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Toxicology of Muconomycins A and B: Inflammatory Properties of Two New Antibiotics: Electrolyte and Protein Characterization of Inflammatory Exudate and Production of a Model State of Ascites in Male Albino Rats

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TOXICOLOGY OF MUCONOMYCINS A AND B: INFLAMMATORY PROPERTIES OF TWO NEW ANTIBIOTICS; ELECTROLYTE AND PROTEIN CHARACTERIZATION OF INFLAMMATORY EXUDATE AND PRODUCTION OF A MODEL STATE OF ASCITES IN MALE ALBINO RATS

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ABSTRACT

Toxicological investigations were conducted on two new antibiotics, Muconomycin A and Muconomycin B. These non-nitrogenous antibiotics were found to be highly toxic and capable of inducing profound inflammation in the peritoneal cavity of male albino rats. Either antibiotic produced large volumes (10-20 ml) of inflammatory exudate even when injected (i.p.) in quantities of 1.6×10^{-10} moles.

An extensive profile of the electrolytes and proteins found in inflammatory exudates was developed. Simultaneous assays of the blood serum of treated rats provided a basis for comparing the concentrations of constituents of serum with those of the exudate. This approach to the study of the inflammatory response has not been previously described in such a comprehensive manner. The results of these assays showed that the exudate contained lower concentrations of sodium and proteins, and greater amounts of potassium, calcium and phosphorus than the serum. Chloride ion concentrations were variable.

It was hypothesized that the electrolyte and protein changes seen in these transudates would be reciprocally related to reported values for inflamed tissues. The data obtained in this research supported this hypothesis except in the case of chloride ion.

The presence of large volumes of peritoneal fluid in these animals presented an opportunity to study water and electrolyte balances in a model state of ascites. This work confirmed the reported decrease in urine volume and electrolyte excretion seen in clinical ascites. A much greater retention of sodium than potassium was seen in this experimental ascites.

The subcellular toxicity of these antibiotics was manifested as potent in vitro inhibition of the enzyme, ATP: creatine phosphotransferase.

I. INTRODUCTION

The process of inflammation has been an appealing subject of investigation for many of the scientific disciplines. Although pharmacologists are quick to acknowledge that the local application of any drug may lead to an inflammatory condition, workers from this discipline have seldom studied this process in detail. It has been recognized that one of the most prominent signs of acute inflammation is the development of edema. In 1964 Dr. L. Szorny, a pharmacologist from the University of Copenhagen, made the statement: "Observations on the chemical composition of the inflammatory edema are scarce, in spite of great efforts to develop experimental methods by which the edema can be studied". The protein constituents of inflammatory exudates have been extensively studied and hence it was decided to consider some other components such as the electrolytes.

There are many agents available which could be used to produce a standard inflammatory response. In fact, "counter-irritants" have been used for centuries on the basis of their ability to induce a regulated inflammatory condition. All the pure organic compounds which cause acute inflammation, and in most cases general toxicity, are known to contain nitrogen as part of their molecular structures. However, heroic quantities of classical irritants must often be used in order to produce enough exudate to carry out the many simultaneous chemical

assays. Therefore, the reports that Muconomycin A and Muconomycin B, two new antibiotics, do not contain nitrogen but do cause severe facial inflammation, became of great interest. It was found that very small quantities of these antibiotics could induce peritoneal exudate volumes of 10 to 20 milliliters per rat. These volumes were obtained after 2 to 3 daily injections of 0.25 mg/kg of either antibiotic; in a rat weighing 300 g, this dose amounts to only 1.6×10^{-10} moles.

In these animals with large amounts of peritoneal exudate, it was desired to compare systemic changes in electrolytes and proteins with exudate constituents. To do this, simultaneous assays were conducted for the following materials in both the exuded fluid and the blood serum of male albino rats: sodium, potassium, chloride, calcium, phosphorus, total protein, and albumin and globulin protein fractions.

These rats also provided a fine opportunity to study electrolyte excretion patterns in a model state of edema and ascites. Water and electrolyte profiles were established for these animals by daily recording or determination of water and food intake, urine volume, urinary sodium, potassium and chloride, and body weight.

There are classic hematologic responses which occur in acute and chronic inflammatory states; therefore, erythrocytes were counted and total and differential peripheral leukocyte counts were made.

This research was designed not only to answer some specific questions about the antibiotics and the inflammatory response, but also to utilize a chemical pathologic approach to study cellular necrosis and subcellular toxicology. Many studies of cellular necrosis have dealt with the constituents of the injured tissue itself. It seemed important, however, to consider the fluid exuded from this injured tissue. The major hypothesis of this work, then, is that the electrolyte and protein changes seen in the transudates are reciprocally related to the changes in the tissue. An ion lost from tissue during injury would therefore appear in increased concentrations in the exudate fluid.

The results obtained in the electrolyte assays and in other experiments done by the author suggested extension of this work to the subcellular level. The effects of these antibiotics on ATP: creatine phosphotransferase, an enzyme of profound importance in cellular energetics, were examined.

II. REVIEW OF THE LITERATURE

A. Early History

From the earliest days of recorded history the subject of inflammation has played a dominant part in medicine. Hippocrates (460-377 B.C.) regarded inflammation as being very closely associated with fever. It was the Hippocratic doctrine that an irritating material was rendered harmless by being deposited in some local area, inducing an abscess. Even at this early point in the history of medicine, both fever and inflammation were considered healing processes. It was also recognized that either of these responses might prove harmful when extended beyond certain limits (Menkin, 1940).

Inflammation was considered by Erasistratos (333-250 B.C.) to be the result of the combined local release, through a wound, of blood from the veins and air from the arteries. The most lucid description of the cardinal signs of inflammation was offered by a contemporary of Christ, Cornelius Celsus (25 B.C.-45 A.D.) Celsus concisely stated the classic definition in the following terms: "Notae vero inflammationis sunt quatuor, rubor et tumor, cum calore et dolore." (Cited in Menkin, 1940).

Galen (130-210 A.D.) considered inflammation to be the most common disease, but one that was nevertheless characterized by numerous manifestations. He seems to have been the first to indicate that inflammation *per se* may be caused by a diversity of unrelated irritants. This idea was expanded by Hunter (1794,

cited in Menkin, 1940), who formulated the classical concept that inflammation represents the reaction to any injury. To Celsus' cardinal symptoms of inflammation, Hunter added etiologic and teleologic significance. He proposed that any injury could cause inflammation. And yet the response, he pointed out, "is almost the same type everywhere, because everywhere it is an effect whose purpose it is to restore the parts to their natural functions." Hunter further proposed that inflammation limited the extent of injury.

To Cohnheim (1889, cited in Florey, 1962) the central point of the process was the increased permeability of the capillary wall. His concept of inflammation is therefore considered as a disturbance in local physiology. He made the important stipulation that the inflammatory response "is not so powerful as to bring about the death of the vessel." Cohnheim clearly pointed out that the increased permeability of the small vessels was independent of any nervous control. This same worker provided some speculations on the etiology of the symptoms enumerated by Celsus. He indicated that the redness can be explained by the "abnormal fullness of the vessels of an inflamed part". Swelling is due both to this abnormal fullness and to transudation of fluid. He related the pain to sensory nerves and observed that the variation in pain depended mostly on the supply of nerve endings. The local heat associated with inflammation, he noted, will never

exceed that of the internal organs. In tribute to this worker it should also be mentioned that although our in vivo methods for studying inflammation have been refined and some of his views had to be modified, little has been added to our knowledge of the visual sequelae of the vascular events which occur under these conditions (Ebert, 1965).

B. Definition of Inflammation

There are several definitions offered for "inflammation". In addition to those given above, the following may be considered:

1. Adami (1909, cited in Menkin, 1940) quoted a definition of inflammation "as the reaction of irritated and damaged tissues which still retain vitality." He also emphasized that "inflammation is a process and not a state".

2. Menkin (1940) provided a broad definition of inflammation: "the complex vascular, lymphatic, and local tissue reaction elicited in higher animals by the presence of microorganisms or non-viable irritants".

3. A very recent definition was provided by Spector and Willoughby (1964). "Inflammation is one aspect of the reaction to injury and consists essentially of the response of the small blood vessels and their contents to insult".

These definitions provide some idea of what inflammation is considered to be.

C. Classification of Inflammation

Two categories are generally used in the description of an inflammatory reaction. All inflammatory responses can be considered as pertaining to the duration of symptoms or to the type of exudation.

1. Duration of Symptoms

Acute inflammation refers to a reaction in which the dominant anatomic changes are vascular and exudative (Oliver, 1957). It is therefore also called exudative inflammation. If this type of inflammation persists for more than a few weeks, it must then be considered to be in a chronic phase (Robbins, 1962).

From a morphologic viewpoint, chronic inflammation is characterized by a proliferative rather than an exudative response, with a predominantly mononuclear cell infiltration. When this condition persists for long periods, considerable scarring and permanent fibrous replacement of functional parenchymal elements occur. Chronic inflammatory reactions can be expected to cause permanent tissue damage.

There is one particular type of chronic inflammation which leads to a unique morphologic pattern called granuloma formation. Such a formation is a tumor-like mass of granulation tissue consisting of actively growing fibroblasts and capillary buds.

2. Character of Exudate

The exudation of fluid is most characteristic in acute inflammation. Exudates are categorized by Menkin (1956) on the basis of the type of fluid or cells present. The fluid consists of plasma and products of cellular injury. Hence, the first type of fluid is referred to as a serous exudate. If the exudate contains a considerable number of red cells, it is known as a hemorrhagic exudate. Fluids containing a large number of dead white cells are called a purulent exudate and if fibrin is present, the exudate is classified as fibrinous.

Although the above classification deals with separate definitions, mixed exudate patterns are commonly observed. For example, an exudate might be termed "serofibrinous".

D. Physiologic and Morphologic Reactions to Injuries

1. Introduction

Whenever cells are injured or destroyed, an immediate protective response occurs in the surrounding tissues. This response is called inflammation (Robbins, 1962). It is common to consider microorganisms as the cause of inflammation, but many non-living agents, such as heat, cold, electromagnetic radiations, electrical or chemical stimuli and simple mechanical trauma like crushing, may also evoke an inflammatory response. Of course, when any of these agents is sufficiently violent to cause death of tissue cells, substances released from the

dead cells also serve as injurious agents and, hence, add to the inflammatory response.

It has been emphasized that the basic character of the inflammatory response is almost always the same, regardless of the nature of the injurious agent or the site of its occurrence (Menkin, 1940, 1956). The utility of this response is that it destroys, dilutes or walls off the injurious agent (Dible, 1950). Although the basic changes of inflammation follow a predictable sequence, the ultimate character, extent and severity of tissue changes vary according to the host and the nature of the irritant.

Repair, even in the Hippocratic era, was an acknowledged component of the inflammatory process. Repair begins during the active phase of inflammation, but concludes only after the injurious influences have been neutralized (Robbins, 1962).

When cells are injured or destroyed, soluble chemical substances are released. These initiate the inflammatory response.

2. Vascular Changes Seen in Inflammation

Since the most obvious changes caused by such released agents are seen in the vascular system, this topic will be discussed now. Cellular injury initiates the release of local chemical mediators which serve as the stimuli for the tissue changes in the inflammatory reaction. Spector and Willoughby

(1964) have listed the following as possible mediators: histamine, serotonin, certain globulins, nucleosides, nucleotides and some catecholamines. These agents cause vasodilatation of arterioles, either by direct action on their walls, or by indirect stimulation of local axon reflex arcs which inhibit vasoconstriction and therefore allow dilation to occur (Cotran and Majno, 1964). These arteriolar changes take place within the first few minutes of exposure to the irritant. Very shortly thereafter the capillaries dilate, presumably due to a direct effect of the humoral agent upon these vessels. Hence, the first hour following injury is marked by vasodilatation and rubor.

In addition to inducing these effects, the mediators of the inflammatory response alter the endothelial linings, causing increased permeability. There appears almost immediately a slow transudation of fluid from the vessels into the injured focus (Menkin, 1940, p. 24).

Robbins (1962) summarized the remaining events seen in the inflammatory response:

> "With the loss of fluid, the red cells become more densely aggregated with the blood (packing) to form a kind of sludge, and consequently the blood becomes increasingly viscous and more resistant to movement. The increased viscosity causes slowing of the blood flow through these altered capillaries. Usually this most evident about an hour after injury. As the chemical agents continue to act upon the capillary walls, the endothelium becomes more leaky and its intercellular substance more porous than normal. Serum is lost more rapidly so that the blood thickens, and this leads to stasis or sometimes total stagnation."

With this decrease in blood flow, white cells appear to adhere to the endothelial lining. After a few hours the vessels in the area of injury are distended with red cells and lined by adherent white cells. This latter process is referred to as pavementing.

Accompanying these vascular changes there is an increased flow of lymph. Pullinger and Florey (1937) showed that as more tissue exudate accumulated, the lymphatics dilate because the fluid stretches the collagen fibers attached to the outside of their endothelial walls and thus holds them open against a pressure greater than the surrounding tissue pressures. Simultaneously the lymphatic walls become more permeable to large molecules and this aids in the removal of the exudate from the inflamed region.

3. Fluid and Cellular Exudation

Under normal conditions, capillaries freely allow water, salts, amino acids, glucose and other small molecules to pass through their walls. Proteins escape in very small amounts except in the intestines and liver (Robbins, 1962).

When the capillary endothelium is damaged under the inflammatory process, proteins pass out freely from the blood. This leakage upsets the pressure relationships, because the vascular osmotic pressure falls with the loss of protein. Hence a fluid exudate accumulates outside the vessels, resulting

in the production of edema. Moreover, breakdown of proteins in the exudate increases the number of molecules in the tissue space; this tends to further increase the osmotic pressure gradient in the exudate (Innerfield, 1957). When the tissue injury is severe, the largest proteins, such as fibrinogen, escape and coagulate to form masses of fibrin.

The altered capillary endothelium which permits fluids to pass out of the vessels, also allows the escape of blood cells into the injured area. Most of the cells appearing in the exudate are polymorphonuclear neutrophils (Florey, 1962). This cell is one of the most important defensive elements of the body (Bostick, 1949). The neutrophil is an actively phagocytic cell and is able to engulf foreign material. These cells are, therefore, the first to be found in acute inflammatory reactions; they are also the most numerous of cells in the exudate.

Eosinophils are believed to emigrate from the blood in increasing numbers when the healing process has already begun. Florey (1962) notes that there does not appear to be an increase of eosinophils in the blood or tissues in acute inflammation. Because mast cells and basophils contain histamine and heparin, their damage causes liberation of these mediators. Basophils occur only rarely in the circulating blood and they have an unknown role in the control of inflammation (Florey, 1962).

The lymphocyte does not appear at the site of injury in the early stages of the response, but does occur later and hence is characteristic of chronic inflammation (Florey, 1962). Monocytes are found in great numbers at the site of injury. As in the case of neutrophils, the monocytes are phagocytic in nature.

There are systemic, as well as local, white cell alterations during inflammation. In the healthy rat the number of leukocytes in the circulating blood averages 9,000 cells per cubic millimeter with a normal range of 6,000-15,000 (D'Amour and Blood, 1954). In significant inflammatory states, leukocytosis occurs. The increase in the quantity of white cells is usually due to an increase in the number of polymorphonuclear neutrophils (Oliver, 1957, p. 123).

E. Functional Significance of the Inflammatory Response

The protective nature of the inflammatory response has already been pointed out. Almost all of the component features of the inflammatory reaction are oriented in the direction of helping the body to rid itself of the injurious agent. By arteriolar and capillary dilatation all the blood protective elements are focused in increased amounts at the site of injury. The serous exudation dilutes the toxic substance, thus lowering its concentration to less lethal levels. Enzymes which digest protein substances are released by the leukocytes and by the damaged tissue.

Welch (1897) urged the use of great caution in interpreting the adaptive processes associated with inflammation. He noted that one must guard against "the conception of something in the nature of an intelligent foresight on the part of the participating cells."

F. Cellular Injury

Inflammation is a manifestation of cellular injury and cellular death. Increased interest in this area lead the CIBA Foundation to organize a symposium, "Cellular Injury" held 2nd-4th July 1963. One major objective of this symposium, and thus of the whole area of study, was stated by Biggers (1964): "The investigation of cellular injury and cell death is an essential part of understanding and controlling various disease processes". At these meetings, Dr. Judah presented a summary of his interesting work on the mechanisms of drug protection of cellular injury. He and his co-workers have formed a hypothesis based on the finding that antihistamines and certain other drugs block mitochondrial swelling and reduce ion transport (Judah and McLean, 1962). Their studies indicate that ion-transport mechanisms are of considerable importance in the events leading to cell necrosis.

Judah and his collaborators placed great emphasis on active transport in the pre-necrotic state. Judah, Ahmed, and McLean (1963) proposed that "the protective drugs may act upon enzymatic pathways involved in the use of energy for the main-

tenance of the intracellular milieu...". Other details of this very fundamental work of the Judah group will be provided in a later section. It is desired at this time to consider Judah's views of the necrotic and pre-necrotic states occuring in a cell under attack from a toxic agent.

Any of the agents mentioned previously as causative agents in the production of inflammation can also cause necrosis. Ischemia, physical agents, chemicals, microorganisms and hypersensitivity reactions can all produce necrosis. According to Judah, a necrotic cell is one that is dying or dead. The irreversibility of this process is therefore implicit. On the other hand he points out that the time needed to reach the necrotic state is unknown. During some phase of the pre-necrotic state there is a point at which some of the effects of injury can be reversed. This site seems to be common to all injured cells. Judah and his collaborators have found that antihistamines and other drugs prevent cell death caused by standard toxicants such as thioacetamide, carbon tetrachloride and viruses. In other words, the injurious agent causes damage which need not go on to cell death if a protective drug is present. It is obvious, then, that in the later stages when the cell is running down, ATP (adenosinetriphosphate) levels fall, K^+ leaks out, water and Na^+ enter, and enzymes leak away. In vivo, these actions may be concealed by vascular changes which are directed toward isolation of the injured part from

the circulation (Judah, Ahmed & McLean, 1963). The proposed site of protective action is seen between stages 1 and 2 in the scheme presented on the following page.

In conclusion, then, the following points are to be emphasized:

1. A variety of agents can produce the same type of injury to a cell.

2. There is a pre-necrotic stage during which the cell is in an altered steady state; water accumulates along with Ca^{++} and Na^{+}; K^{+} and ATP are being lost. Somewhere prior to this stage, drug protection is afforded.

3. Necrosis and cell death are dynamic processes - morphologic changes do not occur suddenly.

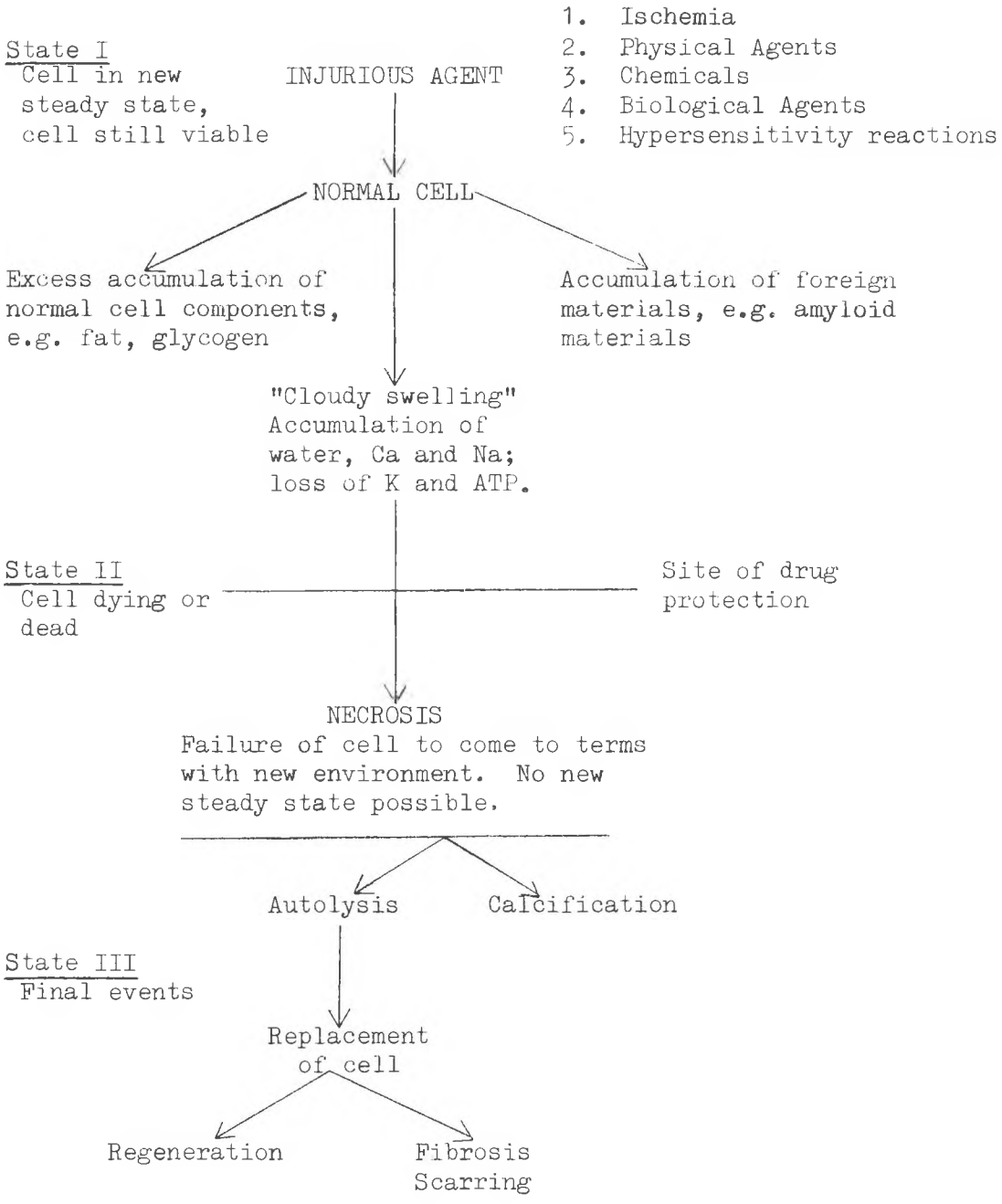
4. After necrosis, there is no new steady state possible. The cell in question must die and will be replaced either by a new cell or by scar tissue.

G. Electrolyte Composition of Blood Serum and Inflammatory Exudate

Before discussing the effects of inflammation on serum and exudate electrolytes, some outlines of the present knowledge of electrolyte distribution in normal tissue must be included. Our starting hypothesis is that the cells and extracellular fluid are in osmotic equilibrium and that water can pass freely across the membranes separating them (Ruch & Fulton, 1960).

The maintenance of high potassium and low sodium levels within cellular fluids is best explained in terms of an hy-

FATE OF CELL UNDER ATTACK AND SITE OF DRUG PROTECTION



Adapted from Judah, Ahmed and McLean (1965).

pothesis based on the electrochemical gradient across the cell membrane and an active extrusion of sodium from the cell. When a cell is at rest sodium remains in the extracellular phase, not because it is unable to penetrate the membrane, but because sodium is continually pumped out of the cell by an active transport process. On the other hand, the high intracellular levels of potassium are maintained by a converse mechanism, i.e., potassium is kept in the cell by active transport (Tosteson, 1964). Some workers have offered evidence that in several tissues the extrusion of sodium is linked to the entrance of potassium (Hodgkin, 1958). It can then be said that the outward movement of sodium occurs against an electrochemical gradient whereas both potassium and chloride can readily pass across the membrane of the resting cell (Ganong, 1963). Some potassium is also transported actively into the cell (Whittam, 1964).

The exact nature of the sodium pump is yet to be established. According to most authors, e.g. Fuhrman, (1952), Ussing, (1959) and Caldwell, (1960), the immediate source of energy for the active sodium transport is probably ATP and/or related compounds. According to Lundgard and Hodgkin (cited in Bourne, 1962), a cytochrome oxidase energy-producing system is involved in the penetration of ions. They have offered evidence that this system requires ATPase, creatine phosphatase (i.e. ATP: creatine phosphotransferase) and cytochrome oxidase.

From muscular and nervous tissue, Kuby, et al. (1954), have isolated an enzyme they designated as a transphorylase. By using creatine phosphate (CP) as a substrate, this enzyme catalyzes the prompt resynthesis of ATP. This reaction is designated as the Lohmann reaction:

$$\text{Creatine Phosphate} + \text{ADP} \rightleftharpoons \text{ATP} + \text{Creatine}$$

Under conditions of rest, the reverse reaction occurs and induces a build-up of the high-energy creatine phosphate. Davies (1965) indicates that the rephosphorylation of ATP and creatine phosphate can be inhibited by means of iodoacetate. It has been established that glycolosis actually is blocked by this inhibitor, via inactivation of triosephosphate dehydrogenase (Davies, 1965). Muscle tissue treated with iodoacetate will contract only about 100 times and then will cease to contract because of the depletion of these energy reservoirs. Hence it is often stated that the maintenance of the activity of nerve and muscle tissue is dependent upon a steady supply of the high-energy phosphate in the form of ATP and CP. The central role of ATP in all cellular energetics has been lucidly reviewed in the recent work of Lehninger (1965). This author indicated that muscle tissue contains three times as much creatine phosphate as ATP.

