPS than the other two species investigated; and, thus, could be involved in the absorption of cations such as molybdenum.

Differences in concentration of ^{35}S-labeled MPS among intestinal segments and kidney medulla and cortex are presented in Appendix Table XXIII. The cecum and colon contained greater ($P<0.05$) quantities of ^{35}S-labeled chondroitin sulfate than other tissues analyzed. The small intestine had slightly larger amounts of MPS labeled with ^{35}S than the kidney. With exception of the pigs, which had more of the labeled compound in the kidney cortex than medulla, the two sections of the kidney contained almost equivalent concentrations of ^{35}S-labeled MPS.

Results obtained from these studies support the original hypothesis that the quantity of sulfated MPS in the mucosal layer of

TABLE X

CONCENTRATION OF ^{35}S-LABELED MUCOPOLYSACCHARIDES IN INTESTINAL WALL AND KIDNEY OF CATTLE, SHEEP AND SWINE (% OF ACTIVITY IN 1 ML. PLASMA/MG. PROTEIN)[a]

Tissue	Species		
	Cattle	Sheep	Swine
Duodenum	0.73±0.37[b]	0.71±0.13[b]	1.43±0.23[c]
Jejunum	0.50±0.31[b]	0.57±0.13[b]	2.28±0.74[c]
Ileum	0.39±0.21[b]	0.74±0.26[b]	2.26±0.35[c]
Cecum	1.96±0.77[c]	2.73±0.14[b]	3.06±0.86[b]
Colon	2.26±0.84[b]	2.54±0.38[b]	4.44±1.08[c]
Kidney			
Medulla	0.39±0.06[b]	0.39±0.07[b]	0.77±0.08[b]
Cortex	0.36±0.12[b]	0.39±0.15[b]	1.25±0.19[c]

[a]Mean ± standard deviation.

[b,c]Means on same line with same superscript are not significantly different ($P<0.05$).

the small intestine and kidney is a factor in influencing absorption and excretory patterns of ^{99}Mo in cattle, sheep and swine. However, it is doubtful if the total difference in ^{99}Mo metabolism among species can be explained by variable MPS concentrations in these tissues as recent studies by Bell, Sneed and Hall (1967) revealed evidence indicating that route of isotope administration and type of ration fed affect ^{99}Mo absorption and elimination in sheep. Animals dosed orally and fed either roughage or concentrate rations excreted most of the ^{99}Mo by fecal excretion, though ^{99}Mo was absorbed more rapidly by sheep receiving the concentrate diet. Sheep fed high concentrate rations and dosed directly into the abomasum excreted approximately 80 per cent of the activity in their urine. However, ^{99}Mo was excreted primarily in the feces by sheep dosed abomasally and fed roughage rations. Sheep intravenously dosed excreted ^{99}Mo similarly to pigs. These researchers offer the explanation that ammonium molybdate may be changed by the rumen bacteria into a form that is toxic and not readily absorbed by the ruminant. Data from these investigations clearly demonstrate the need for additional work to resolve the mechanisms responsible for species differences in ^{99}Mo metabolism.

CHAPTER V

SUMMARY AND CONCLUSIONS

Six calves, six lambs and six pigs were fed natural rations containing equivalent amounts of copper, molybdenum and sulfur for 4 weeks and intraperitoneally administered $Na_2{}^{35}SO_4$ to determine the concentration of total and sulfated MPS in intestine and kidney. In vivo labeling techniques were utilized to establish the amount of chondroitin sulfate in these tissue, however, in order for just interpretation of the data, knowledge about certain conditions which existed inside the body proper at time of labeling, such as plasma radioactive and stable $SO_4^=$ levels and MPS biosynthesis rate, has been obtained. Also, MPS were isolated from protein to insure that the ^{35}S activity was solely representative of the compound being investigated.

Data obtained indicate that the concentration of total MPS is rather similar in cattle, sheep and swine intestinal and kidney tissue. The jejunum and ileum of swine contained significantly ($P<0.01$) greater quantities of MPS than cattle or sheep. The most significant findings from this study are that regardless of which method was employed for expression of ^{35}S-labeled MPS, the small intestine and kidney cortex of these pigs had a greater ($P<0.05$) proportion of their MPS labeled with radioactive sulfur than ruminants. Primarily because of the increased proportion of ^{35}S-labeled chondroitin sulfate to total MPS, the small intestine and kidney cortex of pigs were found to contain a greater

($P<0.05$) concentration of total ^{35}S-labeled MPS as compared to sheep or cattle. The quantity of total and ^{35}S-MPS among these tissue existed in the following relationship: large intestine > small intestine > kidney.

To further substantiate that the concentration of MPS in intestinal and kidney tissue is associated with molybdenum metabolism, experiments could be performed to alter MPS levels in these tissue by hormone or dietary means and then determine if a positive correlation exist between ^{99}Mo absorption and the new established level of MPS.

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APPENDIX

TABLE XI

HEXOSAMINE AND ^{35}S ANALYSES OF TISSUE SAMPLES HOMOGENIZED WITH A WARING BLENDOR

Tissue	Sample no.	μmole hexosamine/ gm. protein	Per cent hexosamine recovered post-dialysis	^{35}S/ μmole hexosamine[a]	
				Pre-dialysis	Post-dialysis
Small intestine	1	68	110	59,233	44,514
	2	83	97	52,488	43,268
	3	101	107	53,856	37,488
	Mean	84	105	55,192	41,756
Cecum	1	112	107	49,359	33,849
	2	102	84	55,271	32,366
	3	90	91	49,546	29,248
	Mean	101	94	51,300	31,821
Colon	1	141	97	65,367	52,644
	2	127	84	65,305	48,498
	3	173	84	54,028	44,108
	Mean	147	88	61,566	48,416

[a]Counts/minute minus background.

TABLE XII

HEXOSAMINE AND ^{35}S ANALYSES OF MUCOSAL CELLS SCRAPED FROM INTESTINAL WALL AND HOMOGENIZED WITH A POTTER-ELVEHJEM HOMOGENIZER

Tissue	Sample no.	µmole hexosamine/gm. protein	Per cent hexosamine recovered post-dialysis	^{35}S/µmole hexosamine[a] Pre-dialysis	^{35}S/µmole hexosamine[a] Post-dialysis
Small intestine	1	120	106	41,047	39,055
	2	100	95	36,816	32,739
	3	114	88	38,058	36,765
	Mean	111	96	38,640	36,186
Cecum	1	122	100	43,678	42,692
	2	164	75	35,465	37,273
	3	126	123	38,770	47,500
	Mean	137	99	39,304	42,488
Colon	1	193	111	63,005	60,250
	2	204	107	70,810	55,142
	3	182	93	62,925	74,481
	Mean	193	104	65,580	63,291

[a]Counts/minute minus background.

TABLE XIII

HEXOSAMINE AND ^{35}S ANALYSES OF TISSUE SAMPLES HOMOGENIZED WITH A POTTER-ELVEHJEM HOMOGENIZER

Tissue	Sample no.	μmole hexosamine/ gm. protein	Per cent hexosamine recovered post-dialysis	^{35}S/ μmole hexosamine[a] Pre-dialysis	^{35}S/ μmole hexosamine[a] Post-dialysis
Small intestine	1	95	83	35,083	32,237
	2	84	98	34,036	31,812
	3	80	100	39,251	34,458
	Mean	86	94	36,123	32,836
Cecum	1	81	110	43,360	34,406
	2	79	98	42,944	38,469
	3	83	101	45,240	33,709
	Mean	81	103	43,848	35,528
Colon	1	148	110	50,577	51,064
	2	137	114	59,647	58,006
	3	135	111	56,406	60,176
	Mean	140	112	55,536	56,415

[a]Counts/minute minus background.