Of great interest is the report of Bueding, et al. (1963). Their preliminary studies of the cellular energetics in intestinal smooth muscle demonstrated the essential nature of both

ATP and creatine phosphate. Their data suggested that after treatment with adrenaline, there occurred increased production of these high energy compounds. These in turn provided more energy "for stabilization of the membrane (Na pump)". Bueding and his co-workers further established that the energy increases were not mediated via the mode usually associated with adrenaline administration, i.e. by increasing phosphorylase activity.

From this brief consideration of cellular energetics, we will turn next to normal electrolyte distribution and redistribution following trauma. In virtually all mammals (Hastings, 1941) the concentration of sodium outside the cell averages 145 mEq/L and inside only about 12 mEq/L. The concentration gradient of potassium lies in the opposite direction; 155 mEq/L inside and 5 mEq/L outside (Guyton, 1961). Hence extracellular fluid contains very large quantities of sodium and chloride, reasonably large quantities of bicarbonate ion, but only small quantities of potassium, calcium, magnesium, phosphate, sulfate and organic acid ions. Plasma contains large amounts of protein while interstitial fluid contains very little.

Intracellular fluid contains only small quantities of sodium and chloride and very little calcium. This cellular fluid does contain very large quantities of potassium and phosphate, and moderate amounts of magnesium and sulfate ions. In addition, cells contain very large amounts of proteins;

about four times as much as in the plasma (Gamble, 1954).

Because extracellular fluid is in constant motion throughout the mammalian body, all cells live in essentially the same environment. Hence we can see why Cannon (1929, 1932) called this fluid "the internal milieu" of the body. By means of this fluid and its constituents, regulations are accomplished so that the cells are bathed continually in a fluid containing the proper electrolytes and nutrients.

In a previous section it was mentioned that fluid accumulation is a component of the inflammatory response. If only plasma accumulated at sites of injury, no change would be expected in the concentration of electrolytes in circulating plasma. However, it is well known that both animals and men have elevated potassium and decreased sodium levels in blood serum after injury, (Fuhrman, 1960). In one of the early demonstrations of electrolytes changes, Manery and Solandt (1943) found that traumatized muscle had a marked loss of potassium and gain of chloride. Thus, the altered tissue state was at least partly the cause of the abnormalities of the circulating plasma. Other evidence supporting the observed hyponatraemia associated with trauma was offered by Tabor and Rosenthal in 1945. They showed that ischemic legs of mice, following tourniquet injury, contained more sodium than could be accounted for by the increased fluid present. Since coincident loss of tissue potassium occurred, it was presumed

that the sodium entered the intracellular phase.

The electrolyte changes reviewed in the previous sections can be explained in terms of failure of the "sodium pump" to extrude sodium as fast as it enters the cell. Conway (1957) has provided a thorough review of the different modes by which cells may lose potassium and gain sodium. The energy for the pump may become inadequate from simple processes such as cold or ischemia. An alternate mode of decreasing the efficacy of this pump is by metabolic inhibition with chemicals. Another effect of "sodium pump" failure is that some sodium may be exchanged for potassium; sodium and chloride enter the cell together and the higher intracellular osmotic pressure results in cellular swelling (Fuhrman, 1960).

There are two main patterns emerging from studies on injured tissues: 1) cells lose potassium; 2) cells gain sodium (Fuhrman and Crismon, 1951). The sodium gain is accompanied by an increase in chloride.

The long-standing problem concerning what anion accompanies potassium was solved by Ling in 1952. He showed that there was a reduction of ATP and other high energy phosphates when potassium ion was released. It was therefore concluded that phosphorus was linked to potassium as chloride was associated with sodium.

By their studies of the reactions leading to cellular necrosis, Judah and co-workers have provided some interesting

views regarding the role of calcium ions in this process. Judah, et al. (1964) have suggested that movements of calcium underlie the initial stages of cellular necrosis. In numerous experiments these workers found that sodium, calcium and drugs that protect animals against liver injury, interact competitively on liver slices. In this interaction, these agents affect potassium accumulation and phosphate turnover in ATP and phosphoproteins (Judah, et al., 1962). It was further shown in their work that calcium ions are powerful inhibitors of cellular ATP and phosphoprotein turnover.

One role that calcium shifts may have in the mechanism of cellular injury is the alteration of membrane permeability. Judah and Ahmed (1963) observed that reduction of the calcium level of the bathing medium results in increased permeability of liver cells to proteins and also in loss of ATP. This loss is dependent upon the presence of sodium in the medium. These workers believe that the permeable cells allow free sodium entry and that the loss of ATP is a manifestation of the sodium- and potassium-activated ATPase. From this model they have constructed the following hypothesis for the initial stages of cell injury (Judah, et al., 1964). Damage to the membrane results in the mobilization of membrane calcium; this in turn causes an increased entry of sodium and a reduction in the level of ATP due to the increased demands upon the sodium pump system. As the process goes on, the cell becomes less and less able to restore its energy supply; consequently

more calcium begins to migrate in from the external medium and an irreversible series of reactions sets in. These workers emphasize that according to this hypothesis the first event is a shift of cell-bound calcium, as opposed to entry of calcium from without. Menkin (1956, p. 92) has noted that of the several ions examined in inflammatory exudate, only calcium occurs in a concentration high enough to induce continuous seepage of fluid into an inflammed area.

H. Ascites, Edema and Proteins

The terms ascites and edema are often used in an ambiguous manner. The definition of the more general term, edema, is given as the presence of abnormally large amounts of fluids in the intercellular tissue spaces of the body. Actually, ascites is a form of edema in which the location is stipulated as the peritoneal cavity. Because of the free space in this cavity, the fluid can flow in an uninhibited manner. In solid tissue edema fluid is localized. There are two major classifications of ascites. Exudative ascites is due to inflammation. Fluid accumulation due to venous obstruction is called dropsical ascities.

Edema is a result of a disturbance in fluid balance. It can be brought about by one or more of the following factors (Wright, 1961):

1. decrease in the plasma proteins;
2. elevation of the capillary pressure;

3. increased capillary permeability to plasma proteins;
4. lymphatic blockade.

The first type of edema listed above is seen under conditions of starvation. The edema develops from the great reduction in plasma proteins and the lowered osmotic pressure of the blood proteins. An example of edema induced by capillary pressure increases was provided by Stead and Varren (1944). They found that the normal subcutaneous fluid contains about 0.2 grams per cent of protein. After elevation of the venous pressure by 30 mm Hg, the protein concentration of this fluid was 1.3 grams per cent.

In inflammatory edema, the integrity of the endothelial wall as a barrier to plasma protein is lost. Plasma and its proteins are then translocated to extravascular areas. Once the plasma is in the interstitial space it is either retained as edema or removed by the lymphatics. Drinker (1946) stated that "the most important function of the lymphatics is the unremitting removal from the tissues of the excess blood proteins". Every 24 hours at least 50 per cent of the total circulating plasma protein leaks out of the capillaries and returns to the circulation via the lymphatics (Zweifach, 1965). The lymphatics can supply 50 per cent of the albumin lost within 24 hours after acute blood loss (Wasserman, et al. (1956).

It is of interest to consider that the large volumes of fluid sometimes found in the peritoneal cavity, originate from some alteration in the lymphatic system. In the rat the thoracic duct is of profound importance in handling lymphatic flow. Abdou, et al. (1952) reported that 94 per cent of intraperitoneally injected plasma protein is returned to the blood by the thoracic duct. Hence the importance of this system is obvious.

Drinker (1946) has favored the concept that fibrin causes lymphatic blockade in inflammatory conditions. He pointed out that edema could be induced by blocking the lymphatic supply to a given area in the dog. If the edema was inflammatory in origin, there was a high concentration of blood proteins in the edema fluid, e.g. 5.5 grams per cent.

Menkin (1956) summarized a most interesting experimental approach to demonstrate the role of the fibrin in localizing an infectious process via lymphatic blockade. Rabbits were pretreated with dicumarol, an anticoagulant. Upon subsequent treatment with a culture of hemolytic streptococci, there was greater mortality in the dicumarol-treated than in the untreated rabbits. Histologic studies indicated that the lack of fibrin in the tissues seemed to be a factor in the spread of an infection. By use of radiographic methods Barer (1952, cited in Menkin, p. 126, 1956) found that lymphatics were obstructed in a fully developed sterile abcess. Similar observations were made by Bangham, et al. (1953, cited in Menkin, p. 127

1956) with radioactive glass particles. These particles were observed to flow readily in the lymphatic vessels during the early stages of inflammation in the peritoneal cavity; but the particles became fixed when the reaction was fully developed.

Gullino, et al. (1964) studied various biological fluids as part of their attempts to characterize the interstitial fluid of solid tumors. Some of the results from rats are summarized below:

Component	Units	Thoracic Duct Lymph	Aortic Serum	Peritoneal Fluid
Total Protein	g/100ml	3.41	5.45	3.87
Sodium	mEq/L	131	140	155
Potassium	mEq/L	6.1	5.0	5.0
Chloride	mEq/L	101	103	99

They found that the concentration of proteins was about 33 per cent lower in the interstitial fluid than in the blood serum of tumors. The albumin-globulin (A/G) ratio of the fluid was equal to that of blood serum. An outstanding conclusion of their extensive research is that the gross composition of tumor fluid is maintained as constant as that of the thoracic lymph, normal peritoneal fluid or subcutaneous fluid, even though numerous necrotic foci were present. It is of value to view these data in light of the previous discussion on cellular injury and the pre-necrotic states of tissue.

In this section we have discussed some of the current views concerning ascites, edema and the proteins in the associated fluids. Another important symptom associated with these conditions involves abnormalities in fluid and electrolyte metabolism. These subjects will be covered in the following section.

1. Water and Electrolyte Metabolism in Ascites

As ascites formation develops, a pattern of physiological disturbances in water and electrolyte metabolism occurs which is the same regardless of the etiology of the underlying liver disease (Laragh and Ames, 1963). When transudation of fluid into the abdominal cavity begins, water and electrolyte retention occurs. Laragh and Gilman (1956) pointed out that renal retention of sodium is essential for the development and maintenance of any state of progressive fluid accumulation. Even on a liberal salt intake the amount of sodium excreted in the urine may be less than 1 mEq per day in patients with liver ascites (Laragh, 1962).

Leiter (1964), in his enumeration of factors involved with fluid accumulation, indicates that one prerequisite is the availability of the necessary ingredients of edema fluid: sodium, chloride and water. Ganong (1963) stated that whenever water is retained, there also is abnormal retention of salt in the body. Even in the early work of Hoberden (1802) it

was clinically observed that persons with such conditions showed a "very tormenting thirst".

Since sodium ions represent about 90 per cent of all the extracellular cations, it is very important that the body regulate the concentration of this ion. The very specific mechanism for regulating this ion is vested in the tubules of the kidneys where active reabsorption occurs. When this reabsorption is blocked, the sodium is lost in the urine (Guyton, 1961). Sodium reabsorption is regulated by aldosterone which is secreted by the adrenal cortices (Gaunt, et al., 1955). Potassium ions, like sodium ions, are continually being reabsorbed by the renal tubules. When aldosterone causes sodium reabsorption, it simultaneously decreases potassium reabsorption (Biglieri, 1963). Increased quantities of potassium can also be secreted by the distal tubules, thereby providing an additional mode of preventing increases of extracellular potassium levels (Guyton, 1961).

In general, the regulation of the total anion concentration is secondary to the control of cations. The main reason for this situation is that each time a cation is absorbed from the tubules, an electrochemical imbalance is created. This gradient immediately causes anions to diffuse through the membrane to provide electrostatic neutrality. Therefore, the same regulatory mechanisms that control cations also promote reabsorption of anions. Since about 75 per cent of the anions

in the glomerular filtrate are chloride ions, it is said that aldosterone promotes chloride absorption via a secondary effect.

Wright (1961) discusses some of the electrolyte and fluid disturbances which are known to occur in hepatic diseases. A demonstration of electrolyte retention in patients with ascites was provided in the studies of Chalmers (1951). From metabolic balance studies, Tremolieres (1960) showed that in ascites conditions there are great increases in the amount of intracellular Na^+. In experiments with simultaneously injected radioactive sodium and radioactive potassium, he showed that the diffusion of K^+ in these circumstances did not differ from that of Na^+. He observed that whereas the injected radioactive electrolytes instantaneously equilibrated with the plasma ions, it was 3 hours before the ascitic fluid compartment was in equilibrium with the plasma. The peritoneal fluid compartment appeared to be largely excluded from the rest of the body.

Obviously, urinary output of electrolytes is related to ionic input. For this reason, proper attention must be paid to food intake.

Selye (1950) provides us with an excellent summary with which to conclude this section.

> "Serous transudates are frequently found in the pleural, pericardial and peritoneal cavities of animals during the A-R (alarm reaction), irrespective of the alarming agent used. The formation of such transudates appears to depend upon many conditioning factors, such as the total salt and fluid intake, the excretory ability of the kidney for water and electrolytes, and the blood protein-osmotic pressure."

J. Methods for Producing and Evaluating Inflammation

Most of the screening methods for evaluating anti-inflammatory drugs are based on the edema stage of the inflammatory response (Szporny, et al., 1964). These screening procedures are severely hampered because "inauspicious conditions are generated throughout by our lack of knowledge of inflammation" (Rosenkilde, 1964). As with all studies of potential therapeutic agents, progress is always enhanced by the presence of a naturally occurring or experimentally induced counterpart of a human disease in another species (Pearson, 1963). Turner (1965) points out that the anti-inflammatory drugs are important "because of their utility, often as lifesaving drugs, in many diseases such as arthritis, lupus erythematosus, pemphigus and rheumatic fever." Of significance is the fact that Turner also considers anti-inflammatory, antiarthritic, and antiedematous agents in one chapter. This is due not only to the similarity of the tests but also because of the great resemblances of the symptoms in these disease states.

1. Standard Irritants

Some of the standard chemical irritants which have been used for the production of inflammation are listed on the following page. It is amazing that such a multiplicity of unrelated materials lead to the same end result. Menkin (1956)

STANDARD IRRITANTS USED IN THE INDUCTION LOCAL INFLAMMATORY RESPONSES

Irritant	Dose	Reference
	Site of injection; rodent paw	
Formalin	0.05 ml of 3.5% solution[1]	Northover & Subramanian, 1962
Serotonin HCl	0.1 ml of 5 μg/ml solution	Parratt & West, 1958
Histamine HCl	0.1 ml of 1 mg/ml solution	Ibid
Dextran	0.5 ml of 6% solution	Turner, 1965
Fresh egg white	0.1 ml of 0.5% solution	Parratt & West, 1958
Mustard powder	0.1 ml of 2.5% suspension	Sturki & Thompson, 1962
Kaolin	0.2 ml of 2% suspension	Turner, 1965
Carrageenin	0.5 ml of 2% solution	Benitz & Hall, 1963
	Site of injection; dog pleural cavity	
Croton Oil	0.5 ml of 5% in olive oil	Menkin, 1956
Turpentine	1.5 ml	Menkin, 1956
	Site of injection; rat pleural cavity	
NaCl	5 ml of 2.7% solution	Gabler, 1964
Gum arabic	5 ml of 1% solution	Ibid
Evans blue & gum arabic	5 ml of solution with 1 g each in 1L saline	Laden, et al., 1958
	Site of injection; rat peritoneal cavity	
Formalin	1 ml of 1.5% solution	Teotino, et al., 1963
NaCl	2 ml/100 g of 0.15 or 0.30M solution	Opie, 1964
Histamine HCl	2 ml/100 g, 0.15-0.40M	Opie, 1964
Sucrose or Glucose	2 ml/100 g, 0.2M solution	Opie, 1965

1. All solutions or suspensions are aqueous.

strongly favors the use of turpentine because of "its capacity of inducing profuse exudation". One ml of turpentine injected into the pleural cavity of dogs induced an exudate volume of 100 ml after three days (Opie, 1964). Most of the other irritants listed here usually induce about 3-6 ml of exudate. The majority of the standard irritants are widely used to induce edema in the feet of rodents (Turner, 1965). This procedure is commonly employed in an additional screening program (Rosenkilde, 1964). It is quite obvious that rodent feet are not the site of choice when one desires to collect large volumes of exudate. Other sites possible for this purpose will be the subject of the following section.

2. Sites for Production of Large Volumes of Exudate

When the granuloma pouch is used, the worker effectively makes a new cavity into which fluids can exude. This technique was discovered by Selye (1954). His original interest was in finding out exactly how stressful was air-injection into the chest-cavity. While preparing for one such injection, Dr. Selye was interrupted by one of his assistants who was escorting a group of visiting Brazilian physicians through the Institute. As Dr. Selye turned around to greet them, his needle slipped out of the chest-cavity and all the air went under the skin. The procedure was refined so that currently the volume of injected air is fixed at 25 ml and this is followed by 0.5 ml

of 1 per cent croton oil in cottonseed oil. After four days a volume of about 6 ml of exudate can be collected from a rat. Adamkiewicz, et al. (1953) took advantage of this method and reported the concentration of some constituents in this exudate. They did not use very many animals and also missed the opportunity to simultaneously assay the blood serum for these same constituents. These workers reported the following mean values: sodium, 146 mEq/L; potassium, 6.3 mEq/L; chloride, 90 mEq/L; phosphorus, 8.84 mg per cent (5.1 mEq/L) and total protein, 5.4 g per cent.

Opie (1964) states that the two largest serous cavities of the body, the pleural and peritoneal cavities, are well adapted for measurement of the changes which occur as the result of injection of irritants into them. The pleural cavity was utilized recently by Weisbach, et al. (1963) to screen a series of anti-inflammatory agents. By means of this test these workers were able to show that aspirin and phenylbutazone caused decreases in the fluid volume in comparison with that of the control animals.

The fundamental research of Opie (1964, 1965) in the chemical production of ascites has provided an excellent basis for the study of inflammation. This "inhibition of ascites" technique is listed by Turner (1965) as a standard anti-inflammatory test. In the hands of Teotino, et al. (1963) this method was used to demonstrate that phenylbutazone (100 mg/kg)

caused a 37 per cent diminution of ascites fluid in rats treated with formalin. Opie (1964) tested the inflammatory responses of both the pleural and peritoneal cavities. Although the fluid volume in the pleural cavity was found to be greater than in the peritoneal cavity, the concentration of protein was usually about two times greater in the latter (Opie, 1964). He pointed out that the larger volumes of fluid found in the pleural cavities could be an artifactual response aided by the negative pressure within the pleural cavity. According to Drinker (1945) pulmonary edema is aggravated not only by this pressure differential but also by respiratory movements themselves.

In his more extensive paper, Opie (1964) employed only the peritoneal cavity as the site of inflammation. Because such serous cavities of the body localize the process of inflammation, Opie (1965) observed that "fluid can be measured, cells counted, and exuded protein determined". He further observed that the activity of the reactions caused by saccharides or by alcohols varied in accord with their molecular weight and other colligative properties. It was concluded by Opie (1964) that the serous cavities of the mammalian body offer opportunity to measure many inflammatory factors.

K. The Antibiotics Muconomycin A and B

Nespiak, et al. (1961) carried out an investigation on the activity of some metabolites of Myrothecium roridum.

They found that metabolites from this agent were potent fungicides. In 1962, it was reported by Kishaba, et al. that some of the compounds produced from this mold possessed insecticidal activity. Harri, et al. (1962) gave mammalian toxicity data for the compounds designated Verrucarin A (Muconomycin A), Verrucarin B and Roridin A. Hence it can be observed that in this family of compounds there is a spectrum of toxicity; this spectrum includes fungi, insects and mammals.

Of the many metabolites isolated from the mold Myrothecium Verrucaria, considerable work has been reported for two of these, Muconomycin A(M-A) and Muconomycin B(M-B). The molecular formulae of these two substances differ only by one water molecule.

Bowden and Schantz (1955) summarized the reports of several Russian workers who described the toxic effects of the mold Stachybotrys alternans. Horses consuming hay on which the mold had been growing displayed severe lesions of the nose, mouth, throat and gastrointestinal tract. Ether extracts of these mold cultures demonstrated dermatitic properties. While handling large quantities of culture fluid from Metarrhizium glutinosum, Brian, et al. (1947) experienced severe facial inflammation which was attributed to a compound produced by the organism. The mold Myrothecium verrucaria has been shown by White and Downing (1947) to be identical with the species Metarrhizium glutinosum. Cultures of this mold were reported

by Brian and McGowan (1946) to cause severe facial and eyelid inflammation, desquamation of skin and local irritation. This publication of Brian and McGowan is of great importance because it is the first reported mold metabolite which can produce severe irritation.

It is interesting to note some other unusual properties which have been observed in Myrothecium verrucaria cultures. This mold can cause the breakdown of cellulose material. Various reports on the cellulolytic activity of these cultures have appeared: cf. Halliwell (1962), Selby, et al. (1963), and Whitaker and Thomas (1963). A highly specific oxalic acid decarboxylase has been found by Lillehoj and Smith (1965) in extracts of Myrothecium verrucaria.

Three constituents isolated from Myrothecium verrucaria cultures by Tamm and Gutzwiller (1962) were designated by them as Verrucarin A, Verrucarin B and Roridin A. The respective LD_{50}'s for these compounds were 1.5, 7.0 and 1.0 mg/kg after intravenous administration in mice. From the work of Vittimberga (1963) it would seem that Muconomycin A and Verrucarin A are identical substances. It is unlikely that Verrucarin B and M-B are the same (Tamburrini, 1965; Vittimberga and Vittimberga, 1965).

The extensive chemical investigations conducted by Harri, et al. (1962); Tamm and Gutzwiller (1962), Vittimberga (1963), Vittimberga and Vittimberga (1965), and Tamburrini (1965) have

yielded the following facts concerning the structures of the antibiotics. Basic hydrolysis of M-A (M.W. 502.5) gave three fragments:

(1) cis, trans - muconic acid;

(2) α - hydroxy - β - methyl - δ - valerolactone;

(3) an alcoholic substance, designated as Alcohol A.

When M-B (M.W. 484.5) was hydrolyzed, three fragments were found:

(1) cis, trans - muconic acid;

(2) Alcohol A;

(3) an oil containing unsaturated carboxylic acids.

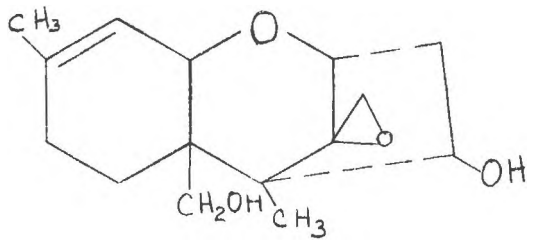
The inconclusive nature of (3) above was resolved by hydrolysis of the octahydro derivative of M-B, thus yielding:

(1) adipic acid;

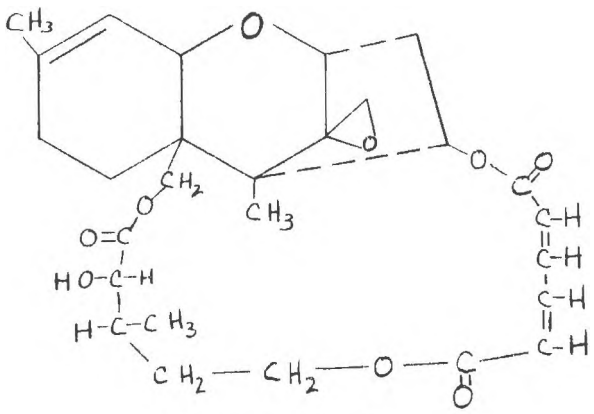
(2) Alcohol A;

(3) β - methyl - δ - valerolactone.

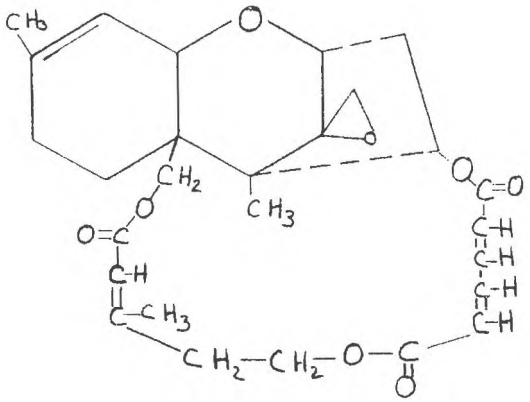
Pertinent formulae appear below:



Alcohol A

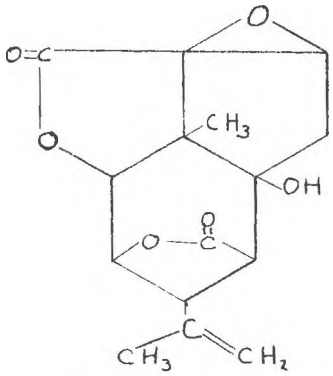


Muconomycin A

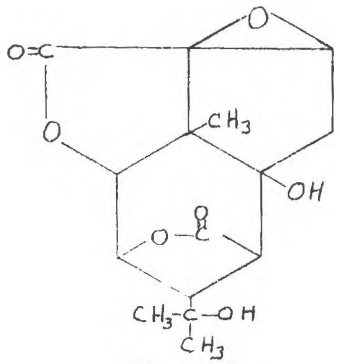


Muconomycin B

In the field of antibiotics, M-A and M-B offer several structural features which are quite novel. Of greatest interest is the fact that neither of these antibiotics contains nitrogen. All of the commercially available antibiotics contain nitrogen (White, 1962). Daniels and Jorgensen (1962) do mention one very toxic, non-nitrogenous compound, picrotoxin; but this is not considered an antibiotic. It is quite interesting, however, to note the ester linkages found in the two degradation products of picrotoxin appearing on the following page:



Picrotoxinin



Picrotin

The triester skeleton is a novel structure in the field of antibiotics (Tamburrini, 1965) and is seen in both M-A and M-B. Erythromycin, carbomycin and oleandomycin each contain one ester linkage in their macro-ring skeletons (White, 1962). This author also points out that the latter two antibiotics contain "a unique exocyclic methylene epoxide". Both M-A and M-B possess this type epoxide. However, erythromycin, carbomycin and oleandomycin all contain nitrogen atoms as part of their molecular structures.

Guarino (1963) reported that the intraperitoneal LD_{50} for M-A was in the range of 0.5 and 0.75 mg/kg in albino mice. This is a very high order of toxicity and is reminiscent of picrotoxin. According to Sollmann (1957), the LD_{50} for picrotoxin in various species is from 0.11 to 0.8 mg/kg. Dogs have an intermediate range of toxicity, i.e. about 0.3 mg/kg.

The high toxicity of M-A and the slow onset of death (animals never died in less than 18 hours) lead the author

to suspect that the toxic action may be exerted through some enzyme system. In addition, there were structural features in M-A which were considered to be similar to those seen in the antibiotic Antimycin. Furthermore, Nason (1960) reviewed some common features found in the formulae of Antimycin and vitamin E. He offered considerable evidence in favor of the antagonistic properties reported for Antimycin and vitamin E in the terminal respiratory chain of mitochondria. In applying, with respectful caution, some structure-biological activity principles, it was noted (Guarino, 1963) that the presence of a large macro-ring in M-A could correspond to the terpene side chain of vitamin E. The presence of three ester linkages in M-A, when compared with the two amide groups in Antimycin, may result in a structure also capable of competing biologically with vitamin E. The similarities of ester and amide reactions are well known both in the areas of pure chemistry (Gould, 1959) and structure-biological activity correlations (Burger, 1963).

Based on the hypothesis, then, that M-A could be a vitamin E antagonist, a pilot experiment was conducted (Guarino, 1963). The method chosen for measurement of this effect was acceleration of the classic avitaminosis-E symptom of heightened creatinuria. In rats treated with 0.25 mg/kg of M-A, some creatinuria was observed. In later experiments, albino rats were deprived of dietary vitamin E for several months and were then

supplemented with graded doses of the pure vitamin for ten days. This method is the same as was used by Hove in 1949 when he demonstrated the anti-vitamin E activity of carbon tetrachloride. When the rats were treated with Mucronomycin A, an enhancement of the urinary creatine output was observed. The amount of creatine appearing in the urine was greatest in those rats which received the least amount of the vitamin supplement. Animals not fed any vitamin E demonstrated a ten-fold increase in creatine output.

Such an imbalance in the relationship between extramuscular synthesis and uptake of creatine by the muscle would result in increased excretion of creatine and this could be due to an increased rate of creatine synthesis, a decreased rate of uptake of creatine by muscle, or to a combination of these factors. Vignos and Warner (1963) pointed out that the creatinuria associated with muscular dystrophy "might occur through excessive breakdown of high energy phosphate compounds". In addition to the possibility of alteration in the rate of creatine synthesis one must consider that the increased loss of creatine by skeletal muscle may be related to a defect in creatine transport across the muscle cell membrane (Gerber, et al. 1962).

The criticism that there is a lack of specificity associated with the symptom of increased creatinuria was answered in the following manner by Butturini (1949):

> "...an increase (of creatinuria) is found in altered muscle biochemistry due to the break in equilibrium between hydrolysis and synthesis of creatine phosphate, with the liberation of creatine and complete non-utilization by the muscle cell. It is, therefore, a symptom of the lack of muscular efficiency that may have a multiple etiology but which always remains subject to a lack of synthesis of creatine phosphate. For the realization of such synthesis, it is necessary that energy be furnished by the oxidation of carbohydrates."

It was postulated that in M-A-treated rats, phosphorylation of creatine was not occurring and hence it was being lost in the urine.

The content of creatine phosphate in muscle is intimately related to ATP through the reversible enzymatic conversion of creatine to creatine phosphate. ATP is required for this reaction and hence any agents affecting the synthesis of ATP will decrease the amount of creatine phosphate formed. If ATP is available, the synthesis of creatine phosphate will occur in the presence of creatine phosphokinase. The recommended name for this enzyme (Enzyme Nomenclature, 1965) is, ATP: creatine phosphotransferase, 2.7.3.2. Bergmeyer (1963) lists the following inhibitors of this enzyme: zinc, copper, p-chloromercuribenzoate, AMP, ADP, thyroxine and malonic acid. Iodoacetate also inhibits this enzyme *in vitro* (Harper, 1965) and may, in addition, alter the synthesis of creatine phosphate *in vivo* since iodoacetate causes creatinuria (Beard, 1941).

III. INVESTIGATION

A. Objectives

Rowden and Schantz (1955) reported the isolation and characterization of some dermatitic compounds produced by the mold, Myrothecium verrucaria. Using procedures similar to theirs, workers at Rohm and Haas Company[1] isolated a highly irritating substance designated Mucronomycin A (M-A). While conducting sub-acute toxicity studies with this antibiotic, this author observed that abdominal distention appeared in the rats after about three days of treatment. At autopsy large volumes of fluid, from 10 to 20 ml, were removed from the peritoneal cavity of each rat. This observation suggested the use of M-A in a study of the inflammatory response, and the availability of an analog of M-A, Mucronomycin B (M-B) offered an opportunity to study these antibiotics on a comparitive basis.

It had also been observed that antibiotic-treated rats did not eat well and therefore it became necessary to preclude the possible effects of this variable. A group of control animals which were deprived of all food (designated "starvation controls"), was therefore included in this research. Daily food consumption was ascertained for each pair of rats and the animals were weighed daily.

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1. Bristol, Pa.

In addition to inclusion of a starvation group of animals, consideration was given to other factors related to formation of peritoneal ascities. When an animal is supporting an inflammatory exudate volume of 10-20 ml, alterations might be expected in the concentration of ion and protein constituents of the body fluids. Consequently, blood serum and inflammatory exudate levels were established for sodium, potassium, chloride, calcium, phosphorus, total protein, albumin and globulin fractions.

In clinical ascities, urinary retention of electrolytes is seen. Hence, in this research, sodium, potassium and chloride determinations were done on all urines collected. Ionic retention can be related to decreased water output and therefore, water balance studies were established for each pair of rats. These latter data are presented separately and then were combined in the UER factor. The urinary excretion rate (UER factor) was calculated as follows: $UER = \frac{Ml\ urine\ excreted/\frac{2\ rats}{day}}{Ml\ water\ consumed/\frac{2\ rats}{day}}$ per 100 g body weight. According to this method of presentation, diuresis is indicated by a high UER factor, while retention is noted if the UER factor is low.

Changes in the numbers and types of circulating blood cells are often associated with the inflammatory process. Total erythrocyes, total leukocytes and differential leukocytes were therefore determined.

In summary then, this report presents, for the first time, data which allow a comparison to be made between the serum of a rat and the inflammatory exudate present in its peritoneal cavity.

B. Materials and Methods

1. General Considerations and Daily Protocols

a. Production of Peritoneal Ascities

In this portion of the study, the rats were divided into five groups as indicated below.

Group	Daily Treatment	Number of Rats
I	M-A, 0.25 mg/kg body weight, i.p.	20
II	M-B, 0.25 mg/kg body weight, i.p.	20
III	Vehicle (10% CW 400[1] in distilled water), 1 ml/kg body weight, i.p.	12
IV	Same as Group III but also starved	6
V	Untreated controls	6

Male, adult albino rats of the Sprague-Dawley strain[2] and weighing 200-400 g, were housed two per cage in standard metabolism cages[3]. The animals were each offered 18 g of Purina[4] rat chow daily. If any food remained after 24 hours, it was weighed to the nearest gram to determine daily food

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1. CW 400 is Carbowax 400, Polyethylene glycol, Union Carbide Chemicals Company, New York, N.Y.
2. Charles River Breeding Farms, North Wilmington, Mass.
3. Wahmann Mfg. Co., Baltimore, Maryland.
4. Ralston Purina Co., St. Louis, Missouri.

consumption. Water was provided for all rats ad libitum, by wiring two jars of 250 ml total capacity into each cage. All animals were weighed to the nearest gram and injected daily between 9-10 A.M. Water consumption was determined at this time by measuring the amount of water required to refill the jars to a previously established mark. The urine volume was determined to the nearest 0.5 ml by means of a graduated cylinder. Also at this time the urine collecting assembly was cleaned, rinsed with distilled water and reset for collection.

The antibiotics were prepared for intraperitoneal injection as follows: sufficient Muconomycin A[1] or Muconomycin B[1] was added to 10 per cent CW 400 to give a concentration of 0.25 mg of antibiotic per ml of suspension. Fresh suspensions were prepared when particle settling was observed. The vehicle was prepared by making a 10 per cent solution of CW 400 in distilled water.

2. Procedures for Obtaining Antibiotics

The following procedure for obtaining Muconomycin A was described in a personal communication to B.M. Vittimberga, University of Rhode Island, Kingston, R.I. The communication was from C. Smythe, Rohm and Haas Co., Bristol, Pa.

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1. Samples of both antibiotics kindly supplied by Dr. B.M. Vittimberga, Department of Chemistry, University of Rhode Island, Kingston, R.I.

"The organism was grown in a medium containing 1.0% glucose, 0.5% rolled oats, 0.1% Bacto-peptone, 0.1% Difco yeast extract, 0.05% K_2HPO_4, and 0.02% $MgSO_4 \cdot 7H_2O$. The medium was adjusted to pH 7.0 with sodium hydroxide, dispensed in 100-ml portions into 1-l. wide mouthed Erlenmeyer flasks and sterilized at 15 p.s.i. for 30 min. After inoculation with 1.0% of aqueous spore suspension derived from a well sporulated potato-dextrose-agar slant of ATCC 13667, incubation was carried out on a shaker rotating at 260 r.p.m. at 26° for 72 hr. The concentration of Muconomycin was about 100 µg/ml. The mycelia was then removed by filtration with the aid of diatomaceous earth and the filtratetreated with about 0.3% of Darco G60. The Darco adsorbate was collected on a filter, dried in air, and the activity eluted with benzene in a Soxhlet extractor. After evaporation of the benzene, a yellow oil remained which was taken up in acetone and induced to crystallize by the addition of water."

The following procedure for obtaining Muconomycin B was described in a personal communication from C. Smythe, Rohm and Haas Co., Bristol, Pa.

"The organism was grown in a medium containing 3.0% glucose, 3.0% rolled oats, 0.1% Difco yeast extract, 0.5% Difco peptone, 0.05% K_2HPO_4, 0.02% $Mg_4SO_47.H_2O$, and 93% H_2O. The isolation procedure for Muconomycin B is similar to that described for Muconomycin A."

3. Urinary Electrolyte Studies

In this phase of the work the effects of the different daily treatments on the daily output of sodium, potassium and chloride in the spontaneously-voided urine of rats were determined.

The urine-collecting funnels were fitted with cotton plugs to aid in keeping food fragments and droppings out of the urine. After measurement of the urine volume, the samples

were stored in stoppered glass test tubes in the freezer (at -40°C) until assay. Urine samples were analyzed for sodium and potassium by means of a flame photometer[1] by the lithium internal standard method.

Stock solutions of NaCl (1000mEq/L) and KCl (100 mEq/L) were prepared using distilled water. From these stock solutions, working standards of Na^+ (10-160 mEq/L) and K^+ (1-10 mEq/L) were prepared by dilution with distilled water. A 1:2000 solution of Li_2SO_4 in distilled water served as the internal standard solution. Standard curves were prepared by using standard concentrations of the ions, diluted 1:100 with the internal standard solutions. Urine was similarly diluted with the lithium sulfate solution: 1:100 for the sodium assay and 1:500 for the potassium assay. Each urine sample was read against the standard curve which had been constructed by plotting concentration of ion vs. resistance units on the instrument dial.

Urinary chloride was analyzed by the mercurimetric procedure of Schales and Schales (1941), as outlined by Boutwell (1961). In this method, the sample is titrated with an acid solution of mercuric nitrate in the presence of a s-diphenyl carbazone indicator.

To determine the total output of the electrolyte in terms of mEq/2 rats/day (i.e., mEq/cage/day), the concentration in

1. Advanced Instruments, Inc., Newton Highlands, Mass.

diluted with Hayem's solution; the solution preparation and method for counting the cells is described by D'Amour and Blood (1954). The diluent for the WBC counts was 1 per cent glacial acetic acid in distilled water. Wright's stain was applied to the dried blood smears, in accordance with the procedures of D'Amour and Blood (1954).

In order to collect the peritoneal exudate an abdominal mid-line incision was made in the rat, extending from the xiphoid process about 5 cm tailward. The exudate was removed by aspiration with a syringe and the total volume was measured to the nearest 0.5 ml by means of a graduated cylinder. The inflammatory exudate was frozen at -40°C. Before any assays were performed on the exudate, the slow-forming colt and cells were removed by centrifugation.

The collection of all terminal samples took less than 5 minutes, and this can be considered to be "simultaneous" sampling (Gullino, et al., 1964).

6. Blood Serum and Peritoneal Inflammatory Exudate Assays

a. Sodium and Potassium

Serum and exudate levels of sodium and potassium were determined in the same manner as in the urinary assays. The only difference in the method was in the range of working standards used: for Na^+, 120-150 mEq/L, and for K^+, 2-10 mEq/L.

the urine aliquot taken for assay was multiplied by the total volume collected for that cage.

4. Weight Gain Studies

The effects of the daily treatments on the individual body weights of rats were determined. All animals used in this study were routinely weighed daily to the nearest gram, on an Ohaus small-animal balance[1]. Records were made of the daily weights of each animal. It was observed by Mendillo (1965), that the most sensitive measure of body weight changes in a study such as this was cumulative daily weight gains from the first day of a study. Hence this method of data analysis was employed.

5. Terminal Protocols

On the last day of the study the animals were anesthetized by allowing them to inhale ether. The thoracic cavity was opened by means of a surgical scissors and the aorta was incised just above the heart. By this method, adequate amounts of blood could be collected so that all the desired chemical assays could be performed on each sample. The blood was allowed to clot, centrifuged and then frozen at -40°C until assay. During the bleeding of each rat, samples were taken for red blood cell (RBC) counts and white blood cell (WBC) counts; slide-smears were also made for differential white cell counts. For RBC counts, the sample was immediately

1. Ohaus Scale Corp., Union, New Jersey

For both the Na^+ and K^+ analysis, 1:100 dilutions of the serum or exudate were prepared using the lithium sulfate solution as the diluent. The values are reported in concentration units, i.e., mEq/L.

b. Chloride

Direct titration of the body fluid with standardized mercuric nitrate was employed here. It is noted by Henry (1964 p. 407) that initial deproteinization is not required if an ultramicro technique is used. Other features of the analytical method are as indicated for the urinary chlorides mentioned previously. Serum and exudate levels of chloride are presented in terms of mEq/L.

c. Calcium

Calcium levels in blood serum and inflammatory exudate were determined by the method of Diehl and Ellingboe (1960). For this assay and most of the following ones, the Beckman[1] Model 150 Ultramicro Analytical System was employed. By the use of this system one is able to analyze for calcium by titration with EDTA (ethylenediaminetetraacetic acid, disodium salt) using calceine as the indicator and only 0.02 ml of biological fluid sample. Calcium values (mg per cent) were converted to mEq/L so that all electrolyte data would be presented in the same units.

1. Beckman Instruments, Inc., Palo Alto, California Technical Bulletin No. 6071D.

d. Phosphorus

The biological fluids in question were also analyzed for phosphorus by use of the Beckman[1] Ultramicro System. This method employes 0.02 ml of sample in the classic method of Fiske and Subbarow (1925). To convert the values obtained in this method (mg P/100 ml) to mEq/L, multiplication by the factor 0.58 was carried out as suggested by Henry (1964, p. 415).

e. Proteins

In another phase of this research, proteins were analyzed in the inflammatory exudate and blood serum of rats treated with M-A or M-B. By the use of the Beckman[2] system one is able to determine total protein, albumin fraction, globulin fraction and albumin/globulin ratio using only 0.005 ml of sample. The total protein, albumin and globulin concentrations are reported as grams of the protein per 100 ml of body fluid.

f. Statistics

Data comparisons were made by means of Student's "t" test (Snedecor, 1956), using the IBM 1420 computer and facilities available at the University of Rhode Island.

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1. Beckman Instrument, Inc., Palo Alto, California Technical Bulletin No. 6079E.
2. Technical Bulletin No. 6074D.

IV. RESULTS

Tables and figures are contained in this section.

TABLE I

CONTROL VALUES OF ELECTROLYTES, PROTEINS AND BLOOD CELLS IN SERUM AND BLOOD OF ALBINO RATS

Constituent	Rat Serum[a]	Sample Size
Sodium	149 ± 8.0[b]	6
Potassium	8.8 ± 1.7	4
Chloride	104 ± 7.0	16
Calcium	5.6 ± 1.1	10
Phosphorus	1.7 ± 0.4	10
Total protein	8.0 ± 0.6	5
Albumin	3.7 ± 0.8	5
Globulin	4.2 ± 1.1	5
	Rat Blood	
Red blood cells	11.8 ± 3.4 x $10^6/mm^3$	6
Total white cells	16.5 ± 5.8 x $10^3/mm^3$	6
Differential white cells		6
	Per Cent	
Polymorphonuclear neutrophiles	27 ± 6	
Lymphocytes	68 ± 6	
Eosinophiles	0 to 5	
Monocytes	0 to 2	
Basophiles	0 to 1	

a: Rat serum electrolytes units are mEq/L. Protein, albumin and globulin units are g/100 ml serum.
b: Mean ± S.D.

TABLE II

NORMAL MEAN VALUES OF ELECTROLYTES, PROTEINS AND BLOOD CELLS REPORTED IN THE LITERATURE[a]

Constituent	Rat Serum[b]	Dog Serum[d]
Sodium	152	151
Potassium	5.9	5.2
Chloride	118	112
Calcium	6.2	5.3
Phosphorus	2.3	1.6
Total protein	5.5[c]	7.0
Albumin	---	3.6
Globulin	---	2.6

	Rat Blood
Red blood cells	$8.9 \times 10^6/mm^3$
Total white cells	$14.0 \times 10^3/mm^3$
Differential white cells	

	Per Cent
Polymorphonuclear neutrophiles	22
Lymphocytes	73
Eosinophiles	2.2
Monocytes	2.3
Basophiles	0.5

a: Except where indicated, values are from Altman (1961).
b: Serum electrolytes units are mEq/L. Protein, albumin and globulin units are g/100 ml serum.
c: From Gullino et al. (1964).
d: Dog serum values are included to provide comparison with another mammal.

TABLE III

LITERATURE AND CONTROL VALUES FOR THE ALBINO RAT; URINE AND WATER DATA

Constituent	LITERATURE mEq/2 rats/day[a,b] Mean	Range	THIS REPORT mEq/2 rats/day Mean	Range	N
Urinary Sodium	2.8	0.4-3.8	1.6	0.7-2.5	30
Urinary Potassium	1.8	0.8-3.8	4.0	2.5-5.4	30
Urinary Chloride	2.6	0.8-3.8	3.1	1.7-4.7	30
	ml/2 rats/day		ml/2 rats/day		
Urine output	24	4.3-48.0	22.9	9.0-39.2	30
Water consumption	--	--------	76.2	65-98	30
UER[c]	--	--------	0.044	0.018-0.078	30

a: Literature values are individual rat values x 2, to conform to method of collection used in this report, i.e. urine collections were done on pairs of rats.
b: From Altman (1961).
c: UER calculates as,

$$\frac{\text{Ml urine excreted}}{\text{Ml water ingested}} \text{ per 2 rats/day/100 gram of body weight.}$$

TABLE IV

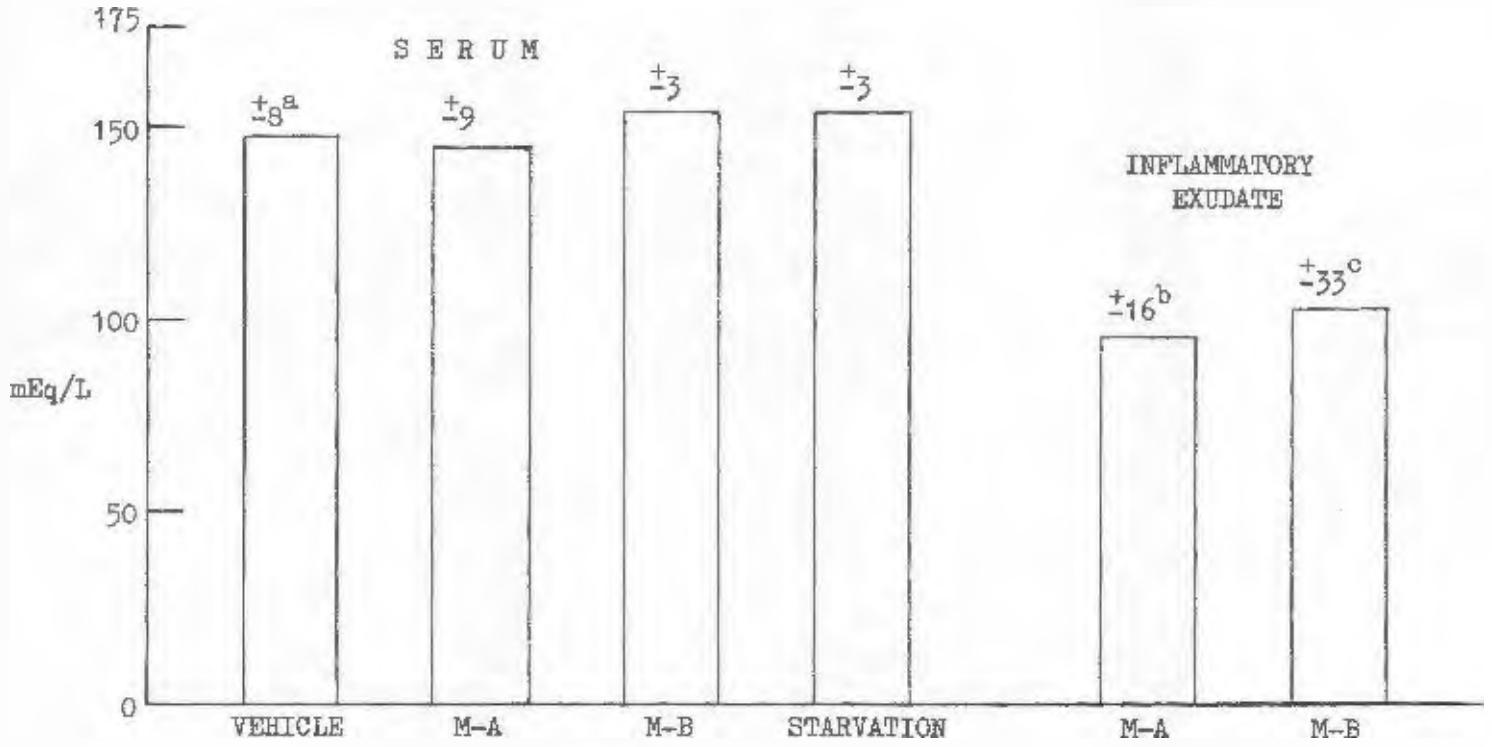
THE EFFECTS OF MUCONOMYCIN A, MUCONOMYCIN B AND STARVATION ON BLOOD SERUM AND PERITONEAL INFLAMMATORY EXUDATE LEVELS OF SODIUM IN MALE ALBINO RATS

Treatment	Blood Serum (mEq/L)	Inflammatory Exudate (mEq/L)
Vehicle	149 ± 8[a] (6)[b]	---
Muconomycin A	147 ± 9 (12), N. S.	96 ± 16 (13), DEC[d]
Muconomycin B	152 ± 3 (17), N. S.	102 ± 33 (18), DEC[e]
Starvation	153 ± 3 (5), N.S.	---

a: mean $\pm$ S. D., concentration of sodium, mEq/L.
b: number of fluid samples assayed.
c: N. S. - not significantly different ($P<0.05$) from Vehicle-treated group.
d: DEC - significantly decreased from Muconomycin A serum.
e: DEC - significantly decreased from Muconomycin B serum.

Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg, Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg. Starvation groups also received Vehicle, 1 ml/kg.

Fig. 1. THE EFFECTS OF MUCONOMYCIN A(M-A), MUCONOMYCIN B(M-B) AND STARVATION ON BLOOD SERUM AND PERITONEAL INFLAMMATORY EXUDATE LEVELS OF SODIUM IN MALE ALBINO RATS.



a: number above each bar is ± S.D.
b: significantly decreased ($P < 0.05$) from M-A serum.
c: significantly decreased from M-B serum.
Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg.
Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg.
Starvation group also received Vehicle, 1 ml/kg.

TABLE V

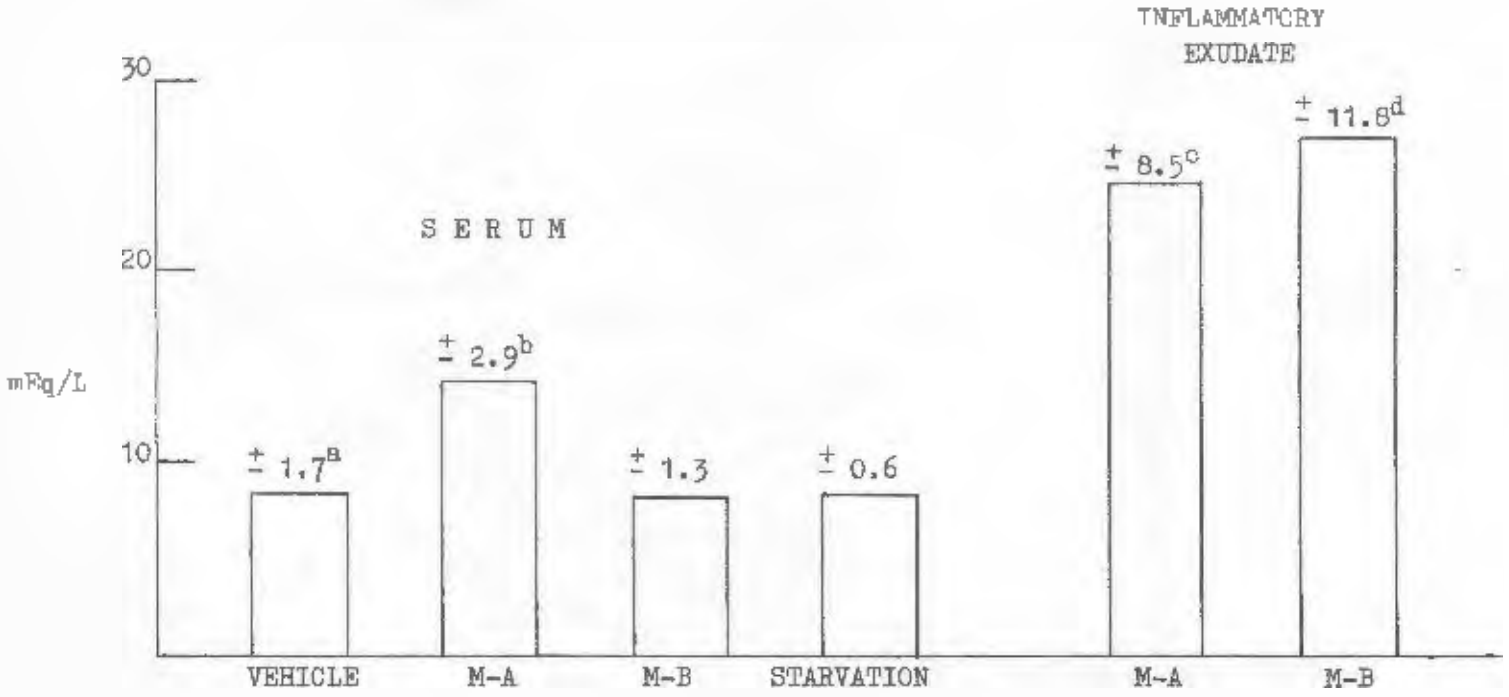
THE EFFECTS OF MUCONOMYCIN A, MUCONOMYCIN B AND STARVATION ON BLOOD SERUM AND PERITONEAL INFLAMMATORY EXUDATE LEVELS OF POTASSIUM IN MALE ALBINO RATS

Treatment	Blood Serum (mEq/L)	Inflammatory Exudate (mEq/L)
Vehicle	8.8 ± 1.7[a] (4)[b]	--
Muconomycin A	14.3 ± 2.9 (8), INC[d]	24.8 ± 8.5 (12), INC[e]
Muconomycin B	8.2 ± 1.3 (13), N.S.[c]	27.0 ± 11.8 (18), INC[f]
Starvation	8.4 ± 0.6 (3), N.S.[c]	--

a: mean ± S. D., concentration of potassium, mEq/L.
b: number of fluid samples. Assay was not done if fluid was hemorrhagic.
c: N.S. not significantly different ($P<0.05$) from Vehicle-treated group.
d: INC-significantly increased from Vehicle-treated group.
e: INC-significantly increased from Muconomycin A serum.
f: INC-significantly increased from Muconomycin B serum.

Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg, Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg. Starvation group also received Vehicle, 1 ml/kg.

FIG. 2. THE EFFECTS OF MUCCNOMYCIN A(M-A), MUCCNOMYCIN B(M-B) AND STARVATION ON BLOOD SERUM AND PERITONEAL INFLAMMATORY EXUDATE LEVELS OF POTASSIUM IN MALE AIRING RATS.



a: number above each bar is ± S.I.
b: significantly increased ($P<0.05$) from Vehicle-treated group.
c: significantly increased from M-A serum.
d: significantly increased from M-B serum.
Doses (i.p.) daily for 7 days: Mucconomycin A or B, 0.25 mg/kg.
Vehicle, Carbowax 400, 10% in distilled water 1 ml/kg.
Starvation group also received Vehicle, 1 ml/kg.

TABLE VI

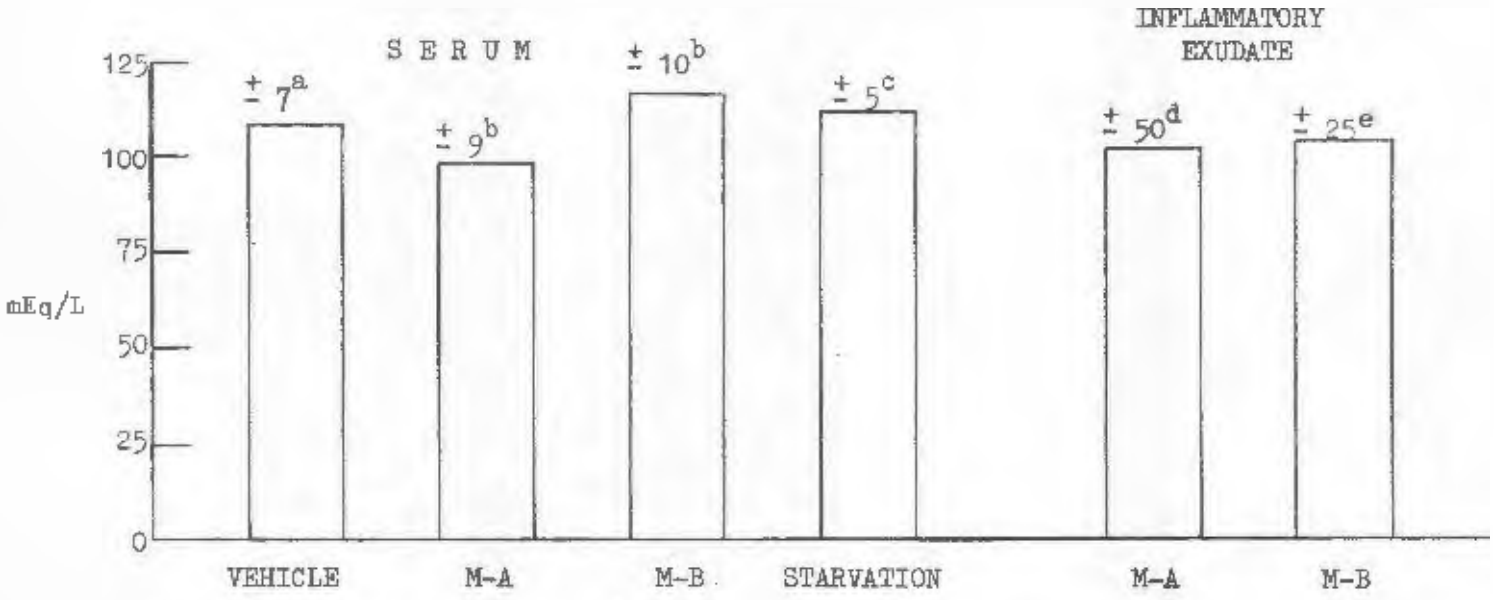
THE EFFECTS OF MUCONOMYCIN A, MUCONOMYCIN B AND STARVATION ON BLOOD SERUM AND PERITONEAL INFLAMMATORY EXUDATE LEVELS OF CHLORIDE IN MALE ALBINO RATS

Treatment	Blood Serum (mEq/L)	Inflammatory Exudate (mEq/L)
Vehicle	107 ± 3[a] (4)[b]	--
Muconomycin A	99 ± 9 (11), DEC[d]	103 ± 50 (12), N.S.[e]
Muconomycin B	117 ± 10 (17), INC[d]	102 ± 25 (16), DEC[f]
Starvation	113 ± 5 (4), N.S.[c]	--

a: mean ± S.D., concentration of chloride, mEq/L.
b: number of fluid samples.
c: N.S. not significantly different ($P<0.05$) from Vehicle-treated group.
e: N.S. not significantly different from Muconomycin A serum.
f: DEC-significantly decreased from Muconomycin B serum.

Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg, Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg. Starvation group also received Vehicle, 1 ml/kg.

FIG. 3. THE EFFECTS OF MUCONOMYCIN A(M-A), MUCONOMYCIN B(M-B) AND STARVATION ON BLOOD SERUM AND PERITONEAL INFLAMMATORY EXUDATE LEVELS OF CHLORIDE IN MALE ALBINO RATS.



a: number above each bar is ± S.D.
b: significantly decreased ($P<0.05$) or increased from Vehicle-treated group.
c: N.S. not significantly different from Vehicle-treated group.
d: N.S. not significantly different from M-A serum.
e: DEC significantly decreased from M-B serum.

TABLE VII

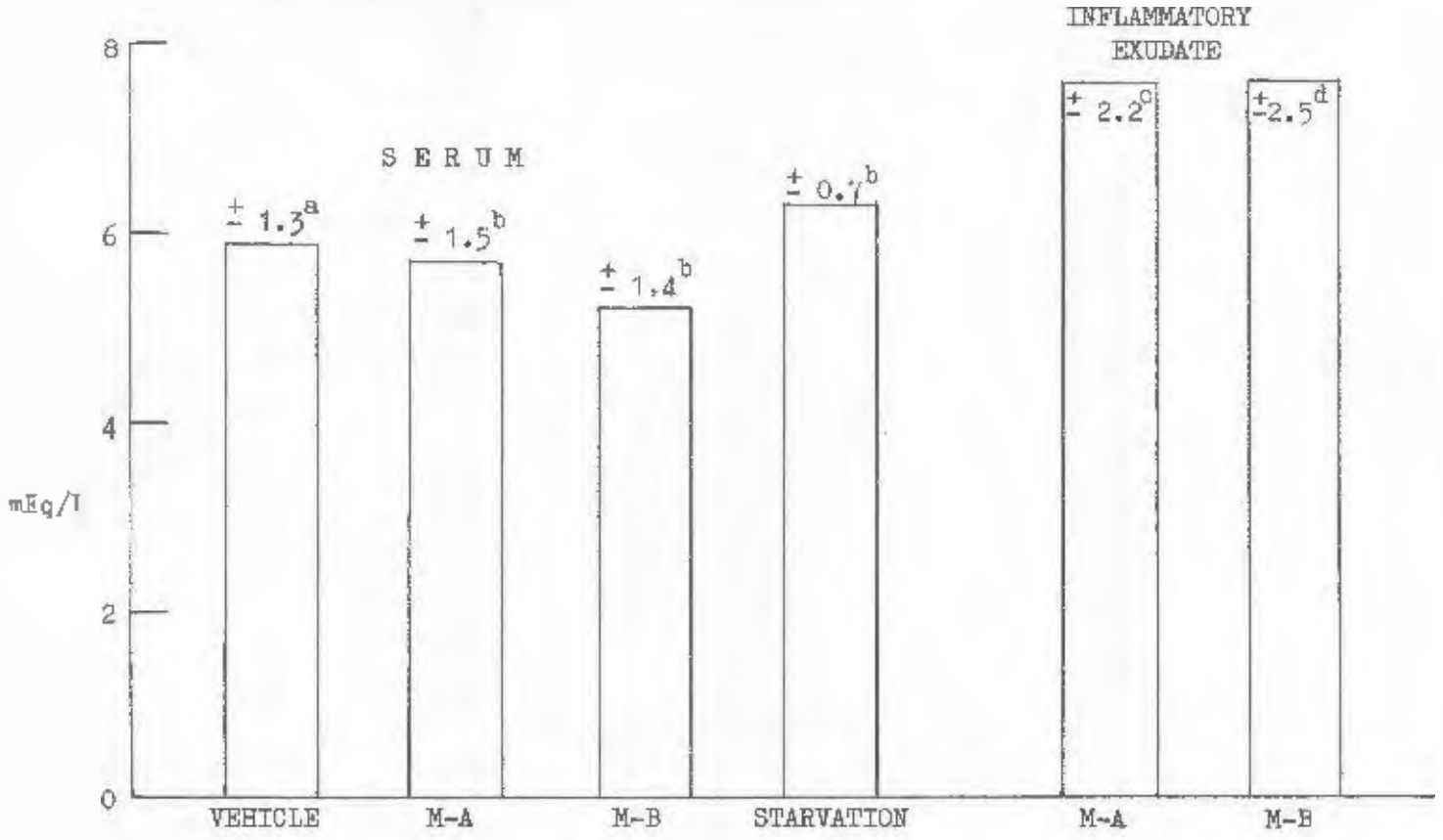
THE EFFECTS OF MUCONOMYCIN A, MUCONOMYCIN B AND STARVATION ON BLOOD SERUM AND PERITONEAL INFLAMMATORY EXUDATE LEVELS OF CALCIUM IN MALE ALBINO RATS

Treatment	Blood Serum (mEq/L)	Inflammatory Exudate (mEq/L)
Vehicle	5.9 ± 1.3[a] (4)[b]	--
Muconomycin A	5.7 ± 1.5 (6), N.S.[c]	7.5 ± 2.2 (12), INC[d]
Muconomycin B	5.2 ± 1.5 (17), N.S.[c]	7.6 ± 2.5 (17), INC[e]
Starvation	6.3 ± 0.7 (4), N.S.[c]	--

a: mean $\pm$ S.D., concentration of calcium, mEq/L.
b: number of fluid samples.
c: N.S.-not significantly different ($P<0.05$) from Vehicle-treated group.
d: INC-significantly increased from Muconomycin A serum.
e: INC-significantly increased from Muconomycin B serum.

Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg
Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg.
Starvation group also received Vehicle, 1 ml/kg.

FIG. 4. THE EFFECTS OF MUCONOMYCIN A(M-A), MUCONOMYCIN B(M-B) AND STARVATION ON BLOOD SERUM AND PERITONEAL INFLAMMATORY EXUDATE LEVELS OF CALCIUM IN MALE ALBINO RATS.



a: number above each bar is ± S.D.
b: not significantly different ($P < 0.05$) from Vehicle-treated group.
c: significantly increased from M-A serum.
d: significantly increased from M-B serum.
Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg.
Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg.
Starvation group also received Vehicle, 1 ml/kg.

TABLE VIII

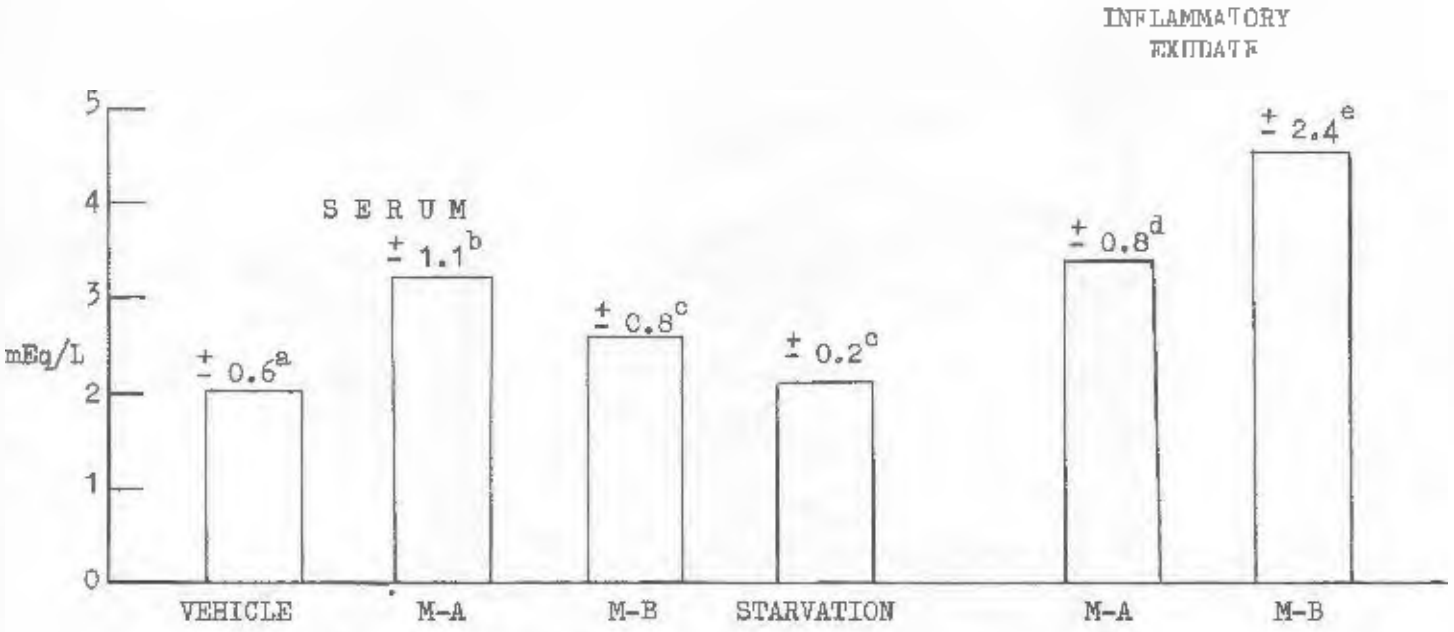
THE EFFECTS OF MUCONOMYCIN A, MUCONOMYCIN B AND STARVATION ON BLOOD SERUM AND PERITONEAL INFLAMMATORY EXUDATE LEVELS OF PHOSPHORUS IN MALE ALBINO RATS

Treatment	Blood Serum (mEq/L)	Inflammatory Exudate (mEq/L)
Vehicle	2.0 ± 0.6[a] (5)[b]	--
Muconomycin A	3.2 ± 1.1 (6), INC[c]	3.4 ± 0.8 (15), N.S.[e]
Muconomycin B	2.6 ± 0.8 (18), N.S.[d]	4.5 ± 2.4 (15), INC[f]
Starvation	2.1 ± 0.2 (4), N.S.[d]	--

a: mean ± S.D., concentration of phosphorus, mEq/L.
b: number of fluid samples.
c: INC-significantly ($P<0.05$) increased from Vehicle-treated group.
d: N.S.-not significantly different from Vehicle-treated group.
e: N.S.-not significantly different from Muconomycin A group serum.
f: INC-significantly increased from Muconomycin B group serum.

Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg, Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg. Starvation group also received Vehicle, 1 ml/kg.

FIG. 5. THE EFFECTS OF MUCONOMYCIN A(M-A), MUCONOMYCIN B(M-B) AND STARVATION ON BLOOD SERUM AND PERITONEAL INFLAMMATORY EXUDATE LEVELS OF PHOSPHORUS IN MALE ALBINO RATS.



a: number above each bar is ± S.D.
b: significantly ($P < C.05$) increased from Vehicle-treated group.
c: not significantly different from Vehicle-treated group.
d: not significantly different from M-A group serum.
e: significantly increased from M-B group serum.
Doses (i.p.) daily for 7 days: Muconomycin A or B, C.25 mg/kg,
Vehicle, Carbowax 4CC, 10% in distilled water, 1 ml/kg.
Starvation group also received Vehicle, 1 ml/kg.

TABLE IX

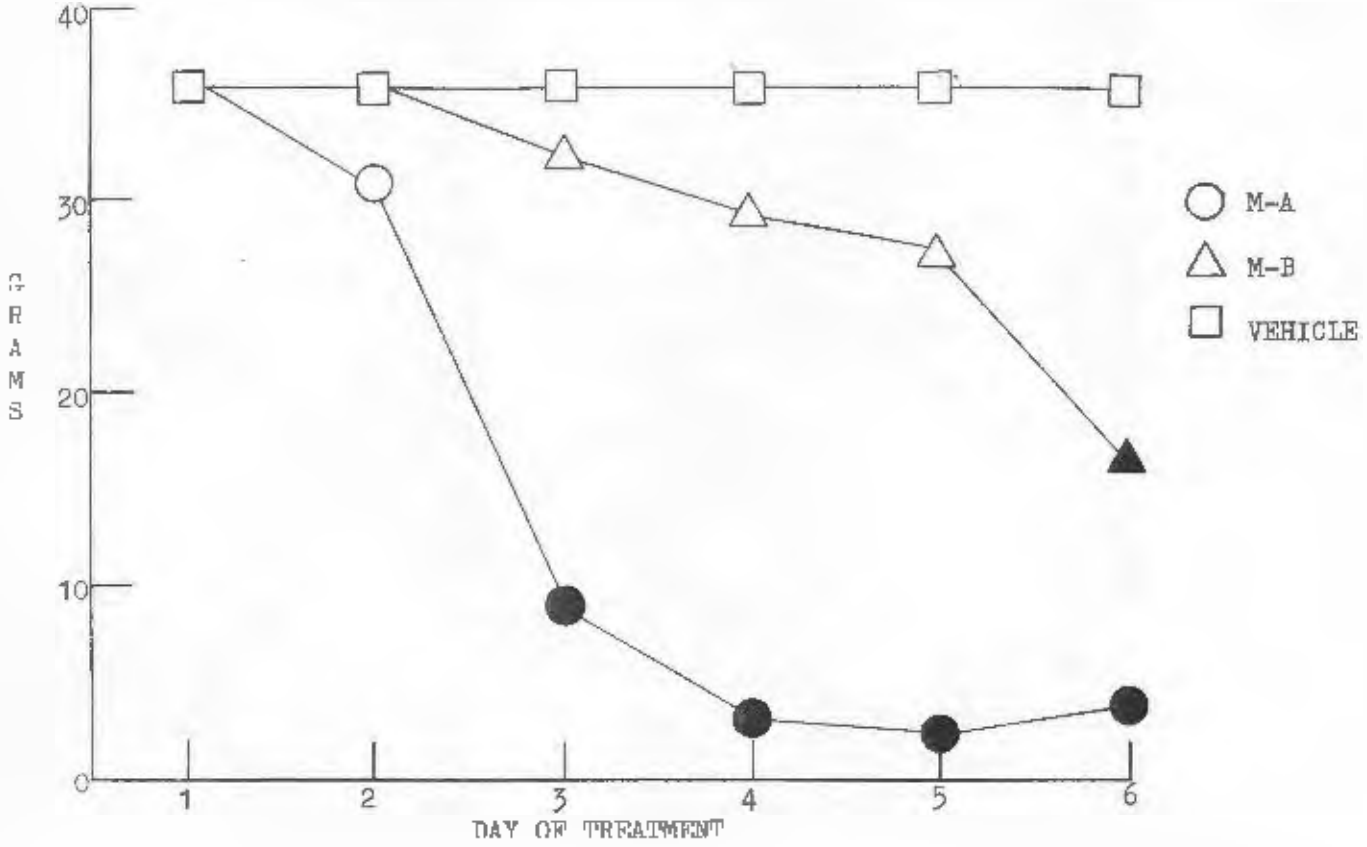
THE EFFECTS OF MUCONOMYCIN A AND MUCONOMYCIN B ON DAILY FOOD CONSUMPTION IN MALE ALBINO RATS

	Food Consumption (G/2 rats/day)		
Day of Treatment	Vehicle	Muconomycin A	Muconomycin B
1	36 (6)[a]	36 (10)	36 (10)
2	36	31 ± 8[b] N.S.[c]	36
3	36	9 ± 12 DEC[d]	32 ± 10 N.S.
4	36	4 ± 13 DEC	30 ± 10 N.S.
5	36	3 ± 4 DEC	28 ± 12 N.S.
6	36	4 ± 7 DEC	17 ± 12 N.S.

a: number of food samples weighed.
b: mean ± S.D. is given when all of food offered was not eaten.
c: N.S.-not significantly ($P<0.05$) different from Vehicle-treated group.
d: DEC-significantly decreased from Vehicle-treated group. Also on days 3, 4, 5 and 6, Muconomycin A-treated group had significantly decreased consumption when compared to Muconomycin B-treated group.

Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg, Vehicle, Carbowax 400, 10% in distilled water 1 ml/kg.

FIG. 6. THE EFFECTS OF MUCONOMYCIN A(M-A) AND MUCONOMYCIN B(M-B) ON MEAN DAILY FOOD CONSUMPTION IN MALE ALPINE RATS. GRAMS/2 RATS/DAY.



Solid symbols indicate significant difference ($P < 0.05$) from Vehicle treated group.
Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg,
Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg.

TABLE X

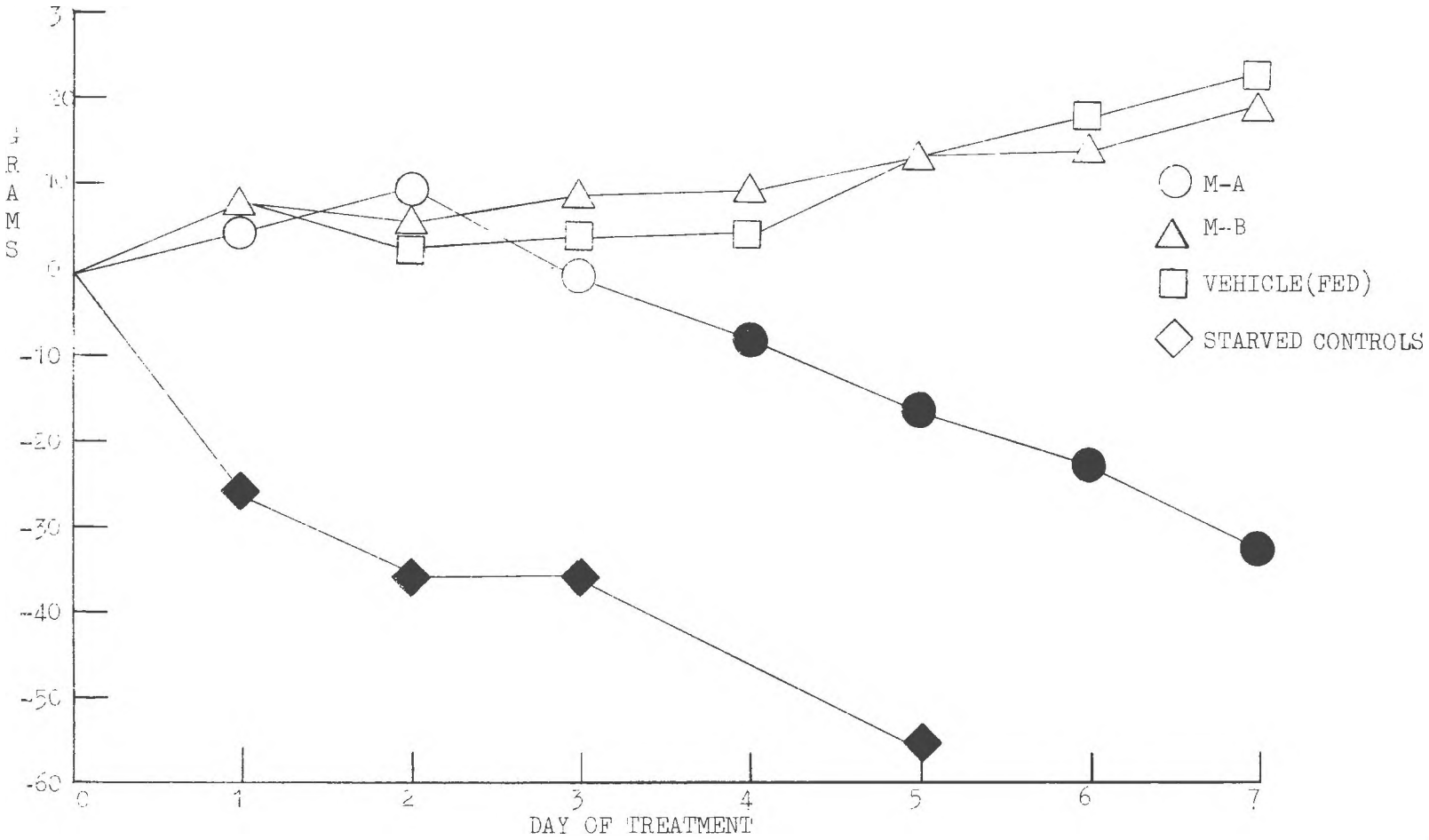
THE EFFECTS OF MUCONOMYCIN A, MUCONOMYCIN B AND STARVATION ON THE CUMULATIVE DAILY WEIGHT GAINS FROM DAY 0 OF MALE ALBINO RATS

Day	Vehicle	Muconomycin A	Muconomycin B	Starvation
1	4 ± 4[a] (12)[b]	4 ± 6 (20), N.S.[c]	6 ± 7 (20), N.S.	-26 ± 8 (6), DEC[c]
2	3 ± 18 (12)	7 ± 8 (20), N.S.	9 ± 8 (20), N.S.	-35 ± 8 (6), DEC
3	3 ± 9 (12)	-1 ± 8 (20), N.S.	8 ± 8 (19), N.S.	-36 ± 9 (6), DEC
4	5 ± 9 (6)	-9 ± 9 (20), DEC	9 ± 9 (19), N.S.	---
5	11 ± 8 (6)	-17 ± 14 (19), DEC	12 ± 10 (19), N.S.	-55 ± 28 (6), DEC
6	15 ± 9 (6)	-23 ± 20 (19), DEC	13 ± 12 (19), N.S.	---
7	21 ± 11 (6)	-34 ± 24 (19), DEC	19 ± 19 (19), N.S.	---

a: mean ± S.D., cumulative weight gains (grams) from day 0.
b: number of rats.
c: statistical comparison at probability, $P<0.05$;
N.S.-not significantly different from Vehicle-treated group.
DEC -significantly decreased from Vehicle-treated group.

Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg, Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg. Starvation groups also received Vehicle, 1 ml/kg.

FIG. 7. THE EFFECTS ON MUCONOMYCIN A(M-A), MUCONOMYCIN B(M-B) AND STARVATION ON THE CUMULATIVE DAILY WEIGHT GAINS FROM DAY ZERO OF MALE ALBINO RATS.



Solid symbols indicate significant difference ($P<0.05$) from Vehicle-treated group.
Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg,
Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg.
Starvation group also received Vehicle, 1 ml/kg.

TABLE XI

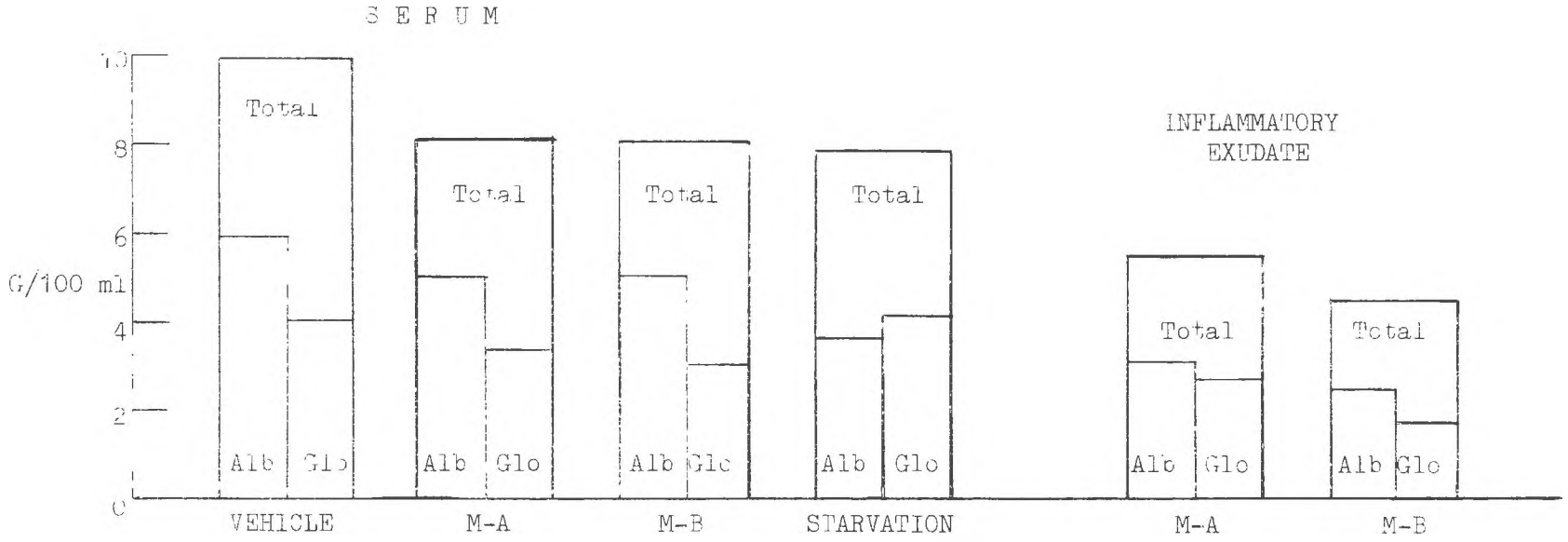
THE EFFECTS OF MUCONOMYCIN A (M-A), MUCONOMYCIN B (M-B) AND STARVATION ON BLOOD SERUM AND PERITONEAL INFLAMMATORY EXUDATE LEVELS OF PROTEINS IN MALE ALBINO RATS

	Protein Concentration (g / 100 ml fluid)			A/G
Treatment	Total	Albumin	Globulin	Ratio
Vehicle	9.9 ± 2.1[a] (2)[b]	5.9 ± 0.6	4.0 ± 1.5	1.6 ± 0.4
M-A Serum	8.2 ± 1.6 (10), N.S.[c]	4.9 ± 1.8 N.S.	3.4 ± 1.3 N.S.	1.7 ± 1.0 N.S.
M-B Serum	8.2 ± 1.4 (5), N.S.[c]	5.2 ± 0.5 N.S.	3.0 ± 1.3 N.S.	2.0 ± 0.8 N.S.
Starvation	7.8 ± 1.7 (5), N.S.[c]	3.6 ± 0.9 DEC[c]	4.2 ± 1.0 N.S.	0.9 ± 0.2 N.S.
M-A Inf. Exudate	5.5 ± 1.3 (13), DEC[d]	2.9 ± 1.2 DEC	2.6 ± 0.9 N.S.	1.5 ± 0.8 N.S.
M-B Inf. Exudate	4.3 ± 1.1 (9), DEC[e]	2.4 ± 1.2 DEC	1.7 ± 0.8 N.S.	1.9 ± 1.8 N.S.

a: mean ± S.D. concentration of protein, g per 100 ml.
b: number of fluid samples.
c: M-A Serum, M-B Serum and Starvation groups compared with Vehicle-treated group.
N.S.-not significantly different ($P<0.05$) from Vehicle-treated group.
DEC-significantly decreased from Vehicle-treated group.
d: M-A Inflammatory Exudate compared with M-A Serum.
e: M-B Inflammatory Exudate compared with M-B Serum.

Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg, Vehicle, Carbowax 400, 10% distilled water, 1 ml/kg.
Starvation group also received Vehicle, 1 ml/kg.

FIG. 8. THE EFFECTS OF MUCONOMYCIN A(M-A), MUCONOMYCIN B(M-B) AND STARVATION ON BLOOD SERUM AND PERITONEAL INFLAMMATORY EXUDATE LEVELS OF PROTEINS IN MALE ALBINO RATS. TOTAL PROTEIN, ALBUMIN AND GLOBULIN FRACTIONS.



Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg, Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg. Starvation group also received Vehicle, 1 ml/kg.
See Table XI for statistical comparisons.

TABLE XII

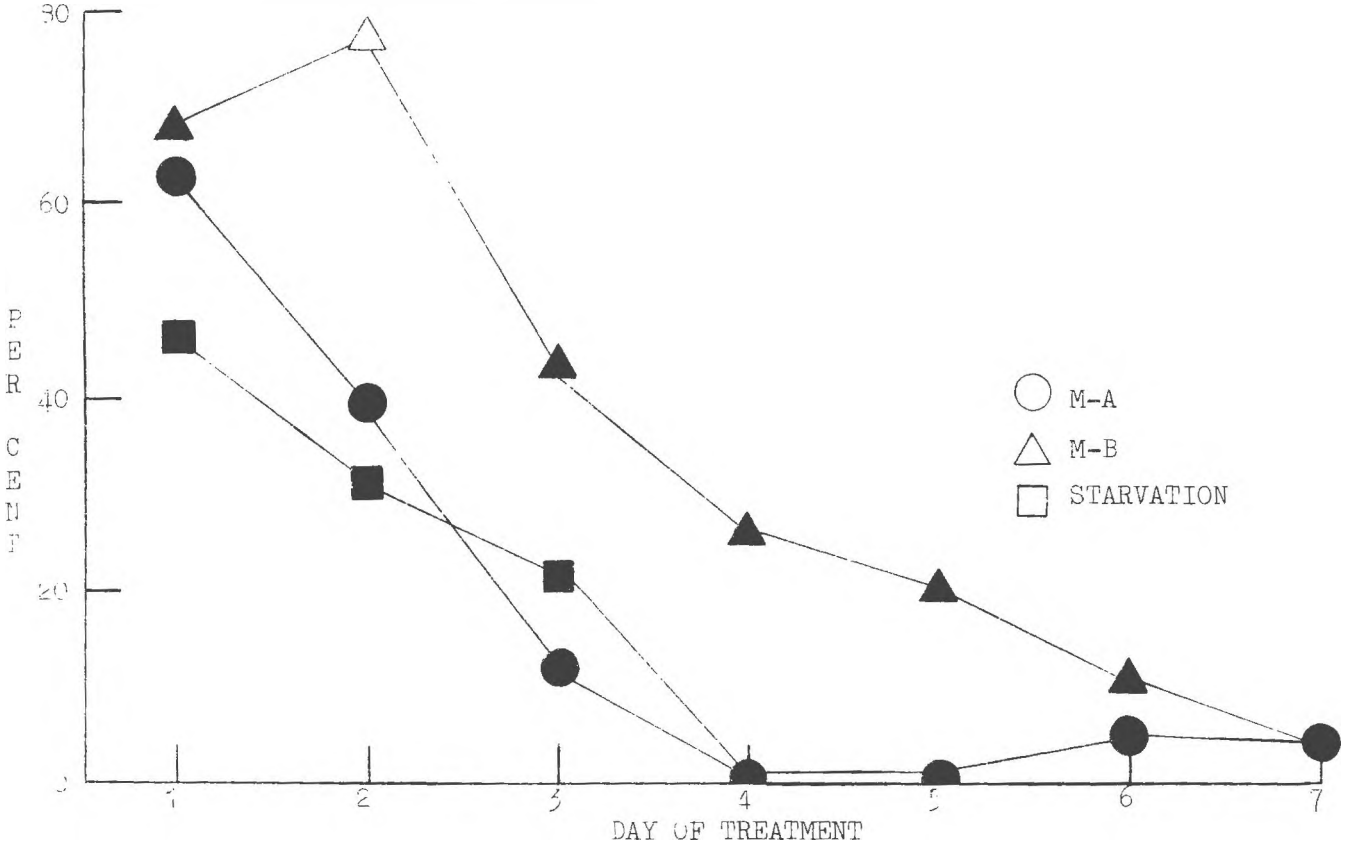
THE EFFECTS OF MUCONOMYCIN A, MUCONOMYCIN B AND STARVATION ON DAILY URINARY EXCRETION OF SODIUM IN MALE ALBINO RATS

Day of Treatment	Sodium Excretion (mEq/2 rats/day)			
	Vehicle	Muconomycin A	Muconomycin B	Starvation
1	1.61 ± 0.40[a] (6)[b]	1.02 ± 0.28 (9), DEC[c]	1.09 ± 0.32 (10), DEC	0.89 ± 0.20 (3), DEC
2	1.33 ± 0.53 (5)	0.52 ± 0.29 (9), DEC	1.04 ± 0.52 (10), N.S.[d]	0.52 ± 0.13 (3), DEC
3	1.78 ± 0.70 (6)	0.22 ± 0.30 (10), DEC	0.78 ± 0.70 (6), DEC	0.44 ± 0.29 (3), DEC
4	1.90 ± 0.30 (3)	0.03 ± 0.05 (10), DEC	0.49 ± 0.53 (10), DEC	Less than 0.02[e] (3), DEC
5	1.69 ± 0.42 (3)	0.04 ± 0.10 (10), DEC	0.33 ± 0.53 (10), DEC	Less than 0.02[e] (3), DEC
6	1.99 ± 0.54 (3)	0.10 ± 0.18 (7), DEC	0.22 ± 0.46 (10), DEC	--
7	1.81 ± 0.57 (3)	0.07 ± 0.07 (9), DEC	0.09 ± 0.19 (9), DEC	--

a: mean ± S.D., mEq/2 rats/day.
b: number of sodium assays completed.
c: DEC-significantly ($P<0.05$) decreased from Vehicle-treated group.
d: N.S.-not significantly different from Vehicle-treated group.
e: mean value was less than 0.02 because some samples read below the sensitivity of the flame photometer.

Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg, Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg.
Starvation group also received Vehicle, 1 ml/kg.

FIG. 7. THE EFFECTS OF MUCONOMYCIN A(M-A), MUCONOMYCIN B(M-B) AND STARVATION ON DAILY URINARY EXCRETION OF SODIUM IN MALE ALBINO RATS. PER CENT OF VEHICLE-TREATED GROUP.



Solid symbols indicate significant difference ($P<0.05$) from Vehicle-treated group.
Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg.
Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg.

TABLE XIII

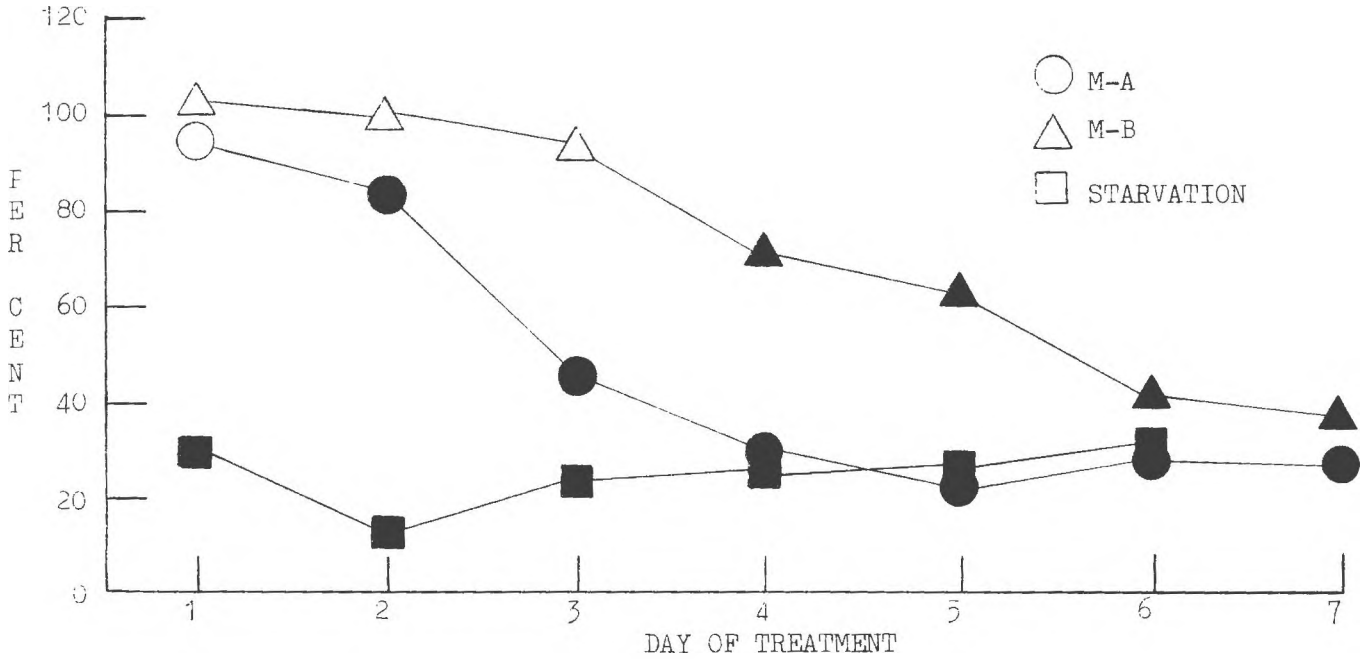
THE EFFECTS OF MUCONOMYCIN A, MUCONOMYCIN B AND STARVATION ON DAILY URINARY EXCRETION OF POTASSIUM IN MALE ALBINO RATS

Day of Treatment	Potassium Excretion (mEq/2 rats/day)			
	Vehicle	Muconomycin A	Muconomycin B	Starvation
1	3.14 ± 0.68[a] (6)[b]	3.01 ± 0.37 (9), N.S.[c]	3.53 ± 1.57 (10), N.S.	1.32 ± 1.17 (3), DEC[d]
2	3.35 ± 0.45 (6)	2.79 ± 0.40 (9), DEC	3.32 ± 0.51 (10), N.S.	0.57 ± 0.43 (3), DEC
3	4.07 ± 0.76 (6)	1.91 ± 0.63 (10), DEC	3.69 ± 0.66 (9), N.S.	1.07 ± 0.06 (3), DEC
4	4.60 ± 0.90 (3)	1.27 ± 0.37 (10), DEC	3.11 ± 1.32 (10), DEC	1.05 ± 0.13 (3), DEC
5	4.08 ± 0.75 (3)	0.83 ± 0.46 (10), DEC	2.45 ± 1.14 (10), DEC	0.83 ± 0.17 (3), DEC
6	4.83 ± 0.89 (3)	1.14 ± 0.23 (8), DEC	2.09 ± 1.16 (10), DEC	---
7	4.02 ± 0.22 (3)	0.98 ± 0.48 (9), DEC	1.53 ± 0.94 (9), DEC	---

a: mean ± S.D., mEq/2 rats/day.
b: number of potassium assays completed.
c: N.S.-not significantly ($P<0.05$) changed from Vehicle-treated group.
d: DEC-significantly decreased from Vehicle-treated group.

Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg, Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg. Starvation group also received Vehicle, 1 ml/kg.

FIG. 10. THE EFFECTS OF MUCONOMYCIN A(M-A), MUCONOMYCIN B(M-B) AND STARVATION ON DAILY URINARY EXCRETION OF POTASSIUM IN MALE ALBINO RATS. PER CENT OF VEHICLE-TREATED GROUP.



Solid symbols indicate significant ($P<0.05$) difference from Vehicle-treated group.
Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg,
Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg.