TABLE XIV

PURIFICATION OF MUCOPOLYSACCHARIDES IN SWINE TISSUE BY PEPSIN AND TRYPSIN DIGESTION DURING PRELIMINARY STUDIES

Tissue	Sample no.	Original homogenate[a] µmole hexosamine/ mg. protein	Original homogenate[a] ^{35}S/ µmole hexosamine	MPS-purified fraction[a] µmole hexosamine/ mg. protein	MPS-purified fraction[a] ^{35}S/ µmole hexosamine	Per cent hexosamine recovered	Per cent protein removed	Purification factor[b]
Small intestine	1			4.63	30,646[c]	37.2	98.2	20.1
	2			2.77	24,647	41.4	96.6	12.0
	Mean	0.23	20,498[c]	3.70	27,646	39.2	97.4	16.0
Colon	1			5.00	25,961	73.1	94.1	12.5
	2			4.09	27,056	53.6	94.7	10.2
	Mean	0.40	25,701	4.54	26,508	63.4	94.4	11.4
Kidney	1			0.70	11,154	21.0	97.8	10.0
	2			0.78	21,054	19.1	98.2	11.1
	Mean	0.07	8,219	0.74	16,104	20.0	98.0	10.6

[a] Samples were dialyzed prior to hexosamine, protein and ^{35}S analyses.

[b] $\frac{\text{µmole hexosamine/mg. protein following enzymatic digestion}}{\text{µmole hexosamine/mg. protein in original homogenate}}$.

[c] Counts/minute minus background.

TABLE XV

PURIFICATION OF MUCOPOLYSACCHARIDES IN SWINE TISSUE BY PAPAIN DIGESTION DURING PRELIMINARY STUDIES

Tissue	Sample no.	Original homogenate[a] µmole hexosamine/ mg. protein	Original homogenate[a] ^{35}S/ µmole hexosamine	MPS-purified fraction[a] µmole hexosamine/ mg. protein	MPS-purified fraction[a] ^{35}S/ µmole hexosamine	Per cent hexosamine recovered	Per cent protein removed	Purification factor[b]
Small intestine	1			2.18	23,183[c]	48.5	94.8	9.5
	2			5.28	22,684	50.0	97.8	23.0
	Mean	0.23	20,498[c]	3.73	22,933	49.2	96.3	16.2
Colon	1			10.56	23,523	52.4	98.0	26.4
	2			18.46	26,876	43.9	99.0	46.1
	Mean	0.40	25,701	14.51	25,199	48.2	98.5	36.2
Kidney	1			1.84	12,435	20.4	99.2	26.3
	2			1.88	9,387	32.1	98.8	26.8
	Mean	0.07	8,219	1.86	10,911	26.2	99.0	26.6

[a] Samples were dialyzed prior to hexosamine, protein and ^{35}S analyses.

[b] $\frac{\text{µmole hexosamine/mg. protein following enzymatic digestion}}{\text{µmole hexosamine/mg. protein in original homogenate}}$.

[c] Counts/minute minus background.

TABLE XVI

INDIVIDUAL DATA FOR HEXOSAMINE CONCENTRATIONS IN TISSUE
(μMOLES HEXOSAMINE/GM. PROTEIN)

Animal no.	Intestine					Kidney	
	Duodenum	Jejunum	Ileum	Cecum	Colon	Medulla	Cortex
			Cattle				
208	93	71	75	140	166	70	49
237	130	74	84	184	194	67	55
182	105	81	82	146	170	58	47
218	96	69	75	134	100	56	55
209	113	79	94	133	164	81	53
205	128	88	60	165	225	76	51
Mean	111	77	78	150	170	68	52
			Sheep				
863	95	49	64	170	166	68	46
854	86	55	51	165	184	72	50
853	86	67	60	192	203	60	60
848	124	72	76	216	187	82	58
842	75	66	69	169	244	65	55
846	170	64	67	194	219	75	56
Mean	106	62	64	184	200	70	54
			Swine				
868	114	110	94	122	192	68	54
867	94	83	128	135	212	78	55
866	119	97	95	121	171	78	58
869	120	111	129	135	165	75	54
871	114	120	138	164	179	83	60
873	142	111	141	147	184	76	55
Mean	117	105	121	137	184	76	56