TABLE XIV

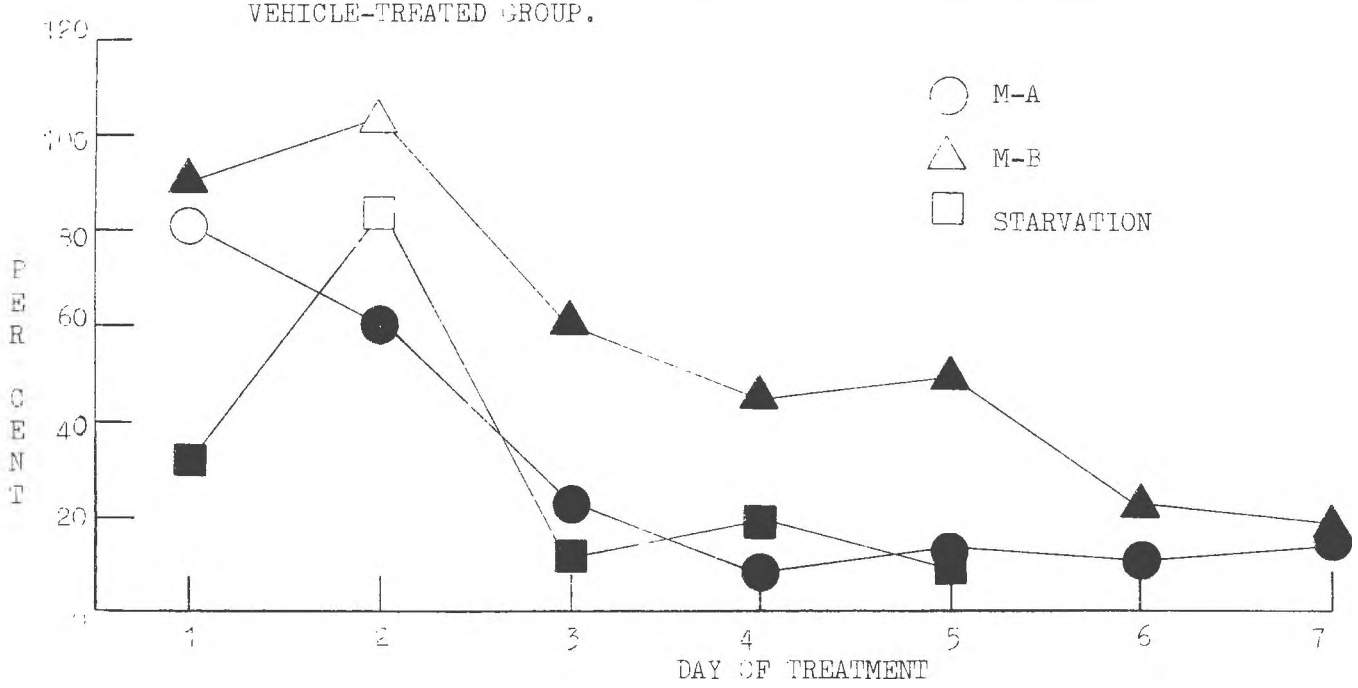
THE EFFECTS OF MUCONOMYCIN A, MUCONOMYCIN B AND STARVATION ON DAILY URINARY EXCRETION OF CHLORIDE IN MALE ALBINO RATS

Day of Treatment	Chloride Excretion (mEq/2 rats/day)			
	Vehicle	Muconomycin A	Muconomycin B	Starvation
1	2.74 ± 0.54[a] (6)[b]	2.22 ± 0.49 (9), N.S.[c]	2.45 ± 0.53 (10), N.S.	1.08 ± 0.52 (3), DEC[d]
2	2.22 ± 0.48 (6)	1.39 ± 0.54 (9), DEC	2.26 ± 0.57 (10), N.S.	2.25 ± 1.50 (3), N.S.
3	3.17 ± 0.77 (6)	0.73 ± 0.72 (10), DEC	2.06 ± 0.83 (9), DEC	0.35 ± 0.04 (3), DEC
4	3.77 ± 0.99 (3)	0.27 ± 0.12 (10), DEC	1.66 ± 1.09 (10), DEC	0.61 ± 0.21 (3), DEC
5	2.67 ± 0.27 (3)	0.21 ± 0.11 (10), DEC	1.26 ± 1.09 (10), DEC	0.16 ± 0.03 (3), DEC
6	3.83 ± 0.83 (3)	0.29 ± 0.19 (8), DEC	0.89 ± 1.09 (9), DEC	---
7	3.50 ± 0.46 (3)	0.39 ± 0.41 (9), DEC	0.55 ± 0.61 (6), DEC	---

a: mean ± S.E., mEq/2 rat/day.
b: number of chloride assays completed.
c: N.S.-not significantly ($P<0.05$) changed from Vehicle-treated group.
d: DEC-Significantly decreased from Vehicle-treated group.

Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg.
Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg.
Starvation group also received Vehicle, 1 ml/kg.

FIG. 11. THE EFFECTS OF MUCONOMYCIN A (M-A), MUCONOMYCIN B (M-B) AND STARVATION ON DAILY URINARY EXCRETION OF CHLORIDE IN MALE ALBINO RATS. PER CENT OF VEHICLE-TREATED GROUP.



Solid symbols indicate significant difference ($P<0.05$) from Vehicle-treated group.
Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg,
Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg.

TABLE XV

THE EFFECTS OF MUCONOMYCIN A, MUCONOMYCIN B AND STARVATION ON DAILY POTASSIUM: SODIUM RATIO IN MALE ALBINO RATS[a]

Day of Treatment	K: Na Ratio			
	Vehicle	Muconomycin A	Muconomycin B	Starvation
1	1.95	2.99	3.51	1.48
2	2.53	5.37	3.79	1.10
3	2.30	8.68	4.74	2.33
4	2.42	42.3	6.33	ca 18[b]
5	2.42	20.8	7.41	ca 14[b]
6	2.42	11.4	9.50	---

a: Data derived from Tables XII, XIII and XIV.
b: These values had to be estimated since some Na values were below the sensitivity of the flame photometer.

Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg. Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg. Starvation group also received Vehicle, 1 ml/kg.

TABLE XVI

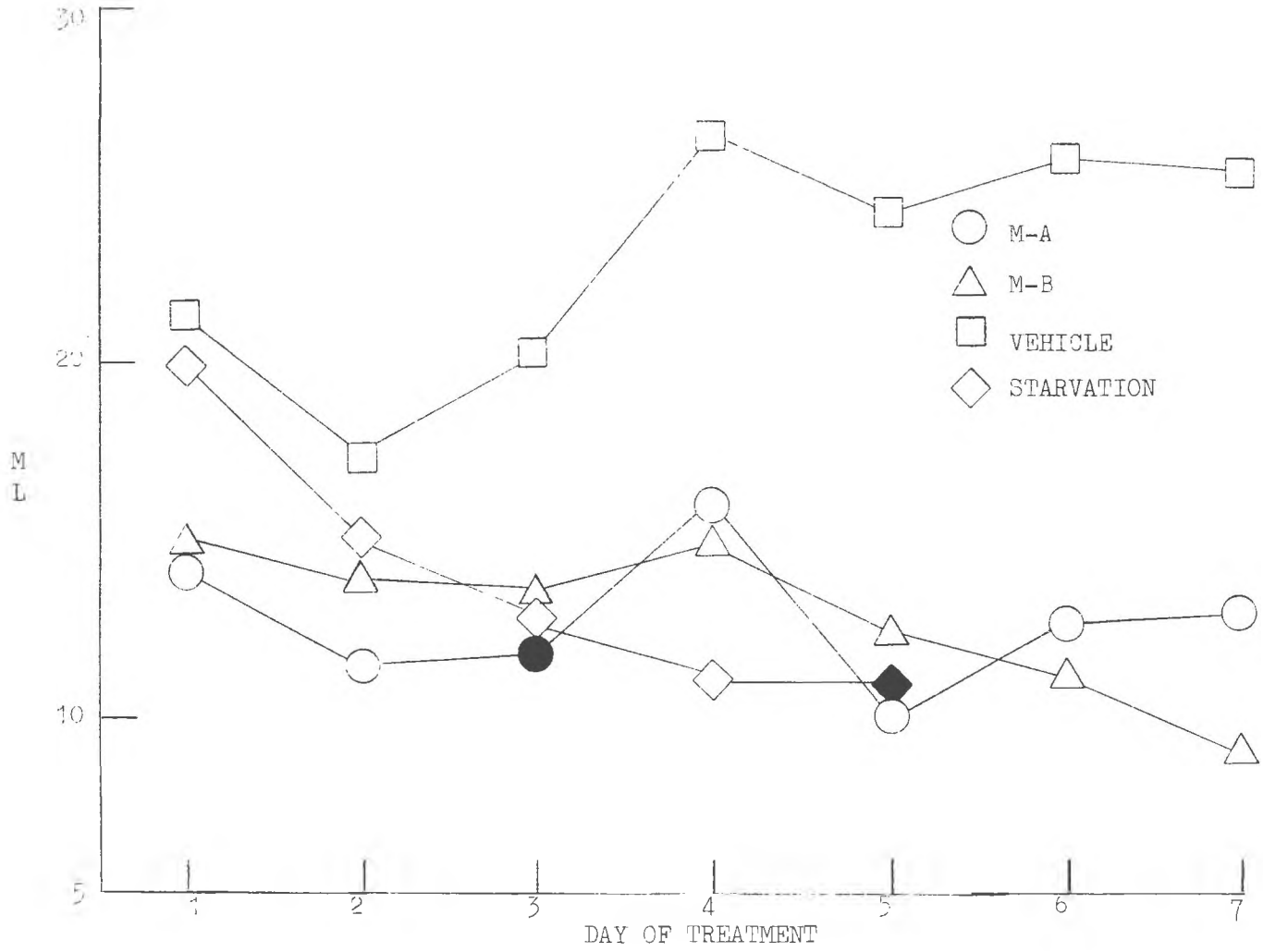
THE EFFECTS OF MUCONOMYCIN A, MUCONOMYCIN B AND STARVATION ON DAILY URINE EXCRETION IN MALE ALBINO RATS

Day of Treatment	Urine Excretion (Ml/2 rats/day)			
	Vehicle	Muconomycin A	Muconomycin B	Starvation
1	21.5 ± 8.0[a] (6)[b]	14.5 ± 4.0 (10), N.S.[c]	15.0 ± 4.0 (10), N.S.	20.0 ± 6.0 (3), N.S.
2	17.0 ± 8.0 (6)	11.5 ± 4.0 (10), N.S.	14.5 ± 4.5 (10), N.S.	14.0 ± 5.2 (3), N.S.
3	20.5 ± 8.0 (6)	12.0 ± 5.5 (10), DEC[d]	13.5 ± 5.5 (10), N.S.	13.0 ± 1.0 (3), N.S.
4	26.5 ± 12.5 (6)	15.5 ± 12.0 (10), N.S.	15.5 ± 7.0 (10), N.S.	11.5 ± 2.5 (3), N.S.
5	24.0 ± 13.5 (3)	10.5 ± 5.5 (10), N.S.	12.0 ± 6.0 (10), N.S.	7.0 ± 2.0 (3), DEC
6	26.0 ± 12.0 (3)	12.5 ± 7.5 (8), N.S.	11.5 ± 7.0 (10), N.S.	—
7	25.5 ± 13.0 (3)	13.0 ± 9.5 (6), N.S.	9.0 ± 4.5 (9), N.S.	—

a: mean ± S.D., ml of urine excreted.
b: number of urine samples measured.
c: N.S.- not significantly ($P<0.05$) different from Vehicle-treated group.
d: DEC-significantly decreased from Vehicle-treated group.

Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg, Vehicle, Carbowax 400, 10% distilled water, 1 ml/kg.
Starvation group also received Vehicle, 1 ml/kg.

FIG. 12. THE EFFECTS OF MUCONOMYCIN A(M-A), MUCONOMYCIN B(M-B) AND STARVATION ON DAILY URINE EXCRETION IN MALE ALBINO RATS. ML/2 RATS/DAY.



Solid symbols indicate significant difference ($P<0.05$) from Vehicle-treated group.
Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg,
Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg.

TABLE XVII

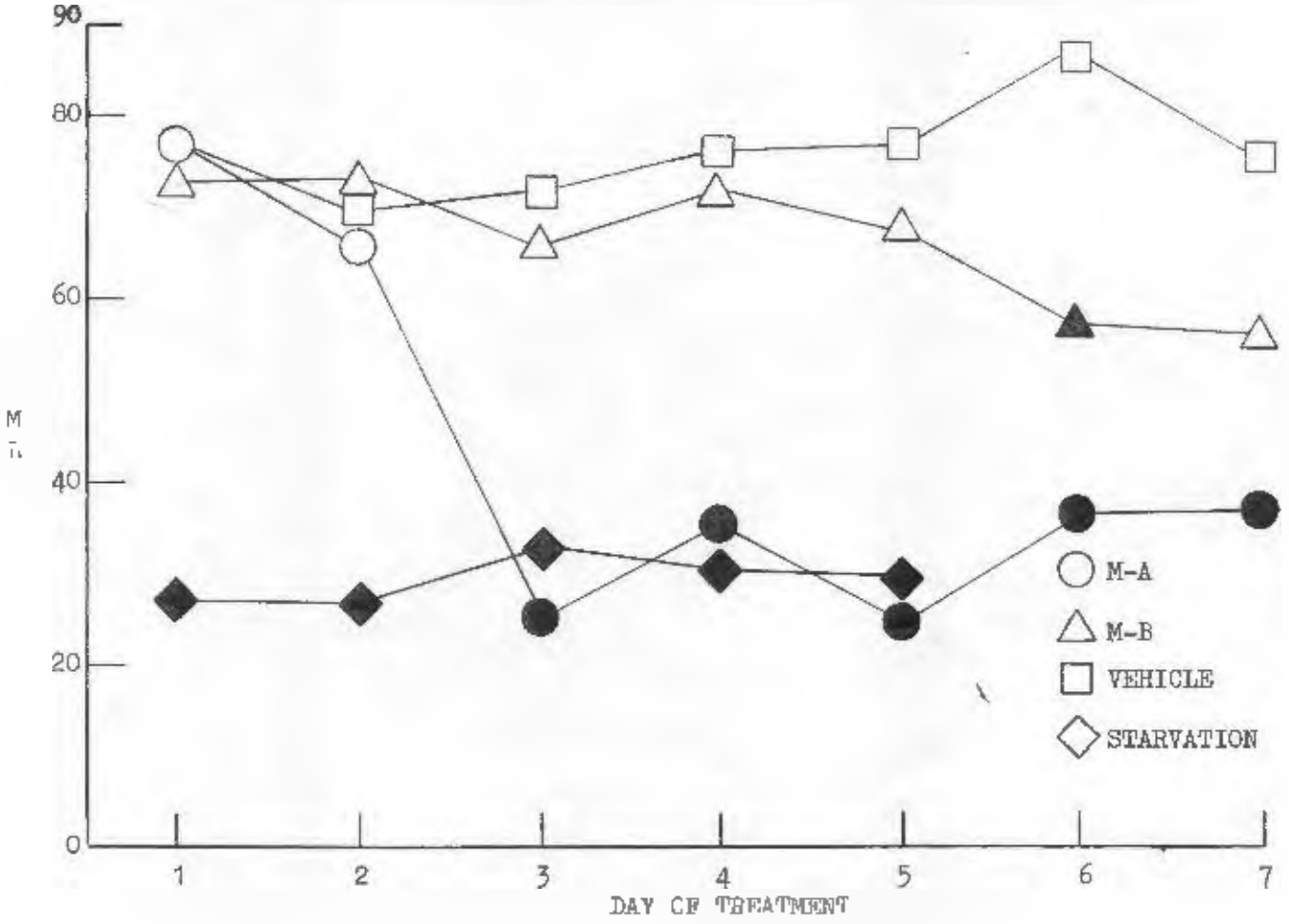
THE EFFECTS OF MUCONOMYCIN A, MUCONOMYCIN B AND STARVATION ON DAILY WATER CONSUMPTION IN MALE ALBINO RATS

	Water Consumption (Ml /2 rats/day)			
Day of Treatment	Vehicle	Muconomycin A	Muconomycin B	Starvation
1	77 ± 13[a] (6)[b]	76 ± 10 (10), N.S.[c]	74 ± 10 (10), N.S.	27 ± 20 (3), DEC[d]
2	70 ± 5 (6)	66 ± 15 (10), N.S.	72 ± 10 (10), N.S.	27 ± 11 (3), DEC
3	72 ± 12 (6)	25 ± 17 (10), DEC	67 ± 14 (10), N.S.	34 ± 3 (3), DEC
4	75 ± 16 (3)	36 ± 18 (10), DEC	75 ± 18 (10), N.S.	26 ± 7 (3), DEC
5	78 ± 18 (3)	25 ± 13 (10), DEC	68 ± 18 (10), N.S.	23 ± 4 (3), DEC
6	88 ± 10 (3)	38 ± 27 (8), DEC	57 ± 15 (9), DEC	---
7	75 ± 17 (3)	37 ± 19 (6), DEC	56 ± 18 (9), N.S.	---

a: mean ± S.D., ml of water consummed.
b: number of water samples measured.
c: N.S. not significantly ($P<0.05$) different from Vehicle-treated group.
d: DEC-significantly decreased from Vehicle-treated group.

Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg, Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg. Starvation group also received Vehicle, 1 ml/kg.

FIG. 13. THE EFFECTS OF MUCONOMYCIN A(M-A), MUCONOMYCIN B(M-B) AND STARVATION ON DAILY WATER CONSUMPTION ON MALE ALBINO RATS. ML/2 RATS/DAY.



Solid symbols indicate significant difference ($P<0.05$) from Vehicle-treated group.
Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg,
Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg.

TABLE XVIII

THE EFFECTS OF MUCONOMYCIN A, MUCONOMYCIN B AND STARVATION ON DAILY URINARY EXCRETION RATIO (UER)* IN MALE ALBINO RATS

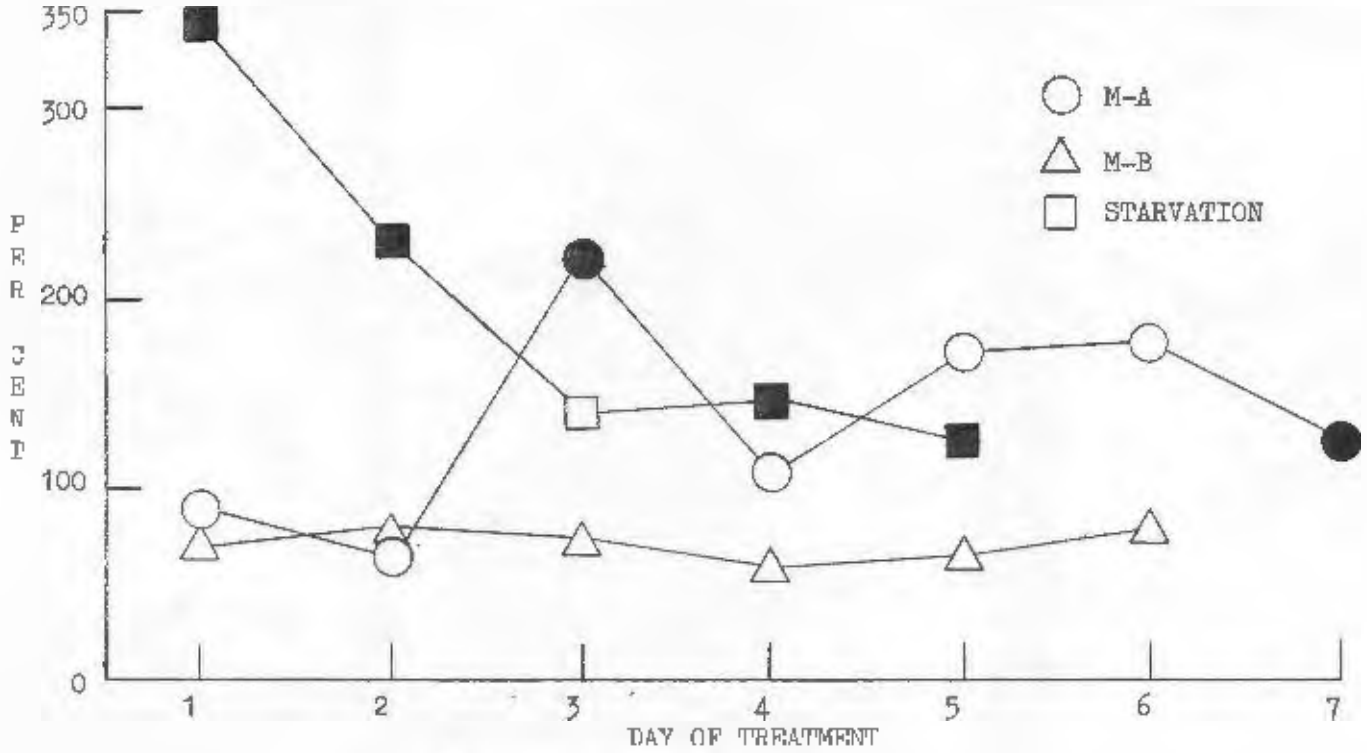
Day of Treatment	Urinary Excretion Ratio x 1000			
	Vehicle	Muconomycin A	Muconomycin B	Starvation
1	40 ± 12[a] (6)[b]	31 ± 9 (10), N.S.[c]	29 ± 6 (10), N.S.	139 ± 70 (3), INC[d]
2	37 ± 19 (6)	28 ± 9 (10), N.S.	28 ± 11 (10), N.S.	86 ± 30 (3), INC
3	43 ± 18 (6)	93 ± 37 (10), INC	36 ± 10 (9), N.S.	59 ± 14 (3), N.S.
4	55 ± 23 (3)	57 ± 27 (10), N.S.	31 ± 12 (9), N.S.	80 ± 26 (3), N.S.
5	46 ± 20 (3)	76 ± 41 (9), N.S.	27 ± 13 (9), N.S.	55 ± 23 (3), N.S.
6	44 ± 20 (3)	75 ± 36 (7), N.S.	31 ± 11 (9), N.S.	---
7	51 ± 20 (3)	63 ± 28 (6), INC	---	---

*UER calculated as, $\frac{\text{Ml urine excreted}}{\text{Ml water ingested}}$ per 2 rats/day/100 g body weight.

a: mean ± S.D., UER/2 rats/day.
b: number of UER's determined.
c: N.S.-not significantly ($P < 0.05$) different from Vehicle-treated group.
d: INC-significantly increased from Vehicle-treated group.

Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg.
Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg.
Starvation group also received Vehicle, 1 ml/kg.

FIG. 14. THE EFFECTS OF MUCONOMYCIN A(M-A), MUCONOMYCIN B(M-B) AND STARVATION ON DAILY URINARY EXCRETION RATIO(UER)* IN MALE ALBINO RATS. PER CENT OF VEHICLE-TREATED GROUP.



*UER calculated as, $\frac{\text{ml urine excreted}}{\text{ml water ingested}}$ per 2 rats/day/100 g body weight.

Solid symbols indicate significant difference ($P<0.05$) from Vehicle-treated group.
Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg,
Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg.

TABLE XIV

THE EFFECTS OF MUCONOMYCIN A (M-A), MUCONOMYCIN B (M-B) AND STARVATION ON RED BLOOD CELL (RBC) AND TOTAL WHITE BLOOD CELL (WBC) COUNTS IN MALE ALBINO RATS

Treatment	RBC ($\times 10^6/mm^3$)	WBC ($\times 10^3/mm^3$)
Vehicle-Fed (V-F)	13.2 ± 3.1[a] (6)[b]	13.1 ± 6.6 (6)
Vehicle-Starvation (V-S)	13.0 ± 4.4 (6)	7.5 ± 1.5 (6)
Muconomycin A	10.3 ± 2.0 (16)	19.8 ± 9.8 (16)
Muconomycin B	10.9 ± 3.4 (16)	14.4 ± 8.8 (18)

Statistical comparisons:[c]

V-F vs M-A	DEC	INC
V-S vs M-A	DEC	INC
V-F vs M-B	DEC	N.S
V-S vs M-B	N.S.	INC
M-A vs M-B	N.S.	DEC

a: mean $\pm$ S.D.
b: number of blood cell counts completed
c: results of t-test at $P<0.05$;
N.S.-not significantly different from compared group.
INC-significantly increased from compared group.
DEC-significantly decreased from compared group.

Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg, Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg. Starvation group also received Vehicle, 1 ml/kg.

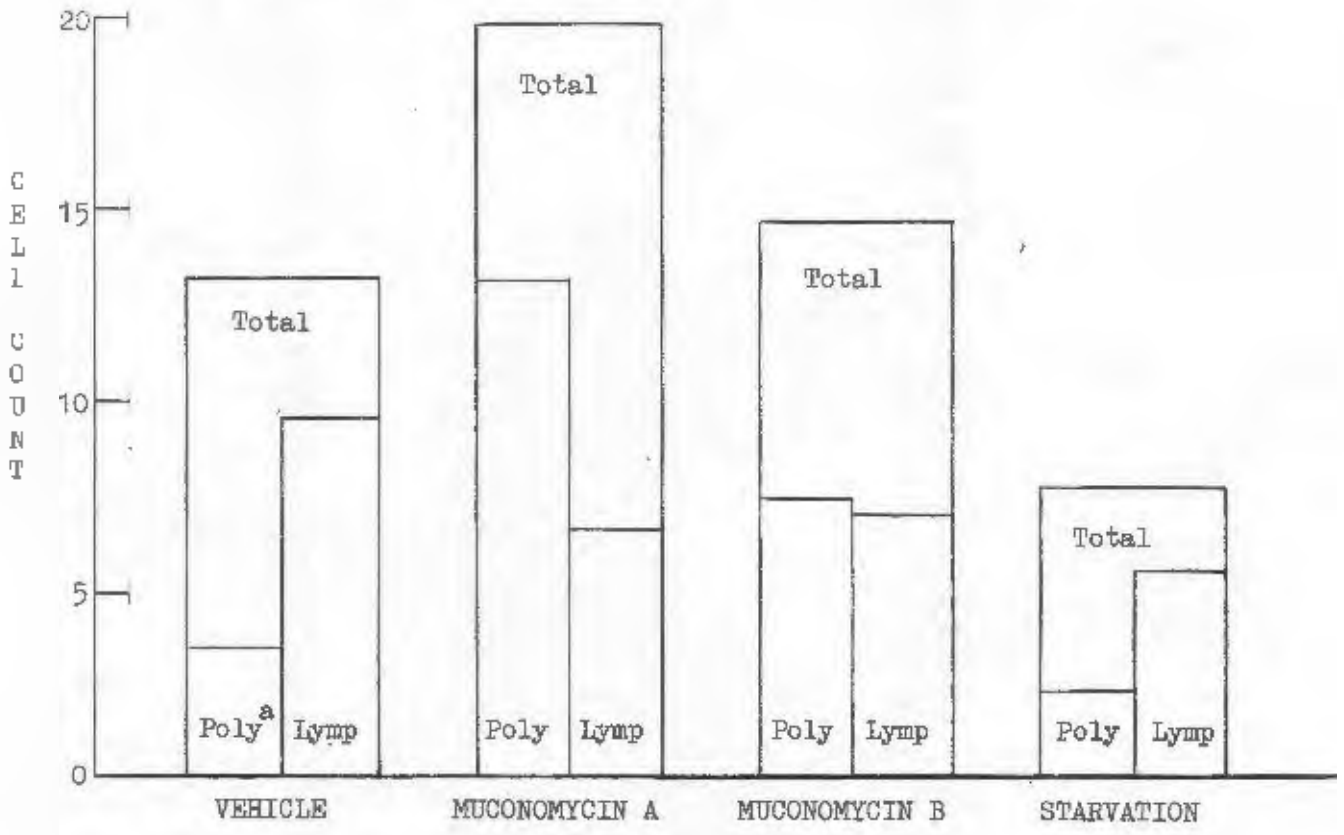
TABLE XX

THE EFFECTS OF MUCONOMYCIN A, MUCONOMYCIN B AND STARVATION ON DIFFERENTIAL LEUKOCYTE COUNTS IN MALE ALBINO RATS

Treatment	Differential Count[a] (%)				
	Poly[b]	Lymph	Eos[c]	Mono	Bas
Vehicle	26 ± 2 (4)[d]	72 ± 3	0-3	C	0-1
Muconomycin A	66 ± 13 (12), INC[e]	33 ± 13 DEC	C-2	C-1	C-2
Muconomycin B	49 ± 9 (18), INC	47 ± 8 DEC	0-6	C-6	0-2
Starvation	28 ± 3 (6), N.S.	71 ± 3 N.S.	C-1	C	0

a: Poly-polymorphonuclear neutrophiles; Lymph-lymphocytes; Eos-eosinophiles; Mono-monocytes; Bas-basophiles
b: Poly and Lymph reported as % ± S.D.
c: Eos, Mono, and Bas reported as ranges observed in slides read.
d: number of differential counts read.
e: results of t-test at $P<0.05$;
N.S.-not significantly different from Vehicle-treated group.
INC-significantly increased from Vehicle group.
DEC-significantly decreased from Vehicle group.

FIG. 15. THE EFFECTS OF MUCONOMYCIN A, MUCONOMYCIN B AND STARVATION ON TOTAL AND DIFFERENTIAL LEUKOCYTE COUNTS IN MALE ALBINO RATS. LEUKOCYTE COUNT X $10^{-3}/mm^3$.



a: Poly-polymorphonuclear neutrophiles; Lymp-lymphocytes.
Doses (i.p.) daily for 7 days: Muconomycin A or B, 0.25 mg/kg.
Vehicle, Carbowax 400, 10% in distilled water, 1 ml/kg.
Starvation group also received Vehicle, 1 ml/kg.
See Table XX for statistical comparisons.

V. DISCUSSION

A. Control Values

At the outset of this research, the reliability of all the methods had to be established. This was done by performing several assays on animals not receiving any drug. The results of these assays were then compared with accepted literature values. The control and literature data appear in Tables I, II, and III. It is unfortunate that most literature values are reported only as means; i.e., no ranges or standard deviations are provided. On the other hand, these accepted clinical methods have an established error range of about 10 per cent. With this fact in mind it can be observed that all the electrolyte data of this report, except for potassium, are within the established ranges. The blood potassium is about 47 per cent higher than the literature values, and (Table III) the mean urinary excretion of potassium is 122 per cent higher than literature values. The author has found that this elevation of blood serum and urinary potassium may be related to an unusually high dietary input of this ion. Ralston Purina Chow which had been standing in distilled water overnight, was assayed by flame photometric methods used elsewhere in this report. As is reported in the Purina Laboratory Chow, Feeding Guide, 1964, this food contained about two times more potassium than sodium. This information should be contrasted

with the reports (e.g. Cizek and Novelli, 1960) that other food brands such as Rockland Rat Diet, contain half as much potassium as sodium.

The blood of control animals was observed to contain quantities of red, white, and differential white cells (Tables I and II) which were within the normal ranges.

The urine and water data, both from the literature sources and from this work, are found in Table III. The daily sodium, chloride and urine outputs are very much the same in the two sources. A possible reason for the high potassium output was mentioned previously. Water consumption and UER data for rats on a fixed caloric input were not found in the literature. These data are presented in Table III.

B. Electrolytes in Rat Blood Serum and Inflammatory Exudate

1. Sodium

Comparative determinations for sodium concentrations in exudate and blood serum are presented in Table IV and Figure 1. None of the sera had significantly different concentrations of sodium. In the sera of the M-A and M-B treated groups, the mean level of sodium was about 150 mEq/L whereas the average level in the exudate was about 100 mEq/L. This 50 per cent reduction in the inflammatory exudate is significant at the 0.05 level. Menkin (1956, p. 91) reported 8 per cent less sodium in pleural exudate than in the blood serum. Using

turpentine as an irritant and the dog as the species under study, Menkin obtained mean values of 146 and 136 mEq/L for serum and exudate, respectively. He did not consider this decrease significant even though he applied no specific tests for significance.

Data from dog studies is included because Hastings (1941) and Lockwood (1964) have reported that the electrolyte profile for the dog and the rat as well as for most mammals, are virtually the same (Table II). Where data are not available for the rat, the author will freely call upon values reported for dogs.

2. Potassium

Some depression of sodium levels in the exudate might be expected if there were a concurrent increase in some other cationic component of the body fluids. In Table V and Figure 2 are found data from potassium flame photometric assays of the body fluids under study in this work. The M-A-treated group exhibited a potassium level that was significantly elevated when compared to any of the other treated groups. The inflammatory exudate showed a dramatic 2-3-fold increase in the potassium content of both antibiotic-treated groups. These data are in accord with the observations of Menkin (1956, p. 85). The mean serum value found by him was 4.7 mEq/L while the exudate level was 9.2 mEq/L. Menkin considers that the

increased concentration of potassium in exudates serves as an index to show that various other materials are "probably likewise extruded for the injured cell at the site of inflammation". On the other hand, he does not explain why the exudate levels do not approach the concentration of K^+ in tissues, i.e., 145 mEq/L.

3. Chloride

After consideration of two of the major cations in the body fluid, some attention must be directed toward the major biologic anion, chloride. Table VI and Figure 3, contain these data. The M-A-treated group had serum chloride levels that were significantly decreased from those of the Vehicle-treated group. The M-B-treated rats showed elevated serum chloride values. There was no significant difference between the serum and exudate levels of chloride in the M-A-treated group but the exudate of the M-B-treated group contained significantly less chloride than did the serum. One reason for the lower concentration of chloride in the exudate of the M-B group may be the greater exudate volume involved. The data on exudate volumes are presented below.

Peritoneal Exudate Volumes (Ml/rat±S.D.)

Muconomycin A Treatment	Muconomycin B Treatment
10.3 ± 6.7 N=13	19.6 ± 12.0 N=18

The difference in exudate volumes is significant at the 0.005 level. One can see, then, that in order to supply almost 20 ml of exudate (M-B group), the blood volume would decrease and the concentration of serum ionic constituents would increase (Goodyer, et al., 1950) more than in the case of a 10 ml exudate volume (M-A group). The mean sodium and chloride values in M-B- treated rat serum show these anticipated trends: i.e., the concentration of both ions is increased. On the other hand, it must be pointed out that the actual decrease in the M-B exudate chloride is only 13 per cent and hence this may not be of any biological significance.

The distribution of chloride in all biological fluids is quite similar. Sodium space is slightly larger than chloride space, indicating that some sodium may be bound to the cell surface (Cotlove, 1954; Manery, 1954). Hence one would not expect any differences between serum and exudate concentrations of chloride. Menkin (1956, p. 92) reported no significant difference between serum and exudate chloride levels in turpentine-treated dogs. Adamkiewicz et al. (1953) reported a rather low mean chloride value, 90 mEq/L, in rat granuloma exudate.

4. Calcium

Menkin (1956, p. 88) found no significant difference between serum and pleural exudate levels of calcium in the early stages of inflammation. However, he did note a signif-

icant elevation of calcium in exudates which became purulent. For example, one exudate contained 8.2 mEq/L while the serum from the same dog had a calcium level of about 5.7 mEq/L. In Table VII and Figure 4, one can see that in this work there is a 30-40 per cent increase of exudate calcium in both antibiotic-treated groups. None of the treatments significantly altered the blood serum levels of calcium.

The appearance of exudate calcium levels in excess of those in sera is of great importance in sustaining the inflammatory condition. Ratnoff (1952) has pointed out that calcium ions accelerate the in vitro activation of proteolytic enzymes. These enzymes are capable of digesting fibrinogen, fibrin, and certain other proteins in vitro. His assays were conducted using calcium concentrations comparable to those in the plasma. This same investigator further pointed out that these facts help in understanding the means by which fibrin deposits are removed in vivo. It is possible that the increased calcium concentration in the transudates may be involved in the healing mechanism by inducing the ultimate removal of the fibrinous network in the terminal stages of the inflammatory reaction.

It has long been known that calcium plays a role in the adhesion of normal tissue cells to one another (Kalant, et al., 1964). Kalant and Miyata (1963) observed that tissue slices cut from normal rat livers and perfused with Ringer solution containing ethylenediaminetetraacetate (EDTA), lose more of

their intracellular potassium, ATP, and protein into the incubation medium than do slices from livers perfused with Ringer solution alone. These effects of perfusions with EDTA do not result from rupture of cell membranes (Leeson and Kalant, 1961) and therefore have been taken as evidence for a role of calcium in the maintenance of normal selective permeability. These results are in agreement with the experiments of Judah and Ahmed (1963) who observed that reduction of calcium in the bathing medium of liver slices results in increased permeability of liver cells. Judah and his co-workers (1964) postulate that the earliest changes associated with cellular injury involve damage to the membrane, resulting in the mobilization of membrane calcium. Hence in the present study such mobilization would be expected to be manifested as an increased level of extracellular calcium. This, indeed, seems to be the case since the exudate calcium concentrations were increased over those of serum.

5. Phosphorus

Inorganic phosphate exists in biological fluids as $HPO_4^{=}$ and $H_2PO_4^{-}$. At normal body pH about 80 per cent occurs in the divalant form (Henry, 1964, p. 415). The assay used in this study gave results in terms of mg P/100 ml and by use of the factor 0.58 provided by Henry (1964, p. 415), all values were converted to mEq/L. These data appear in Table VIII and

Figure 5. Menkin's values (1956, p. 95) were also converted to mEq/L. Menkin found great alterations only in purulent exudates. For example, in one dog, the following values were reported:

Day of Inflammation	Phosphorus Exudate	mEq/L Serum
1	1.0	0.9
2	1.7	1.1
3	1.5	1.6
4	3.9	1.3
5	8.7	2.0

Most of the exudates observed in the present study were not purulent. Those which seemed to possess the characteristic odor of decayed tissue were not assayed.

When one compares the exudate and serum values for phosphorus in the M-A-treated animals, it is seen that both are elevated over the Vehicle-treated blood serum phosphorus levels. This response is similar to that observed by Menkin (1956, p. 95) by the third day of turpentine-induced inflammation in the pleural cavities of dogs. The phosphorus concentrations observed in the M-B-treated group seem to indicate an inflammatory response of a more severe nature, similar to that seen in last two days of Menkin's dog experiment. Menkin also reported one case of a four-fold increase of phosphorus in exudate compared to serum when the inflammation was allowed

to persist for 21 days. Increased blood levels of phosphorus usually are associated with any injury (Tremolieres and Derache, 1960).

The low phosphorus content in the blood of the M-B-treated rats may be related to alterations in food consumption. Haist (1960) fasted rats for 10 hours and then divided them into two groups; one group received 2.0 ml of 25 per cent glucose by stomach tube and the other group did not receive sugar. He found less phosphate in the blood of the rats that had received glucose. The glucose feeding seems to have enhanced the disappearance of inorganic phosphate from the blood. It can be seen from Table IX and Figure 6 that whereas M-A-treated animals did not eat well from the third day on, the M-B-treated animals ate well until the last day of the study.

In conclusion, though, it is important to note that the absolute concentrations of exudate phosphorus were significantly elevated over those in the serum of the Vehicle-treated group.

6. Significance of Electrolyte Changes During the Inflammatory Response

It must be emphasized that the materials of study in the inflammatory response are injured, not dead cells (Opie, 1964). The exudate compositions observed are the result of changes in the injured cell and its fluid environment. Because of the difficulties involved in obtaining adequate quantities of in-

flammatory exudate in animals, most workers have favored assays conducted on inflamed or injured tissues. A major problem in this area lies in the method of presentation of the results. Fuhrman and Crismon (1951) pointed out the great difficulties encountered when various workers freely interchange units such as wet weights, dry weights, fat-free weights and fat-free non-collagen dry weights. In this work it was desired to consider the fluid instead of the tissue involved in the inflammation. It was reasoned that changes in the tissue would be reflected in the surrounding fluid. Opie (1964, 1965) employed the rat in similar, but less extensive studies than those of this work. It was the goal of Opie to "define the characteristic features of inflammation".

The major hypothesis of this work, then, is that electrolyte changes of injured tissue will be reflected in the transudated fluids. An anticipated tissue change will be reflected in a reciprocal manner for the fluid surrounding the injured tissue. In other words a decreased tissue level of potassium would be associated with an increase in exudate potassium.

The first tissue injury to be considered is muscle ischemia. Tremolieres and Derache (1960) reported that the following changes occurred in this kind of injury:

Muscle Electrolyte	Change from Control
Sodium	+ 41 mEq/kg
Potassium	- 36 mEq/kg
Chloride	+ 14 mEq/kg
Phosphorus	- 13 mg atoms/kg

Similar trends are seen in the work of Szpony, et al. (1964) Instead of muscle, these workers used the skin of mice and employed xylene to provoke inflammation. Differences in electrolytes for treated and untreated sections from the skin of the back appear below:

Skin Electrolyte	Change from Control Side
Sodium	+ 23.6 mEq/100 g
Potassium	- 3.5 mEq/100 g
Chloride	+ 17.2 mEq/100 g
Water	+130.0 g/100 g

Neither of the above two studies included calcium assays, but calcium was measured in the work of Kalant and Miyata (1963), Judah and Ahmed (1963) and Menkin (1956). These workers have indicated that in cellular injury, membrane calcium is mobilized. Menkin (1956, p. 89) offered the best in vivo data in this area and these will be presented in the composite tabulation on the next page.

Hence, it would seem that except for the chloride values all the electrolytes in this study provided the profile of changes expected for inflammatory exudates. It is further

REPORTED AND EXPECTED ELECTROLYTE CHANGES
FROM NORMAL TISSUE OR EXUDATE

Study	Reference	Electrolyte Change				
		Na	K	Ca	Cl	P
Muscle Ischemia	Tremolieres & Derache (1960)	INC[1]	DEC	--	INC	DEC
Xylene-treated skin	Szporny, et al. (1964)	INC	DEC	--	INC	--
EDTA-treated liver slices	Kalant & Miyata (1963)	--	--	DEC[3]	--	--
Thioacetamine-treated liver slices	Judah, et al. (1964)	INC	DEC	DEC[3]	INC	DEC[4]
Predicted exudate changes	-----	DEC	INC	INC	DEC	INC
Dog pleural exudate	Menkin (1956)	DEC[2]	INC	INC	N.C.	INC
M-A-induced exudate rat peritoneal cavity	This report	DEC	INC	INC	N.C.	INC
M-B-induced exudate rat peritoneal cavity	This report	DEC	INC	INC	N.C.	INC

1. INC, DEC or N.C.; increased, decreased or no change from normal tissue or blood plasma level.
2. Not cosidered significant by author.
3. Also reported decrease in tissue protein concentration.
4. Based on decreased incorporation into liver phosphoprotein (Ahmed and Judah, 1962).

seen from the above discussions that exudate changes often occurred independent of any alterations in blood serum electrolytes. This latter statement is of importance for two reasons:

1) Inflammatory transudates have often been considered to be derived solely from blood plasma (Szporny, et al., 1964).

2) The experimental animals in this work had symptoms quite similar to the ascites seen in cirrotic patients.

Szporny, et al. (1964) claim that the electrolyte data from xylene-induced edema studies "convincingly illustrates the identity with plasma". These workers presented sodium and chloride plasma assays to support this hypothesis, but they did not include any plasma potassium information. The current research does not support their report of electrolyte change in edema fluid.

The clinical data from cirrotic patients does strengthen the view that ascites fluid is derived from blood plasma. Eisenmenger, et al. (1950) supplied this information on one such patient:

	Na	K	Cl
Serum, mEq/L	137.5	4.9	110
Ascitic fluid, mEq/L	136	4.3	116

Other patients with cirrosis had similar electrolyte profiles. This is not surprizing in view of the fact that experimental ascites can be produced by ligation of hepatic veins (LaLonde,

et al., 1964). The mechanism of ascites in this type of experimental liver disease seems to be related to high hepatic intrasinusoidal pressure which "causes continuous loss of fluid, protein, and electrolyte into the hepatic lymph system, and finally into the free peritoneal cavity itself" (Silver, et al., 1964). In the following sections the inferences of this research will be extended to the subject of edema and ascites.

C. Protein Metabolism in Edema and Ascites

Many of the characteristics common to the conditions of edema and ascites have been mentioned previously. One important feature in these conditions is abnormal capillary permeability. Wright (1961, p. 46) states;

> "When... the capillaries become excessively permeable to protein, plasma protein in varying amounts escapes from the plasma into the tissue spaces and the normal balance of forces is disturbed. As the protein concentration in the plasma falls, its holding power for fluid is decreased and so more tends to escape; at the same time the protein content and the osmotic pressure of the tissue space rise leading to a greater movement of fluid into the tissue spaces and its retention there in excessive amounts, causing oedema."

This reference indicates the emphasis that most researchers place on proteins. Previous studies on the composition of inflammatory edema have been carried out on edema fluid obtained from preformed cavities: e.g. Menkin (1950, 1956), pleural; Opie (1964, 1965), peritoneal; and Adamkiewicz (1953), granuloma pouch. In similar studies Crockett (1956) employed the tech-

nique of direct aspiration of fluids from inflamed areas such as occur in burns and allergic edema. These experiments have been concerned primarily with the quantitative and qualitative determination of proteins. In the previous sections, data have been presented for the electrolyte composition of mucomyin-induced exudates. It is now desired to include the results of protein assays conducted on these exudates and the blood plasma from M-A- and M-B-treated rats. In a later section, electrolyte excretion patterns associated with antibiotic treatment in rats will be presented.

In considering plasma protein levels of the rats in this study, the possible starvation effects are of great importance. It is well documented that under conditions of starvation there occurs a decrease in plasma proteins in which albumin levels are more rapidly lowered than the globulin fraction (Madden, 1950). This is of importance since in the present work there was some decrease in food consumption in both antibiotic-treated groups (Table LX and Figure 6). From these data it is seen that a decrease in food intake was of early onset, occurring on day three in the M-A group whereas there was no significant decrease in food consumption until day 6 in the M-B animals.

The significance of altered food intake becomes more obvious when one considers the cumulative daily weight gains from the first day of the study. This method of presentation employed by Mendillo (1965) is very sensitive to small changes

in food consumption and altered metabolic states. Data presented in this manner are found in Table X and Figure 7. Here one can observe that M-A-treated rats showed a significant decrease in weight gain after the fourth day of the study. M-B-treated animals had weight gains that were as stable as those of Vehicle-treated subjects. The effects of total food deprivation are seen after the first day (starved controls). It would seem then, that some degree of starvation occurred in the M-A-treated animals even though food was available. During this period the rat's intake of electrolytes would, of course, be diminished. In light of the salt-restriction recommended for patients with ascites (Eisenmenger, et al., 1950) one would therefore anticipate some lessening of the ascites in the M-A-treated rats. Indeed, the data on page 93 regarding peritoneal fluid volumes indicate that only one-half as much exudate accumulated in M-A-treated animals. Other details of water and electrolyte metabolism in these rats will be included after discussion of the protein composition of inflammatory exudates.

Under conditions of total starvation (Table XI and Figure 8) it is seen that there is a significant decrease in albumin, no significant change in total protein and, consequently, an inversion of the A/G ratio. This is the protein profile expected under these conditions (Madden, 1950). From these data one also observes that the extent of A/G ratio

inversion seen in food-deprived rats did not occur in the antibiotic-treated rats. Hence, the antibiotics did not cause this symptom commonly observed in starvation.

The concentration of protein found in inflammatory exudate is usually somewhat less than that found in the blood (Yoffey and Courtice, 1956, p. 448). If the protein is derived by lymphatic blockade, the concentration found in the rat fluid is about 60 per cent of that in the blood serum (Drinker, 1946). The exudate from mucomycin-treated animals was 50-60 per cent of that found in the blood serum. In the exudates there appeared to be no differences in the albumin and globulin levels, and the A/G ratio was the same as that of the serum. Since it was also observed that clotting readily took place in the exudate after it was removed from the peritoneal cavity, it can be assumed that all the proteins found in blood plasma were also present in the exudate. This is hardly a precarious assumption since fibrinogen is the largest (in molecular dimensions) of the plasma proteins (Harper, 1963, p. 133). In studies on normal and tumor interstitial fluids, Gullino, et al. (1964) showed that this fluid contained about 70 per cent as much protein as did the plasma, and furthermore, the A/G ratios were the same as for the plasma proteins. In human subjects, Crockett (1956) reported the following protein levels (g per cent): burn blisters, 4.7; allergic edema 4.2; inflammatory edema 4.0-6.0. These authors would classify the anti-

biotic-induced transudation fluid as "quite rich in protein".

D. Electrolyte and Water Metabolism in Edema and Ascites

As mentioned in an earlier part of this work, electrolytes and fluids are required to support this abundant accumulation of exudate in the peritoneal cavity of rats. Goodyer, et al., 1950, in explaining salt retention seen in cirrosis of the liver, provided this information:

> "It has been thought that the salt-containing fluid which escapes from the plasma is rendered 'unavailable' for excretion, or that transudation tends to produce a deficit in the functional plasma volume, which, in as yet unexplained way, stimulates renal tubular reabsorption of salt and water".

During the period of maximum production of ascites, sodium is tenaciously retained by the body (Eisenmenger, et al. (1950). Potassium excretion, like that of sodium, is also very dependent on dietary intake (Henry, 1964, pp. 350, 356).

The daily urine excretion of sodium, potassium and chloride ions are found in Tables XII, XIII, and XIV, and this same information appears in Figure 9, 10 and 11 in terms of per cent of control excretion of electrolyte. In antibiotic-treated rats the rate of electrolyte excretion decreased as the experiment progressed. The gradual decrease in urinary ionic-output corresponded to the appearance of two other phenomena: decreased food intake and formation of peritoneal ascites. The greatest

decrease in ion output occurred under conditions of starvation. Ionic retention appeared to be more severe in the Muconomycin A-treated rats than in those receiving Muconomycin B.

Consideration of the urinary electrolyte data in terms of absolute concentration (Tables XII, XIII, and XIV) or as per cent of control (Figure 9, 10 and 11) is not as informative as other methods of presentation. Eisenmenger, et al. (1950) noted that under conditions of severe fluid accumulation the ratio of potassium to sodium is greatly increased in urine and saliva. A ratio of 300:1 was sometimes seen in urine while saliva ratios were increased from a normal of 2:1 to 6:1 in patients with cirrosis. Table XV contains urinary K:Na ratios for this study. The stable nature of this factor is very obvious in the Vehicle-treated animals. The gradual increase in this factor is expected under starvation since tissue breakdown is taking place with the release of the large amounts of potassium usually contained in body tissues. On the other hand, it is seen that this ratio did not exceed 20 for the period under study. A profound increase in the K:Na ratio is seen in the M-A groups while a smaller increase was found in the M-B-treated rats. This ratio was as high as 42.3 on one day for the Muconomycin A-treated animals while the other antibiotic never induced more than a 9.5 ratio. It is also noteworthy that no profound change in this ratio took place in the starved control group until 4 days passed.

The altered electrolyte excretion seen in ascites and edema is not only due to the fluid accumulation. According to Leiter (1964) liver function may be altered in such a manner that steroid hormones, especially aldosterone, are not effectively catabolized. Laragh and Ames (1963) and Sharp and Leaf (1964) have reviewed the proposals that aldosterone increases potassium excretion while causing sodium retention. According to Laragh and Ames (1963):

> "...increased secretion of aldosterone by the adrenal cortex is a most consistent finding in patients with cirrhosis and ascites, and it appears to play an important or even essential role in the pathogenesis of ascites formation. This increased secretion appears to be associated with intrahepatic congestion, but the mechanisms which elicit this response are not understood... Three direct stimuli to aldosterone secretion are now recognized. These include increases in the plasma levels of (1) potassium ion, (2) angiotensin and (3) ACTH (adrenocorticotropic hormone).