TABLE XVII

DUNCAN'S MULTIPLE RANGE TEST FOR DIFFERENCES IN CONCENTRATION OF HEXOSAMINE AMONG INTESTINAL SEGMENTS AND KIDNEY MEDULLA AND CORTEX OF EACH SPECIES (μMOLES HEXOSAMINE/ GM. PROTEIN)[a]

Item							
			Cattle				
Tissue	Kidney cortex	Kidney medulla	Jejunum	Ileum	Duodenum	Cecum	Colon
Activity	52	68	77	78	111	150	170
			Sheep				
Tissue	Kidney cortex	Jejunum	Ileum	Kidney medulla	Duodenum	Cecum	Colon
Activity	54	62	64	70	106	184	200
			Swine				
Tissue	Kidney cortex	Kidney medulla	Jejunum	Duodenum	Ileum	Cecum	Colon
Activity	56	76	105	117	121	137	184

[a]Means underlined are not significantly different ($P<0.05$).

TABLE XVIII

PROPORTION OF MUCOPOLYSACCHARIDES LABELED WITH ^{35}S IN INTESTINAL WALL AND KIDNEY OF INDIVIDUAL ANIMALS ($\%$ DOSE X $10^3/\mu$MOLE HEXOSAMINE)[a]

Animal no.	Intestine					Kidney	
	Duodenum	Jejunum	Ileum	Cecum	Colon	Medulla	Cortex
			Cattle				
208	5.46	3.72	2.04	6.40	9.55	2.57	4.78
237	2.86	2.39	2.74	5.86	6.70	3.09	3.58
182	2.44	2.91	1.70	5.67	4.92	2.04	2.88
218	6.33	6.23	4.95	10.45	5.83	4.14	4.53
209	2.99	4.64	3.44	8.86	8.42	4.38	3.76
205	2.09	1.13	1.68	6.65	10.26	3.66	3.70
Mean	3.70	3.50	2.76	7.29	7.61	3.31	3.87
			Sheep				
863	3.20	5.52	6.62	8.35	3.92	3.00	4.87
854	4.69	6.47	6.59	9.85	5.69	3.36	2.73
853	4.77	3.30	5.14	7.95	6.68	4.82	6.80
848	2.74	4.68	5.81	7.80	5.73	2.68	3.97
842	5.06	4.54	7.70	8.18	6.98	2.54	2.70
846	2.94	5.45	4.90	6.39	8.64	2.12	2.59
Mean	3.90	4.99	6.13	8.09	6.27	3.09	3.94
			Swine				
868	4.36	6.76	7.79	6.09	6.59	3.72	5.64
867	4.65	5.68	5.20	6.53	6.58	2.75	6.80
866	3.45	6.30	5.35	8.14	11.34	2.79	6.17
869	3.54	8.12	5.72	7.42	5.97	3.38	9.14
871	1.86	3.49	2.37	3.43	3.39	1.32	2.51
873	2.43	4.25	5.05	4.36	6.03	2.81	7.19
Mean	3.38	5.77	5.25	6.00	6.65	2.80	6.24

[a]Per cent dose = ^{35}S activity in relation to the amount of radioactive sulfate administered per kilogram body weight.