Some of the electrolyte changes seen in this work may, therefore, be due to the action of these hormones. Consideration of hormones was not the subject of the current project. It would appear, however, that since these antibiotics can produce a profound ascites by a simple procedure, one could employ these agents in the further study of this medical problem.

It is impossible to separate electrolyte excretion from body fluid balance. In edema and ascites urinary excretion of both sodium and water is minimal (Eisenmenger, et al., 1950). Table XVI and Figure 12 contain data concerning the

urinary excretion of the rats treated with the antibiotics. From these data the profound diminution of urine output in the starved control animals is seen. More subtle decreases are evident in the antibiotic-treated rats. Of course, urinary excretion of water is related to food and water intake. The relationships between water and food ingestion have been well established by several workers (Adolph, 1947; Bolles, 1961, and Cizek and Nocenti, 1965). By calculation of water consumption, food ingestion and urine ratios, the interrelationships of water consumption, food ingestion and urine excretion, were studied. Data was derived from the mean values in Tables IX, XVI, and XVII. As in the work of Cizek and Nocenti (1965), this study shows the parallelism which exists between water and food intakes in the Vehicle-treated animals. It is also noted that these animals had very stable water: food (W/F) ratios. M-B-treated rats also had somewhat constant W/F ratios but an increase occurred later in the study. The great increase in water consumption compared to food intake is seen in the M-A group as an elevation in this ratio. This means that the water intake was no longer contingent on the food eaten; some other factor entered into water metabolism after day three. One other factor which suggests itself is the requirement for more water to support the ascites. On the other hand, it is not clear why the animals did not also eat. It is possible

that the gastrointestinal tract was irritated enough to cause pain and thus the rats would not desire to eat.

WATER:FOOD RATIOS FOR MALE ALBINO RATS

Day	Vehicle	Muconomycin A	Muconomycin B
1	2.14	2.12	2.06
2	1.94	2.13	2.00
3	2.00	2.78	2.09
4	2.08	9.00	2.40
5	2.17	8.39	2.43
6	2.45	9.50	3.35

Another relationship investigated was that of water intake and urinary output. This was also expressed in the form of a ratio, water:urine(W/U). It is well known that there is some degree of water retention associated with the sodium retention of ascites and edema states. The W/U chart shows again the very stable nature of the Vehicle- and M-B-treated groups:

WATER:URINE RATIOS FOR MALE ALBINO RATS

Day	Vehicle	Muconomycin A	Muconomycin B	Starvation
1	3.58	5.43	4.94	1.35
2	4.12	5.74	4.97	1.93
3	3.51	2.08	4.96	2.69
4	2.83	2.33	4.65	2.26
5	3.17	2.48	5.67	--
6	3.38	3.04	4.96	3.28

It is of considerable interest to note the higher ratio in this latter group. Elevation of this ratio can be caused either by an increase in water intake or a decrease in the amount of urine excreted. The ratio increase in this case is due to a greater urinary retention (see Table XVI and Figure 12) compared with the Vehicle-treated animals. In the Mutomycin A group there was urinary retention in the early days of the experiment but for the last days the volume of urine excreted remained constant while the water intake decreased (see Table XVII and Figure 13). This resulted in the initial elevation of the ratio followed by a decrease of the W/U ratio compared to the control group. Starved rats maintained a decreased but consistent water intake, drinking about 30 per cent less water than control animals. Cizek and Nocenti (1965) obtained a similar value, i.e. 31 per cent in a five-day starvation experiment.

In a further effort to explain the above findings in these water metabolism experiments, daily urinary excretion ratios (UER) were calculated and are presented in Table XVIII and Figure 14. The Vehicle-treated animals demonstrated a rather stable ratio for the period under study. Due to the decreased water intake associated with food deprivation (Cizek and Nocenti, 1965) and the increased formation of metabolic water found in starvation (Guyton, 1961, p. 936), a great increase in this

factor is observed in the early phases of starvation (Table XVIII). Toward the end of the study (Figure 14) the M-A group had a UER quite like that of the starved control group. A consistent degree of water retention is seen in the Mucronomycin B-treated rats. This retention corresponds well with the large fluid accumulation observed in the peritoneal cavity of this group of animals. It must be emphasized that consideration of either water intake or urinary output alone did not reveal this retention. The use of this UER factor is therefore, highly recommended in metabolic and renal studies.

E. Hematologic Studies on Mucronomycin A and B Treated Rats

According to Selye (1950, p. 405) the red-cell count rises in the early phase of the alarm reaction but tends to return to or below normal during the resistance phase, presumably due to initial hemoconcentration followed by blood dilution. The formation of large amounts of peritoneal exudate would also be expected to result in an apparent polycythemia due to a decreased effective blood volume. In Table XIX it is seen that both antibiotic-treated groups had mean RBC counts that were significantly below the mean counts of the Vehicle group. The decreased number of erythrocytes associated with starvation (Kimber, et al., 1961, p. 287) seemed to be a factor in the M-A animals but not in the M-B-treated group. Selye (1950, p. 450) cited studies showing that rats kept on a pro-

tein-deficient diet developed neutrophilia accompanied by lymphopenia and, at the same time, the red-cell count fell. These same trends are seen in Tables XIX and XX, and in Figure 15. Again the inclusion of the starved control group in the design of these experiments proved of great importance. When rats were subjected to five days of food deprivation there was a decrease in the total white cells (WBCs) but no significant change from control can be seen in the differential count (cf. Tables XIX and XX). In the report of Selye (1950, pp. 405, 414) peritonitis and other inflammatory states in rats were shown to be accompanied by polymorphonuclear neutrophilia and lymphopenia. To show the close correlation between his work and the data of the current investigation, Selye's information is included below:

Blood-count	Normal	Alarmed
Total WBC	13,000	25,000
% Polymorphs	35	76
% Lymphocytes	62	24

The differential and total WBCs in Figure 15 show there is a less severe neutrophilia and lymphopenia in the M-B group than in the M-A-treated animals. The latter antibiotic may be a more potent irritant than the former. The P derivative caused less of an alteration in the differential WBCs than did the A derivative. The lesser total white count in the

M-P group also supports the view that M-F is less toxic than M-A. There are two other factors to consider in evaluating the difference in the antibiotics: 1) the stage of inflammation under consideration; 2) the possibility that M-A inhibits the lymphocyte responses.

Lymphocytes do not increase in number during the early stages of inflammation; they are more characteristic of chronic inflammation (Robbins, 1962, p. 66). One might then postulate that the daily injections of the highly toxic M-A maintained the inflammatory response in an acute stage. Menkin (1956) used several injections of turpentine to sustain the pleural inflammation in dogs. If Muconomycin F is less irritating, then one would obtain a smaller change in the differential count; this indeed is evident in Figure 15.

The report of Page (1964) presented evidence that drugs which inhibit protein synthesis, e.g. Actinomycin D, 6-mercaptopurine and puromycin, blocked the lymphocyte response to injection of rabbits with nitrogen mustard. Page studied the cells in the area of inflammation and did not report on the changes in circulating WBCs. The effects of the muconomycins have not yet been tested on protein synthesis and hence this possibility must be left open. On the other hand, the hypothesis that the F analog is less toxic seems more tenable at the present time.

The eosinophil count noted for the M-P group in Table XX, is slightly greater than normal. According to Robbins (1962, pp. 64-65) these white cells are more prevalent during the healing process. The recovery phase of the inflammatory response would seem to be inhibited in the Mucomomycin A group since the eosinophile count is not elevated. Both these facts further support the view that the highly toxic nature of the A analog sustained an acute phase, not allowing a recovery phase of inflammation to appear.

No discussion need be included for the monocyte and basophil counts since they occurred within normal ranges.

VI. SUMMARY AND CONCLUSIONS

A. Toxicological investigations were conducted on two new antibiotics, Muconomycin A and Muconomycin B. These highly toxic, non-nitrogenous antibiotics were found to induce profound inflammation in the peritoneal cavity of male albino rats. Either antibiotic produced large volumes (10-20 ml) of inflammatory exudate even when injected (i.p.) in quantities of 1.6×10^{-10} moles. Verification of the inflammatory state of the antibiotic-treated rats was obtained by observation of an increase in the numbers of circulating total white blood cells and polymorphonuclear neutrophiles.

B. An extensive profile of the electrolytes and proteins found in inflammatory exudates was developed. Simultaneous assays of the blood serum of treated rats provided a basis for comparing the concentrations of constituents of serum with those of the exudate. It appears that this approach to the study of the inflammatory response has not been previously described in such a complete manner.

The inflammatory exudates and blood sera from antibiotic-treated rats were assayed for sodium, potassium, chloride, phosphorus, total protein, albumin and globulin. The exudate contained lower concentrations of sodium and proteins, and greater amounts of potassium, calcium and phosphorus than the serum. Chloride ion concentrations were variable.

C. It was hypothesized that the electrolyte and protein changes seen in these transudates would be reciprocally related to reported values for inflamed tissues. The data obtained in this research supported this hypothesis except in the case of chloride ion.

D. The presence of large volumes of peritoneal fluid in these animals presented an opportunity to study water and electrolyte balances in a model state of ascites. This work confirmed the reported decreases in urine volume and electrolyte excretion seen in clinical ascites.

Consideration of urinary K:Na ratios indicated that there was a much greater retention of sodium than potassium in this experimental ascites. Finally, consideration of daily urine volumes and water consumption was found to be not as sensitive in detecting alterations in water balance as was the use of the combined UER factor.

E. Muconomycin A and B were found to be potent inhibitors of the enzyme, ATP: creatine phosphotransferase. A discussion of the importance of this enzyme in cellular energetics is included. The inhibitory effects on this enzyme seem to explain the greatly elevated potassium levels seen in the peritoneal exudates.

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VIII. APPENDIX I

Evidence for Inhibition of Creatine Phosphokinase as a Mode of Subcellular Toxicity for Muconomycin A and B.

A. Introduction

Because of the heightened creatinuria seen in rats injected with Muconomycin A, it was postulated (Guarino, 1963) that this antibiotic may inhibit the enzyme creatine kinase (i.e. ATP: creatine phosphotransferase, creatine phosphokinase, CPK). In addition, the current research shows that the peritoneal exudate which results from injection (i.p.) of M-A or M-B, contains less sodium and considerably more potassium than does the blood serum of rats. This electrolyte alteration can result from a failure of cellular systems involved with ion transport. Furthermore, it has been pointed out (Fuhrman, 1952; Ussing, 1959; Caldwell, 1960; Bourne, 1962 and Bueding, et al., 1963) that ATP and creatine phosphate are of importance in ion transport. It was postulated then, that inhibition of CPK could decrease the body stores of the high energy compounds, ATP and creatine phosphate.

To test this postulate in vitro, assays were conducted on crystalline creatine phosphokinase. The degree of inhibition caused by various concentrations of the antibiotics was determined by the assay method of Tanzer and Gilvarge (1959). These authors pointed out that this method has the advantage of very high specificity "since creatine is the substrate of only one well

characterized enzyme, creatine kinase".

B. Materials and Method

Creatine phosphokinase of very high purity was purchased from Calbiochem, Los Angeles, California. The specific activity of this enzyme is about 1000 units/mg; i.e. 1 mg of enzyme show a conversion of 1000 μmole of NADH (nicotinamide-adenine dinucleotide, reduced) substrate per minute. This enzyme contains only very small amounts (less than 0.01% each) of lactic dehydrogenase (LDH), pyruvate kinase (PK) and ATPase activity. All other biochemical reagents employed in this assay were purchased from Calbiochem, Los Angeles, California. Any dilutions required in these procedures were accomplished by the use of glass distilled water.

The procedure of Tanzer and Gilvarg (1959) for the assay of CPK is based on the following description. Creatine phosphokinase catalyses the reaction:

$$\text{Creatine} + \text{ATP} \rightleftharpoons \text{Creatine phosphate} + \text{ADP} \qquad (1)$$

The ADP (adenosine diphosphate) is phosphorylated by PEP (phosphoenolpyruvate) and PK:

$$\text{ADP} + \text{PEP} \rightleftharpoons \text{ATP} + \text{pyruvate} \qquad (2)$$

The pyruvate which is formed from this reaction then is reduced by NADH and LDH:

$$\text{Pyruvate} + \text{NADH} + H^+ \rightleftharpoons \text{lactate} + NAD^+ \qquad (3)$$

The decrease in NADH concentration, measured by the change in optical density (O.D.) at 340 mμ, is proportional to the creatine kinase activity if a saturating amount of creatine is present.

To facilitate the addition of reagents the following solutions were prepared:

Reagent A, 2 M glycine buffer, pH 9; 8×10^{-4} M NADH; 6×10^{-3} M ATP and 2×10^{-3} M PEP.

Reagent B, contained 2 mg/ml of LDH, 2 mg/ml of PK and 0.5 M of $MgCl_2$.

Reagent C, 0.1 M glycine buffer, pH 9.

Reagent D, 0.1 M glycine buffer, pH 9 and 6.3×10^{-2} M creatine.

CPK, sufficient enzyme was dissolved in glass distilled water to give a change in optical density of about 0.200 per hour.

Inhibitors, M-A or M-B were separately weighed out and dissolved in 95% ethanol.

In a typical experiment the following procedure was employed:

	Cuvette Number			
Solution	1 (Blank)	2 (Control)	3	4
CPK	0.40 ml	0.40 ml	0.40 ml	0.40 ml
Inhibitor	0.05	---	0.05	0.01
Ethanol, 95%	---	0.05	---	0.04

Mix, allow to incubate 10 min. at 30°C.*

Reagent A	0.70	0.70	0.70	0.70
Reagent B	0.05	0.05	0.05	0.05
Reagent C	1.80	---	---	---
Reagent D	---	1.80	1.80	1.80
Total	3.00 ml			

Immediately following the addition of the substrate (Reagent D) a timer was activated. After mixing by inversion, the blank cuvette was set on O.D.=0.300 at 340 mμ on the Beckman DU Spectophotometer**. The other cuvettes were then read and the time and O.D. readings were recorded every few minutes. By plotting optical density vs. time the reaction rates were compared in the presence and absence of inhibitors. Construction of a graph in which per cent inhibition is plotted vs. inhibitor concentration, enables one to graphically calculate an I_{50}, i.e. the concentration of inhibitor which inhibits the enzyme by 50 per cent.

C. Results

Each inhibited reaction was run simultaneously with a control reaction containing the solvent, ethanol but no inhibitor. Control reactions were very reproducible as is indicated by the following five consecutive values obtained; 0.250, 0.240, 0.210, 0.224 and 0.210 O.D. units per hour.

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* Incubations and assays were conducted at 30°C. The temperature inside the DU cuvette chamber was maintained at 30°C ± 0.5° by use of two Beckman Thermospacer (Beckman Instruments, Inc., Fullerton, California) connected to a Haake (Haake, Gerrunder, Berlin) circulating water heater.

** Beckman Instruments, Inc., Fullerton, California.

It can be seen in the below tabulation that both antibiotics inhibited creatine phosphokinase.

MUCONOMYCIN INHIBITION OF CREATINE PHOSPHOKINASE

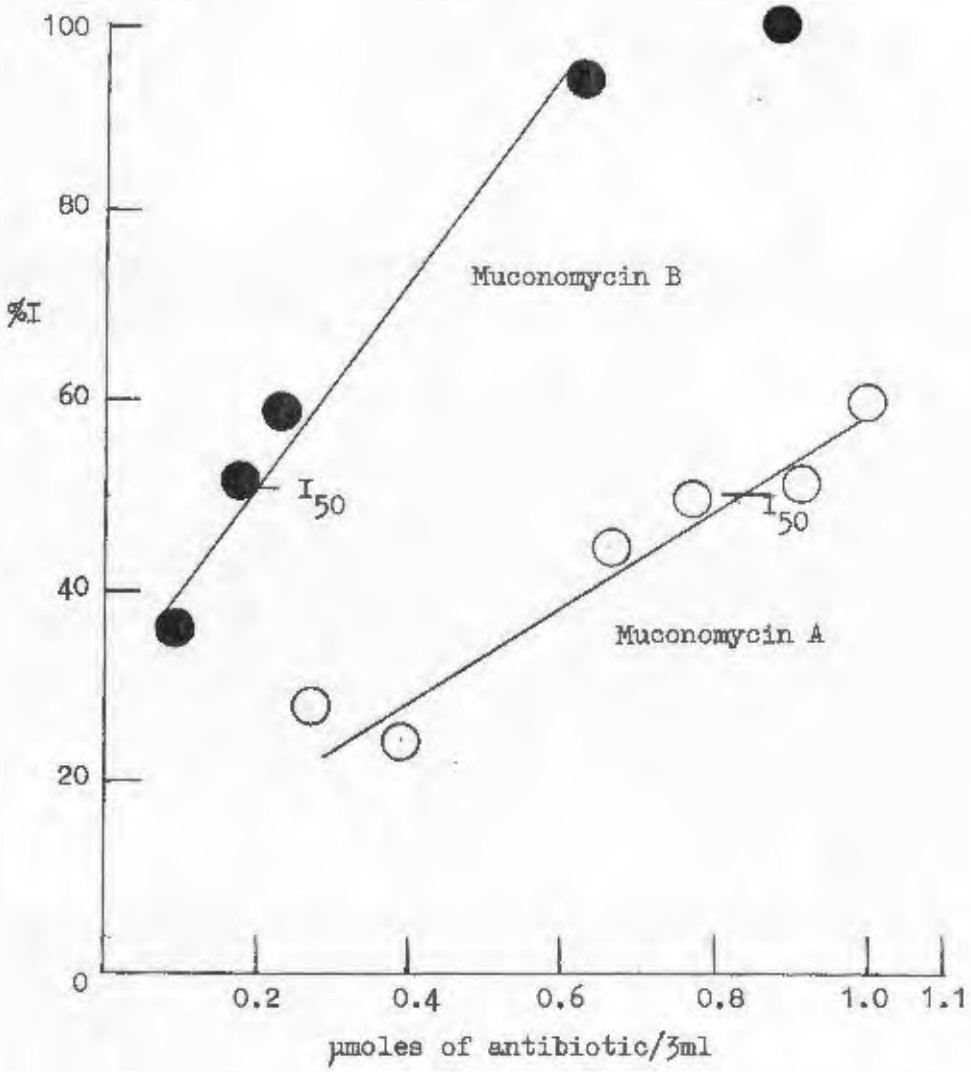
Muconomycin A		Muconomycin B	
Conc. μmole/3 ml	Per Cent Inhibition	Conc. μmole/3 ml	Per Cent Inhibition
0.26	28	0.09	36
0.39	25	0.18	53
0.65	45	0.23	59
0.78	50	0.62	95
0.91	52	0.89	100
1.03	60		

According to the I_{50} calculation on the next page, it would seem that Muconomycin B (I_{50}=0.18 μmoles) is four times more potent an inhibitor as Muconomycin A (I_{50}=0.82 μmoles).

Since this enzyme method employes "coupling" enzymes, the question arose as to the possibility of the antibiotics inhibiting LDH and/or PK. In the absence of CPK and creatine, reactions (2) and (3) previously mentioned were allowed to proceed in the presence of excess ADP. The antibiotics were added to the reaction mixture in concentrations greater than their I_{50}'s and the experiment was conducted as follows:

	Cuvette Number			
Solution	1(Blank)	2(Control)	3(M-A)	4(M-B)
Water	0.40 ml	0.40 ml	0.40 ml	0.40 ml
Inhibitor	0.07(M-B)	---	0.07	0.07
Ethanol	---	0.07	---	---
Reagent B (LDH & PK)	0.05	0.05	0.05	0.05

CALCULATION OF I_{50} FOR MUCONOMYCIN A AND MUCONOMYCIN B ON CREATINE PHOSPHOKINASE



The following components were present in the reaction mixture:

1. 0.7 ml of 2 M glycine buffer, pH 9; 8×10^{-4} M NADH; 6×10^{-3} M ATP and 2×10^{-3} M PEP.

2. 0.05 ml of 0.5 M $MgCl_2$ and 0.1 mg each of LDH and PK.

3. The blank contained 1.80 ml of 0.1 M glycine buffer pH 9 while the reaction cuvettes contained this buffer plus 6.3×10^{-2} M creatine in the same volume of solution.

4. All cuvettes contained 1.68 μg of creatine phosphokinase.

5. Antibiotics were dissolved in 95% ethanol; control cuvette contained an equal volume of ethanol.

Total volume was 3.0 ml in quartz cells, light path=1.0 cm.

Mix, allow to incubate 10 min. at 30°C.

Reagent A	0.70	0.70	0.70	0.70
ADP(40 μM)	---	0.30	0.30	0.30
Reagent C (Buffer)	1.48	1.48	1.48	1.48
Water	0.30	---	---	---
Total	3.00			
O.D. units/hr	---	112	117	102

It would therefore seem that neither antibiotic inhibited the coupling enzymes at concentrations which did cause inhibition of creatine phosphokinase.

D. Discussion

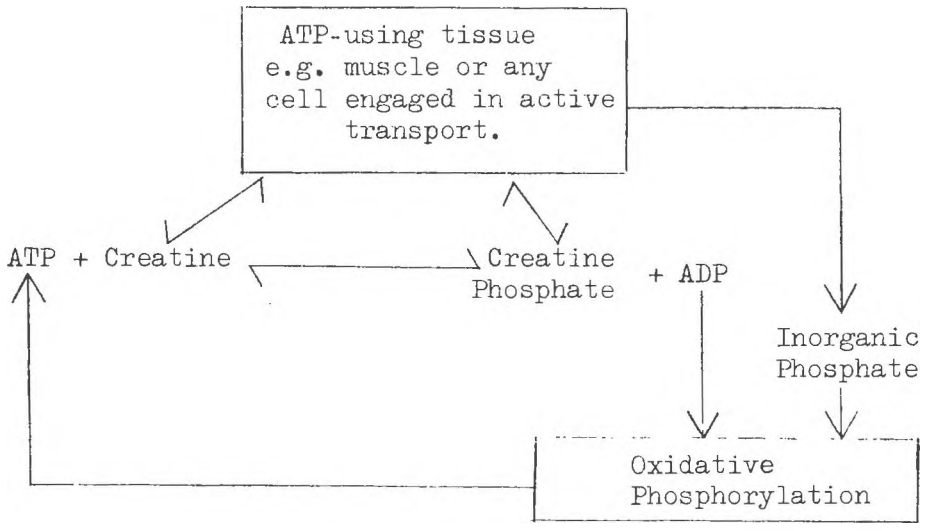
Skeletal muscle contains greater creatine phosphokinase activity than any other mammalian tissue (Tanzer and Gilvarg, 1959). Because of the presence of this enzyme no one had been able to demonstrate changes in the concentration of ATP during a single muscle contraction. As the ATP was consumed it was rapidly regenerated from creatine phosphate by the enzyme creatine phosphokinase (Cain and Davies, 1962). Only after inhibition of this enzyme by 1-fluoro-2,4-dinitrobenzene were these workers able to demonstrate the long-sought-for changes in ATP.

Not only are CPK and creatine phosphate involved with energy production in the muscle, but also they are involved with ion transport (Judah and Ahmed, 1964). Even in the squid

axon where phosphoarginine serves in place of creatine phosphate, Caldwell (1959) has shown that this high energy compound is involved with sodium ion transport.

Jacobs, et al. (1964) presented data which demonstrated a constant proportion of CPK activity to respiratory chain activity. Because they found separate extra- and intramitochondrial isoenzymes they inferred that there may be an unknown pathway of phosphate metabolism. Such a pathway could lead to high energy phosphate transfer between intra- and extramitochondrial compartments.

Chance (1965) has provided an excellent diagram (somewhat revised here) which clearly demonstrates the interrelationships discussed above.



The effects of inhibitors of CPK, then, are quite obvious. In an earlier report Guarino (1963) had postulated that such an inhibition could cause enhanced excretion of creatine in urine. This seems consistent with the proposed cause of creatinuria in muscular dystrophy, viz. excessive breakdown of high energy phosphate compounds (Vignos and Warner, 1963). The reviews of Caldwell (1959), Judah and Ahmed (1964) and Chance (1965) have indicated the role of creatine phosphate and other high energy phosphate compounds in ion transport. Since creatine phosphate stores are maintained by CPK, inhibition of this enzyme would be expected to cause mammalian cells to lose potassium and gain sodium. This report also has shown that M-A and M-P cause these ionic alterations. The absolute amounts of potassium in the inflammatory exudates are so high, it is thought that ion transport is being inhibited in these animals. If we assume an approximate blood volume of 15 ml in a 300 g rat, the M-P-treated animal would have 15 ml x 8 mEq/1000 ml (Table V), or 0.120 mEq of potassium in the blood. Since the mean peritoneal fluid volume in these animals was about 20 ml, then 20 ml x 25 mEq/1000 ml (Table V), there was about 0.500 mEq of potassium in the exudate. This seems to be too large a difference to be explained in terms of "altered permiability" and hence inhibition of ion transport seems to occur in Mucronomycin A and Mucronomycin B treated rats.