TABLE XIX

PROPORTION OF MUCOPOLYSACCHARIDES WITH ^{35}S IN INTESTINAL WALL AND KIDNEY OF INDIVIDUAL ANIMALS (% OF ACTIVITY IN 1 ML. PLASMA/μMOLE HEXOSAMINE)

Animal no.	Intestine					Kidney	
	Duodenum	Jejunum	Ileum	Cecum	Colon	Medulla	Cortex
			Cattle				
208	9.29	6.32	3.46	10.88	16.22	4.37	8.12
237	4.40	3.69	4.22	9.04	10.34	4.77	5.53
182	9.00	10.74	6.27	20.94	18.16	7.54	10.62
218	12.75	12.56	9.92	21.07	11.75	8.33	9.13
209	2.63	4.90	3.63	9.36	8.88	4.63	3.97
205	2.82	1.54	2.28	8.88	13.96	4.98	5.04
Mean	6.82	6.59	4.96	13.36	13.22	5.77	7.07
			Sheep				
863	5.66	9.77	11.72	14.78	6.93	5.32	8.63
854	8.04	11.09	11.30	16.89	9.77	5.76	4.68
853	7.65	5.30	8.23	12.74	10.70	7.73	10.91
848	5.41	9.23	11.46	15.38	11.30	5.29	7.83
842	11.50	10.34	17.52	18.62	15.88	5.78	6.14
Mean	7.22	9.18	11.44	14.90	11.57	5.59	7.10
			Swine				
868	14.10	21.85	25.18	19.66	21.30	12.03	18.22
867	16.28	19.85	18.19	22.84	22.99	9.62	23.79
866	10.79	19.25	16.75	25.49	35.49	8.73	19.32
869	11.11	25.50	17.95	23.29	18.73	10.60	28.68
871	15.13	28.37	19.26	27.93	27.61	10.74	20.76
873	7.78	13.60	16.17	13.97	19.31	8.99	23.01
Mean	12.53	21.40	18.92	22.20	24.24	10.12	22.30

TABLE XX

DUNCAN'S MULTIPLE RANGE TEST FOR DIFFERENCES IN PROPORTION OF MUCOPOLYSACCHARIDES LABELED WITH ^{35}S IN INTESTINAL SEGMENTS AND KIDNEY MEDULLA AND CORTEX OF EACH SPECIES (% DOSE X 10^3/μMOLE HEXOSAMINE)[a]

Item							
				Cattle			
Tissue	Ileum	Kidney medulla	Jejunum	Duodenum	Kidney cortex	Cecum	Colon
Activity	2.76	3.31	3.50	3.70	3.87	7.29	7.61
				Sheep			
Tissue	Kidney medulla	Duodenum	Kidney cortex	Jejunum	Ileum	Colon	Cecum
Activity	3.09	3.90	3.94	4.99	6.13	6.27	8.09
				Swine			
Tissue	Kidney medulla	Duodenum	Ileum	Jejunum	Cecum	Kidney cortex	Colon
Activity	2.80	3.38	5.25	5.77	6.00	6.24	6.65

[a]Means underlined are not significantly different ($P<0.05$).

TABLE XXI

CONCENTRATION OF ^{35}S-LABELED MUCOPOLYSACCHARIDES IN INTESTINAL WALL AND KIDNEY OF INDIVIDUAL ANIMALS ($\%$ DOSE X 10^4/MG. PROTEIN)[a]

Animal no.	Intestine					Kidney	
	Duodenum	Jejunum	Ileum	Cecum	Colon	Medulla	Cortex
			Cattle				
208	5.64	2.64	1.53	8.94	15.86	1.79	2.34
237	3.71	1.78	2.30	10.77	12.83	2.30	1.99
182	2.56	2.34	1.39	8.29	8.34	1.17	1.34
218	6.05	4.30	3.72	14.01	5.85	2.34	2.50
209	2.80	3.66	3.22	11.77	13.84	3.54	1.99
205	2.68	1.00	1.02	10.80	23.08	2.78	1.90
Mean	3.91	2.62	2.20	10.73	13.30	2.32	2.01
			Sheep				
863	3.05	2.70	4.23	14.25	6.49	2.03	2.25
854	4.02	3.58	3.37	16.29	10.49	2.44	1.38
853	4.12	2.20	3.08	15.29	13.54	2.90	4.07
848	3.39	3.36	4.42	16.81	10.69	2.19	2.31
842	3.81	3.00	5.29	13.85	17.02	1.65	1.48
846	5.00	3.51	3.28	12.38	18.96	1.59	1.45
Mean	3.90	3.06	3.94	14.81	12.86	2.13	2.16
			Swine				
868	4.96	7.46	7.31	7.44	12.63	2.55	3.05
867	4.39	4.71	6.66	8.81	13.96	2.13	3.73
866	4.10	5.96	5.07	9.87	19.34	2.19	3.61
869	4.24	8.99	7.39	10.02	9.85	2.54	4.98
871	2.12	4.18	3.26	5.62	6.08	1.10	1.52
873	3.47	4.73	7.14	6.42	11.10	2.13	3.96
Mean	3.88	6.00	6.14	8.03	12.16	2.11	3.48

[a]Per cent dose = ^{35}S activity in relation to the amount of radioactive sulfate administered per kilogram body weight.

TABLE XXII

CONCENTRATION OF ^{35}S-LABELED MUCOPOLYSACCHARIDES IN INTESTINAL WALL AND KIDNEY OF INDIVIDUAL ANIMALS (% OF ACTIVITY IN 1 ML. PLASMA/MG. PROTEIN)

Animal no.	Intestine					Kidney	
	Duodenum	Jejunum	Ileum	Cecum	Colon	Medulla	Cortex
			Cattle				
208	0.96	0.45	0.26	1.52	2.69	0.30	0.40
237	0.57	0.28	0.35	1.66	1.98	0.36	0.31
182	0.94	0.87	0.52	3.06	3.08	0.43	0.50
218	1.22	0.87	0.75	2.82	1.18	0.47	0.50
209	0.30	0.39	0.35	1.24	1.46	0.37	0.21
205	0.36	0.14	0.14	1.47	3.14	0.38	0.26
Mean	0.73	0.50	0.39	1.96	2.26	0.39	0.36
			Sheep				
863	0.54	0.48	0.75	2.52	1.15	0.36	0.40
854	0.69	0.61	0.58	2.79	1.80	0.42	0.24
853	0.66	0.35	0.49	2.45	2.17	0.46	0.65
848	0.67	0.66	0.87	3.31	2.11	0.43	0.46
842	0.87	0.68	1.20	3.15	3.87	0.38	0.34
846	0.86	0.60	0.56	2.13	4.16	0.27	0.25
Mean	0.71	0.57	0.74	2.73	2.54	0.39	0.39
			Swine				
868	1.60	2.41	2.36	2.40	4.08	0.82	0.99
867	1.53	1.65	2.33	3.08	4.88	0.74	1.31
866	1.28	1.87	1.59	3.09	6.06	0.68	1.13
869	1.33	2.82	2.32	3.15	3.09	0.80	1.56
871	1.73	3.40	2.65	4.57	4.95	0.89	1.24
873	1.11	1.52	2.29	2.06	3.55	0.68	1.27
Mean	1.43	2.28	2.26	3.06	4.44	0.77	1.25

TABLE XXIII

DUNCAN'S MULTIPLE RANGE TEST FOR DIFFERENCES IN CONCENTRATION OF ^{35}S-LABELED MUCOPOLYSACCHARIDES IN INTESTINAL SEGMENTS AND KIDNEY MEDULLA AND CORTEX FOR EACH SPECIES (% DOSE X 10^4/MG. PROTEIN)[a]

Item							
	Cattle						
Tissue	Kidney cortex	Ileum	Kidney medulla	Jejunum	Duodenum	Cecum	Colon
Activity	2.01	2.20	2.32	2.62	3.91	10.73	13.30
	Sheep						
Tissue	Kidney medulla	Kidney cortex	Jejunum	Duodenum	Ileum	Colon	Cecum
Activity	2.13	2.16	3.06	3.90	3.94	12.86	14.81
	Swine						
Tissue	Kidney medulla	Kidney cortex	Duodenum	Jejunum	Ileum	Cecum	Colon
Activity	2.11	3.48	3.88	6.00	6.14	8.03	12.16

[a]Means underlined are not significantly different ($P<0.05$).